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Expression of cyclooxygenase-2 in the kidney of fetal rats following intrauterine distress

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Abstract: Objective To examine the mRNA and protein expressions of cyclooxygenase-2 (COX-2) and the kinetic changes of three metabolites (PGI_2, PGE_2 and TXA_2) of COX-2 in kidney tissues of fetal rats after intrauterine distress (ID), and to explore the possible roles of COX-2 in the pathogenesis of kidney impairment of fetal rats after ID. **Methods** A model of intrauterine ischemia and hypoxia of fetal rats was made by occluding one side of vessels supplying two horn uteri. The fetal rats in the other side were used as Sham-operation group ($n = 22$). After 30 minutes of blood occlusion, reperfusion started. Pups were removed 0, 0.5, 2, 6, 12, 24 and 30 hrs post-reperfusion respectively ($n = 20$ for each time point). After the renal tissues of fetal rats were homogenized, RT-PCR, Western blotting and radioimmunoassay methods were used to detect the mRNA and protein expressions of COX-2 and the kinetic changes of PGI_2, PGE_2 and TXA_2 concentrations. At the same time, hematoxylin-eosin staining was used to observe the histopathological changes of the kidney. **Results** After intrauterine ischemia, hypoxia and reperfusion, the expressions of COX-2 protein and gene in fetal rat kidney were significantly up-regulated. Compared with the Sham-operation group, the concentrations of 6-*keto*-$PGF_{1\alpha}$ and PGE_2 began to increase following 2 hrs of reperfusion, and reached a peak 12 hrs and 24 hrs after reperfusion respectively. The TXB_2 concentration in the Ischemia-reperfusion group was not different from the Sham-operation group at any reperfusion time point. **Conclusions** Intrauterine ischemia, hypoxia and reperfusion can induce the up-regulation of COX-2 expression in fetal kidney at the transcriptional level. COX-2 may play a protective role in ischemic impairment of fetal kidney through PGI_2 and PGE_2. It is suggested that COX-2 inhibitors should not be used for kidney impairment patients during the perinatal period. [**Chin J Contemp Pediatr, 2005, 7(1): 15-19**]

Key words: Fetal distress; Cyclooxygenase; Dinoprostone; Epoprostenol; Kidney; Fetal rat

宫内窘迫胎鼠肾组织环氧化酶-2 表达的实验研究

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[**摘 要**] **目的** 缺血缺氧性肾损伤是一个复杂的病理生理过程,炎症反应在其中占有重要的作用。该文旨在观察宫内窘迫后胎鼠肾组织炎症介质环氧化酶-2(COX-2)蛋白和基因表达及其代谢产物前列环素 I_2(PGI_2),前列腺素 E_2(PGE_2)和血栓素(TXA_2)的动态变化,初步探讨 COX-2 在宫内窘迫胎鼠肾损伤发病机制中的作用。**方法** 制备胎鼠宫内缺血缺氧再灌注模型(缺血缺氧组:缺血缺氧 30 min;再灌注组:缺血缺氧 30 min 后,分别再灌注 0.5 h,2 h,6 h,12 h,24 h and 30 h)。各时间点分别取胎鼠 20 只,假手术组胎鼠 22 只,将肾组织匀浆后采用 RT-PCR,Western 印迹杂交和放免法进行检测。同时苏木精-伊红染色观察肾组织病理学改变。**结果** 宫内窘迫后胎肾组织 COX-2 蛋白和基因表达上调,PGI_2 的稳定代谢产物 6-*keto*-$PGF_{1\alpha}$及 PGE_2 均于再灌注 2 h 开始增高($P < 0.05$)。其中 6-*keto*-$PGF_{1\alpha}$增加迅速,于再灌注 12 h 达高峰($P < 0.01$),PGE_2 于再灌注 24 h 达最高水平($P < 0.01$),而 TXB_2 增加幅度不大。**结论** 宫内缺血再灌注从转录水平诱导胎肾 COX-2 蛋白表达增强,COX-2 的主要代谢产物是 PGI_2 和 PGE_2。COX-2 可能通过 PGI_2 和 PGE_2 对缺血性胎肾损伤具有保护作用,因此,在围产期肾损伤不宜应用 COX-2 抑制剂。 [**中国当代儿科杂志,2005, 7(1): 15-19**]

[**关 键 词**] 宫内窘迫;环氧化酶;前列腺素;肾脏;胎鼠
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Cyclooxygenase (COX), known as a key enzyme in the synthesis of prostaglandin from arachidonic acid (AA), exists in two subtypes in the human body, COX-1 and COX-2. COX-1 is expressed constitutively in a wide variety of tissues. In contrast, the expression of COX-2 is low under physiological conditions but is dramatically up-regulated by a variety of stimuli such as hypoxia, lipopolysaccharide (LPS), interleukin-1 (IL-1), tumor necrosis factor (TNF) and epidermal growth factor (EGF). It has been reported that the expression of COX-2 protein is up-regulated following ischemia and reperfusion in adult renal tissues, but little is known about the effect of intrauterine distress on COX-2 expression in fetal rat kidney. In this study, Western blotting assay, RT-PCR and radioimmunoassay were used to examine the changes of protein, mRNA of COX-2 and metabolites of COX-2 in rat fetal kidney so as to study the roles of COX-2 in kidney impairment of fetal rats after intrauterine distress.

Materials and methods

Animals and reagents

Animals: Sprague-Dawley (SD) rats were purchased from the Department of Experimental Animal, China Medical University. The average body weight of female SD rats was 250 g ± 20 g, and 380 g ± 20 g for male SD rats. The female rats were housed with breeder males in a ratio of 4∶1. The day on which sperm was observed in a vaginal smear was designated as day zero of pregnancy. Pregnant rats were then housed in individual cages for 21 days.

Reagents: Goat anti-rat polyclonal antibodies of COX-2 were purchased from Santa Cruz. Rabbit anti-goat horseradish peroxidase-conjugated secondary antibody was from Beijing Zhongshan Bio-Tech Company. Total RNA was extracted by Trizol (Promega). Reverse Transcription Kits were from Takara. The primer pairs for COX-2 and β-actin were chosen on the basis of published standards [1] and were synthesed by Shanghai Shenggong Bio-Tech Company. The sequences were as follows: COX-2: upstream: 5′ ACA CTC TAT CAC TGG CAT CC 3′, downstream: 5′ GAA GGG ACA CCC TTT CAC AT 3′; β-actin: upstream: 5′ GAG ACC TTC AAC ACC CCA GCC 3′, downstream: 5′ TCG GGG CAT CGG AAC CGC TCA 3′. ^{3}H-PGE$_2$ kit, ^{125}I-TXB$_2$ and ^{125}I-6-*keto*-PGF$_{1\alpha}$ kits were purchased from Beijing Immune-Tech Research Institute.

Animal model and grouping

The animal model of intrauterine asphxia was established as follow[2]: Briefly, 21-day-old pregnant rats were anesthetized with 2.5% pentobarbital sodium (50 mg/kg). A small incision was made in the midline lower abdominal to expose two horn uterus and vessels supplying the uterus and ovary. An arterial clamp occluded one side of the vessels supplying the two horn uteri. The fetal rats in the non-occluded side were used as the Sham-operation group. After 30 minutes of blood occlusion, reperfusion started. The reperfusion times were 0, 0.5, 2, 6, 12, 24 and 30 hours respectively and then an experimental model of different time reperfusion after intrauterine hypoxia was established. After reaching the prescribed time, the uterus horn was opened rapidly and pups were removed. Twenty fetal rats were used for each time point in the Ischemia-reperfusion group. Twenty-two fetal rats were used for the Sham-operation group. The pups were sacrificed and kidneys were removed and stored in -70°C. Samples for RT-PCR were stored in sterilized, RNAase free Eppendorf tubes. Meanwhile renal tissues from three fetal rats were removed, fixed in buffered solution of 10% formaldehyde to observe the morphological changes of kidney.

Hematoxylin-eosin staining

Renal tissues from fetal rats were removed, fixed in a buffered solution of 10% formaldehyde, and embedded in paraffin. Longitudinal 5μm sections were cut and mounted on glass slides. Hematoxylin-eosin staining was performed as routine. Morphological changes in the kidney were observed under a microscope.

Western blotting assay

After fetal renal tissues were homogenized, membrane protein was extracted and diluted to equal concentration. Samples of 200 mL were mixed with mixed with 50 μL of 5 × sample buffer, and then boiled for 5 minutes. After electrophoresis in a 7.5% SDS-PAGE gel, the protein was transferred on to nitrocellulose membrane, washed with TBS for 10 minutes and blocked with TBS containing 5% bovine serum albumin for 1 hour. After being washed with TBS twice for 5 minutes each, the membrane was incubated with goat antibody to COX-2 (at 1∶400 dilution) overnight at 4 ℃. The next day blots were washed again and incubated with rabbit anti-goat ALP-conjugated second IgG antibody (at 1∶2 000 dilution) at room temperature for 2 hours. Finally the substrate of ALP O-dianidine and β-naphthy acid phosphate were added to the membrane

and were stained for 15 minutes. The blots were analyzed with Chemilmager-5500 electrophoresis gel image system based on optical density scanning.

RT-PCR

Total RNA of fetal kidneys was isolated using Trizol reagent according to manufacture's instructions. A total of 2 μL of sample RNA (2.5 μg) was used for first strand cDNA synthesis, which was performed in 4 μL of 2mM $MgCL_2$, 2 μL of RT buffer (10 ×), 1 μL of dNTPs (10 mM each), 1 μL of Bca BEST polymerase (2 U/μL), 1 μL of Oligo dT primer, 0.5 μL of Rnase Inhibitor (40 U/μL) and 8.5 μL of DEPC water in a total volume of 20 μL, incubated first at 45℃ for 60 minutes and then 70℃ for 10 minutes. The reaction product was then subjected to PCR amplification, which was performed in a total volume of 20 μL in the presence of 4 μL of cDNA, 1.25 μL of $MgCl_2$, 2 μL of buffer (10 ×), 0.3 μL of dNTPs, 0.1 μL of Tag DNA polymerase, 10.35 μL of DEPC water and 1 μL of COX-2 (or β-actin) upstream and downstream primer. The amplification condition was: denaturation, 94℃, 3 minutes; annealing, 60℃, 45 seconds; and elongation, 72℃, 1 minutes for 30 cycles. Ten microliters of PCR products were electrophoresised on a 10% polyacrylamide gel, then stained with ethidium-bromide, and observed using ultraviolet light and photographed. Electrophoresis bands were analyzed by Chemilmager-5500 gel image system.

Radioimmunoassay

The renal tissues were obtained at different time points. The concentrations of 6-*keto*-$PGF_{1\alpha}$, TXB_2, and PGE_2 (metabolites of COX-2) in fetal renal tissues were detected by specially trained technicians according to the manufacture's instructions.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS 10.0. The data were expressed as $\bar{x} \pm s$ and were analyzed by two-way analysis of variance for multiple comparisons between groups. The Dunnett *t*-test was used to assess differences between individual means. Differences were considered significant at $P < 0.05$.

Results

Histopathologic changes in renal tissues

Cloudy swelling of renal tubules occurred following 30 minutes ischemia without reperfusion, including swelling of epithelial cells in proximal convoluted tubule, whose cavity got narrow, and cytoplasm was filled with pink staining materials. Six hours after reperfusion, proximal convoluted tubule dilated slightly, renal glomerulus got hyperemia and vacuolation occurred (Figure 1A); when reperfusion was continued, proximal convoluted tubule dilated visibly and there was exudation in cavity. Twenty-four hours after reperfusion, vacuolation was more serious in proximal convoluted tubule, the cavity disappeared, and sheet necrosis occurred locally. After 30 hours of reperfusion, the above mentioned changes were aggravated, large pieces of tissue necrosis occurred in proximal convoluted tubule, the structure of renal tubule was not clear, and the pathologic changes were most severe (Figure 1B).

Changes of COX-2 proteins in renal tissues

COX-2 proteins were expressed in normal renal tissues of fetal rats at a low level. Following 30 minutes ischemia and 2 hours reperfusion, COX-2 proteins expression was up-regulated significantly ($P < 0.05$), and reached a peak 24 hours post-reperfusion ($P < 0.01$). Thirty hours after reperfusion it was still above the Control group ($P < 0.01$). See Figure 2 and Table 1.

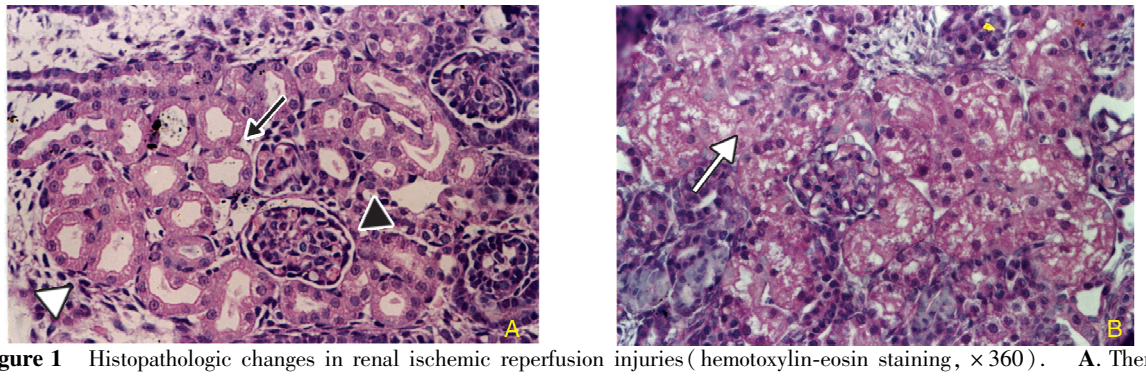

Figure 1 Histopathologic changes in renal ischemic reperfusion injuries (hemotoxylin-eosin staining, ×360). **A**. There were evident histopathologic changes in fetal kidney tissues 6 hrs after reperfusion: Proximal convoluted tubule dilated slightly (dark arrow), renal glomerulus got hyperemia (dark triangle) and vacuolation (white triangle) occurred. **B**. 30 hrs post-reperfusion, large pieces of necrosis occurred in proximal convoluted tubule (white arrow), the structures of renal tubule were not clear, and the pathologic changes were most severe.

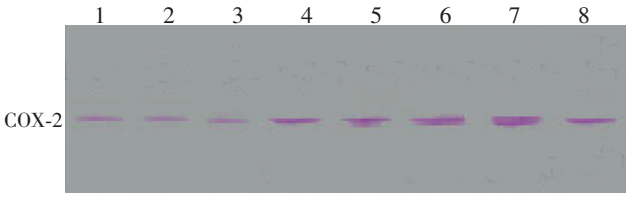

Figure 2 Effects of acute intrauterine ischemia and reperfusion on expression of COX-2 protein in fetal rat kidney.

Lane 1: Control group. lane 2: Ischemia 0.5 hr group. lane 3: Reperfusion 0.5 hr group. lane 4: Reperfusion 2 hrs group. lane 5: Reperfusion 6 hrs group. lane 6: Reperfusion 12 hrs group. lane 7: Reperfusion 24 hrs group, lane 8: Reperfusion 30 hrs group.

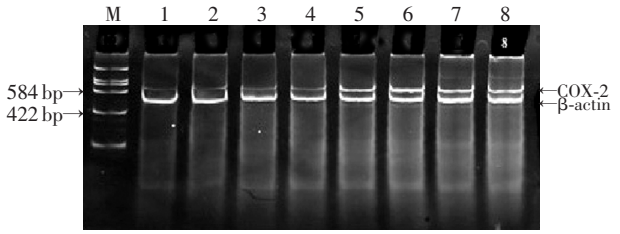

Figure 3 Expression of COX-2 mRNA in fetal rat kidney after acute intrauterine ischemia and reperfusion by RT-PCR. M: marker DL 2000. lane 1: Control group. lane 2: Ischemia 0.5 hr group. lane 3: Reperfusion 0.5 hr group. lane 4: Reperfusion 2 hrs group. lane 5: Reperfusion 6 hrs group. lane 6: Reperfusion 12 hrs group. lane 7: Reperfusion 24 hrs group. lane 8: Reperfusion 30 hrs group.

Expression of COX-2 mRNA in fetal rat kidney

In the normal controls, COX-2 mRNA was detected in renal tissues of fetal rats, but the expression was less. During the reperfusion process following 30 minutes ischemia, the COX-2 mRNA expression increased gradually. Compared with the Sham-operation group, the COX-2 mRNA expression began to increase in renal tissues 30 minutes post-reperfusion ($P < 0.05$), and reached a peak 12 hours later ($P < 0.01$), then decreased, but it remained higher until 30 hours post-reperfusion ($P < 0.01$). See Figure 3 and Table 1.

Kinetic changes of 6-*keto*-PGF1α, TXB_2 and PGE_2 concentrations

Compared with the Sham-operation group, the concentrations of 6-*keto*-$PGF_{1\alpha}$ and PGE_2 began to increase following 2 hours reperfusion, and reached a peak 12 hours and 24 hours after reperfusion respectively. The TXB_2 concentration in the Ischemia-reperfusion group was not different from the Sham-operation group at any reperfusion time point (Table 1).

Table 1 6-*keto*-$PGF_{1\alpha}$, TXB_2, PGE_2, COX-2 concentrations and COX-2mRNA expression after intrauterine ischemia and reperfusion in fetal rat kidneys ($\bar{x} \pm s$)

Group		6-*keto*-$PGF_{1\alpha}$ (pg/mg) ($n=8$)	TXB_2 (pg/mg) ($n=8$)	PGE_2 (pg/mg) ($n=8$)	COX-2 ($n=6$)	COX-2 mRNA ($n=6$)
Sham- operation		165 ± 84	126 ± 32	3360 ± 1292	30 ± 6	0.60 ± 0.10
30 min ischemia		180 ± 84	128 ± 16	3462 ± 1504	32 ± 7	0.66 ± 0.09
Reperfusion	0.5 hr	230 ± 121	135 ± 28	3764 ± 1537	35 ± 8	0.76 ± 0.09[a]
	2 hrs	402 ± 185[a]	161 ± 20	5596 ± 1551[a]	43 ± 7[a]	0.79 ± 0.06[b]
	6 hrs	534 ± 113[b]	170 ± 23	5898 ± 1460[b]	54 ± 9[b]	0.97 ± 0.10[b]
	12 hrs	995 ± 216[b]	179 ± 22	5953 ± 1371[b]	66 ± 12[b]	1.09 ± 0.10[b]
	24 hrs	822 ± 221[b]	189 ± 15	6850 ± 2109[b]	70 ± 11[b]	1.96 ± 0.07[b]
	30 hrs	456 ± 267[a]	166 ± 36	5824 ± 1692[b]	52 ± 7[b]	0.82 ± 0.06[b]

Compared with the Sham-operation group (a $P < 0.05$, b $P < 0.01$)

Discussion

Perinatal asphyxia that can result in multi-organ impairments is one of the leading causes of death and disability in neonates. The kidney is energy-consuming, and shares much blood flow from the body. So it is especially sensitive to ischemia and hypoxia. The incidence of kidney impairment following asphyxia is high, reaching as high as 56%[3]. As there are no early and special clinical symptoms after kidney impairment, it is difficult to make an early diagnosis and so research should focus on the pathogenesis, prevention and treatment of the disease.

COX-2 is a key enzyme in the synthesis of prostaglandin. It has been shown that COX-2 can be induced in renal tissues after ischemia and reperfusion but until now the function of COX-2 in the ischemic renal impairment has not been clear. Abassi[4] has reported that in rats with renal ischemia caused by experimental heart failure, COX-1 and COX-2 are abundantly expressed in the renal medulla, with a level that is significantly higher than that in the cortex. Along with the advances of heart failure, the expressions of COX-2

mRNA and protein becomes much higher, and selective COX-2 inhibitor can obviously lower the blood flow in renal medulla and thus lead to much more severe renal impairment by ischemia. Therland[5] has reported that in ischemic renal failure cases, COX-2 is up-regulated in vascular smooth muscles and glomerular mesenchymal cells, so it can be deduced that the COX-2 metabolic products is important in maintaining the glomerular filtration rate and protectfing the renal function. Recently, some studies have shown that COX-2 is also expressed in normal human kidney[5]. This suggests that it is possible for COX-2 inhibitors to suppress the renal function and cause acute renal failure in the patients who have already had chronic renal function deficiency[6]. In the present study, Western blotting was adopted to study the expression of COX-2 protein in fetal rat kidney following intrauterine distress. The results showed that COX-2 protein was increased after ischemia for 30 minutes. The COX-2 protein began to increase at 2 hours of reperfusion, peaked by 24 hours and remained at high levels 30 hours after reperfusion. These results were consistent with the report of Abassi[4].

COX-2 is inducible. Under physiological conditions, it is expressed at a low level in most tissues. COX-2 mRNA can be induced by ischemia, LPS, IL-1, TNF and many other inflammatory factors. This study showed that COX-2 mRNA began to increase at 30 minutes of reperfusion, peaked at 12 hours and remained at high levels 30 hours after reperfusion. This suggestes that the elevated COX-2 protein was regulated by COX-2 mRNA at the transcriptional level.

The main functions of COX-2 are performed by its metabolic products. Now it is certain that the metabolic products in renal tissue are 6-*keto*-$PGF_{1\alpha}$, TXB_2 and PGE_2. This study showed that in fetal renal tissues, 6-*keto*-$PGF_{1\alpha}$ and PGE_2 began to increase at 2 hours of reperfusion, peaked between 12-24 hours and remained at high levels 30 hours after reperfusion. TXB_2 increased only slightly and showed no significant difference compared with the Sham-operation group.

Many studies have shown that PGI_2 and PGE_2 are the protective factors for acute ischemic renal injury. Animal experiments indicate that the administration of PGI_2 analogues can lessen the medulla impairment caused by acute renal failure. PGE_2 can inhibit the synthesis of TNF-α by activated macrophages in adult renal ischemia-reperfusion impairment and thus suppress the inflammation[7]. In vitro studies testified that PGI_2 and PGE_2 can lessen the injury in renal medulla epithelial cells and exert a protective effect when they were administrated into the cell culture media in advance[8]. Recently in adult renal ischemia-reperfusion animal experiments, Matsuyam[9] found that large pieces of necrosis occurred in renal tubules 12 hours after reperfusion following 30 minutes ischemia and that the COX-2 protein expression was up-regulated in normal renal tubular epithelial cells before necrosis occurred. The present study also showed that the major metabolic products of COX-2 were PGI_2 and PGE_2 in ischemic fetal renal tissues. Both these studies demonstrated that COX-2 may exert a protective effect on ischemic renal injury. It was found that the peak expression of COX-2 protein appeared prior to the peak pathologic injury of renal medulla, which indicated that COX-2 may have protective effects on fetal renal ischemic impairment. It is suggested that COX-2 inhibitors should not be administered in the treatment of asphyxia in perinatal period.

[References]

[1] Yang T, Singh I, Pham H, Sun D, Smart A, Schnermann JB, et al. Regulation of cyclooxygenase expression in the kidney by dietary salt intake [J]. Am J Physiol, 274(3 Pt 2):F481-489.

[2] Wu J, Yan L, Wei K-L. Expression of intercelluar adhesion molecule21 in the fetal kidneys of rats after intrauterine acute ischemia and hypoxia (in Chinese) [J]. Chin J Contemp Pediatr, 2004, 6(3):199-202.

[3] Perlman JM, Tack ED, Martin T, Shackelford G, Amon E. Acute systemic organ injury in term infants after asphyxia[J]. Am J Dis Child. 1989, 143(5):617-620.

[4] Abassi Z, Brodsky S, Gealekman O, Rubinstein I, Hoffman A, Winaver J. Intrarenal expression and distribution of cyclooxygenase isoforms in rats with experimental heart failure[J]. Am J Physiol Renal Physiol, 2001, 280(1): F43-53.

[5] Therland KL, Stubbe J, Thiesson HC, Ottosen PD, Walter S, Sorensen GL, et al. Cycloxygenase-2 is expressed in vasculature of normal and ischemic adult human kidney and is colocalized with vascular prostaglandin E2 EP4 receptors[J]. J Am Soc Nephrol. 2004, 15(5):1189-1198.

[6] Perazella MA, Eras J. Are selective COX-2 inhibitors nephrotoxic? [J]. Am J Kidney Dis, 2000, 35(5):937-940.

[7] Kunkel SL, Spengler M, May MA, Spengler R, Larrick J, Remick D. Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression[J]. J Biol Chem, 1988, 15;263(11):5380-5384.

[8] Paller MS, Manivel JC. Prostaglandins protect kidneys against ischemic and toxic injury by a cellular effect[J]. Kidney Int, 1992, 42(6):1345-1354.

[9] Matsuyama M, Nakatani T, Hase T, Kawahito Y, Sano H, Kawamura M, et al. The expression of cyclooxygenases and lipoxygenases in renal ischemia-reperfusion injury[J]. Transplant Proc, 2004, 36(7):1939-1942.

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