Di (2-ethylhexyl) phthalate facilitates the progression of alcoholic fatty liver via mediating oxidative stress injury and lipid peroxidation in rats

Qiang Xu
Anhui Medical University
Ke Ji
Anhui Medical University
Zongbiao Zhao
Anhui Medical University
Song Huang
Anhui Medical University
Xiang Zhang
University of Science and Technology of China
Weiping Xu
University of Science and Technology of China
Wei Wei (wwei@ahmu.edu.cn)
Anhui Medical University

Research Article

Keywords: Alcoholic fatty liver, DEHP, oxidative stress, steatosis, CYP2E1, SIRT1

Posted Date: November 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-109220/v1

Di (2-ethylhexyl) phthalate facilitates the progression of alcoholic fatty liver via mediating oxidative stress injury and lipid peroxidation in rats

Qiang Xu [a, #], Ke Ji [a, #],Zong-biao Zhao [a], Song Huang [a], Xiang Zhang [b], Wei-ping Xu [b,*], Wei Wei [a,**]

[a] Institute of Clinical Pharmacology of Anhui Medical University, Key Laboratory of Anti-inflammatory and Immune Medicine of Education Ministry, Anhui Collaborative Innovation Center of Anti-inflammatory and Immune Medicine, Hefei, 230032, Anhui, China.

[b] The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Anhui Provincial Key Laboratory of Tumor Immunotherapy and Nutrition Therapy, Hefei, 230001, Anhui,China.

#Contribute equally to the manuscript.

*Corresponding author. Tel.: +86 0551 6228 3106; Fax: +86 0551 6228 3106

**Corresponding author. Tel.: +86 0551 6516 1209; Fax: +86 0551 6516 1209

E-mail addresses: xu_weiping666@163.com (W-P Xu) and wwei@ahmu.edu.cn (W. Wei).

Abstract

***Background*:** Long-term excessive drinking can cause various harms to the human body. Alcoholic fatty liver is the manifestation of early liver lesions mainly induced by alcohol. Di-(2-ethylhexyl) phthalate, as one of the environmental internal-secretion interfering-substance, is widely used in industrial processing and various consumer goods. Large quantities of epidemiological investigations and studies have shown that frequent exposure to high concentrations of DEHP may be a potential risk factor for liver function.

Methods: We used DEHP to expose alcohol-induced fatty liver rats to study the effect of DEHP on alcoholic fatty liver.

***Results*:** The results found that DEHP exposure prominently accelerated hepatic steatosis, inflammation and oxidant stress, and activates signaling pathways involved in liver inflammation and oxidative stress-related proteins expression. Studies have suggested that cytochrome P450 2E1 and silent information regulator-1 are closely

related to the occurrence of alcoholic fatty liver disease. Interestingly, the trend observed in the LO-2 cells assay was consistent with the in vivo conditions. ***Conclusions*****:** DEHP may promote or aggravate the progression of alcoholic fatty liver disease through CYP2E1, SIRT1 and p38 mitogen-activated protein kinase (p38MAPK) / nuclear factor-kappa B signaling pathways. And this experimental study warns us that the environmental pollutants DEHP and its potential toxicity hazards to the human body are worthy of attention.

Keywords: Alcoholic fatty liver, DEHP, oxidative stress, steatosis, CYP2E1, SIRT1

1. Introduction

At present, phthalate (PAE) is currently the most commonly used plasticizer. As a kind of polymeric material additives, it has been widely used in industrial production[1]. Plasticizer mainly includes phthalates, aliphatic dibasic acid esters and fatty acid esters etc [2]. Phthalates have become the main component of plasticizers in the past decade; PAE is found in plastics, concrete, packaged goods, cosmetics and some medical equipment, especially in PVC plastics, content of which accounts for 50% of the product sometimes[3]. Evidence shows that the content of plasticizers in water resources, atmosphere and soil exceeds the standard by 100 fold[4]. DEHP is one of the most common members of phthalate family in society, and its toxicity is known to be among the most detrimental to human's health. Since the exposure of Taiwan's "cloud-forming agent " incident on May 24, 2011, the toxicity of DEHP has attracted the attention of the scientific community. Studies have confirmed that DEHP is an environmental internal-secretion interfering-substance, which has different degrees of effects on reproductive, digestive, respiratory, nervous and immune systems[5, 6]. An investigation has revealed that the harm of long-term DEHP exposure to human health has become the focus of attention in recent times.

Alcoholic liver disease, is a general term for alcohol-related liver diseases, which begins with alcoholic steatosis (fatty liver), alcoholic hepatitis developing to alcoholic liver fibrosis and ultimately leading to alcoholic liver cancer[7]. It is reported that the number of people who died of alcohol abuse in the United States has increased year by year, with nearly 200,000 deaths attributed to ALD in 2010. And fatty liver disease caused by excessive drinking is not only the earliest manifestation, but also the starting point and foundation for subsequent alcohol-related liver damages. The liver, as the main metabolic organ of alcohol, is also the primary target organ for alcohol

damage[8]. What is more, most alcoholics tend to develop fatty liver. Although some advances have been made in the exploration of the complex mechanisms of alcohol-induced fatty liver (involving lipid accumulation, inflammation and oxidative stress) in cellular systems and animal models, but there is still scanty information about the detailed mechanisms[9].

A gene knockout experiment implied that the production and development of chronic ethanol-deficient fatty liver (AFLD) is closely related to the SIRT1-Lipin-1 signaling pathway[10]. SIRT1, a NAD-dependent protein deacetylase, is a member of the sirtuin protein family that regulates hepatic lipid metabolism, inflammation, and oxidative stress by modifying histones and transcription factors[11]. It has been proved that SIRT1 is a crucial target of ethanol in liver and plays an important role in the formation and development of AFLD[12]. Long-term exposure in ethanol will directly curb the expression of SIRT1, which stimulates fat production and reduces fatty acid β oxidation, causes inflammatory reactions, increases reactive oxygen species etc[10, 13, 14].

Similarly, during alcohol metabolism, ethanol is firstly metabolized by alcohol dehydrogenase (ADH) in the cytoplasm and CYP2E1 in the endoplasmic reticulum to form acetaldehyde, then rapidly metabolized to acetic acid by mitochondrial aldehyde dehydrogenase (ALDH). Clinical studies postulate that the pathogenesis of alcoholic liver disease has a close relationship with the expression of CYP2E1[15]. The induction of CYP2E1 by ethanol can aggravate the liver toxicity of ethanol metabolites, change the window of hepatic endothelial cells, leading to increased hepatic cells fat uptake and promote fatty formation of liver[16]. Meanwhile, CYP2E1 is considered to be one of the major contributors to oxidant stress-related ROS production during ethanol metabolism. CYP2E1 enhances local in mitochondria and cellular oxidative stress and serves as an important pathogenic agent in alcoholic liver disease[17].

An anterior study suggested that long-term accumulation of DEHP *in vivo* might affect the balance of oxidation and anti-oxidation, promote inflammation, and even destroy cells or organs[18]. It is found that DEHP may aggravate non-alcoholic fatty liver disease (NAFLD) by regulating the activity of PPAR α and SREPP-1c[19].Furthermore, it is showed that high-doses DEHP exposure can mediate liver oxidation damage by up-regulating the levels of TGF-β1/Smad and p38MAPK/NF-κB, thereby aggravating liver fibrosis development[20]. To sum up,

DEHP exposure has been demonstrated to be a risk of stimulating hepatotoxicity or chronic liver injury. Thus far, there has been scanty research on the toxic effects of DEHP on alcoholic fatty liver, hence, the purpose of the current study is to investigate whether DEHP can promote or accentuate alcohol-induced fatty liver and further explore possible molecular mechanisms.

2. Materials and methods

2.1 Animals and experimental design

SPF (Specific Pathogen Free) adult male SD rats (weight 160-180g) were obtained from Experimental Animal Center of Anhui Medical University. The study was approved by the Animal Experimental Ethics Evaluation Committee of the Institute of Clinical Pharmacology, Anhui Medical University. All methods were performed in accordance with the relevant guidelines and regulations. At a temperature of 18-26 °C and a relative humidity of 40-70 %, the animals were kept in 12 hour cycle of dark and light with free access to chow and water. After approximately 7 days of adaptation, SD rats were randomly assigned to 6 groups: Group 1(normal), saline gavage and normal chow diet; group 2 (alcoholic fatty liver model), moderate high-fat diet and white wine by gavage; Groups 3, 4 and 5 (DEHP exposed groups), were fed with moderate high-fat diet, white wine and exposed to DEHP (0.05, 5, 500 respectively) mg/kg by gavage; group 6, only DEHP 500 mg/kg was given intragastrically. DEHP was mixed with corn oil and given at 9:00 a.m. while liquor was given at 3:00 p.m. The experiment lasted for 8 weeks. In week 9, SD rats were deprived of food for 12-24 h. After anesthesia with ether, blood and liver samples were collected for further studies.

Based on previous findings, the proportion of high-fat diet selected for the current study shortens the formation time of alcoholic fatty liver[19]. We chose three doses of 0.05, 5, 500 mg/kg to study the effect of DEHP on alcoholic fatty liver toxicity which correspond to daily exposure, high risk job exposure, and hepatotoxic dose. The high, medium and low doses were in line with the "real world" range of DEHP concentrations [20].

2.2 Body weight and corresponding liver index of SD rats

Body weight of rats was checked once a week during the experiment. Based on ethics, the body weight of SD rats was first weighed, before the liver was quickly dissected and washed with cold PBS and weighed. Finally, a portion of the liver tissue was fixed with 4 % paraformaldehyde for routine histopathology, and the remaining

liver samples were stored at -80 °C until needed.

2.3 AST and ALT activities in serum samples

Rats'blood were taken and allowed to stand at 4 °C for 2 h. Blood was then centrifuged at 2500 rpm for 15 min at 4 °C. The supernatant (serum) was harvested and store at -20 °C. Determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities by microplate was by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4 Analysis of hepatic lipid content

Liver tissues homogenate were collected and triglyceride (TG) levels were determined using the GOP-PAP-triglycerid kit (Nanjing Jian cheng Bioengineering Institute, Nanjing, China).

2.5 Liver histopathological analysis

The liver tissue was immersed in 4% paraformaldehyde for one week, and after dehydration treatment, it was embedded in paraffin to prepare a 5 μm thick section. Pathological sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT) for routine morphological examination of the tissue. In addition, pathological section was one of the "golden indicators" for evaluating rat's lipid accumulation and collagen fiber infiltration.

2.6 Assaying of oxidative stress indicators in liver tissue

Weighing the appropriate amount of liver tissue, then add 0.9% physiological saline (1:9, w: v) for 15 minutes to grind into homogenate. Homogenates were centrifuged (2500 r/min, 15 min, 4 °C) and the supernatant was extracted. The hepatic homogenate supernatant was assayed for SOD activity, and the content of MDA was determined by the thibabituric acid (TBA) method. Determination of total protein concentration of the sample was carried out by bicinchoninic acid (BCA) method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7 Distribution and expression of CYP2E1 and SIRT1 proteins were detected by laser scanning confocal microscopy (LSCM)

Firstly, the paraffin sections were heated at 60 °C for 2 hours, then dewaxed and hydrated in graded xylene and ethanol, and washed three times with PBS. Secondly, it was with 0.5% Triton X-100 for 30 minutes, covered with EDTA antigen repair solution for 10 minutes, and the sections were blocked in 3% BSA for 1 hour at room temperature, and then the anti-rabbit CYP2E1 and anti-mouse SIRT1 antibody were incubated at 4 °C, over night. After rewarming from 4 °C, the sections were washed 3

times with PBS for 3 minutes each time, and the sections were incubated with goat anti-mouse-Alexa Fluor 594 and goat anti-rabbit-Alexa Fluor 488 secondary antibody for 1 hour at 25 °C in the dark. Finally, DAPI (Beyotime) counterstained the nuclei for 10 minutes, washed three times with PBS, and mounted with a fluorescent quencher and observed with LSCM.

2.8 Western Blotting

In the first step, a mixture of RIPA containing a protease inhibitor and a phosphatase inhibitor was used to dissolve liver tissue and cells and the proteins were extracted. Secondly, protein levels were detected by a BCA protein quantification kit (Biyuntian, China). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, Massachusetts, USA) on Western Blot technology. The PVDF membrane was incubated with the corresponding primary antibody for 15 hours at 4 °C with 5% skim milk. Finally, the second antibody was incubated and the corresponding protein expression was detected by Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.9 In vitro

The normal human hepatic stellate cell line (LO-2) was obtained from China Cell Culture Center (Shanghai, China). The cells were cultured in DMEM medium (HyClone, USA) containing 10% fetal bovine serum (FBS), with simultaneous addition of 100 units/ml penicillin and 100 μg/ml streptomycin inhibits bacterial growth. Cells were incubated at 37 °C and 5% CO_2. After the cells were cultured to logarithmic growth phase, each test was carried out.

2.10 CCK-8 cell viability assay

LO-2 cells were seeded in 96 well cell culture cluster at a density of 2000-3000 cells per well and incubated for 8 hours in DMEM containing 10% FBS. And LO-2 cells were divided into 8 groups: normal group, anhydrous ethanol group (100 mM/L), DEHP (6.25, 12.5, 25, 50, 100, 200 μmol/L) and ethanol groups. Each group had 5 duplicate wells. Detection of the effect of DEHP on cell proliferation was by the CCK-8 kit. After 48 hours of treatment, 10 μL of CCK-8 solution was added to each well, and the cells were continuously cultured at 5% CO_2 and 37 °C for 1-3 hours. The optical density (OD) of each well was measured every 0.5 hours at a test wavelength of 450 nm.

2.11 Lipid accumulation experiment of LO-2 cells

Well-growing LO-2 cells were spread out in six well cell culture cluster and

incubated for 12 hours[21]. The lipid accumulation model *in vitro* was established by using anhydrous ethanol (100 mM/L) combined with oleic acid (90 μM/L) for 48 hours[22]. Simultaneously, different concentrations of DEHP were added to assess the effect of plasticizer on lipid accumulation. Each group was stained with Oil Red O staining solution, which the lipid accumulation of each group was observed and evaluated by optical microscope.

2.12 Detection of cellular ROS levels

To examine the oxidative stress damage of DEHP to LO-2 cells, intracellular ROS levels were determined by flow cytometry using a reactive oxygen species assay kit. By entering the cell with the fluorescent probe DCFH-DA, the ROS in the cell would generate fluorescent DCF from the non-fluorescent DCFH. The oxidative fluorescence intensity mediated by the probe is used to determine the production of reactive oxygen species in the organelles and cytoplasm. Six-well cell culture plates were divided into six groups (normal group, ethanol 100mM/L group, ethanol plus DEHP 6.25, 25, 100 μmol/L three groups, DEHP 100 μmol/L control group), and LO-2 cells were treated with stimulant for 48 hours. After the treatment, more than 1 ml of 10 μmol/L DCFH-DA probe was added to each well for 30 minutes. Then, the DCF fluorescence intensity was measured using a flow cytometer (BECKMAN COULTER, INC. 4300 N. HARBOR BLVD FULLERTON, CA 92835 USA) under the parameter setting of FITC.

2.13 Statistical analysis

The experimental data was presented as mean ± SD, meanwhile, a histogram is drawn using Graphpad Prism, v 6.0. And SPSS 16.0 software is used for statistical analysis of multiple data of each group. Comparisons between experimental groups were analyzed by one-way ANOVA and Duncan's test. Values of $P < 0.05$ were considered to have significant statistical differences.

3. Results

3.1 Effects of DEHP on body weight and liver index in rats

As shown in Fig. 1A, the body weight of the alcohol group was significantly lower than that of the normal group from day 7 to day 56 as time progressed. In the alcohol+DEHP 5 mg/kg group and alcohol+DEHP (500 mg/kg) group showed a significant decrease in body weight compared with the alcohol group from 35 day. Nevertheless, there was no significant change in body weight of rats with alcohol+DEHP 0.05 mg/kg and DEHP control group. We examined the effect of

DEHP on rats liver index (Fig. 1B). Compared with the normal group, the results indicated that the hepatic index of the alcohol group and the DEHP control group was significantly higher; and the liver index of the alcohol+DEHP 500 mg/kg group was higher than that of the other alcohol groups.

3.2 Effects of DEHP on serum AST and ALT activities

The activity levels of AST and ALT in serum of rats belonged to the biochemical indexes of liver injury that reflected the basic status of liver function. In Fig. 1(C, D), the alcoholic fatty liver was induced by alcohol plus high fat diet. Compared with the normal group, serum AST and ALT activity levels were significantly increased in the alcohol group and the DEHP control group. Compared with the alcohol group, the ALT level increased significantly with the dose of DEHP exposure; AST levels increased significantly at the high dose of DEHP.

3.3 Effects of DEHP on TG content and lipid accumulation in hepatic of rats with alcoholic fatty liver

To probe into the effect of DEHP on triglyceride metabolism in liver, we measured liver triglyceride (TG) levels (Fig. 2B). Compared with the alcohol group, the liver of triglyceride levels were markedly increased after exposure to medium and high doses of DEHP; there was no marked change in the DEHP control group compared with the normal group. At the same time, rat liver sections were stained with oil red O dyeing liquid (Fig. 2A). Histopathological examination revealed that there were occasional lipid accumulation in the liver cells of the normal group, and partial lipid piled up in alcohol group and DEHP control group; hepatocytes of the alcohol combined with DEHP (5,500 mg/kg) groups were filled with a considerable amount of lipids, among which the accumulation of lipids was the highest and most severe in the plasticizer high dose group.

3.4 Effects of DEHP on MDA content, SOD vitality in chronic alcoholic liver

SOD was an active agent that could eliminate harmful substances in the metabolism of organisms. It had the function of scavenging oxygen free radicals to protect cells from oxidative damage. Oxygen free radicals were produced by the body could attack polyunsaturated fatty acids in biofilms, trigger lipid peroxidation and finally form lipid peroxides. Pharmacologically, the consequence of MDA and SOD were often combined to reflect the severity of oxidative stress damage in living organisms.

Our result as demonstrated in Fig. 2(C, D) shows that white wine combined with

the high-fat diet group increased MDA and notably reduced the activities of SOD compared to the normal group. However, the deterioration tendency was exacerbated in high dose of DEHP exposure. Long-term exposure to large quantity of DEHP led to a severe imbalance between oxidation and antioxidant systems in the liver, which could damage liver cells through oxidative stress pathways.

3.5 The effect of DEHP on liver and pathological changes in rats

Fig. 3 shows the histopathological examination of hematoxylin-eosin (H&E) under microscope. In Fig. 3A, the liver was mostly dark red and the smoothness decreased except for normal group. Moreover, many fine particles were visible on the surface of the liver. In Fig. 3B, there was no significant lesion manifestation in the normal group. In the DEHP control group, inflammatory cells aggregated, and coarse particles were deposited in the cytoplasm. In the model and the low dose group of DEHP, the volume of intracellular fat vacuoles increased and the amount was increased. It could be seen that the large fat vacuoles squeezed the nucleus to one side and the nucleus became smaller. The diagnosis shows fatty liver formation, indicating that the alcoholic fatty liver model was successfully established. The nucleolus of hepatocytes in the DEHP 5 mg/kg group was different in size, and a large amount of coarse particles were deposited in the cytoplasm (suspected for a large amount of lipid deformation and accumulation), and infiltration of inflammatory cells around the blood vessels accompanied by mild fibrosis. Special of DEHP 500 mg/kg combined with alcohol group, reveals nuclear fragmentation and dissolution, cytoplasmic loose cytoplasmic lysis; a large number of collagen fibers around the blood vessels infiltrated outward, showing mild and moderate liver fibrosis, massive cell necrosis accompanied by inflammatory infiltration. Thus, long-term exposure to high doses of DEHP might aggravate liver lipid accumulation and inflammation. The masson trichrome staining (MT) was analyzed under an optical microscope. Fig. 3C shows that the model group, DEHP 0.05 mg/kg and DEHP 5mg/kg groups were suspected to have mild liver fibrosis characteristics. The DEHP control group exhibited inflammatory cells clustering around blood vessels. On the contrary, DEHP 500 mg/kg+alcohol group had a surge of inflammatory cells and collagen deposits around the blood vessels, and typical burr-like collagen fibers radiate around. There was a clear presence of moderate hepatic fibrosis.

3.6 Long-term exposure of DEHP, to detect changes in CYP2E1, SIRT1, P-p38, P-p65 protein expression in liver tissues and LO-2 cells

Long-term heavy drinking causes liver lipid production and metabolic imbalance, and fat accumulation around the liver cells gradually forms alcoholic fatty liver. Recent studies have demonstrated that CYP2E1 and SIRT1 play a key role in chronic ethanol-induced fatty liver disease. In the current study, we sought to find out whether CYP2E1 and SIRT1 play a role in alcohol consumption-induced AFL. Also, the effect of DEHP on the expression of CYP2E1 and SIRT1 protein in fatty liver induced by ethanol combined with high fat-diet was investigated. Results from Fig 4 depicts that there was a conspicuous change in CYP2E1, SIRT1, P-p38 and P-p65 levels in model group and DEHP control group compared to normal group. Furthermore, compared with model group, the expression levels of CYP2E1, P-p38 and P-p65 proteins were up-regulated by DEHP medium and high doses treatments, especially in high doses. As shown in Fig. 4B, there was a statistically significant difference in SIRT1 protein expression between the DEHP 500 mg/kg+alcohol group and the model group. The results of Fig. 5 represents tissue immunofluorescence pattern of CYP2E1 and SIRT1 as photographed by laser scanning confocal microscopy (LSCM). It can be seen that the fluorescence intensity of CYP2E1 protein markedly increased with the increase of DEHP doses, and there was no conspicuous change in the expression of SIRT1 protein in cytoplasm.

In order to verify changes in protein levels, normal liver cell LO-2 was used for in vitro western blot experiments. The results of Fig. 7B by cck-8 kit revealed that low concentration of DEHP had no effect on cell proliferation, and higher concentration of DEHP significantly inhibits cell viability. The research team selected low, medium and high three concentrations of DEHP (6.25, 25, 100μmol/L) for in vitro studies. As shown in figure 6, there were statistically arresting differences between the normal group and the model group. After treatment with DEHP 25 and 100μmol/L, the expression of CYP2E1, P-p38 and P-p65 increased, and SIRT1 decreased. This suggests that high doses of plasticizers might accelerate the development of these trends. Combined with animal experimental data, the changes of protein levels in vivo and in vitro were basically consistent.

3.7 DEHP accelerated lipid accumulation and intracellular ROS production in LO-2 cells

Fluorescence flow cytometry (DCFH-DA) was used the changes of ROS content in LO-2 cells after DEHP exposure. In Fig. 7A, the peak fluorescence of ROS fluorescence in ethanol-stimulated LO-2 cells was shifted to the right compared to the

normal group. Fluorescence intensity was enhanced with increasing dose of DEHP exposure, especially in the DEHP (25, 100μmol/L) dose group. *In vivo* experiments (Fig. 2A) insinuated that DEHP promoted intracellular fat accumulation. Lipid accumulation model *in vitro* was established by absolute ethanol combined with oleic acid stimulation, followed by different concentrations of DEHP. After oil red O staining, red lipids assembled around the cell membrane under an inverted microscope As DEHP exposure augmented, so did the aggregation of lipid. In the most severe group, red lipids were more widely deposited and had the most lipids content (Fig. 8).

4. Discussion

PAEs participate in the formation of polymer and non-polymer products which have unstable chemical properties when combined[20], and are transferrable to human life environment, such as: industrial production, heating, packaging, filling process etc. More importantly, with the development of material civilization, the abuse of PAEs are especially serious. It is noteworthy that the contamination of DEHP had developed into a "global" catastrophe. Evidence suggests that long-term intake of "estrogen-like hormones" (DEHP) could affect human reproductive development[23, 24], induces obesity, place a premium on asthma[25], produces toxicity in multiple organs such as liver and kidney, disturb the balance of glucose metabolism[26], of which reproductive toxicity and hepatotoxicity are the main concerns[27]. However, the pharmacological mechanism of DEHP causing hepatotoxicity remains unclear. The short-term toxicity of DEHP may not be obvious when it enters the human body through liver metabolism, but sundry toxicities may appear over time and accumulation of toxicity persists. It is emphasized that the toxicity of DEHP can be amplified or participated in the process of disease occurrence if the liver is in a certain disease state or in the stage of disease development and occurrence. In this research, we have linked DEHP to the formation of alcoholic fatty liver to explore the effects of DEHP on AFLD.

Basically, our study focused on 6 concentrations of DEHP to assess the activity of LO-2 cells *in vitro*[21], and found that from the dosage of 50 μM/L, DEHP significantly inhibited the proliferation of LO-2 cells, suggesting the existence of cytotoxicity induced by DEHP[28]. Subsequently, the relationship between DEHP and AFLD was studied by using the corresponding high, medium and low concentrations (6.25, 25, 100 μM/L). Weight, liver index and ALT/AST were objective indexes for evaluating liver status in rats. According to the results of classical biochemical

indicators, serums ALT and AST levels in SD rats increased significantly under the influence of plasticizer, and the increase of liver index was accompanied by weight loss, which reflected that the rats might be in a state of severe liver injury (Fig. 1). In contrast, DEHP exposed aggravates the damage of AFLD. It is well known that fatty liver caused by chronic alcohol consumption was inseparable from lipid accumulation in hepatocytes[9]. Abundance of TG accumulation in hepatocytes is one of the necessary conditions for the formation of fatty liver. Studies have shown that plasticizers (such as DEHP) may interfere with normal lipid metabolism and destroy the secretion of adipocytes, closely related to lipid metabolism disorders and energy metabolism instability[29, 30]. In general, triglyceride (TG) levels in liver homogenates were usually measured to determine whether there was abnormal lipid metabolism. In this research, after treatment with DEHP and alcohol, the TG content in the liver of the corresponding rats increased significantly, which was consistent with the previously reported results that DEHP might cause disorders of fat metabolism. Combined with the results of lipid accumulation test of LO-2 cells *in vitro*, it was observed that as the concentration of DEHP increases, not only the area of red lipid accumulated near the cells membrane was enlarged, but also accumulated lipid were more extensive. These findings confirm that plasticizer intake could induce lipid accumulation in hepatocytes and might play a role in the formation of AFL. Afterwards, the analysis of H&E pathological sections at week 8 showed that the number of lipid droplets in the treated group increased, but the volume became smaller compared with the model group, which was contrary to the anticipated ideas. Although no increase in the volume of fat vacuoles in plasticizer group was observed in pathological sections, it was not that DEHP could alleviate the accumulation of fat in alcoholic fatty liver. It was undeniable that the inflammation and lesion damage of cells in tissues gradually exasperated at the doses of DEHP (5 mg/kg, 500 mg/kg), especially DEHP 500 mg/kg combined with alcohol group even showed liver fibrosis and cells necrosis in the eighth week. Moreover, the results of oil red O staining of rat liver slices in eighth week hinted that the area and extent of intracellular lipid accumulation after DEHP exposed were substantially enlarged than of the alcohol group. Although the fatty liver under pathological examination was typically characterized by the presence of bulky fat vacuoles in the cells, there were also manifestations of diffuse small lipid droplets that were widely distributed and sometimes more severe than classical fatty liver.

The important role of oxidative stress injury in the pathogenesis of acute and chronic ALD has been widely recognized by the scientific community. Typical characteristics of oxidative stress are imbalance of endogenous and exogenous levels of reactive oxygen species (ROS) and disorder of antioxidant system (SOD and MDA), which induce lipid peroxidation, especially when lipid droplets occupy the cytoplasm, hepatocytes are more susceptible to oxidative stress[31, 32]. It was found that liver lipid metabolism was accelerated and ethanol-mediated oxidative stress was alleviated, thereby protecting liver of rats from damaging by ethanol[33]. Recent studies suggest that DEHP may induce organ oxidative stress by interfering with the defense mechanism of Nrf 2 signaling pathway or by altering its downstream gene transcription[34, 35]. A large amounts of data believed that DEHP may cause apoptosis via ROS and oxidative stress[36]. On the one hand, Long-term alcohol intake or being in the state of alcoholism, impaired liver metabolic function, imbalance of steady-state system of lipid synthesis and metabolism in hepatocytes, accumulation of intracellular lipid droplets, excessive production of reactive oxygen species, oxidative stress injury and lipid degeneration might be the decisive factors to generate alcoholic fatty live disease[37]. As demonstrated In Fig. 2(C, D), the SOD activity of liver homogenate decreased and the MDA content increased significantly after ingestion of DEHP 500 mg/kg, suggesting that DEHP enhanced alcohol-induced hepatic oxidative stress. Besides, fluorescence flow cytometry (DCFH-DA) analysis of ROS fluorescence intensity in LO-2 cells *in vitro* found that the DEHP (5,500 mg/kg) groups memorably activated the level of ROS in ethanol group (Fig. 7A). Both results further certify that DEHP exposure in DEHP exacerbated the imbalance of AFL oxidation and antioxidant systems, and the oxidative stress mediated by positive feedback was aggravated. That is to say, the promotion or aggravation of DEHP on AFL may be closely related to oxidative defense. On the other hand, excessive alcohol consumption could promote the formation of new hepatic fat and accelerate the deterioration of fatty liver. Ethanol and its metabolites directly damage hepatocytes, producing an inflammatory response and aggravating liver damage[38]. It has been widely recognized that P38MAPK and p65 proteins were involved in regulating various inflammatory signaling pathways, and their activation not only stimulated the production of pro-inflammatory mediators (including IL-6 and TNF-α), but also affect oxidative stress and important functions in inflammatory cytokines[39, 40]. It was found that DEHP could facilitate the secretion of inflammatory factors

IL-6 and TNF-α, interfere with lipid metabolism, and significantly up-regulate pro-inflammatory cytokine levels and improve HSCs via p38MAPK/NF-κB signaling pathway to aggravate liver fibrosis[20]. In our study, the degree of inflammatory induced by alcoholic fatty liver was associated with the expression of P-p38 and P-p65 protein, and the degree of damage was more severe with increasing exposure to DEHP. Similar to previous studies, our study confirms that DEHP may up-regulate the expression of p38MAPK/NF-κB to accelerate liver injury.

Some studies suggested that lipid degeneration of nonalcoholic fatty liver might be directly related to the expression of PPAR and SREP-1c proteins[26, 41]. Nevertheless, the presence of fatty vacuoles and inflammatory cells infiltration in hepatocytes following long-term alcohol consumption and high fat intake was a typical marker of alcoholic fatty liver disease[37, 38]. The formation and evolution of alcoholic fatty liver is inseparable from the ROS, inflammatory factors and oxidative stress induced by the liver during the metabolism of ethanol. The scientist knocked out the SIRT1 gene in the liver of mice fed with ethanol, activated downstream signal molecule Lipin-1 activity[10], and then up-regulated SREBP-1c and NFATc-4 proteins, eventually liver steatosis, inflammation and oxidative stress were evidently exacerbated[42]. Recent findings postulate that SIRT1 might be a specific target for ethanol to act in liver, and SIRT1-Lipin-1 mediated signaling plays a key role in the development of AFL[43, 44]. Other studies have shown that CYP2E1 was a member of the CYP450, the most important mixed functional oxidase in liver microsomes. And it is responsible for the metabolism of ethanol. Ethanol activated cytochrome P450 2E1 and catalyzes the production of ROS during the conversion of ethanol to acetaldehyde, which was one of the major contributors to hepatocytes reactive oxygen species and damage. Stimulated by ethanol, CYP2E1 mediated oxidative stress impairment protein kinase B (PKB/AKT) activity, which promoted fat accumulation and inflammation in liver[33, 41]. In our results, alcohol intake significantly up-regulated the expression of CYP2E1, P-p38, P-p65 proteins, and inhibited the activity of SIRT1 in the nucleus; interestingly, DEHP significantly promoted the development of this trend after medium and high doses of DEHP treatment. According to our experiment results, DEHP plays a major role in promoting and aggravating the formation and development of alcoholic fatty liver. On one side, long-term ingestion of DEHP can be stored in liver and its toxicity during metabolism may directly destroy hepatocytes to produce oxidative stress, causing some

inflammatory and lipid degeneration[19, 20]. On other side, oxidative stress, inflammation and lipid metabolism disorders may be achieved by affecting CYP2E1 and SIRT1 signaling pathways to continuously accelerate or aggravate the pathogenesis of AFLD. All in all, CYP2E1 and SIRT1 are two emphatic etiological factors in the occurrence and development of AFLD, and DEHP exposure may have a negative impact on them[10, 45].

5. Conclusion

The current study insinuates that DEHP dramatically facilitate or aggravate the occurrence and evolution of alcoholic-induced fatty liver by disrupting the liver oxidation defense systems. DEHP may promote liver oxidative stress damage, induce hepatocytes lipid metabolism obstruct and inflammatory response. Although the experiment initially explored that DEHP may play a role in AFLD by up-regulating the levels of CYP2E1, p38MAPK/NF-κB and inhibiting the SIRT1 signaling pathway, the detailed mechanism still needs further investigation(Fig. 9). In practice, the daily exposure of DEHP in real life has little effects on humans' health, but the exposure doses of DEHP (corresponding to the doses DEHP in our experiment) for people working in high risk (such as painter and plastic processing) which is dozens or even hundreds of times higher than normal peoples. When exposed for a long time, the toxicity of DEHP may stack continuously, which can induce the occurrence and development of various potential diseases. Therefore, it is also important to study the toxicity of DEHP to human beings in real life. As this study shows, DEHP has a significant role in the formation and development of alcoholic fatty liver, and its potential harm deserves further research attention. Finally, the discussion of AFLD signaling pathway in this experiment could provide basis for future prevention and treatment of fatty liver.

Abbreviations

AFLD, Alcoholic fatty liver disease; DEHP, Di (2-ethylhexyl) phthalate; CYP2E1, Cytochrome P450 2E1; LO-2 cell, Human normal liver cells; SIRT1, Silent information regulator-1; ALD, Alcoholic liver disease; ROS, Reactive oxygen species; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; MDA, Malondialdehyde; TG, Triglycerides; CCK-8, Cell Counting Kit-8; P-p65, Phosphorylated nuclear transcription factor; P-p38, Phosphorylated P38 mitogen-activated protease.

Acknowledgments

Not applicable.

Authors' contributions

Qiang Xu and Ke Ji participated in all the experiments of the project and XQ wrote manuscript. Zongbiao Zhao participated in part of the experiment, Xiang Zhang and Song Huang processed part of the data, Wei Wei and Weiping Xu provided guidance for the entire experiment and gave detailed instructions for manuscript.

Funding

This work was financially supported by the Anhui Natural Science Foundation Project (China, No.1608085MH176) and National Natural Science Foundation Project (China, No.51672004).

Availability of data and materials

The analyzed datasets generated during the study are available from thecorresponding author upon reasonable request.

Ethical approval

The study was approved by the Animal Experimental Ethics Evaluation Committee of the Institute of Clinical Pharmacology, Anhui Medical University. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests regarding this study.

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Figure Legends:

Figure 1. (A, B): Changes of body weights and liver index of SD rats after DEHP intervention for eight weeks (n=7-8); (C, D): Effects of DEHP on activity of AST and ALT in SD rats serum. (Date represent the mean ± standard deviation (SD), #P<0.05, ##P<0.01 vs normal group, *P<0.05, **P<0.01 vs alcohol group).

Figure 2. DEHP increased the levels of lipid accumulation and oxidative stress in rats in alcohol groups. (A): Changes of oil red O staining images in liver tissue slices after DEHP treatment for eight weeks (400×); (B, C, D): Effects of DEHP on the contents of triglyceride, SOD and MDA in liver homogenate. (Date represent the mean ± standard deviation (SD), #P<0.05, ##P<0.01 vs normal group, *P<0.05, **P<0.01 vs alcohol group).

Figure 3. DEHP aggravates alcohol-induced fatty liver degeneration in rats. (A)Apparent changes in fresh liver samples of rats in each groups after 8 weeks of DEHP treatment. (B)The changes of hepatic histology in each groups of rats exposed to DEHP for 8 weeks were observed respectively by H&E staining (400×); (C): MT staining to evaluate the effect of DEHP on hepatic collagen in rats at the eighth week (400×).

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Figure 6. Effects of DEHP on the levels of proteins expression in LO-2 cells. (A) CYP2E1, (B) SIRT1, (C) P-p38/p38, (D) P-p65/p65 (Date represent the mean ± standard deviation (SD), #P<0.05, ##P<0.01 vs normal group, *P<0.05, **P<0.01 vs ethanol group).

Figure 7. Changes of ROS and viability of LO-2 cells in vitro under the action of DEHP (48 hours). (A): Flow cytometer was utilized to measure ROS levels of LO-2 cells under the influence of DEHP by DCFH-DA fluorescence intensity;(B): The effect of DEHP on the inhibition of LO-2 cells was analyzed using CCK-8 kit.

Figure 8. Lipid accumulation model in LO-2 cells induced by anhydrous ethanol (100 mM/L) and oleic acid (90 mM/L) in vitro. Under the stimulation of oleic acid and ethanol for 48 hours, DEHP was administered at the same time with 6.25,25,100 μM/L. As the concentration of DEHP increased, the lipid accumulation near the cell membrane of the model group increased significantly (100×). (A) Normal group, (B) Ethanol group, (C) Ethanol+DEHP 6.25 μM/L, (D) Ethanol+DEHP 25 μM/L, (E)

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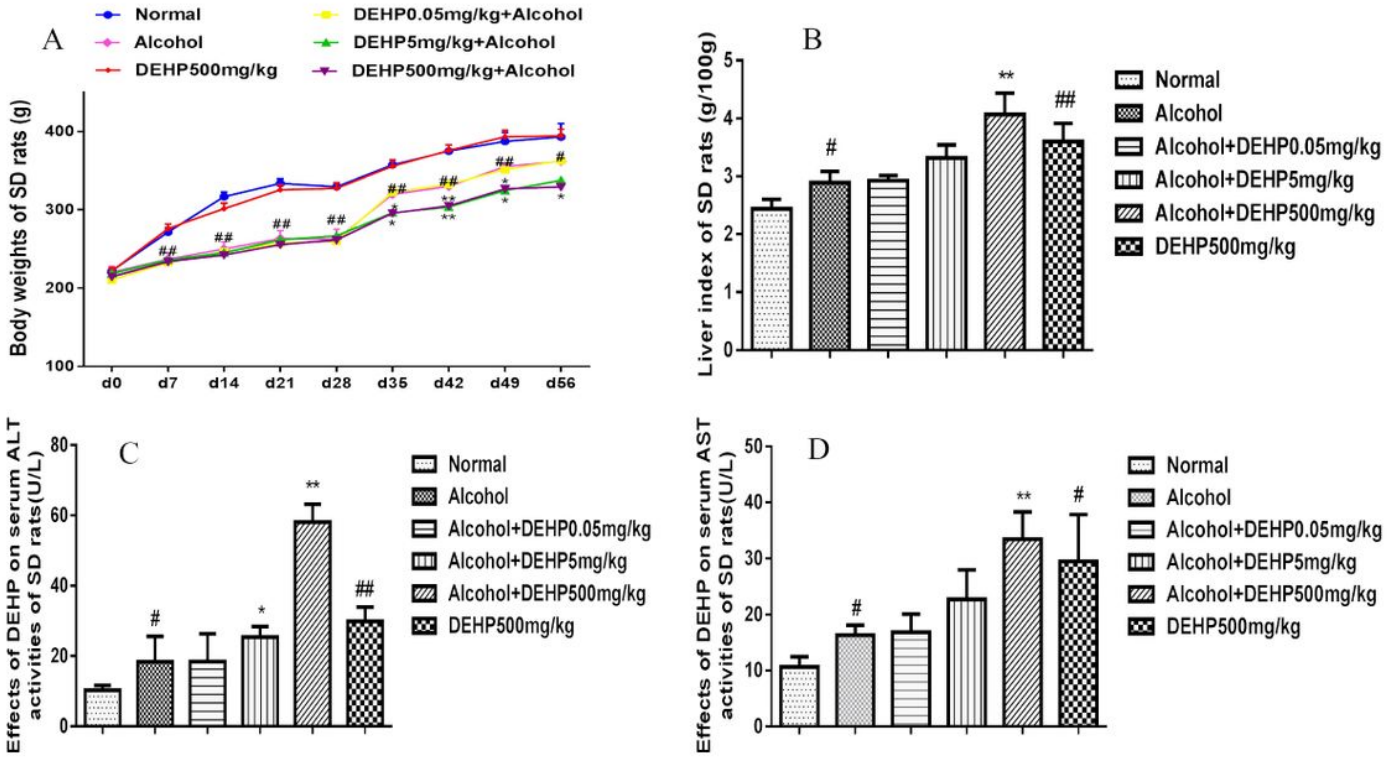
Figures



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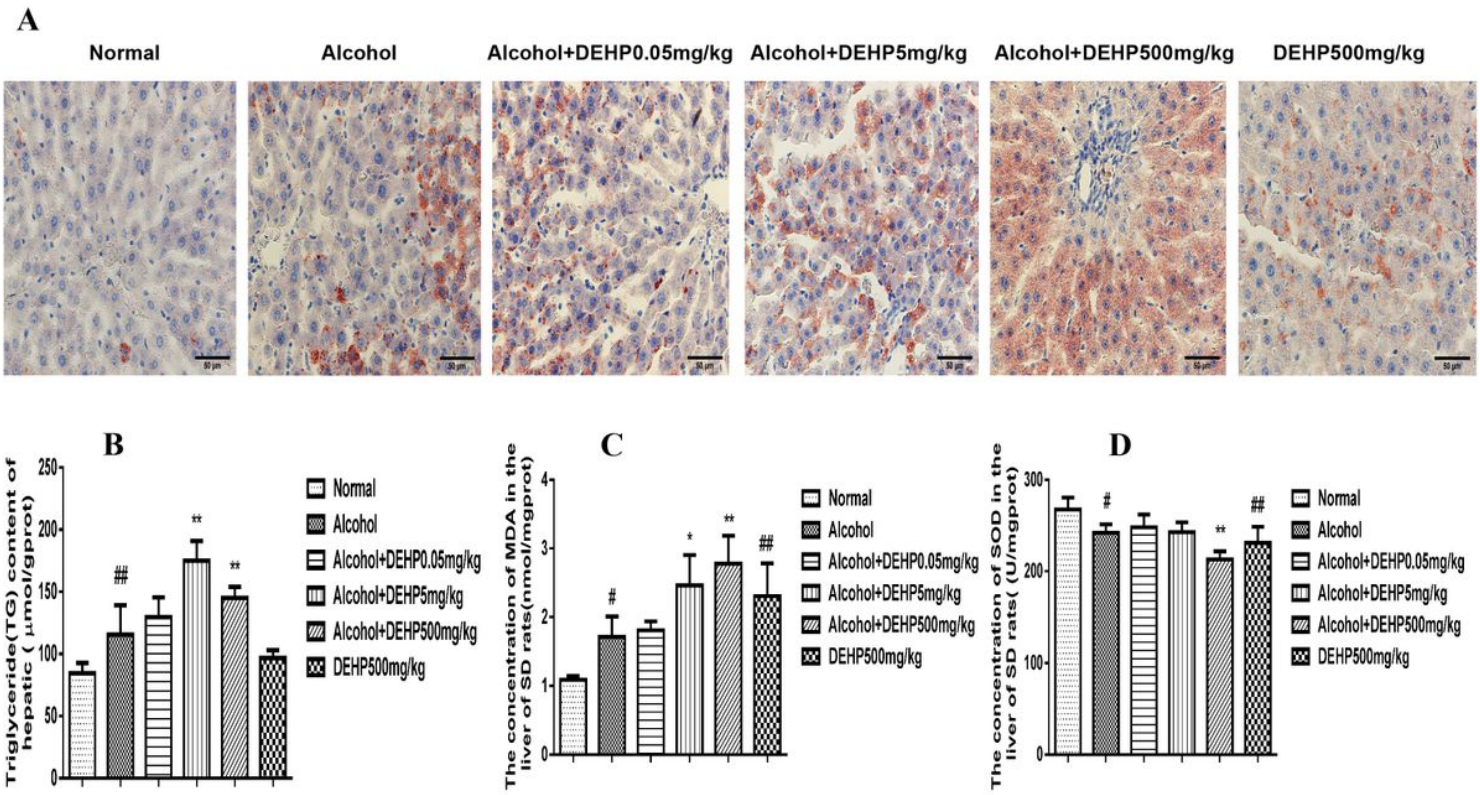

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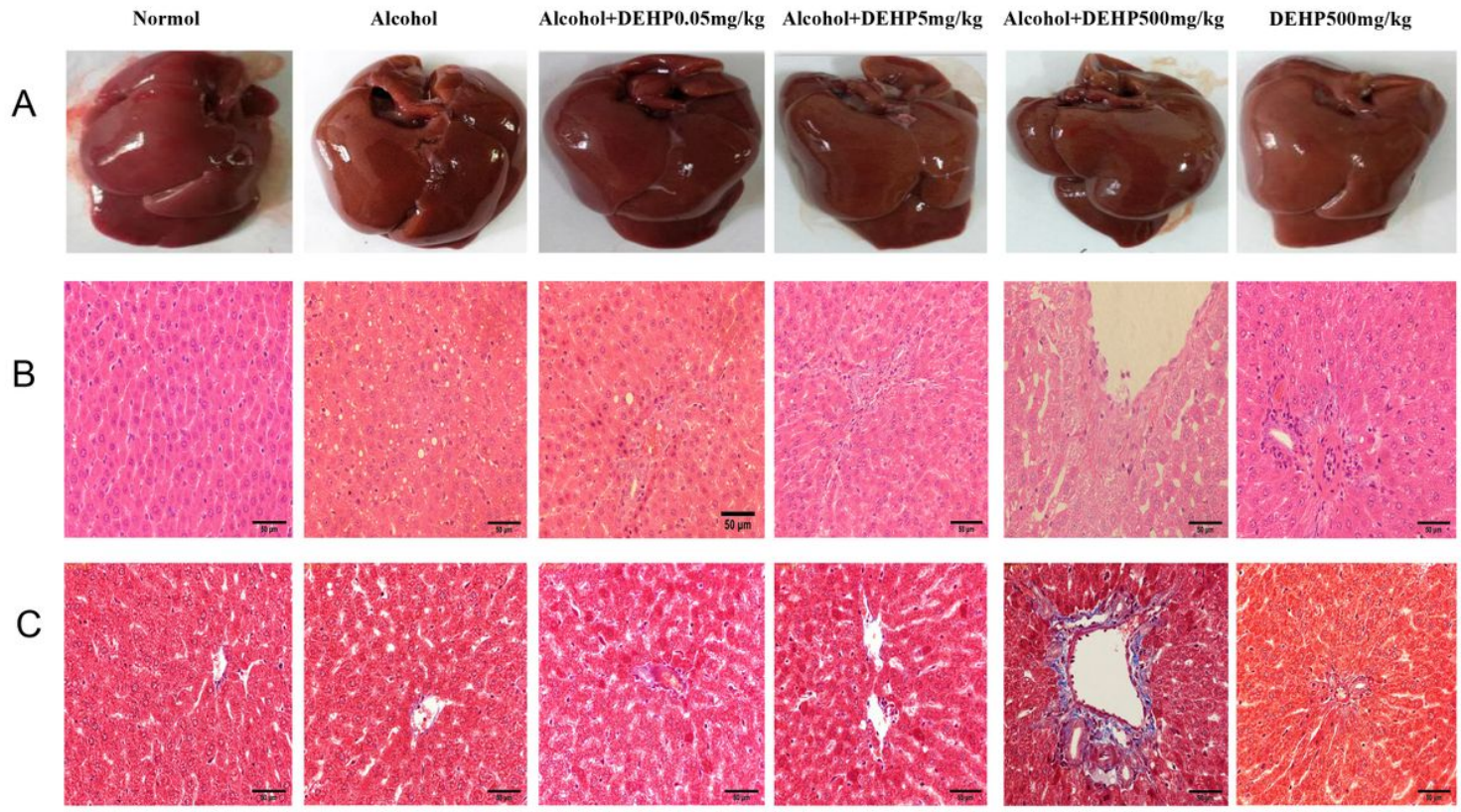

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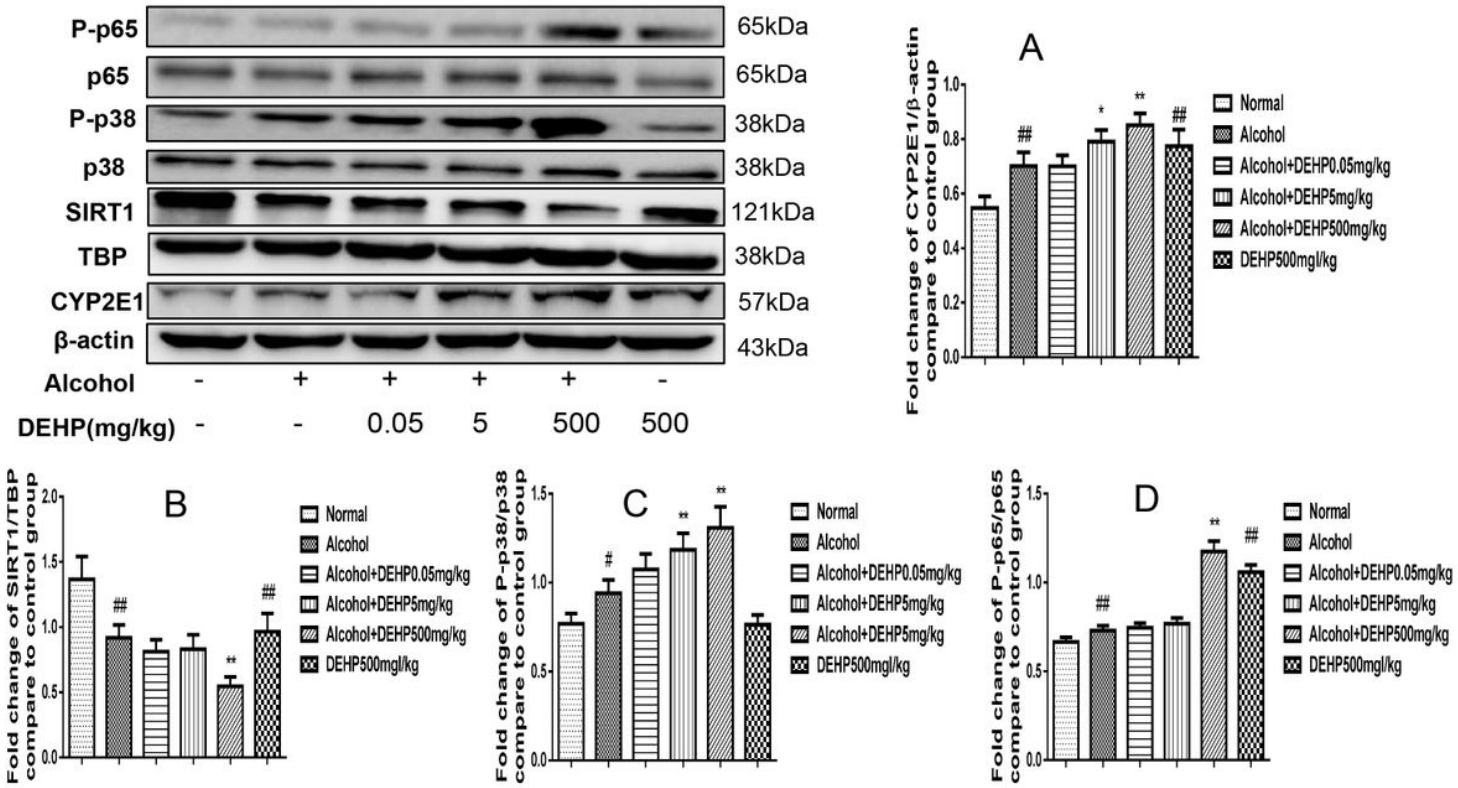

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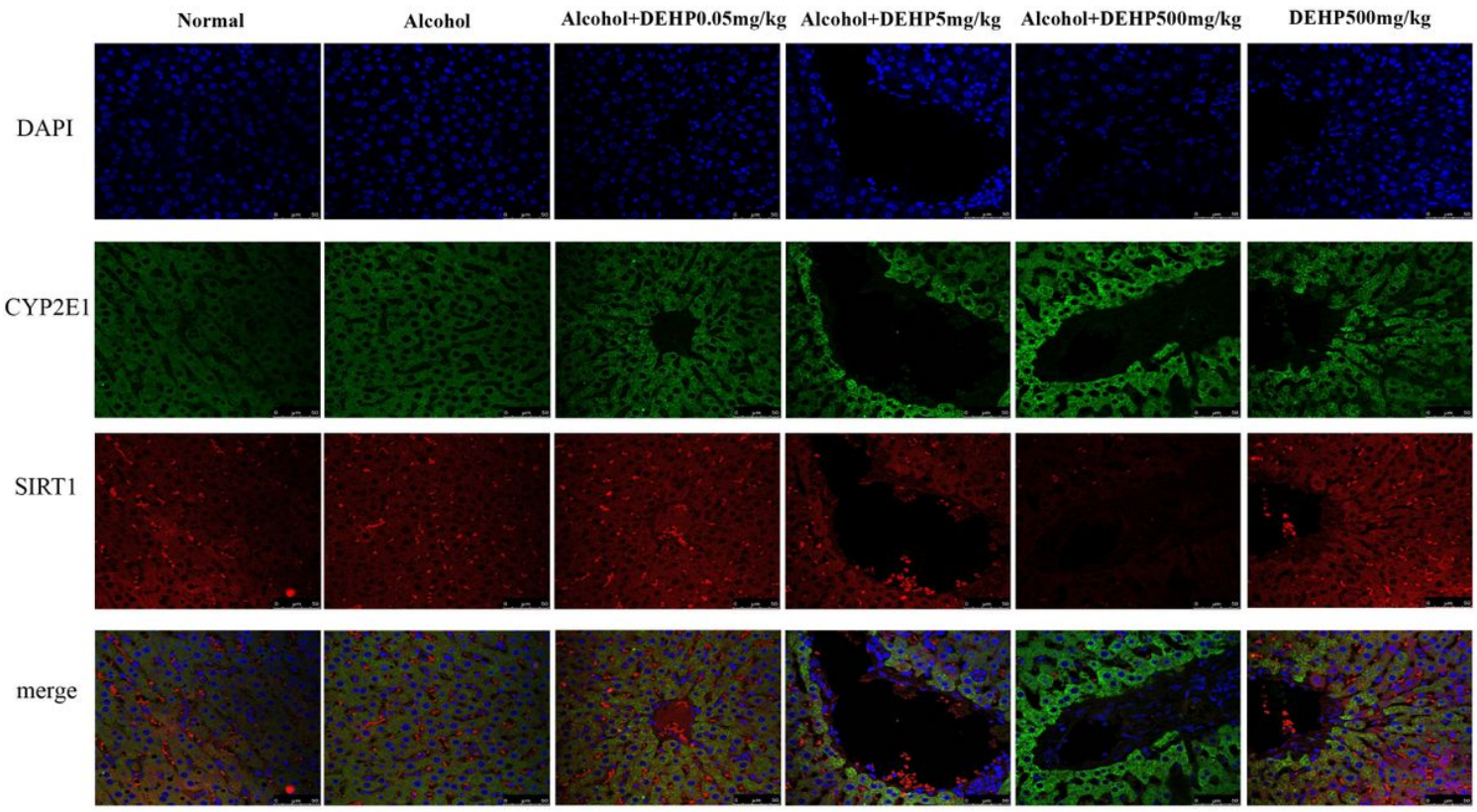

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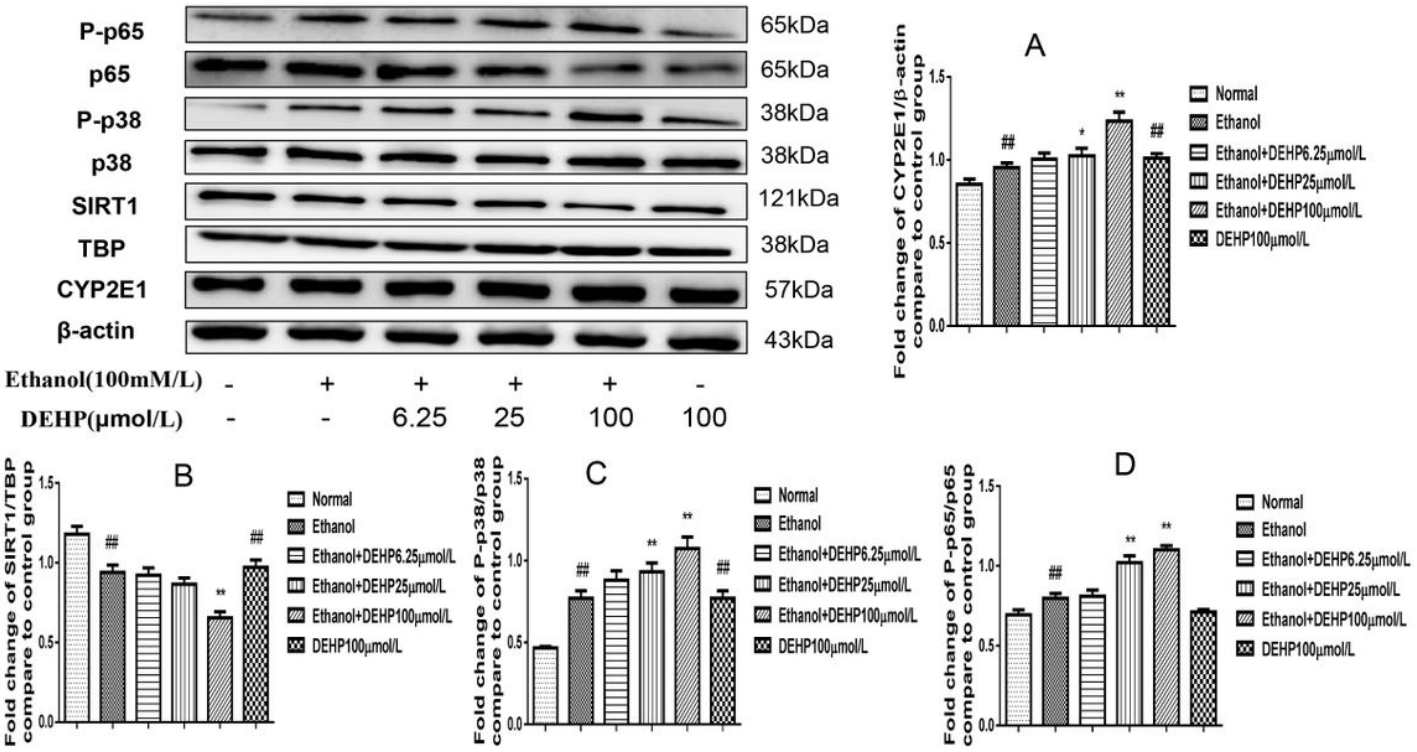

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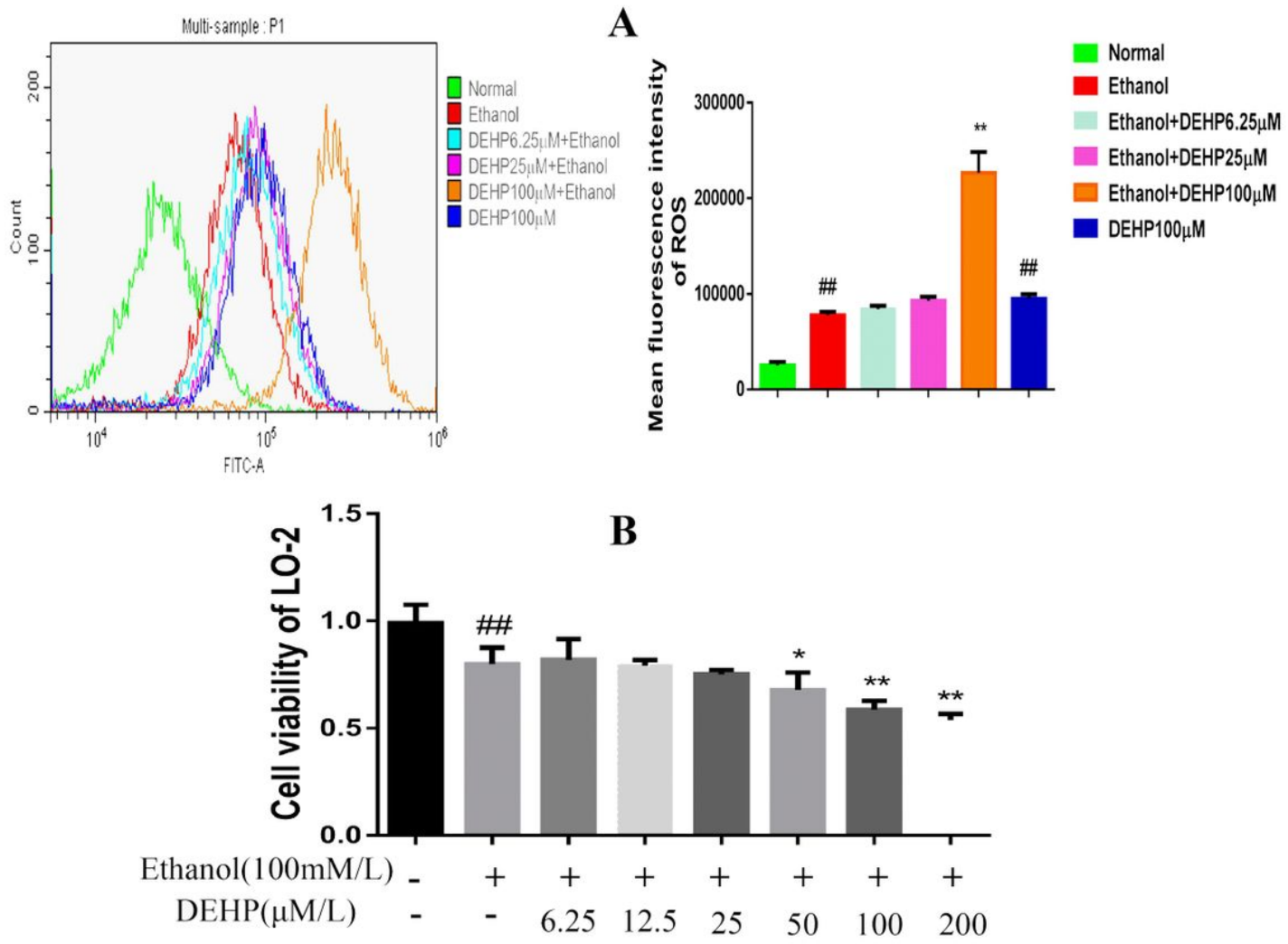

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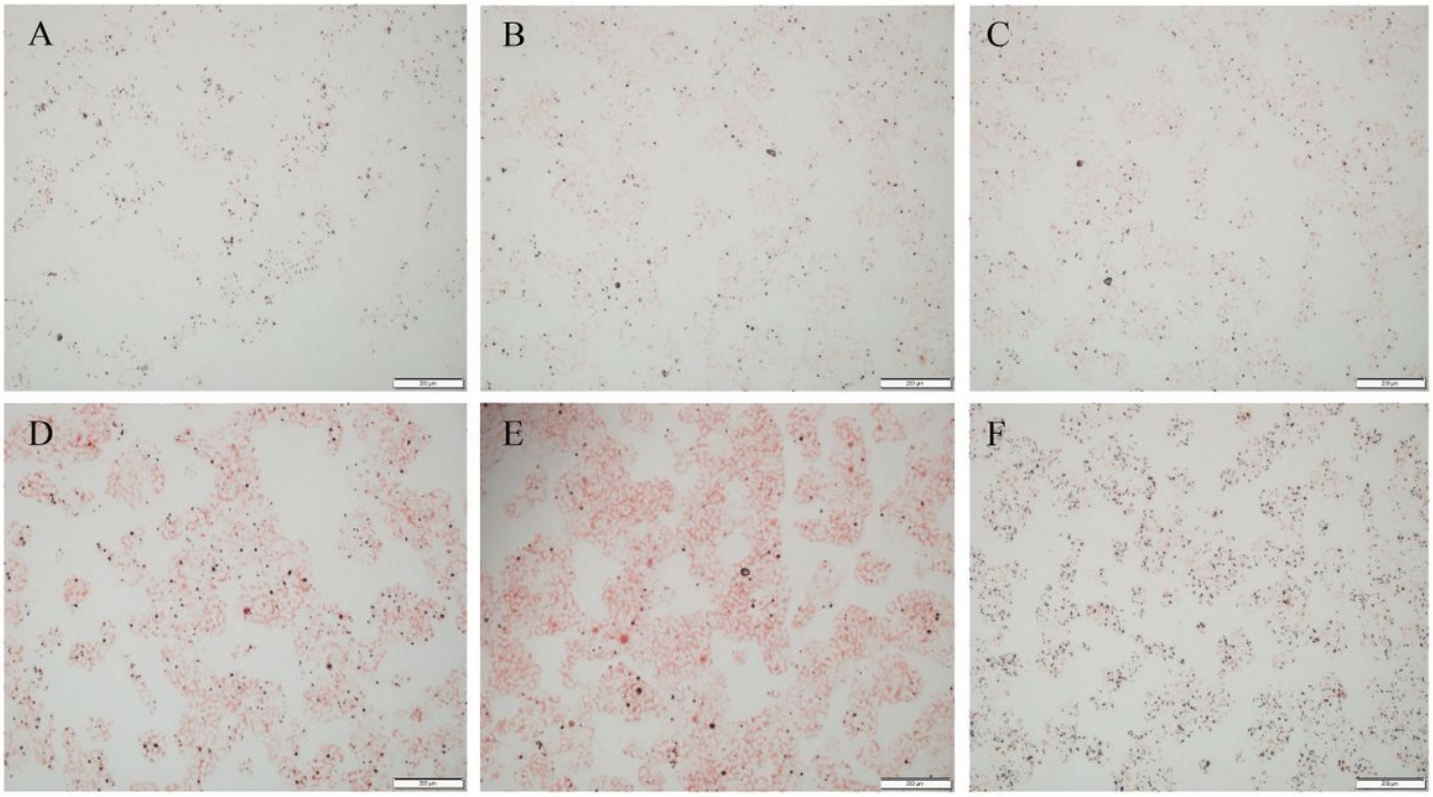

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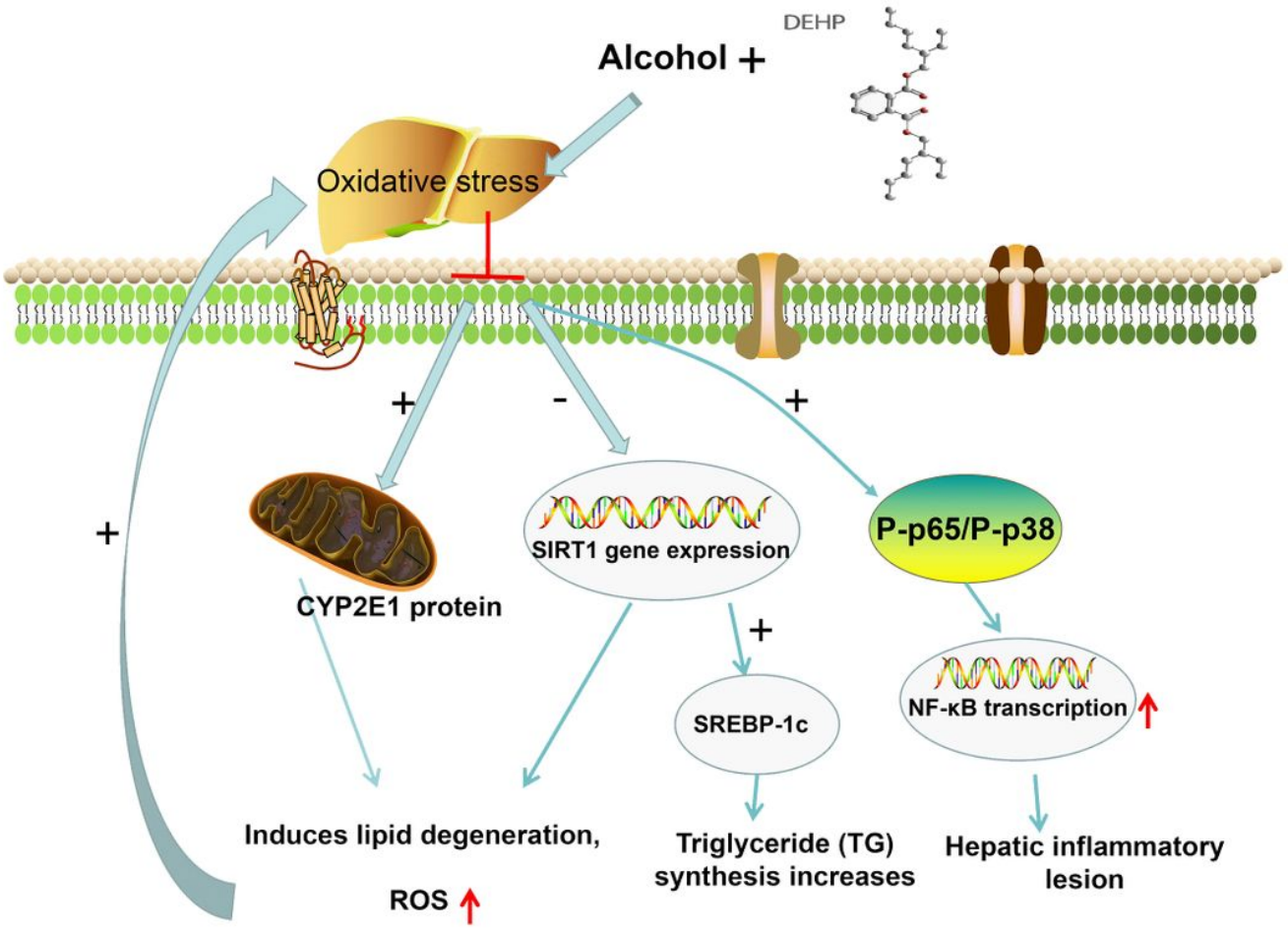

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