Universidad Autónoma Metropolitana

Unidad Iztapalapa

El consumo de una dieta alta en fructosa potencia la progresión del carcinoma hepatocelular en ratones.

The consumption of a high fructose diet enhances the hepatocellular carcinoma progression in mice.

Tesis

Para obtener el grado de Maestra en Biología Experimental

Presenta

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Ciudad de México, 26 de Noviembre de 2020

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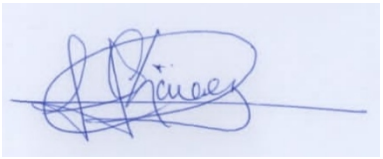

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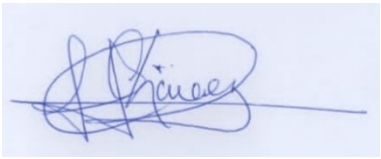

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To my parents,

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Resumen

Introducción y Antecedentes

El carcinoma hepatocelular (HCC) constituye la cuarta causa de muerte relacionada con cáncer a nivel mundial y la tasa de incidencia de este continúa en ascenso, tanto en hombres como en mujeres. La principal causa ha sido el consumo de dietas hipercalóricas ricas en carbohidratos como la fructosa. La dieta alta en fructosa se relaciona con el desarrollo de la NAFLD, uno de los principales factores de riesgo para la aparición del HCC con una alta incidencia en la región americana. La NAFLD se ha clasificado como la enfermedad crónica del hígado más común en los últimos 30 años y es una de las principales causas de trasplante hepático. La NAFLD se define como un conjunto de patologías que pueden desarrollarse debido a la acumulación de lípidos en forma de esteatosis macrovesicular en más del 5% de los hepatocitos y que no se encuentra relacionado con el alto consumo de alcohol. Esta enfermedad puede comenzar como simple esteatosis que puede evolucionar a NASH, fibrosis, puede pasar o no por cirrosis, hasta llegar a la aparición del HCC.

México presenta un alto consumo de alimentos ricos en azúcares añadidos, consumo de los cuales se ha relacionado con el riesgo de padecer cáncer. Actualmente, el país ocupa el 5to lugar en consumo de bebidas azucaradas y se preveé un aumento en el consumo de los mismos hacia los próximos 5 años. El principal edulcorante utilizado para la fabricación de estos refrescos es el jarabe de maíz con alto contenido en fructosa (HFCS por sus siglas en inglés), el cual tiene una composición de fructosa:glucosa entre 55%:45% o 65%:35%, respectivamente.

Ante un consumo elevado de fructosa en la dieta, aproximadamente el 70% de su metabolismo se lleva a cabo en el hígado. Se ha visto que la fructosa es un metabolito altamente lipogénico que entra al hepatocito a traves del transportador GLUT-2 y GLUT-8. Una vez dentro, la fructosa es rápidamente fosforilada en la posición 1 por la principal enzima de la ruta: la cetohexocinasa (KHK). La Fructosa-1-fosfato (F-1-P) es escindida por la Aldolasa B, dando lugar a los metabolitos intermediarios gliceraldehído-3-fosfato y dihidroxiacetona fosfato (G-3-P y DHAP), los cuales se incorporan a la ruta glicolítica pasando por puntos de regulación importantes de esta ruta (Glucocinasa y Fosfofructocinasa-1). Como resultado se obtienen metabolitos precursores de bases nitrogenadas o que se incorporan a la vía lipogénica a partir del citrato, ocasionando un incremento en la lipogénesis y la acumulación de lípidos en el hígado. Además, también se conoce que tanto la fructosa como sus metabolitos activan la expresión de genes implicados en la degradación de la fructosa y rutas lipogénicas (*glut-2, khk, acly, acaca, fasn*, *chrebp* y *srepb1*) y reprimen la expresión de genes relacionados con la oxidación de lípidos (*cpt1a* y *fgf2*1), lo cual potencia la esteatosis en el hígado y la activación de rutas pro-inflamatorias.

Por tanto, el alto consumo de fructosa en la dieta constituye un factor de riesgo importante para la aparición, progresión y agresividad del HCC.

Pregunta de investigación

¿Cómo influyen una dieta alta en fructosa en la progresión del hepatocarcinoma celular?

Hipótesis

La dieta occidental tiene una alta composición de carbohidratos, entre los que se encuentran los edulcorantes artificiales o azúcares. El edulcorante más utilizado debido a la accesibilidad de los precios, es la fructosa en forma de jarabe de maíz con alto contenido de fructosa (HFCS) y está relacionada con la enfermedad de hígado graso no alcohólico (NAFLD). Por lo tanto, un alto consumo de fructosa en la dieta puede potenciar la agresividad del HCC.

Objetivo General

- Determinar el efecto de la dieta alta en fructosa sobre la progresión del carcinoma hepatocelular.

Objetivos específicos

- Determinar el efecto de la fructosa (33% de contenido) sobre la proliferación, el metabolismo y la tumorigénesis utilizando las línea celular de HCC (Huh7) como modelo.
- Determinar el efecto de la dieta alta en fructosa (33% de contenido) sobre la progresión del carcinoma hepatocelular mediante enfoques celulares y moleculares en ratones C57BL/6j.

Materiales y Métodos

Para desarrollar estos objetivos se utilizó la cepa de ratones C57Bl/6j (ambos sexos) y se establecieron cuatro grupos de tratamiento:

- **Grupo CW** Dieta normal (Chow 5001) tratados con solución salina isotónica (SSI) (24 ratones)

- **Grupo CW/HCC** Dieta normal (Chow 5001) tratados con N-Dietilnitrosamina (DEN) (carcinogénico) en SSI (24 ratones)
- **Grupo Fru** Dieta normal (Chow 5001) con fructosa 33% *ad libitum* en el agua de beber y tratados con SSI (24 ratones)
- **Grupo Fru/HCC** Dieta normal (Chow 5001) con fructosa 33% *ad libitum* en el agua de beber y tratados con DEN en SSI (24 ratones)

La suplementación con Fructosa se inició a los 15 días de edad, dos días después se inyectó el DEN (10 µg/Kg, i.p) y se llevó a 32 semanas con el fin de evaluar el papel de la fructosa en la progresión tumoral mediante pruebas histológicas y bioquímicas (Protocolo aprobado por la comisión de ética de la UAM).

Por otro lado, con la finalidad de evaluar los efectos a nivel celular, se utilizó como modelo la línea celular de HCC Huh7 y se aplicaron tres tratamientos, solo Fructosa o Glucosa a diferentes concentraciones (0.65 mM, 0.68 mM y 0.72 mM) y la combinación de ambas (Fructosa:Glucosa) (0.58 mM:0.14 mM, 0.61 mM:0.11 mM y 0.67 mM: 0.05 mM) para llevar a cabo pruebas de viabilidad, proliferación, y funcionalidad celular. También se evaluaron parámetros tumorigénicos como la formación de esferoides y formación de colonias.

Resultados

Con el objetivo de determinar el efecto de la dieta alta en fructosa *in vivo* se les suministró a los ratones de la cepa C57Bl/6j agua *ad libitum* con un 33% de fructosa. Los ratones fueron sacrificados a las 12, 20 y 32 semanas. Al analizar la composición corporal de los ratones se obtuvo que los que tenían la dieta suplementada con fructosa (**Fru**) tenían un mayor contenido de tejido adiposo respecto al control (13.1%

vs. 6.7% respectivamente) (Fig. 5). Posteriormente al analizar el tejido hepático se observaron cambios en el peso y la morfología del mismo (Fig. 6 y Fig. 7), con un mayor número de tumores en el grupo **Fru/HCC** (Fig. 8A), actuando la fructosa como un promotor tumoral en el hígado. También se observaron otras lesiones tanto en pulmones como en las patas de los ratones. En el caso de los pulmones, los nódulos se presentaron en mayor cantidad en el grupo **Fru/HCC** a las 32 semanas de tratamiento en ambos sexos (Fig. 8B y 9). Sin embargo, en el caso de los tumores de las patas, tanto anteriores como posteriores, solo se obtuvieron en los ratones de sexo hembra que tenían una dieta suplementada con fructosa (**Fru** y **Fru/HCC**) a las 32 semanas de tratamiento (Fig. 10). Luego se analizó el tejido por medio de hematoxilina-eosina y se obtuvo que el grupo con una dieta suplementada con fructosa (**Fru**) presentaba esteatosis y un aumento del infliltrado inflamatorio (Fig. 11).

Posteriormente se hizo un análisis del perfil bioquímico del suero, y se obtuvo un mayor nivel de CHO y menor nivel de TG en suero en los grupos **Fru** y **Fru/HCC** (Fig. 12). Luego se prosiguó a analizar la composición lipídica del tejido y se obtuvo completamente lo contrario, ya que los niveles de TG eran significativamente mayores en los grupo suplementados con fructosa (Fru y Fru/HCC) vs el grupo control. En el caso del CHO no se observaron diferencias significativas entre tratamientos. Para corroborar estos resultados se analizó el contenido proteico de las principales enzimas implicadas en la síntesis de lípidos. Se obtuvo que el contenido de las enzimas relacionadas con la síntesis de ácidos grasos era significativamente mayor respecto a las implicadas en la ruta del mevalonato (Fig. 13).

Posteriormente se evaluó en un modelo experimental *in vitro* el efecto de la fructosa sobre la línea celular Huh-7. Se obtuvo que la fructosa aumenta tanto la proliferación como la funcionalidad de la línea cancerosa sin afectar la viabilidad (Fig. 14). Además se obtuvo que la fructosa potencia la agresividad de la línea celular (Fig. 15).

Conclusiones

Estos resultados evidencian que la fructosa actúa como un compuesto tóxico en las células del hígado potenciando la progresión hacia HCC a través del aumento de la agresividad de las células cancerosas y la formación de tumores. Además, el alto consumo de fructosa provoca la activación de FASN y, con ella, la lipogénesis aberrante, factor que se correlaciona con la promoción de tumores y la agresividad del cáncer.

Abstract

Hepatocellular carcinoma (HCC) is the fourth cause of cancer-related death and its incidence has been increasing in both men and women. One of the main concerns has been the consumption of hypercaloric diets mainly rich in carbohydrates such as fructose. High fructose diet is related to the development of Non-Alcoholic Fatty Liver Disease (NAFLD) and the progression of HCC since it potentiates the lipogenic pathway and the accumulation of lipids. The aim of the study is to determine the effect of a high fructose diet on the progression and aggressiveness of HCC. We used a murine model (C57Bl/6j mice strain, both sex) with a high Fructose diet (**Fru**) (33% of Fructose in the drinking water, *ad libitum*). Fru supplementation started with 15 days old mice, two days after N-Diethylnitrosamine (DEN) was injected (10 µg/Kg, i.p) and the treatment was ended 32 weeks later to evaluate the role of fructose in tumor progression by histological and biochemical tests. The protocol was approved by the UAM ethics commission. Also, we used Huh-7 HCC cell line and applied three treatments, Fructose or Glucose alone at different concentrations (0.65 mM, 0.68 mM and 0.72 mM) and the combination of both (Fructose: Glucose) (0.58 mM: 0.14 mM, 0.61 mM: 0.11 mM and 0.67 mM: 0.05 mM) to carry out tests of viability, proliferation, and cellular functionality. Tumorigenic parameters such as spheroid formation and colony formation were also evaluated. Fructose consumption increases the adipose tissue in Fru and Fru/HCC groups, and also enhance tumor formation. The major number of tumors were found in the Fructose+DEN **(Fru/HCC)** mice group *vs*. only

DEN (**CW/HCC**) mice group. Triglyceride levels (TG) was evaluated in the serum with no differences between treatments; however, in the liver tissue the **Fru** and **Fru/HCC** groups showed significantly higher TG content. On the contrary, the Cholesterol (CHO) levels were significantly higher in the serum of dietary fructose group and had no differences in the tissue. The protein content in tissue followed the same observed pattern, since significance was only found in fatty acid synthase (FASN) with a higher protein content in the groups with dietary fructose. Curiously, we noticed tumors in the lungs. In vitro model did not show significant changes with sugar concentrations, so 0.68 mM fructose was continued for 24 h. Following this experimental model, a greater number of spheroids and colonies were obtained in the cells treated with fructose compared to the control. The data strongly suggests that the high consumption of Fru in the diet induces effects in liver tumor promotion, in a mechanism dependent on FASN and independent of CHO, enhancing the aggressiveness of the HCC.

INDEX

Introduction

Cancer is classified as a non-communicable disease and it is also known as malignant tumors or neoplasms. Cancer includes a group of characteristics like abnormal and uncontrolled cell growth, the evasion of cell growth suppressors, resistance to apoptosis and replicative immortality, induction of angiogenesis, activation of invasion into new tissues and organs (metastasis). Another acquire functional capability of the transformed cells is the metabolic reprogramming, this characteristic allows cancer cells to survive and proliferate (Hanahan and Weinberg, 2011). Tumors are defined as complex tissues composed of multiple cell types that participate in heterotypic interactions with each other (Hanahan and Weinberg, 2011).

In recent years the incidence of non-communicable diseases has increased, among them, cancer is thought to be the leading cause of death in the 21st century. According to data reported by the World Health Organization (WHO), it was estimated that this disease could affect approximately 9.6 million people worldwide in 2018 (https://www.who.int/cancer/en/). Mexico shows a total of 190,667 new cases and 83, 476 deaths, and 471, 497 five years prevalent cases (https://gco.iarc.fr/today/data/factsheets/populations/484-mexico-fact-sheets.pdf Consulted: 15/November/2020). The incidence of liver tumors has also been increasing, being within the top 10 of the main diagnosed cancers worldwide (Bray et al., 2018).

The liver is one of the first organs to form in the embryo stage. It is composed of various cell types including hepatocytes, cholangiocytes, sinusoidal endothelial cells, dendritic cells and stellate cells. Due to its morphology, this organ performs various functions allowing the maintenance of homeostasis in the body. In the liver is carry out the metabolism of proteins, lipids and carbohydrates. It also stores, exports and/or excretes metabolic products and neutralizes antigens and foreign microbes of the intestine (Irwin et al., 2009). That is why a failure in the functioning of the organ can lead to an imbalance in the body and even death (Alwahsh and Gebhardt, 2016).

In the liver, two types of malignant tumors have been generally classified: primary malignant tumors and secondary malignant tumors (when referring to metastasis) (Ahmed and Lobo, 2009). Primary malignant tumors can originate from epithelial, mesenchymal, muscular tissue or have a heterogeneous origin. The epithelial malignant tumors have the highest incidence, and among them, hepatocellular carcinoma and cholangiocarcinoma (Ahmed and Lobo, 2009).

Hepatocellular carcinoma (HCC) originates from the hepatocyte cell type and comprises between 75% - 85% of reported cases of liver cancer (Bray et al., 2018). This type of tumor has ranked sixth in incidence and is the fourth leading cause of cancer-related death worldwide. Around 841,000 cases and 782,000 deaths are reported annually, and it has been seen to have a higher incidence in men than women (Bray et al., 2018). Mexico shows similar trend being one of the top ten cancers with a high mortality in the country. Around 7,265 new cases and 6868 deaths were reported

in 2018 (https://gco.iarc.fr/today/data/factsheets/populations/484-mexico-fact-sheets.pdf Consulted: 15/November/2020).

There are several risk factors for the development of this type of cancer such as chronic infection with Hepatitis B and C viruses, alcoholism, aflatoxin B1, non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Alwahsh and Gebhardt, 2016; Llovet et al., 2016). These risk factors vary by geographic region, being Asian and Sub-Saharan Africa region the one with the highest incidence of HCC due to Hepatitis virus infection and alcoholism, while in America the greatest risk factor is obesity and NAFLD (Llovet et al., 2016).

NAFLD is defined as a set of pathologies that can develop due to the accumulation of lipids in more than 5% of hepatocytes and it is not related to high alcohol consumption. NAFLD is associated with obesity, insulin resistance, type II diabetes, and hyperlipidemia, that is why it has been defined as the hepatic manifestation of metabolic syndrome (Kleiner and Makhlouf, 2015). NAFLD can begin as simple steatosis that can evolve to NASH, fibrosis, cirrhosis, until reaching the appearance of HCC (Kleiner and Makhlouf, 2015; Cobbina and Akhlaghi, 2017). NASH is characterized by accumulation of lipids in addition to hepatocytes ballooning, lobular inflammation, and, in most cases, fibrosis. Cirrhosis is the terminal stage of liver failure and can lead to HCC (Cobbina and Akhlaghi, 2017).

The consumption of calories in the diet (both lipids and carbohydrates) has increased over the years and hypercaloric diets have been shown to be implicated in the prevalence of NAFLD (Alwahsh and Gebhardt, 2016; Kroemer et al., 2018).

Carbohydrates have a lower price in the market compared to fats or proteins, thus increasing their commercial use even more (Kroemer et al., 2018). Among them, the most used as a sweetener in beverages and food is High Fructose Corn Syrup (HFCS) with a Fructose:Glucose composition between 55%:45% or 65%:35%, respectively (Alwahsh and Gebhardt, 2016: Jensen et al., 2018). The consumption of ultra-processed foods rich in added sugars is linked to the appearance and development of NAFLD (Alwahsh and Gebhardt, 2016; Jensen et al., 2018; Kroemer et al., 2018).

Fructose is a six-carbon monosaccharide, and it is naturally found in fruits and honey; however, its consumption in the form of HFCS has increased in the Western diet. The use of HFCS as a sweetener began in the United States between the 60´s and 70´s, and then it was spread to the rest of the world (Windemuller et al., 2016; Kroemer et al., 2018). It has been reported that a low consumption of fructose in fruits is associated with a loss of weight (Sharma et al., 2016), however with a high consumption of industrial foods there is a strong link with obesity and NAFLD (Ouyang et al., 2008; Alwahsh and Gebhardt, 2016; Horst and Serlie, 2017).

Fructose is a highly lipogenic metabolite that is absorbed in the small intestine by means of specific transporters (GLUT-5). The 70% of its metabolism occurs in the liver (Charrez et al., 2015; Li et al., 2016) and it enters the hepatocyte through GLUT-2 (Riveros et al., 2014) or GLUT-8 (Horst and Serlie, 2017). The main enzyme involved in fructose metabolism is Ketohexokinase (KHK). This enzyme has a high affinity for fructose, it is not negatively regulated by product concentration and phosphorylates fructose in position 1. It is subsequently metabolized by Aldolase B to

Dihydroxyacetone phosphate (DHAP) and Glyceraldehyde-3-phosphate (G-3-P). Intermediate metabolites can be incorporated into the glycolytic pathway avoiding important points of regulation of this mechanism (Glucokinase and Phosphofructokinase) and yielding intermediate metabolites to give precursors of nitrogenous bases or incorporate them into the lipogenic pathway from of citrate, causing an increase in lipogenesis and the accumulation of lipids in the liver (Riveros et al., 2014; Charrez et al., 2015; Li et al., 2016).

Background

The Liver

The liver is one of the first organs to form in the embryo stage, and in adults it weights between 1,300 to 1,700 grams depending on gender of the person. According to its anatomical definition, the liver is defined as a highly vascular parenchymal mass, which is penetrated by tunnels or gaps composed of interdigital networks of efferent and afferent vessels. It is composed of various cell types including hepatocytes, cholangiocytes, sinusoidal endothelial cells, dendritic cells and stellate cells. Due to its morphology, this organ performs various functions allowing the maintenance of homeostasis in the body. The liver stores, exports and/or excretes metabolic products and neutralizes antigens and foreign microbes of the intestine. Also, in the liver is carry out the metabolism of proteins, lipids and carbohydrates (Irwin et al., 2009). That is why a failure in the functioning of the organ can lead to an imbalance in the body and even death (Alwahsh and Gebhardt, 2016).

Fructose metabolism

Fructose is a highly lipogenic monosaccharide that is absorbed in the small intestine by means of specific transporters for it (GLUT-5) and 70% of its metabolism occurs in the liver (Charrez et al., 2015). However, it has been seen that when low doses of fructose are consumed (<0.5 g / kg in mice), the intestine is in charge of metabolizing it and converting it to glucose, which will reach the liver (Fig. 1). The main enzyme of this fructose degradation pathway has two isoforms, specifically KHK-C is expressed mainly in the intestine, liver and kidney and has a high affinity for fructose; while isoform A is present in all tissues and has a lower affinity. KHK-C phosphorylates fructose in position 1 (F-1-P), which is subsequently cleaved by Aldolase B into DHAP and G-3-P, intermediary's metabolites capable of activating the expression of genes related to the fructose metabolism itself and lipogenic pathways (Charrez et al., 2015; DiStefano, 2019).

In the intestine, fructose increases the expression of its own transporter (GLUT-5) (Zwarts et al., 2019). Once in the enterocyte, the intermediate metabolites (F-1-P, DHAP, G3P) are capable of activating the Liver X Receptor (LXR) transcription factor, which increases the membrane expression of the transporter. It has been seen that the promoter of the gene that codes for GLUT-5 has elements of responses to LXR (LXRE), so this allows the increase in the expression of this transporter in the membrane (Zwarts et al., 2019). Likewise, it has been seen that the carbohydrate response element binding protein (ChREBP) can increase the expression of GLUT-5 due to the

fact that have been identified carbohydrate response elements (ChoRE) in the promoter.

Then, following a high-fructose diet (>1g / kg in mice), the fructose that was not metabolized in the enterocyte exits through the GLUT-2 transporter located in the basolateral membrane and into portal blood (Merino et al., 2020). Once there, it enters the hepatocyte through the same transporter (Riveros et al., 2014), and the intermediate metabolites can be incorporated into the glycolytic pathway, passing the two main regulatory steps of this. Subsequently, these metabolites constitute the carbon skeleton for de novo lipid synthesis (Charrez et al., 2015).

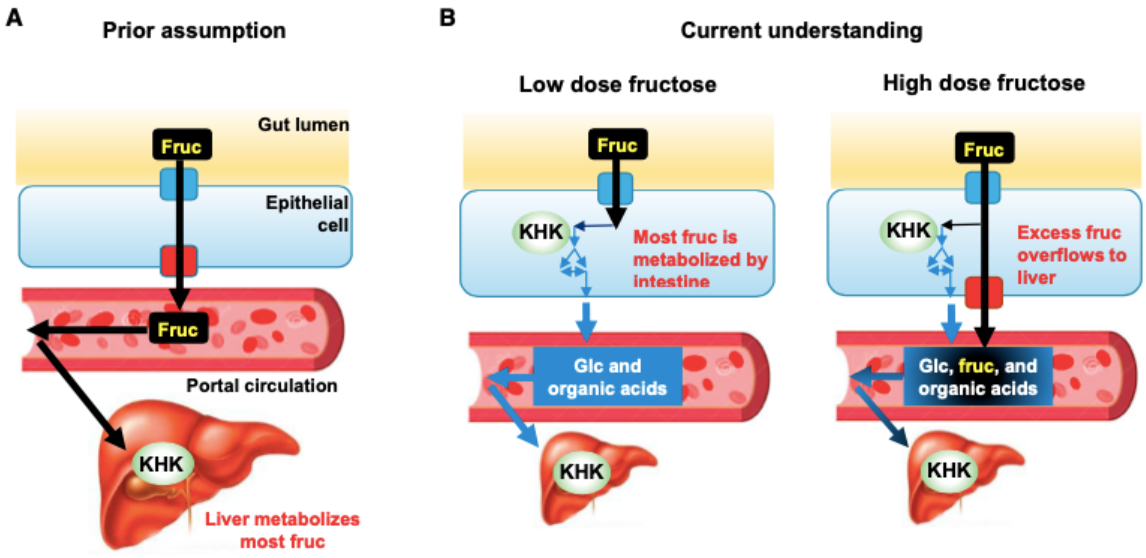


Figure 1. Prior and current understandings about fructose metabolism in the liver (Taken from Jang et al., 2018)

Intermediate metabolites of fructose degradation induce the activation of transcription factors such as Sterol Response Element Binding Protein 1 c (SREBP1c) and ChREBP, which in turn activate the transcription of genes involved in the lipogenic

pathway (Riveros et al., 2014; Softic et al., 2017). In studies carried out in rats fed a high-fructose diet, an increase in ChREBP and SREBP1 and genes related to lipid synthesis was observed, as well of steatosis in the liver (Koo et al., 2009). ChREBP activation is carried out by post-translational modifications regulated by fructose itself. Xylulose-5-phosphate (Xu-5-P) is an intermediate metabolite of the pentose phosphate pathway and can also be obtained from fructose. This metabolite activates protein phosphatase 2A (PP2A) which dephosphorylates the P1 (Ser196) and P3 (Thr666) sites of the transcription factor ChREBP allowing its translocation to the nucleus and binding to the ChoRE sites in the promoter (Lee and Cha, 2018).

Fructose consumption and de novo lipogenesis

Due to the current lifestyle, eating habits have changed by increasing the consumption of fat and sweets, which is related to the increasing incidence of NAFLD, positioning it as the most common chronic liver disease in the last 30 years (Mikolasevic et al., 2018). In studies carried out using mice as animal models, diets high in fat and fructose have been shown to promote insulin resistance, obesity, hypertriglyceridemia, liver steatosis, fibrosis, and the spontaneous generation of HCC (Dowman et al., 2014; Asgharpour, et al., 2016).

Consumption of high fructose diets has been linked to obesity and NAFLD (Ouyang et al., 2008; Alwahsh and Gebhardt, 2016; Horst and Serlie, 2017). Many authors argue that this is due to the rapid metabolism of fructose in hepatocytes since it avoids the main points of regulation of glycolysis, functioning as an unregulated source of intermediate metabolites for *de novo* lipid synthesis (Riveros et al., 2014; Li et al.,

2016). *De novo* lipogenesis is the process by which the cell synthetizes the fatty acids through Acetyl CoA. Aberrant lipogenesis has been related with tumor promotion and aggressiveness, and it is now recognized as a phenotype of the cancer cells (Calvisi et al., 2011). Studies carried out with cell lines (HepG2 and Huh-7) demonstrate the same lipogenic effect of fructose (Lanaspa et al., 2012; Li et al., 2016; Windemuller et al., 2016).

Transformed cells obtain many of the building blocks for the cellular macromolecules through this reprogramming. Fructose and its metabolites can reprogram cancer cells metabolism to enhance proliferation and growth. Fructose metabolism is associated with the expression of genes related to *de novo* lipogenesis. As I mentioned before fructose is related with ChREBP expression and translocation to the nucleus. Some of the genes that are implicated in the metabolism of fructose itself and lipids have ChoRE sites in the promoter (*glut-5, glut-2, khk, acly, acaca, fasn)* (Lee and Cha, 2018). Also, in a study conducted by DiStefano (2019), demonstrated that consumption of fructose is involved in the expression of genes related to lipid metabolism, enhancing expression of genes involved in the lipogenic route (*acaca, acly, fasn, chrebp*) and the repression of genes related to lipid oxidation (*cpt1a* and *fgf2*1).

The mTOR protein is also involved in lipid synthesis (Guri et al., 2017; Hindupur et al., 2018). Studies carried out with an mTOR-dependent model of HCC in mice reported that mTOR2 promotes the development of hepatosteatosis, liver damage and tumor development with an increase in fatty acid synthesis similar to that observed in patients with NAFLD who progress to HCC (Guri et al., 2017). Also, it is reported that histidine

phosphatase (LHPP) is a tumor suppressor that is regulated by the mTOR complex, so once mTOR is active, LHPP is silenced. Among the target molecules that have been proposed for this phosphatase is the enzyme ATP Citrate Liase (ACLY), related to the synthesis of fatty acids (Hindupur et al., 2018). Furthermore, it has been reported that mTOR2 can be activated by FASN and at the same time to activate AKT/mTOR pathway, classified as a tumor promoter pathway (Calvisi et al., 2011). FASN expression have been associated with carcinogenic processes, metastasis, proliferation, aberrant lipogenesis, and a poor prognosis in HCC patients (Calvisi et al., 2011; Li et al., 2015; Che et al., 2019).

Due to the high affinity of Ketohexokinase for fructose, there is a rapid decrease in the ATP levels of the cell, generating an excess of uric acid inside the hepatocyte, which leads to oxidative stress in the mitochondria through of NADPH oxidase 4 (NOX4) (Lanaspa et al., 2012). In a study carried out in the HepG2 cell line, the influence of uric acid in the decrease of the mitochondrial membrane potential was demonstrated, thereby affecting the electrochemical potential of the membrane and favoring the production of ROS and mitochondrial disfunction. On the other hand, the Aconitase enzyme, which was involved in the isomerization of citrate to isocitrate, is highly sensitive to the levels of ROS generated in the mitochondria, therefore it decreases its activity and leads to the accumulation of citrate in the mitochondrial matrix and the posterior lipid synthesis. The authors reported an increase in the synthesis and accumulation of lipids in HepG2 cells due to the incorporation of citrate into the lipogenic pathway (Lanaspa et al., 2012). Also, mitochondrial disfunction lead to the

increasement of Warburg effect in the cell to obtain ATP and building blocks to the main macromolecules of the cell (Nakagawa et al., 2020).

Lipid metabolism from carbohydrates in liver tumors can be regulated by various proteins and transcription factors (Lanaspa et al., 2012; Riveros et al., 2014; Softic et al., 2017; Guri et al., 2017; Hindupur et al., 2018). Studies carried out in mice fed diets high in fat and fructose, show similarities in the transcriptomic profile between the murine model with NAFLD and patients with this disease (Asgharpour et al., 2016); however, no information is available on the contribution of only fructose or only fats in this transcriptomic profile. Due to this and that there are no specific markers in the early diagnosis of this type of cancer, the relevance of our research is based on identifying the contribution of fructose in the progression and aggressiveness of HCC.

Justification

Recently, there has been a slight decrease in cancer-related death rates between 2012 and 2016 (1.8% and 1.4%, in men and women respectively); however, some types of cancer have increased their incidence, including cancer of the liver and intrahepatic bile ducts, with an increase rate of 1.1% and 1.9%, in men and women, respectively (https://seer.cancer.gov/report_to_nation/statistics.html). NAFLD is one of the most important risk factors for the development of HCC, and it has been classified as the most common chronic liver disease in the last 30 years, being one of the main causes for liver transplantation (Mikolasevic et al., 2018). HCC comprises between 75% - 85% of reported cases of liver cancer (Bray et al., 2018). Around 841,000 cases and 782,000

deaths are reported annually worldwide, which is why, according to these data, it ranks sixth in terms of incidence and constitutes the fourth cause of cancer-related death (Bray et al., 2018). Liver cancer is third cause of cancer-related death in Mexico, with a total of 6,868 deaths reported in 2018 (https://gco.iarc.fr/today/data/factsheets/populations/484-mexico-fact-sheets.pdf Consulted: 15/November/2020). The high mortality rate reported in this type of cancer is due to the high price of existing treatments and the failure of the HCC detection in early stages of the disease. That is why one of the scientists' main objective is to find new tools to the early diagnosis and prognosis of the disease.

The high fructose diet is related to the increase in lipogenesis, liver steatosis and the development of NAFLD (Ouyang et al., 2008; Alwahsh and Gebhardt, 2016; Horst and Serlie, 2017), and with it the progression towards HCC (Li et al., 2016). The consumption of ultra-processed sugary foods has increased and the relation between its consumption and cancer incidence has been proved (Fiolet et al, 2018). Particularly in Mexico, the consumption of added sugars is high and the demand and consumption of sugary drinks continues to increase, being one of the 5 countries with the highest consumption of soft drinks (https://www.statista.com/outlook/20020100/100/carbonated-soft-drinks/worldwide Consulted: 03/07/2020). Therefore, the high consumption of fructose in the diet constitutes an important risk factor for the HCC progression and aggressiveness.

Research question

How does a high fructose diet influence the progression of hepatocellular carcinoma?

Hypothesis

Western diet has a high composition of carbohydrates, among which are artificial sweeteners or sugars. The most widely used sweetener due to price accessibility is Fructose in the form of High Fructose Corn Syrup (HFCS) and it is related with the Non-Alcoholic Fatty Liver Disease (NAFLD). So, a high consumption of fructose in the diet can enhances the HCC aggressiveness.

Objectives

General Objective

- To determine the effect of a diet high in fructose on the progression of hepatocellular carcinoma.

Specific objectives

1. Determine the effect of fructose (33% content) on proliferation, metabolism and tumorigenesis using the HCC cell line (Huh-7) as a model.
2. To determine the effect of the high fructose diet (33% content) on hepatocellular carcinoma by cellular and molecular approaches in C57BL6j mice.

Materials and Methods

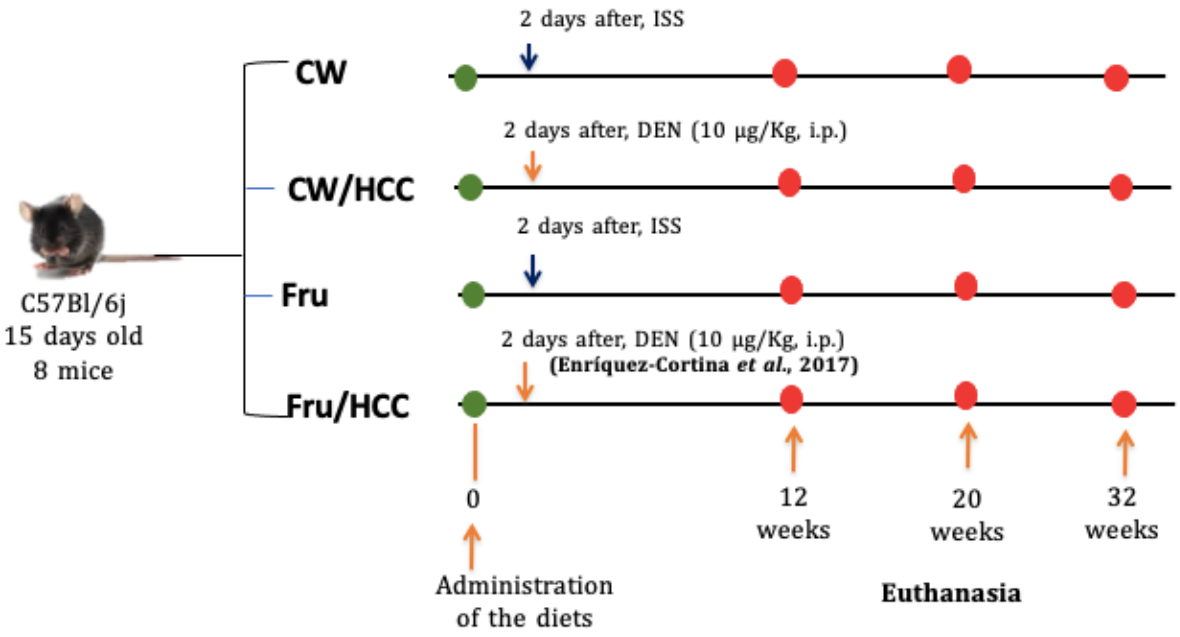
In vivo experimental model

In the current study we used 96 C57Bl/6j mice strain (males and females) with 15 days old and they were kept with the mother until 21 days when weaning was performed. This mice strain is one of the most frequently used in the investigation of NASH and hepatocarcinogenic processes (Takami et al., 2007). The strain has some favorable characteristics to the study: to have a good reproduction, being long-lived and little susceptible to the generation of tumors; however, the expression of spontaneous or induced mutations is possible, that is why once the tumors are generated in our model, their aggressiveness is guaranteed. Furthermore, the complete genome of the strain is sequenced, which allows us to carry out transcriptomic studies (https://www.jax.org/strain/000664#jump-nav-2). The mice were obtained from the National Institute of Cardiology Ignacio Chávez´s animal housing. The mice were kept in a temperature range of 20°C - 25°C, humidity between 40% - 70% and a light/dark cycle of 12/12 hours. The experimental protocol was prepared following the NIH Guide for the care and use of laboratory animals and NOM-062-ZOO-1999 and was presented to the Committee for the Care and Use of Laboratory Animals (CICUAL) of the National Institute of Cardiology Ignacio Chávez.

To study the effect of fructose on HCC, we used a carcinogenic model induction through intraperitoneally injection of mice with N-Diethylnitrosamine (10 µg/Kg, i.p) (DEN, Sigma-Aldrich, St. Louis, MO, USA) (Enríquez-Cortina et al., 2017). This is a carcinogen metabolized by Cytochrome P450 2E1 (CYP2E1) and whose

biotransformation products cause DNA damage, death of hepatocytes, and the proliferation of those hepatocytes that contain the mutations, which gives rise to HCC (Tolba et al., 2015). Mice were divided in four groups of treatments (Fig. 2):

- **Group CW** Normal diet (Chow 5001) treated with isotonic saline solution (ISS) (24 mice).
- **Group CW/HCC** Normal diet treated with DEN (10 µg/Kg, i.p) in ISS (24 mice).
- **Group Fru** Normal diet with fructose 33% ad libitum in drinking water and treated with ISS (24 mice).
- **Group Fru/HCC** Normal diet with fructose 33% ad libitum in drinking water and treated with DEN (10 µg/Kg, i.p) in ISS (24 mice).



The diet was obtained commercially (CHOW 5001).

Figure 2. In vivo experimental model to determine the effects of a high fructose diet in HCC progression.

The Fructose (Sigma-Aldrich, St. Louis, MO, USA) was added *ad libitum* to the drinking water of the treatment groups of mice **Fru** and **Fru/HCC** with 15 days old. The treatment with DEN started two days after the diets and it was dissolved in ISS (unique dose). The control group received only ISS as previous reports (Takami et al., 2007; Enríquez-Cortina et al., 2017).

The mice were euthanized at 12, 20 and 32 weeks after the beginning of the treatments. To develop the procedure was used Avertin (2,2,2- Tribromoethanol) (2%, Sigma-Aldrich, St. Louis, MO, USA) injected intraperitoneally. Then a V-shaped incision was made in the abdominal region to recover the organs: heart, liver, pancreas, spleen, intestine (duodenum and jejunum), lungs, kidneys and gallbladder, which were weighed and made a photographic record. Blood samples were also taken to analyze serum biochemical parameters. The number of lesions or tumors larger than 1 mm were counted, then fragments of the tumors as well as the surrounding tissue were frozen. A part of the organs was fixed in Formalin 10% to perform histology and immunohistochemistry studies, and the other part were frozen at -80^{o}C for RNA isolation and processing for qRTPCR in posterior studies.

Dual-energy X-ray Absorptiometry

To determinate the body composition, we evaluated the bone, fat and fat free bone free mass (FFBF) (muscle) using Dual-energy X-ray Absorptiometry (DXA). To develop this assay, we used the DXA scan (Serie Discovery QDR, Hologic ® Discovery) with the small species configuration. The mice were drugged with Avertin by intraperitoneal injection and were positioned with their limbs extended on an acrylic plate. The total fat

and total bone mass were obtained by a densitometric analysis (with emphasis on the abdominal region). To obtain the total fat free bone free mass (FFBF) was required to make a differential between the total body mass and the bone and fat composition. For the latter case, bone and fat free mass determinations had an emphasis on the mice´s limbs.

Serum biochemical profile

Once the animals were under the anesthesia, blood was recovered from the orbital venous plexus. The samples were centrifuged at 3000 rpm for 10 min at 4^{o}C. Serum biochemical parameters were measured: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total serum cholesterol (T-Cho) and Triglycerides (TG) using the SpotChem EZ automated analyzer (ARKRAY, USA Clinical Diagnostics).

Histology

Liver left lobe sections was fixed in formalin solution (37%) at least for 48 hours. The immersion in paraffin was performed following the next instructions:

- Dehydrate in increasing concentrations of ethanol (70%, 96% and 100%) for 1h each one.
- Clear in ethanol – xylene (1:1) and two times in to changes of xylene for 1h.
- Paraffin embedding: three times in three changes of paraffin for 1h the first two and the last one 30 min. Each block was cut in sections of 3 micron with a microtome.

To develop Hematoxylin – Eosin (H&E) staining, we followed the next protocol:

- De-wax with heat (10 – 15 min at 60ºC) and wash with xylene over 5 min.
- Rehydrate in decreasing concentrations of ethanol (100%, 96% and 70%) for 5 min each one.
- Wash with distilled water
- Pass to Hematoxylin solution for 4 min.
- Wash again with distilled water and added acid ethanol (1%) for 2 seg
- Wash with distilled water
- Immerse the slides in Eosin 10 times
- Immerse the slides once in ethanol (96%), absolute ethanol, ethanol-xylene (1:1) and xylene solutions, and mount in a resinous medium.

To perform Sirius Red staining, we followed the next protocol:

- De-wax with heat (10 – 15 min at 60ºC) and wash with xylene over 5 min.
- Rehydrate in decreasing concentrations of ethanol (100%, 96% and 70%) for 5 min.
- Wash with distilled water
- Stain nuclei with Weigert´s hematoxylin for 8 min, and then wash the slides for 10 min with water.
- Stain in pico-sirius red for 1h
- Wash in two changes of acidified water
- Remove most of the water from the slides
- Dehydrate in three changes of ethanol (100%)
- Clear in xylene and mount in a resinous medium

Protein extraction and Immunoblotting

To carry out the immunoblotting assay, 30 mg of liver tissue were homogenized in 500 μL of PBS. The cellular pellet was resuspended in 200 – 250 μL on lysis buffer (T-Per supplemented with proteases and phosphatases inhibitors). Lysis was performed on ice for 30 min with vortex every 5 min. At the end, cell debris were removed with centrifugation at 13 000 rpm for 10 min and supernatant was recovered. Total protein in the liver samples was quantified using bicinchoninic acid kit mixing BCA (reactive A) and $CuSO_4 \cdot 5H_2O$ (reactive B) in the following composition:

- (**X**) x 200 μL of Master Mix= Total Volume (μL). (**X**): Number of wells

Master Mix:

- 1/51 x (**X**)= Volume of reactive B (μL)
- 50/51 x (**X**)= Volume of reactive A (μL)

The plate was incubated for 30 min at 37ºC and read at 595nm.

The protein samples were analyzed by SDS – PAGE using 10% gel. The samples (100 μg) were previously treated with β-mercaptoethanol and then were heated 7 min at 95ºC. The electrophoretic run was done at 120 V at room temperature. Proteins were transferred to a PVDF membrane (Sigma-Aldrich, St. Louis, MO, USA) at 120 V for 2h at 4ºC. The membrane was blocked with fat free milk (1%) dissolved in TBS – Tween for 1h. Immunoblotting was performed using the next antibodies: FASN (Cell Signaling, 3181S, Rabbit), ACLY (Abcam, ab40793, Rabbit), HMGCoAR (Abcam, Ab174830,

Rabbit), MVK (Santa Cruz, sc-390669, Mouse), SQS (Santa Cruz, sc-365101, Mouse), β-actin (Sigma, A2066).

Triglycerides determination in liver tissue

For triglycerides (TG) determination, we used 100 mg of liver tissue. The samples were homogenized in 1 mL solution of 5% TRITON – X100 and slowly heated at 80 – 100°C in water bath for 2 – 5 min until the TRITON – X100 becomes cloudy. The samples were cooled in room temperature and then centrifugated for 2 min at top speed to remove insoluble material. We did a 10 – fold dilution with ultrapure water before assay. To develop the assay, we used the Triglyceride Quantification Kit (Sigma-Aldrich, St. Louis, MO, USA) and followed the manufacturer´s instructions.

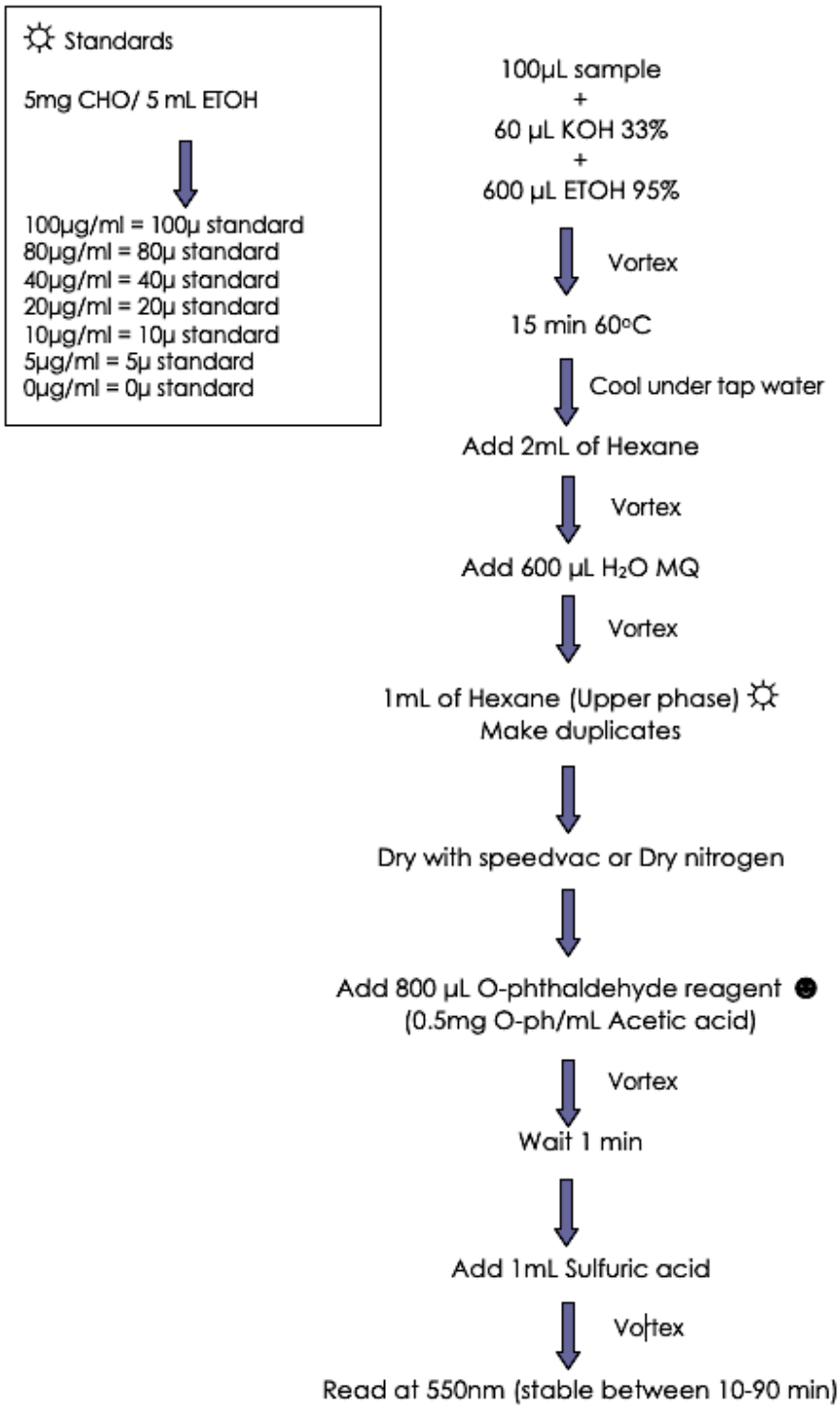
Cholesterol determination in liver tissue using O-phthaldehyde method

For total cholesterol (CHO) determination we used a homogenate of 30 mg of liver tissue dissolved in PBS, and 100 μL of the sample were separated to carry out protein quantification. We used a cholesterol control curve, prepared a stock solution (5 mg of CHO/ 5 mL of ethanol) and we used the next final concentration in each point: 100 μg/mL, 80 μg/mL, 40 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL and 0 μg/mL. Also, we used an O-phthaldehyde (O-ph) solution (25 mg of O-ph with 50 mL of acetic acid and protect from light).

Then, 100 μL of the sample were mixed with 60 μL of KOH (33 %) and 600 μL of ethanol (95 %). The mix were stirred and left for 15 minutes at 60°C. Later, the mixtures were cooled with tap water, 2 mL of Hexane were added to each one and stirred. After

vortexing the mixtures, we added 600 μl of H_2O, vortex again and took 1 mL of the upper phase. The samples were dried with vacuum centrifugation (Speedvac) for 1h and later we added 800 μL of O-ph reagent. We vortexed the samples, incubate 1 minute (take care of the light). Finally, 1 mL of Sulfuric acid was added. The samples were vortexed, incubated for 10 minutes and read at 550 nm.

To continue the assay, we followed the next steps (Fig. 3):



● 25mg of O-phthaldehyde + 50mL of Acetic acid. **Protect from light**

Figure 3. procedure for cholesterol determination assay using the O-phthaldehyde method.

In vitro experimental model

The Huh-7 cell line was used, which is a tumorigenic cell line of epithelial-type differentiated HCC hepatocytes with the TP53 gene mutated. This line was obtained from a liver tumor from a 57-year-old Japanese patient in 1982 (https://huh7.com/). The cell line was obtained from the American Cell Culture Collection (ATCC) and was cultured in Williams E medium (Appendix 1: Composition) supplemented with 10% fetal bovine serum (FBS, Hy-Clone, Logan, UT, USA), 100 U/mL ampicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), maintained at 37ºC, 5% CO_2 and 90% atmospheric humidity. The tests were carried out with Treatment medium (Williams medium without Fetal Bovine Serum – FBS).

Three treatment groups were applied at different concentrations, both Glucose (Glc, Sigma-Aldrich, St. Louis, MO, USA), Fructose (Fru, Sigma-Aldrich, St. Louis, MO, USA), and an isomolar combination of Fructose: Glucose (Fru: Glc) according to Windemuller et al. (2016) (Table 1). Three application times of the treatments was established: 24h, 48h and 72h (Fig. 4).

Table 1. Concentration of the different treatments used in the in vitro experimental model.

Concentrations	Glucose (Glc)	Fructose (Fru)	Fructose:Glucose (Fru:Glc)
1	0.65 mM	0.65 mM	0.58 mM:0.14 mM
2	0.68 mM	0.68 mM	0.61 mM:0.11 mM
3	0.72 mM	0.72 mM	0.67 mM:0.05 mM

Figure 4. In vitro experimental model to determine the effects of a fructose in proliferation, viability and functionality in Huh-7 cell line.

Proliferation assay

The cell proliferation assay was carried out using the cell counting kit -8 (CCK-8, Dojindo Lab, Kumamoto, Japan), following the manufacturer's instructions. To develop the assay, 10,000 cells were seeded per well using 96-well plates and we used the treatments whit Glc, Fru and Fru:Glc at 24h, 48h and 72h.

Viability assay

The viability test was carried out by staining the cells with Crystal Violet (Sigma-Aldrich, Saint Louis, MO, USA) and 10,000 cells were seeded per well using 96-well plates following the previously reported protocol by Gerardo-Ramírez et al. (2019). Briefly, cells were allowed to proliferate for the established times, then the medium was removed and washed with 50 µL of PBS. Subsequently they were fixed with 100 µL of cold methanol for 10 min. After this time, remove the methanol and add 100 µL of Crystal Violet solution for 20 min. The plates were washed to remove the excess Crystal and they were left to dry and then carried out the dye extraction. For this, 100 µL of 2%

Sodium Dodecyl Sulfate (SDS) weas used in each well and washed for 20 min. The plates were then centrifuged at 4500 rpm for 5 min, 50 µL of the supernatant were recovered to read at 620nm. The optical density value of the cells that received no treatment was taken as 100% viability.

Cellular functionality assay

Cellular functionality was performed using a commercial mitochondrial functionality assay (Vybrant MTT Cell Proliferation Assay) (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's directions.

Spheroid formation assay

The assay was performed by seeding 1000 cells per well in low-adhesion plates (Millipore-Sigma, Saint Louis MO, USA). The treatments (Fru) were applied 4h after the cells are seeded and were left for 6 days without Fetal Bovine Serum (FBS). The spheroids were counted and photographed using the Carl Zeiss VERT.A1 microscope.

Clonogenicity assay

The test was carried out by sowing 1000 cells per well in high adherence plates of six wells, the treatments were applied and were left for 6 days (Fru) without FBS. Subsequently the cells were stained with Violet Crystal, counted and photographed.

Statistical analysis

All the tests were carried out at least in triplicate, and the data was presented as the mean ± SD. Normality and variance homogeneity tests were carried out to verify the data comply with a normal distribution. The t-Student test was performed for

comparison between means, two-way ANOVA to compare between treatment groups and the Tukey-Kramer test. The Graphpad Prism 8.2.1 Software for Mac Os X, GraphPad Software, San Diego, California USA, www.graphpad.com was used for data processing and a significance value $p \leq 0.01$ was used.

Results

1. Gross inspection

1.1. Fructose induces adipose tissue accumulation

In order to determine the effect of the high fructose diet *in vivo*, the mice of the C57Bl/6j strain were given water *ad libitum* with 33% fructose. At 32 weeks of treatment, it was decided to analyze the body composition of the mice in the **CW** and **Fru** groups by means of dual energy X-ray absorptiometry (DXA) examination. It was found that the mice that had a diet supplemented with Fructose showed a significantly higher percentage of adipose tissue (13.1% ± 2.4) compared to the **CW** control group (6.7% ± 2). Regarding the percentage of muscle tissue, significant differences were also obtained, since the **Fru** group showed lower muscle mass (83.2% ± 2.5) compared to the **CW** (88.3 ± 2.2) (Table 2 and Fig. 5); however, the percentage of bone tissue did not vary, which was indicating that fructose was inducing an increase in adipose tissue in these mice.

Table 2. Average of the parameters measured in the DXA analysis in the 32-week-old C57Bl/6j mice.

	%fat	SD	%bone	SD	%muscle	SD
Control	6.7	2.0	3.4	0.2	88.3	2.2
Fructose	13.1	2.4	3.7	0.2	83.2	2.5

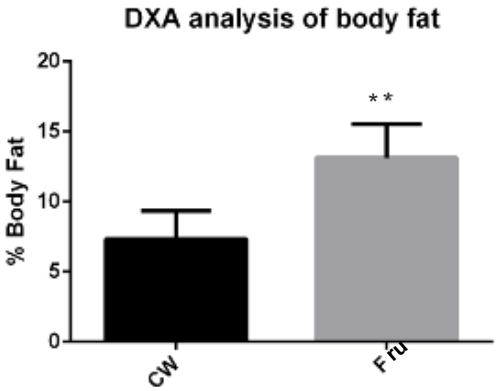


Figure 5. Analysis of the body composition of C57Bl/6j mice with 32 weeks of treatment. (A) Adipose tissue mass. ***(** p≤ 0.01 vs. CW)***

1.2. Fructose consumption induces changes in the liver

Euthanasia was carried out at 12, 20 and 32 weeks. The macroscopic inspection of the recovered organs of the mice sacrificed at 12 weeks showed significant differences regarding the weight of the liver, and it was obtained that the mice with a diet supplemented with Fructose (**Fru** and **Fru/HCC**) had a lower liver; however, no differences were observed regarding the weight of the animals. The differences observed were reflected in the ratio of Liver Weight and Mouse Weight, with the mice of the **Fru** group being the ones that showed smaller liver size compared to the control; however, the liver of **Fru/HCC** mice showed larger size compared to the **Fru** group (Fig. 6A).

At 20 weeks of treatment, no differences were obtained between the groups (Liver weight, Body weight and Liver/Body weight) (Fig. 6B). However, 12 weeks later the groups showed significant differences. Regarding the weight of the liver, it was obtained that only the mice of the **Fru** group showed significant differences compared

to the control with a greater weight. Likewise, regarding the weight of the mouse, the groups to which fructose was supplied in the diet had a higher weight. When performing the quotient analysis, it was observed that the **Fru** group was the one with the largest organ size, indicating that the fructose treatment alone at 32 weeks of treatment was inducing liver damage (Fig. 6C).

Morphological changes in the liver were also observed between the different treatment groups. After 20 weeks of treatment, vascularized and rigid livers were observed in some mice of the **Fru/HCC** group. This pattern was observed at 32 weeks in the caudate lobe, with a more marked effect in the group supplemented with Fructose, with tumors that occupied the entire lobe were observed. Likewise, adhesions between the lobes were observed in the **Fru/HCC** group (Fig. 7). Furthermore, the liver and kidneys of the mice of the **Fru** group had a paler coloration compared to the other experimental groups.

Regarding adipose tissue, it had previously been observed that the **Fru** group had a higher percentage of adipose tissue, which was verified at the time of euthanasia. Fat accumulation was observed in the abdominal region and around the heart, being accentuated in the **Fru** and **Fru/HCC** groups with respect to the **CW** and **CW/HCC** at 32 weeks of treatment.

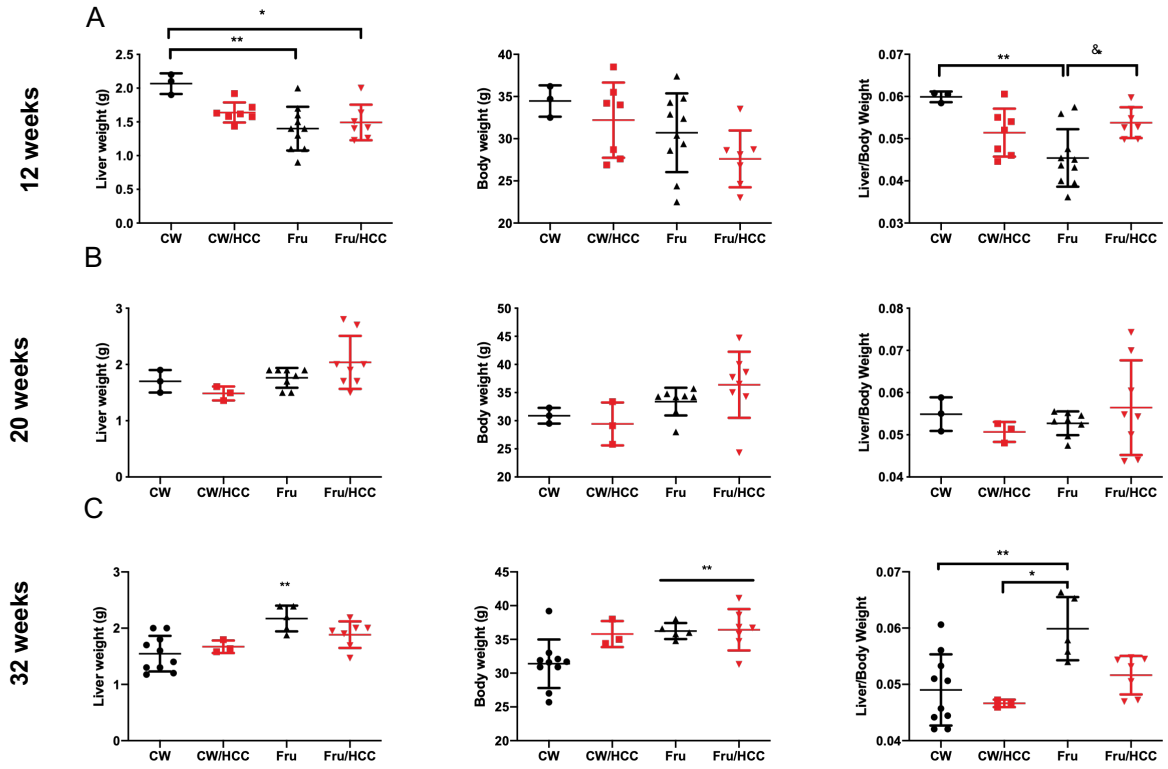
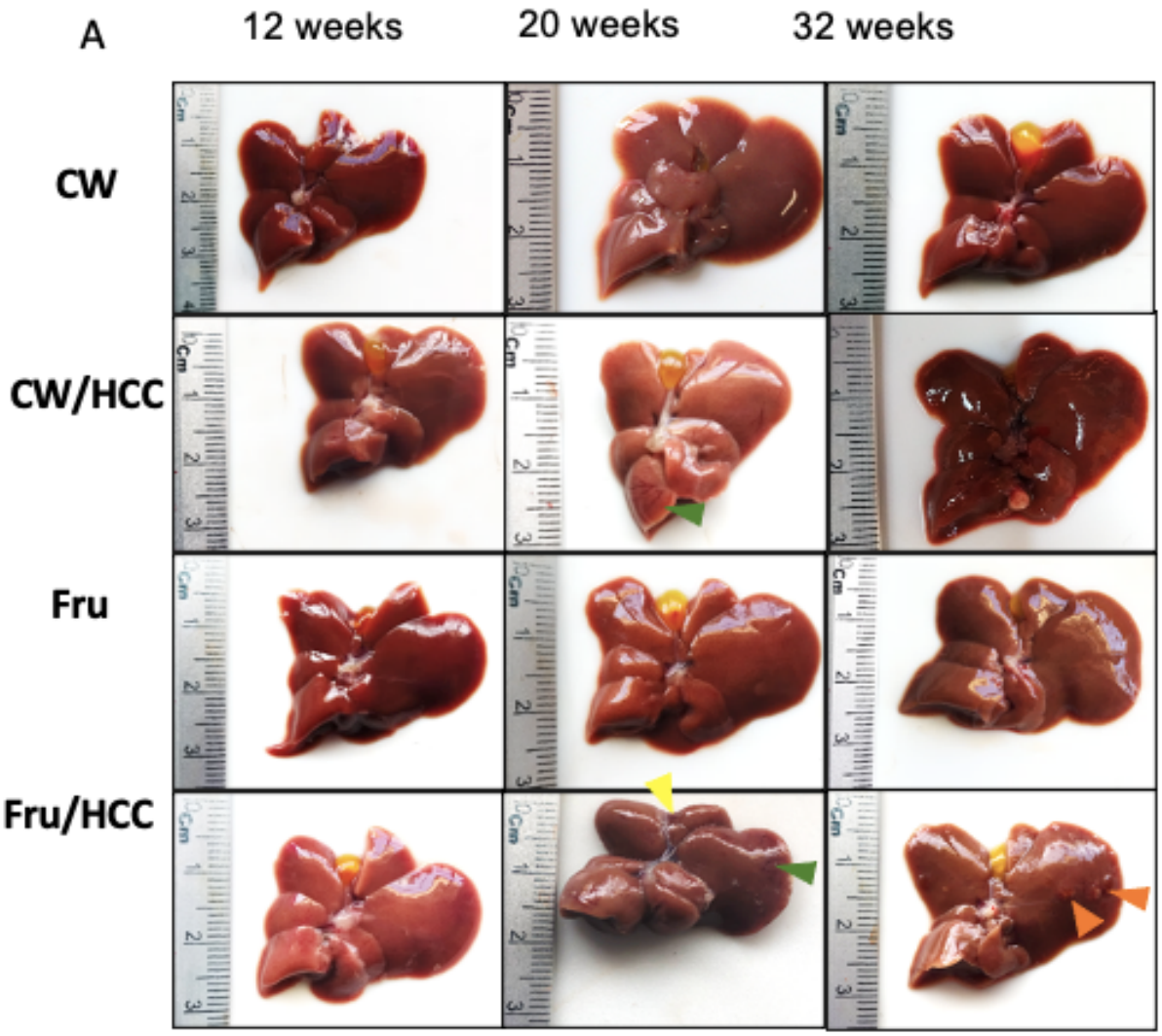


Figure 6. The fructose does not influence the weight of the body and the liver of C57Bl/6j mice strain. (A) Liver weight, Mouse weight and Liver / Mouse weight at 12 weeks of treatment; (B) Liver weight, Mouse weight and Liver / Mouse weight at 20 weeks of treatment; (C) Liver weight, Mouse weight and Liver / Mouse weight at 32 weeks of treatment. ***(* p≤ 0.05 vs. CW) (** p≤ 0.01 vs. CW) (& p≤ 0.05 vs. Fru).***


A
12 weeks
20 weeks
32 weeks
CW
CW/HCC
Fru
Fru/HCC

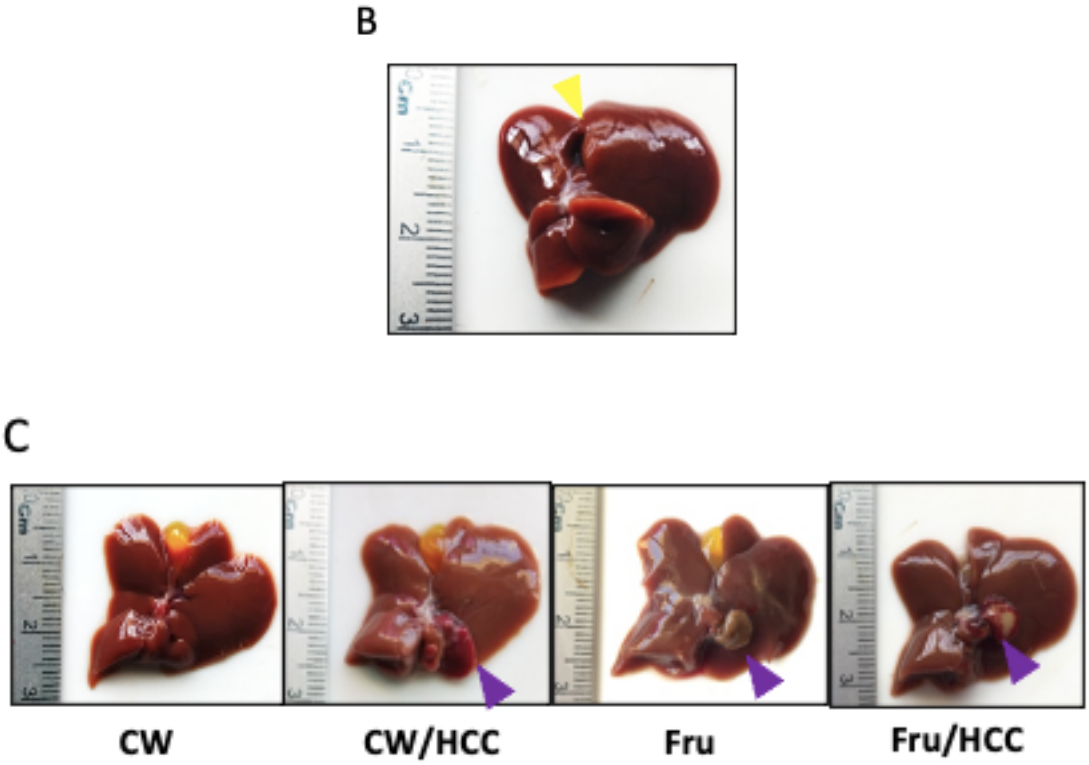


Figure 7. Macroscopic inspection of tissue from C57Bl / 6j mice sacrificed at 12, 20 and 32 weeks of treatment. (A) Macroscopic view of the liver at 12, 20 and 32 weeks of treatment; (B) Adhesions between mouse lobes from 20 weeks of treatment from the Fru / HCC group; (C) Rigid and vascularized mouse caudate lobe from 32 weeks of treatment. Yellow arrowhead (●): adhesion between lobes; green arrowhead (●): Vascularization; violet arrowhead (●): rigid and vascularized caudate lobe; red arrowhead (●): tumors.

1.3. Fructose consumption enhance tumor progression in the liver and other organs

Then, the presence and number of tumors were examined macroscopically. The livers showed tumors up to 32 weeks of treatment in the **CW/HCC** and **Fru/HCC** groups. The **Fru/HCC** group was the one with the highest number of tumors (Fig. 7A and Fig. 8A).

Nodules were also found in the lungs from 12 weeks of treatment in the **CW/HCC** and **Fru/HCC** groups, and up to 32 weeks of treatment in the **Fru** group (Fig. 8B and Fig. 9). This type of nodules had not appeared in the different diets previously applied by Simoni-Nieves, MSc., Nor even in the group with the Western + DEN diet (**W/HCC**). The **Fru/HCC** group had the highest number of nodules in the lungs. Furthermore, tumors were also found only in the females of the **Fru** and **Fru/HCC** groups near the fore and hind legs (Fig. 10).

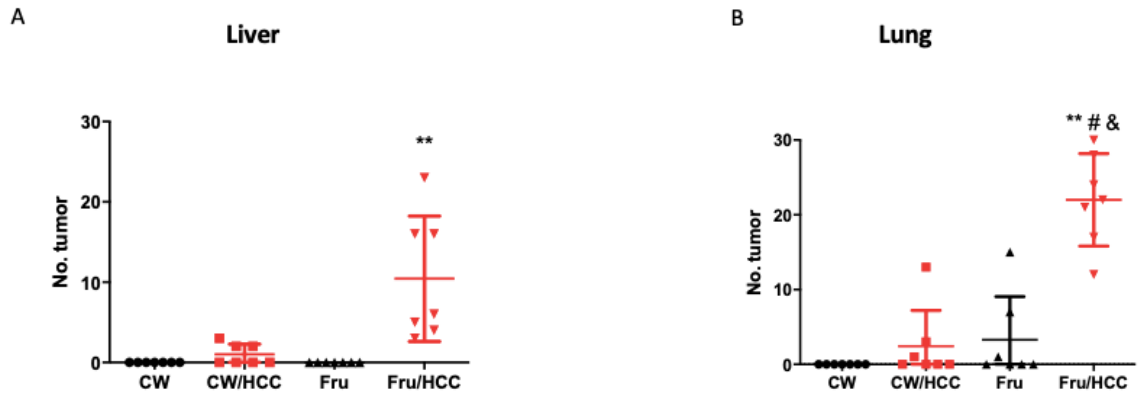


Figure 8. Fructose influences the number of tumors. (A) Number of tumors in the liver of mice with 32 weeks of treatment; (B) Number of nodules in the lungs of mice with 32 weeks of treatment. ***(** p≤ 0.01 vs. CW); (# p≤ 0.01 vs. CW / HCC); (& p≤ 0.01 vs. Fru).***

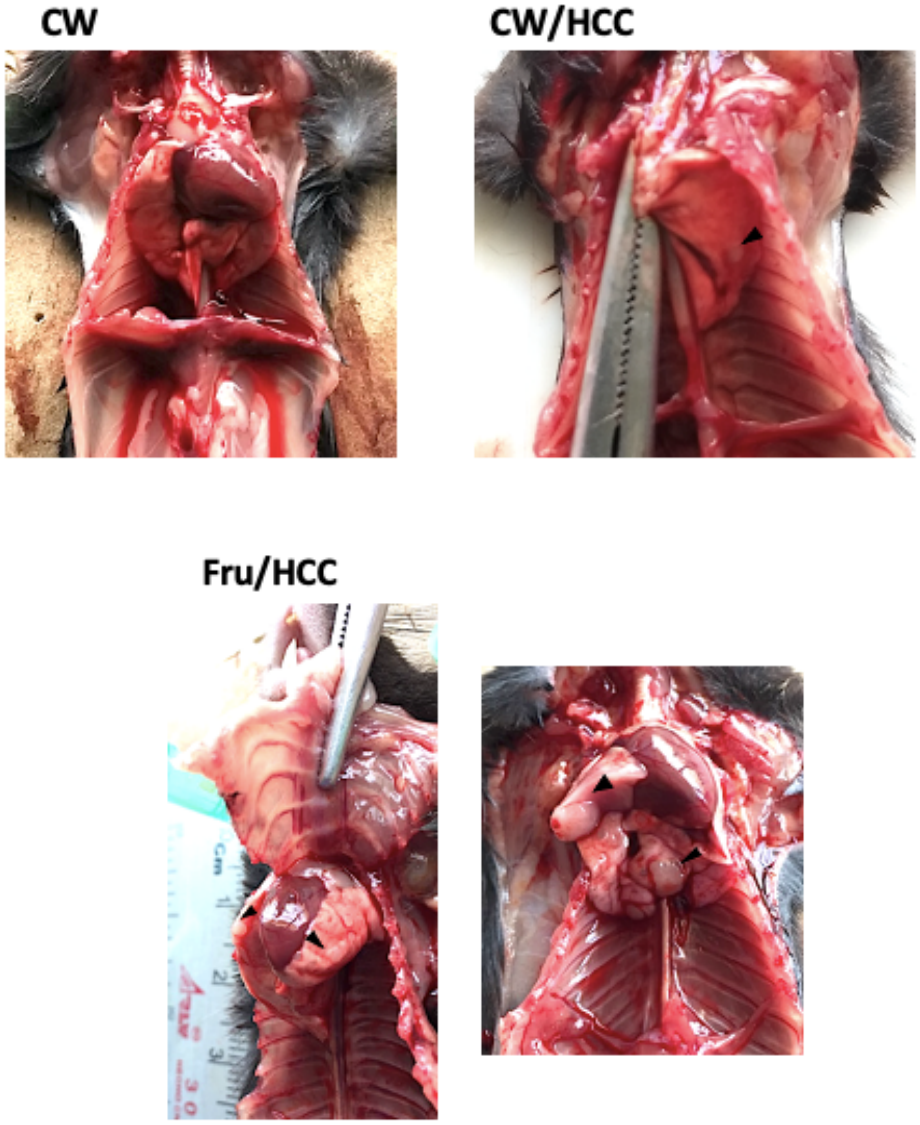
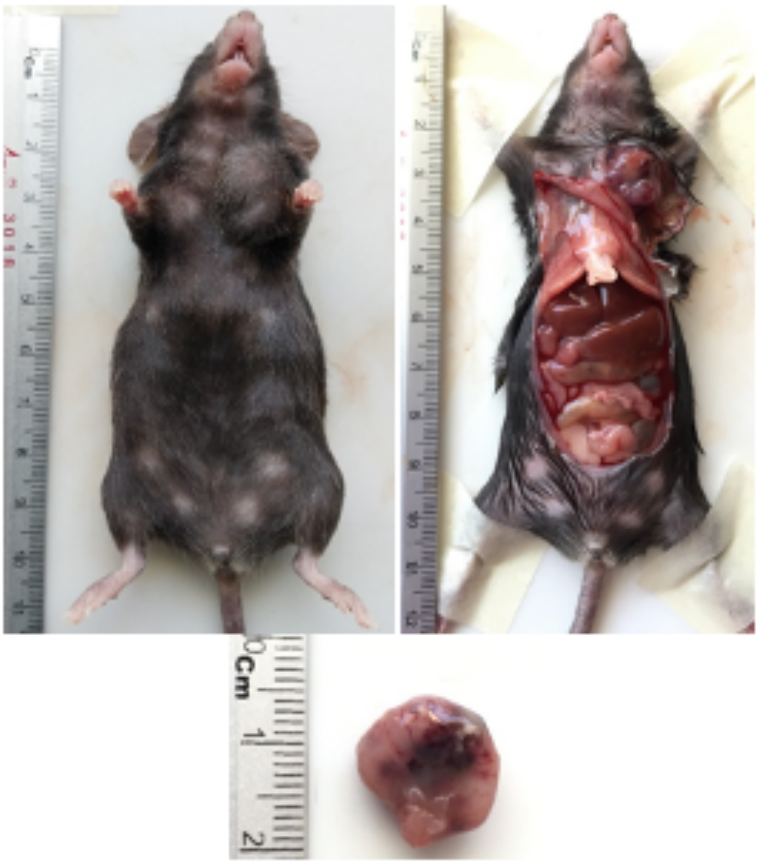


Figure 9. Fructose influences the generation of nodules in the lungs. CW mouse normal lung with 32 weeks of treatment; CW / HCC mouse lung with 32 weeks of treatment; Fru / HCC mouse lung with 32 weeks of treatment. ***Black arrowhead****: nodules in the lungs.*

A **Fru**



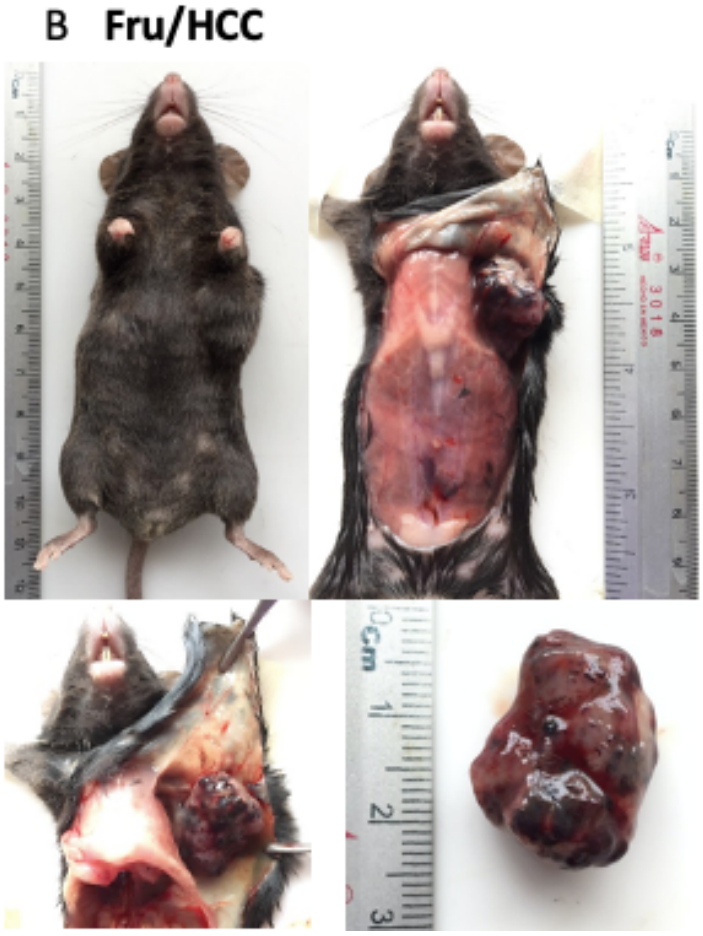


*Figure 10. Tumors in the fore and hind legs of female mice treated with the high fructose diet. (A) Tumor in the foreleg of a female mouse from the **Fru** group with 32 weeks of treatment; (B) Tumor in the foreleg of a female mouse from the **Fru/HCC** group with 32 weeks of treatment.*

1.4. Fructose consumption enhances liver fat accumulation and inflammatory infiltrate

Subsequently, the liver tissue samples were evaluated using the H&E technique. It was observed that the mice with a diet supplemented with fructose presented fat accumulation in the hepatocytes and also areas with a greater inflammatory infiltrate (**Fru** group), which was not observed in the mice that were on a control diet (**CW** and **CW/HCC**). Mice in the **Fru/HCC** group showed a marked difference between the tumor

area and the surrounding tissue, in addition to having an increase in the number of bile ducts which was indicating liver tissue damage (Fig. 11).

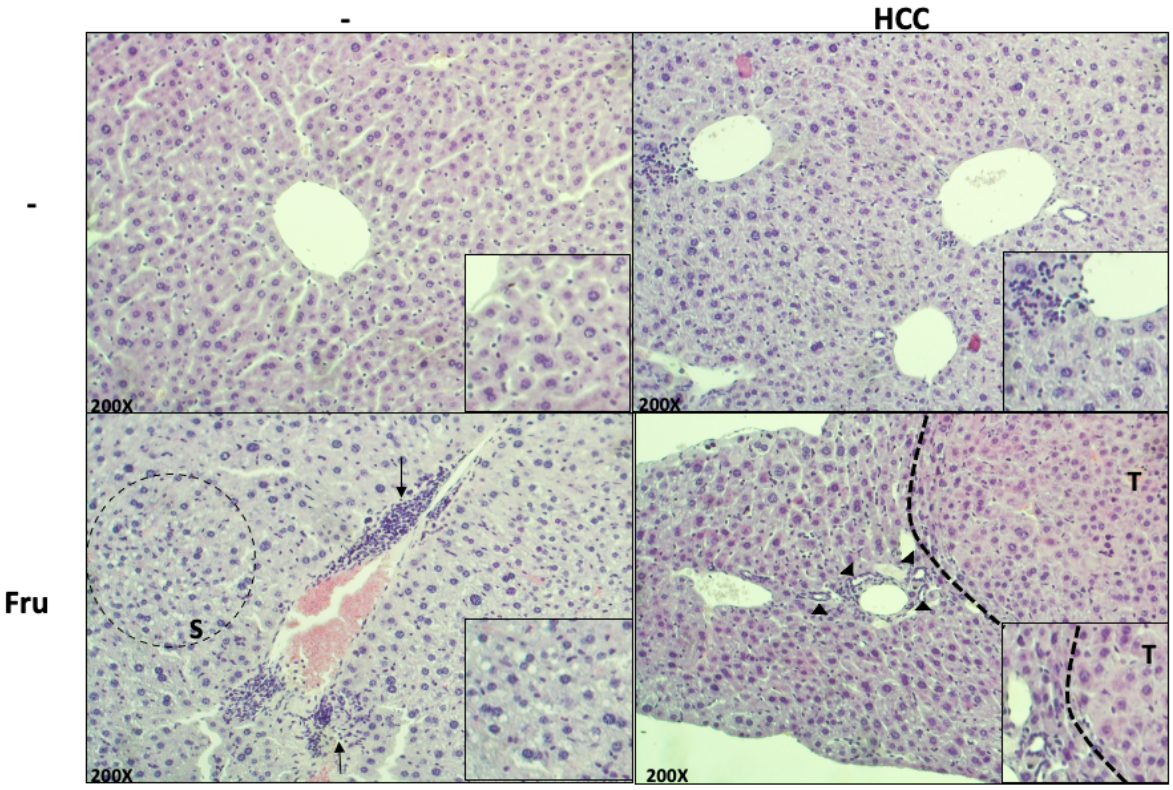


Figure 11. Histological analysis of the livers of C57B1/6j mice at 32 weeks of treatment. ***S****: steatosis;* ***Black arrow****: Inflammatory infiltrate;* ***T****: Tumor Zone;* ***Black arrowhead****: bile ducts. (200X)*

1.5. Fructose consumption causes changes in serum lipids composition

Once the serum was obtained, the biochemical profile was analyzed at 32 weeks of treatment. The levels of the transaminase enzymes (AST and ALT) were no detectable most of the mice treated. Then the lipid composition of the serum was analyzed. In the case of TG, serum levels were not significative different between treatments (Fig. 12A). Regarding serum cholesterol (CHO), significant differences were found between the

experimental groups when total Cholesterol (T-Cho) levels were measured, being the **Fru** and **Fru/HCC** groups the ones with the highest serum cholesterol levels (Fig. 12B).

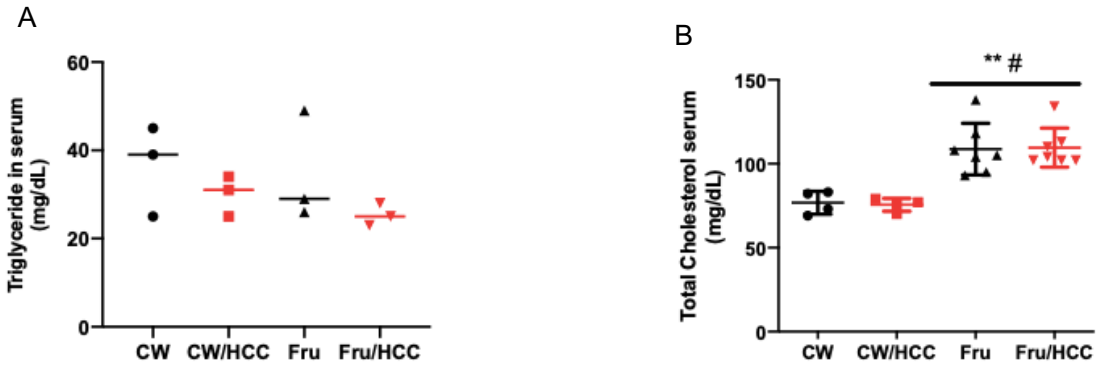


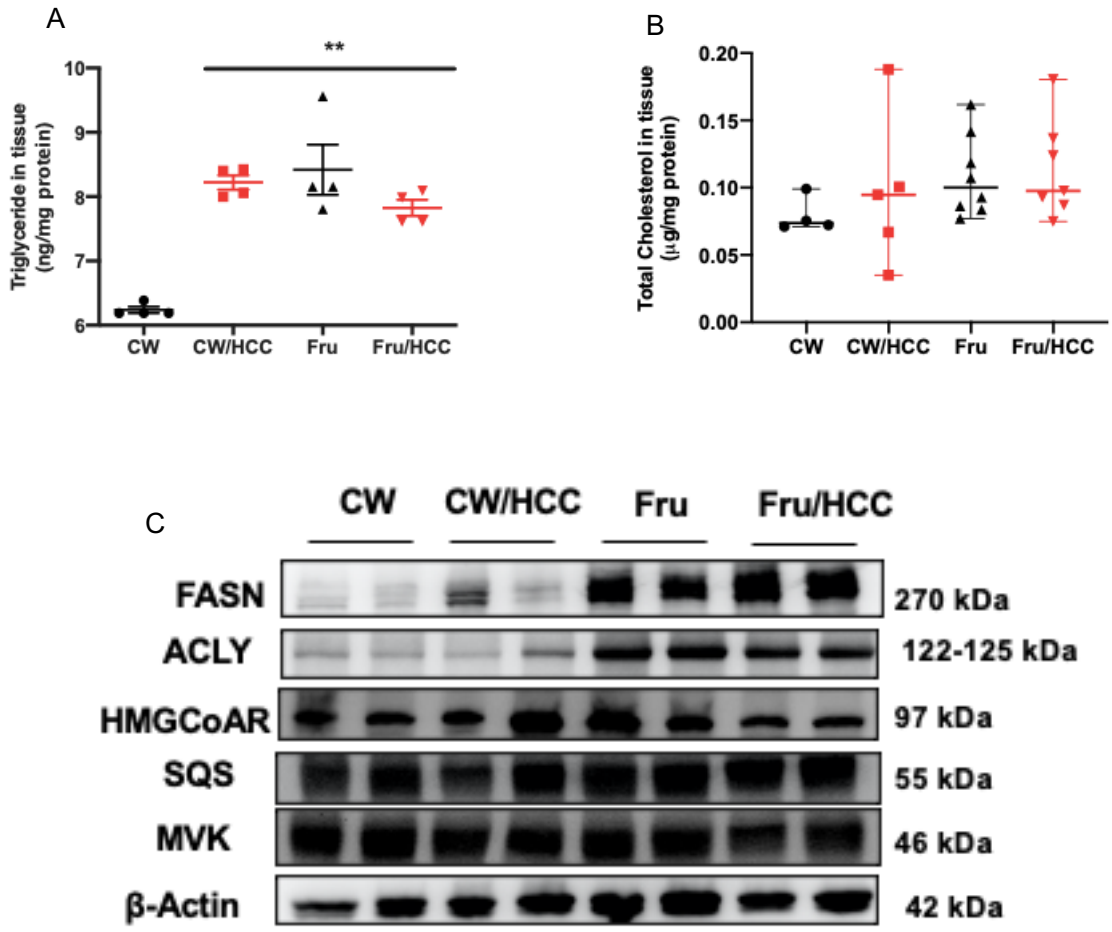
Figure 12. Biochemical profile of the serum of mice with 32 weeks of treatment. (A) Triglyceride content in serum (B) Total cholesterol content in serum. ***(** p≤ 0.01 vs. CW) (# p≤ 0.01 vs. CW/HCC)***

2. Fructose consumption increases the protein content of fatty acids-related enzymes.

Subsequently, the content of both TG and CHO in the liver of mice with 32 weeks of treatment was analyzed. In the case of TG, the triglyceride kit (SIGMA) was used to quantify it, and the opposite was observed to the results obtained in serum. The TG level was significantly higher in the **CW/HCC**, **Fru** and **Fru/HCC** groups. However, the CHO levels were quantified by means of the O-phthalaldehyde technique, and no significant changes were observed between the different treatments (Fig. 13A and B).

To corroborate these results obtained in the tissue, it was decided to evaluate the protein content of some enzymes related to the mevalonate and fatty acid synthesis

pathways and the following proteins were evaluated: Fatty Acid Synthase (FASN), ATP-Citrate Lyase (ACLY), 3-Hydroxy-3-Methyl-Glutaryl-CoA reductase (HMGCoAr), Mevalonate Kinase (MVK), Squalene Synthase (SQS,). In the case of enzymes related to the mevalonate pathway, no differences were observed in terms of their protein content between the different groups. However, when evaluating FASN, a significantly higher content was observed in the groups with fructose in the diet, corresponding to what was found previously (Fig. 13C and D). These results indicate that the fructose-supplemented diet diverts lipogenic metabolism towards the synthesis of fatty acids and not cholesterol.



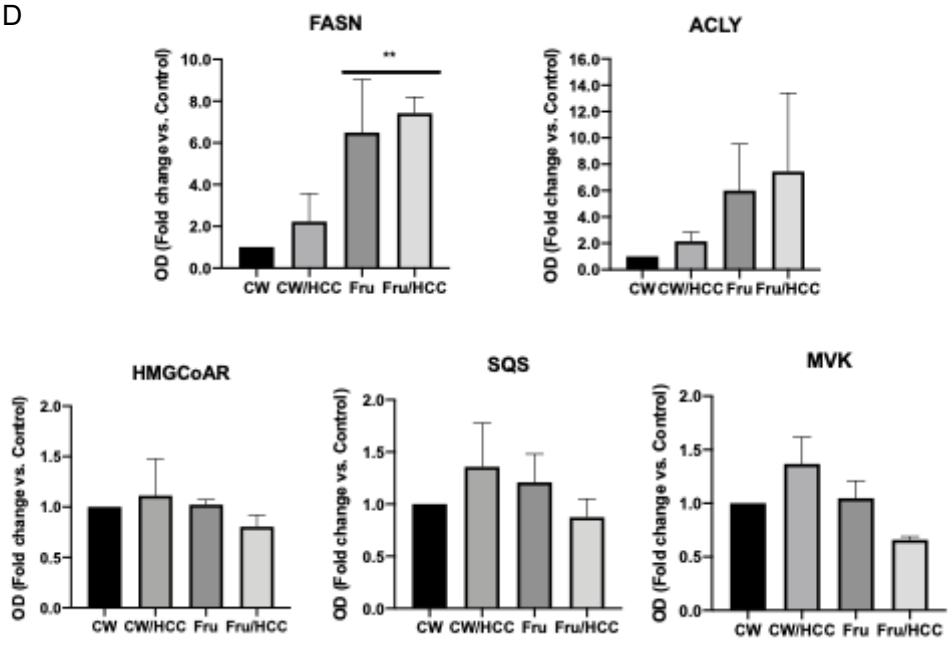


Figure 13. Lipid composition of the serum and tissue of the C57Bl/6j mice sacrificed with 32 weeks of treatment. (A) Triglyceride content in the liver; (B) Total cholesterol content in the liver; (C) Western Blot of the main enzymes related to lipid metabolism; (D) Quantification of the protein content of each evaluated enzyme ***(** p≤0.01 vs. CW).***

3. Fructose treatments increases the aggressiveness of Huh-7 cell line

3.1. Fructose increases the proliferation, viability and functionality properties of the Huh-7 cell line

In order to determine if carbohydrates, not only fructose, enhanced proliferation and viability and if they affected the functionality of the Huh-7 HCC cell line, different treatments (Glucose, Fructose and Fructose: Glucose) were evaluated at different concentrations. In all the treatments evaluated, an increase in the proliferation of Huh-7 cells was observed after 48 h of treatment. However, the treatment with the highest

proliferation was the combination of Fru:Glc, which shows that the increase in the concentration of fructose also increases cell proliferation. In the case of cell viability and functionality, no significant differences were observed between treatments. Once the proposed treatments have been evaluated, we choose the 0.68 mM Fructose treatment (F2) for 48 h as an experimental model because the objective of the work is to find the effect of only fructose in the cell line (Fig. 14).

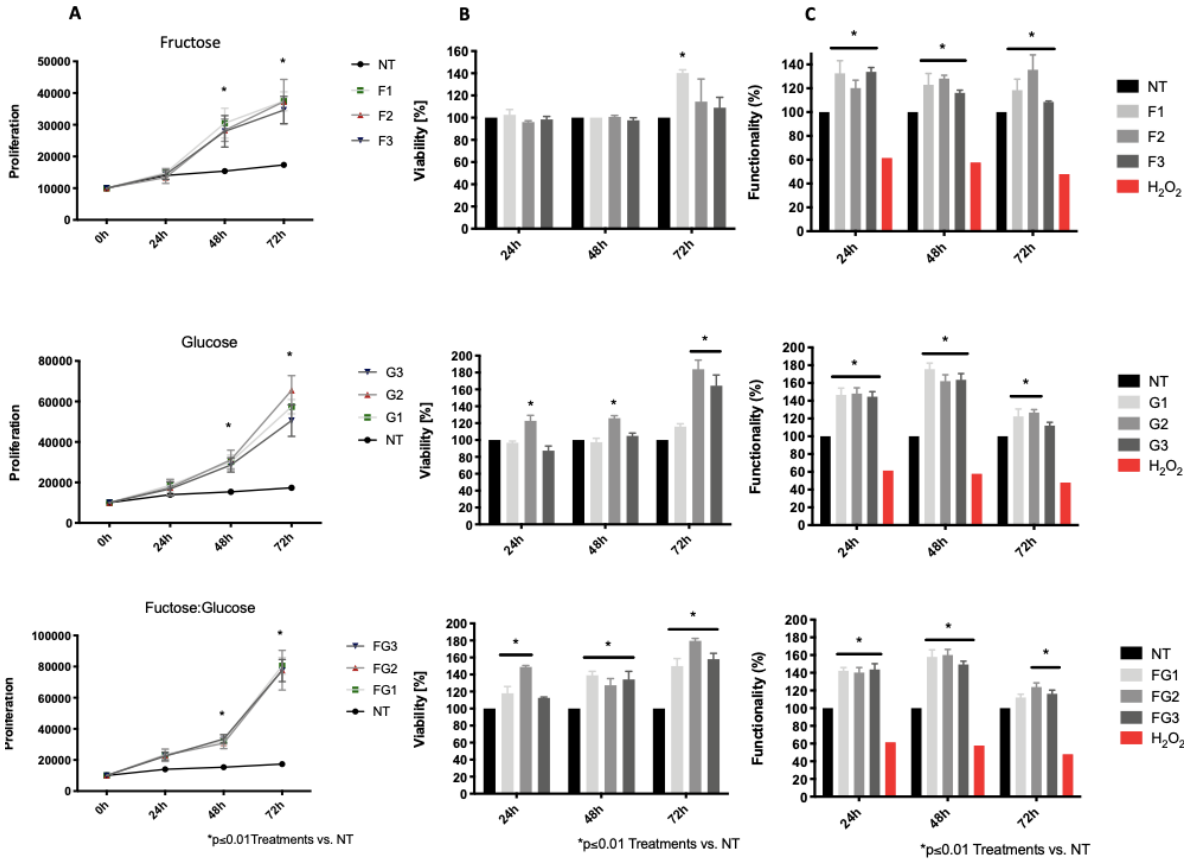


Figure 14. Proliferation, viability and functionality tests in the Huh-7 cell line with the Fructose (F), Glucose. (G) treatments and the combination of both, Fructose: Glucose (FG), with the three-concentration proposed (1, 2 and 3). (A) Proliferation assay; (B) Feasibility Test; (C) Test of Functionality. ***(** p≤0.01 vs. NT)*** *(See In vitro experimental model for more details)*

3.2. Fructose enhances the tumorigenic properties in Huh-7 cell lines

Following this experimental model, then we wanted to determinate if the fructose enhances the aggressiveness of the HCC cell line, that is why its effect on the tumorigenic properties of the Huh-7 cell line was evaluated. For this, a spheroidal formation and clonogenicity test was carried out, and a higher number of spheroids and colonies were obtained in the cells treated with fructose compared to the control (Fig. 15A and B). In the case of spheroids, an increase in their size and forming clusters was also observed (Fig. 15B).

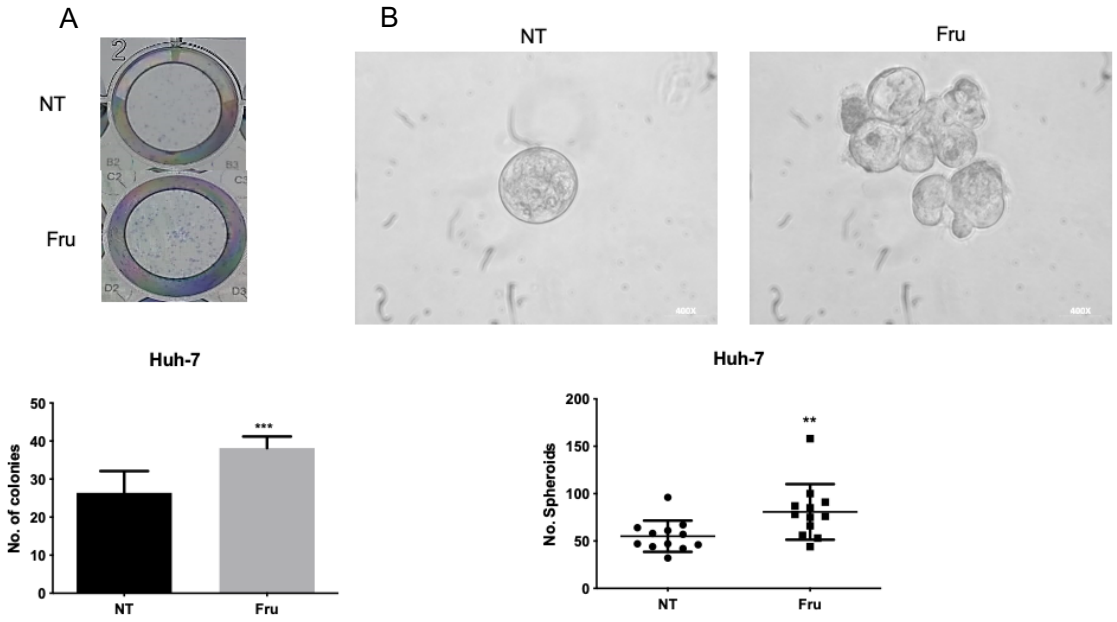


Figure 15. Tumorigenic properties of the Huh-7 cell line treated with Fructose (0.68 mM). (A) Clonogenicity test; (B) Spheroid formation test. There were seeded 1000 cells per well and the assays were conducted in triplicates ***(*** p≤ 0.001 vs. NT) (** p≤ 0.01 vs. NT)*** *(See Clonogenicity assay and Spheroid formation assay for more details)*

Discussion

Hepatocellular carcinoma is the fourth leading cause of death worldwide. In 2018, around 841,000 new cases and approximately 782,000 deaths were reported (Bray et al., 2018). The incidence of liver and intrahepatic bile duct cancer has increased, both men and women, compared to another cancer´s type like lungs and bronchus, ovary and brain and other nervous system which has diminished over the years (Henley et al., 2020). One of the main risk factors is NAFLD, which is reported as the most common chronic liver disease in the last 30 years (Mikolasevic et al., 2018). Due to the current lifestyle, the consumption of hypercaloric diets has increased, especially the consumption of ultra-processed food with sugar added. Mexico is the fifth country with the highest sugary drinks consumption, and this industry will be continue increasing in the next five years from now (https://www.statista.com/outlook/20020100/100/carbonated-soft-drinks/worldwide Consulted: 03/07/2020). The consumption of ultra-processed food rich in added sugars has been related with the development of NAFLD: hepatic steatosis, the progression to NASH until the appearance of HCC. One of the main sweeteners used in the food market is HFCS, and among its main components is fructose, a six-carbon monosaccharide that can represent between 42% and 65% of the product's composition. Fructose has been reported to contribute to the onset and development of NAFLD since it is a highly lipogenic metabolite (Ouyang et al., 2008; Alwahsh and Gebhardt, 2016; Horst and Serlie, 2017).

The results obtained show the effect of fructose in the hepatic tissue C57Bl/6j mice strain treated for 32 weeks with a high fructose diet (**Fru** and **Fru/HCC**). Mice under a high fructose consumption had the bigger adipose tissue accumulation being the groups with the most corporal weight (**Fru** and **Fru/HCC**). Many authors have reported that a high fructose diet is related with the increasing of the adipose tissue and the development of obesity (Dowman et al., 2014; Asgharpour et al., 2016; Jensen et al., 2018; Todoric et al., 2020). Also, Todoric et al. (2020) reported that the supplementation of the drinking water with fructose enhance the accumulation of adipose tissue in C57Bl/6j mice strain compared to the inclusion of fructose in a solid diet. However, the group only supplemented with fructose in water (**Fru**) showed hepatomegaly at 32 weeks of treatment, suggesting that the fructose diet by itself could damage the liver and promote the tumor consolidation without the exposure to a carcinogenic compound.

Then morphological changes were observed in the liver of **Fru/HCC** group after 32 weeks of treatment. Among the main changes, the mice in the **Fru/HCC** group had adhesion between the lobes of the liver. These lobes adhesions are similar like the ones observed in mice with partial hepatectomy surgeries, which could indicate an inflammatory process in the organ. Another change observed is the rigid appearance of some livers belonging **Fru/HCC** group, where the cirrhotic process could already be taking place, and accelerating the tumor formation. Specially the **Fru/HCC** group had the highest number of tumors in the liver. These results are consistent with previous studies where dietary fructose consumption and the development of NAFLD have been

linked. In studies carried out using mice as animal models, diets high in fat and fructose have been shown to promote insulin resistance, obesity, hypertriglyceridemia, liver steatosis, fibrosis, and the spontaneous generation of HCC (Dowman et al., 2014; Asgharpour et al., 2016). These changes not only occur in combination with fat diets, but also it has been seen that the only consumption of high fructose diets enhances the tumor formation in the liver of mice (Todoric et al., 2020).

The nodules seen in the lungs and the tumors seen in the feet of the females still need to be subjected to histological analysis. In both cases, this type of analysis would be very useful to know the nature of the nodules and whether they are primary or secondary tumors. This type of nodules in lungs had not appeared in the different diets previously applied by Simoni-Nieves, MSc., nor even in the group with the Western + DEN diet (**W/HCC**). In the case of the leg's tumors in the females, it must be confirmed if it is due to the effect of the diet in the female or to some characteristic of the strain that could be influencing it.

One of the hallmarks that the transformed cell uses to its advantage is metabolic reprogramming. This adaptation allows the transformed cell to survive, proliferate and spread to other tissues in the body. Fructose has been shown to be involved in the metabolic reprogramming of cancer cells. Faced with a high consumption of fructose, aberrant lipogenesis is increased, a recognized phenotype of a transformed cell that has been related to its aggressiveness.

Many authors argue that this is due to the rapid metabolism of fructose in hepatocytes, since it avoids the main points of regulation of glycolysis, functioning as an unregulated

source of intermediate metabolites for *de novo* lipid synthesis and with it, the accumulation of lipids in hepatocytes (Riveros et al., 2014; Li et al., 2016). The truth is that the fructose consumption has been linked to the development of the hepatic steatosis and inflammation. At first, it produces an increase in *de novo* lipid synthesis and with it an imbalance in lipid metabolism in the liver, which results in liver steatosis. Fructose itself as its metabolites can activate the expression of genes related with *de novo* lipogenesis (*acly, acaca, fasn, chrebp, srepb*) and suppresses the expression of genes related with lipids β-oxidation (*cpt1a, fgf21*) (DiStefano, 2019). Furthermore, increasing the expression of Acetyl CoA carboxylase a (ACACA) is involved in obtaining higher levels of Malonil-CoA. The excess in the cytoplasm of this metabolite inhibits the Carnitine Palmitoyl transferase 1 (CPT1a) transporter in mitochondria and thereby inhibits lipid β-oxidation, leading to the first stage of the NAFLD (Pereira et al., 2017).

Once this process is carried out, the second aggression is a chronic inflammation as a result of oxidative stress, lipid peroxidation and the activation of pro-inflammatory cytokines, which promotes perisinusoidal fibrosis and with-it apoptosis and death of hepatocytes, resulting in NASH (Lim et al., 2010). An increase in Acetyl CoA levels can lead to the obtaining of Diacylglycerol (DAG), which results in the activation of PKCε. This protein phosphorylates the N-terminal c-Jun kinase 1 (JNK1) on Serine 129, which enhances subsequent phosphorylation by MKK4 and MKK7 (Tyr185 and Thr183 respectively) (Lopez-Bergami and Ronai, 2008; Pereira et al., 2017; Merino et al., 2020). JNK1 activates c-Jun, forming the complex c-Jun-c-Fos (activating protein 1:

AP-1 transcription factor), which is related to the transcription of genes implicated in pro-inflammatory pathways (Pereira et al., 2017) which could be explaining the inflammation observed in the histology **Fru** group. Also, the inflammation has been linked to the lipid synthesis through the weakening of the tight junctions in the enterocytes, promotion of intestinal dysbiosis and the passage of LPS into the blood. Endotoxemia causes an increase in the expression of genes related to pro-inflammatory pathways and this has been shown to also enhance *de novo* lipogenesis in hepatocytes (Tododric et al., 2020). We observed steatosis in the liver tissue of the same group of treatment (**Fru**).

Fructose has also been related to metabolic reprogramming in transformed cells and it is due to the increase levels of uric acid. The high affinity of KHK to fructose causes a rapid decrease in the ATP levels of the cell, generating an excess of uric acid inside the hepatocyte, which leads to oxidative stress in the mitochondria through NADPH oxidase 4 (NOX4) (Lanaspa et al., 2012). In a study carried out in the HepG2 cell line was demonstrated the influence of uric acid in the decrease of the mitochondrial membrane potential, thereby favoring the production of ROS and the mitochondrial disfunction. Aconitase, which is an enzyme that is involved in the isomerization of citrate to isocitrate, is highly sensitive to the levels of ROS generated in the mitochondria. Therefore, high levels of ROS decrease the aconitase activity and lead to the accumulation of citrate in the mitochondrial matrix. Consequently, the authors reported an increase in the synthesis and accumulation of lipids in HepG2 cells (Lanaspa et al., 2012).

As a result of this metabolism also a reprogramming through favoring the Warburg effect has been reported (Nakagawa et al., 2020). Cancer cells take advantage of this effect to obtain reducing power and building blocks to cellular division through the pentose phosphate pathway. Also, another consequence of the Warburg effect is the acidification of the tumor microenvironment due to the high levels of Lactate, which causes the modification of the tumor stroma favoring metastasis to other tissues (Liberti and Locasale, 2016). Once this stage of the disease has been reached, the progression to cirrhosis and subsequently HCC occurs more easily and, in less time (Lim et al., 2010).

The levels of the transaminase enzymes were not detectable between the treated groups. This may be due to the fact that after 32 weeks of treatment these levels will normalize as a mechanism of homeostasis of the organism itself. In the case of serum cholesterol, significantly different high levels were obtained in the mice of the **Fru** and **Fru/HCC** group compared to the **CW** and **CW/HCC** groups; however, serum triglyceride levels had not differences between groups. In this case, it is known that one of the characteristics of most types of cancer is aberrant lipogenesis to obtain energy, construction of new membranes and signaling routes, and it is related to the cancer aggressiveness (Calvisi et al., 2011). To further investigate the dependence of HCC to triglyceride and not to cholesterol, we evaluated the levels of TG and CHO in the liver tissue and the protein content of the enzymes implicated in the lipogenic pathways, and notice that both TG levels and the content of FASN and ACLY were elevated in the liver of **Fru** and **Fru/HCC**. In contrast levels of CHO and HMGCoAr, MVK and SQS

were no significative between treatments. In our case, the tumors formed could be preferring triglycerides and not cholesterol as fuel for the progression of HCC.

One of the main enzymes related with TG synthesis is FASN, and in our in vivo experimental model we obtained high levels of this protein. In patients with HCC, FASN expression is higher in the tumor zone compare with the lower expression in the surrounding and normal tissue. The overexpression in the tumor tissue can lead to the overactivation of the AKT/mTOR pathway, leading to an upregulation of lipogenic pathways (Calvisi et al., 2011). Recent studies have demonstrated that when FASN is activated there is a strong synthesis of TG, diacylglycerols and free fatty acids. Also, FASN activation inhibits Srebp2 translocation to the nucleus and reduces Mevalonate pathway (Che et al., 2020). A strong relation has been demonstrated between the AKT/mTOR pathway and FASN activation, reporting that activating of FASN can enhance the activity of mTORc2 and increased the sustain activation of AKT leading to the promotion to HCC in a genetic murine model. Also, it has been shown that there is a subtype of HCC patients with a high content of FASN in the tumor tissue, which correlates with a high content of AKT, classifying FASN as a tumor promoter in this murine model and subtype of HCC patients (Calvisi et al., 2011; Li et al., 2016; Che et al., 2019). For this reason, in the murine model used during this study, FASN could be enhancing the aggressiveness of this type of cancer by inducing the aberrant lipogenesis phenotype.

By last, we also analyzed the fructose treatment effect in the HCC cell line Huh-7. We obtained the highest proliferation rate in the combined treatment (Fru:Glc) which had a

main composition of fructose instead of glucose. This result evidences that with an increase in Fructose the proliferation and functionality of the cell line also increases without affecting the viability. Also, when we evaluated the tumorigenic parameters, we found that the treatment of the cell line with fructose enhances these properties (spheroids formation and clonogenicity). In the case of spheroids formation this is a 3D model to simulate a tumor of the same cell type in a cell plate. Tumors as spheroids has hypoxic zones (1% - 5% de O2) and in these conditions, hypoxia-inducible factor 1-α (HIF-1-α) is expressed and the activation of genes related to proliferation, metastasis, angiogenesis and stemness (Dengler et al., 2014). Fructose metabolism can enhance these effects through the aberrant lipogenesis pathway activation and ROS production. Oxidative stress is related to the stabilization HIF-1-α (Guzy et al., 2005), and the exacerbation of these effects, indicating an increase in the cancer cell line aggressiveness.

Conclusion

In conclusion, these results evidence that fructose acts as a tumor promotor compound in the liver cells increasing the progression to HCC through enhancement of cell aggressiveness and tumor formation. Also, high fructose consumption causes the activation of FASN and with-it aberrant lipogenesis, a factor that is correlated with tumor promotion and cancer aggressiveness.

The present work presents an undoubtable evidence that a high fructose diet could be promoting liver carcinogenesis and tumorigenesis. The information presented here could be very useful for the design of new nutritional and health politics that could impact, in the near future in the population wellness, not only avoiding the overweight problem but cancer as well.

Perspectives

It is necessary to carry out studies at the transcriptomic level to determine which genes are mainly activated in the presence of a high fructose consumption in HCC. These results could be used as early diagnostic and prognostic markers in this type of cancer, as well as a possible target for therapeutic intervention. Furthermore, it is important to use other HCC lines as an experimental *in vitro* model in order to compare the effects of fructose obtained in this work.

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Appendix

Appendix 1. Williams E medium composition

	W4125
	[powder]
COMPONENT	g/L
Inorganic Salts	
$CaCl_2$ (anhydrous)	0.2
$CuSO_4 \cdot 5H_2O$	0.0000001
$Fe(NO_3)_3 \cdot 9H_2O$	0.0000001
$MgSO_4$ (anhydrous)	0.0977
$MnCl_2 \cdot 4H_2O$	0.0000001
KCl	0.4
$NaHCO_3$	—
NaCl	6.8
NaH_2PO_4 (anhydrous)	0.122
$ZnSO_4 \cdot 7H_2O$	0.0000002
Amino Acids	
L-Alanine	0.09
L-Arginine (free base)	0.05
L-Asparagine • H_2O	0.02
L-Aspartic Acid	0.03
L-Cysteine (free acid)	0.04
L-Cystine	0.02
L-Glutamic Acid	0.0445
L-Glutamine	0.292
Glycine	0.05
L-Histidine (free base)	0.015
L-Isoleucine	0.05
L-Leucine	0.075
L-Lysine • HCl	0.08746
L-Methionine	0.015
L-Phenylalanine	0.025
L-Proline	0.03
L-Serine	0.01
L-Threonine	0.04
L-Tryptophan	0.01
L-Tyrosine • 2Na • $2H_2O$	0.05045
L-Valine	0.05
Vitamins	
Ascorbic Acid • Na	0.00227
D-Biotin	0.0005
Calciferol	0.0001
Choline Chloride	0.0015
Folic Acid	0.001
myo-Inositol	0.002
Menadione ($NaHSO_3$)	0.00001
Niacinamide	0.001
D-Pantothenic Acid • ½ Ca	0.001
Pyridoxal • HCl	0.001
Retinol Acetate	0.0001
Riboflavin	0.0001
Thiamine•HCl	0.001
(+/–)-α-Tocopherol phosphate • 2Na	0.00001
Vitamin B_{12}	0.0002
Other	
D-Glucose	2.0
Glutathione (reduced)	0.00005
Methyl Linoleate	0.00003
Phenol Red • Na	0.0107
Pyruvic Acid • Na	0.025
Add	
L-Glutamine	—
$NaHCO_3$	2.2
Grams of powder required to prepare 1 L	10.768

Appendix 2. Research results

XIV National Congress of Hepatology (2019)

Dietary cholesterol has been shown to play a role in the development of steatohepatitis. However, the mechanisms by which cholesterol promotes HCC development is unclear.

Methods. The RNA-seq of 16 patients with HCC with no history of primary risk factor, and 12 samples of NHCC (NAFLD-HCC), were retrieved from The Cancer Genome Atlas (TCGA) database. 64 14-days old male mice (C57/BL6) were randomly separated: i.) Fed with a high cholesterol diet (HC); ii.) HC diet and single intraperitoneal (ip) injection of 10 μg/kg body weight of N-Nitrosodimethylamide (DEN) (HCD); iii.) Fed with Western (W) diet; iv.) W diet and DEN (WD); Chow diet (CW) with v or without vi. DEN. After 8 months mice were euthanized. Data are reported as the average ± standard error (SEM). For the comparison of means of different groups, an analysis of variance (ANOVA) was used, followed by multiple comparisons by the Tukey test. The level of significance was $p \leq 0.05$.

Results. In order to identify the gene expression profile related to NAFLD/NASH-related HCC, we proceeded to the analysis NAFLD-HCC samples, and HCC samples with no history of primary risk factors. Interestingly, the analysis of HCC samples versus NAFLD-HCC samples, exhibited 325 differentially expressed. In order to validate the findings in human samples, we established a mice model. Histological studies revealed high lipid accumulation under experimental diets, the Western diet exhibited both micro and macrovascular lipid accumulation. This preliminary data suggest a differential expression profile between different diets.

Conclusions. Increasing evidence supports that in cancer, lipids overload is overacting to support the tumor growth. Suggesting that cholesterol could be positioned as a key element in HCC progression. Dietary lipids play a significant role, because they provide the ultimate resources for growth and survival.

Conacyt: Fronteras de la Ciencia 1320.

21. Fructose Effect on the Functionality of Cells Derived from a Human Hepatocarcinoma

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Background and aim. Hepatocellular carcinoma (HCC) comprises between 75% - 85% of reported cases of primary liver cancer and is the fourth cause of cancer-related death in men mostly. The consumption of hypercaloric diets mainly rich in carbohydrates such as fructose has increased in recent years. High fructose diet is related to the development of Non-Alcoholic Fatty Liver Disease (NAFLD) and the progression of HCC since it potentiates the lipogenic pathway and the accumulation of lipids. The aim of the study is to determine the effect of fructose on the main tumorigenic properties in cells derived from a human hepatocellular carcinoma in order to characterize the role of fructose in the HCC.

Methods. We used Huh7 HCC cell line and applied three treatments, Fructose or Glucose alone at different concentrations (0.65 mM, 0.68 mM and 0.72 mM) and the combination of both (Fructose: Glucose) (0.58 mM: 0.14 mM, 0.61 mM: 0.11 mM and 0.67 mM: 0.05 mM) to carry out tests of viability, proliferation, and cellular functionality. Tumorigenic parameters such as spheroid formation, colony formation and cell migration with phalloidin staining were also evaluated.

Results. No significant changes were found with sugar concentrations, so 0.68 mM fructose was continued for 24 h. Following this experimental model, a greater number of spheroids and colonies were obtained in the cells treated with fructose compared to the control. Regarding migration, wound closure was faster in the presence of fructose.

Conclusions. The data show that fructose increases the proliferation of Huh7cancer cell line without affecting its viability and functionality and enhances its tumorigenic properties.

This work has been partially founded by Conacyt: Fronteras de la Ciencia 1320 and by the UAM.

23. Follow-Up of Patients with Hepatocellular Carcinoma on an Oncology Center vs. Hepatology Center

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Background. Liver cancer is the second and third cause of cancer-related mortality worldwide and in Mexico, respectively. Liver transplant (LT) is the best treatment for early stage HCC. Recently, an alpha-fetoprotein (AFP) model was introduced as a better predictor of HCC recurrence and survival after LT.

Methods. We studied 126 patients, with HCC and cholangiocarcinoma diagnosis confirmed by biopsy, image study or elevated AFP(HCC), between 2012-2018. 62 (49%) patients from the Hepatology Center (HC), and 64 (51%) from the University Center Against Cancer (UCAC) from "Dr. José Eleuterio González" University Hospital. 31(25%) were cholangiocarcinoma. We present only HCC (n = 95). 64 (67%) males (65.09 ± 9.69). 100% cirrhotic; NASH 33(39%), ASH 29 (34%), HCV 18 (21%), PBC/AIH 1(1%) and Non-determined 4 (5%). Okuda's and BCLC classifications to stage the patients (A-early, B-intermediate, C-advanced, D-end stage). Milan criteria and University of California, San Francisco expanded criteria (UCSF) to identify candidates for LT. Population was divided in low (≤ 2) and high (> 2) recurrence risk according to AFP model.

Results. Cirrhotic patients undergo screening (US + AFP) every 6 months in the HC for early diagnosis. Average size of the biggest tumor was 73 mm (16–167). 20 (21%) patients had extrahepatic metastasis at diagnosis. Patients with Okuda I-II and BCLC A-B were more prevalent in HC, whereas, more advanced HCC was diagnosed in UCAC by the same classifications. There were more patients within MC and UCSF in the HC. A larger number of patients had major recurrence risk in the UCAC. 15 (27%) patients from HC were evaluated for LT, only 6 received it (4 HCC progression, 2 economic issues, 2 still on evaluation, 1 denied LT).

HC (n = 56)	OKUDA	BCLC	MC	UCSF	AFP Model
	I-II*	A + B**	Yes 17/49	Yes 26/49	> 2 26/43
	44/48 (92%)	34/49 (69%)	(35%)***	(53%)****	(60%)*****
UCAC	II-III*	C + D**	Yes 3/26	Yes 6/26	> 2 19/22
(n = 39)	24/29 (83%)	22/36 (61%)	(12%)***	(23%)****	(86%)****

*p = 0.013; **p = NS; ***p = NS; ****p = 0.024; *****p = 0.024.

Conclusions. Patients whom received regular screening for HCC, showed early disease. 40% had low risk of HCC recurrence after LT. 35–53% of the patients could have received LT, which reflects the national problem of lack of access to transplants.

24. Intra-Hospital Tests Overestimates Outpatients Physical Activity in Liver Cirrhosis Patients Evaluated for Liver Transplantation

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5

The fructose enhances the HCC progression in mice under a high intake of fructose in dieT

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Background and aim: Hepatocellular carcinoma (HCC) is the fourth cause of cancer-related death and its incidence has been increasing in both men and women. One of the main concerns has been the consumption of hypercaloric diets mainly rich in carbohydrates such as fructose. High fructose diet is related to the development of Non-Alcoholic Fatty Liver Disease (NAFLD) and the progression of HCC since it potentiates the lipogenic pathway and the accumulation of lipids. The aim of the study is to determine the effect of a high fructose diet on the progression of HCC, induced by DEN, in C57Bl/6J mice strain.

Materials and methods: We used C57Bl/6J mice strain (both sex) with a high Fructose diet (Fru)(33% of Fructose in the drinking water, *ad libitum*). Fru supplementation started with 15 days old mice, two days after DEN was injected (10 µg/Kg, i.p) and the treatment was ended 8 months later to evaluate the role of fructose in tumor progression by histological and biochemical tests. The protocol was approved by the UAM ethics commission.

Results: The major number of tumors were found in the Fructose + DEN (FD) mice group *vs.* only DEN (CWD) mice group. Triglyceride levels (TG) was evaluated in the serum with no detectable values; however, in the liver tissue the FD group showed significantly higher TG content. On the contrary, the Cholesterol (CHO) levels were significantly higher in the serum of dietary fructose group and had no differences in the tissue. The protein content in tissue followed the same observed pattern, since significance was only found in Fatty acid synthase (Fasn) with a higher protein content in the groups with dietary Fructose. Curiously, we noticed tumors in the lungs. Conclusion. The data strongly suggests that the high consumption of Fru in the diet induces effects in liver tumor promotion, in a mechanism dependent on FASN and independent of CHO. High consumption of Fru should be considered as a liver toxic factor.

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Conflicts of interest: The authors have no conflicts of interest to declare.

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6

Gallbladder adenomyomatosis

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Background and aim: Adenomatous hyperplasia of the gallbladder or adenomyomatosis is a benign neoplasia characterized by epithelium hyperplasia with invaginations into the subserose forming intramural diverticula (Rokitansky-Aschoff sinuses). It is reported in 1 to 8.5% of cholecystectomies and 7% of autopsies. It has been associated with cholelithiasis in 80% of cases and may have an asymptomatic course or present with biliary cholic. According to its site it can be localized, annular, diffuse or segmental; the later associated with cancer in 3.2%.

Material and methods: Retrospective review of medical records of patients with pathology study diagnosis of adenomyomatosis from January 1st, 2015 through December 31st, 2019.

Results: Twenty-four cases were found, with 58.3% of women and mean age of 51 years. Elective cholecystectomy was found in 26% of cases. Most frequent symptoms were abdominal pain, nausea and, vomit with 75%, 41.7%, and 33.3%, respectively. Duration of symptoms was less than 24 hours in 21.1%, and 7 days to 3 months in 57.9% of cases. Smoking was reported in 58.3%, alcohol consumption in 12.5% and dyslipidemia in 20.8% of cases. Murphy's sign was reported in 37.5% and the most frequent clinical diagnosis was acute cholecystitis in 66.7% of cases. Mean alkaline phosphatase was 105.6 ± 76.0 UI/L and mean gamma-glutamyl transpeptidase was 113.2 ± 161.3 UI/L. In abdominal ultrasound, the gallbladder had a thin wall in 50%, thick wall in 8.3%, polyps in 20.8% and stones in 54.2% of cases. In pathology studies, mean thickness of adenomyomatosis was 90.5 mm and the location were localized (fundus) in 58.3%, diffuse in 20.8% and annular in 4.2% of cases.

Conclusions: Female sex (estrogens), chronic inflammation (cholecystitis) and cholelithiasis are the few associated factors for the development of adenomyomatosis. Most ultrasound findings are non-specific and, therefore, presurgical diagnosis is difficult. In most of the cases, the diagnosis of adenomyomatosis was an incidental finding associated with acute cholecystitis.

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7

HGF induces a protective response in a preclinical model of nephropathy induced by acute cholestasis

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Background and aim: The relationship between the liver and the kidneys in some hepatic diseases is well known. Hepatorenal syndrome usually occurs in chronic damage but has also been observed in the acute one. Therapeutic approaches remain limited and poorly optimized, especially to address the commitment of both organs. HGF induces protection in various organs, but its effects are unknown in a scenario of multi-organ compromise, as in the case of hepatorenal syndrome or colemic nephropathy. The aim of this investigation was to determine the mechanism induced by HGF to counteract liver and kidney damage in a preclinical model of systemic damage induced by intrahepatic cholestasis in a setting of colemic nephropathy.

Casa abierta al tiempo
UNIVERSIDAD AUTÓNOMA METROPOLITANA

ACTA DE EXAMEN DE GRADO

No. 00219
Matrícula: 2183802586

THE CONSUMPTION OF A HIGH FRUCTOSE DIET ENHANCES THE HEPATOCELLULAR CARCINOMA PROGRESSION IN MICE

Con base en la Legislación de la Universidad Autónoma Metropolitana, en la Ciudad de México se presentaron a las 12:00 horas del día 26 del mes de noviembre del año 2020 POR VÍA REMOTA ELECTRÓNICA, los suscritos miembros del jurado designado por la Comisión del Posgrado:

DRA. ROXANA URI MIRANDA LABRA
DR. IVAN URIEL BAHENA OCAMPO
DR. JULIO CESAR ALMANZA PEREZ
DR. ALBERTO GABRIEL LOPEZ REYES

Bajo la Presidencia de la primera y con carácter de Secretario el último, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRA EN BIOLOGIA EXPERIMENTAL

DE: LISETTE CHAVEZ RODRIGUEZ

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

APROBAR

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

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ALUMNA

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