Effect of insulin-sensitizing agents in combination with ezetimibe, and valsartan in rats with non-alcoholic fatty liver disease

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Abstract

AIM: To assess whether treatment with insulin-sensitizing agents (ISAs) in combination with ezetimibe and valsartan have greater effect on hepatic fat content and lipid peroxidation compared to monotherapy in the methionine choline-deficient diet (MCDD) rat model of non-alcoholic fatty liver disease (NAFLD).

METHODS: Rats (n = 6 per group) were treated with different drugs, including MCDD only, MCDD diet with either metformin (200 mg/kg), rosiglitazone (3 mg/kg), metformin plus rosiglitazone (M+R), ezetimibe (2 mg/kg), valsartan (2 mg/kg), or combination of all drugs for a total of 15 wk. Liver histology, lipids, parameters of oxidative stress and TNF-alpha were measured.

RESULTS: Fatty liver (FL) rats demonstrated severe hepatic fat infiltration (> 91% fat), with an increase in hepatic TG (+1263%, P < 0.001), hepatic cholesterol (+245%, P < 0.03), hepatic MDA levels (+225%, P < 0.001), serum TNF-alpha (17.8 ± 10 vs 7.8 ± 0.0, P < 0.001), but a decrease in hepatic alpha tocopherol (-74%, P < 0.001) as compared to the control rats. Combination therapy with all drugs produced a significant decrease in liver steatosis (-54%), hepatic TG (-64%), hepatic cholesterol (-31%) and hepatic MDA (-70%), but increased hepatic alpha tocopherol (+44%) as compared to FL rats. Combination therapy with ISA alone produced a smaller decrease in liver steatosis (-32% vs -54%, P < 0.001) and in hepatic MDA levels (-55% vs -70%, P < 0.01), but a similar decrease in hepatic lipids when compared with the all drugs combination. TNF-alpha levels decreased significantly in all treatment groups except in ISA group.

CONCLUSION: Combination therapies have a greater effect on liver fat content as compared to monotherapy. Rosiglitazone appears to improve hepatic steatosis to a greater extent than metformin.

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Key words: Fatty liver; Rosiglitazone; Metformin; Ezetimibe; Valsartan; Methionine choline-deficient diet; Insulin resistance

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INTRODUCTION

The clinical implications of fat accumulation in the liver are derived mostly from its occurrence in the general population and it has potential to progress to fibrosis, cirrhosis and hepatocellular carcinoma[1-3]. Non-alcoholic fatty liver disease (NAFLD) is a component of the metabolic syndrome with a clinical spectrum ranging from simple FL to steatohepatitis, bridging fibrosis and cirrhosis[4]. Obesity and diabetes type 2 are considered the most powerful predisposing risk factors for the development of more severe manifestations of NAFLD[5]. The primary event of non-alcoholic steatohepatitis (NASH) is the accumulation of triglycerides in hepatocytes which seem to be determined by insulin resistance[6]. These fats stem mainly from increased splanchnic lipolysis of visceral and subcutaneous abdominal fat and from continuous delivery of free fatty acids to the liver after ingestion of fatty foods, both of which increase hepatic insulin resistance[7]. The secondary event is hepatocellular injury which includes factors, such as oxidative stress, pro-inflammatory cytokines, mitochondrial dysfunction, iron overload, bacterial overgrowth and genetic predisposition[8]. A recent study by Wanless and Shiotai[9] describes an inflammatory injury to hepatic veins due to release of fat followed by venous obstruction with secondary collapse...
and ultimately bridging fibrosis and progressing from fatty liver to NASH to cirrhosis. More recently, deregulated adipocytokines such as adiponectin, interleukin-6 and TNF-α have been examined as causative candidates of insulin resistance[7,10] and it was reported that oxidative stress in accumulated fat causes deregulated adipocytokine production[11].

The methionine choline-deficient diet (MCDD) model of fatty liver in rats is characterized by increased insulin resistance, hypertriglycerideremia, and increased lipid per oxidation[12,15]. Moreover, it has been shown that in the MCDD model of steatohepatitis, there is down-regulation of insulin signaling with decreased phosphorylation of IRS-1, IRS-2 and Akt and increased oxidative stress with over-expression of CYP2E1[19]. In light of these findings, it seems possible that NAFLD can be successfully treated by the reduction of fat absorption and delivery, oxidative stress and systemic inflammation, as well as reduction of insulin resistance. To date, no consistently effective therapy for FL disease has been identified. The need to use drugs with different and complementary mechanisms of action frequently arises in daily clinical practice. Possible therapeutic agents for fat-induced hepatic insulin resistance include metformin, which acts by decreasing gluconeogenesis and by enhancing peripheral glucose uptake[13] and thiazolidinediones, a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist that acts by increasing insulin sensitivity in adipose tissues, shifting fat away from the liver into fat stores (adipocytes)[10].

The combination of metformin and rosiglitazone may be complementary. Other therapeutic agents for insulin resistance include valsartan, an angiotensin 11 type-1 receptor blocker which stimulates the insulin signaling cascade and enhances glucose transporter type 4 (GLUT 4) translocation[17] and ezetimibe, the cholesterol absorption inhibitor which improves insulin resistance by decreasing low-density lipoprotein (LDL) tendency to peroxidation[18]. In the present study, we aimed to examine the impact of these different therapeutic interventions, alone or in combination, on hepatic fat content, hepatic lipid composition and parameters of oxidative stress, antioxidant capability and systemic inflammation in FL rats fed by MCDD.

**MATERIALS AND METHODS**

**Animal and protocol**

This study consisted of sixty male Sprague Dawley rats (Harlan Laboratories Limited, Jerusalem, Israel), weighing 200-280 g. Rats were housed in regular cages situated in an animal room at 22°C, maintained on standard rat chow diet (Koffolk, Tel Aviv, Israel) and given tap water to drink ad libitum. All animal studies were conducted according to the regulations for the use and care of experimental animals and treatment groups.

FL was induced in rats fed by MCDD diet for 9 wk as previously described[19,20]. The rats were randomly divided into nine groups. Group 1 comprised of 6 rats serving as control group and was maintained on standard chow diet (control liver, CL). Group 2 (FL group or NAFLD group) was given MCDD only for 9 wk (MCDD Harlan Tecklad, Madison, WI). The following groups were given various pharmaceutical interventions for additional 6 wk: Group 3 received MCDD diet with metformin (200 mg/kg); Group 4 received MCDD diet with rosiglitazone (3 mg/kg); Group 5 was fed MCDD diet with metformin + rosiglitazone; Group 6 received MCDD diet with ezetimibe (2 mg/kg); Group 7 were fed MCDD diet with valsartan (2 mg/kg); Group 8 received MCDD diet with metformin + rosiglitazone + valsartan; and Group 9 received MCDD diet with metformin + rosiglitazone + valsartan + ezetimibe. All treatment groups were treated for a total of 15 wk (9 + 6 wk). The dosage selected for each intervention was based on the results of previous studies using these agents in various liver diseases and the dose of each drug was controlled by semi-quantitative analysis[16-19]. Diets were supplied in pellet form. The medication was given with food and in drinking water. Simply the MCD diet was supplemented with the respective drug and not with a semi-purified diet. All the drugs were water soluble and there was no need for additional solvents. The drugs were monitored daily and were supplied by the local pharmacy of the Sieff Hospital, Safed, Israel and not by pharmaceutical companies. Rats were then sacrificed and liver histology, hepatic and plasma lipid content, parameters of oxidative stress, and TNF-α were measured.

**Biochemistry**

Serum triglyceride (TG) concentration was measured with an automated analyzer (Olympus AU2700; Hamburg, Germany). Additional biochemical parameters, such as serum total cholesterol (TC) and glucose, were measured using an automatic biochemical analytical system. Alanine (ALT) transaminase activities were performed spectrophotometrically using a commercially available kit test (ALT Reagents, Sigma-Aldrich). Tumor necrosis factor alpha (TNF-α) was assayed by rat-specific kit (R&D Systems). Insulin was assayed by a rat-specific RIA kit (Incstar, Stillwater, MN). Blood insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) derived from the following equation: IR = (fasting plasma glucose level mg%/0.055) × (fasting plasma insulin level mU/L) /22.5). This insulin resistance reflects both peripheral and hepatic insulin resistance[21].

**Determination of hepatic lipid composition**

Total hepatic lipids were extracted from freeze-dried samples by chloroform: methanol (2:1) and measured as previously described by Folch et al[22]. Triglycerides, TC and phospholipids in hepatic tissue extract were determined enzymatically in an auto analyzer (Hitachi 736, Tokyo, Japan) using commercial kits (Triglycerides, Infinity Cholesterol Reagent and Reagents for inorganic phosphorous assay, Sigma-Aldrich) as previously described[23-26].

**Determination of hepatic pro-oxidant and anti-oxidants**

Tissue tocopherol-alpha was determined following the methods described by Gowenlock et al[27]. Hepatic maleic dialdehyde (MDA) level was determined as previously described by Yagi et al[28].
Histology
A 5-μm thick section of liver tissue was cut from the center of each hepatic lobe and fixed in 40 g/L buffered formaldehyde, processed by standard techniques and embedded in paraffin. The sections were stained with hematoxylin-eosin. Fat extension, necroinflammatory grade and stage of fibrosis were assessed as previously described by Brunt et al.[29]. Steatosis was assessed by a morphological semiquantitative approach and graded as follows: mild = 5%-30%, moderate = 30%-60%, and severe > 60% of hepatocytes affected. The specimens were also examined for histological features of Mallory’s bodies, ballooning degeneration, acidophilic necrosis, sinusoidal fibrosis and polymorph nuclear infiltrates. The histological evaluation of the liver sections was performed blindly.

Statistical analysis
Results are expressed as mean ± SE. Analysis of variance was used to compare the means of multiple groups, followed by the Newman-Keuls test to determine statistical significance between two groups. When the data were not normally distributed, the Kruskal-Wallis test was performed to compare the means of multiple groups, followed by Mann-Whitney test. Correlation analysis was performed using spearman rank correlation. The statistical comparisons were performed using the unit values rather than percentages. P < 0.05 was considered statistically significant. The statistical analysis was performed using the Winstat program for windows (Kalma, MA).

RESULTS

Effect of methionine choline-deficient diet
The methionine choline FL model used in this experimental study showed features of the fatty liver observed in humans, including hypertriglyceridemia, increased oxidative stress, increased insulin resistance and increased ALT and TNF-α levels (Tables 1 and 2). FL induced by the MCDD diet had a 68% increase in the liver weight/rat weight ratio when compared to CL (Figure 1, P < 0.02). Hepatic TG and hepatic cholesterol concentrations were significantly higher in the rats fed MCDD diet than that in the control rats (+1263% increase, P < 0.001 and +245% increase, P < 0.03, respectively). Triglycerides represented the highest proportion of lipid components of the fat vesicles. FL rats showed a 100% increase in plasma triglycerides and 157% increase in HOMA-insulin resistance index (P < 0.05 and P < 0.001, respectively). FL rats had significantly lower concentrations of alpha-tocopherol when compared with CL group (P < 0.03). The hepatic levels of MDA were significantly greater and hepatic alpha-tocopherol/MDA ratio was significantly lower in FL group as compared with CL group (2.01 ± 0.4 vs 0.6 ± 0.1; P < 0.03 and 0.03 vs 0.05, P < 0.03, respectively). Specimens from the rats fed with methionine choline-deficient diet showed massive fatty infiltration (> 91%), predominantly macrovesicular. Features of steatohepatitis, including ballooning degeneration and pericellular fibrosis, were seen mildly but no pronounced fibrosis.

Table 1 Liver-related parameters, plasma biochemistry and cytokines in rats fed with methionine choline-deficient diet (MCDD) or choline-supplemented diet (Chow diet) (mean ± SD, n = 6)

| Parameters                  | CL Chow diet | FL MCDD diet | P values |
|-----------------------------|--------------|--------------|----------|
| Baseline rat mass (g)       | 227 ± 7      | 226 ± 6      | 0.5      |
| Final rat mass (g)          | 406 ± 19     | 257 ± 12     | 0.03     |
| Final liver mass (g)        | 10.2 ± 0.3   | 10.8 ± 0.8   | 0.4      |
| Liver/body mass ratio       | 0.025 ± 0.003| 0.042 ± 0.003| 0.02     |
| % change                    | 68           |              |          |
| Liver triglycerides (mg/l)  | 1.6 ± 0.3    | 21.8 ± 1.0   | < 0.001  |
| % change                    | 1263         |              |          |
| Liver cholesterol (mg/l)    | 0.5 ± 0.0    | 1.6 ± 0.2    | < 0.03   |
| % change                    | 245          |              |          |
| Serum HOMA-IRI              | 7 ± 0.3      | 18 ± 2.0     | < 0.001  |
| Liver tocopherol/MDA ratio  | 0.05         | 0.003        | < 0.03   |
| FL infiltration (%)         | 0            | 91 ± 5       | < 0.001  |
| ALT (nkat/L)                | 667 ± 83     | 800 ± 33     | < 0.05   |

Effect of pharmacologic intervention
All the drug regimens included in this study significantly decreased the liver weight/body weight ratio as compared to FL rats fed with the methionine choline-deficient diet alone (range 24%-33%, Figure 1).

Effect of insulin-sensitizing agents
Rosiglitazone improved insulin resistance (-39%, P < 0.03) and reduced hepatic and plasma triglycerides by -54% (P < 0.01) and -59% (P < 0.001), respectively, but not hepatic cholesterol as compared to FL group. Moreover, this decrease in hepatic TG was obviously greater than that with metformin monotherapy (P < 0.01). It increased the hepatic tocopherol-alpha/MDA ratio by 233% compared to FL group (P < 0.01), and most importantly, reduced hepatic fat content by 46% (P < 0.01). Rosiglitazone reversed the liver weight/body weight ratio approximately close to CL because of the decrease in the liver weight rather than increase in total body weight (Table 2). A
significant inverse correlation ($r = -0.6$) was found between hepatic alpha-tocopherol and hepatic MDA concentrations in the rosiglitazone group. Rosiglitazone did not significantly reduce the TNF-α levels as compared to FL group.

Metformin markedly reduced insulin resistance (-33%, $P < 0.03$), decreased hepatic TG (-40%, $P < 0.01$) and most effectively improved the tocopherol-alpha/MDA ratio (+833%, $P < 0.03$) as compared to FL group. However, it only mildly decreased the hepatic fat content (-14%, $P < 0.05$). Metformin decreased serum TNF-α levels by 44% $(P < 0.01)$ when compared to FL group, and reduced hepatic TG to a lesser extent than rosiglitazone and had no effect on hepatic cholesterol content.

The combination of metformin and rosiglitazone was beneficial in many ways: it reduced insulin resistance by -50% $(P < 0.03)$ and decreased hepatic and plasma TG by -64% $(P < 0.02)$ and by -50% $(P < 0.03)$ respectively; decreased hepatic cholesterol by -27% as compared to FL group $(P < 0.01)$ and a significant increase in tocopherol-alpha/MDA ratio (566%, $P < 0.01$) as well as a 32% reduction in hepatic fat $(P < 0.03)$ were observed. Surprisingly, this reduction of liver fat content was less important than rosiglitazone monotherapy (32% vs 46%, $P < 0.01$) but greater than metformin monotherapy (32% vs 14%, $P < 0.001$). And also, the combination of ISAs significantly increased TNF-α levels when compared to FL alone $(P < 0.02$, Tables 2 and 3).

**Effect of ezetimibe**

Ezetimibe, a cholesterol absorption inhibitor, reversed the liver weight/body weight ratio and improved insulin resistance by -28% $(P < 0.03)$, improved oxidant-antioxidant status, reduced TNF-α levels and mildly decreased the hepatic fat content (-14%). Ezetimibe also decreased hepatic and plasma triglycerides by 53% $(P < 0.01)$ and 56% $(P < 0.03)$, respectively when compared to FL rats. In addition, a 25% decrease $(P < 0.03)$ in hepatic cholesterol and a 39% decrease $(P < 0.02)$ in plasma cholesterol were observed when compared to CI group, suggesting an apparent effect on cholesterol absorption. Of all aforementioned drugs, ezetimibe monotherapy exerted the most powerful antioxidant effect, whereby it caused the highest increase in the alpha-tocopherol/MDA ratio (900%, $P < 0.001$) followed by metformin monotherapy (+833%). Ezetimibe also decreased TNF-α level by 32% $(P < 0.01)$ and hepatic fat content by 14% $(P < 0.01)$ (Tables 2 and 3). This mild reduction in hepatic fat content might be partially related to a significant effect on cholesterol absorption.

**Effect of valsartan**

Although valsartan had no significant effect on insulin resistance, it decreased hepatic and plasma triglycerides by -54% $(P < 0.02)$ and -50% $(P < 0.01)$, respectively and improved the tocopherol-alpha/MDA ratio by +266% $(P < 0.02)$. However, it had no beneficial effect on hepatic
cholesterol (+19%). Finally, it significantly decreased hepatic fat content (−28%) when compared to FL fed with MCDD (P < 0.01). Valsartan did not add much to the effect of metformin and rosiglitazone combination therapy regarding fat content but had significant effect on TNF-α levels (−49%, P < 0.01). The dosage used in this study was low and demonstrated no significant effect on blood pressure (Tables 2 and 3).

Effect of combined drug therapy

Combined drug therapy was most effective in improving the insulin resistance index (−55%, P < 0.001) followed by the combination of insulin-sensitizing agents alone (−50%, P < 0.02). The combined administration of drugs caused similar decrease in hepatic and plasma triglycerides as the combination of ISA alone (Tables 2 and 3). Furthermore, the combination therapy decreased hepatic and plasma cholesterol by 31% (P < 0.02) and 21% (P < 0.03), respectively when compared to FL. They demonstrated a powerful antioxidant activity by decreasing hepatic MDA levels and increasing tocopherol-alpha/MDA ratio by 1 900% (P < 0.01 and P < 0.02, respectively). Finally and most importantly, the combination of all the above drugs improved hepatic steatosis to a greater extent than ISA alone (54% vs 32%, P < 0.001) and greater than rosiglitazone monotherapy (54% vs 46%, P < 0.01). Combined therapy also decreased the serum TNF-α level by −27% (Tables 2 and 3, Figure 2). The combination of all drugs had a greater effect on hepatic tocopherol-alpha, hepatic MDA, and hepatic cholesterol than the combination of three drugs (R+M+V) (Table 3). The effect of various drugs on hepatocellular injury is shown in Table 4.

### DISCUSSION

There are currently no approved therapeutic regimes for treatment of NAFLD. Given the strong associations of NAFLD with features of the metabolic syndrome, including insulin resistance, hyperlipidemia and hypertension, it is reasonable to suppose that agents ameliorating these conditions might also attenuate the development of NAFLD, and considering also that NAFLD is likely to be multifactorial in etiology, it is also sound to predict that combination treatment may be more effective than monotherapy. The present study clearly indicates that combination of insulin-sensitizing agent, cholesterol absorption inhibitor and angiotensin antagonist have greater effect on liver fat content and on lipid peroxidation than monotherapy in the MCDD rat model of NAFLD. Hepatic steatosis and hepatic TG accumulation lead to hepatic insulin resistance by stimulating gluconeogenesis and by activation of protein kinase PKC and JNK-1, which may interfere with tyrosine phosphorylation of IRS-1 and IRS-2 and impair the ability of insulin to activate glycogen synthase and diminish the ability of the liver to store glucose as glycogen[30]. Rosiglitazone appears to improve hepatic steatosis to a greater extent than metformin and the same effect was observed when examining hepatic triglyceride but not hepatic cholesterol content. The present study is in keeping with previous data showing a similar decrease in liver fat using either pioglitazone[31] or rosiglitazone[32,33]. In a human study, rosiglitazone decreased liver fat content by 22% as assessed by magnetic resonance spectroscopy[34]. Several mechanisms may underlie the ability of rosiglitazone to reduce liver fat content. First, it has been suggested that rosiglitazone doubles fatty acid uptake into subcutaneous adipose tissue and decreases hepatic fatty acid uptake into liver by 40% and in muscle by 30%, i.e., “the lipid steal mechanism”[35]. Second, rosiglitazone activates PPAR-gamma which decreases free
fatty acid availability for hepatic lipogenesis and reduces the release of TNF-α cytokine by adipocytes[36,34]. Finally, rosiglitazone has also been shown to increase insulin sensitivity by improving downstream insulin signaling by increasing insulin stimulation of IRS-1 tyrosine phosphorylation and p85 association with IRS-1[37]. Consistent with the reduction of free fatty acids flux to the liver is the decrease in hepatic TG concentration[38] and the improvement in insulin sensitivity. Rosiglitazone may also increase adiponectin expression which in turn increases hepatic insulin sensitivity, activates fatty acid oxidation and inhibits phosphoenolpyruvate carboxykinase expression[39]. Unfortunately, concentrations of adiponectin and free fatty acid were not measured in this study.

We found metformin sensitizes the liver but has little effect on hepatic fat content. The ability of metformin to increase hepatic insulin sensitivity has been documented in a previous study[39], but liver fat content was not measured. In isolated hepatocytes and metformin-treated rats, lipogenesis was inhibited because of adenosine 5’ monophosphate-activated protein kinase (AMPK)-induced inactivation of acetyl-CoA carboxylase and suppression of lipogenic enzyme and transcription factor expression[40]. Additionally, metformin has been shown to inhibit oxidative phosphorylation and lower cellular ATP levels at high doses[41]. The dose used in the present study (200 mg/kg) was smaller than that used in the study by Zhou et al[42] (286 mg/kg). In ob/ob mice with FL, metformin was found to reverse hepatomegaly and steatosis[41]. The therapeutic mechanism of metformin likely involves hepatic expression of TNF-α and TNF-α-inducible factors that promote hepatic lipid accumulation and ATP depletion[43]. In the current study, plasma TNF-α decreased with metformin when compared to untreated FL. It remains to be determined whether metformin also increases adiponectin levels or impacts on free fatty acid uptake and distribution. Although rosiglitazone and metformin activate different signaling pathways, there was no additive effect of the combination of both drugs on liver fat content compared to rosiglitazone monotherapy. In contrast, there was an additive effect of the combination of ISA on hepatic triglyceride, hepatic cholesterol content, and on anti-oxidant/oxidant ratio (Table 3); the reason for this is unclear. It could be related to up-regulation of TNF-α expression which was observed in the R+M group. There was no significant increase in body weight by combined rosiglitazone and metformin treatment. This effect may have been counterbalanced by metformin treatment.

Ezetimibe inhibits the transport of dietary and biliary cholesterol across the intestinal wall[44]. It was chosen rather than a statin because statins are not very effective in the treatment of NAFLD (atorvastatin) and because ezetimibe is safe and may help in reducing the cholesterol absorption from food intake. The other rational for using ezetimibe was the high corn oil component (100 g/kg) in the MCD diet. Ezetimibe decreased hepatic fat content with an apparent mild decrease in cholesterol absorption, suggesting that its antioxidant and anti-inflammatory effects are via other mechanisms for reducing liver fat. The dose used in our experiment is considered high dose because the effective dose at which 50% inhibition is observed (ED50) for rat is 0.0016 mg/kg. This dose was chosen in order to capture any effect ezetimibe might have on the hydrolysis of cholesterol ester[45]. Usually, ezetimibe does not affect the acute absorption of TG[44]. In our study, ezetimibe, however, reduced hepatic and plasma TG, which is in accordance with the companion study in which ezetimibe decreased the plasma concentration of TGs[46].

Treatment with valsartan had modest antioxidant activity as compared to ezetimibe. The molecular mechanism was not investigated in this study, but Siuchi et al[37] have postulated that valsartan treatment exaggerates the insulin-induced phosphorylation of IRS-1, the association of IRS-1 with the p85 regulatory subunit of PI3-K activity and translocation of GLUT 4 to the plasma membrane. Whether valsartan has a selective additional PPAR-γ modulating activity as Telmisartan remains to be determined[46]. The therapeutic efficacy of another angiotensin 11 receptor antagonist in patients with NASH has recently been documented[47]. The antifibrotic effect of valsartan was not seen in our rats because rats were fed with the MCDD for a short time (9 wk) and did not produce enough fibrosis.

Abrogation of oxidative stress improves whole body insulin resistance[48]. In this respect, our study showed that alpha-tocopherol/MDA ratio improved mostly with the combination of all drugs, followed by ezetimibe and by metformin (+1900, +900%, +833%, respectively). Hypertriglyceridemia and increased hepatic TG were prominent features in rats fed with MCDD[49,50]. Nevertheless, reduction of TG did not improve liver fat content in all study groups. For example, metformin reduced hepatic TGs and hepatic steatosis by 40% and 17%, respectively. This implies that another mechanism of hepatic fat reduction is implicated in addition to lowering hepatic triglycerides. The mean levels of plasma TNF-α were relatively low in all treatment groups because rats were examined in early phase of the disease progression and with mild overt NASH with inflammation, ballooning and pericellular fibrosis. The reason for increased TNF-α in rats fed with MCDD diet with rosiglitazone plus metformin is unclear, but appears to be unrelated to overweight or to lipid peroxidation in any case. Whether it is related to an early activation of NF-kappa B or to a potentiation of IL-6 and IL-8 expression by rosiglitazone plus metformin treatment remains unknown[49].

Although the MCDD model of fatty liver is the most commonly used in experimental studies, one limitation of our study is that MCDD model is not the ideal model for studying insulin-sensitizing drugs and an extensive number of additional experiments would be required to perform in order to derive definitive data in insulin-resistant models[51]. Another concern is that rats fed with MCDD for 9 wk did not develop the same phenotype as previous reports of MCDD-induced NASH in mice (as short as 10 d of MCDD). The phenotype described here largely consisted of marked steatosis with mild inflammation and mild fibrosis[51].

In conclusion, the current study shows for the first time that combination therapy of rosiglitazone and metformin with ezetimibe and valsartan in parallel has a greater effect on the extent of fatty infiltration and on lipid
peroxidation compared to insulin-sensitizing agents only and to monotherapy with either drug. Improving insulin resistance together with decreasing TNF-α and reduction of oxidative stress remains the best targeted treatment to date for NAFLD.

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