Rosiglitazone protects against alcohol toxicity in non alcoholic fatty liver

Thesis by

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I thank my parents and other family members who always pray and support for me and my colleagues for their technical assistance.

Lastly, I really thank the great Jesus Christ.

By author Tae Woo Jung

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Rosiglitazone, a peroxisome proliferator activated receptor (PPAR)-γ agonist and originally developed as an anti-diabetic agent, have been known to have a protective effect against alcoholic toxicity in patients with non-alcoholic fatty liver. To investigate the underlying mechanisms of this protective effects of rosiglitazone, several *in vitro* experiments including the measurement of cell viability, mitochondrial aldehyde dehydrogenase (ALD2) expression, anti-oxidant enzymes activities, the mitochondrial apoptotic cascades and *in vivo* experiments including histopathologic examinations of

liver by hematoxylin & eosin and TUNEL staining and blood chemistry were performed. We found that ALD2 and anti-oxidant enzymes expressions were inhibited in HepG2 cells under hyperlipidemic condition, and rosiglitazone reversed the expression of these genes. Sole acetaldehyde and acetaldehyde-free fatty acids (FFAs) impaired both ALD2 and anti-oxidant enzymes expressions. However, these conditions were reversed by rosiglitazone treatment indicating that rosiglitazone may regulate ALD2 and anti-oxidant enzymes in HepG2 cells. It also prevented apoptotic cascades including Bax and Bcl-2 ratio, cytochrome *c* release, and caspase-3 activation. In *in vivo* experiment, we found that high fat diet and alcohol-induced aspartate aminotransferase, alanine aminotransferase, triglycerides, free fatty acids, and total bilirubin were significantly decreased and hepatic apoptosis was prevented by rosiglitazone treatment. Finally, we propose that rosiglitazone treatment may provide therapeutic strategy for the prevention of alcohol toxicity in non-alcoholic fatty liver via recovery of ALD2 and anti-oxidant enzymes.

Key Words: non-alcoholic fatty liver, rosiglitazone, mitochondrial aldehyde dehydrogenase, apoptosis

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I. Introduction

Non-alcoholic steatohepatitis (NASH) is a type of hepatic steatosis which is one of the major contributors to overall obesity-related morbidity and mortality. [1] Although several predisposing factors such as obesity, diabetes, and drugs have been related to non-alcoholic fatty liver, pathogenesis of non-alcoholic fatty liver and its progression to fibrosis and chronic liver disease is still unclear. [2]

Recently, the incidence of fatty liver has significantly increased due to

increased food consumption, especially high calorie and high fat food with deceased physical activity. In this environment, alcohol consumption can be more harmful. If a person suffering fatty liver disease drinks alcohol continuously, alcoholic liver diseases such as alcoholic hepatitis, cirrhosis will be accelerated. Eighty five percent of obese population suffers hepatic steatosis, but non-obese population suffers hepatic steatosis only in 19%. While 40% of obese people suffer steatohepatitis, non-obese people suffer steatohepatitis only in 3%. [2] These data support one of the hypothesis of the present study that alcohol drinking can accelerate hepatic injuries.

Acetaldehyde is the first and the most toxic metabolite created by alcohol metabolism. It is a potential carcinogen, which is also found in cigarette smoke, car exhausts and embalming fluid. Acetaldehyde has been proved to be more toxic to the body than alcohol itself. In addition to hangovers, acetaldehyde has been shown to increase the risk of cirrhosis, several types of cancer, and alcoholism. [3] One of the underlying mechanism of acetaldehyde mediated hepatic injury is that it causes hepatocyte death through mitochondrial dysfunction. [3]

Acetaldehyde is catalyzed by mitochondrial aldehyde dehydrogenase (ALD2). ALD2 is one of 19 members of the human ALD2 gene family of $NAD(P)^+$-dependent enzymes. [4] ALD2 is the principal enzyme involved in

acetaldehyde oxidation at the physiological concentrations typically found when a person consumes alcohol.

Alcohol enhances oxidative stress conditions and it has been suggested that protective effects of anti-oxidant enzymes against alcohol-induced liver disease in the baboon might be due to against lipid peroxidation. [5]

Members of the thiazolidinedione (TZD) class of insulin-sensitizing drugs are highly selective ligands and activators of PPAR-γ and act as potent regulators of genes contained peroxisome proliferator-response elements (PPRE) and related to adipocyte development. Rosiglitazone is a member of TZD class of anti-diabetic agents, and it enhances glycemic control by increasing insulin sensitivity. [6] In addition to anti-diabetic effects, other effects of rosiglitazone have been reported. For example, this agent had functions to repair liver injuries, [7] did not provoke hepatoxicity differently other thiazolidinediones, [8] and was able to decrease serum lipid [9] recover blood pressure. [10] It was also reported that the anti-inflammatory effects of rosiglitazone may contribute to cardiovascular risk reduction [11] and prevent neuronal cell death. [12-14]

Recently, the relationships between fatty liver and decreases of aldehyde dehydrogenase 2 (ALD2), an eliminator of acetaldehyde, expression in obese mouse liver have been demonstrated. [15]

In the present study, we hypothesized that rosiglitazone can reduce alcohol toxicity in non-alcoholic fatty liver by induction of ALD2 and anti-oxidant enzymes. Therefore alcohol toxicity on non-alcoholic fatty liver and the effect of rosiglitazone were analyzed in HepG2 cells and experimental animals.

II. Materials and Methods

1. Chemicals

Fetal bovine serum (FBS), Dulbecco`s Modified Eagle Medium (DMEM), trypsin-EDTA, and antibiotics for cell culture were from Gibco-BRL-Life Technologies (Grand Ismand, NY, USA). Rosiglitazone maleate was from SmithKline Beecham Pharmaceuticals (West Sussex, Unite Kingdom). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

2. Cell culture and treatment

The HepG2 and Hur7 line of human hepatoma cells (Korean Cell Line Bank, Seoul, Korea) were maintained in 10 cm culture plates with 5 mL of DMEM containing 100 units/mL penicillin, 0.1 mg/mL streptomycin, 1 mM pyruvate, and 10% heat-inactivated FBS (complete DMEM) incubated under an atmosphere of 95% air and 5% CO_2 at 37℃. Cells were subcultured at a 1:4 ratio twice a week. The culture medium was replaced every 24 h with fresh medium. A 200 mM stock solution of acetaldehyde was prepared daily in PBS. The culture plates were kept tightly sealed with parafilm to prevent evaporation of acetaldehyde. The cells were treated with the indicated

concentrations of FFAs (2:1 oleate/palmitate), rosiglitazone, acetaldehyde, and calcium cyanamide as ALD2 inhibitor for 48 h without FBS. Especially, HepG2 cells were pretreated with rosiglitazone and calcium cyanamide for 12-24 h before the administration of acetaldehyde. In some experiments, the concentration of acetaldehyde was monitored and maintained by compensation for losses (initially 180 μM with three or four additions in 24 h).

3. Messenger RNA analysis

The messenger RNA (mRNA) levels of Bcl-2 and Bax were assessed by semi-quantitative RT-PCR analysis using beta actin as a house keeping gene. Total RNA was extracted from HepG2 and liver using Trizol (Invitrogen, Carlsbad, CA, USA). Contaminating DNA was removed by treatment of each sample with DNAse I, according to the manufacturer`s instructions (Promega, Madison, WI, USA). cDNA was prepared using SuperScript II™ first strand synthesis system, according to the manufacturer`s instructions (Invitrogen). PCR primers were designed as follows: Bcl-2 sense, 5'-actttgcagagatgtccagt-3'; Bcl-2 anti-sense, 5'-cggttcaggtactcagtcat-3'; Bax sense, 5'-actggacagtaacatggagc-3'; Bax anti-sense, 5'-tcttcttccagatggtgagt-3'; β-actin sense, 5'-gacctgacagactacctca-3'; β-actin anti-sense, 5'-

tctcttgctcgaagtctagg-3'. RT-PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide and bands were visualized by UV light.

4. Enzyme assay in HepG2 cells

Catalase activity assay was performed as describes previously. [16] HepG2 cells were harvested by scrapping. The cells were suspended 20 mM potassium phosphate (pH 7.5) buffer and sonicated. Catalase activity was measured at 240 nm for 3 minutes adding 30% H_2O_2. SOD activity assay was measured using kits purchased from Dojindo (Kumamoto, Japan). Caspase-3 activity assay was carried out following user's manual of assay kit (Peptron, Korea).

5. Immunoblot analysis

Immunoblot analysis was performed using whole cell lysates or cytosolic fractions. Total extracts were prepared with cell lysis solution (PROPREP, Intron, Korea). In preparation of cytosolic fractions, after cells were mixed with reagent buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and reagent buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% triton-X), they

were incubated in ice for 15 min and centrifuged (13,000 rpm, 4℃, 1 min). Supernatant was used as cytosolic fractions. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was incubated with peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the bound antibody was visualized using a chemiluminescent (ECL, Intron) and X-ray film (FUJI FILM, Japan). Primary antibodies were mouse anti-ALD2 (1:1000, Peptron, Korea) and mouse anti-cytochrome *c* (1:1000, BD Sciences, San Jose, CA, USA).

6. Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in PBS solution at concentration of 5 mg/ml and filtered through a 0.22 μm filter to sterilize and remove insoluble residues then stored in the amber vials at 4℃ for a month. After 48 and 72 h incubation, 25 μl of the MTT solution was added to each well of 96-well plates and incubated for 4 h at 37℃ in a humidified atmosphere of 5% CO_2. At the end of the incubation period, the media were discarded using a suction pump. The extraction buffer of 20% w/v sodium dodecyl sulfate (SDS) in a solution of 50% of

N,N-dimethylformamide (DMF) in demineralized water (50:50, v/v) was prepared at pH 4.7 and filtered through a 0.22 μm filter to remove insoluble residues. The absorbance was determined at 570 nm. The A570 was taken as an index of the cell viability and the activity of mitochondria. [17] The net absorbance from the plates of cells cultured with the control medium (not treated) was considered as 100% of the cell viability and the mitochondrial activity.

7. Nuclear staining with Hoechst 33258

After being treated with FFAs, acetaldehyde and/or rosiglitazone for 48 h, the cells incubated with DNA fluorochrome 3 μg/ml of Hoechst 33258 for 20 min. Then, cells were washed with PBS and analyzed by fluorescent microscopy. Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptosis.

8. Light microscopic examination, TUNEL, and blood chemistry

4-weeks-old male Spraque Dawley (SD) rats were purchased from Samptako (O San, Korea) and were housed (n = 2 per cage) under 12-hour light/dark cycles with *ad libitum* access to food and water. The 10-weeks-old

rats were allowed to age of 25 weeks on standard pellet mouse chow. After the animals were treated with 5 mg/kg/d of rosiglitazone for 1 weeks delivered by oral administration and standard lab chow *ad libitum*, they were treated with high-fat diet (15% sucrose, 1% orotic acid, and 10% lard oil) to induce non-alcoholic fatty liver and/or 3 g/kg/day alcohol for 8 weeks. After experiments were ended, their liver tissue samples were prepared. Small sections of liver from each SD rats were fixed 10% buffered formalin, and the sections were stained with both hematoxylin & eosin (H&E) and TUNEL. [18, 19] In the case of H&E staining, deparaffinization and rehydration were performed on some sections by dipping xylene and graded ethanols (100, 90, and 70%) for 3 min, 2 times and washing in DW for 3 min, 2 times. These sections were stained by H&E mixture solution (Sigma). In the case of TUNEL, pretreatment with proteinase K was performed for 15 min followed by washing in D/W for 2 min, 4 times. In order to block the endogenous peroxidase activity, all sections were incubated in 2% H_2O_2 at room temperature (RT) for 5 min and then washed in D/W. Sections were immersed for 10 min at RT in TdT buffer (30 mm Tris-HCl, pH 7±2, 140 mm sodium cacodylate, 1 mM cobalt chloride) and incubated in TdT (50 U, 50 ml; Boehringer Mannheim) and biotinylated dUTP (50 nM, 50 ml) in TdT buffer at 37 °C for 1 h in a humid atmosphere. The reaction was stopped by

transferring the sections to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The sections were subsequently rinsed in D/W and blocked in 2% bovine serum albumin in phosphate buffered saline (PBS) for 10 min at RT. After rinsing in D/W and immersion in PBS for 5 min, the sections were covered with extravidin-horseradish peroxidase (Sigma) diluted 1:20 in PBS for 30 min at RT, rinsed in PBS twice (5 min each) and stained for no more than 10 min using diaminobenzidine (DAB) as the chromogen. Blood chemistry (serum alanine aminotranferease, aspartate aminotransferase, free fatty acids, triglycerides, total Bilirubin) was carried out by Neodin (Seoul, Korea). Diameters of lipid droplets were manually measured by using scale bar. Apoptotic intensity was measured in same area by using densitometer program (SCION). All experiments using rats were performed in compliance with The UFAW Handbook on the Care and Management of Laboratory Animals (Web: www.ufaw.org.uk/pubs.htm#Lab).

III. Results

1. The effect of rosiglitazone treatment on viability of HepG2 cells and Hur7 cells under different metabolic conditions

In HepG2 cells, cell viability under FFAs-acetaldehyde treatment decreased dramatically compared with under sole acetaldehyde, FFAs treatment, and control for 48 h. Sole FFAs treatment did not affect cell viability. But, sole acetaldehyde and FFAs-acetaldehyde treatment directly decreased cell viability. These results indicated that FFAs-acetaldehyde treatment group was more damaged than sole treatment groups by an additive effect. But, interestingly, cell viability of HepG2 cells was moderately reversed after rosiglitazone treatment (Fig. 1A). When we used ethanol instead of acetaldehyde, cell viability of Hur7 expressing alcohol dehydrogenase was similar to that of HepG2 cells (Fig. 1B).

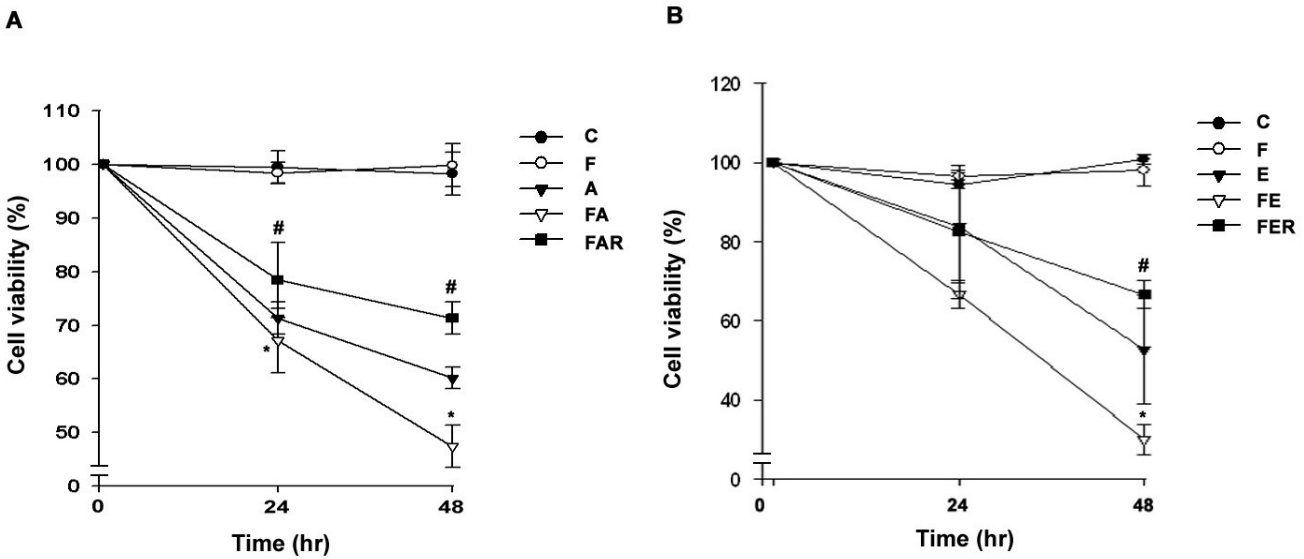

Fig. 1. The effect of rosiglitazone treatment on viability of HepG2 cells and Hur7 cells under different metabolic conditions. Rosiglitazone recovers decreased cell viability in HepG2 (A) and Hur7 cells (B). Cell viability was assessed by MTT assay as described in the materials and methods. Cells are treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence or presence of 5 μM rosiglitazone for 48 h. Data are expressed as the percentage of values in untreated control cultures and are means ± SD (n = 4). *P<0.05, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}$P<0.05, FFAs-acetaldehyde or ethanol treatment (FA or FE) vs. FFAs-acetaldehyde or ethaol-rosiglitazone treatment (FAR or FER).

2. The effect of rosiglitazone in impaired expression of ALD2 in HepG2 cells

To elucidate the underlying mechanism of impaired aldehyde metabolism, expression of ALD2 was assessed by semi-quantitative RT-PCR and Western blot analysis under several conditions in HepG2 cells. ALD2 expression decreased to 72%, 81%, and 51% in HepG2 cells treated with FFAs or acetaldehyde, FFAs-acetaldehyde, respectively, but was near fully recovered by rosiglitazone treatment (Fig. 2).

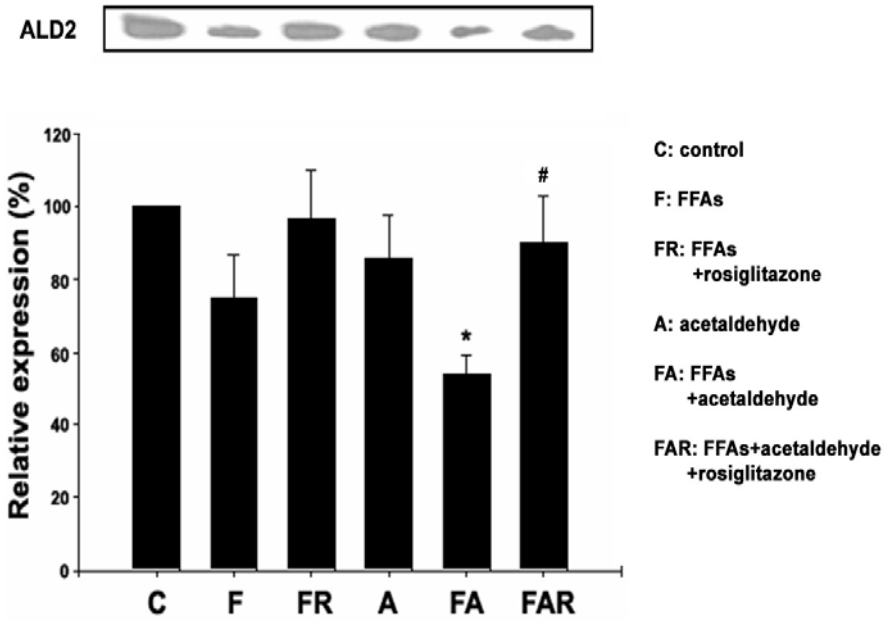

Fig. 2. The effect of rosiglitazone in impaired expression of ALD2 in HepG2 cells. Rosiglitazone recovers decreased level of ALD2 expression in HepG2 cells. Cells were treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone for 48 h, and crude extracts were collected for Western blot analysis. The levels of ALD2 were quantitated by densitometric analysis. Data are means ± SD (n = 3). *$P<0.05$, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}P<0.05$, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR).

3. The change of nuclear morphology after FFAs-acetaldehyde and rosiglitazone treatment

As shown in Fig. 3, the control and FFAs treated HepG2 cell`s nuclei had a regular and oval shape. However, apoptotic nuclei, characterized by nuclear condensation and fragmentation, appeared after exposure to acetaldehyde and FFAs-acetaldehyde for 48 h. But, rosiglitazone blocked the FFAs-acetaldehyde-induced nuclear morphological change.

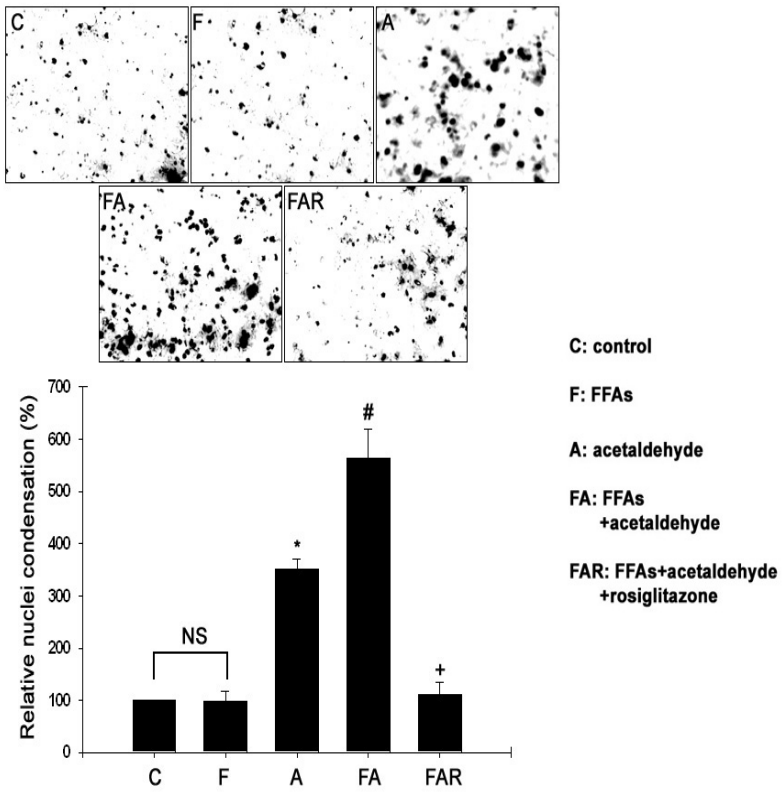

Fig. 3. The change of nuclear morphology after FFAs-acetaldehyde and rosiglitazone treatment. Rosiglitazone prevents nuclear condensation in HepG2 cells. The figures show the fluorescence micrographs of Hoechst 33258 stained nuclear morphology. Percentage of condensed nuclei as assessed Hoechst 33258 staining of adherent and floating cells by using square densitometry program. HepG2 cells were untreated, treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone for 48 h. *$P<0.05$, control (C) vs. acetaldehyde treatment (A). $^{\#}P<0.05$, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{+}P<0.05$, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR). Not significance (NS).

4. The effect of rosiglitazone on oxidative stress in HepG2 cells under different metabolic conditions

The effects of rosiglitazone in FFAs-acetaldehyde-induced oxidative stress were examined, because increased oxidative stress may contribute to hepatic damage and inflammation. [20] In HepG2 cell lines under FFAs, acetaldehyde treatment resulted in significantly lower levels of SOD and catalase compared with those of the other groups and these effects were reversed by rosiglitazone treatment. As expected, production of ROS was induced in the presence of FFAs, acetaldehyde, and FFAs-acetaldehyde but its production was decreased by rosiglitazone treatment (Fig. 4). Taken together, these results suggest that fatty liver treated with alcohol is exposed to higher levels of oxidative stress and rosiglitazone can reduce it.

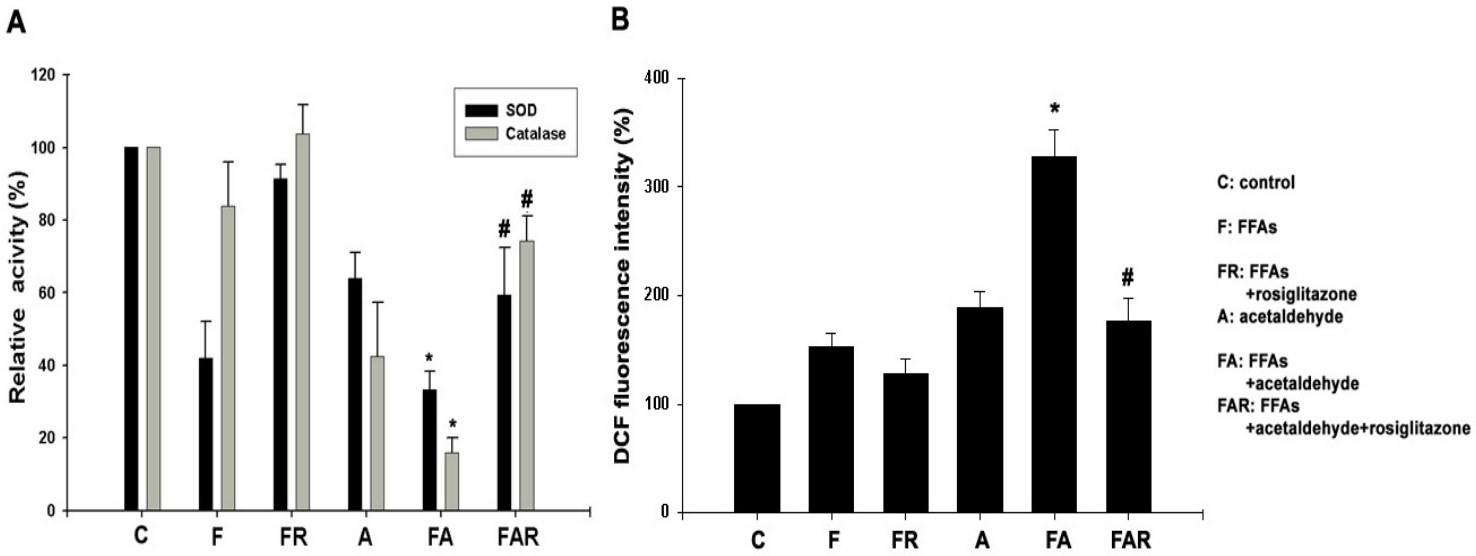

Fig.4. The effect of rosiglitazone on oxidative stress in HepG2 cells under different metabolic conditions. Rosiglitazone induces the activation of anti-oxidant enzymes and inhibited the production of ROS in HepG2 cells. The activities of anti-oxidant enzymes and the production of ROS were measured as described in the materials and methods. Cells were treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone for 48 h. Data are means ± SD (n = 3). *P<0.05, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}$P<0.05, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR).

5. The effect of rosiglitazone on the expression of Bax and Bcl-2 in HepG2 cells

The highest expression of Bax, which is known to contribute to mitochondrial permeability transition (MPT) activation, was observed in HepG2 cells treated with FFAs-acetaldehyde by using semi-quantitative RT-PCR. Contrast to Bax, decreased level of Bcl-2 was observed in FFAs-acetaldehyde group compared with control (Fig. 5). Because this protein is known to inhibit MPT activation, its decreased expression contributes to enhanced MPT activation. [21] Consequently, Bax/Bcl-2 ratio was the highest in FFAs-acetaldehyde treatment group but was fully reversed by rosiglitazone treatment (Fig. 5).

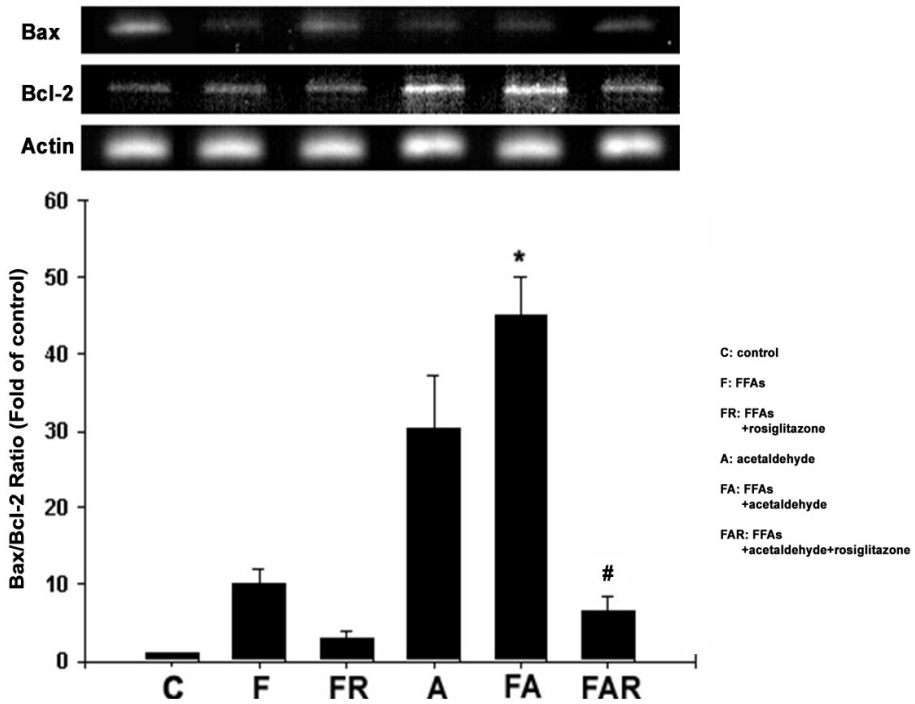

Fig. 5. The effect of rosiglitazone on the expression of Bax and Bcl-2 in HepG2 cells. Rosiglitazone decreased Bax/Bcl-2 ratio in HepG2 cells. Cells were treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone for 48 h, and total RNA and crude extracts were collected for semi-quantitative RT-PCR. The levels of Bax and Bcl-2 were quantitated by densitometric analysis. Data are means ± SD (n = 4). *$P<0.05$, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}P<0.05$, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR).

6. The effect of rosiglitazone on acetaldehyde-induced cytochrome *c* release in HepG2 cells

Using Western blot analysis, we investigated the possible effect of rosiglitazone on FFAs-acetaldehyde-induced cytochrome *c* release from mitochondria. As shown in Fig. 6, FFAs-acetaldehyde treatment significantly induced cytochrome *c* release, to approximately 6.6-fold of that of the control. However, the induction of cytochrome *c* release was inhibited in the presence of rosiglitazone. The release of cytochrome *c* was inhibited by 42.4% compared to FFAs-acetaldehyde treatment group by additional rosiglitazone treatment. From our observation, an increase in cytochrome *c* release correlates well with an increase in the Bax/Bcl-2 ratio, as pro-apoptotic Bax is thought to be upstream of cytochrome *c* release in the mitochondria-mediated apoptosis pathway.

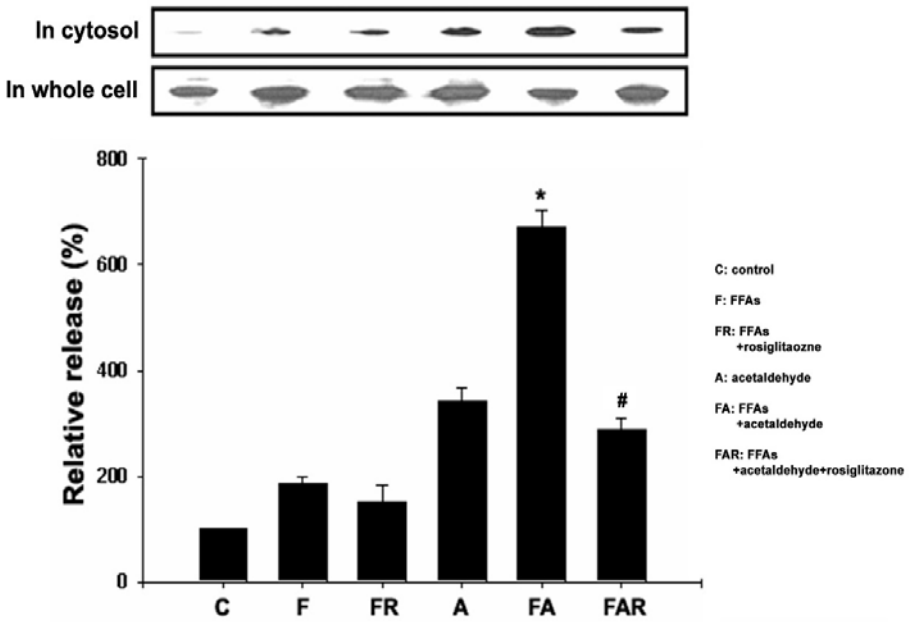

Fig. 6. The effect of rosiglitazone on acetaldehyde-induced cytochrome *c* release in HepG2 cells. Rosiglitazone inhibited the release of cytochrome *c* from mitochondria in HepG2 cells. Cells were treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone for 48 h, and crude extracts and cytosolic extracts were collected for Western blot analysis. The release of cytochrome *c* were quantitated by densitometric analysis. Data are means ± SD (n = 3). *$P<0.05$, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}P<0.05$, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR).

7. The effect of rosiglitazone on FFAs-acetaldehyde-induced caspase-3 activity in HepG2 cells

Because caspase-3 is an important apoptotic biomarker of the apoptotic, [22] its activity was measured in this study. The effect of rosiglitazone on FFAs-acetaldehyde-induced caspase-3 up-regulation is shown in Fig. 6. Following 48 h treatment of HepG2 cells with FFAs-acetaldehyde, we detected a dramatic increase in caspase-3 activity. Addition of rosiglitazone attenuated acetaldehyde-induced caspase-3 activity. However, cyanamide, an ALD2 inhibitor, treatment blocked rosiglitazone mediated attenuation of acetaldehyde-induced caspase-3. To confirm the effects of rosiglitazone by inducing ALD2 expressions in an activity of caspase-3, we also treated cells with cyanamide as ALD2 inhibitor and it eliminated the effect of rosiglitazone.

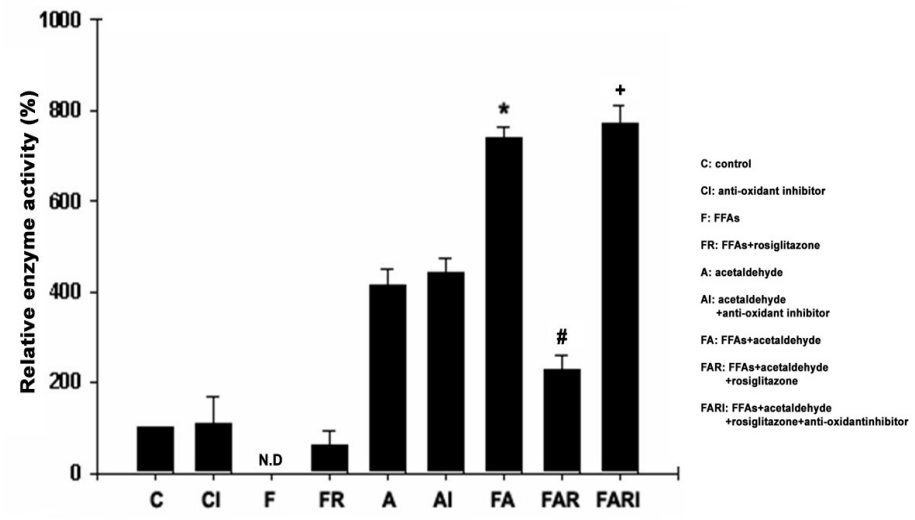

Fig. 7. The effect of rosiglitazone on FFAs-acetaldehyde-induced caspase-3 activity in HepG2 cells. Rosiglitazone inhibited caspase-3 activity in HepG2 cells. Cells were treated with 0.3 mM FFAs and 100 μM acetaldehyde in the absence and presence of 5 μM rosiglitazone and 25 μM ALD2 inhibitor for 48 h, and crude extracts were collected for enzyme activity assay. Data are means ± SD (n = 3). *P<0.05, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). $^{\#}$P<0.05, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR). $^{+}$P<0.05, FFAs-acetaldehyde-rosiglitazone treatment (FAR) vs. FFAs-acetaldehyde-rosiglitazone-anti-oxidant enzyme inhibitors treatment (FARI). N.D, not detected.

8. The effect of rosiglitazone on high fat diet induced fatty liver change and blood chemistry in SD rats

Fatty liver disease was induced after 5 weeks of high fat diet in SD rats. In H&E staining analysis, hepatic lipid droplets were formed in liver treated with high fat diet. Alcohol consumed SD rats liver also has lipid droplets, but smaller (average 80% of high fat diet group) than those in high fat diet treated liver. The liver treated with alcohol and high fat diet has more lipid droplets than other groups. However, rosiglitazone recovered impaired conditions by decreasing lipid droplets. In TUNEL staining analysis, hepatic apoptosis induced after 8 weeks of alcohol and high fat diet feeding were significantly increased to 3.42 times of control in SD rats with fatty liver. Rosiglitazone also shows a possibility that it is able to block apoptosis procedure (Fig. 8).

Indeed, serum triglycerides levels were significantly increased to 300% in SD rats after high fat diet-alcohol treatment compared with control groups. But, they were significantly decreased to 40% in SD rats fed high fat diet-alcohol-rosiglitazone compared with those of high fat-alcohol group. Free fatty acids (FFAs) levels were increased to 1.8 times of control group by treating alcohol and high fat diet-alcohol. However, FFAs levels were decreased to levels of control by rosiglitazone treatment. Serum aspartate

aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were significantly increased to 2 and 5.8 times of control group, respectively in SD rats fed high fat diet-alcohol. Level of the total bilirubin was also elevated to 1.3-1.5 mg/dl in SD rats fed sole high fat diet, alcohol compared with control, and the highest level in high fat diet-alcohol group was observed. However, rosiglitazone significantly recovered moderately elevated level of the total bilirubin (Fig. 9). These results indicate that combination of high concentration alcohol and high fat diet consumption results in more severe hepatocyte damage than sole treatment. But, rosiglitazone is able to protect this additive damage.

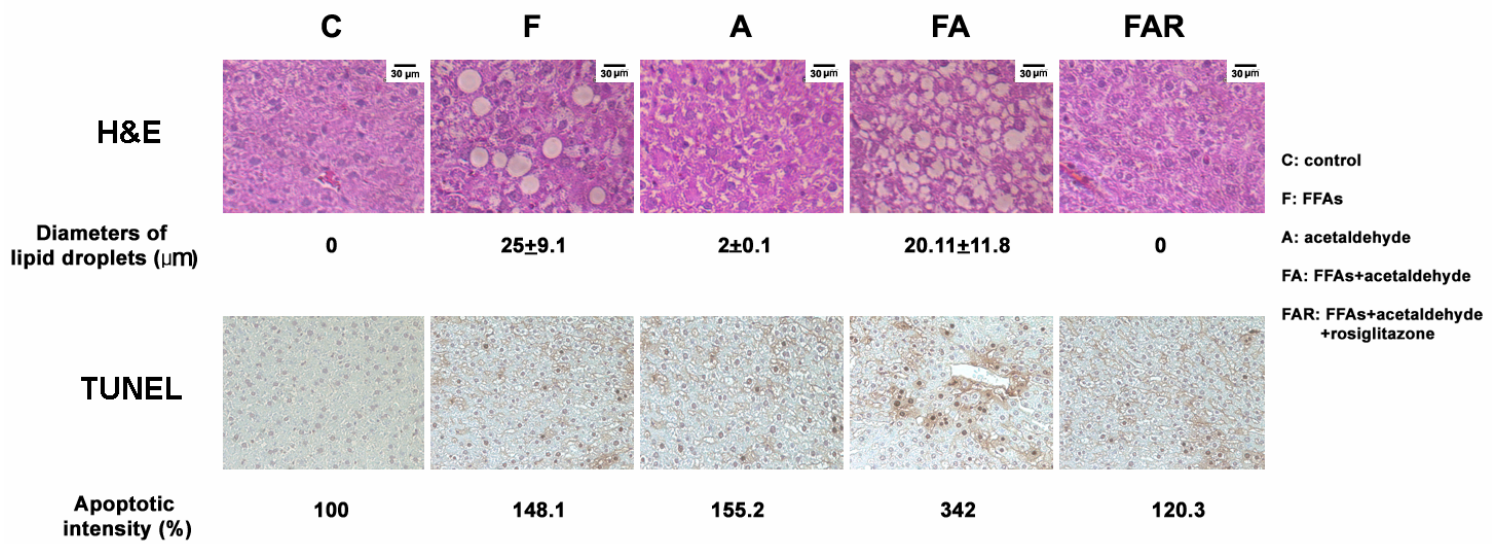

Fig. 8. The effect of rosiglitazone on high fat diet induced fatty liver change in SD rats. Phenotypical changes in liver of experimental animals. Light micrographs of liver stained by hematoxylin and eosin (H & E) and TUNEL method were observed, respectively. Scale bar on the higher right of the higher photo, 30 μm. Abbreviation used: C, control; F, high fat diet treatment; A, ethanol treatment; FA, high fat diet-ethanol treatment; FAR, high fat diet-ethanol-rosiglitazone treatment.

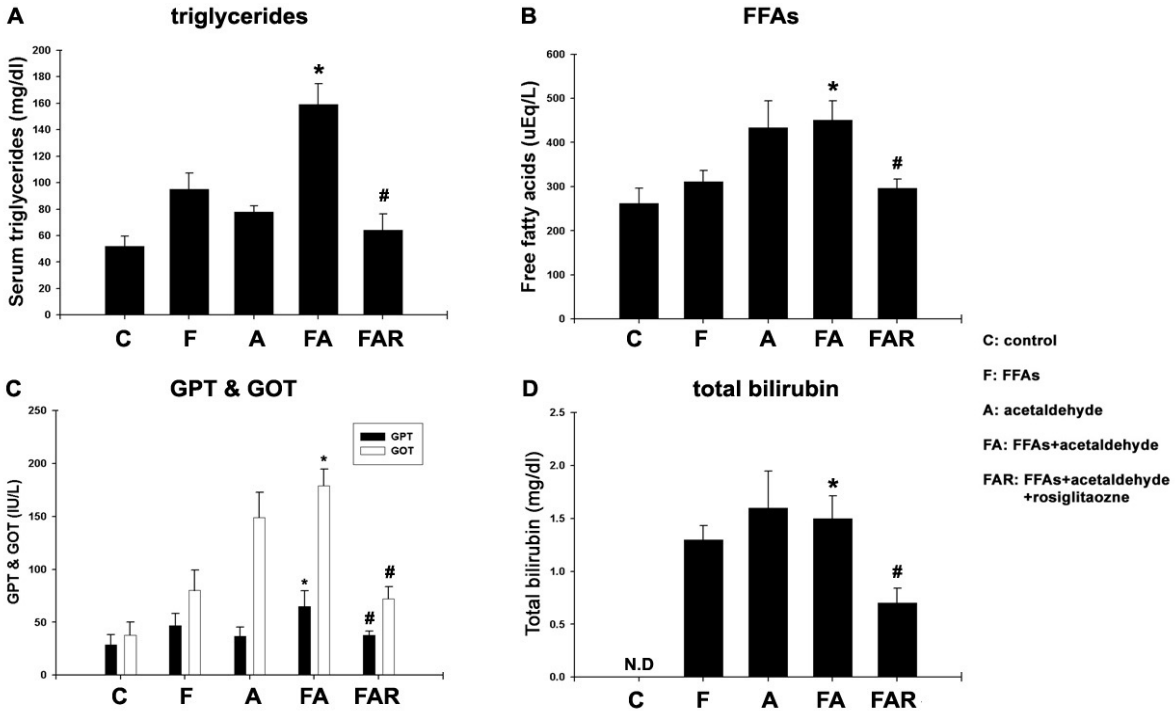

Fig. 9. Blood chemistry value of experimental animals. Serum triglycerides (A), serum free fatty acids (B), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), (C) and serum total bilirubin (D) in experimental animals. Data are means ± SD (n = 3). *P<0.05, FFAs treatment (F) vs. FFAs-acetaldehyde treatment (FA). #P<0.05, FFAs-acetaldehyde treatment (FA) vs. FFAs-acetaldehyde-rosiglitazone treatment (FAR). N.D, not detected.

IV. Discussion

Although middle aged people have lower prevalence of alcohol drinking than young people, [1] they suffer liver disease more frequently than young because of a higher incidence of fatty liver in middle aged people. Many middle aged people with excess fat in the abdominal area have a fatty liver. In this condition, the liver has stopped burning fat and has turned into a fat storing organ decreasing its function. Fatty acids or fatty acyl CoA may inhibit genes involved in liver metabolism including ALD2. [8]

To examine the cell death induced by acetaldehyde, we used Hur7 cells which are normally expressing alcohol dehydrogenase and treated them with alcohol. Acetaldehyde in HepG2 cells and alcohol in Hur7 cells decreased cell viability in same pattern.

The results from a present study suggest that adverse hepatic changes, such as accumulation of triglycerides and apoptotic bodies are the results of elevated cellular toxicity mediated by impairment of expressions of ALD2 and anti-oxidant enzymes. The first possibility is consistent with the finding that increased fatty acid levels suppress ALD2 expression through the inhibition of nuclear receptors in obese mice models. [15] ALD2 promoter includes PPRE. [23] ALD2 expression may be important for the protection of

liver, because appropriate ALD2 expression is able to close the door leading to cell death by eliminating acetaldehyde. The second possibility is supported by the facts that catalase and Cu, Zn-SOD promoters have PPRE [24] and these enzymes can be potentially regulated by PPAR-γ. [12-14] PPAR-γ also regulates production of the ROS. It is well known that mitochondria lies at the center of the process of cell death regulation. Induction of apoptosis often converges on the mitochondria to induce MPT and release of apoptotic proteins into the cytoplasm, resulting in a biochemical and morphological alteration of apoptosis. Although the precise mechanism by which the Bcl-2 family acts remains unclear, it has been established that the Bcl-2 family plays a key role in the mitochondrial apoptotic pathway. [25] Bax and Bcl-2, the two main members of Bcl-2 family, affect the permeability of the mitochondrial membrane. Bax is a pore-forming cytoplasmic protein, translocates to the outer mitochondrial membrane, influences its permeability. It induces cytochrome *c* release from the intermembrane space of the mitochondria into the cytosol, and subsequently leads to apoptosis. [26] Bcl-2, which has anti-apoptotic properties, is associated with the outer mitochondrial membrane where it stabilizes membrane permeability. It preserves mitochondrial integrity, thus suppresses the release of cytochrome *c* and inhibits apoptosis. [27] Cell survival in the early phases of the apoptotic

cascade depends mostly on the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family. In this regard, the Bax/Bcl-2 ratio may be a better predictor of apoptotic fate than the absolute concentrations of either Bax or Bcl-2 alone. [25] In this study, we confirmed that FFAs-acetaldehyde-induced apoptosis is associated with Bax/Bcl-2 pathway. The opening of the MPT pores causes a release of apoptogenic substances such as cytochrome *c* from mitochondria into the cytosol. [28] Cytochrome *c* release from mitochondria was proven to play a critical role in apoptosis. [29] In the mitochondrial pathway, a variety of stimuli can trigger the MPT and the release of cytochrome *c*. The opening of the MPT pores is associated with collapse of the membrane voltage. [30] Given the key role of the ratio between Bax and Bcl-2 levels in the apoptotic cascade, it is not surprising that in our experiments treatment with rosiglitazone is also associated with the prevention of the downstream apoptotic signaling pathways, finally preventing release of cytochrome *c* from mitochondria. In this study, the involvement of mitochondria in FFAs-acetaldehyde-induced apoptosis and its recovery were investigated by measuring released cytochrome *c*. Using Western blot analysis, we investigated the possible effect of rosiglitazone on acetaldehyde-induced cytochrome *c* release from mitochondria and caspase-3 expression. As expected, rosiglitazone inhibited both cytochrome *c* release

and caspase-3 expression. Lastly, we applied this study to SD rats as an *in vivo* study. In accordance with the results of *in vitro* study, rosiglitazone improved fatty liver and alcohol toxicity. It also decreased levels of serum FFAs, triglycerides, AST/ALT, and total bilirubin.

Although the precise mechanism underlying the increased hepatic apoptosis resulting from alcohol treatment in SD rats with fatty liver can not be determined results from the present studies could provide one of the pathogenic mechanisms although several common factors can work in concert to cause this type of liver damage.

In conclusion, we confirmed one of the underlying mechanisms of alcohol toxicity in non-alcoholic fatty liver. Rosiglitazone moderately protects fatty liver from alcohol toxicity by preventing apoptosis. Further detailed studies of liver protective mechanisms of rosiglitazone will be necessary before definite conclusions can be drawn. Based on the protective role of rosiglitazone on alcohol toxicity in fatty liver, our results presented in this report may open up a new clinical perspective in alcohol toxicity in fatty liver.

V. Conclusion

To assess the protective effect and its underlying mechanisms of rosiglitazone against alcohol toxicity in non-alcoholic fatty liver, several *in vitro* experiments including the measurement of cell viability, ALD2 expression, antioxidant enzymes activities, and apoptotic cascades in HepG2 cells and in vivo experiments including morphologic and TUNEL analysis of livers and blood chemistry are performed.

We obtain the following results.

1. Rosiglitazone protects HepG2 cells from toxicities of acetaldehyde and additive FFAs.
2. Rosiglitazone recovers impaired ALD2 and antioxidant enzyme expressions under FFAs-acetaldehyde in HepG2 cells.
3. Rosiglitazone prevents inappropriate apoptosis by FFAs-acetaldehyde in HepG2 cells
4. Rosiglitazone inhibits formation of lipid droplets and local apoptosis induced by high fat diet and alcohol treatment in rats
5. Rosiglitazone decreases serum AST, ALT, total bilirubin, triglycerides, FFAs are elevated by high fat diet and alcohol treatment in rats.

According to the results observed in the study, we can conclude that rosiglitazone may protect non-alcoholic fatty liver from additive alcohol toxicity. This protection seems to be mainly through the induction of ALD2 which eliminates acetaldehyde and antioxidant enzymes which digest ROS produced from mitochondrial dysfunction by acetaldehyde.

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국문요약

비 알콜성 지방간 모델에서 rosiglitazone의 알코올 독성으로부터 간 보호작용

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정 태 우

Peroxisome proliferator activated receptor (PPAR)-γ agonist인 rosiglitazone은 혈당 조절 작용 외에 항염증, 활성산소 생성 억제와 세포 사멸을 억제하는 효과를 지닌 것으로 알려져 있다. 본 연구에서 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 세포사멸에 관련한 유전자 발현양상 확인, 그리고 간의 조직소견과 TUNEL 분석에 의한 세포사멸 측정을 통하여 rosiglitazone의 알코올에 의한 비알코올성 지방 간 손상방지 기전을 분석하였다.

세포 실험인 경우, 고지방상태의 간세포 (HepG2)에서

아세트알데하이드를 무독화시키는 mitochondrial aldehyde dehydrogenase (ALD2)와 항 산화효소의 발현이 감소된 것을 rosiglitazone의 처리에 의한 ALD2와 항 산화 효소의 회복을 확인하였다. 아세트알데하이드 단독투여와, 고지방과 아세트알데하이드 동시 투여 모두 ALD2, 항 산화효소의 감소를 유발하였고 rosiglitazone의 처리로 인한 회복을 확인하였다. 동물실험인 경우, Rosiglitazone을 투여한 군의 혈액을 분석한 결과 고지방식과 에탄올로 인하여 높아진 혈중 aspartate aminotransferase, alanine aminotransferase, 중성지방, 유리지방산, 총 bilirubin의 농도의 감소를 확인하였고, 각 군 간의 조직소견을 분석하였을 때 고지방식과 에탄올로 인한 지방간의 호전과 간세포사멸의 감소를 관찰하였다.

위와 같은 연구결과로 rosiglitazone의 사용은 지방간 상태에서 알코올 독성을 완화하는 작용을 지니고 있는 것을 확인 하였고 이에는 ALD2와 항 산화효소의 발현 유도가 한 기전이라고 사료된다.

핵심 되는 말: 지방간, 알코올, rosiglitazone, 아세트알데하이드, 세포사멸, mitochondrial aldehyde dehydrogenase, 항산화 효소