Study of pentoxifylline drug effect on Bax gene expression changes in kidney after ischemic/reperfusion in rat

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ABSTRACT

Ischemia Reperfusion injury is the tissue damage caused when blood supply returns to the tissue after a period of ischemia or lack of oxygen. Ischemia Reperfusion induces cell death and endemic reaction that is one of the most important clinical problems with acute renal failure and renal transplantation. In this study, the effect of pentoxifylline on rat kidney function and cell injury following Ischemia Reperfusion were evaluated. In this experimental study, 20 male wistar rats with average weight of 250-300g were selected and then were accidently divided them on two tenth group of control and treatment groups. In the control group, celiotomy was performed by ventral midline incision. The left kidney was isolated, and then both the renal artery and vein were obstructed. After 60 minutes of warm ischemia, vessel obstruction resolved and the right kidney was removed. 72 hours after reperfusion, tissue samples were taken from left kidney for histopathology. All these steps in treatment group were exactly repeated after administration of 45 mg/kg/PO pentoxifylline (3 hours before operation) and in this group treatment was continued every 12h until 3 days. In this research quantitative real-time PCR is used for the detection expression Bax gene in ischemia group and PNT drug group and compared to normal sample .The results showed the gene dosage ratio of 1.24 for ischemia groups and 0.64 for drug group. The results showed the expression Bax gene in PNT group decline than to ischemia group. Therefore, quantitative real time PCR could be used as a direct method for detection of Bax gene expression in tested and normal samples

Keywords: Ischimia; reperfusion; apoptosis; Bax gene; Real-Time PCR

INTRODUCTION

Ischemia-Reperfusion is a kind of complex clinical syndrome that in particular situation it might impress different body's organs, as it is apparent from its name it has one level of reduction or obstruction of perfusion to the tissue and after a while reperfusion to that organ [1].If the cells involved in the reversal injury, reperfusion can improve the cells. However, in certain conditions blood recirculation to the ischemia tissues which are otherwise healthy repugnantly causes aggravation and worse damages in them. Consequently, In addition to the cells that were irreversibly damaged until the end of ischemia, the other cells already destroy in tissues. This state that called the ischemia-reperfusion injury, it's an important clinical process that has a significance role in tissues destruction but it can be controlled with medical interventions [2,3]. Although, the exact mechanisms of injury are not known, one of the causes of injury (ischemia- reperfusion) stated as follow. Further blood perfusion in ischemia-reperfusion resulted in exacerbation locally absorption of inflammatory cells. These cells release large amounts of oxygen-derived active radicals and promote the membrane destruction process and mitochondrial permeability. Increased permeability of mitochondria and formation of holes in mitochondrial membrane decrease the membrane potential, adenosine triphosphate

production and swelling of mitochondria. Increase the permeability of mitochondrial outer membranes causes the releasing initiator inducer of apoptosis, the C cytochrome, into the cell cytosol. This process continued and moves on the proteolytic events and induces the apoptosis [4]. Ischemic acute renal failure is a clinical syndrome that occurs following obstruction or reduction in renal blood flow. Despite preventive measures and treatment of this disease is associated with high mortality [5]. Tissue damage begins from the same ischemic phase. Necrosis and apoptosis are two forms of cell injury. In necrosis, the cell elements come out of it and damage adjacent tissues. During apoptosis that is a programmable cell death, nucleus elements and cytoplasm are located within the vacuoles and then swallowed by macrophages without damaging to surrounding tissues [6, 7]. Reperfusion flowing to the initial phase of ischemia generates new type of injury to the body. According to indicated reason, these two sets together are called Ischemic Reperfusion (IR) [7]. New findings in this field emphasize the important role in ischemia reperfusion injury (1, 2). Several edematous mediators are released in the tissue which necrosis factor alpha (TNF-α), interleukin 1 and interleukin 6 are the most important one. The mediators generate and spread inflammatory phase in reperfusion. By increasing number of connection molecules (ICAM-1) on the surface of endothelial cells, the multi-nuclear cells are being entered into ischemic tissue. The entrance of multi-nuclear inflammatory cells is associated with the production of myeloperoxidase enzyme and then combination of nitric oxide with superoxide radicals and production of nitrite proxy individually causes the addition of oxidative stress to the inflammatory process that extend the range of ischemic injury and this reflects the pivotal role of inflammation in injury following ischemia reperfusion [8, 9]. In this study, the role of pentoxifylline drug on Bax gene expression changes in renal cells was examined.

MATERIALS AND METHODS

Animals

In this experimental study, 20 adult wistar rats were selected and healthily function of renal of these animals was determined by measuring creatinine and blood urea nitrogen. Rats were randomly allocated into two control group (n=10) and treatment with pantoxyfylline group (n=10). In control group,for inducing anesthesia about 12 mg/kg Ketamine intraperitoneal by injection method was used. Celiotomy was performed by ventral midline incision the left kidney was isolated, and then both the renal artery and vein were obstructed. After 60 minutes ischemia, the vessels opened and reperfusion was performed. Then the right kidney was removed by using the nephrectomy. The time of blood reperfusion in this study was 3 days. After surgery, the animals were allowed to have free access to water. In treatment group with pantoxyfylline, all the actions above were done except t that this group had used pantoxyfylline dose of 45mg/kg orally 3 hours before surgery. This treatment was continued once every 12 hours for 3 days. For microscopic studies, at the end of experiment and 72 hours after reperfusion, left kidney of rats separated by dislocation in neck vertebra.

RNA isolation ,DNA digestion and reverse transcription

Tissue samples were treated with TRI Reagent (SIGMA-Aldrich) as recommended by the manufacturer. The extracted RNA was further purified using RNeasy mini kit (Qiagen)The concentration and purity of the purified RNA were determined by spectrophotometry. High quality RNAs (A260/280≥1.8) were selected and kept at −80 °C until used for cDNA synthesis. Up to 1 μg RNA was converted to cDNA using Quantitect® reverse transcription kit (Qiagen) according to the manufacturer's instruction. To verify the integrity of the cDNA, a PCR experiment was performed using GAPDH

specific primer. The primer for quantitation PCR of BCL2and GAPDH genes expression were designed by the Primer Express v.3.0 software (Applied Biosystems, Foster City, USA).

Real-time PCR with SYBR green I

The selected primers underwent an extensive search using BLAST tool (www.ncbi.nlm.nih.gov/blast) .The characteristics of the primers used in this study are summarized in Table 1.

Real-time PCR was carried out in optical grade 96-well plates (Micro amp, Applied Bio systems, Singapore) at reaction volume of 25 macro Liter including SYBR Green Master mix(primer design12/5)300nM primer and 5ng genomic template DNA. All samples were run in duplicate Thermal cycling was performed on the Applied Bio systems 7300 real-time PCR system using the following cycling conditions: 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each complete amplification stage was followed by a dissociation stage; at 95 °C for 15 s, 60 °C for 30 s, then temperature was ramped up from 60 °C to 95 °C (at the rate of 0.03 °C/s), and fluorescence intensity data was collected continuously over the ramping stage for 20 min. Melting curve analysis was performed according to the dissociation stage data and reactions with a single peak at expected Tm were considered for further analysis.

Data analysis

Quantitative analysis was performed by the measurement of CTvalues during the exponential phase of amplification. The Ct parameter was defined as the cycle number at which the amplification plot passed a fixed threshold. In each assay, m Ct was the mean Ct value of duplicate amplifications. Relative quantity of Bax gene was determined using comparative Ct method and Δ Ct was calculated as the difference between the Ct values of theBcl2 and the Ct value of GAPDH gene. The data were analyzed using the formula: Gene dosage ratio=2−ΔΔCt, where −ΔΔCt = [m Ct Bax(normal sample)−m Ct GAPDH gene (normal sample)]−[m Ct Bax (test sample)–m Ct GAPDH gene (test sampl)(29). gene dosage ratios were determined relative to the mean Δ Ct value of these samples Data processing was performed using the ABI Prism 7300Sequence Detection System and the SDS software Ver. 1.2.3(Applied Bio systems, UK). Statistical analysis and graph preparation were performed using Microsoft® Excel 2007 an d RJS Graph 3.90.10 software.[10]

Table1. Characteristics of the primers used in the real-time PCR assay

Gene	Sequence	(Tm)
rat-BAX-F	AGGGTGGCTGGGAAGGC	56.8
rat-BAX-R	TGAGCGAGGCGGTGAGG	57.7
rat-GAPDH-F	AAGTTCAACGGCACAGTCAAGG	57.8
rat-GAPDH-R	CATACTCAGCACCAGCATCACC	56.7

RESULTS

pentoxifylline drug effect on Bax gene expression changes in kidney after ischemic/reperfusion in wistar rat were tested by multiplex PCR . pentoxifylline drug effect on Bax gene expression changes in hippocampus after ischemic/reperfusion was campared with ischemia group . Using this method tested sample and normal sample were analyzed. As expected, there was significant difference between the tested and normal samples in multiplex PCR .Optimization and validation of real-time PCR assays (Figure1) . The gene dosage ratios obtained in each group were as follows:

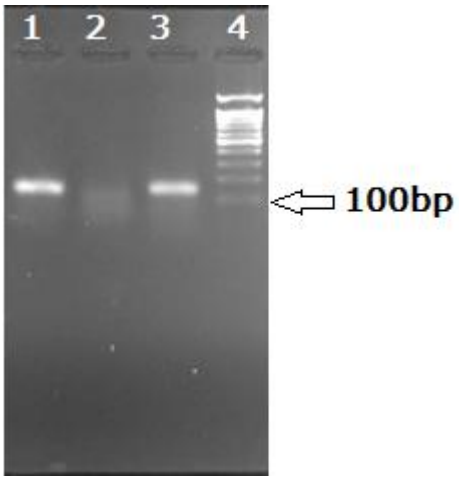

Figure 1. Results of Real-time PCR analysis for expression of *Bax* and GAPDH genes.
1,Bax gene
2,NTC, non-template control.
3.GAPDH gene
4.M, DNA Size marker

the ischemia group of bcl2 gene expression showed the ratios of 1.24.group drug of the Bax gene expression experiments had the ratios of 0.64 ($p<0.01$).

DISCUSSION

Apoptosis, or programmed cell death, which can be distinguished on the basis of morphologic and biochemical criteria,is different from necrosis (11–13). The mechanism responsible for post-I/R apoptosis is attributed to the increasing activity of endonuclease by elevation of calcium entry into cells (14), or the release of reactive oxygen species (ROS) (14 –16).

ROS induce apoptosis by causing DNA damage, oxidation of lipid membranes, and/or direct activation and expression of the genes/proteins responsible for apoptosis (17). For example, increases in the Bax/Bcl-2 ratio (18), expression of caspase and its activity (19,20), and caspase-mediated cleavage of poly- (ADP-ribose)-polymerase (PARP) (21) have been found in organs subjected to I/R injury or in cells after a cytotoxic insult.

The sources of ROS generated after I/R may be circulatory macrophages/neutrophils (22) or the resident cells (23).

In the kidney, demonstration of increased renal venous ROS after I/R by electron spin resonance and the efficacy of antioxidants or free-radical scavengers in minimizing I/R injury (14,16) suggest a role of ROS in I/R injury. However, the precise origin of ROS in the I/R kidney has yet to be defined.

The inflammatory response partially mediates the damage of reperfusion injury. As pentoxifylline is reducing inflammatory processes; therefore, it directly reduces the production of growth factors produced from leukocytes in the inflammatory process that the higher expression of FGF & IGF In the process of ischemia-reperfusion of other pathways causes cell death. FGF from MAPK way and ERK cause the activation of Bax pathway and formation of mitochondrial channels and by facilitating the exit of C cytochrome, induces cell death. IGF o through PI3K also causes the activation of AKT/PKB and the function of Bax protein and BCL2 .Therefore, the pathway of cell death is going to be started, by forming mitochondrial membrane channel and exiting of C cytochrome.

The role of Fas ligand in the induction of cell death after reperfusion should not be forgotten which exert its cell death effect through FADD pathway and caspases 1, 3, 4, 6, 7&8. Because all the agents that been mentioned are effective in cell death of renal tubules, it's clear that the effects of pentoxifylline on cellular and vascular events, edematous process, inhibition of banding molecules, inhibition of inflammatory cells out, reduction of edematous mediators' production, cytokines (IL-α, TNF-α , IL-8 , IL-6 ,IL-1β,I) and growth factors can be effective in reducing and inhibiting cell death in the treatment group [24].

CONCLUSION

In this study we have designed and optimized quantitative real-time PCR assays based on SYBR Green I chemistry for determining the PTX drug effect on Bax gene expression changes in kidney after ischemic /reperfusion in rat.

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