

# Study of klotho gene transfer for the protective effect of the coronary of diabetic rats

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**Abstract:** The authors model diabetes in rats, and inject the rats with Klotho gene. Then the levels of serum lipoprotein are tested, measuring the thickness of coronary artery intima and the ratio of intima and media to see whether Klotho gene has protective effect of coronary artery in diabetes rats. Methods: Extracting the Klotho gene from kidney tissue in the normal SD rats amplifies the target gene by PCR and uses adenovirus as carrier. Then SD rats were randomly divided into model group, control group and the treatment group for diabetes modeling. Transferred the Klotho gene into treatment group and blank adenovirus into control group by the experimenters. Nothing was done for model group. Rats were killed after a successful modeling in the twelfth week, then tested blood low-density lipoprotein, high density lipoprotein, and the coronary artery intima-media thickness. After doing these, intima-media thickness ratio was tested. Results: The high density lipoprotein is  $0.67\pm 0.06$  mmol/L in treatment group,  $0.48\pm 0.10$  mmol/L in control group,  $0.47\pm 0.10$  mmol/L in model group. The treatment group, control group and model group respectively two independent sample tests. There is statistical significance between treatment group and the other group  $p<0.01$  in treatment group. The low density lipoprotein is  $0.44\pm 0.08$  mmol/L in treatment group,  $0.45\pm 0.10$  mmol/L in control group,  $0.44\pm 0.05$  mmol/L in model group. Respectively two independent sample test show that there is no statistical significance between treatment group and the other group ( $p>0.05$ ). Intima thickness is  $1.74\pm 0.05$   $\mu$ m in treatment group,  $2.23\pm 0.06$   $\mu$ m in control group,  $2.15\pm 0.05$   $\mu$ m in model group. There is statistical significance between treatment group and the other group ( $p<0.01$ ). The ratio of intima and media is  $0.237\pm 0.097$  in treatment group,  $0.308\pm 0.023$  in control group,  $0.316\pm 0.037$  in model group, and t test, there is statistical significance between treatment group and the other group ( $p<0.01$ ). Conclusion: There is protective effect on coronary after Klotho gene was transferred into diabetic rats.

**Keywords:** Klotho gene; lipoprotein; coronary atherosclerosis; diabetes; gene transfer

## INTRODUCTION

At present, the most common method of treating cardiovascular disease are: rebuilding anatomical structure (percutaneous coronary stent implantation and aorto-coronary bypass grafting); Treatment aim at the pathogenesis of acute cardiovascular (such as antiplatelet, anti-freezing, etc.); Controlling of the controllable hazards (improve the mode of life, regulate the lipid, control blood glucose, quit smoking, restrict alcohol, etc.) To the patients to whom the acute cardiovascular event has occurred, we resist the heart reconstitution and prevent the complication from happening. Though treated actively, there is still chance for the patient to suffer coronary artery restenosis and recurrence of acute cardiovascular. In 1996, Kuro-o and *et al*, found a new gene-Klotho gene (standard by KL gene in the following) which is relevant with senium. Research showed that the genotype and the expression product had a lot to do with the pathogenesis of diabetes, hypertension, atherosclerosis chronic renal failure, etc (Xiaolin *et al.*, 2010). The treatment aimed at its genotype examination and target spot provided a new method for the clinical diagnose and treatment of angiocardopathy, and provided another way for the prevention of angiocardopathy. KL gene is the

gene which has great relationship with body senium, life span and diseases response to the aged (Kuro *et al.*, 1997). Besides, the protein KL gene coded (KL protein) plays important role in several metabolisms. The variation of KL gene's expression is closely relevant to the progress and prognosis of some pathological states (Arking *et al.*, 2003). Considering the homology (81% resemble) of rat's KL gene and human's, we used rat to model diabetes and lead in KL gene thus to study the protective function of Klotho gene to the rat's coronary artery.

## MATERIALS AND METHODS

### Material

73 clean SD rats, whose weight are 150-170g, male (purchased from Shanghai & K universal experimental animal Co., Ltd.); Sigma-aldrich; transfection reagent Lipofectamine 2000 (bought from *In vitro* gen company); adenovirus (bought from Biowit company); pMD18-T-Klotho plasmid (purchased from Hangzhou Wehbe Technology Co. Ltd.); Plasmid Extraction Kit (Generay company); fidelity PCR reagent LA Taq (TaKaRa); restriction endonuclease, T4 DNA ligase (Fermentas); DNA Gel Extraction Kit (Axygen company); PVDF membrane (Millipore company); lipoprotein cholesterol assay kit (north of Beijing Kangtai clinical Reagent Co. Ltd.); diabetic model feed (Nantong Tero Feifeed

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Technology Co., Ltd., 20130720); ECL Plus light kit, SDS-PAGE protein sample buffer (5×), Western and IP cell lysate, PMSF, BCA protein assay kit (beyotime company).

### **Experimental groups**

The experimental animals were randomly divided into normal group and diabetic group. Adapted to feeding after 1W, the normal group was fed with regular diet, diabetes group with high fat diet. After a period of time, diabetic group were given intraperitoneal injection of streptozotocin. The normal group was injected with the same volume of citric acid sodium citrate buffer liquid. Satiety tail vein blood glucose (FBG), full FBG was greater than 16.7 mmol/L as the diabetic. The diabetic rats were randomly divided into 3 groups. 3 groups were diabetic model group, diabetic model and transfection group (treatment group), diabetic model + negative carrier group (control group)

### **Methods**

(1) Amplification of the KL gene construct: 1) extracting gene: 2μL RNA solution in the high accuracy spectrophotometer detection optical density value, to observe the protein absorption peak at A260/A280, A260/A230 ratio and continued wavelength absorption peak, and calculated the concentration of RNA. A260/A280>2.0 and <2.3 standards. The extracted RNA was about 1μg, Oligo (dT) 18 primers was a centrifuge tube in 2μL. Coke two ethyl carbonate water 10μ L was put in centrifuge tube. After the centrifugal treatment, cDNA was created by adding drugs dry bath. According to the design objective gene sequences extracted primers, KL gene was amplified by PCR amplification system. The KL gene was cloned into pMD18-T vector, and the overnight culture plates included white clones from 37°C, liquid LB cultured in 4ml containing 50g/ml ampicillin medium, 37°C /220rpm oscillation overnight culture. Plasmid DNA was extracted by Plasmid Extraction Kit, then granulating preparation and the recombinant plasmids were identified by restriction analyzes. 2) To construct a recombinant adenovirus packaging and carrier: In liquid nitrogen jar , 293 cell cryopreservation was gotten out, and 37°C water bath melted quickly, then the experimenters inoculated in the culture bottle, added DMEM culture medium (containing 10% fetal bovine serum). The cells grew into a uniform monolayer cells and more than 90% aggregation condition. Culture medium was diluted into DNA with 4μg DNA, which would be added to the transfection complexes containing cells and medium 6 well plates by gently mixing. After 4~6 hours culture medium was changed, they were cultivated at 37°C, 5% CO2 incubator. When the cells were covered with a Petri dish, the cells passaged in 25cm<sup>2</sup> cells were observed in every dish. The cells grew to fully the bottom of the bottle, and then transferred to 75cm<sup>2</sup> cell culture flasks, the toxic signs were observed

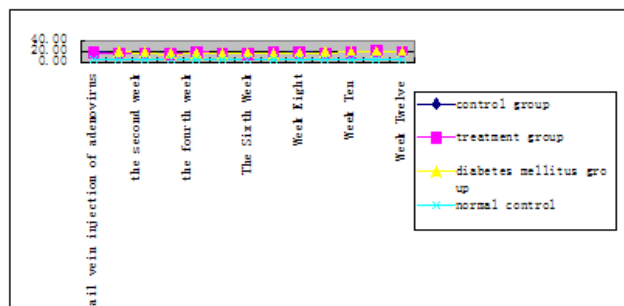
by the experimenters. Culturing HEK293 cells amplification, when cell confluence reached 80%-90%, added to the first generation of virus AD-rklotho. 48h infection was collected after centrifugation, and 10min viral supernatant was collected and viral amplification to P3 generation with adenovirus purification kit P3 virus. Titration of virus could be detected in the sample from the AD-rklotho virus with 3405bp, which showed that the target gene had been successfully integrated into the virus genome. (2) Animal model was created: The experimental animal was randomly divided into normal group and diabetic group, adapted to feeding in 1W. The normal group was fed with regular diet, diabetes group fed with high fat diet. After the diabetes group with 30 mg/kg dose in period of time in intraperitoneal injection, streptozotocin in diabetic model group was cultured. The normal group was only with equal volume of citric acid citrate sodium buffer solution. Animals were maintained on diet for 12 W. The diabetic SD rat model45 was divided into three groups. After being divided, model group was without treatment, while the treatment group was given a single intravenous injection of Ad-rklotho, and the control group was given an injection of Ad-EGFP. The average rat adenovirus injection quantity was 2 × 10<sup>9</sup> TU. (3) Sample collection: The experimenters continued to raise 12W, and rats were anesthetized, opened the abdominal cavity, then collected abdominal aortic blood to do biochemical detection, serum was collected for biochemical detection. Take heart, along the coronal ditch down the heart is divided into two parts, the upper part is soaked in 10% formalin solution, the lower part of frozen by liquid nitrogen, -80°C ultra low temperature refrigerator used for RT-PCR, WB detection. (4) The serum biochemical detection: using serum lipoprotein kit serum fractionation, after centrifugation and corresponding reagent mixing, the supernatant determined cholesterol. The RI 10ml RII solution, with a working fluid, stable 10min, 96 well plate added 10μL and 100μ l supernatant liquid, mixing 37 degrees after the reaction of 5min, the absorbance was measured numerical HDL microplate absorbance values 500nm, LDL520nm, HDL and LDL content. (5) Coronary section staining: rat coronary slices, whose thickness was 4 μ m, after Masson staining, collagen fiber was blue green color which indicated the outer membrane. Smooth muscle was bright red indicating membrane, and internal elastic membrane was white or colorless. Cut-off point for the film was calculated in the intima; intima media was thickness with micrometer.

### **STATISTICAL METHODS**

The data were used mean value ± standard deviation (x ± s) to mark. The application of the SPSS statistics software processing (SPSS19 Chinese version) was done to deal. Two independent samples t test was experimented data which was gotten in the test, setting p<0.05 to have statistical significance.

## RESULTS

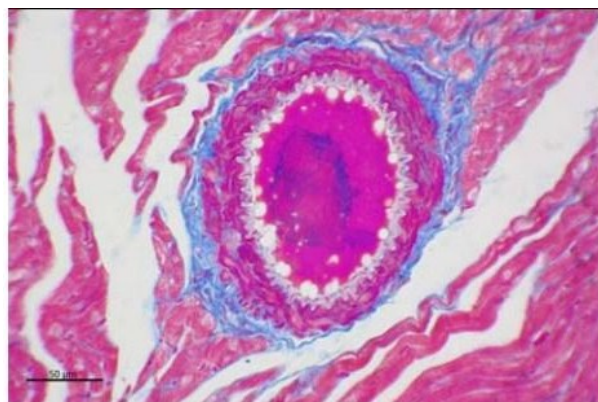
Blood glucose detection: Glucose was 5.7mmol/L in normal group, and diabetic model group was 17.3 mmol/L, control group was 16.7mmol/L, treatment group was 16.1mmol/L. Diabetic rat groups had no difference ( $p>0.05$ ), as seen in fig. 1.



**Fig. 1:** Blood glucose detection

### *Diabetic model group lipoprotein data was shown in table 1*

The content of HDL in the treatment group was higher than those in the model group and control group. After treatment group and model group two independent samples t test, the treatment group and the control group were statistically significant ( $p<0.05$ ), model group and control group had no significant difference. The normal group was  $0.67\pm 0.06$ mmol/L.



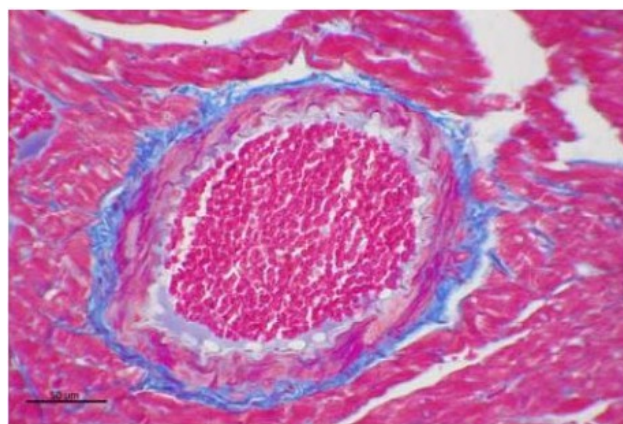
**Fig. 2:** The model group

The content of LDL in diabetes group was significantly increased. Two independent samples t test showed no significant difference between these groups ( $P>0.05$ ) ( $0.1\pm 0.01$ ) mmol/L.

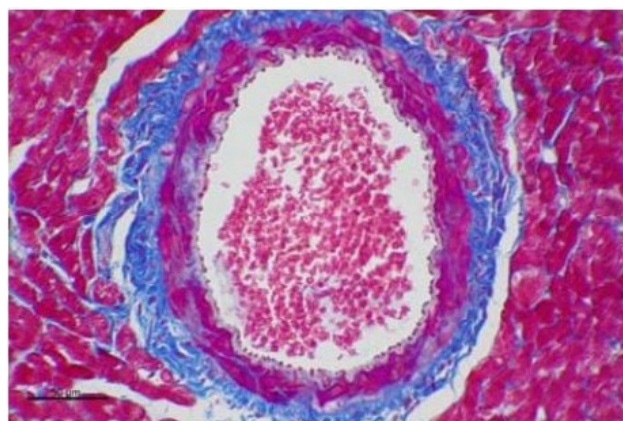
### *Coronary artery atherosclerotic plaque*

After Masson staining of collagen fibers was blue green color which indicated the outer membrane, smooth muscle was bright red indicating membrane, and internal elastic membrane was white or colorless. Cut-off points for inner membrane, diabetic rats in the experimental group were shown in fig. 2, 3, 4:

Foam cells with lipid groups of diabetic rats were observed in endothelial cells (foamy cell, FC FC), large volume, cytoplasmic vacuolization. FC mainly comes from the immigration intimal smooth muscle cells (SMC) phagocytosis lipid formation. In addition, a small amount of endometrial lymph cells was also neutrophils. The film structure becomes loose in the arteries. In the membrane of smooth muscle cells, characteristics changed. A synthetic cell type and cell migration reduced elastic fibres. No obvious pathological changed in membrane.



**Fig. 3:** The Control group



**Fig. 4:** The treatment group

### *Micrometer calculated intima-media thickness*

The treatment group was  $1.74\pm 0.05\mu$  m; model group was  $2.15\pm 0.05\mu$  m; control group was  $2.23\pm 0.06\mu$  m. The endometrial thickness of therapy group was clearer reduced than model group and control group. Endometrial thickness was significantly reduced by two independent samples t test. When treatment group and the control group were  $p<0.01$ , treatment group and model group were  $p<0.01$ , they were statistically significant; While the model group and the control group were  $p>0.05$ , there was no statistical significance. No significant difference between groups in the membrane thickness.

### *Three groups of rats intimal media thickness ratio is shown in table 2*

The normal group was  $0.238\pm 0.062$ . Treatment group was respectively in the model group and the control group

**Table 1:** Diabetic rats' lipoprotein

	The model group	The Control group	The treatment group
High density lipoprotein (mmol/L)	0.47±0.10	0.48±0.11	0.61±0.06
Low density lipoprotein (mmol/L)	0.44±0.04	0.45±0.10	0.44±0.09

**Table 2:** Groups than in the intimal thickness

	The model group	The Control group	The treatment group
Mean ± SD	0.316±0.037**	0.308±0.023	0.237±0.097

Note: compared with the normal group, model group, intima-media thickness ratio (IMT) expression were significantly different, \*\*P<0.01

undergoing t examination, and both were statistically significant (p<0.01). The results showed that the KL gene was led into the intima media thickness ratio close to the normal level, lower than that of model group and control group, and intima thickness decreased.

## DISCUSSION

Study on KL gene in recent years, it was found in the molecular level interventions such as multiple metabolic, lipid, NO, calcium and phosphorus metabolism. And the experimenters participated in the occurrence and development of coronary artery atherosclerosis (Hongqi *et al.*, 2004). In China, the incidence of coronary heart disease has been rising day by day. The KL gene got much more attention. Complications of diabetes is the most common disease, and the incidence of coronary atherosclerosis plays a role in promoting development, dome statistics, the number of diabetic patients in China has ranked first in the world (Wenyong *et al.*, 2010). This effect is caused by the disorder of lipid metabolism. The machine is the two important lipoprotein LDL. HDL is significant important indexes to be observed in the body metabolism, as seen in the following.

Degradation of LDL in plasma about 1/3 in the peripheral by phagocytic clearance, foam cell was formed, into the intima after becoming part of atherosclerotic plaque. In the presence of insulin resistance in liver cells, the secretion of LDL increased, and the sterol regulatory element protein (Srebps) expression decreased, eventually LDL receptor and lipase expression decreased (Socrates and Angela, 2008; Sudha *et al.*, 2008). Dissociate blood LDL increased hepatic glucose output and fatty acid decomposition accompanied by hyperinsulinemia. Blood free fatty acid increased.

HDL is a lipoprotein, whose platelet aggregation in endothelial cells. Smooth muscle cells (SMC) come from the middle to the inner layer down. At the same time, peroxidation enhanced cholesterol atherogenic capacity (Matthew and Prediman, 2002). HDL is mainly through the process of intervention against atherosclerosis process, which also has the transfer function to the early intimal

lower cholesterol. It can reverse the development of atherosclerosis. Insulin resistance in liver cells reduces the production of HLD that causes the liver to output LDL at the same time. Compared with the KL treatment group and control group and the model group, LDL had no statistical significance (p>0.05). High density lipoprotein (HDL) was statistically significant (p<0.01), which was higher than that of the control group and model group. Two kinds of lipoprotein in the control group and the model group showed no statistical difference (p>0.05). The level of LDL as a risk factor of coronary atherosclerosis was not due to KL gene showed significantly reduced. There were two main reasons: (1) diabetes that serum LDL levels rose, there was no inter action between diabetes status and KL gene; (2) KL gene was with polymorphism (Yasuhiko *et al.*, 2009), its expression did not have a clear role on LDL. For the second aspect, the KL gene expression had several different correlations between intensity and LDL level. But the exact role had not been studied, suggesting a protective role for KL was not on coronary atherosclerosis which was realized by the concentration of LDL effect.

In this experiment, HDL of KL gene therapy group was higher than the negative control group and model group. But compared with the normal control group, it was close to normal rats HDL level after KL introduction. Negative control group and model group HDL level of KL gene decreased below normal. Previous research indicated in the domestic population, 63% of patients with coronary atherosclerosis and low HDL (Hulin *et al.*, 2010). Therefore the KL gene can maintain the individual HDL level in coronary heart disease.

Staining in rat heart tissue after coronary artery intima media thickness, measured in light microscope, the degree of coronary artery stenosis can be judged. KL gene the treatment group compared with the negative control group and model group of intimal thickening decreased significantly, indicating that KL gene on intimal protection and its role on lipoprotein levels in rats. Confirmed that the KL gene had protective effect on coronary diabetic rats.

This research suggested that the polymorphism of KL gene has obvious correlation with coronary heart disease. KL protein has relationship with angiocardopathy and the relevant hazards such as chronic renal failure, diabetes, high uric acid, hypertension, dyslipidemia and etc.. At present, there are lots of drugs which can enhance KL gene's expression (Tang *et al.*, 2011). What's more, Chinese scholar has, for the first time, realized the recombination of natural hsKL protein. These provide basis for the application of KL protein in treatment (Ning, 2012). The knowledge about the polymorphism provides expansive prospect for pertinence treatment to particular people and even for new gene therapy. As the research of KL gene's polymorphism and KL protein's action mechanism goes more and more thorough, it will provide new method for the diagnose and treatment of angiocardopathy.

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