Both Rosiglitazone and Oleate Protect MCD-induced Non-alcoholic Fatty Liver Disease

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Directed by Professor Bong-Soo Cha

The Master's Thesis

Submitted to the Department of Medical Science

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

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December 2007

This certifies that the Master's Thesis
of Ji-Young Lee is approved.

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December 2007

Acknowledgements

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Feeding mice a methionine choline deficient (MCD) diet leads to the development of steatohepatitis with fibrosis and serves as an animal model for non-alcoholic fatty liver disease (NAFLD). Rosiglitazone, a class of insulin-sensitizing thiazolidinedione (TZD) drugs, has been widely used for treating hyperglycaemia and improving insulin resistance. Oleate, the major monounsaturated fatty acid of olive oil, has been known to show various anti-atherogenic, and to beneficial effects on liver antioxidant enzyme. In this study, we compared the efficacy of rosiglitazone versus oleate against MCD

diet-induced NAFLD mice model. Our results suggest that rosiglitazone and oleate play important roles in the prevention of NAFLD but via different mechanism. Oleate provided significant improvement in both hepatic clearance of fatty acids and pro-inflammatory cytokine-mediated hepatocyte injury. Rosiglitazone did not influence fat accumulation but protected fat derived inflammatory response. These suggest that both rosiglitazone and oleate may be useful therapeutic agents for treatment of NAFLD in different aspects.

Key words: Non-alcoholic fatty liver disease (NAFLD), Methionine choline deficient diet (MCD), Rosiglitazone, Oleate.

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Ⅰ. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of disorders characterized by hepatic fat acculmulation ranging from simple steatosis to severe steatohepatitis with centrilobular necroinflammation[1]. It is a complex metabolic condition in which both life style and genetic factors have a pathogenic role[2] and has been increasingly recognized as a major cause of liver-related morbidity and mortality[3].

Although the pathogenesis of non-alcoholic steatohepatitis is not well understood, a two-hit theory has been proposed. According to this theory, hepatic steatosis is mainly caused by metabolic syndrome: the first hit. Then,

the hepatic steatosis develops into non-alcoholic steatohepatitis due to the effects of oxidative stress, reactive oxygen species, lipid peroxidation and/or any cytokine: the second hit[4].

Feeding mice with a methionine choline deficient (MCD) diet leads to the development of steatohepatitis with fibrosis and serves as an animal model for NAFLD[5, 6]. Rodents fed an MCD diet have been shown to have higher levels of TNF-alpha and inflammatory cytokines. MCD diet impairs mitochondrial ß-oxidation and leads to induction of steatosis[7].

Rosiglitazone has been widely used for treating hyperglycaemia and improving in sulin resistance. Peroxisome proliferator-activated receptor-γ (PPARγ), a member of the nuclear hormone superfamily, is expressed in many tissues[8]. PPARg has endogenous and pharmacological ligands including 15-deoxy-$_{D}$12,14-prostaglandin J2 and two thiazolidinediones (TZD), rosiglitazone and pioglitazone, which enhance insulin sensitivity and are used clinically to treat type-2 diabetes mellitus[9].

Oleic acid, the richest source of fatty acid in olive oil, is a monounsaturated fatty acid (MUFA, OA; 18:1n-9)[10]. Recent studies have shown that diets rich in monounsaturated fatty acids have effects on inflammation[11]. The strongest evidence that monounsaturated fatty acids such as oleate may influence recovery of antioxidant enzymes in liver[12]. Steatosis recovery after treatment

with a olive oil-based diet involve with involvement of stellate cell[13].

Thus, pharmacologic agents that reduce inflammatory cytokines and mitochondrial ß-oxidation in the liver may prevent the progression from steatosis to steatohepatitis, and provides a potentially rational therapy for the treatment of NAFLD.

The purpose of this study was to investigate the protective effects of rosiglitazone or oleate on MCD diet induced hepatotoxicity in C57BL/6J mice in order to find a possible therapeutic application to degenerative disease, as well as provide new strategies for prevention and treatment of non-alcoholic fatty liver disease.

Ⅱ. MATERIALS AND METHODS

1. Chemicals

Rosiglitazone was obtained from GlaxoSmithKlin (Avandia, USA). Oleate was purchased by Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma Aldrich (St. Louis, MO, USA).

2. Experimental conditions

8-week-old C57BL/6J mice were purchased from Samtako (O San, Korea) and weighing 20 g to 21 g underwent experiments performed in accordance with the guidelines for Animal Research from AAALAC International and approved by Department of Yonsei Animal Research Center. The animals were housed in cages under 12/12-h light/dark cycles. The mice received a chow diet and MCD diet with tap water and libitum through the experiment for 4 weeks. At first, all mice were fed the normal diet during a 1-week quarantine and acclimation period. Then, at 8 weeks of age, mice displaying no abnormal findings at the end of the quarantine and acclimation period were randomly divided into four groups (n=10/group): chow diet, MCD diet, rosiglitazone with MCD diet and oleate with MCD diet. All experimental procedures were performed under sterile conditions. Rosiglitazone (20

mg/Kg) or oleate (0.5 mg/g) were given orally once daily to mice fed with a MCD diet for 4 weeks.

3. Biochemical examination

Blood was collected from the heart puncture. Blood samples were collected for the measurement of plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and high density lipoprotein (HDL)-cholesterol concentrations were measured using the standard techniques of clinical chemistry laboratories. Triglyceride concentrations were measured using a thermo triglyceride test kit according to the manufacturer's instructions (Thermo Fisher Scientific, U.S.A).

4. Histopathological analysis

Livers were weighed to allow relative liver weights (with respect to body weight) to be calculated. Livers were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 3–4μm. Standard hematoxylin-eosin and trichrome stains (TRC) staining was performed. The fresh frozen tissue will be frozen immediately after the animal was dissected. Place tissue in pre-labeled base molds filled with OCT. Store frozen tissue block in -80°C freezer until sectioning. Routine section are cut at 7 μm and picked up onto slides.

Frozen sections were stained with Oil red O. For the evaluation of hepatic steatosis, sections were stained with Oil-red O and TRC, and the average area (%) of the fat droplets and fibrosis within hepatocytes in 3 fields (×100 magnification) was measured with the aid of a image analyzer.

5. mRNA analysis

The hepatic levels of the messenger RNA (mRNAs) for monocyte chemoattractant protein (MCP) 1, tumor necrosis factor (TNF)-α, and matrix metalloproteinase (MMP)9 and acyl-CoA oxidase (ACO) were assessed by semi-quantitative RT-PCR analysis using GAPDH as a house keeping gene. Total RNA was extracted from livers using Trizol (Invitrogen, Carlsbad, CA). Contaminating DNA was removed by treatment of each sample with DNase I, according to the manufacturer's instructions (Promega, Madison, WI). cDNA was prepared using SuperScript II™ first strand synthesis system, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RT-PCR products were electrophoresed on a 1%(w/v) agarose gel, stained with ethidium bromide and bands were visualized by UV light.

6. Statistical analysis

Data are presented as means ± SD. Statically significant difference between

groups calculated by student t-test. A value of $P < 0.05$ is considered significant.

Ⅲ. RESULTS

1. Body weight changes and relative liver weight

Body weight was measured from the beginning and the end of the experiment, at the terms of 2 days. Body weight in MCD-diet fed mice show significantly decreased compared to chow-diet-fed mice. MCD diet intake had significantly impact on body weight loss both rosiglitazone treated mice and oleate treated mice, respectively (Fig. 1A, B).

Liver weight/body weight ratio in MCD diet fed mice significantly decreased compared to chow diet fed group. Nearly 23% decrease in liver weight / body weight were found in MCD diet fed mice compared to chow diet fed mice (Fig. 1C).

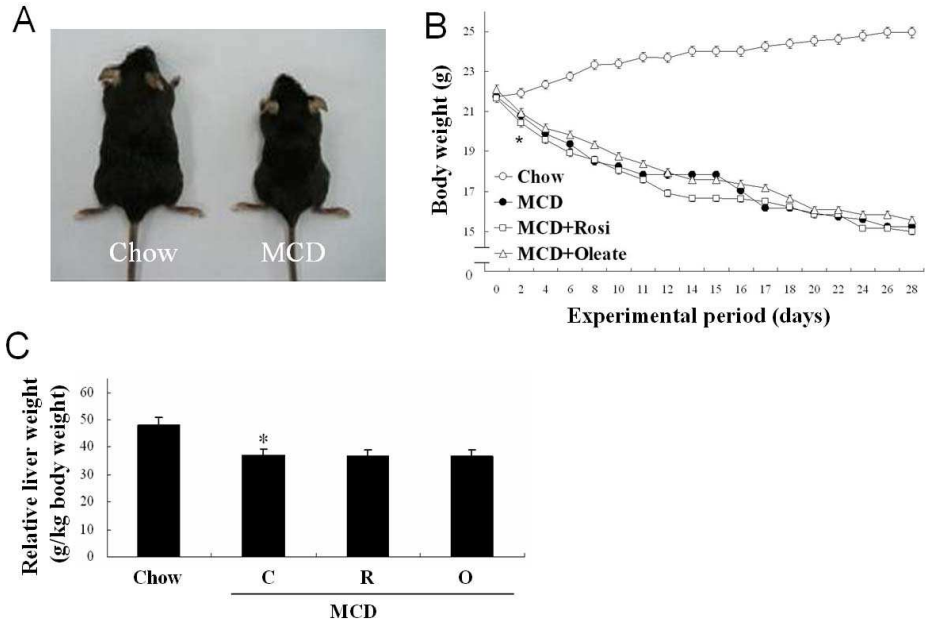

Fig. 1. Body weight changes and relative liver mass. (A) Photographs of mice are shown (Chow vs. MCD: C57BL/6J mouse on left was treated with chow diet for 4 weeks. C57BL/6J mouse on right was treated with MCD diet for 4 weeks.) (B) Effect of Chow, MCD, MCD + rosiglitazone and MCD + oleate supplemented diets on body weight. (C) Relative liver weight (g/Kg BW). Data represent means ± S.D. for 10 mice in each group. * $P < 0.05$ vs. chow. Chow: Chow-diet-fed mice; MCD: MCD-diet-fed mice; C: MCD control; R: Rosiglitazone, 20 mg/Kg/day; O: Oleate, 0.5 mg/g/day.

2. Effects of rosiglitazone and oleate on biochemical parameters

Plasma triglyceride, HDL-cholesterol concentrations were lower in MCD-diet-fed control mice than in chow-diet fed mice. Both rosiglitazone and oleate treated mice, had significant decrease effect on triglyceride concentration compare to chow diet mice (Fig. 2). Triglyceride showed as 151.9 ± 18.9 mg/dL in chow-diet-fed mice, but greatly decreased in MCD-diet-fed mice (82.1 ± 4.8 mg/dL), rosiglitazone treated mice (89.4 ± 16.9 mg/dL) and oleate treated mice (90.1 ± 11.1 mg/dL), respectively.

MCD diet fed, both rosiglitazone and oleate treated mice, had significant decrease effect on HDL cholesterol level compare to chow diet group. HDL-cholesterol showed as 50.4 ± 0.55 mg/dL in chow-diet-fed mice, but greatly decreased in MCD-diet-fed mice (16.8 ± 0.45 mg/dL), rosiglitazone treated mice (13.2 ± 0.45 mg/dL) and oleate treated mice (17.2 ± 0.84 mg/dL), respectively.

3. Rosiglitazone and oleate inhibited NAFLD **on plasma AST and ALT**

Mice fed the MCD diet for 4 weeks developed severe steatohepatitis, with an associated elevation in the plasma AST and ALT. Plasma AST concentration showed in chow-diet-fed mice was 83.4 ± 0.89 IU/L but greatly increased in MCD-diet-fed mice (477.6 ± 5.68 IU/L). Plasma AST increased in MCD diet fed mice, but decreased 19.8% and 39.8% by treatment with rosiglitazone (383.2±43.36 IU/L) and oleate (287.6±53.37 IU/L), respectively. Treatment with rosiglitazone and oleate inhibited this elevation in the plasma AST and ALT concentration (Fig. 2).

Likewise, plasma ALT in MCD-diet-fed mice was shown 652.0±7.58 IU/L significantly increased compared to chow-diet-fed mice (38.6±0.55 IU/L). Plasma ALT increased in MCD-diet-fed mice, but decreased 29.1% and 32.1% by treatment with rosiglitazone (462.2±11.63 IU/L) and oleate (443.4±6.23 IU/L). Plasma ALT and AST levels were significantly lower both rosiglitazone treated mice and oleate treated mice as compared with MCD-diet-fed mice (Fig. 2).

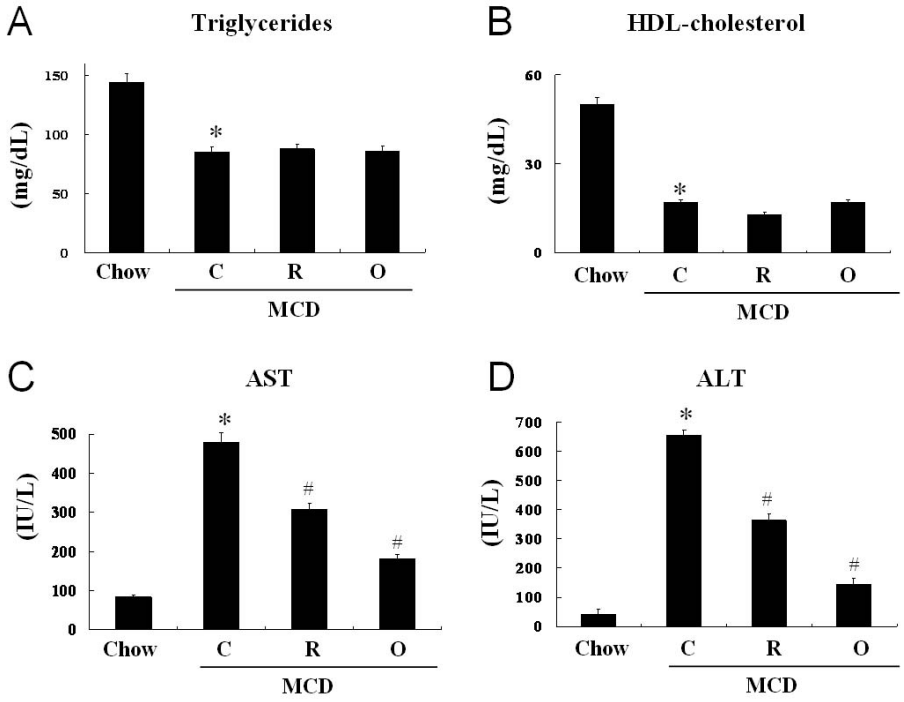

Fig. 2. Effects of rosiglitazone and oleate on plasma triglyceride, HDL-cholesterol, AST and ALT levels. (A) Hepatic triglycerides level (B) Hepatic HDL-cholesterol level (C) Plasma AST level (D) Plasma ALT level. Data represent means ± S.D. for 10 mice in each group. * $P < 0.05$ vs. chow. $^{\#}P < 0.05$ vs. MCD control. Chow: Chow-diet-fed mice; MCD: MCD-diet-fed mice C: MCD control; R: Rosiglitazone, 20 mg/Kg/day; O: Oleate, 0.5 mg/g/day.

4. Histopathological analysis

H & E, Oil-red-O stain and TRC were carried out. In MCD-diet-fed mice, numerous fat droplets were observed within hepatocytes and numerous swollen cells were observed in liver (Fig. 3). Percent of the sum of fat droplets area to total area showed that increases in fat droplets area in MCD-diet-fed mice (Fig. 4). Pericellular fibrosis is shown. Numerous collagen fibers were observed in liver from MCD-diet-fed mice (Fig. 5). Rosiglitazone treated mice, fat droplets area does not changed as compared with MCD-diet-fed mice but have no presence of cell swelling in hepatocyte. Interestingly, fat droplets area was almost completely absent in oleate treated mice. Rosiglitazone treated mice, the numbers of collagen fibers was no higher than in chow fed mice. Oleate treated mice, the numbers of collagen fibers was no higher than in chow fed mice.

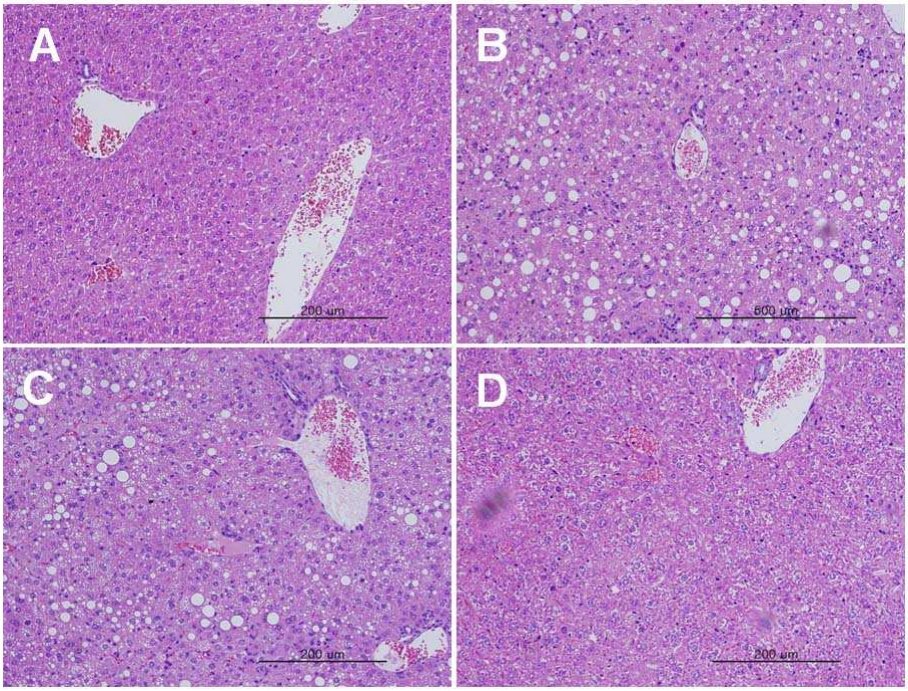

Fig. 3. Histology (H&E, Hematoxylin and eosin stain, × 100) of liver paraffin embedded section. (A) chow-diet-fed mice (B) MCD-diet-fed mice (C) Rosiglitazone (20 mg/Kg/day) with MCD-diet-fed mice (D) Oleate (0.5 mg/g/day) with MCD-diet-fed mice. 200-μm scale bar is shown in the panel.

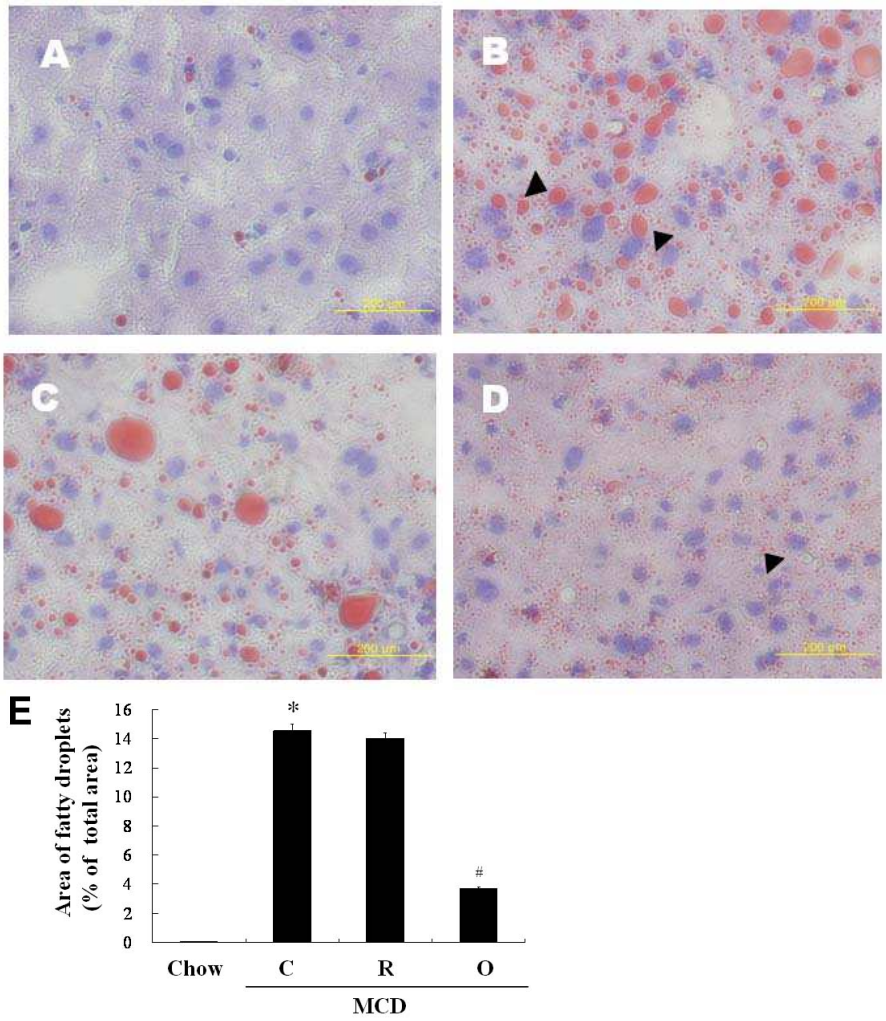

Fig. 4. Effect of rosiglitazone and oleate on lipid accumulation in liver frozen section was determined by Oil-Red-O staining (×100). (A) Chow-diet-fed mice (B) MCD-diet-fed mice (C) Rosiglitazone (20 mg/Kg/day) with MCD-diet-fed mice (D) Oleate (0.5 mg/g/day) with MCD-diet-fed mice. 200-μm scale bar is shown in the panel. Numerous fat droplets (▶) were observed in liver from MCD fed mice. (E) The areas of hepatic fat droplets were measured and compared. Data represent means ± S.D. * $P < 0.05$ vs. chow. # $P < 0.05$ vs. MCD control. Chow: Chow-diet-fed mice; MCD: MCD-diet-fed mice C: MCD control; R: Rosiglitazone, 20 mg/Kg/day; O: Oleate, 0.5 mg/g/day.

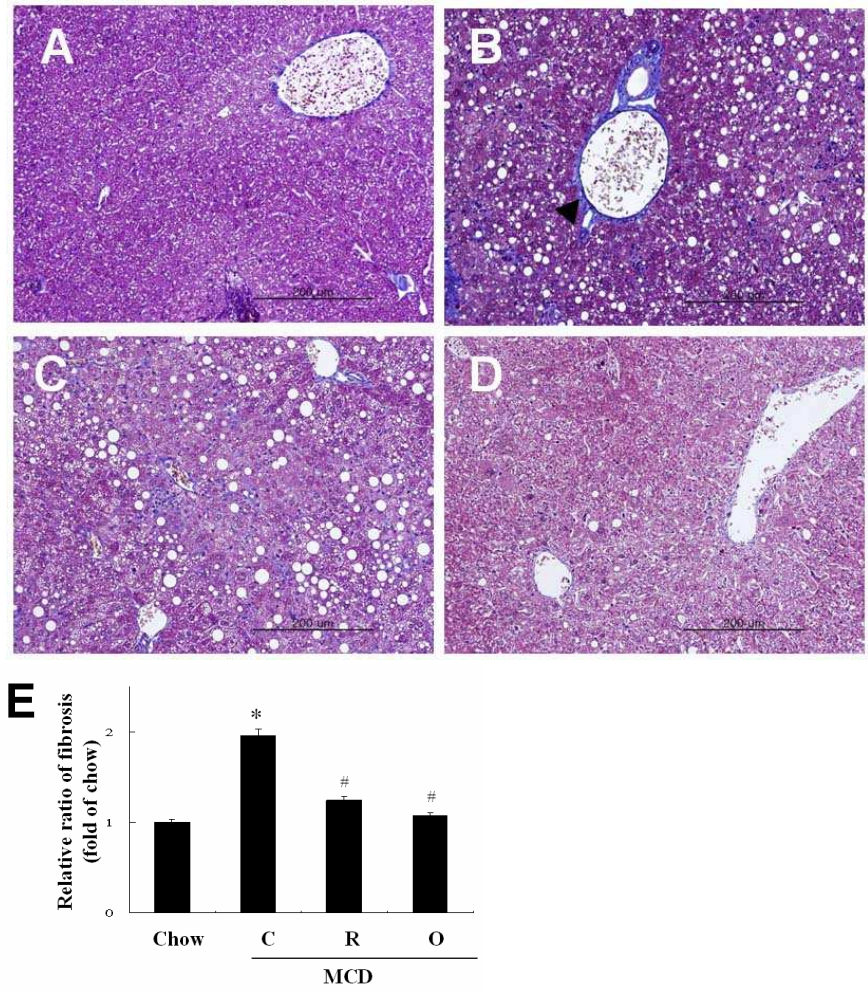

Fig. 5. Histology (trichrome stain, ×100) of liver paraffin embedded section. (A) Chow-diet-fed mice (B) MCD-diet-fed mice (C) Rosiglitazone (20 mg/Kg/day) with MCD-diet-fed mice (D) Oleate (0.5 mg/g/day) with MCD-diet-fed mice. 200-μm scale bar is shown in the panel. Numerous collagen fibers (▶) were observed in liver from MCD fed group. After treatment the liver with rosiglitazone and oleate has returned to a normal appearance. (E) The areas of hepatic fibrosis were measured and compared. Data represent means ± S.D. * $P < 0.05$ vs. chow. # $P < 0.05$ vs. MCD control. Chow: Chow-diet-fed mice; MCD: MCD-diet-fed mice C: MCD control; R: Rosiglitazone, 20 mg/Kg/day; O: Oleate, 0.5 mg/g/day.

5. mRNA analysis

In liver tissue, MCD diet had significant effects on inflammatory cytokine regulation. There are remarkable increases in MCP 1, TNF- α and MMP 9 mRNA expression.

MCP 1 mRNA expression increased 1.96 *fold* in MCD-diet-fed-mice compared to chow-diet-fed mice. MCP 1 mRNA expression decreased 35.2% in rosiglitazone treated mice (1.28 ± 0.22 *fold* compared to chow-diet-fed mice) and 22.2% in oleate treated mice (1.52 ± 0.23 *fold* compared to chow-diet-fed mice) compared to MCD-diet-fed mice. TNF- α mRNA expression increased 1.85 *fold* in MCD-diet-fed-mice compared to chow-diet-fed mice. TNF- α mRNA expression decreased 38.5% in rosiglitazone treated mice (1.15 ± 0.19 *fold* compared to chow-diet-fed mice) and 37.3% in oleate treated mice (1.17 ± 0.22 *fold* compared to chow-diet-fed mice) compared to MCD-diet-fed mice. MMP 9 mRNA expression increased 1.59 *fold* in MCD-diet-fed-mice compared to chow-diet-fed mice. MMP 9 mRNA expression decreased 24.0% in rosiglitazone treated mice (1.21 ± 0.18 *fold* compared to chow-diet-fed mice) and 24.2% in oleate treated mice (1.22 ± 0.31 *fold* compared to chow-diet-fed mice) compared to MCD-diet-fed mice. ACO mRNA expression levels significantly decreased in MCD-fed-mice (0.88 ± 0.98) compared with chow-diet-fed mice. Rosiglitazone (0.89 ± 0.16 *fold*

compared to chow-diet-fed mice) treated mice did not effect on ACO mRNA expression compared to MCD-diet-fed-mice (0.88 ± 0.16 *fold* compared to chow-diet-fed mice). Otherwise, oleate treated mice (0.98 ± 0.23 *fold* compared to chow-diet-fed mice) showed significantly increase in ACO mRNA expression compared to MCD-diet-fed mice (Fig. 6).

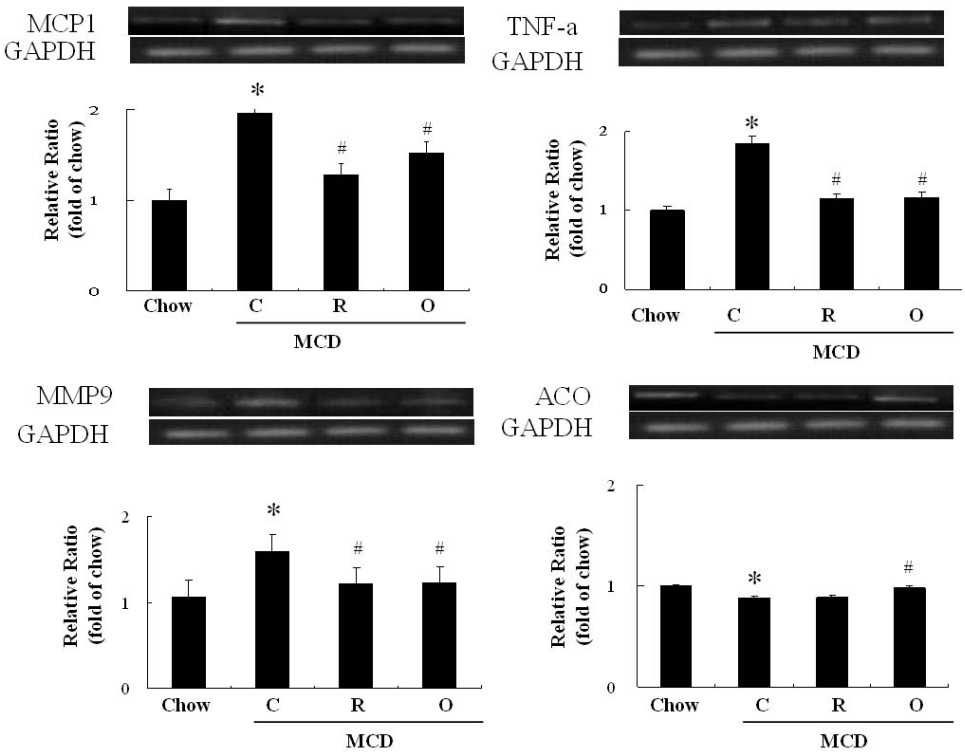

Fig. 6. The effect of rosiglitazone and oleate on monocyte chemoattractant protein (MCP) 1, tumor necrosis factor (TNF)-α, matrix metalloproteinase (MMP)9 and acyl-CoA oxidase (ACO) mRNA expression. Data are based on individual normalized gene expression (relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data represent means ± S.D. for 10 mice in each group. * $P < 0.05$ vs. chow. # $P < 0.05$ vs. MCD control. Chow: Chow-diet-fed mice; MCD: MCD-diet-fed mice C: MCD control; R: Rosiglitazone, 20 mg/Kg/day; O: Oleate, 0.5 mg/g/day.

Ⅳ. DISCUSSION

In the present study shows that methionine and choline deprivation has pronounced effects on hepatic lipid metabolism that go beyond inflammation, fibrosis and the failure of ß-oxidaion[14-16]. The role of dietary choline deficiency in promoting hepatic steatosis and reduced plasma triglycerides levels is well established in the literature[17]. In this study, our results shown that methionine and choline deficiency diet effectively induced non-alcholic fatty liver disease.

Increased hepatocyte accumulation of triglyceride (TG) is the hallmark of NAFLD. Hepatic TG accumulation results from alterations of factors (hepatic and systemic), which control the balance between hepatic lipid input (uptake and synthesis) and output[18]. We have shown that a MCD-diet increases liver fat droplets. Although rosiglitazone did not affect on this fatty droplets increase attenuation. However, within the liver, we show that rosiglitazone result in different fat accumulation pattern. MCD-diet treated mice shown unstable fat accumulation with cell destruction like swelling but rosiglitazone treated mice shown stabilized fat accumulation without cell destruction like swelling. Oleate treated mice shown that remarkably decreased fat droplets area.

Increasing liver enzyme levels, particularly aspartate aminotransferase

(AST) and alanine aminotransferase (ALT), predicts NAFLD[19]. We have shown that a MCD-diet increases these enzyme levels. Both rosiglitazone and oleate treated mice were shown that decreased AST and ALT levels of enzyme involved in NAFLD.

Fibrotic change in liver begins from lipid peroxidation and accumulation of extracellular matrix (ECM) including collagen, proteoglycan and adhesive glicoproteins, which are principally produced by activated hepatic stellate cells. In this study, trichrome stained sections of liver tissue displayed a evidence of fibrosis[20]. MCD control mice show that increased fibrotic change. Both rosiglitazone and oleate treated mice shown that clearly decreased fibrotic change in liver.

A recent study in humans showed that increased hepatic insulin sensitivity with rosiglitazone treatment is associated with decreased in liver fat content[21]. PPARs are known to regulate variety of physiological processes including glucose metabolism and adipogenesis. Recent studies implicated the role of various PPAR isoforms in inflammation; in particular PPAR-g and PPAR-ɣ were reported to regulate the inflammatory response[22].

However, Richard H *et al*. reported that developed cholestatic hepatitis in association with rosiglitazone use in human with type 2 diabetes patients[23]. Rosiglitazone, up to now, has not been known clearly whether it has positive

effect or negative effect on NAFLD. However, our results suggest that function of rosiglitazone critically is involved in anti-inflammatory effect of cytokines such as MCP 1, TNF-α, MMP 9 in NAFLD.

It was shown that administration of olive oil allowed recovery of antioxidant enzymes in liver[24]. Recovery from steatosis after treatment with olive oil-based diet is associated with perisinusoidal stellate cells[25]. Liver fatty acid composition were changed in mice fed olive oil enriched diet[26]. Diet with olive oil modulates several genes related to lipolysis or lipogenesis and newly identified responders from other metabolisms[27]. Several studies have emphasized the importance of dietary composition in the treatment of fatty liver. Monounsaturated fatty acids (MUFA) have traditionally been thought to be good substrates for the ß-oxidation reaction[28]. This has led to the hypothesis that increased dietary levels of specific fatty acids can induce in ß-oxidation capacity. Our results indicate that oleate can increase ß-oxidation capacity by inducing ACO mRNA expression. Our data also suggest that oleate effectively prevent MCD-diet induced lipid accumulation. These data is the first study showing that oleate improves hepatic inflammation by inhibiting MCP-1, TNF-α, MMP9, increase ß-oxidation by enhancing ACO.

The major findings of this study as follows. These observations show that 1) rosiglitazone and oleate play important roles in the prevention of NAFLD

but via different mechanism. 2) Oleate provided significant improvement in both hepatic clearance of fatty acids and pro-inflammatory cytokine-mediated hepatocyte injury. 3) Rosiglitazone did not influence fat accumulation but protected fat derived inflammatory response.

Ⅴ. CONCLUSION

To assess the protective effect and its underlying mechanisms of rosiglitazone and oleate against hepatotoxicity induced with methionine choline deficient diet in non-alcoholic fatty liver, in vivo and in vitro experiments including and blood chemistry are performed.

We obtain the following results.

1. Rosiglitazone normalized elevated serum AST and ALT which are elevated by methionine choline deficient diet in mice.
2. Rosiglitazone inhibits formation of local fibrosis and cell swelling which are induced by methionine choline deficient diet in mice.
3. Rosiglitazone inhibits expression of inflammatory cytokine but not modulating β oxidation failure which are induced methionine choline deficient diet in mice.
4. Oleate normalized elevated serum AST and ALT which are elevated by methionine choline deficient diet in mice.
5. Oleate inhibits not only formation of lipid droplets but also local fibrosis and cell swelling induced by methionine choline deficient diet in mice.
6. Oleate inhibits expression of inflammatory cytokine and also modulates β oxidation failure induced methionine choline deficient

diet in mice.

According to the results observed in the study, we conclude both rosiglitazone and oleate may be useful therapeutic agents for treatment of NAFLD in different aspects.

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Abstract(in Korean)

비알콜성 지방간 동물모델에서 Rosiglitazone과 Oleate의 효과 비교

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이 지 영

필수지방산인 methionine과 choline이 결핍 된 사료 투여 시, 간에 특이적으로 지방이 축적되고 염증이 발생하여 지방간을 유도할 수 있다. 이에 in vivo 실험을 통하여 rosiglitazone과 oleate가 가지는 비알콜성 지방간 억제 효과에 대하여 알아 보고자 하였다. 8주령의 C57BL/6J마우스를 정상식이군, MCD-diet 대조군, MCD-diet와 rosiglitazone (20mg/kg, 처리군 R), MCD-diet와 oleate (0.5mg/g, 처리군 O)로 하여 4주간 사육 후 측정 그 결과를 비교하였다. AST, ALT 수치는 대조군과 비교하여 처리군에서 모두 유의하게 감소하였다(처리군 R: 19.8% 및 처리군 O: 39.8%, 처리군 R: 29.1%

및 처리군 O: 32.1%). Oil red-O 염색 결과, 대조군과 비교하여 처리군 O에서는 fat droplets이 거의 감소 하였으며, 처리군 R에서는 fat droplets area의 변화는 없으나 cell swelling은 관찰 되지 않았다. RT-PCR 결과, MCP1, TNF-α, MMP9 의 발현은 대조군과 비교하여 처리군 R (by 35.2%, 38.5% 및 24.0%), 처리군 O (22.2%, 37.3% 및 24.2%) 모두에서 유의하게 감소하였다. ACO의 발현은 대조군과 비교하여, 처리군 O에서 유의적으로 증가하였으나 처리군 R에서는 차이를 보이지 않았다. Rosiglitazone은 지방 축적에서 대조군과 차이를 보이지 않았으나 지방 유래 염증반응은 현저하게 감소 됨을 확인 할 수 있었다. Oleate는 간에서 지방산 상화를 원활히 하였으며 pro-inflammatory cytokine 에 의한 간 독성을 억제하였다. 본 연구는 rosiglitazone과 oleate가 작용 기전에서는 차이를 보이지만 모두 비알콜성 지방간 억제효과가 있음을 보여준다.

핵심되는 말: 비알콜성 지방간, 메티오닌 콜린 결핍 식이, rosiglitazone, oleate.