의학석사 학위논문

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Streptozotocin로 인한 당뇨병 신증에서의 bilirubin의 triglyceride 합성과정에 대한 연구

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이 논문을 의학석사 학위논문으로 제출함

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The effect of bilirubin on the regulations of triglyceride synthesis in Streptozotocin-induced diabetes mellitus nephropathy

by

Xu jianwei

A thesis submitted to the Department of medicine in partial fulfillment of the requirements for the Degree of Master of Philosophy in medicine at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor Dong Wan Chae **Chairman**

Professor Sung Kwan Kim **Vice chairman**

Professor Dong Ki Kim

Abstract

Introduction: Modest level of bilirubin is a major physiologic cytoprotectant by antioxidation, anti-inflammation and others. Also our previous experiment confirmed hyperbilirubiemia's protective effect of kidney in DM rat. And we tried to go further and reveal the effect of bilirubin on kidney and triglyceride synthesis in DM nephropathy by employing a new model which bilirubin was treated after STZ-induction.

Methods: Eighteen male SD rats were prepared, and 13 (DM group and Bil group) of them were induced DM by single injection of STZ at a dose of 60mg/kg and others' were treated with vehicle citrate buffer PH 4.5. After 8 hours, 5 rats which were randomly selected from the 13 DM rates were treated with 0.3mg/ml bilirubin and marked as Bil group. Five weeks later basal data were collected, kidney and liver were harvested. PMC model: Primary mesangial cells were also prepared, and divide into three groups (control, DM, Bil). First all groups were cultured in normal glucose environment, then 1 day later, Bil group and DM group were changed to high glucose culture, and 1 day later, Bil group was treated with Bilirubin , finally the cells were harvested . Hepatoma cells model: Hepatoma cells were treated in the same way as PMC model. iRNA transfection model: Expressions of target gene were silenced in order to determine the effect of bilirubin on TG regulating genes by employing siRNA transfection procedure in hepatoma cells model ,and cells were harvested .

Results: 5 weeks after STZ –induction, diabetic symptoms appeared in the DM model. Bilirubin improved these symptoms to some extent. And blood TG

concentration in Bil group was lower than that in DM group. Bilirubin decreased the 24 hour urine albumin excretion and also urine H_2O_2 production in Bil group. In kidney tissue, bilirubin decreased NADPH oxidase dependent oxidative end products. The fibrosis and apoptosis situation of glomerular in Bil group was much better than that in DM group. Bilirubin decreased TGF-beta synthesis in kidney tissues, and also decreased a series of gene expressions which were involved in triglyceride synthesis. In liver tissue, the amount of TG in DM group was less than that in Bil group, and bilirubin also inhibited the gene expressions which were involved in triglyceride synthesis. PMC model and hepatoma cells model confirmed the same results. In iRNA transfection model, Bilirubin acted as a dual regulator. It maintained the expression of genes which involved in TG synthesis in a certain lower level.

Conclusions: Bilirubin alleviated proteinuria by maintaining the integrality of the glomerular filtration membrane and promoting renal proximal tubules viabilities through apoptosis improvement and might also by its vasodilation effect, then delayed the progression of diabetic nephropathy. Bilirubin also reduced the lipid-induced TGF-beta production and improved the fibrosis situation in kidney tissue. Bilirubin didn't only decrease triglyceride synthesis in DM, but also maintain the triglyceride in a lower level through elaborate regulations.

Keywords: Bilirubin, DM nephropathy, Triglyceride synthesis, antiapoptosis, antioxidants, TGF-β

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Bil: bilirubin

TG: Triglyceride

PCT: proximal convoluted tubule

Ccr: creatinine clearance rate

DMEM: Dulbecco's modified eagle's medium

FBS: fetal bovine serum

HG: high glucose

HO: heme oxygenase

PAS: periodic acid-schiff

PMC (or MC): primary mesangial cells

REBM: renal epithelial cell basal medium

ROS: reactive oxygen species

SE: standard error

STZ: streptozotocin

TUNEL: transferase-mediated dUTP-biotin nick and labeling

DM: diabetes mellitus

Bil: Bilirubin-treated group

Control (normal): normal condition

Bil(3),Bil(10),Bil(15): 0.3, 1.0 and 1.5 mg/ml bilirubin-treated

HX,BilX(X=C,S,L..): HC, BilC—Control . “H” means high glucose, “Bil” means Bilirubin treated

iRNA transfected : HS, BilS—SREBP-1 iRNA transfected ; HL, BilL--- LXR-α iRNA transfected

aveBilX(X=C,S,L…): average of SREBP-1 iRNA, LXR-α iRNA or control iRNA transfected group treated with bilirubin.

The effect of bilirubin on the regulations of triglyceride synthesis in streptozotocin-induced diabetes mellitus nephropathy

Introduction

Bilirubin is an end product of heme metabolism. Heme is known as a constituent of hemoglobin, which is released in association with the breakdown of aging red blood cells. Heme also is contained in a wide range of enzymes whose turnover also leads to free heme release. Free heme can be toxic, so is involved a family of heme oxygenase enzymes to degrade heme[1-2]. These enzymes cleave the heme ring to form biliverdin, iron, and a 1-carbon fragment as carbon monoxide CO is increasingly appreciated as a neurotransmitter and iron, itself toxic[3-4], is excreted from cells by a recently characterized pump[5-6]. Biliverdin would seem to be an appropriate end product of the pathway, being readily excreted in the bile to enter the intestine and leave the body in the feces. Indeed, in birds, reptiles, and amphibians, biliverdin is the predominant end product of heme degradation [7]. For reasons that until now have seemed obscure, in mammals, biliverdin undergoes additional metabolism, being reduced by biliverdin reductase (BVR) to bilirubin. As bilirubin is more hydrophobic and insoluble than biliverdin, it is glucuronidated to facilitate excretion into the bile, costing additional cell resources. The combined evidence from animal and human studies indicates that modest level of bilirubin is a major physiologic cytoprotectant by antioxidation, anti-inflamination [8], and others studies also have proved cytoprotection and reduction of oxidative stress effect of bilirubin in a ischemia

reperfusion kidney [9]. Elevated serum bilirubin has been associated with reduced risk of cardiovascular disease (CVD). Also serum bilirubin is also related with several potential confounders related to CVD, such as obesity [10]. Our previous studies tried to clear the effect of bilirubin on diabetics and diabetic nephropathy by employing Gunn rat for the DM model, and Gunn rats were resistant to fasting hyperglycemia and impaired glucose tolerance in STZ induced type 1 diabetes partly attributing to hyperbilirubinemia, bilirubin also improved destruction of pancreatic beta-cells, recovery insulin secretion, and bilirubin also delayed kidney sclerosis. However, the DM model was built based on a hyperbilirubiemia rat, when it came to the preventative effects or therapeutic effects of bilirubin on the model, it might be a little confused. Thus, a new model was applied, in which bilirubin was treated after DM model was built. We would try to verify its antioxidation, anti-inflamination and others in our new model to certificate its feasibility. Excess of a variety of lipoproteins and lipids worsens glomerular injury, promotes tubulointerstitial fibrosis, and accelerates progression of diabetic nephropathy [11]. Some clinic trials also demonstrated the inverse relationship of serum bilirubin levels and LDL. [12] As a component of LDL, Triglyceride levels might be affected by bilirubin too. The effects of bilirubin on Triglyceride amount in kidney tissues of DM nephropathy were studied in our experiment. Furthermore, the probable effects of bilirubin on triglyceride synthesis in various levels (tissue, cell, molecular, gene) in diabetic nephropathy were also investigated in the experiment.

Methods and Materials:

1. Animals

Male homozygous SD rats were obtained from Harlan (Indianapolis, IN, USA) and raised them in an animal facility where temperature is controlled and 12h light and 12h night shift in Seoul National University Bundang hospital. All the animals were supplied with standard rat chow and enough water. 24h urine was collected according to the baseline and after STZ injected in metabolic cage. At 5 weeks, being anesthetized, rats are sacrificed. Blood was collected, and plasma was obtained by blood centrifuged at 3500 rpm, 4C ℃for 20min and then preserved in -20C °for further application. Kidney and liver collected were fixed in 10% formalin for 24h and then embedded in paraffin for or frozen in -80C °for further use.

2. STZ-injection and grouping

18 SD rats were chosen after optimizing body weight, basal albumin excretion and FBG level to make sure that no statistical difference was existed among the subjects, after 2 weeks of acclimatization, those rats were divided into 3 groups (Control, n=5 ;DM, n=8; Bil n=5). After 8h fasting, STZ (60mg/kg body weight; sigma, St Louis MO, USA) was injected into the abdomen of DM, Bil groups. And 8h later, Bil group was treated with 0.3mg/ml bilirubin (0.3mg/ml bilirubin dissolved in dimethylsylphoxide, DMSO) and our previous study confirmed that this concentration of bilirubin would not induce severe toxicities, other groups were treated with equal solution in the same way [13].

3. Cells

(1)Hepatoma cells model

Hepatoma cells were purchased from ATCC company (CRL-1830) and cultured in DMEM(glibco by life technologies) normal glucose (1g/L D-glucose) / high glucose(4.5g/L D-Glucose) both supplemented with 10% heated inactivated fetal calf serum and 1% antibiotics (streptomycin 10mg/ml, penicillin G 10,000UI/ml) in a humidified environment with 5% CO_2-95% air at 37C °. After controlling external conditions, 4 medium dishes of hepatoma cells growing in normal glucose media were obtained after 1 week, and those dish were marked as [control , HG ,Bil(3) Bil(10)] , then 8h later, DM, Bil groups were changed to High glucose media , another 8h later. Bil(3) and Bil(10) groups were treated with 0.3mg/ml and 1mg/ml bilirubin [bilirubin dissolved in dimethylsylphoxide, DMSO] each, other groups were treated with the same amount of sodium citrate solution.

Finally, 8h later, all the cells were harvested for protein and for further use

(2) Mesasngial cells model

MC cells obtained from C57BL6j mouse were cultured in Dulbecco's modified Eagle's medium (DMEM) also supplemented with10% heated inactivated fetal calf serum and antibiotics (streptomycin 10mg/ml, penicillin G 10,000UI/ml) in a humidified environment with 5% CO_2-95% air at 37C °. After controlling external conditions, 4 medium dish of PMC cells growing in normal glucose media were obtained after 1 week, and those dishes were marked as [control , HG ,Bil(3) Bil(15)] ,

then 8h later, DM, Bil groups were changed to High glucose media , another 8h later. Bil(3) and Bil(15) groups were treated with 0.3mg/ml and 1.5mg/ml bilirubin [bilirubin dissolved in dimethylsylphoxide, DMSO] each, other groups were treated with the same amount of sodium citrate solution,

Finally, 8h later, all the cells were harvested for protein and for further use.

4. Liver tissue TG measurement

100mg live tissue was obtained. And the amounts of triglyceride per 100 mg tissue were measured among control, DM and Bil groups by triglyceride quantification kit: ab65336 (Abcam corp. CA USA), and the instructions were followed.

5. Plasma creatinine and creatinine clearance measurement

Plasma creatinine levels were determined by the quantichrom creatinine assay kit DICT-500 from bioAssay systems (Hayward. CA, USA), the instructions were followed. Plasma creatinine concentrations were expressed as mg/dl, and creatinine clearance was calculated as creatinine excreted in the urine (24 h period) divided by creatinine concentration in plasma, and the results were expressed as ml/min normalize to kidney weight.

6. Urine albumin

Urine albumin was determined by using a commercially available kid specific

for rat urine albumin (Nephrat; Exocell,Philadelphia,PA,USA) according to the manufacturer's instructions [14].

7. H_2O_2 measurement

H_2O_2 production in kidney tissue homogenate was determined by using DCFH (2', 7'-Dichlorofluorescin diacetate; sigma), briefly samples (urine or plasma 100ul,) were incubated with 10umDCFH-CA at a total volume of 200ul at 37℃ for 30 min and then the fluorescence intensity was measured. Background activity had been subtracted by setting up blank control wells.

8. Kidney and liver protein preparation and western blot

Kidneys and livers from our models were homogenized (1:10 wt/vol) in 10mM Tris buffered saline (20mM Tris-HCL, pH 7.4 0.25M sucrose, and 1mM EDTA), with the presence of a cocktail of protease inhibitors and centrifuged, and proteins were extracted [15] and quantified by Bradford essay, aliquots of 80ug were loaded on SDS-polyacylamide gel, then transferred to nitrocellulose paper for electrophoresis. Later, the membrane was incubated in 5% non-fat milk with primary antibody over night. Second anti-body was added on the second day at room temperature. Beta-Actin was used as internal control. The selected bands were scanned with an Imaging Densitometry (GS-700, Bio-Rad, Hercules, CA, USA). And the density was determined by Imagine J.

9. Kidney histology analysis

TUNEL staining terminal deoxynucleotidyl transfertase-mediated dUTP-biotin nick end labeling .TUNEL staining was performed on paraffin sections of kidney using in situ cell death detection kit (Roche) according to the manufacturer's instructions. Negative controls were included, sections were dewaxed in xylene and rehydrated in a graded series of ethanol and double-distilled water, sections were also heated for 5min in sodium citrate buffer (0.1M, PH 6.0) by using a microwave oven, risen with PBS, and incubated for 60min with terminal deoxynucleotidyl transferase and a label solution of a nucleotide mistune in reaction buffer. Negative controls with label solution only, sections were then rinsed with PBS for three times, coverslipped by using VectaShield hard mount with DAPI and observed with a fluorescence microscopy equipped with digital camera. Percentage of apoptosis of each glomerular was calculated as follows: percentage of apoptosis = (number of apoptosis cells /total cells). 10 glomerulars were analysised in each slide, and 20 PCT were analysised in each slide.

10. Kidney tissue pathology analysis

Kidney sections, 3um in thickness, were stained with periodic acid-Schiff (PAS). A computer-assisted image analysis system was applied to quantitate the tubulointerstitial structure in the renal cortex [16], sections were viewed with a microscopy, and the images were digitalized onto a computer screen by a video camera linked to image analysis software, to quantitate mesangial expansion. Mesangial area/glomeruli area ratios were determinate. Mean values were calculated

from each of 30 glomeruli.

11. siRNA transfection procedure

One six-well tissue culture sample plate was prepared .We seeded $2x10^5$ cells per well in 2ml normal growth medium with high glucose concentration, incubated the cells at 37C° in a CO_2 incubator until the cells are 60-80%confluent overnight, divided 6 groups HC, BilC, HS BilS, HL and BilL. After 8 hours BilC, BilL, BilS were treated with bilirubin (0.3mg/ml) [bilirubin dissolved in dimethylsylphoxide, DMSO]. Then after 8 hours ,we followed the instructions of siRNA transfection regment : SC29528(santa cruz biotechnology.inc)and added the siRNA duplex solution to Control group (HC,BilC) and transfected group (HS BilS, HL, BilL), incubated cells 5-7hs later and then collected the cells, isolated cell protein for western blot ,and isolated mRNA for PCR.

12. Statistics

The results are expressed as Mean±Standard error (SE). Statistical comparisons between animal groups were performed by excel and SPSS. Differences were analyzed by students't-test. A *P* value ≤0.05 was considered as a represented statistical significance.

Results

1. STZ- induced DM model and data of the model.

The data which was collected at the beginning of the experiment demonstrated that merely differences were existed among the three groups (Control , DM, Bil) (Table1).

The data which was collected 5 weeks later revealed some statistical differences between DM and control group in nearly all subjects. Water intake, food intake and 24h urine volume were increased significantly in DM group when compared to the initial data, however, there were no differences seen between the initial data and the data collected 5 weeks later in control group, which indicate an available type 1 DM model. Diabetic symptoms were improved in two subjects: food intake and 24 hours Urine volume in Bli group, compared to that in DM group. Body weight was also increased with statistically significance nearly 1 week after STZ- induction (DM: 321.5, Bil: 352.4615. Figure 1) in Bil group. In other subjects between Bil group and DM group, an improving trend could be observed, but there was no statistical significance. (Table 2A)

In T1DM model, 24h urine albumin in Bil group was significantly attenuated by bilirubin, meanwhile Ccr in Bil group seemed increased when compared to that in DM group, but lack of statistic significance. (Table 2 and Figure 2).

In blood samples, the concentration of total cholesterol and triglyceride in Bil group were less than that in DM group, however, although bilirubin seemed decrease

the concentration of blood free fat acid in Bil group, no statistic differences existed in these two subjects were found between Bil group and DM group (Figure 3, Table2B), therefore, Bilirubin may affect triglyceride synthesis.

Table1. Physiological parameters at baseline

Day 0	Control	DM	Bil
Water inta(g).	25.47 ±4.39	22.02 ±3.65	21.29 ±3.01
Food inta.(g)	17.99 ±2.72	15.04 ±1.61	18.32 ±1.39
UV(ml)	14.84 ±4.01	11.05 ±1.37	11.82 ±0.81
BWT(g)	223.80 ±2.46	218 ±1.28	220.17 ±1.82
FBS(mg/dl)	151.80 ±7.59	141.6 ±2.57	141.92 ±3.43
HbA1c (%)	3.02 ±0.07	3.04 ±0.027	3.08 ±0.04
Hb(g/dl)	19.88 ±0.44	20.22 ±0.32	20.38 ±0.38

Table 2(A). Physiological parameters at 5 weeks after STZ-induction

5 weeks	Control	DM	Bil
water intake(g)	20.29 ±1.83	*171.73 ±83.16	138.97 ±76.09
food intake(g)	14.62 ±2.77	*41.07 ±12.63	#35.37 ±13.21
UV(ml)	14.50 ±2.00	*103.12 ±74.08	#87.68 ±47.95
FBS(mg/dl)	141.00 ±6.97	*484.75 ±79.30	496.37 ±66.81
HbA1c (%)	3.58 ±0.07	*8.66 ±0.75	8.52 ±0.92
24h Albumin in urine (mg)	130.76 ±18.84	489.79 ±165.93	# 283.60 ±44.13
Ccr(ml/min)	2.99 ±0.21	1.67 ±0.32	1.84 ±0.30

Table 2(B). Physiological parameters at 5 weeks after STZ-induction

5weeks	Control	DM	Bil
Total-Chole. (mg/dl)	4.13 ±83.40	9.55 ±95.25	# 4.64 ±73.2
Triglyceride (mg/dl)	8.38 ±52.25	122.87 ±41.01	# 9.97 ±134.60
FFA (mg/dl)	710.4 ±15.46	1357.25 ±359.64	# 975.4 ±145.05
H2O2 urine Inflorescence unit/400 (ul)	11342.93 ±870.27	10347.05 ±732.78	#7918.631 ±1135.33

***Compared to control group, p≤0.05, # compared to DM group, *p*≤0.05.**

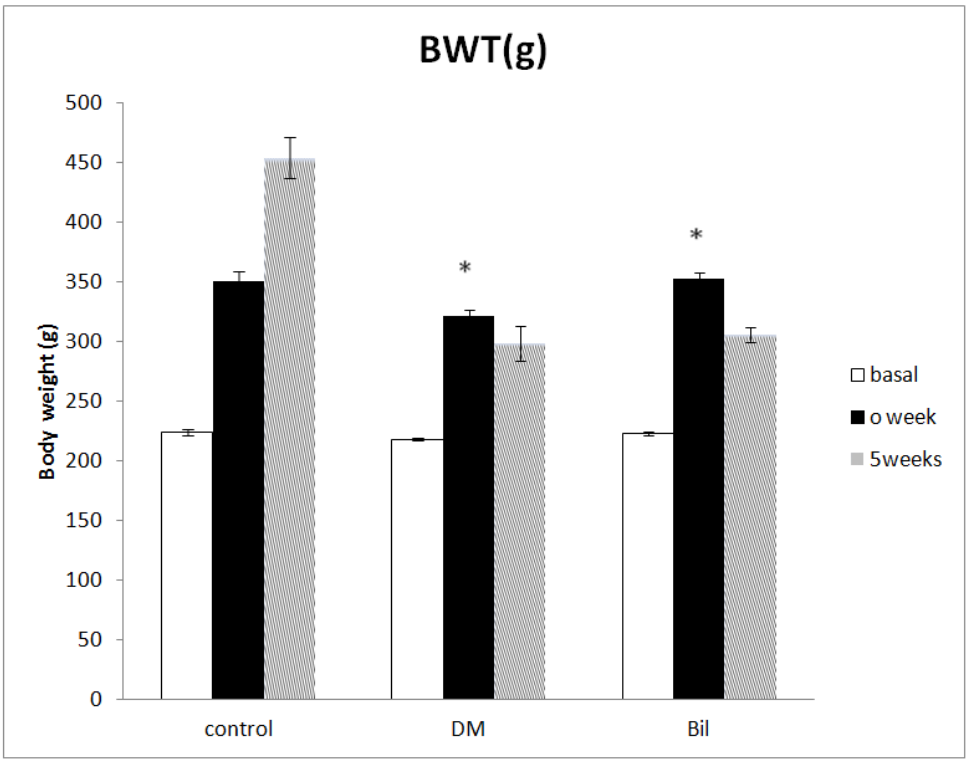


Figure1. Body weight at baseline, nearly one week after STZ-induction, and 5 weeks after STZ-induction, * P≤0.05

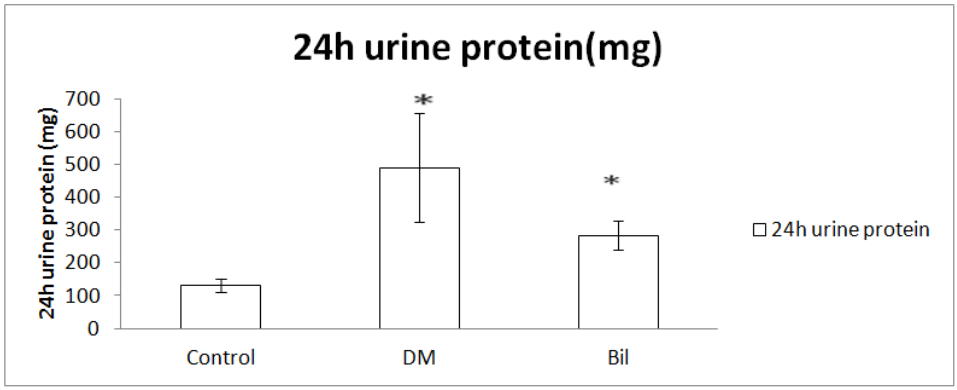
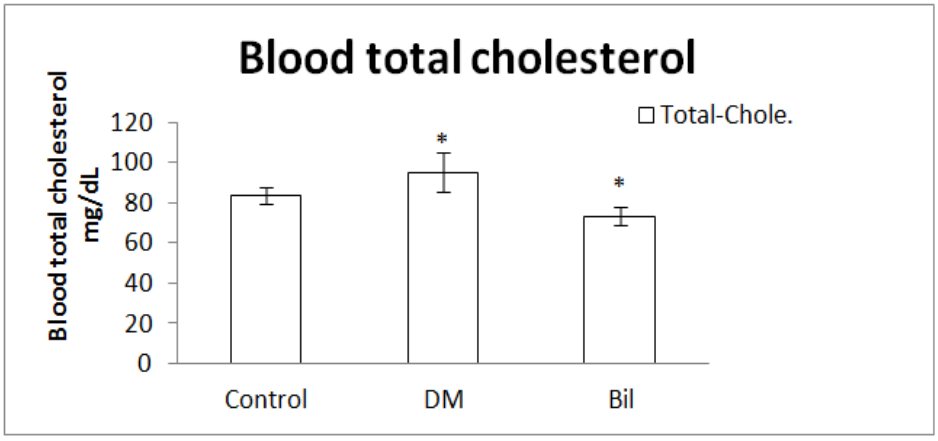


Figure2. 24h urine protein 5 weeks after STZ-induction, *p≤0.05

A



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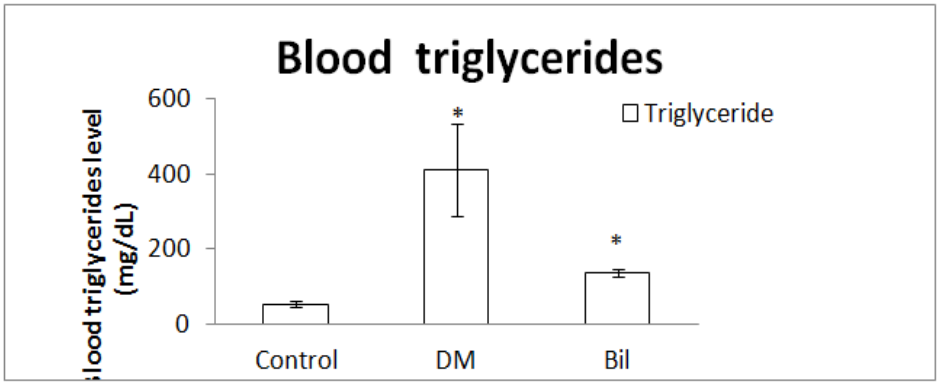

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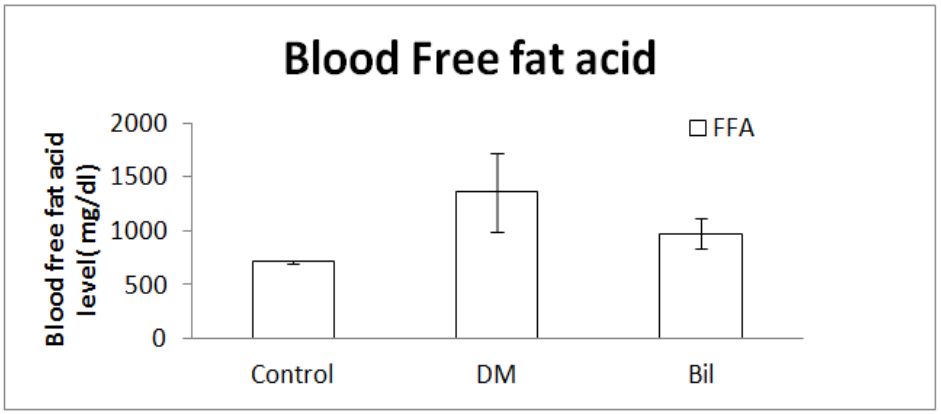


Figure3. Blood total cholesterol (A), triglyceride (B) and free fat acid concentration (C) 5 weeks after STZ-induction, * $p \leqslant 0.05$

2. Kidney tissue for DM model

Significant changes were observed in mesangial cells proliferation (% of glom area) in DM group through PAS staining between Bil group and DM group. Bilirubin lessened the mesangiail cells proliferation and inhibited Collagen IV synthesis in kidney tissue and meangial cells (Table 3, Figure 5), which attenuated the renal fibrosis of DM nephropathy. (Table 3 Figure 4)

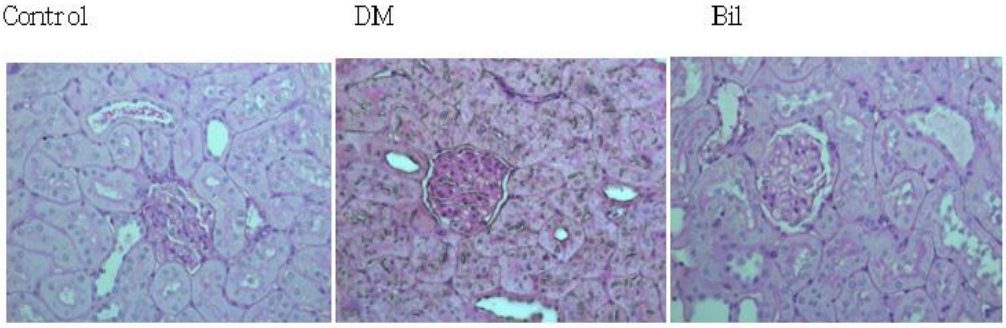
The apoptosis situation in DM nephropathy was also investigated by using TUNEL staining, and more glomeruli and PCT cells death were detected in DM group than that in Control group, and bilirubin improved apoptosis situation in Bil group (Figure 6 and Table 3). Bilirubin also lowered activated AKT expression in kidney (Figure 7 and Table 3) . The AKT pathway plays an important role in regulating apoptosis [17]. Activated AKT is associated with tumor cell survival, proliferation, and invasiveness. Bilirubin might also act as a pro-apoptotic factor for those unrepairable cells. As a whole, bilirubin may have the effect of anti-apoptosis and improve viability of cells.

Table 3 Data from kidney tissue 5 weeks after STZ-induction

	Control	DM	Bil
MS-ratio(% of glomeruli area)(PAS staining)	▼0.140±0.003	0.160±0.004	●0.154±0.004
P-AKT/AKT(%)(WB)	0.153±0.083	0.153±0.052	●0.045±1.111
Apoptosis cells ratio (% of total glomeruli	▼0.121±0.004	0.222±0.007	●0.166±0.006
Apoptosis cells ratio(% of PCT) cells)[TUNEL staining]	▼0.151±0.0054	0.232±0.0085	●0.186±0.0067
Collagen IV(% of actin) (WB)	▼0.172±0.092	0.672±0.122	●0.702±0.113
pNFKb/NFKb(%)(WB)	▼0.183±0.103	3.593±0.423	●2.663±0.543
NOX4(% of actin) (WB)	▼0.213±0.123	2.623±0.313	●0.623±1.263
P22phox(% of actin) (WB)	▼0.183±0.093	1.762±0.203	●1.223±1.312
P47phox(% of actin) (WB)	▼0.392±0.224	3.394±0.324	●2.283±0.453
SCD-1(% of actin) (WB)	▼0.0012±0.00028	0.011±0.0020	●0.002±0.00060
FAS (% of actin) (WB)	▼0.48±0.08	1.54±0.13	●0.53±0.06
SREBP-1(% of actin) (WB)	▼0.15±0.013	0.46±0.018	●0.25±0.028
LXR-α(% of actin) (WB)	▼0.18±0.015	0.42±0.014	●0.32±0.011
TGF-beta (pg/ml)/100mg tissue(ELISA)	6.02±0.24	5.93±0.42	●3.53±0.064

●▼Compared to DM group, $p \leq 0.05$.

A



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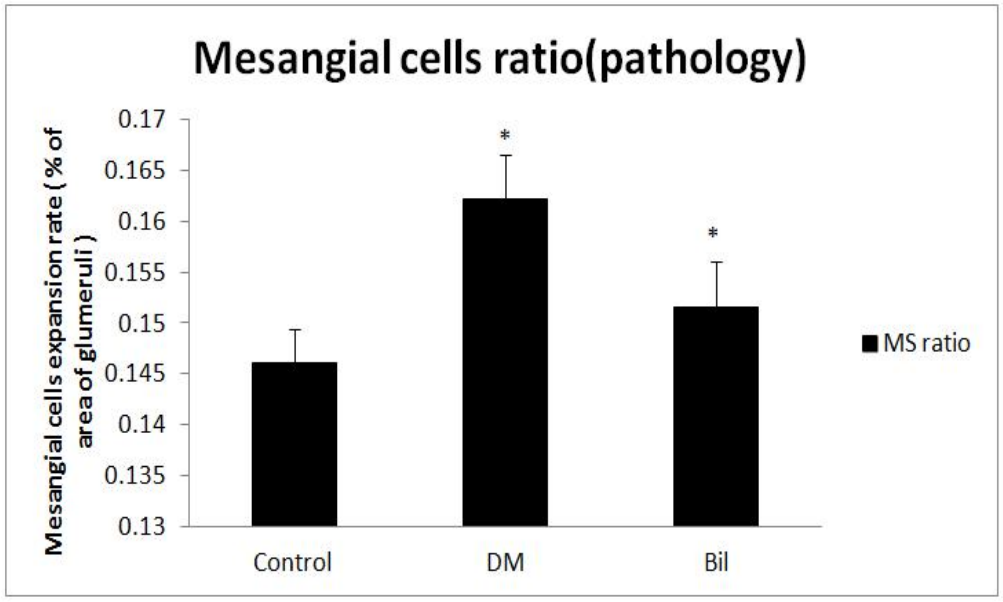
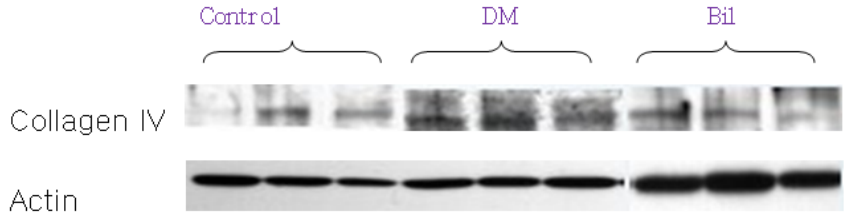


Figure4. Mesangil cells ration (ratio= mesangial area/glomeruli area) in kidney tissue at 5 weeks after STZ injection *A* PAS staining of glomeruli of different groups of kidney tissue (magnification * 400). We could observe that fibrosis situation in Bil group is much better than that in DM group *B* mesangial cell ratio (ratio= mesangial area/glomeruli area) among different groups, *p≤0.05

A



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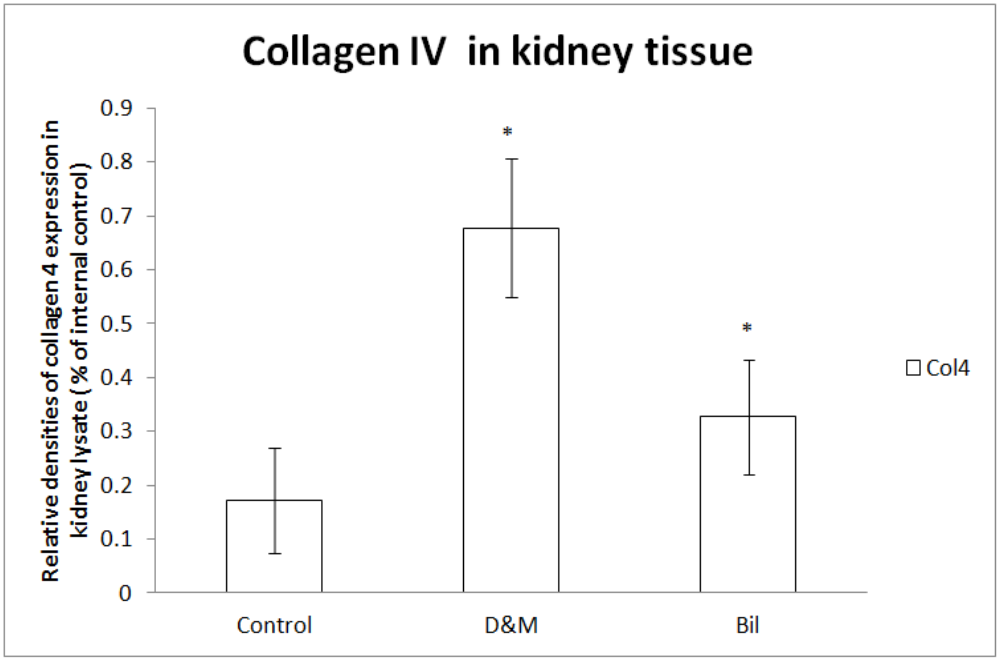
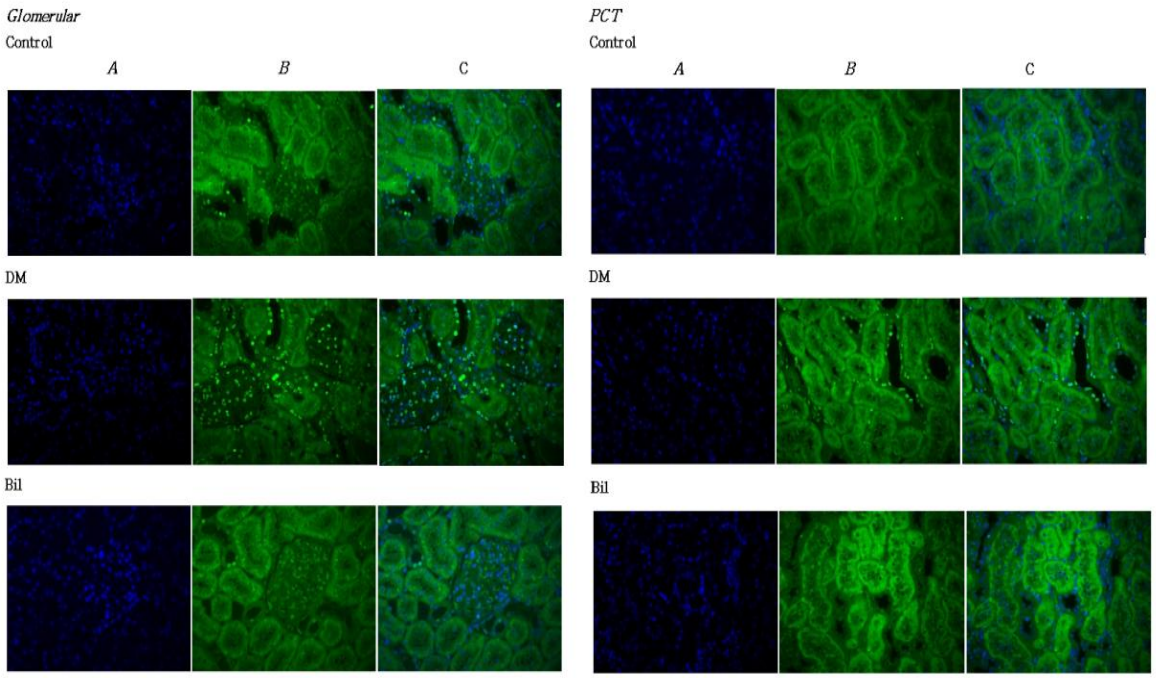


Figure5. Collagen IV expressions in kidney tissue at 5 weeks after STZ-injection *A* wester n blot for Collagen 4 expression in varies groups .*B* Collagen 4 expression was increased i n DM group compared in control group, however, Bilirubin decreased Collagen IV expressi on and promoted renal fibrosis,* P≤0.05



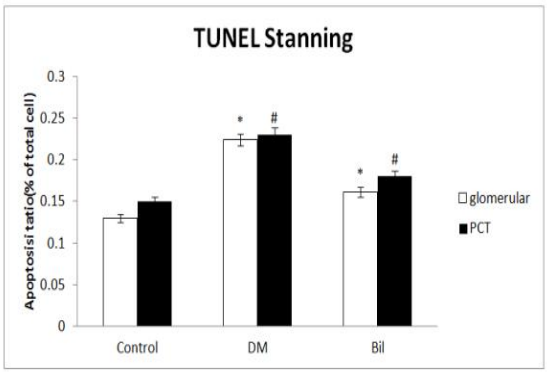
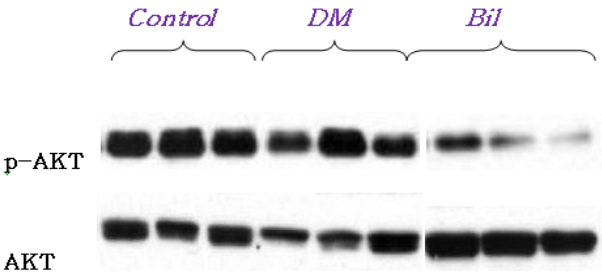


Figure6. Apoptosis cells ratio in glomeruli and PCT of kidney (ratio=apoptosis numbers/total cell numbers) in kidney tissue at 5 weeks after STZ injection ,tunnel staining of glomeruli and PCT of different groups of kidney tissue *A*. DAPI staining for nucleus (magnification X200); *B*. TUNEL staining with FITC labeled antibody (magnification X200); *C*. Merged image of A and B. the blue staining show normal cells and the blue-green nucleus show apoptotic cells (Magnification X200): we could observe that apoptosis situation in Bil group is much better than that in DM group. *# P<0.05

A



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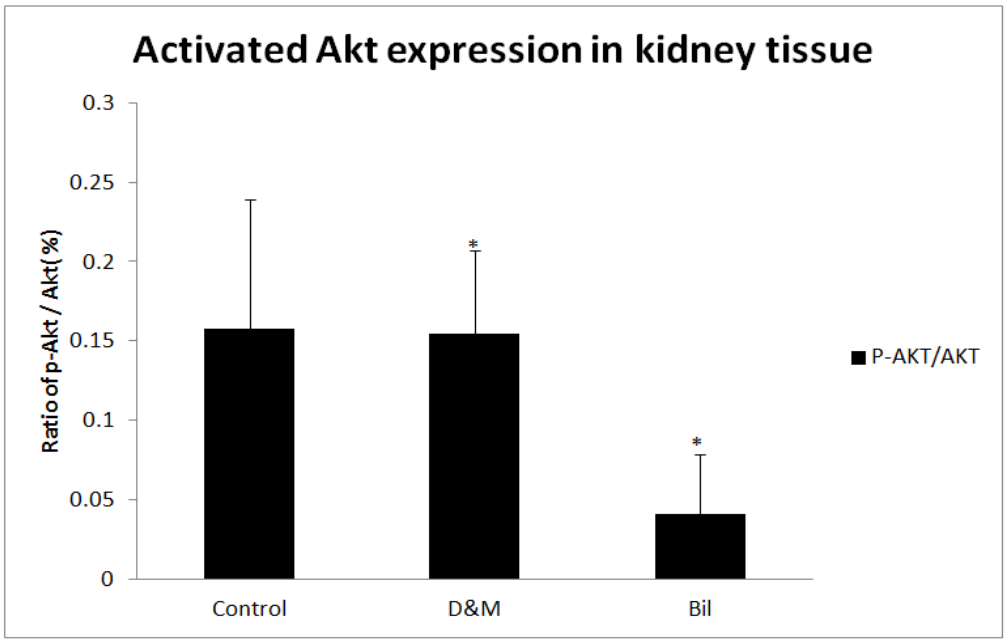
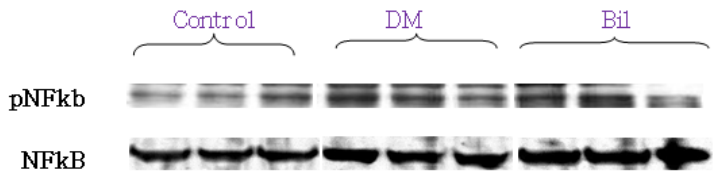


Figure7. Activated AKT expression in kidney tissue at 5 weeks after STZ -injection *A* western blot for phosphated AKT and AKT expressions .*B* less activate AKT expression were seen in Bil group compared to DM group (activate AKT expression= amount of p hosphate AKT / amount of AKT). * P≤0.05

A



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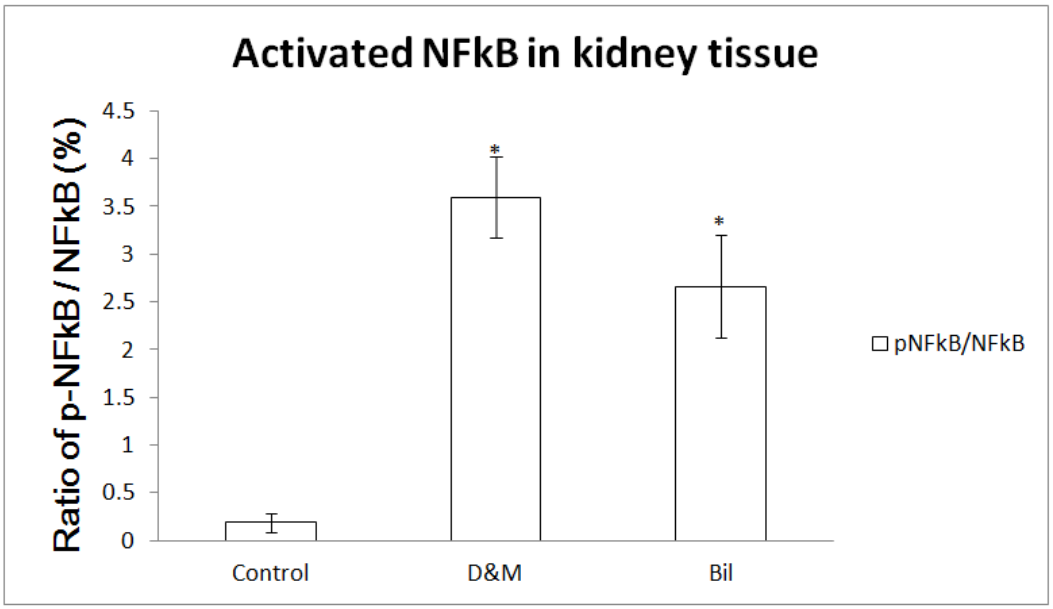
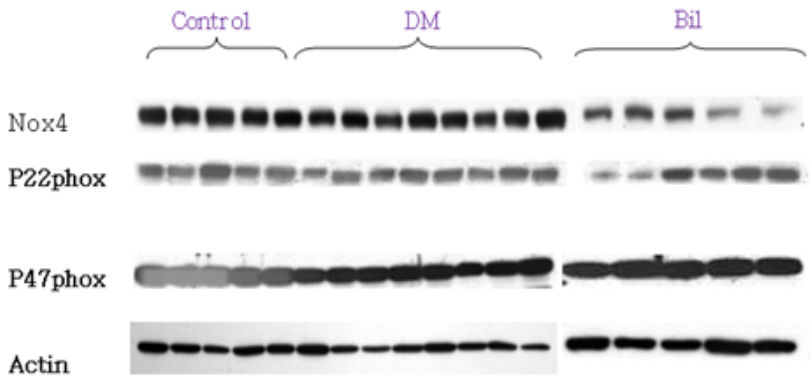


Figure8. Ratio of the amount of phosphated NFkB expressed divided by the amount of NF kB in kidney tissue at 5 weeks after STZ- injection. *A* western blot for phosphated NFkB and NFKB in kidney tissue homogenate, *B* showed that the expression of activated NFkB was less in Bil group than that in DM group with statistic significance, * P≤0.05.

NADPH oxidase is the most important source of ROS production in vascular

tissues and cells [18], the phagocytic NADPH oxidase complex comprises a membrane-associated cytochrome b558 composed of GP91 phox (NOX2) and p22phox and several cytosolic regulatory subunits(P47phox P67phox and Rac1 Rac 2). As a homolog of gp91phox in the kidney, it has been implicated that NOX4 is a major source of ROS production[19-20]. In our experiment, NOX4, p22phox, p47phox protein expressions were higher in DM group than that in control group, NOX4, p22phox and p47phox protein expressions were lower in Bil group when compared to that in DM group in kidney tissues (Figure 9 and Table 3). The amount of H_2O_2 measured in kidney tissue homogenate was also less in Bil group compared to that in DM group (Table2). Bilirubin decreased the protein expression of activated NFκB in kidney tissues of DM model. (Table 3, Figure 8). NFκB is a family of transcription factors and is of central importance in flammation and immunity [21], and NFκB is also associated with ROS production. Bilirubin may reduce the degree of oxidase stress by inhibition of NFκB activation and reduction of ROS.

A



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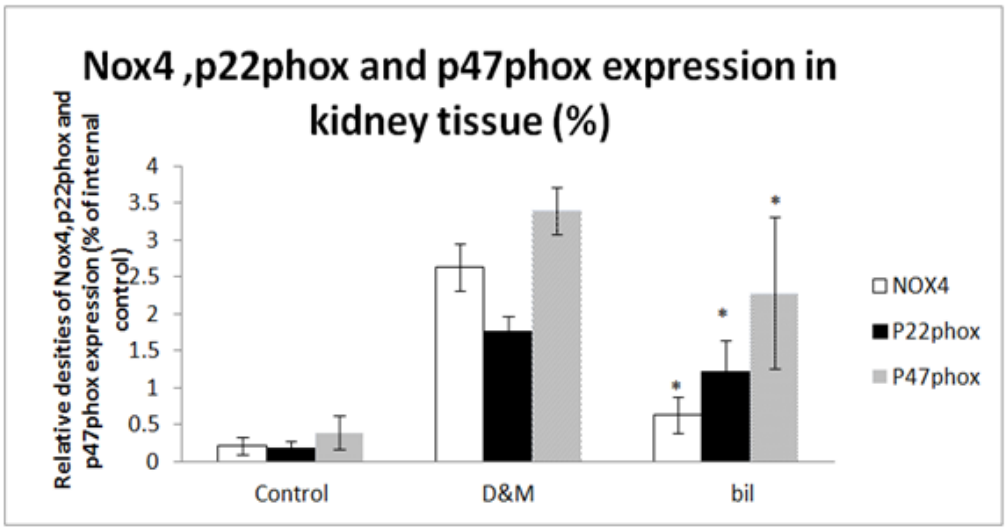


Figure9. NOX4, p22phonx and p47phox expression in kidney tissue at 5 weeks after STZ injection *A* western blot for NOx4, p22phox, p47phox in kidney tissue homogenate *B* NOX 4, p22phox, p47phox expressions were higher in DM group than control, NOX4, p22phox and p47phox expressions were lower in Bil group compared to DM group. * Compared to DM group, $P \leq 0.05$.

Three sterol regulatory element–binding proteins (SREBP-1a, -1c, and -2) stimulate transcription of a number of genes involved in the synthesis and receptor-mediated uptake of cholesterol and fatty acids. Results to date support the notion that SREBP-1 primarily activates the fatty acid, triglyceride, and phospholipid

pathways, while SREBP-2 is the prominent isoform supporting cholesterol synthesis and uptake In fatty acid biosynthesis, proteases release nuclear SREBP-1c (the major SREBP-1 isoform in the liver of animals), which activates transcription of the major genes of fatty acid synthesis including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl CoA desaturase-1 (SCD-1), glycerol-3-phosphate acyltransferase, and others [22] . Studies also confirmed that hypertriglyceridemic effect was associated with LXR-α agonist-dependent induction of the SREBP-1 lipogenic program [23]. In our experiment, Bilirubin inhibited Triglycerides synthesis by down-regulating LXR-α, SREBP-1, SCD-1 and FAS protein expressions. Bilirubin also lowered TGF-beta expression in DM nephropathy, which may play an important role in lipid-induced renal injury (Table 3 Figure9-10).

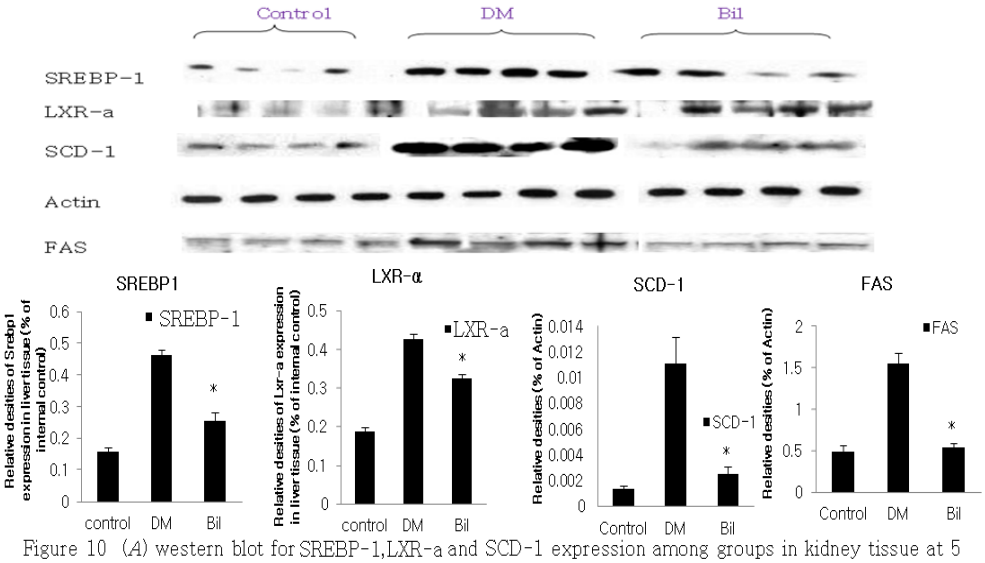


Figure 10 (*A*) western blot for SREBP-1,LXR-a and SCD-1 expression among groups in kidney tissue at 5 weeks after STZ-injection.(*B*) SREBP-1, LXR-a,SCD-1,FAS expression ratio (ratio= the amount of target protein/the amount of Actin) among groups in kidney tissue at 5 weeks after STZ-injection. *. Compared to DM group, P≤0.05.

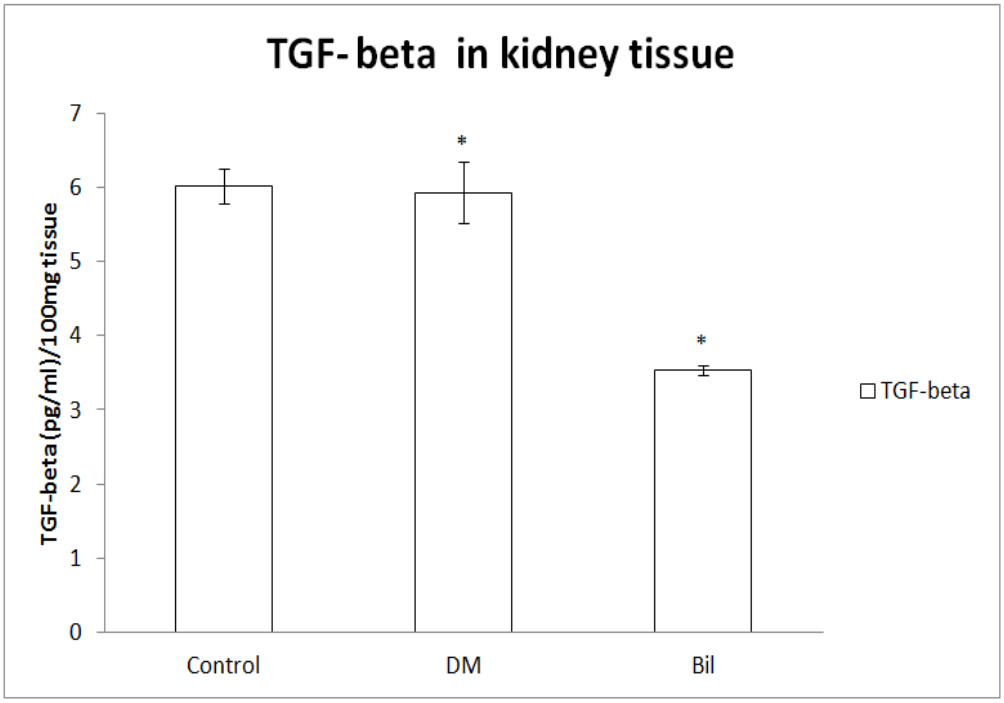


Figure11. The amount of TGF-beta in kidney tissue 5 weeks after STZ-induction
*p≤0.05.

3. Liver tissue for DM model

Table 4 Data from liver tissue at 5 weeks after STZ-induction

	control	DM	Bil
liver TG(mg/100mg tissue)	0.086±0.0019	0.092±0.0035	◆0.085±0.0033
SREBP-1(% of actin)	0.21±0.011	0.43±0.028	◆0.19±0.009
LXR-α(% of actin)	1.26±0.083	2.18±0.079	◆1.56±0.082
FAS(% of actin)	0.051±0.017	0.158±0.011	◆0.129±0.009
SCD-1(% of actin)	0.013±0.0066	0.103±0.0195	◆0.049±0.0065

◆Compared to DM group, P≤0.05

Triglyceride production was measured in liver tissue and triglyceride production was higher in DM group than that in Control group, however, bilirubin inhibited TG synthesis in Bil group (Table 4 and Figure 12). It was also confirmed in cell model, the protein expression of LXR-α, SREBP-1, SCD-1 and FAS were less in Bil group than that in DM group in both hepatoma cells and PMC. (Table 4 and Figure 13)

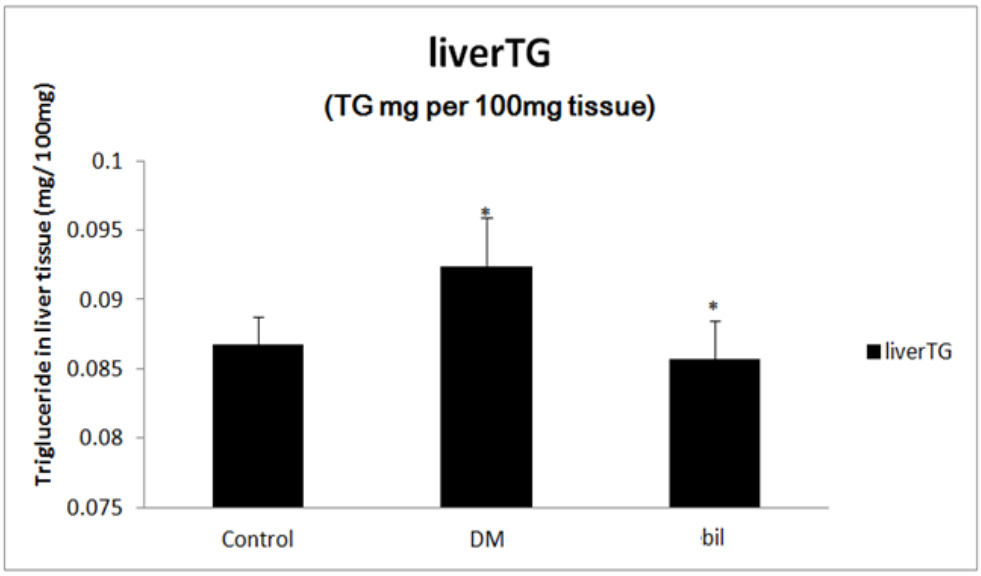
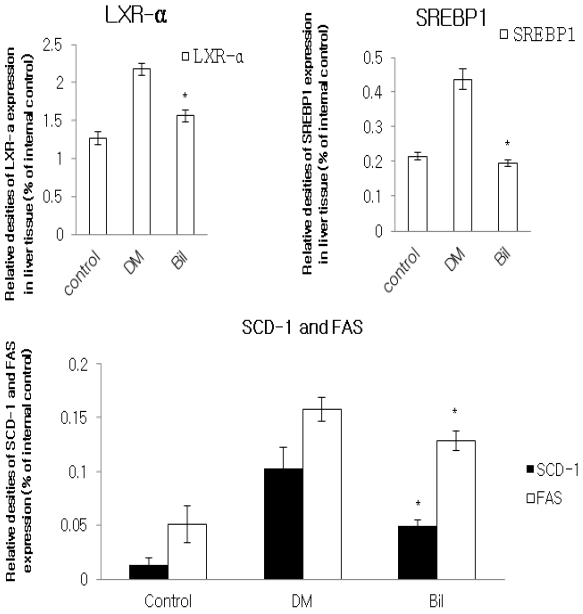


Figure12. TG amount in liver tissue among various groups at 5 weeks after STZ-injection. TG amount in Bil group was less than that in DM group. *p≤0.05.



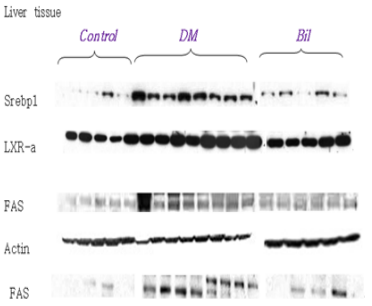


Figure 13 (A) Western blot for SREBP1,LXR-a, SCD-1 and FAS expression among groups in liver tissue at 5 weeks after STZ-injection.(B) REBP1,LXR-a, SCD-1 and FAS expression ratio (ratio= the amount of target protein/the amount of Actin) among groups in liver tissue at 5 weeks after STZ-injection, which showed that less SREBP1.LXR-a, SCD-1 and FAS expression were observed in Bil group than that in DM group. * compared to DM group, p≤ 0.05.

4 . MC cells model

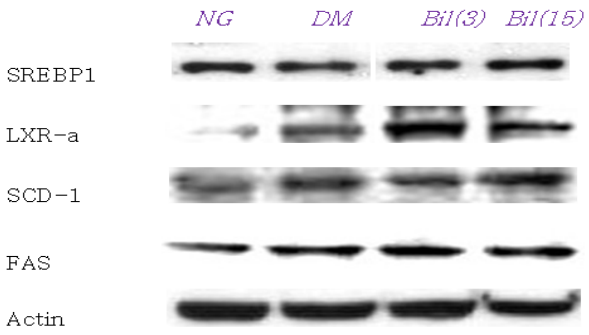
SREBP-1, SCD-1 Collagen IV and FAS expressions in Bil(15) were lower than that in HG group and SREBP-1, Collagen IV and FAS expressions in Bil（3） were lower than that in Bil(15). However , the expression of PPAR-r were higher in Bil(3) group than that in HG group, but lower in Bil(15) group than that in HG group, which may indicate a dual regulation of bilirubin. (Table 4 Figure 14-16).

Table 5 Data from PMC model.

Mesangial cells (% of actin)	NC	HC	Bil(3)	Bil(15)
SREBP-1(% of actin) (WB)	1.18±0.046	1.28±0.005	▼1.14±0.008	▼0.98±0.030
LXR-α(% of actin) (WB)	0.68±0.006	0.97±0.064	1.0±0.004	0.85±0.043
PPAR-r(% of actin) (WB)	2.46±0.019	2.43±0.02	▼2.87±0.057	1.71±0.403
SCD-1(% of actin) (WB)	3.34±0.338	4.11±0.371	3.49±0.296	▼3.06±0.41
FAS(% of actin) (WB)	0.50±0.052	0.80±0.071	0.68±0.004	▼0.58±0.003
Collagen IV (% of actin) (WB)	69.21±1.665	106.851±2.125	▼62.44±1.566	▼34.20±0.897

▼ Compared to DM, p≤0.05

A



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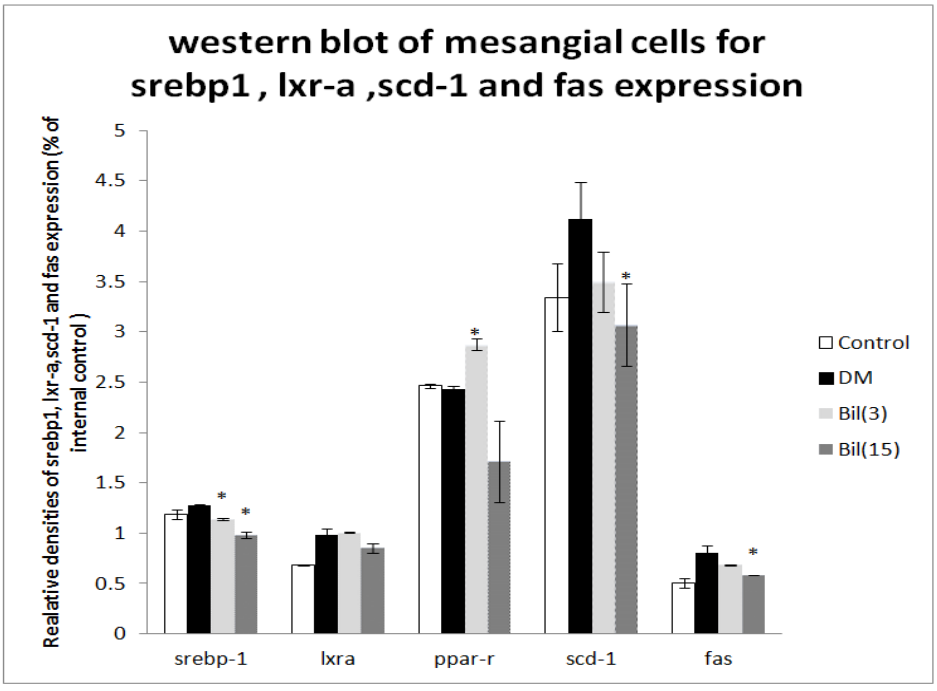
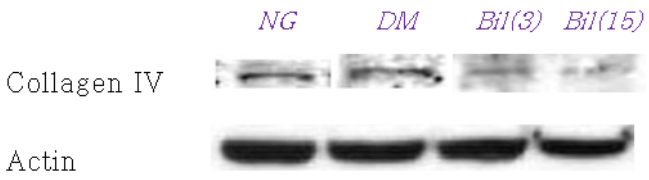


Figure14. (A) Western blot for SREBP1,LXR-a SCD-1 and FAS expression among groups in PMC.(B) REBP1,LXR-a, SCD-1 and FAS expression ratio (ratio= the amount of target protein/the amount of actin) among groups in liver tissue at 5 weeks after STZ-injection. *Compared to DM group, *p≤0.05.

A



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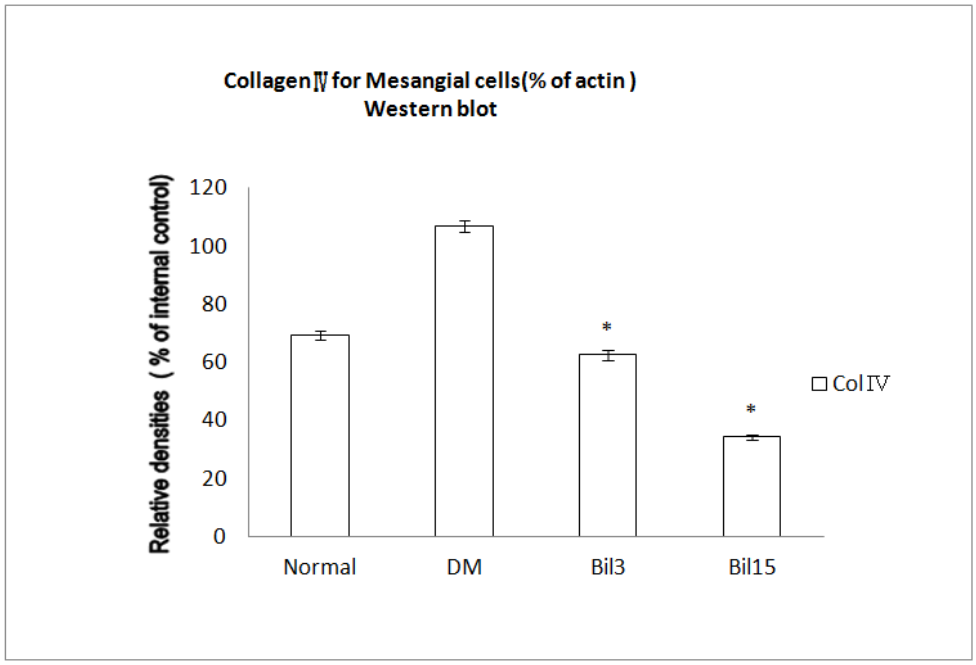


Figure15. Showed collagen Ⅳ expression of mesangial cells among the groups. *A* western b lot for collagen Ⅳ expression. *B* Collagen Ⅳ expressions was increased in DM group comp ared in control group, however, Bil3 and Bil5 decreased collagen Ⅳ expression, when com pared to DM group. This also showed that 1.5mg/ml bilirubin was more effectively than 0.3 mg/ml bilirubin. So Bil seemed promoted renal fibrosis. *Compared to DM group, *p≤0.05.

5. Heptoma cells model and iRNA transfected model

LXR-α, SREBP-1 and SCD-1 protein expression were lower in Bil (3) and Bil(10) than that in HG group. SREBP-1 protein expression in Bil(3) was lower than that in Bil(15), but LXR-α and SCD-1 protein expression in Bil(3) were lower than that in Bil(15) group, which also indicate a dual regulation of Bilirubin (Table 6, figure 16)

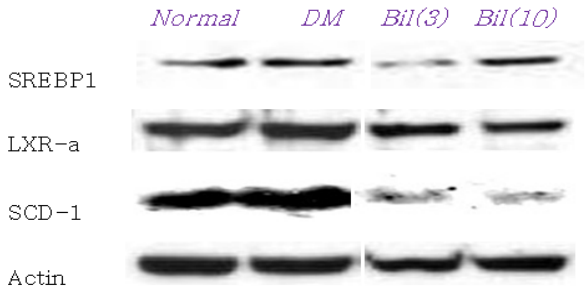
In SREBP-1-transfected hepatoma cells model, the transfection ratio of SREBP-1 iRNA was 74% In HG group, while in bilirubin group, the ratio was 88%. As a positive up-stream of SREBP-1, LXR-α protein expression was elevated for a compensation (HB group LXR-α protein expression increased 27%, while HG group increased 5.3%). In LXR-α-transfected hepatoma cells model, the transfection ratio of LXR-α was 79% in HG group while the ratio was 86% in bilirubin group. Meanwhile, bilirubin also tried to reduce the SREBP-1 expression decreasing rate for a compensation relatively (HB group SREBP-1 expression decreasing rate was 12%, while HG group was 31%). The same phenomena was also confirmed in gene expression by PCR. (Table 7-8, figure 17-18)

Table 6 Data from Hepatoma cells Model

% of actin	Normal	HG	Bil(3)	Bil(10)
SREBP-1	189.92±7.70	383.34±7.54	*91.71±1.79	#203.73±2.25
LXR-α	1893.39±52.80	4317.56±128.81	*1903.30±24.43	#31425.54±65.60
SCD-1	12.18±0.41	31.26±0.76	*11.53±0.20	#12.89±0.22

***#. Compared to DM group, P≤0.05**

A



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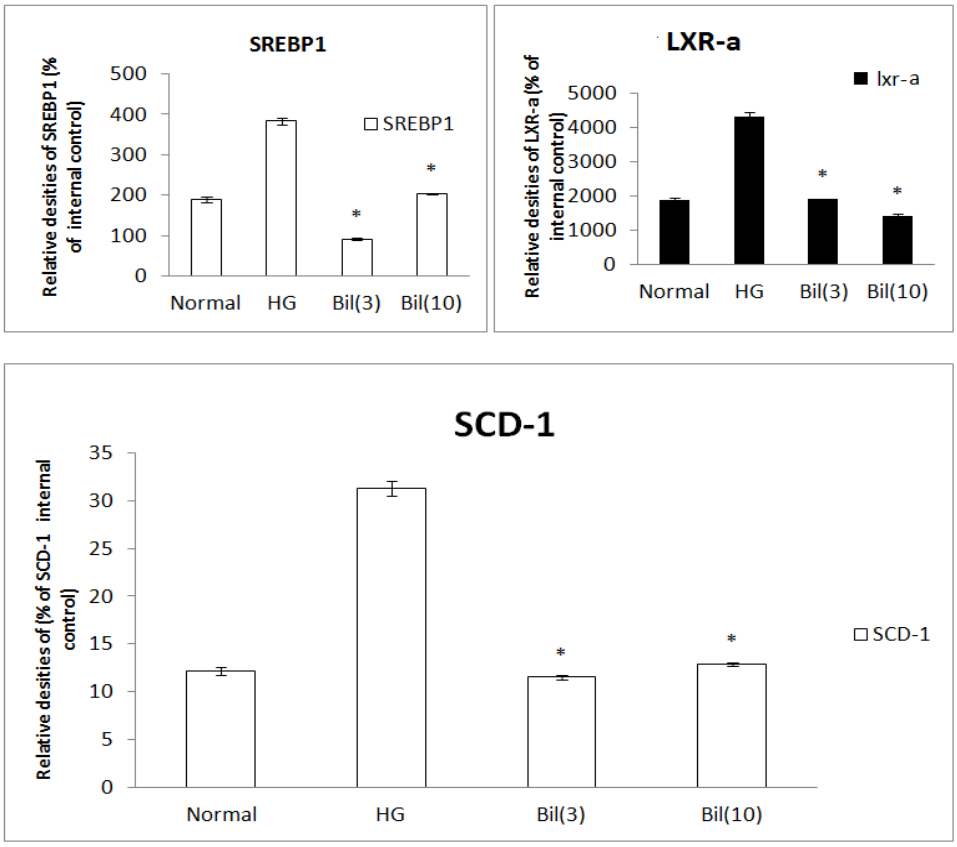

Figure16. (A) Western blot pictures (B) SREBP1 LXR-a and scd-1 expression for hepatoma cells * Compared to HG group, $p \leq 0.05$.

(1) Western blot for SREBP-1 transfected hepatoma cells and LXR-α transfected hepatoma cells

Table 7 Data from iRNA transfected Hepatoma cells Model

Western blot(Relative densities % of actin)	HC	HS	BilC	BilS
SREBP-1	0.45±0.0086	▼0.33±0.0034	0.26±0.0036	●0.23±0.0065
LXR-α	0.57±0.0075	▼0.60±0.0057	0.41±0.0032	●0.52±0.0066
	HC	HL	BilC	BilL
LXR-α	1.81±0.015	▼1.43±0.037	1.49±0.013	●1.29±0.022
SERBP1	3.08±0.0299	▼2.11±0.033	2.90±0.083	●2.55±0.100
PCR (Relative densities % of actin)	HC	HS	BilC	BilS
SREBP-1	2.79±0.036	▼2.72±0.032	2.77±0.074	●2.76±0.016
LXR-α	0.20±0.0046	▼0.25±0.0072	0.18±0.0107	●0.27±0.0044
	HC	HL	BilC	BilL
LXR-α	513.57±6.03	▼454.05±5.23	367.02±4.40	●345.75±2.52
SREBP-1	4.10±0.069	▼3.54±0.043	3.85±0.055	●3.65±0.051

▼ compared to HC. P<=0.05. ● compared to BilC, $p \leq 0.05$

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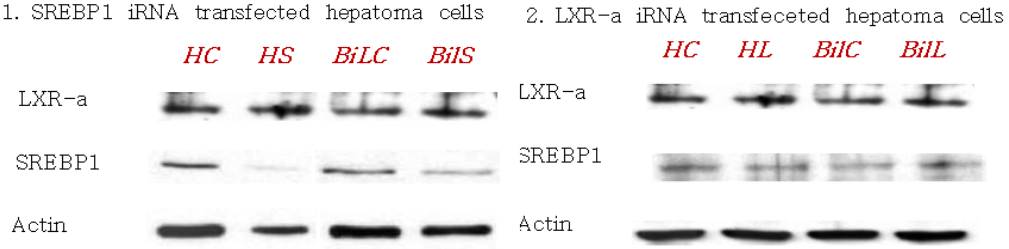

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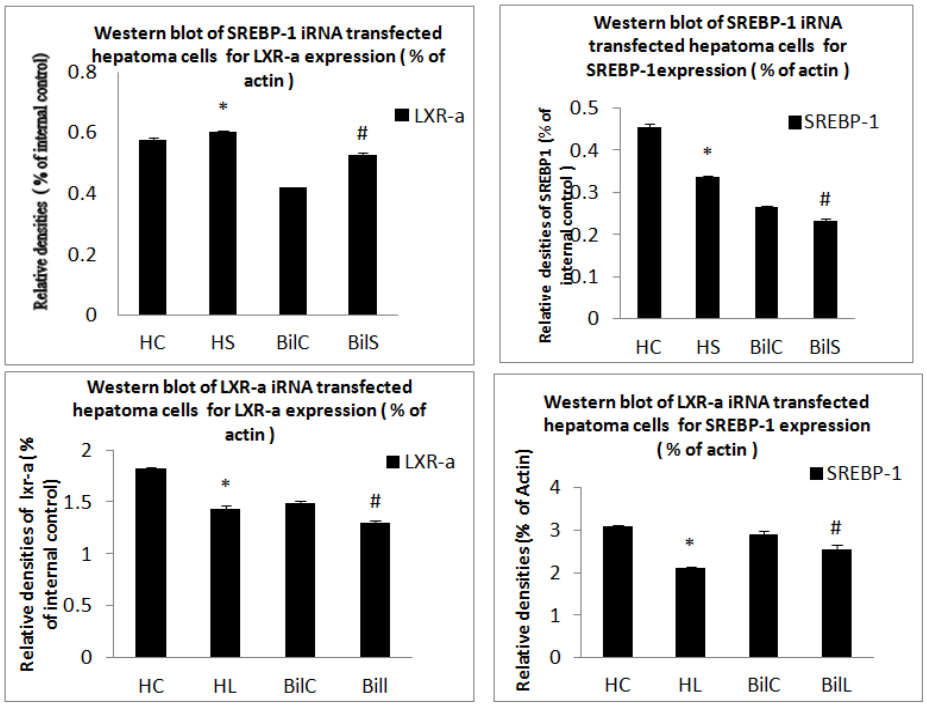

Figure17. Western blot for SREBP1 and LXR-a expression in siRNA SREBP-1 transfected hepatoma cells model and in siRNA LXR-a transfected hepatoma cells model. (*A*) Western blot pictures (*B*) SREBP1 nd LXR-a expression ratio (ratio= the amount of target expressio n/the amount of actin expression). * compared to HC, $p \leqslant 0.05$. # compared to BilC $p \leqslant 0.05$.

(2) PCR for SREBP-1 transfected hepatoma cells and LXR-α transfected hepatoma cells

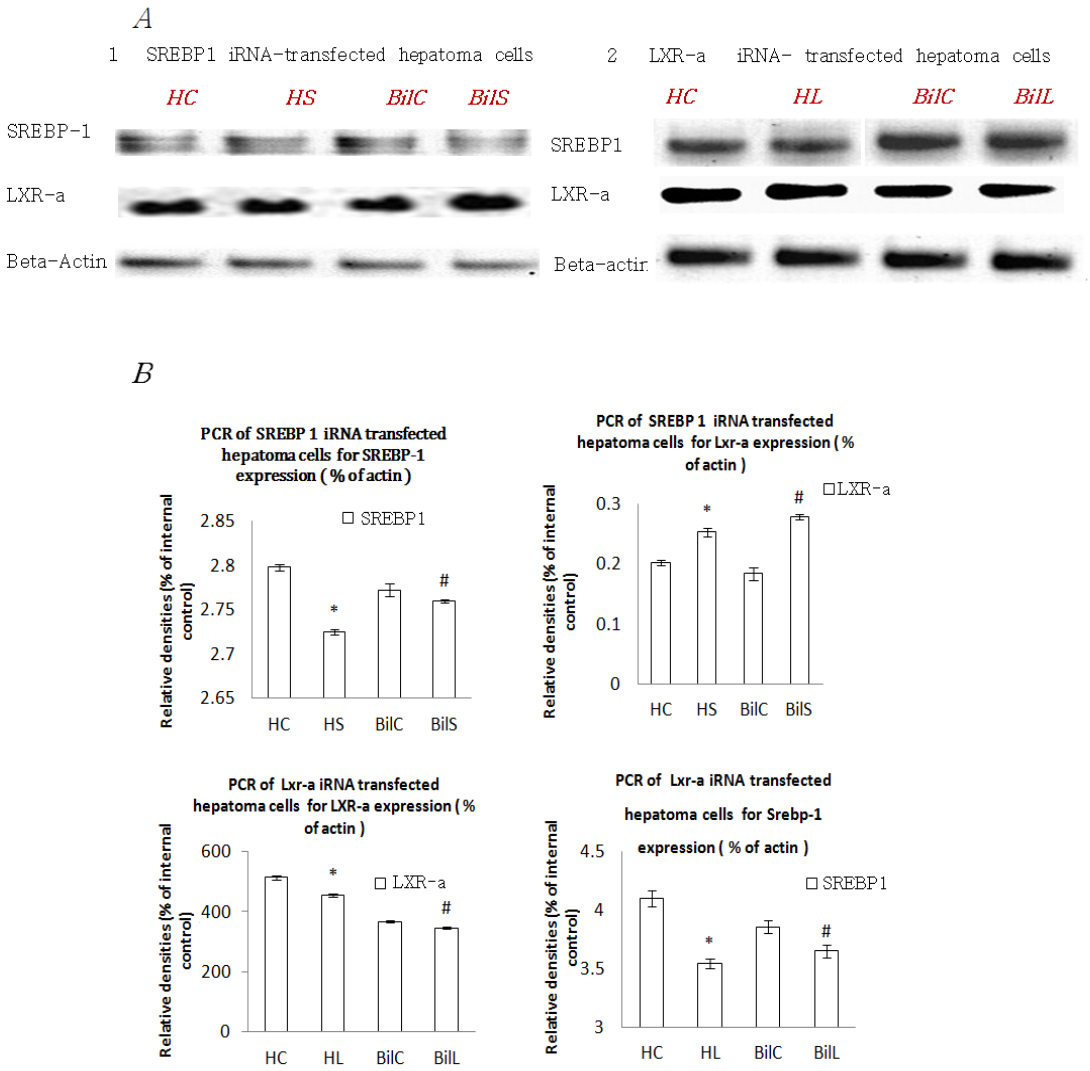

Figure 18 PCR for SREBP-1 and LXR-a expression in siRNA SREBP1-transfected hepatom a cells model. And in siRNA LXR-a transfected hepatoma cells model (*A*) western blot pict ures (*B*) SREBP1 and LXR-a expression ratio (ratio= the amount of target protein/the am ount of actin) * compared to HC, $p \leqslant 0.05$, # compared to BilC $p \leqslant 0.05$.

Table 8(A) Data analysis from iRNA transfection model

Expression decreasing rate (%)=(DM-average Bil)/DM	kidney tissue 5weeks after STZ-injection	liver tissue 5 weeks after STZ-injection	hepatoma cells	mesangial cells
SREBP-1	0.39	0.38	0.63	0.17
LXR-α	0.12	0.16	0.48	-0.32

Table 8(B) Data analysis from iRNA transfection model

Transfection rate =aveX/C(HX/HC or aveBilX/aveBilC) (%)	western blot		PCR	
	SREBP-1 iRNA transfected SREBP-1 transfection rate	LXR-α iRNA transfected LXR-α transfection rate	SREBP-1 iRNA transfected SREBP-1 transfection rate	LXR-α iRNA transfected LXR-α transfection rate
HX/HC	0.74	0.79	0.97	0.88
aveBilX/aveBilC	0.88	0.86	0.98	0.94
	Corresponding LXR-α changing rate	Corresponding SREBP-1 changing rate	Corresponding LXR-α changing rate	Corresponding SREBP-1 changing rate
(HX-HC)/HC	0.053	-0.31	0.25	-0.14
(aveBilX-aveBilC) /aveBilC	0.27	-0.12	0.5	-0.052

Discussion and analysis

Bilirubin and proteinuria in DM nephropathy

Previous studies about billirubin effects on various DM model have demonstrated that Blirubin had some benefits on kidney function protection on diabetic nephropathy in a Gunn rat model [24]. In our new DM model, these findings were also confirmed. Three groups were divided: Control, DM , Bil. An intraperitoneal injection of STZ causes an immediate destruction of pancreatic beta-cells, resulting in hyperglycemia and rapid progression of nephropathy which is similar to human diabetic glomerulonephropthy [25], this technique was employed to create type 1 DM model. Control means normal rats; Bil means that a proper amount of bilirubin was treated after STZ-injection. 5 weeks later , Polyuria, Polydipsia, Polyphagia and weight loss appeared in DM group. In our experiment we found that proper concentration may reveal reno-protection and delay the progression of DM nephropathy.

Diabetic nephropathy is a nephrotic syndrome, which the detection of increased amounts of protein in urine provides an index of diagnostic, prognostic and pathogenesis significance [26-31]. Increasing rates of urinary protein excretion, more accurately, albumin excretions in diverse nephropthies predict more rapid decline in renal function. In our studies, we found that, In the DM nephropathy model, Bilirubin

decreased 24 hour urine albumin excretion. Usually, renal glomerular filtration membrane integrity, renal proximal tubule cell reabsorption and filtration pressure would affect proteiuria pathogenesis. These days, we use ACEI drugs to reduce kidney protein excretion by decreasing filtration pressure in order to alleviate the damage of kidney.

Bilirubin has been proved to have dose-dependent vasodilatory effect. Studies showed that ANG II induces vasoconstriction, at least in part, by stimulating NADPH oxidase and generating reactive oxygen species, and also by inhibiting Heme oxidase-1, which would decrease the production of bilirubin. Also Enhancement of Glomerular heme oxigenase-1 expression was observed in DM nephropathy. So we may contribute vasodilatory effect of bilirubin to its interfering of ANGII's vasoconstriction. Studies also supported this hypothesis [32-33]. As we described above that, NADPH oxidase is the most important source of ROS production in vascular tissues and cells, which includes NOX2 (a homolog of NOX4), p22phox and p47phox. In our study we found that (Billirubin decreased the amount of H2O2 expression in Urine in DM nephropathy model.) Bilirubin decreased the protein expression of Nox4, p22phox and p47phox, which may alleviate the vasoconstriction of AT II. Some studies also confirm this opinion [33-34]. Our study also showed that the apoptosis situation of glomerular cells in Bil group was better than that in DM group, which may indicate a good condition of glomerular filtration membrane. Our studies still affirmed that bilirubin increased PCT cells viabilities in Bil group, and PCT cells were the major part of renal proximal tubule cells responsible for reabsorption, which meant Bilirubin may maintain reabsorption functions of proximal tubule cells. Thus,

Bilirubin may impede the ANG II's vasoconstriction by antioxidation and maintain integrity of fitting membrane by alleviating glomerular apoptosis and improve reabsroption by increasing viabilities of renal proximal tubule cells, which would reduce the amount of albumin excretion and delay the progression of DM nephropathy.

Bilirubin and TG synthesis in kidney in DM nephropathy

Data from basic and clinical studies strongly indicate that in patients with diabetes, an excess of a variety of lipoproteins and lipids worsens microvascular and macrovascular disease, worsens glomerular injury, promotes tubulointerstitial fibrosis, and accelerates progression of diabetic nephropathy [35]. TGF-β is upregulated in the glomerular of both rats with streptozotocin -induced diabetes and in the glomerular of humans with diabetic nephropathy [36]. Activation of TGF-β mechanistically links hyperlipidemia with glomerular disease. Several lines of evidence indicate that lipids may induce renal damage through activation of the TGF-β pathway by inducing the ROS production and pro-fibrosis [37-39].

In our studies, bilirubin decreased protein expression of SREBP-1 in kidney tissues from DM nephropathy model. SREBP-1 stimulated the synthesis of free fatty acids and increased the concentration of triglycerides in the kidney. SREBP-1 also simulated synthesis of TGF-beta [40], which was also observed in our experiment. Activated TGF-β acts in an autocrine fashion through the TGF-β type II receptor to upregulate messenger (m)RNA and protein expression of extracellular matrix proteins, such as fibronectin, laminin, type I collagen, type iv collagen,[41] and biglycan.[42] The

expression of these factors is stimulated by smaD3 signaling and suppressed by smaD7 signaling [43]. Activated TGF-β also increases levels of extracellular matrix protease inhibitors, such as plasminogen activator inhibitor-1 (Pai-1) and the tissue inhibitors of metalloproteinase, therefore, decreased expression of TGF-beta would decrease the synthesis of extra cellular matrix protein and increase extracellular matrix degradation, which also observed in our experiment by PAS staining of kidney tissues [44]. Studies also demonstrated that TGRL lipolysis products stimulate the production of ROS in human aortic endothelial cells via activation of nox4 [45]. The involvement of ROS in TGF-β activation may in part explain the ability of antioxidant therapy to attenuate the induction of fibronectin expression by nox4 [46]. So bilirubin may delay the sclerosis and fibrosis of DM glomerular by inhibit lipid-induced TGF-beta expression. 2003 study also suggested that lower levels of the latent TGF-β binding protein may slow the progression of diabetic nephropathy [47].

Bilirubin and Triglyceride synthesis in liver in DM

Studies have proved the inverse relationship between serum blirubin levels and small dense LDL [48] , Triglycerides are the major part in LDL, so blirubin may affect triglyceride synthesis. In our experiment, the concentration of serum triglyceride in Bil group was less than that in DM group. And also less Triglyceride synthesized in Bil group compared to that in DM group in liver tissue.

SREBP-1 primarily activates the fatty acid, triglyceride, and phospholipids pathways, SREBP-1c activates transcription of the major genes of fatty acid synthesis including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl CoA

desaturase-1 (SCD-1), glycerol-3-phosphate acyltransferase, and others, also SREBP-1 acts downstream of LXR-α as we described above. In our study we found that bilirubin decreased SREBP-1, LXR-α SCD-1 and FAS expression and increased PPAR-r expression to varies extent in kidney tissue, liver tissue, hepatoma cells and mesangil cells. And we also found that the reduction rate of SREBP-1 expression by bilirubin was more than that of LXR-α expression. Since that SREBP-1 acts downstream of LXR-α, we could assume that bilirubin indirectly decreased SREBP-1 expression through its directly decreasing LXR-α expression. Then, we tried to demonstrate our hypothesis by using siRNA transfection procedure to silence either LXR-α or SREBP-1. After running a series of western blot to detect target proteins, we found that Bilirubin would try to reduce the transfection ratio of LXR-α and SREBP-1 to a certain extent in hepatoma cells, although bilirubin was thought to inhibit the expression of these genes. Bilirubin seemed to maintain triglyceride level within certain limits. (Table 8)

As a whole, Blirubin seems to work as a dual elaborated regulator and maintain triglyceride level in a range of values.

Conclusions:

Bilirubin exerted protection to the diabetic nephropathy through therapeutic functions. Bilirubin might alleviate proteinuria by maintaining the integrality of the glomerular filtration membrane and promoting renal proximal tubules viabilities through apoptosis improvement and may also by its vasodilatoy effect, which delayed the progression of sclerosis of diabetic nephropathy. Bilirubin also decreased the triglyceride amount of kidney tissue in DM nephropathy and reduced the lipid-induced TGF-beta production, which improved the fibrosis situation in kidney tissue. bilirubin may also affected the synthesis of triglyceride in liver and blood, and it didn't only lower the blood triglyceride but also maintain the Triglyceride in a proper level, which was benefit for health and may indicate therapeutic value for triglyceride regulation. Further study is needed to reveal this regulation in detail.

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개요

도입: 적당한 빌리루빈은 항산화 및 항염증 작용 등을 통해 생리학적으로 중요한 세포보호작용을 한다. 그리고 전에 우리는 DM Rat을 이용한 실험에서 빌리루빈이 신장의 고빌리루빈혈증 예방작용도 한다는 것을 확인하였다. 본 실험에서 우리는 STZ 유도 후에 빌리루빈을 처리한 새로운 모델을 사용하여 당뇨병성 신장증에서 빌리루빈이 신장과 triglyceride의 합성에 어떤 영향을 주는지 확인하려고 시도하였다.

방법: 본 실험에 18마리의 male SD Rats를 사용하였고 그 중에서 13마리는 60mg/kg 용량으로 STZ를 주사하여 DM을 유도하였으며 기타 Rats은 vehicle citrate buffer(PH 4.5)로 처리하였다. 8시간이 지난 후 13마리 DM Rats에서 5마리를 임의로 선택하여 0.3mg/ml bilirubin 처리를 하고 이들을 Bil group이라고 명명하였다. 5주 후 Rats의 몸무게, 물 섭취량, 음식 섭취량 등 기본적인 자료를 수집하였고 간과 신장을 적출하였으며 PAS 염색, TUNEL 염색, ROS 측정 그리고 western blotting을 실시하였다. PMC 모델: 초기혈관사이세포를 준비하고 모두 세 그룹(control, DM, Bil)으로 나누었다. 먼저 모든 그룹은 정상치의 글루코스 환경에서 배양하다가 하루가 지난 후 DM 그룹과 Bil 그룹은 고농도 글루코스 환경으로 바꿔 배양하였으며 또 하루가 지난 뒤 Bil 그룹은 빌리루빈 처리를 하였다. 마지막에 이 세포들을

이용하여 western blotting을 실시하였다. 간암세포모델은 PMC 모델과 동일한 방법으로 만들었다.

iRNA transfection model: 빌리루빈이 TG regulating gene에서의 효과를 확인하기 위하여 iRNA transfection 방법을 이용하여 간암세포에서 target gene의 발현을 억제시킨 후 세포를 거둬들여 PCR과 western blotting을 진행하였다.

결과: STZ 유도 실시 5주 후 DM 모델에서 당뇨병증상이 나타났다. 빌리루빈은 이런 증상을 일정한 정도에서 개선하며 Bil 그룹에서 혈중 TG 농도는 DM 그룹보다 낮았다. 그리고 Bil 그룹에서 빌리루빈은 24시간 내의 소변 알부민의 배설과 H_2O_2 생성을 감소시켰다. 신장조직에서 빌리루빈은 NADPH 산화효소에 의존하는 oxidative end 산물을 감소시킨다. Bil 그룹은 DM 그룹보다 사구체의 섬유화와 세포사멸이 훨씬 적게 나타났다. 신장조직에서 빌리루빈은 TGF-beta 합성과 triglyceride 합성에 관련된 일련의 유전자 발현을 감소시킨다. 간 조직에서 TG의 양은 DM 그룹이 Bil 그룹보다 적으며 빌리루빈은 역시 triglyceride 합성에 관련된 유전자의 발현을 억제한다. PMC 모델에서도 간암세포 모델과 동일한 결과를 확인했다. iRNA transfection 모델에서 빌리루빈은 이중 조절인자이며 일정한 낮은 레벨에서 TG 합성에 관련된 유전자의 발현을 유지한다.

결론: 빌리루빈은 사구체의 여과막을 완전하게 유지하는 것을 통해 단백뇨를 완화하고 세포사멸의 개선과 혈관확장작용을 통해 proximal tubules의 생존능력을 제고하며 따라서 당뇨병성 신장증의 진행을 지연시킨다. 빌리

루빈은 지질에 의한 TGF-β 생산을 감소시키고 신장 조직의 섬유화 진행을 개선하였다. 빌리루빈은 DM에서 triglyceride의 합성을 감소할 뿐만 아니라 정교한 조절을 통해 Triglyceride의 낮은 수준을 유지해 준다.

키워드 : , 빌리루빈, 당뇨병신장병, triglyceride의 합성 , antiapoptosis, 항산화, TGF-β

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