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Full Length Research Paper

Intervention study of *Ginkgo biloba* extract in rat model of lipid-induced insulin resistance

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Our research focused on the correlation between IR and glucolipid metabolism, vascular endothelial dysfunction and steatohepatitis. Then to provide evidence of *Ginkgo* on early-stage prevention of IR related diseases in a rat model. Forty male SD rats were equally randomized to four groups: normal group, model group, Ginkgo group and rosiglitazone group. Blood samples and serum biochemical indicators were collected and detected. Insulin sensitive index (ISI) was selected to evaluate the model. Compared with normal group, ISI, NO, T-AOC and SOD significantly decreased, while serum glucose, lipid, ET-1, MDA, and other indicators significantly increased in model group (P <0.05 or P <0.01). But compared with model group, it was opposite for both Ginkgo and rosiglitazone groups (P <0.05 or P <0.01). Fatty streaks and typical athesclerotic plaques exhibited early-stage injuries of arterial atherosclerosis emerged in the fourth month, while they were improved remarkably in Ginkgo group. The hepatocytes exhibited diffused steatosis, hepatic lobular inflammation and abnormal mitochondria in model group, but were smaller and only tiny lipid droplets were observed in both Ginkgo and rosiglitazone groups. We can make the conclusion IR may lead to endothelial dysfunction and steatohepatitis. (2) *Ginkgo biloba* extract can improve glucolipid metabolism and may have protective effects on hepatocytes and endothelial function of blood vessels and partly or remarkably reverse endothelial dysfunction and steatohepatitis due to IR, and may have related intervention on IR.

Key words: Insulin resistance, endothelial dysfunction, steatohepatitis, *Ginkgo biloba* extract, disease models, rats.

INTRODUCTION

Reaven proposed the well-known "X syndrome" in 1988, which was a new milestone in understanding insulin resistance (IR) (Reaven, 1988). Recently, there is an increasing interest in IR as a key-point in the syndrome. So, the early-stage prevention and cure of IR related diseases seems particularly important. In many studies, IR is reported to be the basic pathogenesis of Type 2 diabetes mellitus (T2DM), metabolic syndrome (MS) and atherosclerosis (AS), and it also plays an important role in steatohepatitis. UKPDS and a series of pathology researches show that T2DM athesclerotic macroangiopathy happens before the raise of blood glucose, and its occurrence and development are closely related to IR. Thus, it is very important to investigate the correlation between IR and diabetic macroangiopathy for early-stage prevention. Based on the construction of HF-IR rat model, our research focused on the inner correlation between IR and endothelial dysfunction and steatohepatitis through detecting the amount of glucolipid metabolism, ISI, endothelial related substances (NO, NOS, ET-1, AngⅡ) and hepatocyte related substances (TG, FFA, ALT, AST) and observing the morphology of aortic wall and liver. Through investigating the effect of *Ginkgo biloba* extract on endothelial dysfunction and steatohepatitis, we can reveal its potential in curing IR. And then to provide experimental evidence on early-stage prevention and cure of MS, and reduction of the incidence rate and death rate of cardiovascular disease for clinical

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use.

MATERIALS AND METHODS

Experimental animal

40 male SD rats with the weight of 200 to 250 g were bought from Animal Experimental Center, Shanghai Second Military Medical University. We raised these rats under the circumstances with the constant temperature of 22°C, relative humidity of 50 to 60% and photoperiod of 12 h for 1 week. Then we went on with the experiment.

Experimental drugs

The basal feed was bought from Animal Experimental Center, Shanghai Second Military Medical University. The high-fat feed was made by ourselves, formulation: the energy from lipid is 59% (the lard occupies 39%), from protein is 21% (casein occupies 31%) and the other 20% comes from the carbohydrates (cornstarch occupies 30%), furthermore, we need to add the necessary vitamins and minerals. Rosiglitazone, 4 mg/tablet, was from GSK. *G. biloba* extract (Ginkgo flavonoids ≥ 44%, *G. biloba* Extract 50, GBE50) was from Shanghai Xinling Technological Pharmaceutical Co., LTD.

Experimental reagents

NO, T-AOC, SOD, MDA, FFA, ALT/GPT, AST/GOT and Coomassie brilliant blue protein kits were from Nanjing Jiancheng Bioengineering Institute. Ins kit was from Linco Co., America. ET-1 and Ang kits were from Beijing East Asian Institute of Immune Technology. TG, TC and HDL-C kits were from Wenzhou Jinma Biotechnological Co., LTD. Glucose enzymatic determination kit and LDL-C kit were from Shanghai Kexin Institute of Biological Technology. Insulin RIA kit was from Beijing North Institute of Biological Technology.

Experimental apparatus

In the experiment, we used Hitachi 7600 automatic biochemical analyzer, automatic RIAγcomputing apparatus from China, LABSYSTEMSM- LTISKAN MS (353) microplate reader from Finland, 80-2 low speed centrifuge from Shanghai and UV-2100 spectrophotometer from SHIM- ADZU, Japan.

Model and group

After 1 week feeding to accommodate these rats to our experimental circumstances, we equally randomized the 40 rats to four groups: normal control group, model control group, Ginkgo group and rosiglitazone group. We fed the normal control group with basal feed, the model control group with high-fat feed, the Ginkgo group with high-fat feed and *G. biloba* extract and the rosiglitazone group with high-fat feed and rosiglitazone.

The rats were separated into 5 rats/cage, and raised under the standard circumstances with the photoperiod of 12 h, the temperature of 18 to 25°C and the relative humidity of 50 to 60% and they were free to consume water and food. Except the normal control group, all the other 3 groups were fed with high-fat feed (basal feed 67.8%, sucrose 20%, lard 10%, cholesterol 2% and cholate 0.2%). Successively feeding for 8 weeks, we detected the FPG and 2 h PG of the rats and with the help of HOMA-IR, we found that the indexes of 80% of the model rats were reliable, which indicated that our rat model was reliable the other way round.

Drug administration

The same volume of normal saline was administered by gavage to both the normal control and model control group, while to the Ginkgo group we added an extra amount of Ginkgo flavonoids (0.1 $g \cdot kg^{-1} \cdot d^{-1}$) and to the rosiglitazone group, an extra amount of rosiglitazone (3 $mg \cdot kg^{-1} \cdot d^{-1}$) was added. The drugs were administered once a day for successively 8 weeks.

Specimen collection

Successively feeding for 12 weeks, the rats were applied to abrosia but free to consume water for 12 h. Then we took blood samples from their jugular vein, and we collected the serum after centrifugation to detect related indexes. At last, we needed to dissect the rats to get the aorta and liver for morphological observation and detecting the amount of hepatic lipid and enzyme.

Analysis indicators and methods

Biochemical indicators

We used glucose oxidation method to determine FBG, enzymatic method to determine TG, TC, HDL-C and LDL-C in serum, Insulin RIA kit to determine serum insulin, Ins kit to determine ET-1, nitrate reduction method to determine NO, spectrophotometry method to determine FFA, thiobarbituric acid method to determine MDA, Fe^{3+}/Fe^{2+} reduction method to determine T-AOC and enzymatic method to determine the hepatic lipid (TG, TC, HDL-C, LDL-C and FFA) and hepatic enzyme (ALT, AST, MDA, SOD and LDH). Then, we calculated ISI with the equation $ISI = \ln(1/FBG \times FINS)$. For its abnormal distribution, we took its natural logarithm for analysis. At last, we had to calculate $HOMA - IR = FPG \times FINS / 22.5$. Lee's index reflects the obesity of the experimental rats, it is useful and reliable to evaluate rats' obesity $Lee's\ index = \sqrt[3]{weight \times 10^3 / length(cm)}$ (The Heart Outcome Prevention Evaluation Study Investigators, 2000)

Visceral morphology and related indicators

We cut down the aortic wall and liver for morphological observation with optical microscopy. We detected and recorded the wet weight of the rats' heart and liver and then to calculate the cardiac index (wet weight of heart/body weight × 100%) and hepatic index (wet weight of liver/body weight × 100%).

Statistical analysis

All the data were represented in the form of average ± standard deviation ($\bar{x} \pm s$) or percentage. We also did the Z test and homogeneity of variance test to all data. To those abnormal distributions and unequal variances, we converted them to their natural logarithm. To those equal variances, we did t test between the two groups. And variance analysis was chosen when it came to

Table 1. Cardiac index and hepatic index (n=8, $\bar{x}$ ±s).

Groups	Body weight (g)	Heart weight (g)	Cardiac index	Liver weight (g)	Hepatic index
Normal	468.43 ± 25.68[d]	1.41 ± 0.08	0.0030 ± 0.0002[c]	11.93 ± 1.50	0.0254 ± 0.0018[c]
Model	549.2 ± 39.34[b]	1.50 ± 0.20	0.0027 ± 0.0003[a]	26.08 ± 2.53	0.0465 ± 0.0032[a]
GBE	535.84 ± 42.49[b]	1.45 ± 0.15	0.0027 ± 0.0003[a]	24.75 ± 2.21	0.0456 ± 0.0038[a]
Rosiglitazone	539.51 ± 37.35[b]	1.42 ± 0.13	0.0027 ± 0.0003[a]	18.32 ± 2.54	0.0341 ± 0.0026[a]

Notes: Compared with normal control group, [a]P < 0.05, [b]P < 0.01; Compared with model control group, [c]P < 0.05, [d]P < 0.01.

Table 2. Comparison of FBG, IR, ISI etc. (n=8, $\bar{x}$ ± s).

Groups	FBG (mmol/L)	FINS (mIU/L)	HOMA-IR	ISI	HbA1c (%)
Normal	6.09 ± 0.51[d]	17.79 ± 1.89[c]	4.79 ± 0.51[d]	-4.67 ± 0.11[d]	1.61 ± 0.08[d]
Model	7.31 ± 0.44[b]	21.62 ± 2.22[a]	7.38 ± 0.90[b]	-5.10 ± 0.12[b]	3.41 ± 0.05[b]
GBE	5.74 ± 0.38[d]	20.65 ± 2.89[c]	6.46 ± 0.92[c]	-4.77 ± 0.23[c]	3.17 ± 0.63[c]
Rosiglitazone	5.89 ± 0.46[d]	19.26 ± 5.53[d]	5.36 ± 1.30[d]	-4.97 ± 0.14[a]	2.84 ± 0.62[d]

Notes: Compared with normal control group, [a]P < 0.05, [b]P < 0.01; Compared with model control group, [c]P < 0.05, [d]P < 0.01.

multi-group comparison. Logistic regression was used to do multivariate analysis, while χ^2 test was used to do group rate comparison. Among different indicators, we took linear correlation analysis, and the correlation coefficient and significance test were taken to evaluate the correlation between two indicators. We did all the statistical analysis with SAS6.12.

RESULTS

General situation

In normal control group, the rats with quick movements and shiny furs gained weight remarkably. Compared with normal control group, the rats in model control group presented huddled body, obviously overweight, straight and lusterless furs, dull in action and the symptoms of polydipsia and polyphagia. While compared with model group, the rats in Ginkgo group and rosiglitazone group seemed much healthier. This indicated that the *G. biloba* extract can significantly relieve the polydipsia, obesity and other symptoms of the IR rats.

Cardiac index and hepatic index

After feeding for 12 weeks, we detected the weight of the rats' heart and liver, and then calculated the cardiac index and hepatic index. We found that the rats in model control group showed the symptoms of cardinomegaly and heptamegaly, and its cardiac index and hepatic index were significantly larger than normal control group (P < 0.01). Compared with model control group, the body weight, cardiac index and hepatic index declined obviously in both Ginkgo group and rosiglitazone group (P < 0.05, Table 1).

Comparison of serum glucose and IR

We detected FINS and ISI of the rats in model control group after feeding 12 weeks. Compared with normal control group, FBG, FINS and HOMA -IR increased but ISI decreased significantly in model control group. With the treatment of Ginkgo flavonoids and rosiglitazone, FBG and IR declined remarkably. We also detected HbA1 c which was 1.61 ± 0.08% in normal control group, but in model control group it was 3.41 ± 0.45% (Table 2).

Comparison of serum lipid metabolism

The rats in model control group were small but fat which led to obviously increase in Lee's index. There were statistically differences when compared with other groups (P < 0.01). Related analysis showed: changes in Lee's index was directly proportional to weight gained, abdominal fats and serum TC, TG and LDL-C (P < 0.01), but highly inversely proportional to HDL –C (P < 0.01).

The atherogenic index of plasma (AIP) in model control group was higher than that in normal control group (P < 0.01). With the treatment of Ginkgo flavonoids and rosiglitazone, serum lipid metabolism and AIP improved prominently, and rosiglitazone seemed better than Ginkgo flavonoids (Tables 3 to 5).

Comparison of ET-1, NO and AngII

Serum ET-1 and AngII increased but serum NO

Table 3. Comparison of Lee's index and abdominal fat weight (n=8, $\bar{x}$ ±s).

Groups	Body weight (g)	Body length (cm)	Lee's index	Fat weight (g)
Normal	468.43 ± 25.68[d]	25.28 ± 0.56[d]	307.22 ± 4.15[d]	4.98 ± 0.91[d]
Model	549.23 ± 39.34[b]	26.31 ± 0.67[b]	311.13 ± 6.65[b]	8.34 ± 1.05[b]
GBE	535.84 ± 42.49[b]	26.33 ± 0.64[b]	308.31 ± 1.29[d]	7.79 ± 1.00[d]
Rosiglitazone	539.51 ± 37.35[b]	26.24 ± 0.53[b]	310.54 ± 1.95[c]	8.14 ± 1.21[c]

Notes: Compared with normal control group, [a]P < 0.05, [b]P < 0.01; Compared with model control group, [c]P < 0.05, [d]P < 0.01.

Table 4. Comparison of serum lipid and AIP (n=8, $\bar{x}$ ±s).

Groups	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	AIP
Normal	1.98 ± 0.07[d]	0.93 ± 0.14[d]	0.85 ± 0.17[d]	1.005 ± 0.081[d]	0.164 ± 0.072[d]
Model	3.24 ± 0.91[b]	1.23 ± 0.08[b]	2.06 ± 0.13[b]	0.622 ± 0.106[b]	4.776 ± 1.541[b]
GBE	2.45 ± 0.37[bc]	0.90 ± 0.10[d]	1.43 ± 0.22b[d]	0.812 ± 0.129[bd]	2.534 ± 0.937[bc]
Rosiglitazone	2.69 ± 0.34[ad]	0.61 ± 0.06[bd]	1.62 ± 0.30[bd]	0.735 ± 0.100[ad]	0.994 ± 0.465[bd]

Notes: Compared with normal control group, [a]P < 0.05, [b]P < 0.01; Compared with model control group, [c]P < 0.05, [d]P < 0.01.

Table 5. Correlation analysis of Lee's index and serum lipid (n=8, $\bar{x}$ ± s).

	TC	TG	LDL-C	HDL-C	Body weight	Fat weight
Correlation factor	0.7290*	0.7612*	0.7048*	0.7504*	0.8044*	0.8583*

Note:*P < 0.01.

Table 6. Comparison of serum ET and NO (n=8, $\bar{x}$ ± s).

	ET-1 (pg/ml)	NO (μ mol/L)	Ang Ⅱ (pg/ml)
Normal	323.79 ± 86.70[d]	58.31 ± 2.13[c]	71.31 ± 22.43[d]
Model	381.26 ± 73.25[b]	29.75 ± 4.06[b]	241.82 ± 49.62[b]
GBE	329.36 ± 74.55[bd]	40.22 ± 3.00[bd]	151.17 ± 29.18[bd]
Rosiglitazone	334.43 ± 67.28[bd]	35.07 ± 2.16[bd]	136.26 ± 25.46[bd]

Notes: Compared with normal control group, [a]P < 0.05, [b]P < 0.01; Compared with model control group, [c]P < 0.05, [d]P < 0.01.

decreased in model control group compared with normal control group (P < 0.01), with statistical significance. With the use of Ginkgo flavonoids and rosiglitazone, serum ET-1 and AngII decreased (P < 0.01) and serum NO increased (P < 0.05) (Table 6).

Comparison of hepatic biochemical indicators

Hepatic TG, FFA, ALT and AST increased significantly in model control group compared with normal control group. While in Ginkgo group and rosiglitazone group, hepatic TG and FFA decreased remarkably but still higher than that in normal control group. There were no significant difference between Ginkgo group and rosiglitazone group (P > 0.05). Compared with normal control group, hepatic MDA increased and T-AOC and SOD decreased significantly in model control group (P < 0.01). While compared with model control group, hepatic T-AOC and SOD increased and MDA decreased in both Ginkgo group and rosiglitazone group (P < 0.01). After the treatment with Ginkgo flavonoids or rosiglitazone, T-AOC/MDA increased which indicated the improvement of the comprehensive oxidation stress state of the IR rats. And

Table 7. Comparison of biochemical indicators in liver (n=8, $\bar{x}$ ± s).

Groups	TG (mg/g)	FFA (μ mol/g)	MDA (nmol/mg prot)	SOD (U/mg prot)	AST (Carmen's unit)	ALT (Carmen's unit)
Normal	70.2 ± 15.36^{d}	60.35 ± 7.22^{d}	15.79 ± 0.43^{d}	198.65 ± 32.49^{d}	169.31 ± 23.47^{c}	149.82 ± 27.15^{c}
Model	138.1 ± 27.59^{b}	156.3 ± 18.25^{b}	29.85 ± 0.91^{b}	106.85 ± 23.87^{b}	247.52 ± 34.22^{b}	251.35 ± 34.52^{b}
GBE	104.7 ± 24.28^{bd}	125.1 ± 19.76^{bd}	24.17 ± 0.65^{bd}	152.17 ± 28.23^{bd}	191.26 ± 26.39^{bd}	158.62 ± 33.72^{bd}
Rosiglitazone	110.2 ± 25.31^{bc}	137.3 ± 17.14^{bd}	19.42 ± 0.72^{bd}	148.52 ± 31.46^{bd}	209.63 ± 35.84^{bd}	160.46 ± 23.61^{bd}

Notes: Compared with normal control group, $^{a}P < 0.05$, $^{b}P < 0.01$; Compared with model control group, $^{c}P < 0.05$, $^{d}P < 0.01$.

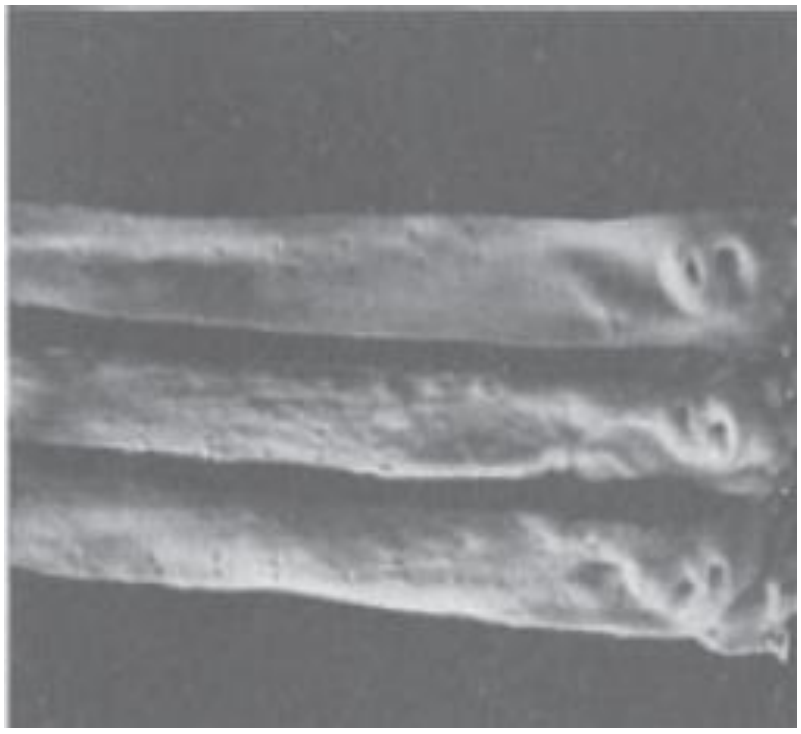

Figure 1. Morphological change in aortic wall. The upper strip represented the aortic wall in normal control group which is smooth and with no lipid deposition, while the middle and lower strips represented the model control group and cured group respectively. From the picture above we could see atherosclerosis obviously in the model group but not in the cured group.

T-AOC was inversely proportional to MDA (r = -0.312, P < 0.01) (Table 7).

Logistic regression analysis of HOMA-IR

We chose normal and model control groups to do this regression. We did the Logistic regression with HOMA-IR as dependent variable, body weight, FBG, FFA, FINS, 2 h INS, TC, TG, LDL-C, NO, ET-1 and AngII as independent variables, and we found body weight, TG, FFA and FBG were the independent risk factors of IR (B were 0.523, 0.135, 0.264 and 0.039; SE were 0.184, 0.027, 0.089 and 0.015; OR were 0.263, 0.416, 0.220 and 0.194; P were 0.006, 0.000, 0.004 and 0.009).

Regression equation = Body weight × 0.523 + *TG* × 0.135 + *FFA* × 0.264 + *FPG* × 0.039 – 1.808

Morphological observation

The morphological change in aortic wall was shown in Figure 1, and in liver was shown in Figure 2.

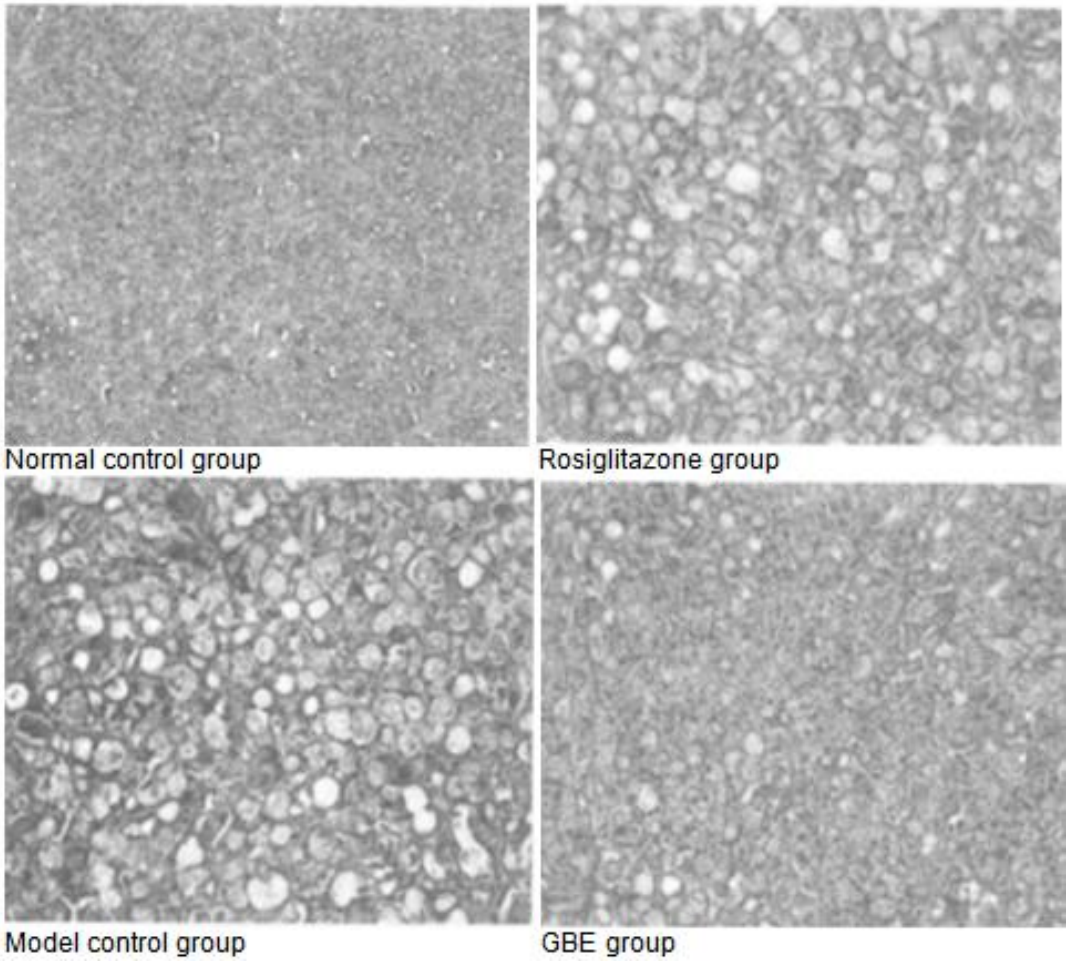

Figure 2. Morphological changes in liver (×200). The heptacytes were arranged in order with big and round nucleus in the middle in normal control group. While in model control group, there existed liver cells turgescence and lipid vacuoles, and the hepatocytes were bigger than those in normal control group. In cured groups, the hepatocytes were smaller than those in model control group and only tiny lipid droplets were seen.

DISCUSSION

With the development of social economy and change of lifestyle, the number of patients with T2DM, coronary disease, hypertension, obesity and other modern chronic diseases increases rapidly. At the year of 1995, Stern proposed the well-known “common soil” theory which indicated that those diseases mentioned above were all related to IR. And recently, it was confirmed by HOPE experiment (HOPE Research Group, 2000).

The mechanism of IR is very complicate which contains three different states: pre-receptor, receptor and post-receptor (Zeng et al., 2000; Terasaki et al., 1998). It is also closely related to hereditary and environmental factors. It is reported that IR is widespread in peripheral tissues (liver, skeletal muscle, etc.) when feed the normal rats with high-fat diet (HF) for 4 to 8 weeks. HF-IR animal models especially for rat model have been widely used in studying the mechanisms of IR (Storlien et al., 1986; Li et al., 2000; Storlin et al., 2000; He and Shi, 2002; Du et al., 2002; Choudhury and Sanyal, 2004; Chung et al., 1998). The three indexes of symptom, hyphology and biochemical indicators in our model are correspond with that in clinical IR and diabetes research. For its low cost, easy and relatively cheap to construct, our model seems to be an ideal model to investigate IR.

NO is known as the most effective relaxing factor synthesized by VEC; and ET-1 is known as the most effective vasoconstrictor synthesized and secreted by VEC when it comes to the hypoxic ischemic condition. Under the physiological conditions, the balance between NO and ET-1 is needed to keep the normal vasomotoricity. And in patients with IRS, this kind of balance is broken.

It has been reported that independent RAS exists on some part of the vascular wall. And ACE produced by RAS through autocrine or paracrine way has strong and

persistent effect on vascular contraction. In our research, we found the activation of RAS is widespread in IR rats. There may exist a positive feedback regulation between AngII and ET-1. And the unbalance between NO and ET-1 which give rise to ED could be chosen as an important index to evaluate ED in IR. Our study shows that in IR rats, the level of serum TG and LDL-C are very high but of serum HDL-C is very low which would lead to obviously damage on EC dependent vasodilatation. And *G. biloba* extract can cure this kind of damage significantly.

For its specific role in glucose metabolism, it is of great importance to study liver in diabetes and glucolipid metabolism disorders. Numerous studies have shown that nonalcoholic fatty liver disease (NAFLD) has the symptoms of obesity, lipid metabolism disorders, hypertension, glucose metabolism disorders etc., which are also the characters of IR. We still have no idea about the mechanism of NAFLD, but most researchers believe that IR is the main cause of NAFLD (Miller et al., 2002).

In our study, we found that body weight, FBG, TG and FFA are independent risk factors of IR. Similar to recent reports, we found IR could play an important role in the formation of fatty liver, and then the fatty liver participate in the occurrence and development of MS. Our results also show that, the rise of hepatic enzyme is positively related to several components of MS.

Our study shows HOMA-IR in NAFLD rats is much higher than that in normal control group, this indicates that IR exists in NAFLD. The multivariate analysis indicates that IR is the independent risk factor of NAFLD. In IR state, the regulation of insulin to lipid metabolism is weakened which would lead to an increase in absorbing FFA and synthesizing TG in blood, fatty deposition in hepatocytes and then give birth to fatty liver.

The correlation between IR, ED and NAFLD has become a hot spot in recent years (Cooke, 2000; Chen and Zhang, 1999; Drieu et al., 2002). These studies would be helpful for searching effective drugs against ED, NAFLD and IR. *G. biloba*, which is also known as “maidenhair tree” belongs to ancient relic plant. And 70% of *G. biloba* are grown in China. The main components of *G. biloba* extract (GBE) are Ginkgo flavonoids and Ginkgolides. It has been reported that GBE has the effect on clearing oxygen radicals and inhibiting lipid peroxidation (Chen and Zhang, 1999; Drieu et al., 2002). It can also inhibit ACE, promote the liberation of NO and relax the blood vessel obviously. Our study further confirms that GBE can improve SOD activity and decrease MDA in early-stage diabetes which indicates early treatment with GBE could delay the occurrence of diabetes fatty liver, improve IR and ISI, lower hepatic lipid and hepatic enzyme, partly or significantly reverse fatty liver due to IR, and may have related intervention on IR.

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