Title page

Functional, metabolic, and dynamic mitochondrial changes in the rat cirrhosis-hepatocellular carcinoma model and the protective effect of IFC-305.

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Abbreviations: diethylnitrosamine (DEN), dynamin-related protein 1 (DRP1), electron transport chain (ETC), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), mitofusin-2 (MFN-2), poly(ADP-ribose) polymerase 1 (PARP-1), peroxisome proliferator-activated receptor γ-coactivator 1α (PGC-1α), sirtuin-1, -3 (Sirt-1, -3).

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ABSTRACT

Background: Mitochondrion is an important metabolic and energetic organelle which regulates several cellular processes. Mitochondrial dysfunction has been related with liver diseases including hepatocellular carcinoma. As a result, the energetic demand is not properly supplied and mitochondrial morphologic changes has been observed resulting in an altered metabolism. We previously demonstrated the chemopreventive effect of the hepatoprotector IFC-305. Aim: In this work we aimed to evaluate the functional, metabolic, and dynamic mitochondrial alterations in the sequential model of cirrhosis-hepatocellular carcinoma induced by diethylnitrosamine in rats and the possible beneficial effect of IFC-305. Methods: Experimental groups of rats were formed to induce cirrhosis-hepatocellular carcinoma and to assess the IFC-305 effect during cancer development and progression through the evaluation of functional, metabolic and dynamic mitochondrial parameters. Results: In this experimental model, dysfunctional mitochondria were observed and suspension of the diethylnitrosamine treatment was not enough to restore them. Administration of IFC-305 maintained and restored the mitochondrial function and regulated parameters implicated in metabolism as well as the mitochondrial dynamics modified by diethylnitrosamine intoxication. Conclusion: This study supports IFC-305 as a potential hepatocellular carcinoma treatment or as an adjuvant in chemotherapy.
1. INTRODUCTION

Mitochondria are organelles responsible for most of the energetic metabolism in eukaryotic cells. As an integral part of ATP production, through the oxidative phosphorylation (OXPHOS), the tricarboxylic acid cycle (TCA) donates electrons to the electron transport chain (ETC), which consists of five complexes (I-V), where complex I is the electron entry site for NADH and generates NAD\(^+\) and complex V is in charge of ATP synthesis. The ability of mitochondria to regulate the energetic, redox state and metabolism of the cells could result in the production of epigenetic intermediates, point them out as a major therapeutic target because mitochondrial dysfunction is involved in several diseases including cancer (Mughal et al., 2012; Boland et al., 2013).

Studies have revealed several metabolic alterations in liver diseases including modifications in energy supply (Hernandez-Munoz et al., 1991; Hernandez-Munoz and Chagoya de Sanchez, 1994). Otto Warburg suggests that mitochondria from tumor cells supply the energetic demand, through the glycolytic flux because of the lack of oxygen or due to genetic-epigenetic alterations that affect the oxidative metabolism (Wallace and Fan, 2010). In fact, metabolic alterations and some anti-apoptotic proteins such as BCL-K\(_L\) reduce the acetyl-CoA level (Ac-CoA) (Yi et al., 2011); this intermediate also plays an important role as a signal transducer and gene expression (Pietrocola et al., 2015). Oxidative stress diminishes oxidative metabolism flux, which includes TCA enzymes such as isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH). Indeed, studies have identified mutations in IDH producing an oncometabolite in different tumor types (Dang et al., 2016). A good indicator of the mitochondrial redox state is the NAD\(^+\)/NADH ratio and if it is correctly regulated, an efficient ETC activity is possible; as shown in the limitation of breast tumor growth (Santidrian et al., 2013). Previously our research group
demonstrated the ability of adenosine to maintain the energetic and redox state of the cell (Hernandez-Munoz et al., 1978; Hernandez-Munoz et al., 1987). In addition to the metabolic role of NAD$^+$, it participates in the activity of multiple enzymes. According to this, Sirtuin-1 (Sirt-1), a NAD$^+$-dependent protein and a member of class III histone deacetylase, targets several transcription factors including the peroxisome proliferator-activated receptor $\gamma$-coactivator 1$\alpha$ (PGC-1$\alpha$). The latter coactivates major transcription factors involved in mitochondrial and nuclear gene expression directing the complex program of mitochondrial biogenesis (Finley and Haigis, 2009). However, Sirt-1 and PGC-1$\alpha$ have been found over-expressed in HCC and are related to defective mitochondrial accumulation (Chen J, 2012; Boland et al., 2013). Another NAD$^+$-dependent protein is PARP-1 (Poly(ADP-ribose)) polymerase-1 whose activity modulates transcription and DNA repair; nevertheless, over-expression and increased activity of PARP-1 have been found in HCC and has been considered as a cancer hallmark (Hanahan and Weinberg, 2011).

Mitochondria are not static organelles; their dynamism depends, at least in part, on the fission and fusion phenomena that determine their shape. Mitochondrial fusion requires polarized mitochondrial membrane and the activity of proteins such as mitofusin 1 and 2 (MFN 1, 2); this process promotes cristae integrity and OXPHOS. Mitochondrial fission is induced by different kinds of stress and requires proteins such as the dynamin-related protein 1 (DRP1); membrane depolarization is observed during fission and, if the membrane potential is not recovered, mitochondria are targeted to autophagy (Boland et al., 2013). Altered mitochondrial fission and fusion have been observed in tumor cells mainly due to increased DRP1 and decreased MFN-2 expression (Rehman et al., 2012).
Our research group demonstrated the hepatoprotective effects of IFC-305, an adenosine-derived compound (Perez-Carreon et al., 2010; Velasco-Loyden et al., 2010; Chagoya de Sanchez et al., 2012; Velasco-Loyden, 2016); this compound has been evaluated in a diethylnitrosamine (DEN)-induced sequential rat model of cirrhosis-HCC, where it inhibited pre-neoplastic lesions development in comparison to DEN-treated groups. The chemopreventive effect was associated with the reduction of the expression of collagen, thymidylate synthase, the tumor marker γ-glutamyl transferase, the hepatocyte growth factor, and the induction of the cell cycle inhibitor p27 expression (Velasco-Loyden, 2016).

In this work we evaluated the mitochondrial alterations in the sequential cirrhosis-hepatocellular carcinoma model, previously described by Schiffer et al. (Schiffer et al., 2005), and the possible beneficial effects of the hepatoprotector IFC-305 during HCC development and progression on mitochondrial function, metabolism, and dynamics. We measured the mitochondrial function through the respiratory quotient, membrane potential, complex I activity, and ATP synthesis. The activity of the malate-aspartate shuttle, IDH and MDH, and the level of Ac-CoA and lactate were determined as metabolic parameters. Dynamic mitochondrial proteins were determined to evaluate the morphology and biogenesis; electron microscopy was also used for morphology studies.
2. MATERIALS AND METHODS

2.1 Chemicals

IFC-305 is the aspartate salt of adenosine prepared with adenosine free base (MP Biomedicals, LLC, Illkirch, France) and L-aspartic acid (MP Biomedicals, Inc, Eschwege, Germany) as described (Patent No. MX220780; MX 207422; US 8,507,459 B2) (Chagoya de Sanchez, 2002; Chagoya de Sanchez, 2004; Chagoya de Sanchez, 2013). Dietthynitrosamine, sucrose, EDTA, Trisma base, KCl, MgCl₂, glutamate, ADP, rhodamine 123, HEPES, EGTA, cyclosporine A, valinomycin, succinate, 2,6-dichlorindophenol, ATP, NAD. NADH, acetophenone were purchased from the Sigma Chemical Company (St. Louis, MO, USA).

2.2 Animal treatment and experimental groups

Male Wistar rats (weighing 200 g) were obtained from and housed at the Animal Facility of the National Autonomous University of Mexico (UNAM), and all procedures were conducted according to our institutional guidelines for the care and use of laboratory animals. Groups of rats (n = 6, each group) were divided in two different schemes: hepatocellular carcinoma (HCC) and cancer progression (CP). These were treated as follows: a) the HCC groups were injected with DEN (Sigma-Aldrich, St. Louis, MO) at 50 mg/kg body weight i.p., once a week and saline solution or IFC-305 at 50 mg/kg body weight i.p., 3 times weekly (HCC+IFC-305) for 16 weeks plus 2-weeks wash out; b) the CP groups were administered DEN at 50 mg/kg body weight i.p., once a week for 16 weeks and then received saline solution or IFC-305 (CP+IFC-305) at 50 mg/kg body weight i.p., 3 times weekly during 6 weeks (Table 1). To euthanize animals, a lethal dose of sodium pentobarbital was used and the liver was removed.

2.3 Mitochondrial function
2.3.1 Mitochondria isolation and oxygen uptake

Liver samples were homogenized (1:10 w/v) in a medium containing 250 mM sucrose, 1 mM EDTA, 10 mM Trizma base, and 0.1% BSA, pH 7.3. The tissue homogenate was centrifuged at 755 g for 5 min to remove nuclei and plasma membrane fragments. The supernatant was filtered through organza fabric and centrifuged at 8400 g for 10 min to obtain the mitochondrial pellet. Mitochondria were resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM Trizma base, pH 7.3. Mitochondrial respiration was recorded polarographically with a Clark-type oxygen electrode in 3 mL of a medium containing 250 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 10 mM potassium phosphate, 10 mM Trizma base; 10 mM glutamate and malate were used as substrates for site I. Mitochondrial state 3 was initiated by adding 226 µM ADP (final concentration).

2.3.2 Mitochondrial membrane potential

The mitochondrial membrane potential was determined according to Baracca et al. (Baracca et al., 2003). Briefly, a calibration curve was done by plotting rhodamine 123 fluorescence against the membrane potential calculated through the Nerst equation. To determine the mitochondrial membrane potential 0.15 mg of mitochondria in a medium containing 250 mM sucrose, 10 mM HEPES, 100 µM EGTA, 2 mM MgCl₂, 4 mM KH₂PO₄, pH 7.4, and 10 mM sucrose and 2.5 U hexokinase. Then, 1 µg/mL of 33 nM cyclosporine A and 0.1 mM ADP were added. Finally, rhodamine (50 nM) fluorescence was measured in the presence of 20 mM succinate.

2.3.3 Complex I activity

The 2,6-dichloroindophenol (DCPIP) reduction was used to determine complex I activity in mitochondria isolated from the liver. Briefly, 1 mg of mitochondrial protein was added to a medium containing 0.25 M sucrose, 1 mM EDTA, 0.1% BSA, 100 µM KCN, and 10 mM
glutamate as substrate, and 152 μM rotenone. The absorbance was followed at 600 nm and the activity was calculated using the DCPIP extinction coefficient (21 mM⁻¹cm⁻¹).

2.3.4 Determination of the ATPase activity and the content of the αβ subunits

Rat liver mitochondria (2 mg) were resuspended in different volumes of the mitochondrial preparation medium. Mitochondria were centrifuged at 180 g for 10 min at 4°C. The mitochondrial pellet was resuspended in 50 μL of 250 mM sucrose and 1 mM MgCl₂ and centrifuged in an air-driven ultracentrifuge at 5 249 g at 10 psi. The pellet was resuspended in a medium containing 50 mM NaCl, 50 mM imidazole, 1 mM EDTA, 2 mM aminocaproic acid, 0.66 mg digitonin /mg mitochondria. The suspension was centrifuged at 9 860 g at 22 psi for 5 min. The supernatant was used for protein determination with first dimension blue native PAGE (BN-PAGE) and second dimension SDS PAGE.

First dimension. Protein extracted with digitonin (100 μg) was mixed with 10 μL buffer 3X (1.5 M 6-aminocaproic acid, 150 mM Bis-Tris) plus serva blue G (7 μg/μL stock serva blue G in 1.5 M 6-aminocaproic acid) (final concentration, 30 ng serva blue/1 μg protein). The sample was charged to a 3.5-11% linear BN-PAGE and electrophoresis was performed at 70 V for 2 h and 100 V for 8 h at 4°C. ATPase activity was identified by incubating the first dimension gel in a preincubation-solution containing 35 mM Tris, 250 mM glycine, pH 8.3. Then, the gel was stirred and incubated for 1 h and 2 h at 37°C with 5 mM ATP, 5 mM MgCl₂, 0.15 % (w/v) lead acetate, and 150 mM glycine, pH 8.3. After that, the gel was stirred and incubated at room temperature for 24 h. Monomer and dimer bands with dark background were scanned at 1, 2, and 24 h. Monomer bands were read at 1, 2, and 24 h, with Image J software.

2.4 Metabolic evaluation
2.4.1 Malate-aspartate shuttle

The activity of the malate-aspartate shuttle was determined using the assay previously described for Scholz et al. (Scholz et al., 1998). Briefly, 50 μL of mitochondrial suspension were mixed with 2 mL of 300 mM mannitol, 10 mM potassium phosphate, 10 mM Tris, 10 mM KCl, 5 mM MgCl₂, 2 mM aspartate, 2 mM ADP, and 0.14 mM NADH, pH 7.4. Then, 2 IU/mL of AST was added and basal oxidation of NADH was followed at 340 nm at 37°C for 4 min. The shuttle activity was started with the addition of 4 mM malate and 4 mM glutamate. Oxidation of NADH was followed at 340 nm for 4 min at 37°C.

2.4.2 Isocitrate dehydrogenase and malate dehydrogenase activities

Isocitrate dehydrogenase and malate dehydrogenase activities were estimated using the following commercial kits: Isocitrate Dehydrogenase Activity Assay Kit (Cat. MAK062) and Malate Dehydrogenase Assay Kit (Cat. MAK196) following the instructions provided by the manufacturer (Sigma-Aldrich, Mexico).

2.4.4 Acetyl-CoA, 3-hydroxybutyric acid, and lactate determination

The levels of acetyl-CoA, 3-hydroxybutyric acid, acetoacetate and lactate were determined using the following commercial kits: Acetyl-Coenzyme A Assay Kit (Cat. MAK039, Sigma-Aldrich, Mexico); Acetoacetate Colorimetric Assay Kit (Cat. MAK199); EnzyChrom Ketone Body Assay Kit (Cat. EKBD-100, Hayward, CA, USA). The instructions provided by the manufacturer were followed.

2.4.5 PARP-1 enzymatic activity

The PARP-1 enzymatic activity was determined according to Karson et al. (Putt and Hergenrother, 2004). Briefly, liver samples were homogenized in a buffer containing 50 mM
Tris, 2 mM MgCl₂, pH 8.0, and protease inhibitors cocktail (Roche Diagnosis, Indianapolis, IN, USA). The tissue homogenate was centrifuged at 13 700 g for 10 min at 4°C. The supernatant was separated and protein content was determined by the Bradford method (Bio-Rad, Laboratories, Inc. USA). A DNA sample was obtained from the control liver tissue through a modified salt extraction method described by Lopera-Barrero et al. (Lopera-Barrero, 2008). Briefly, 20 µL of 100 µM NAD⁺ was added to the plate; subsequently, 30 µg of protein and DNA (at 12.5 µg/mL), previously activated with UV irradiation, were added. The plate was incubated at room temperature for 20 min and the amount of NAD⁺ present was determined by the addition of 10 µL of 2 M KOH and 10 µL of 20% acetophenone (in ethanol). The plate was incubated at 4°C for 10 min. Finally, 45 µL of 88% formic acid was added and the plate incubated at 110°C for 5 min. The plate was allowed to cool and was read at 378 nm. To quantitate the NAD⁺, absorbances were interpolated in a NAD⁺ calibration curve, previously performed.

2.5 Evaluation of mitochondrial dynamics

2.5.1 Nuclear protein extraction

The isolation of intact and stable nuclei consisted in an iso-osmotic lysis procedure, as reported by Dyer and Herzog (Dyer and Herzog, 1995). With this lysis procedure, the nuclear envelope remained intact even during further manipulations of washing, freezing, and ultracentrifugation, and provided nuclear protein extract. Briefly, 0.8 g of liver tissue was homogenized in 1.6 mL of buffer containing 0.25 M sucrose, 0.05 M Tris-Cl, 0.005 M KCl₂. The homogenate was filtered and supplemented with 3 mL of a 2.3 M sucrose solution to increase the homogenate’s density. Then, the sucrose gradient was created by adding 1.5 mL of a 2.3 M sucrose solution to the bottom of the tube containing the homogenate. The nucleus was isolated by centrifugation for 30 min at 4°C at 255 000 g. After the isolation, nuclear lysis was performed by incubating and
stirring with a hypotonic and hypertonic solution. The nuclear extract protein was determined by the Bradford method and used for the corresponding assays.

2.5.2 Total protein extraction

Liver samples were homogenized with RIPA buffer (100 mg of tissue per milliliter of buffer) containing protease and phosphatase inhibitors (Roche Diagnostics Corp.). The homogenate was centrifuged at 16,000 g for 10 min at 4°C, the supernatant was utilized as total liver homogenate.

2.5.3 Western blot assays

Mitochondrial, nuclear, and total protein extracts were used as corresponding. Volumes equivalent to 50 µg of protein were electrophoresed on 12% polyacrylamide gel (20% polyacrylamide for histones); separated proteins were transferred onto PVDF (Immobilon P). Next, blots were blocked with 5% skim milk and 0.05% Tween-20 for 30 min at room temperature, and independently incubated overnight at 4°C with selective antibodies (dilution 1:1000) against PGC-1α (MAB 1032 from Chemicon Int. Inc.), Sirt-1, Drp-1, Sirt-3, Mfn-2 (Santa Cruz Biotechnology sc-15404, sc-32898, sc-99143, sc-50331, respectively), H4ac, Hsp60, H3, H4 (06-946, MAB 3844, 06-755, 04-858 from Millipore, respectively), as appropriate. On the following day, the membranes were washed and then exposed to a secondary peroxidase-labeled antibody at dilution 1:10000 (Jackson ImmunoResearch) in the blocking solution for 1 h at room temperature. Blots were washed and protein was developed using the ECL detection system. Densitometric analyses of bands were performed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.5.4 Electron microscopy
Liver samples for electron microscopy were fixed with glutaraldehyde (6%) and stained with osmium tetroxide (1% phosphate buffered saline solution) according to Mascorro et al. (Mascorro JA, 1976).

2.6 Statistical analysis

Data are expressed as mean values ± standard error of the mean (SEM). Comparisons were carried out by analysis of variance, followed by Tukey's test, as appropriate, using Graph Pad Prism 5.0 (Graph Pad Software Inc, La Jolla, CA, USA) for Windows. Differences were considered statistically significant when \( p < 0.05 \).
3. RESULTS

3.1 IFC-305 treatment was helpful to maintain and recover the mitochondrial function that was altered in the sequential model of cirrhosis-hepatocellular carcinoma.

Mitochondria are the main source of energy and act as an energy modulator to orchestrate their physiological responses. For this reason, we decided to evaluate some parameters that reflect the mitochondrial function in the sequential model of cirrhosis-hepatocellular carcinoma in the rat and the effect of IFC-305 treatment. The respiratory quotient was obtained from the state 3 and state 4 ratio. Chronic treatment with DEN induced an increased oxidation of glutamate in the absence of ADP (state 4) and a lower response for ADP (state 3) resulting in a diminished respiratory quotient in the HCC and CP groups compared to the control group (Table 2); the IFC-305 treatment prevented and reversed this alteration as seen in the HCC+IFC-305 and CP+IFC-305 groups. Moreover, there was a significant decrease in ATP synthesis in the groups treated with DEN that was not restored even in the CP group, and the beneficial effect of IFC-305 to prevent and reverse the damage caused by DEN can be observed in the groups receiving IFC-305 (Table 2). It can be observed a significant reduction in the complex I activity in the HCC and CP groups and the beneficial effect of the IFC-305 treatment which was able to reverse this alteration to the normal level. Mitochondrial membrane potential provides an electrochemical gradient required to regulate mitochondrial function. We found important changes in this parameter; depolarization was observed in mitochondria from HCC and CP groups and a tendency to prevent and reverse this effect can be observed in groups HCC+IFC-305 and CP+IFC-305 (Table 2). These results clearly indicate the functional impairment due to the chronic intoxication with DEN and the improvement effect exerted by the IFC-305 treatment.
Considering the aforementioned findings, we evaluated complex V, which is in charge of ATP synthesis as an integral part of the mitochondrial function. Through the isolation of the F1F0 complex from samples at week 22, we evaluated its ability to hydrolyze ATP. In this approach, we observed that the ATPase activity remained lower in the CP group than in the control one. The IFC-305 treatment restored this activity, which became higher at 24 h (Figure 1A). Moreover, the absence of the dimeric form of the F1F0 complex was seen the DEN intoxication even after it had been suspended (CP group); the control group and that treated with the IFC-305 compound (CP+IFC-305 group) revealed the presence of the dimeric form of the F1F0 complex (Figure 1B). Similar results were obtained through the monomer quantification in blue gel and Coomassie staining and, the αβ subunits content (Supplemental Figure 1). These findings support the IFC-305 beneficial effect on the αβ ATPase subunits.

The mitochondrial function was damaged in the HCC and CP groups; as it was previously mentioned, the suspension of DEN not recovered mitochondrial functionality but the IFC-305 compound helps to maintain mitochondrial integrity.

3.2 The hepatoprotector improved the metabolic alterations generated by dysfunctional mitochondria.

Due to the previously described results, we decided to evaluate parameters that could evidence metabolic modifications. As seen in Figure 2A, the malate-aspartate shuttle activity is decreased in preparations obtained from HCC and CP groups; IFC-305 treatment inhibited the effect of DEN and restored the activity of this shuttle, HCC+IFC-305 and CP+IFC-305, respectively. From the levels of 3-hydroxybutyric acid and acetoacetate we calculated the NAD+/NADH ratio. Mitochondria isolated from the HCC and CP groups had a marked diminution of the
mitochondrial NAD+/NADH ratio. In the HCC+IFC-305 group small protection against this damage was observed and the CP+IFC-305 group reached the control value (Figure 2B).

Because we observed alterations in the NAD+ mitochondrial regulation, we decided to evaluate the activity of two NAD+-dependent enzymes that participate in the TCA cycle, IDH and MDH. As shown in Figure 2C, IDH activity did not change significantly when it was compared to the control group. Nevertheless, treatment with IFC-305 increased the activity of this enzyme compared to the HCC and CP groups, respectively. MDH activity of HCC and HCC+IFC-305 is similar to the control group. In the CP group, the MDH activity decreased but the treatment with IFC-305 tended to reverse this effect (Figure 2D). The impaired IDH and MDH activity suggest an altered TCA function due to DEN administration, IFC-305 treatment improved mainly the IDH activity possibly related with the increase in redox potential.

As mentioned in the Introduction, the experimental model used in this study is associated with HCC and because we found modifications in mitochondrial function, we determined the level of hepatic lactate as an indicator of metabolic adaptation. Chronic administration of DEN increased the amount of lactate in the liver and it did not return to the control level six weeks after the toxic had been suspended as shown in the HCC and CP groups (Figure 3). IFC-305 treatment significantly decreased the level of lactate (HCC+IFC-305) suggesting metabolic adaptation in response to recuperated mitochondrial function.

Another intermediate that depends on mitochondrial energetics is Ac-CoA. DEN administration had no significant effect on the amount of Ac-CoA in both groups, HCC and CP, but IFC-305-treatment significantly increased Ac-CoA levels in the HCC+IFC-305 and CP+IFC-305 groups (Figure 4A) consequently to the recuperation of mitochondrial function. Previous studies showed the ability of IFC-305 to modulate some epigenetic modifications including histone acetylation.
(Rodríguez-Aguilera, 2015). Thanks to this and considering the increased level of this metabolic intermediate, we decided to determine this post-translational modification because Ac-CoA is a required cofactor for histone acetyltransferases. Diminution of $H4ac$ was observed in the HCC and CP groups; an increment trend was observed in HCC+IFC-305 and CP+IFC-305 groups (Figure 4B and 4C), suggesting Ac-CoA availability. This result could be important because it might establish a link between mitochondrial function and epigenetic control of gene expression.

Besides the mitochondrial alterations, other enzyme activities could be modified, dependent, at least in part, on mitochondrial integrity and functionality. Specifically, PARP-1 over-expression and over-activation have been shown to be a characteristic of HCC. The PARP-1 activity is dependent on $NAD^+$ and Figure 5 shows a significant increment in the CP group, whereas the CP+IFC-305 group revealed the IFC-305 ability to decrease it. The over-activation of PARP-1 obtained in the DEN-intoxicated groups could be the cause of the diminution of $NAD^+$ level previously described in Figure 2B.

3.3 IFC-305 treatment was beneficial to avoid and recover the altered mitochondrial dynamics induced by DEN intoxication.

The interplay between the mitochondrion and the nucleus is relevant in the regulation of the mitochondrial response to stress and PGC-1$\alpha$ and Sirt-1 are proteins involved in this process. The nuclear amount of both proteins was increased by DEN treatment, even in the CP group, as compared to the control (Figure 6A and 6B). The pharmacological treatment with IFC-305 in, HCC+IFC-305 and CP+IFC-305, reduced the nuclear content of both, Sirt-1 and PGC-1$\alpha$ suggesting a diminution of mitochondrial stress induced by DEN.
Mitochondrial networks determined by fission and fusion cycle were evaluated through the mitochondrial level of proteins regulating this process. Figure 7 shows the effect of DEN and IFC-305 on mitochondrial localization of three important proteins related to fission and fusion processes. DRP-1, required for mitochondrial fission by pinching them off into smaller fragmented mitochondria, was induced in HCC and CP groups and it was prevented by the hepatoprotector (Figure 7A). Figure 7B shows no significant differences in the MFN-2 content between the experimental groups; however, a tendency to increase this protein in the HCC group can be seen. However, when the DRP-1/MFN-2 ratio was determined (Figure 7C), an important increase in this parameter was observed in the CP group reflecting a predominance of the fission process. This alteration was not observed in the CP+IFC-305 group.

Until now important mitochondrial alterations were observed, but we wanted to know if there were changes in mitochondrial content of Sirt-3, a NAD⁺-dependent deacetylase, that would be indicative of a correlation between mitochondrial dynamics and metabolic function. The progressive damage, observed when the toxicant was suspended, induced a decreased in mitochondrial Sirt-3 (CP group) and the administration of IFC-305 increased Sirt-3 level (CP+IFC-305), even more than the control group (Figure 7D). The up regulation in the presence of the compound could be related to the improved mitochondrial redox state. Mitochondrial modifications prompted us to obtain liver slices to observe the changes in mitochondrial morphology through electron microscopy. The control group depicted mainly elongated mitochondria. Circular shaped mitochondria were abundant in HCC and CP groups (Figures 8B and 8D) and elongated shape was observed in groups receiving IFC-305 (Figures 8C and 8E). As well as the altered mitochondrial shape, altered cristae were observed in the HCC and CP groups, whereas the IFC-305-treated groups maintained and recovered cristae An important observation
is the large amount of ribosomes observed in the slices from the HCC+IFC-305 and CP+IFC-305
groups marked with black arrows (Supplemental Figure 2). To appreciate better these effects, in
Supplemental Figure 2 mitochondria can be observed in an amplified size than in Figure 8. This
observations support the previous observations in mitochondrial functions, metabolism and
dynamic.
4. DISCUSSION

This study shows that mitochondria from the liver of DEN-treated rats underwent alterations resulting in decreased respiratory quotient which means that uncoupled mitochondria with low phosphorylating capacity depicted lower ATP synthesis probably because of the altered $\Delta \Psi_m$ and complex I activity (Table 2). Moreover, ATP synthase plays an essential role in energy metabolism as it is in charge of ATP synthesis; the dimeric and oligomeric ATP synthase complexes increase its stability and promote the formation of mitochondrial cristae (Garcia-Trejo and Morales-Rios, 2008; Couoh-Cardel et al., 2010). The regeneration of the dimeric form of the F1F0 ATPase is an indicator that the mitochondrial energetic machinery and morphology of cristae were recovered with IFC-305 administration to the CP. Previously, the sensitivity of the ETC complex I to the hepatotoxic action of CCl$_4$, ethanol, and DEN had been demonstrated; for DEN, this effect is attributed to the inhibition of NAD$^+$-linked respiration at this site (Cederbaum et al., 1974; Schilling and Reitz, 1980; Boitier et al., 1995). The malate-aspartate shuttle maintains appropriate NADH equivalents, and its intermediates are coupled to the TCA cycle. The activity of this system was diminished in mitochondria from DEN-treated rats (Figure 2A), supporting the idea that mitochondria are the site for DEN-induced damaged. As mentioned before, the treatment with IFC-305 improved the mitochondrial redox state damaged by DEN. Mitochondrial function requires an optimal NAD$^+$/NADH ratio to be efficient. The ability to restore the mitochondrial NAD$^+$/NADH ratio was achieved when the IFC-305 was given after the discontinuation of DEN injection (CP+IFC-305 group) (Figure 2B). The maintenance of this ratio allowed the correct supply of electrons to complex I and the right function of mitochondria. The decreased $\Delta \Psi_m$ in the DEN-treated rats was expected because of the alterations previously mentioned. These results demonstrate relevant mitochondrial function impairment induced by
DEN and the beneficial effects of IFC-305 to maintain the mitochondrial function supporting the previously mentioned adenosine findings which is the base molecule of the compound studied in this work (Hernandez-Munoz et al., 1978).

IDH and MDH are mitochondrial TCA enzymes coupled to the mitochondrial redox potential. IDH activity is decreased in patients with chronic liver injury after several years of alcohol abuse and in rats treated with DEN (Suresh et al., 2013; Popov et al., 2014). We found a tendency to diminish the IDH activity by DEN intoxication and its increased activity by IFC-305-treatment (Figure 2C). This compound might promote the correct function of mitochondrial enzymes by modulating the redox state as we mentioned above. A similar effect was seen in MDH activity in the CP scheme.

The PARP-1 enzyme activity consists in the poly-adenosine diphosphate ribosylation (Poly(ADP-ribosyl)ation) of different substrates and there are evidences of increased PARP-1 expression and auto-poly(ADP-ribosyl)ation in HCC and cirrhosis (Nomura et al., 2000; Shiobara et al., 2001; Shimizu et al., 2004). Here, we demonstrated an increased PARP-1 activity in the liver of rats treated with DEN and this result correlates with the diminution of the NAD⁺/NADH ratio, suggesting depletion in NAD⁺ content. The IFC-305 compound prevented PARP-1 activation by DEN as previously was showed in the presence of adenosine (Jagtap et al., 2004). This result favor the NAD⁺ availability and contributing to the maintenance of the redox state.

Mitochondrial changes affect the cytosolic redox state and, hence, metabolic alterations such as lactate production. It has been suggested the aerobic glycolysis as the main source of energy in cancer (Linehan and Rouault, 2013). In this context, glucose metabolism produces lactate. Here, we found an induction of high levels of lactate with DEN even 6 weeks after intoxication was
stopped. This metabolic adaptation was inhibited and reversed with the IFC-305 compound (Figure 3). Probably the source of energy became glycolysis-dependent because of the altered mitochondrial function observed in the DEN-treated rats; the beneficial effect of IFC-305 treatment could be explained by its ability to maintain mitochondrial integrity.

The synthesis of Ac-CoA, a carbon donor in anabolic reactions, is altered by mitochondrial dysfunction (Wellen and Thompson, 2012). Moreover, it represents the link between pyruvate and the mitochondrial TCA cycle and ETC; thus, it might reflect the mitochondrial energetic supply. Besides its energetic role, Ac-CoA represents availability of the substrate for acetylation reactions; it is important in the nucleus to modulate the gene expression through acetylation of histone lysines in DNA-binding proteins reducing the protein-DNA interaction (Wallace and Fan, 2010). Even though DEN did not significantly change the Ac-CoA level respect to the control group, IFC-305 treatment did change it (Figure 4A); moreover, we observed a tenuous and not significant increased H4ac induced by IFC-305 suggesting that Ac-CoA levels regulated by IFC-305 might affect the mark of histones acetylation (Figure 4B). In cancer cells, Ac-CoA is ectopically synthesized in the nucleus and is involved in cell cycle progression and DNA replication (Comerford et al., 2014). However, our results show Ac-CoA from mitochondria, indicating that the source of this metabolite is from the normal glycolytic flux through pyruvate, β-oxidation, and the catabolism of amino acids. These results confirm IFC-305’s ability to increase the energy status of the liver and to improve the mitochondrial integrity. Possibly, H4ac allowed the transactivation of genes involved in growth and replication, it must be considered that the experimental model used implies cirrhosis, i.e., the whole liver was affected by the administration of DEN. Further studies are necessary to test if replication of healthy hepatocytes
is facilitated in this experimental model by IFC-305, as we previously reported in the CCl₄ model (Chagoya de Sanchez et al., 2012).

The diminished Sirt-3 mitochondrial content obtained with DEN reflects the inability to control the redox state and the metabolic activity. Recently, Sultana et al. demonstrated the protective effect of Sirt-3 on the mitochondrial function probably by the oxidative stress modulation through the mitochondrial antioxidant enzymes such as Mn-SOD (Sultana et al., 2016). Moreover, some authors have demonstrated downregulation of Sirt-3 in human HCC, suggesting that this pattern is associated with differentiation and tumor multiplicity (Xiong et al., 2016). Our results suggest a role of Sirt-3 in the mitochondrial effects of the IFC-305 compound possibly by deacetylation of the ATPase and complex I subunits (Vassilopoulos et al., 2014). Further studies are needed to demonstrate this. Overexpression of Sirt-3 induces apoptosis in HCC culture cells through mitochondrial translocation of the Bcl-2-associated X protein (BAX) (Zhang and Zhou, 2012; Song et al., 2015). The transcriptional co-activator PGC-1α and Sirt-1 are considered important inducers of mitochondrial biogenesis through nuclear-encoded mitochondrial genes. Sirt-1 maintains PGC-1α in a deacetylated active state, promoting its transcriptional activity (Gerhart-Hines et al., 2007). Our results suggest the ability of IFC-305 to inhibit the activation of PGC-1α, at least in part, to maintain the Ac-CoA levels and the decreased nuclear amount of Sirt-1. This effect allows us to explain the lower accumulation of dysfunctional mitochondria. Additionally, Sirt-1 has been linked to tumorigenesis and its depletion inhibits proliferation of HCC cells by cellular senescence or apoptosis (Chen J, 2012). The nuclear localization of both proteins is increased in the rats intoxicated with DEN as compared to the control group (Figure 4). The IFC-305 compound was able to decrease the nuclear content of both, Sirt-1 and PGC-1 α.
It is suggested that biogenesis induced by PGC-1α is tumor promoting, but production of new healthy mitochondria could be tumor suppressive (Boland et al., 2013).

Mitochondria are dynamic organelles to maintain their correct functionality and morphology. DRP1 regulates mitochondrial fission through scission of the outer mitochondrial membrane (OMM). Mitochondrial fusion is regulated by MFN-1 and -2, which facilitates binding of the OMM of different mitochondria (Dhingra and Kirshenbaum, 2014). According to this, small and fragmented mitochondria have been found in mice with reduced levels of MFN-2, but when this protein is restored, the normal mitochondria phenotype is obtained (Liesa et al., 2008). Here, we show that DEN intoxication induces important changes in the DRP-1/MFN-2 ratio 6 weeks after DEN administration was suspended. This alteration indicates a predominance fission over fusion. The mitochondrial morphology observed by electronic microscopy corroborates these results, since those isolated from HCC and CP groups depicted a circular shape instead of the elongated phenotype shown by the control and those receiving IFC-305 treatment. These results support the idea that mitochondrial shape regulates their function and integrity and that the hepatoprotector IFC-305 favors the fusion process promoting the functional mitochondria.

We conclude that chronic DEN intoxication induces mitochondrial alterations favoring the establishment of liver damage resulting in abnormal mitochondrial accumulation and cellular behavior. The damage with DEN had been established for 16 weeks and, according to the results, it continued even after the insult had been stopped. IFC-305 maintains and restores the mitochondrial activity damaged by DEN and probably allows mitochondria to be repaired. The beneficial effects of IFC-305 reveals an integrative effect of the compound between mitochondrion and nucleus. This study demonstrated the mitochondrial impairment through functional, metabolic and dynamic alterations in the sequential model of cirrhosis-hepatocellular
carcinoma and the hepatoprotector IFC-305 helps to repair them, supporting its use as a potential hepatocellular carcinoma treatment or as an adjuvant in chemotherapy.
5. ACKNOWLEDGMENTS

The authors dedicate this work to the memory of Dr. Armando Gómez-Puyou one of initiator of Bioenergetic in Mexico.
6. AUTHORSHIP CONTRIBUTIONS

*Participated in the research design:* Enrique Chávez, Marietta Tuena de Gómez-Puyou and Victoria Chagoya de Sánchez.

*Conducted experiments:* Enrique Chávez, María Guadalupe Lozano-Rosas, Jesús Rafael Rodríguez-Aguilera, Mariana Domínguez-López, Gabriela Velasco-Loyden and Concepción José-Nuñez.

*Performed data analysis:* Enrique Chávez, María Guadalupe Lozano-Rosas and Victoria Chagoya de Sánchez.

*Wrote or contributed to the writing of the manuscript:* Enrique Chávez and Victoria Chagoya de Sánchez.
7. REFERENCES


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8. **FOOTNOTES**

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LEGENDS FOR FIGURES

**Figure 1.** Changes in ATPase activity and determination of the monomeric and dimeric F1F0 ATPase complex in mitochondria. A) Monomer F1F0-ATPase activity quantification at 1, 2, and 24 h in digitonin mitochondrial extracts in gradient blue native gels. Optical density data were normalized taking the control group as 100%. Each bar represents the mean of each group ± SE. a, statistical significant difference vs. control; b, significant difference vs. CP group (p < 0.05). B) Monomer and dimer F1F0-ATPase at 24 h in digitonin mitochondrial extracts from four different samples in blue native gels.

**Figure 2.** Metabolic mitochondrial parameters evaluated in isolated mitochondria from the liver in the different experimental groups. A) Malate-aspartate shuttle (*P<0.05); B) NAD+/NADH ratio (**P<0.001); C) isocitrate dehydrogenase activity (**P<0.01), and D) malate dehydrogenase activity (**P<0.001). Values are expressed as means ± SE. a, significant difference vs. Control; b, statistical significant difference vs. HCC group; c, significant difference vs CP group.

**Figure 3.** Lactate level in the liver as an indicator of metabolic adaptation. Perchloric extracts from the liver were obtained to determine the levels of lactate. Values are expressed as means ± SE. a, significant difference vs. Control; b, statistical significant difference vs. HCC group; c, significant difference vs. CP group (**P<0.01).

**Figure 4.** Changes in the mitochondrial amount of Ac-CoA might reflect mitochondrial dysfunction and epigenetic changes. A) Ac-CoA determined with a commercial kit in mitochondria isolated following manufacturer’s instructions (**P<0.001). B) Densitometric analysis of the acetylated H4, western blot normalized respect to H4 (*P<0.05). C)
Representative western blot of B). a, significant difference vs. Control; c, statistically significant difference vs. CP group (p < 0.05).

**Figure 5.** Enzymatic activity of PARP-1, a NAD⁺-dependent enzyme. This parameter was determined in liver slices through the consumption of NAD⁺ in presence of DNA previously activated. Values are expressed as means ± SE. a, statistically significant difference vs. Control; c, significant difference vs. CP group (**P<0.01).

**Figure 6.** Changes in the nuclear amount of proteins implicated in mitochondrial biogenesis. A) PGC-1α and B) Sirt-1; the bars correspond to the densitometric analysis. Values are expressed as means ± SE. a, significant difference vs. Control; b, significant difference vs. HCC group; c, significant difference vs. CP group (**P<0.001). C) Representative western blot of A) and B).

**Figure 7.** Level of proteins related to the mitochondrial dynamics determined by western blot. A) DRP-1 (*P<0.05), B) MFN-2, C) DRP-1/MFN-2 ratio (*P<0.05), and D) Sirt-3 (**P<0.001) (*P<0.05). The bars correspond to the densitometric analysis. Values are expressed as means ± SE. a, significant difference vs. Control; b, means significant difference vs. HCC group; c, means significant difference vs. CP group. E) Representative western blot of each protein.

**Figure 8.** Morphologic changes evaluated through electron microscopy. Each panel is representative of each group. Liver slices were fixed with glutaraldehyde and stained with osmium tetroxide. The square in the right inferior border represents a magnification of a mitochondrion. A) Control; B) HCC; C) HCC+IFC-305; D) CP; E) CP+IFC-305.
Table 1. Experimental groups design.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>16 weeks</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCC</td>
<td>DEN + saline solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCC+IFC-305</td>
<td>DEN + IFC-305</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>DEN</td>
<td></td>
<td>Saline solution</td>
</tr>
<tr>
<td>CP+IFC-305</td>
<td>DEN</td>
<td></td>
<td>IFC-305</td>
</tr>
</tbody>
</table>

Five experimental groups (n=6) were formed as indicated. HCC (hepatocellular carcinoma), CP (cancer progression). The control group received saline solution only. DEN was administered at 50 mg/kg i.p., once a week for 16 weeks and the IFC-305 compound at 50 mg/kg i.p. 3 times weekly during the DEN intoxication (HCC+IFC-305) or 6 weeks after DEN administration was stopped (CP+IFC-305).
Table 2. Effect of DEN and IFC-305 administration on the mitochondrial respiratory quotient, ATP synthesis, complex I activity, and membrane potential.

<table>
<thead>
<tr>
<th>Group</th>
<th>State III/State IV</th>
<th>ATP synthesis (nmol/min/mg)</th>
<th>Complex I activity (µmol/min/mg)</th>
<th>ΔΨm (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.39 ± 0.62</td>
<td>171.60 ± 22.01</td>
<td>20.16 ± 3.03</td>
<td>163.77 ± 11.17</td>
</tr>
<tr>
<td>HCC</td>
<td>2.98 ± 1.43a</td>
<td>100.27 ± 24.61a</td>
<td>1.62 ± 0.76a</td>
<td>58.29 ± 7.19a</td>
</tr>
<tr>
<td>HCC+IFC-305</td>
<td>6.13 ± 0.55b</td>
<td>173.46 ± 17.27b</td>
<td>4.46 ± 1.30a</td>
<td>100.55 ± 13.94ab</td>
</tr>
<tr>
<td>CP</td>
<td>4.86 ± 1.39a</td>
<td>125.02 ± 4.18a</td>
<td>6.96 ± 2.17a</td>
<td>67.01 ± 8.46a</td>
</tr>
<tr>
<td>CP+IFC-305</td>
<td>7.80 ± 1.25c</td>
<td>174.94 ± 12.81c</td>
<td>17.57 ± 3.70c</td>
<td>102.19 ± 15.65ac</td>
</tr>
</tbody>
</table>

Isolated mitochondria were used to determine the respiratory quotient with glutamate plus malate as substrates (*P<0.05), complex I activity (**P<0.01), and mitochondrial membrane potential (ΔΨm) (**P<0.001). ATP synthesis was calculated from data obtained in the oximetry analysis in the presence of glutamate plus malate (state III X ADP/O) (*P<0.05). Values are expressed as mean ± SE.

a. significant difference vs. control; b, significant difference vs. HCC; c, significant difference vs. CP.
Figures

Figure 1.

A) % of monomer

<table>
<thead>
<tr>
<th></th>
<th>1h</th>
<th>2h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>a</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>CP-IFC</td>
<td>a,b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) Dimeric F1F0 complex
Monomer F1F0 complex

Control    CP    CP-IFC
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.

A) Control  B) HCC  C) HCC+IFC-305

D) CP  E) CP+IFC-305
SUPPLEMENTAL METHODS

Coomassie staining. First dimension was stained with Coomassie solution (0.1% Coomassie Blue R-250, 45% methanol, and 10% glacial acetic acid), then stirred at room temperature for 2 h and discolored with a solution containing 30% methanol and 10% glacial acetic acid. The gel was scanned and monomer bands were analyzed by using the Image J software.

Second dimension. The Coomassie gel was denatured with a solution containing 1% SDS, 5 mM DTT for 1 h. The band containing the monomer was cut off from the Coomassie gel with a razor blade. The band was rotated 90° and placed on 15% Schägger and von Jagow-SDS-PAGE and electrophoresis was performed at 70 V for 2 h and 100 V for 8 h at 4°C. The second dimension gel was dyed and de-stained as mentioned above for Coomassie gel. The gel was scanned and α+β subunits were analyzed by using the Image J software.
Supplementary Figure 1.

Supplementary Figure 2.

A) CONTROL  
B) HCC  
C) HCC+IFC-305

D) CP  
D) CP+IFC-305
SUPPLEMENTARY LEGEND TO THE FIGURES

Supplemental Figure 1. Monomer quantification in the blue gel, Coomassie staining and, αβ subunits content. Optical density data were normalized taking the control group as 100%. Each bar represents the mean of each group ± SE. a, statistical significant difference vs. control; b, significant difference vs. CP group (p < 0.05).

Supplemental Figure 2. Morphologic changes evaluated through electron microscopy. Each panel is a magnified mitochondrion and is representative of each group. Liver slices were fixed with glutaraldehyde and stained with osmium tetroxide. A) Control; B) HCC; C) HCC+IFC-305; D) CP; E) CP+IFC-305.