Journal of Animal and Veterinary Advances 11 (24): 4617-4624, 2012
ISSN: 1680-5593
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Protective Effect of Kinetin on Iso-Induced Myocardial Ischemia *in vitro* and *in vivo*

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Abstract: In this study, for the first time, researchers used kinetin, a plant hormone to treat myocardial ischemia. Researchers detected the effect of kinetin on myocardial ischemia by combining *in vitro* and *in vivo* experiments in physiological and cellular levels. After analyzing the results of kinetin on ECG, cardiac physiology forms, cultivation of rat myocardial cells, NO content, activities of myocardial and antioxidant enzymes. As the result, researchers found that kinetin protected heart from myocardial ischemia by scavenging the free radical, inhibiting the lipid peroxidation and apoptosis and maintaining the structure of myocardium, myocardial cells and vascular endothelial.

Key words: Kinetin, myocardial ischemia, antioxidation, apoptosis, *in vitro* and *in vivo*

INTRODUCTION

Myocardial ischemia is a kind of cardiovascular disease caused by an imbalance between coronary blood flow and myocardial needs and the most fundamental factor for it is hypoxia. When myocardial ischemia happens, the enhancement of mitochondrial energy metabolism will lead to the production of a large number of oxygen free radicals which are far beyond the scavenging capacity of the endogenous scavenging system (Calvillo *et al*., 2003; Kaminski *et al*., 2002; Pearl *et al*., 2001).

Kinetin (Kn), a purine compounds was found by Miller *et al*. (1955). As a plant hormone, Kn can promote plant cell division, differentiation and growth (Jianping and Yuqin, 2002). In recent years, a large number of tests have confirmed the antioxidant and other biological activities of Kn on human and animals. Rattan and Clark (1994) found the aging phenomenon in the cell morphology, the growth rate, cell volume, the cytoskeleton and the molecular synthesis were all delayed by Kn. Berge *et al*. (2006) also found that Kn can suppress the growth of human keratinocyte. In the comparative experiment of clinical local efficacy trial, Kn was found to have anti-aging effects (Chiu *et al*., 2007). Jablonska-Trypuc and Czerpak (2009) observed the effect of Kn on cell growth.

They proved Kn as an important constituent in oxidative stress system, maintains the viability of cells by allowing them to release excess free radicals. Last year, Liu *et al*. (2011) found Kn can improve antioxidant status of the cultured astrocytes and mouse brain cells and protect them against lipid peroxidation.

The causes of the myocardial ischemia and the abilities of Kn suggest that Kn may have the protective effects on the myocardial ischemia. In this study, for the first time, researchers confirm demonstrated this assumption and investigated the underlying mechanisms of Kn on myocardial ischemia.

MATERIALS AND METHODS

Kinetin, from Sigma (K3378, USA) was made into solution by 0.06 mol L^{-1} HCl.

Animals: Healthy New Zealand rabbits were 50 and evenly composed of male and female (the female rabbit was not pregnant) and weighing 2.5±0.2 kg (provided by the Experimental Animal Center of Northwest A&F University).

Reagents and instruments: SOD, GSH-pX, MDA, AST, CK, LDH and NO kit were from Nanjing JianCheng Bioengineering Institute (China); Isoprenaline (Iso) was

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from Shanghai Harvest Pharmaceutical Co., Ltd. (China). Tacho-sleep II was from Changchun Quartermaster University Veterinary Research Institute (China). DMEM was from Gibco Company (USA). Rabbit anti Bcl-2 polyclonal antibody and Goat Anti-Rabbit IgG were from Beijing Biosynthesis Biotechnology Co., Ltd. (China). Rabbit anti NF-κB polyclonal antibody was from Wuhan boster biological technology Co., Ltd. (China).

Twenty four well plates were purchased from Costar (USA). Spectrophotometer (UV-2450) was purchased from Shimadzu Co., Ltd. (Japan). Electrocardiograph (6511) was purchased from Shanghai Kohden Co., Ltd. (China). Refrigerated centrifuge (J2-MC) was purchased from Beckman Co., Ltd. (China). Automatic biochemistry analyzer (CL8000) was purchased from Shimadzu Co., Ltd. (China). Analytic balance (BS224S) was purchased from Sartorius Instrument Systems Co., Ltd. (China). AO histotome was purchased from Warner-Lambert Tech. Inc. (USA). Normal microscope (BX1) was purchased from Olympus Co., Ltd. (Japan). Transmission electron microscope: H-600 purchased from Hitachi, Ltd. (Japan). Thermostat water bath: HH-6, purchased from Guohua Electric Appliance Co., Ltd. (China).

Experimental methods

Mice models: They were randomly divided into 5 groups: the control group, the model group, Kn low dose group, Kn middle dose group and Kn high dose group. According to the pre-test results, the control and the model group were intragastric administration (ig) 0.06 mol L^{-1} HCl, simultaneously, Kn low dose group ig Kn 20 mg kg^{-1}, Kn middle dose group ig Kn 40 mg kg^{-1} and Kn high dose group ig Kn 80 mg kg^{-1} to intervene respectively at the dosage of 20 mL kg^{-1} to rabbit twice a day for 14 days. The model group, Kn low dose group, Kn middle dose group and Kn high dose group were made into myocardial ischemia model by intraperitoneal injection (ip) Iso at the dosage of 10 mL kg^{-1} twice a day after 2 h of the last ig and there was 12 h between two ip. The model was improved from Xu *et al.* (1982). The control group was ip isovolumetric saline at the same time.

Cell culture: Myocardial cells from Sprague-Dawley (SD) neonatal rat were cultured *in vitro* by using the method improved from Zhang (2002). The myocardial cells were inoculated into 24-well plates and adjusted the density to 5×10^5 mL^{-1}. The myocardial cells were cultured in DMEM containing 100 g L^{-1} fetal bovine serum, 100 000 U L^{-1} penicillin and 100 mg L^{-1} streptomycin in the carbon dioxide incubator of 5% CO_2, 37°C for 72 h. Unicellular suspension was dropped on the coverslip for HE staining and immunohistochemical staining.

After a 72 h culture, the myocardial cells were divided into five groups: the control group, the model group, Kn low dose group, Kn middle dose group and Kn high dose group. Each group was separately treated with: none, 100 mg L^{-1} Iso, 100 mg L^{-1} Iso + 10 mg L^{-1} Kn, 100 mg L^{-1} Iso + 20 mg L^{-1} Kn and 100 mg L^{-1} Iso + 40 mg L^{-1} Kn, respectively. All groups were then cultured for another 24 h.

Cell density and cell survival rate assay: Cultured myocardial cells were observed by HE staining. The cell suspension of each group was prepared to count by the inverted microscope and then calculate cell density. Trypan blue staining was employed to determine the death of myocardial cells:

$$\text{The cell survival rate} = \frac{\text{The total number of the cells} - \text{The number of the stained cells}}{\text{The total number of the cells}} \times 100\%$$

Electrocardiogram (ECG) assay: In the myocardial ischemia rabbits model, the model group, Kn low dose group, Kn middle dose group and Kn high dose group were intramuscular injected tacho-sleep II (0.1 mL kg^{-1}) after the second time of ip Iso. The rabbits in anesthetic were bound to dissecting table with an overhead position. The needle electrodes were inset in limbs of the rabbits and the electrodes were connecting to the electrocardiograph to record II lead electrocardiogram of limb. Researchers set standard voltage in 1 mV = 20 mm, chart speed in 50 mm sec^{-1}; simultaneously, the same test was done on control group after the second saline injection. The heart rate and the change of the J point were observed.

Biochemical indicators assay: After 24 h of the earlier administration, all rabbits of each group were killed by exsanguination respectfully. Blood was collected and isolated serum. The hearts were taken out rapidly to make homogenate. The suspension was homogenized for 10~15 min and centrifuging at 14000 ram at 4°C for 15 min. SOD, GSH-pX, MDA and NO of the serum, SOD, GSH-pX, MDA, AST, CK and LDH of myocardium and myocardial cells culture medium of each group were assayed according to the instructions of kits, respectively.

Pathological histology: A part of the ventricular myocardium of each group rabbits were taken and put into 4% paraformaldehyde then were made into paraffin sections and observed by HE staining. The heart tissues of each group were cut into pieces of 70 nm thick and observed by the Transmission Electron Microscope (TEM).

Statistical analysis: Statistical analyses were conducted using the Statistical Package for Social Science (SPSS) Version 19.0 (SPSS Inc., IL, USA). Differences among groups were evaluated by one-way ANOVA. Values were expressed as means±Standard Deviation (X±SD).

RESULTS AND DISCUSSION

Effect of Kn on the ECG: Compared with the control group, ECG of the model group was manifested by tachycardia, j-point rise, t wave from positive turn to negative or positive and negative two-way. After given Kn, ECG of myocardial ischemia rabbits was improved and the improvement showed a dose effect relationship.

It can be seen from Fig. 1, after 5 and 30 min of ip Iso, the heart rate of the model group was faster than that of the control group significantly ($p<0.01$). After 5 min of ip Iso, compared with the model group, the rabbits' heart rates of the Kn each dose group have varying degrees of slowdown (Fig. 2). Kn middle dose group and the model group were significantly different ($p<0.05$). After 30 min ip Iso, compared with the model group, the heart rate of Kn low dose group was slower significantly ($p<0.01$).

Compared with the control group, J point of the model group was significantly higher ($p<0.01$). J point rise was subject to varying degrees of inhibition by given different doses of Kn. After ip Iso for 5 min, compared with the model group, J point of Kn each dose group was significantly reduced ($p<0.01$ or $p<0.05$). After ip Iso for 30 min, compared with the model group, J point of Kn high dose group was significantly reduced ($p<0.01$) (Fig. 3).

Myocardial ischemia can change the excitability, the conductivity and the self-discipline of myocardium. These register as ECG abnormalities on the whole. In this experiment with the continuous high dose ip Iso, the rabbits' heart rate significantly speeded up and J point significantly heightened. After given Kn, the heart rate of the rabbit slowed down, the ups and downs of J-point were lessened. It showed Kn obviously improved the excitability, the conductivity and the self-discipline of myocardium which were influenced by myocardial ischemia.

Effect of Kn on histopathology and ultrastructure of myocardium: To observe the pathology with HE staining, a great degree of the myocardium pathological have changed in the model group: the cytoplasm of the focal myocardial fibroblasts were acidophilic stain; the wide-bound cardiac muscle fibers were swelling, broken and lost; the interstitial edema was occurred with massive infiltration of the inflammatory cell; the portion of the capillaries were hemorrhaging and the thrombus appeared. In Kn low dose group: the cytoplasm of the focal myocardial fibroblasts were acidophilic stain; parts of the cardiac muscle fibers were swelling and broken; the interstitial edema was occurred with lots of infiltration of the inflammatory cell. In Kn middle dose group: less acidophilic stain of the focal myocardial fibroblasts cytoplasm than the model group; a little cardiac muscle fibers were swelling and broken; the interstitial edema was occurred with a little infiltration of the inflammatory cell. In Kn high dose group: the level of pathological damage was abated obviously compared with the model group and just had a little infiltration of the inflammatory cell (Fig. 4).

Myocardium of the rabbits was observed by TEM. In the control group: the sarcomeres were neat and isometric; myofilament arranged orderly; Z line, I and A bands were clear; the mitochondrion was compact and tidy arrangements along with the myofilament axis; the mitochondrial membrane was intact and clear; crest

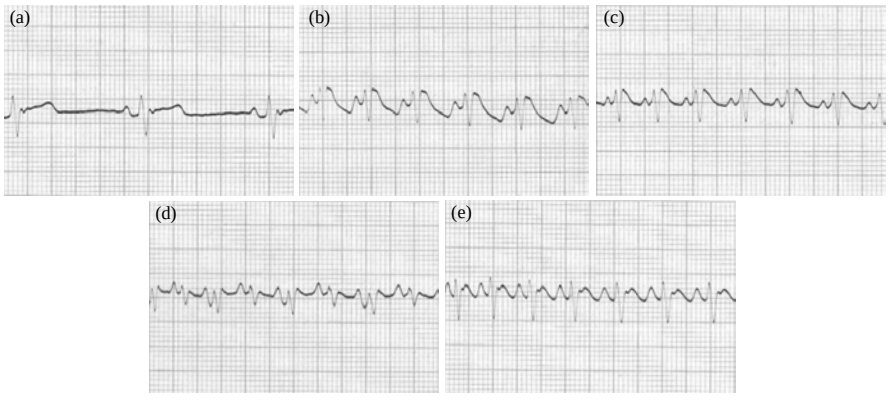

Fig. 1: Effect of Kn on ECG; a) ECG of the control group; b) ECG of the model group; c) ECG of Kn low dose group; d) ECG of Kn middle dose group and e) ECG of Kn high dose group

arranges orderly. In model group: the sarcomeres were contracture and varying length; Z lines were thick and blurry; I and A bands were not clear; the myofilaments were loose, arranged disorderly and dissolve; the mitochondrials were disorderly; the mitochondria were swelling; some of the mitochondria membranes had local damage; the cristae were broken resulting in the formation of the large vacuoles or cavities in mitochondria. In Kn low dose group: the sarcomeres were fuzzy and different in length; Z line, I and A bands were fuzzy; the myofilaments were arranged a little irregularly; some of the myofilaments were loose, broken or dissolved; the mitochondria arranged loosely along with the myofilament axis; the mitochondrial membranes were relatively complete; some of the mitochondria were swelling; part of the cristae fractured and the hollow region of cristae appeared. In Kn middle dose group: sarcomeres were fuzzy and different in length; Z lines was mildly fuzzy; I and A bands were relatively clear; myofilaments were arranged in neat rows; mitochondrion were arranged compactly and tidily along with the myofilament axis and a hollow region appeared in the individual mitochondrion. In Kn high dose group: sarcomeres were neat and isometric; Z line, I and A bands were clear; the myofilament was arranged orderly; the mitochondrion were arranged compactly and tidily along with the myofilament axis; the mitochondria membrane was relatively complete; a small part of the mitochondria swelled and most of the crest was arranged orderly but a few were fractured (Fig. 5).

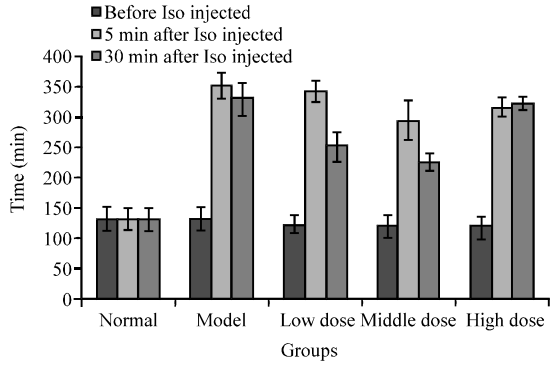

Fig. 2: Effect of Kn on heart rates of the myocardial ischemia rabbit ($\bar{X}$±SD)

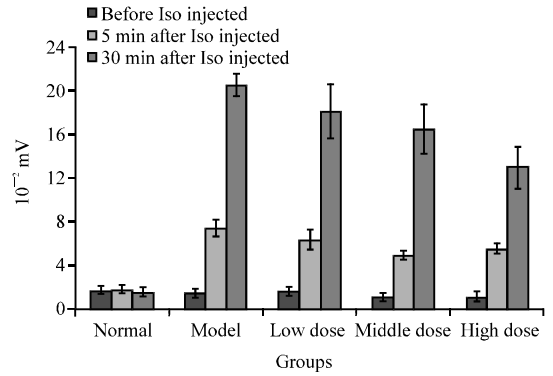

Fig. 3: Effect of Kn on J spot of the myocardial ischemia rabbit ($\bar{X}$±SD)

The integrity of the cell structure and cell organ were the basic guarantee of vital movement. When myocardial ischemia damaged it, the related diseases appeared in consequence. The result showed, Kn can inhibit the changes in microstructure and ultrastructure levels which were led by myocardial ischemia, maintained the integrity of cells and cell organelles.

Effect of Kn on the form of cells, cell density and cell survival rate *in vitro*: In the control group, the myocardial cells showed a fibroblast-like growth *in vitro* culture

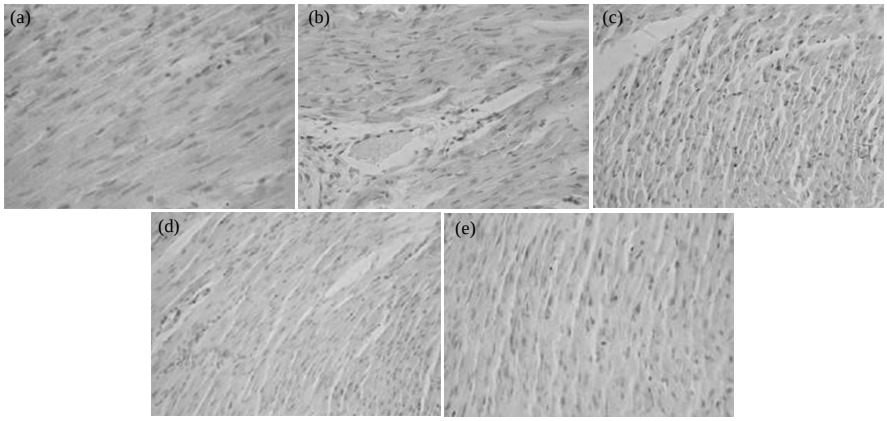

Fig. 4: Effect of Kn on microstructure of myocardium; a) Microstructure of the control group (x200); b) Microstructure of the model group (x200); c) Microstructure of Kn low dose group (x200); d) Microstructure of Kn middle dose group (x200) and e) Microstructure of Kn high dose group (x200)

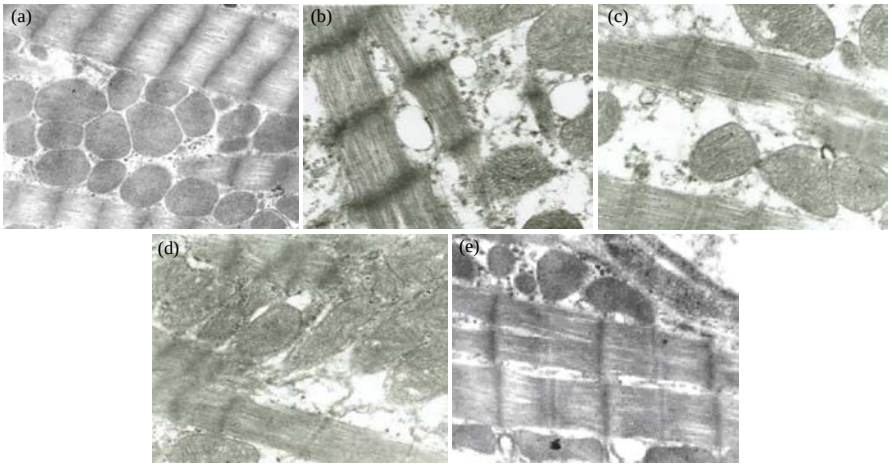

Fig. 5: Effect of Kn on ultrastructure of myocardium; a) Ultrastructure of the control group (x17,000); b) Ultrastructure of model group (x17,000); c) Ultrastructure of Kn low dose group (x17,000); d) Ultrastructure of Kn middle dose group (x17,000) and e) Ultrastructure of Kn high dose group (x17,000)

conditions. Attached to the bottom of the culture vessel, the cells were spindle-shaped, irregular triangular or fan-shaped; the nucleus was orbicular-ovate and was located in the center of the cytoplasm. Some of the cells were single growth and the cytoplasm was pseudopodia-like. Others experienced intensive growth and arranged in a spiral shape, rediatiform or palisade. Added Iso 24 h later, the cells refractivity of the model group decreased significantly, the cell were swelling. The pseudopods decreased and became round. A few cells dropped off the culture flask and died. The connections between cells reduced and the intracytoplasmic particles increased. The cell connections and the adherent cells of Kn each dose group increased significantly than that of the model group and showed a dose effect relationship (Fig. 6).

The cell density and the cell survival rate of the control group were 91.2±4.0% and (40.5±3.6)×10^4 mL^{-1} separately and that of the model groups were 58.5±7.3% and (18.8±2.0)×10^4 mL^{-1} separately. Compared with the model group, Kn each groups all had varying degrees of increase in the cell density and the cell survival probability, Kn middle and high dose groups increased significantly ($p<0.01$).

The effect of Kn on serum NO content of Iso-induced myocardial ischemia rabbits: NO plays an important role in resisting myocardial ischemia injury and in recovery of myocardial function (Fig. 7). It can effectively maintain coronary blood flow and inhibit vascular smooth muscle cell proliferation and prevent thrombosis so it has a regulatory role on coronary vasomotion (Yang, 2006).

The result showed that the serum NO content of the model group was significantly lower than that of the control group ($p<0.01$). The serum NO content of the middle dose group and Kn high dose group was significantly higher than that in the model group ($p<0.05$ and $p<0.01$) (Fig. 8). This suggested Kn plays a protective role on vascular endothelial by improving the content of NO which could be one of the protections mechanisms of Kn on myocardial ischemia.

Effect of Kn on SOD, GSH-pX and MDA: The myocardial SOD activity of the model group was significantly lower than that of the control group ($p<0.01$). Compared with the model group, the myocardial SOD activities of Kn each groups all had varying degrees increase and Kn middle and high dose groups were increased significantly ($p<0.05$ and $p<0.01$).

The serum SOD activity of the model group was significantly lower than that of the control group ($p<0.01$). Compared with the model group, the serum SOD activities increased significantly of Kn low dose group ($p<0.05$), Kn middle dose group ($p<0.01$) and Kn high dose group ($p<0.01$). In the cell culture medium, compared with the control group, the SOD activity of the model group decreased significantly ($p<0.01$). Compared with the model group, the SOD activities of Kn middle and high dose groups increased significantly ($p<0.01$).

The myocardial GSH-pX activity of model group was significantly lower than that of the control group ($p<0.01$). Compared with the model group, the myocardial GSH-pX activities of Kn middle and high dose groups increased significantly ($p<0.05$ and $p<0.01$). The serum GSH-pX activity in the model group was significantly lower than that of the control group ($p<0.01$). The serum GSH-pX activities of Kn middle and high dose groups were significantly higher than that of the control group ($p<0.01$).

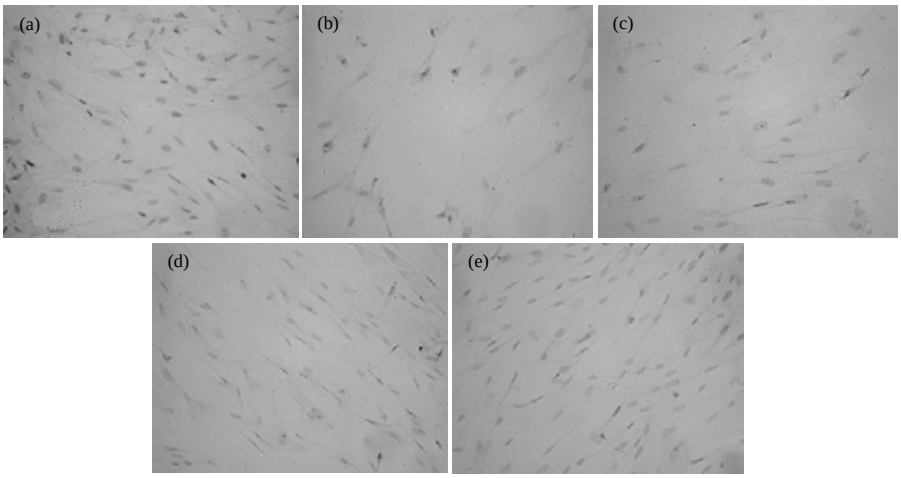

Fig. 6: Effect of Kn on cell culture; a) The control group (x200); b) The model group (x200); c) Kn low dose group (x200); d) Kn middle dose group (x200) and e) Kn high dose group (x200)

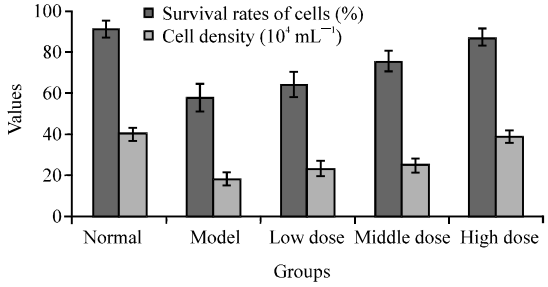

Fig. 7: Effect of Kn on cell density and cell survival rate of cells ($\bar{X}$±SD)

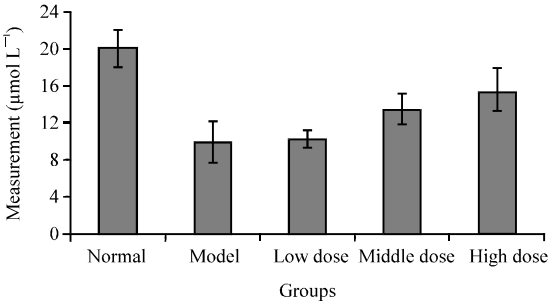

Fig. 8: Effect of Kn on serum NO content of Iso-induced myocardial ischemia rabbits ($\bar{X}$±SD)

In the cell culture medium, the GSH-pX activity of the model group was significantly lower than that of the control group ($p<0.01$). Compared with the model group, the GSH-pX activities of the Kn low, middle and high dose groups were increased significantly ($p<0.05$, $p<0.05$ and $p<0.01$).

Compared with the control group, the content of myocardial MDA in the model group were increased significantly ($p<0.01$). Compared with the model group, the contents of the myocardial MDA in Kn middle and high dose groups lowered significantly ($p<0.01$). The serum MDA content of the model group was significantly higher than that of the control group ($p<0.01$). Compared with the model group, the serum MDA content of Kn middle and high dose group lowered significantly ($p<0.01$).

In the cell culture medium, compared with the control group, the MDA content of the model group was increased significantly ($p<0.01$). Compare with the model group, MDA content of Kn high dose group significantly decreased ($p<0.05$). When myocardial ischemia happens, the aerobic metabolism gets disordered. It leads to the lack of energy of myocardium and then the explosive of the oxygen free radicals generates (Huang *et al*., 1997). The free radical damage is considered to be the main mechanisms of myocardial ischemia injury. The main toxic effects of free radicals generated in the myocardial ischemia are biofilm damage. Researchers suggest that free radicals can start a chain reaction. It is so easy to react with a variety of unsaturated fatty acids and cholesterol response on the cell membrane. Such a direct oxidative damage on cells will lead to apoptosis (Chu *et al*., 2003), membrane lipid peroxidation and myocardial cell membrane structural changes. Finally, myocardial was damaged.

The level of SOD's vitality indirectly shows the free radical scavenging ability of the body (Gauduel and Duvelleroy, 1984). GSH-pX specifically catalyzes the reduction reaction of glutathione of the hydrogen peroxide to eliminate intracellular metabolites and block the lipid peroxidation chain reaction so that to protect the cell metabolism. MDA can reflect the degree of the free

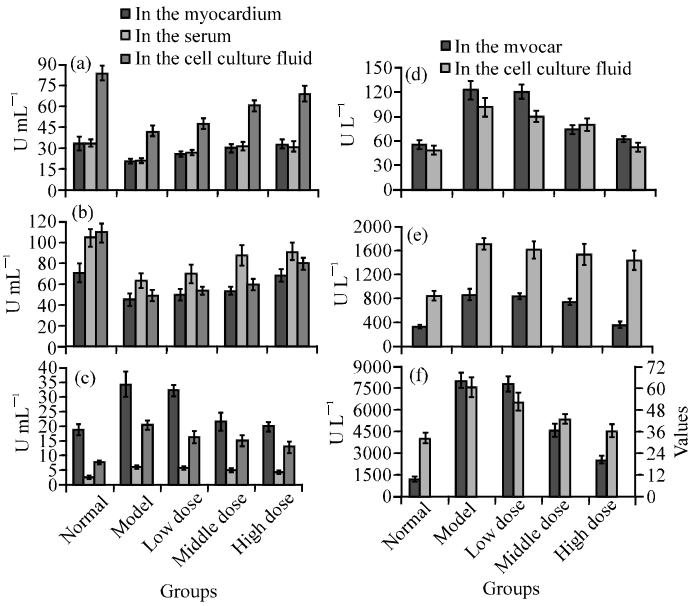

Fig. 9: Effect of Kn on SOD, GSH-pX, MDA, AST, LDH and CK; a) Effect of Kn on SOD ($\bar{X}$±SD); b) Effect of Kn on GSH-pX ($\bar{X}$±SD); c) Effect of Kn on MDA ($\bar{X}$±SD); d) Effect of Kn on AST ($\bar{X}$±SD); e) Effect of Kn on LDH ($\bar{X}$±SD); f) Effect of Kn on CK ($\bar{X}$±SD)

radical damage on the body and the cell. Therefore, the free radical damage on the body can assess by the detection of the activities of SOD and GSH-pX and the content of MDA (Fig. 9).

In the model group, the result hinted that a lot of free radicals were formed in the process of myocardial ischemia injury. This is consistent with earlier studies (Zheng *et al*., 2003). Also, in the cell culture medium, the positive inotropic effect of Iso on the myocardium increased the consumption of the myocardial oxygen and the membrane permeability. As a result, myocardial cells were in a ischemic state (Jin *et al*., 1995).

In this experiment, the activities of SOD and GSH-pX were significantly reduced and the content of MDA was significantly increased in the myocardium and the serum of iso-induced myocardial ischemia model. The same condition also appeared in Iso-induced myocardial ischemia injury cells of the SD neonatal rat *in vitro*. The results showed that the protection of Kn associated with its two abilities: reducing the lipid oxidation products and enhancing the vitality of the antioxidant enzymes.

Effect of Kn on CK, AST and LDH: The results showed that the enzyme activities of Creatine Kinase (CK), the Aspartate aminotransferase (AST) and the Lactate Dehydrogenase (LDH) in the model groups were significantly higher than those of the control group in serum ($p<0.01$) and the r ise of three enzyme activities were all inhibited by given different doses of Kn. Compared with the model group, three enzyme activities of Kn high dose group were all significantly lower ($p<0.01$).

In the cell culture medium, the similar tendency was showed. The enzyme activities of CK, AST and LDH of the model group were significantly higher than those of the control group ($p<0.01$). Compared with the model group, the activities of three enzymes of Kn each dose group has varying degrees of decrease: In Kn low dose group, CK activity decreased significantly ($p<0.05$): in Kn middle dose group AST and CK activity decreased significantly ($p<0.01$ and $p<0.05$). In Kn high dose group, the activities of AST, CK and LDH decreased significantly ($p<0.01$, $p<0.01$ and $p<0.05$).

CK, AST and LDH are often clinically considered as a diagnostic marker of myocardial injury. The increase in CK activity is considered to be one of the most sensitive indicators of the myocardial cell injury. AST, an aminotransferase, plays an important role in the metabolism of amino acids. The activity of LDH can be used to reflect the extent of cell membrane damage.

When myocardial ischemia happens, the myocardial cell metabolism is disordered so the harmful products cannot be cleared. It will lead to the changes in physiological and biochemical. One of the changes is the activities increases of CK, AST and LDH. This is caused by the changes in membrane permeability or the membrane destruction which lead to a large number of these three enzymes releasing into the blood. However, to

maintain the integrity of the myocardial cell membrane is the premise of cell survival. When cell membrane is destructive or the permeability of the cell increases, the cell will rupture and die. So, the activities increases of CK, AST and LDH can be used as the criteria of cell necrosis.

The results showed that the increase of three enzyme's activities in the model were inhibited by Kn. This indicates that Kn can reduce the damage to cell membranes in myocardial injury, maintaining the integrity of the cell membrane, reducing the release of intracellular enzymes and maintaining the normal function of the myocardial cells.

CONCLUSION

In this study, *in vivo* and *in vitro* tests were carried out to measure the protective effect and discussed the mechanism of Kn on myocardial ischemia. The results showed that Kn can protect the heart from myocardial ischemia by scavenging the free radical inhibiting the lipid peroxidation and apoptosis and maintaining the structure of myocardium, myocardial cells and vascular endothelial.

ACKNOWLEDGEMENT

This study was supported by National Natural Science Foundation of China (No. 31101779).

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