Brain death affects the renal morphology and function of Ba-Ma mini pigs

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Abstract

Background. How brain death affects kidney is still not clear and its mechanism needs further study. *Methods.* Ten Ba-Ma mini pigs were randomized into 2 groups: brain-dead group (n = 5), and control group (n = 5). Brain-dead model was established by increasing intracranial pressure. At 3, 6, 12, 18 and 24 hours after the initial brain death, serum BUN, Cr, TNF-α, IL-1β, and IL-6 were determined. At 6, 12, and 24 hours, renal tissues were taken, the changes of renal tissues were observed by HE staining, the expression of PKC-α and NF-κB by immnohistochemistry, and PKC-α mRNA and NF-κB mRNA by RT-PCR. The ultrastructure changes of renal cells were observed under electron microscope. *Results.* Serum BUN and Cr of group B began to increase at 12 hours after initial brain death. Since 3 hours after the initial brain death, IL-1β, IL-6, and TNF-α of group B began to increase, and increased with time. Protein expression of PKC-α and NF-κB for group B showed significantly higher than those of group C at 6, 12, and 24 hours. PKC-α mRNA and NF-κB mRNA expression for group B began to increase since 3 hours. After 12 hours, changes of renal cells could be found in group B and no change was found in group C. *Conclusions.* Brain death may activate PKC-α mRNA and NF-κB mRNA transcription and increase the level of inflammatory mediators in kidney, thus release inflammatory factors, and disrupt both the renal function and morphology of kidney. [Life Science Journal. 2007; 4(4): 15 – 20] (ISSN: 1097 – 8135).

Keywords: brain death; Ba-Ma mini pigs; inflammatory factors; kidney

1 Introduction

It is an effective solution to meet the shortage of grafts by utilizing the kidney organs of the brain-dead donor (BBD). The success of the transplantation from the BBD is not better than that from living donor both in the short-term and long-term although the technologies and immunosuppression have improved much. The only difference between these two is brain death[1–3]. There are few studies on how the protein kinase C (PKC) and nuclear factor kappa B (NF-κB) impact on the kidney of brain-dead donors. In this research, we established the brain-dead model with Ba-Ma mini pigs by increasing intracranial pressure (ICP) to observe the change of kidney morphology and function, and try to reveal the underlying mechanism.

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2 Materials and Methods

2.1 Animals

Ten healthy Ba-Ma mini pigs (aged 6 months and weighted approximate 24 kg) were provided by Experimental Animal Centre of the Third Military Medical University (Chongqing, China). Prior to the study, all protocols were approved by our institution's animal welfare regulatory committee and all protocols conform with the *the Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86 – 23, 1996 edition).

2.2 BD model

Pigs were fasted for 12 hours with free access to water. Anesthesia was accomplished with ketamine (20 mg/kg, intramuscularly), benzodiazepine (10 mg, intramuscularly), and atropine (0.03 mg/kg, intramuscularly); 20 minutes later, thiopental sodium (10 mg/kg), pancuronium (0.15 mg/kg), and ketamine (2 – 5 mg/kg) injected intravenously in the auricle vein.

After animals were anaesthetized, and craniotomy was performed, Foley catheter were placed in the skull, and they were randomly assigned to 2 groups: the BD group (group B, $n = 5$) and control group (group C, $n = 5$).

2.3 The establishment of brain-dead model with Ba-Ma mini pigs

The brain-dead model was established by the improved method of Pratschke[4] and Lanza[5]. The pigs in both group were generally anesthetized through intravenous injection. The respiration was supported by the anaesthetic machine and HR and BP were monitored and recorded by the electrocardiogram monitor. The pigs were fixed in left recumbent position, establishing the venous access at the same time. Craniotomy was carried out along the skull median line. A Foley 18F catheter was placed in intracranially epidural space. In the group B, the catheter was infused with saline slowly to increase the pressure of intracranial pressure (ICP), meanwhile monitoring the changes of HR and BP, to establish the brain-dead model. The conformation standard of brain-dead were: (1) A deep coma. Excluding reversible factors such as anesthesia and low temperature, and so on. (2) Absence of oculopupillary reflex and corneal reflex, repeated twice. (3) Loss of spontaneous breathing. (4) No EEG activity continuously recorded for over 30 minutes. (5) Atropine test negative, that was, giving intravenous injection of 1 mg atropine, and HR did not accelerate when monitoring per 5 – 15 minutes (if heart rate accelerated by 20% – 40%, the test was positive). (6) The "tic reaction" disappeared. (7) Observation time of confirming BD: through 12 hours' observation after first verification accorded with criteria (1), (2), (3), (4) and (6), no changes happened and BD could be verified.

2.4 Specimen collection

The samples of serum and kidney tissues were taken at 3, 6, 12, 18, and 24 hours after the first verification of BD. Blood sample (5 ml) taken from superior vena which was deposited for 30 minutes and then centrifuged to detect serum BUN and Cr, by automatic biochemistry analyzer. TNF-α, IL-1β, and IL-6 were measured by ELISA. The renal tissues were to detect PKC-α mRNA, by RT-PCR.

2.4.1 Detection of PKC-α mRNA and NF-κB in renal tissue by RT-PCR. Total RNA was extracted from renal tissues by TRIzol assay (Invitrogen Co., USA). PCR amplificated after reverse transcription by one-step assay kit (Bao Bioengineering Co., Dalian, China). The primer was designed according to the gene sequence from GenBank and synthesized by Bioengineering Corporation. The primers of PKC-α (220 bp) were: lateral upstream 5'-CGCTTCGCCCGCAAAGG-3', downstream: 5'-GCAGGTGTCACATTTCATCCC-3'; medial upstream: 5'-TGCACCGACTTCATCTGGG-3', downstream: 5'-GATAAGTCCATAGAGCAGGG-3'; Tropnin β (β-actin, 480 bp) was the internal reference. The primers were: upstream, 5'-CATCCTGCGTCTGGACCT-3'; downstream 5'-TCAGGAGGAGCAATGATCTTG-3'. The product of PCR was placed on the 2% agarose gel (stained by 0.5 μg/ml ethidium bromide), observed under ultraviolet ray projectoscope. The pictures were integral absorbance analyzed by gel picture analysator.

2.4.2 Detection of PKC-α by immunohistochemistry. The procedure of SP-immunohistochemistry followed the kit maunal. Positive cells are brown-yellowish with granular and patchy staining patterns. PKC mainly confined in cytoplasm and cell membrane, while NF-κB mainly located in nucleus and cytoplasm. The positive cells in 5 microscope visions were counted. The positive rate was calculated as: the positive rate = the positive cell population / the total cell population, taking PBS as negative control to replace first antibody.

2.4.3 Morphology observation. Tissues taken from upperpole of kidney stained with HE were observed under light microscope, and those fixed with glutaraldehyde were observed under the electron microscope.

2.5 Statistic analysis

The quantitative variables were expressed as mean ± standard deviation ($\bar{X} \pm s$). Using SPSS 10.0 software, ANOVA and one-way ANOVA were applied. Significant level $\alpha = 0.05$.

3 Results

3.1 The changes of kidney function

The serum BUN and Cr in group C kept stable during the 24 hours ($P > 0.05$). Twelve hours after BD, serum BUN and Cr in group B began to increase. The serum BUN and Cr in group B were more than in group C from 12 hours to 24 hours after brain death ($P < 0.05$). At the same time, since 12 hours after brain death the serum BUN and Cr kept increasing in the following 12 hours ($P < 0.05$). The data were shown in Table 1.

3.2 Changes of inflammatory mediators

The levels of serum IL-1β, IL-6 and TNF-α kept stable in control group during the whole detection ($P > 0.05$). Three hours after BD, the levels of serum IL-1β, IL-6 and TNF-α in group B began to increase. Three hours after brain death the serum IL-1β, IL-6 and TNF-α of group

Table 1. The parameter changes of the levels of liver function ($\bar{X}$ ± s, mmol/L)

	Group	Measuring time (hours)				
		3	6	12	18	24
BUN	C	2.31 ± 0.09	2.33 ± 0.06	2.38 ± 0.02	2.30 ± 0.02	2.37 ± 0.04
	B	2.41 ± 0.11	2.40 ± 0.10	3.68 ± 0.02[ab]	4.79 ± 0.02[abc]	5.76 ± 0.02[abcd]
Cr	C	42.00 ± 0.32	44.60 ± 1.21	42.80 ± 0.73	42.80 ± 0.73	43.60 ± 0.68
	B	42.40 ± 0.51	43.40 ± 1.40	79.80 ± 0.80[ab]	85.60 ± 0.80[abc]	93.60 ± 1.03[abcd]

$n = 5$, [a]: *vs*. C group, $P < 0.05$; [b]: $P < 0.05$ *vs*. the 6 hours; [c]: $P < 0.05$ *vs*. the 12 hours; [d]: $P < 0.05$ *vs*. the 18 hours;

B were more than those of group C, and continued to increase with time. Twenty-four hours later, serum IL-1β, IL-6 and TNF-α of group B were high above those of group C. The data were shown in Table 1.

3.3 PKC-α protein and NF-κB p65 protein in kidney

Figure 1 showed that PKC-α proteins of group C were confined to the cytoplasm of normal renal cells and a small quantity of PKC-α protein could be found under light microscope. The PKC-α protein of group C almost kept at the same level in the 24 hours. In group B, 6 hours after BD, more positive cells appeared in the kidney tissues, and more with the time. At 6, 12, 24 hours the positive cells in group B were more than those of group C ($P < 0.05$). The data were shown in Table 2.

Figure 2 showed that in group C, the NF-κB p65 proteins were mainly within the cytoplasm of normal renal cells, and was rare in the nucleus. Similarly, the NF-κB p65 protein expressed mainly in the cytoplasm during the 24 hours. Although the nuclear NF-κB p65 protein in group B were more than those in group C from 6 hours after brain death, the difference were of no significance. Comparision within the B group revealed that more and more NF-κB p65 protein were found with time passed, but the increase were so small that it was not statistically different.

3.4 PKC-α mRNA and NF-κB mRNA in renal tissues

Figure 3 showed that the PKC-α mRNA didn't change much in group C. In group B, the transcriptional levels of PKC-α mRNA began to increase three hours after brain death, and increasd in the following hours ($P < 0.05$). From 3 hours after brain death, PKC-α mRNA expressed in group B were more than in group C ($P < 0.05$). The data were shown in Table 3.

3.5 Morphology changes

In group C, the shape of kidney was normal. Under light microscope, renal tubular cells were lined up in order, the cells were stained equaly, glomeruli were almost normal. Under the electron microscope, affluent chondriosome in the renal tubular cells, and the structure of chondriosome crista was clear and complete.

Six hours after BD the renal shape was normal in group B, and observation under light microscope and electron microscope were similar with the group C except that the chondriosome of proximal convoluted tubule swelled, and the structure of chondriosome crista was not so clear.

Twelve hours after BD, light microscope observation showed that the renal tubular cells were slightly cloudy swelling, the cells of proximal convoluted tubule swelled, the lumens became thinner, the number of cells in glomerulus increased, Bowman's renal capsule shrank with red blood cells and pink-stained substances inside. The proximal convoluted tubule were generally denatured. There were vacuolus and cell organelle in the glomerular capillary became some brush borders of proximal convoluted tubule fractured, and the structure of chondriosome crista became vague even disappeared. Electron microscope revealed that the chondriosome of renal tubular cells swelled and the crista disordered.

At 24 hours the swelling of cells in group B became more obvious under light microscope, and part of proximal convoluted tubule cells got swelled heavily and vacuolar degenerated. Part of proximal convoluted tubules were obstructed. There were no obvious changes of glomcrulus, and the inflammatory cells infiltrated interstitial tissue. The number of cells in glomeruli increased, and some capsules of glomerulus became small, others became dilated, with a lot of RBCs and equably pink-stained substance inside. The proximal convoluted tubules generally denatured and even became necrosis. Under electron microscope, there were vacuolus and cell organelles in the glomerular capillary because some brush borders of proximal convoluted tubules fractured, and the structure of chondriosome crista became vague even disappeared. Cell edema, mitochondia swelling, crista disappearing and parts of chondriosome membrans dissolving were also observed. The cell nucleolous extruded to renal tubule lumens. The arrangement of chondriosome that sur-

Table 2. The changes of the levels of inflammatory mediators at each time point ($\bar{X}$ ± s, pg/ml)

	Group	Measuring time (hours)				
		3	6	12	18	24
IL-1β	C	5.91 ± 0.17	5.91 ± 0.10	6.06 ± 0.12	6.08 ± 0.12	6.10 ± 0.15
	B	8.26 ± 0.21[a]	9.17 ± 0.08[ab]	10.30 ± 0.11[abc]	12.53 ± 0.35[abcd]	16.37 ± 0.16[abcde]
IL-6	C	17.35 ± 0.59	18.04 ± 0.23	17.99 ± 0.83	17.77 ± 1.05	17.62 ± 0.67
	B	29.50 ± 1.92[a]	37.92 ± 1.69[ab]	46.32 ± 1.70[abc]	56.62 ± 1.95[abcd]	66.68 ± 2.11[abcde]
TNF-α	C	17.35 ± 0.59	18.04 ± 0.23	17.99 ± 0.83	17.77 ± 1.05	17.62 ± 0.67
	B	29.50 ± 1.92[a]	37.92 ± 1.69[ab]	46.32 ± 1.70[abc]	56.62 ± 1.95[abcd]	66.68 ± 2.11[abcde]

n = 5, [a]: B group *vs*. C group, $P < 0.05$; [b]: $P < 0.05$, *vs*. the 3 hours; [c]: $P < 0.05$, *vs*. the 6 hours; [d]: $P < 0.05$, *vs*. the 12 hours; [e]: $P < 0.05$, *vs*. the 18 hours.

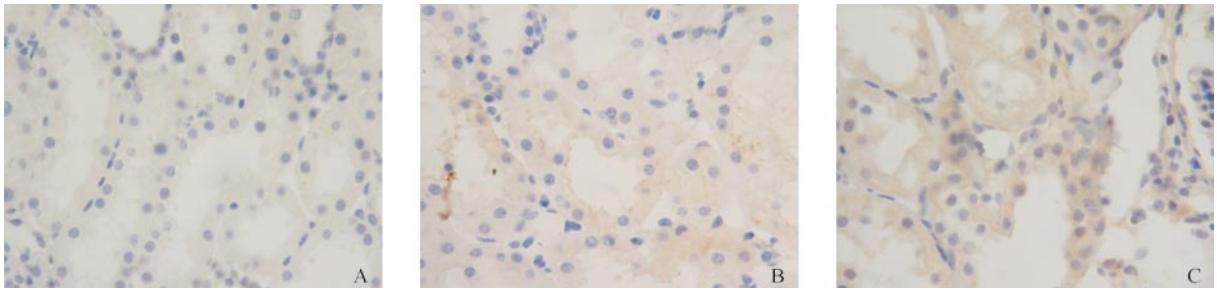

Figure 1. A: Expression of PKC-α protein in group C, × 400; B: Expression of PKC-α protein in group B at 6 hours, × 400; C: Expression of PKC-α protein in group B at 24 hours, × 400.

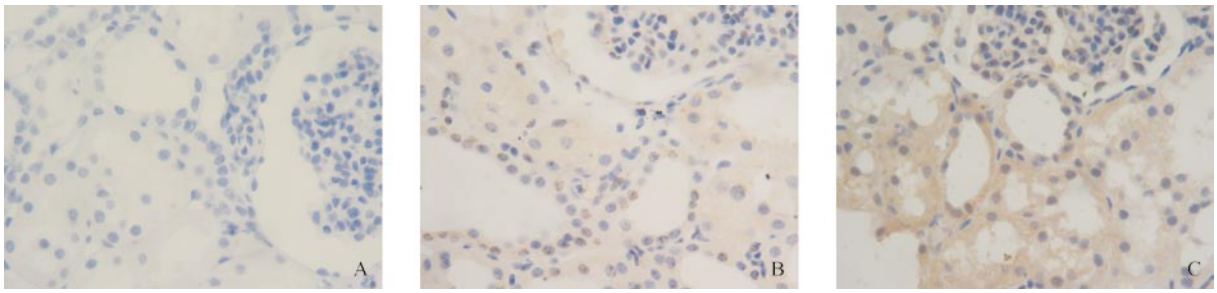

Figure 2. A: NF-κB P65 in group B, × 400; B: NF-κB P65 in group B at 6 hours, ×400; C: NF-κB P65 in group B at 24 hours, ×400.

Table 3. The PKC-α and PKC-α mRNA at different time after BD ($\bar{X}$ ± s)

	Group	Measuring time (hours)		
		6	12	24
PKC-α (pg/ml)	C	0.09 ± 0.01	0.10 ± 0.02	0.11 ± 0.02
	B	0.22 ± 0.04[a]	0.38 ± 0.04[ab]	0.52 ± 0.06[abc]
PKC-α mRNA	C	0.87 ± 0.03	0.85 ± 0.02	0.86 ± 0.04
	B	1.10 ± 0.02	1.16 ± 0.05[ab]	1.55 ± 0.05[abc]

n = 5, [a]: *vs*. C group, $P < 0.05$; [b]: *vs*. the 6 hours, $P < 0.05$; [c]: *vs*. the 12 hours, $P < 0.05$.

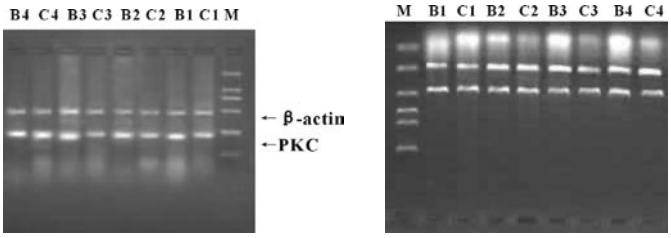

Figure 3. Left: PKC-α mRNA in all groups; Right: NF-κB mRNA in all groups.

rounded microvilli became disordered (Figures 4 and 5).

4 Discussion

The donors of BD have become the main source of kidney transplantation donors in many countries. With the advance of the BD legislation in our country, the transplantation will be possible by utilizing the BD donors. But recent researches showed that the non-immune factors such as BD have an important impact on the function of grafts, and have a close relationship with the chronic disfunction of grafts. At present the changes and the mechanism of renal lesion in the state of BD are not exactly clear, so it will be significant to discover the changes and the mechanism of renal lesion of BD.

Du *et al*[7] established the cat BD model to observe the kidney structure changes during BD. It was observed under microscope the proximal convoluted tubule cells got swelled heavily, vacuolar and granula degeneration, the proximal convoluted tubule obstructed, and the number of glomeruli of kidney cell increased, in which there were juxtaglomerular apparatus and macula densa hyperplasy. It was also observed that there were injured chondriosome, vague crista, dilated endocytoplasmic reticulum and cell death under electron microscope. Six hours after the first verification of BD, Van der Hoeven[8] observed that there were inflammatory cells infiltrated in the renal tissue in the rat BD model. The experiment showed that the glomeruli of kidney were almost normal, proximal convoluted tubule slightly denatured and the chondriosome of proximal convoluted tubule swelled, and the structure of crista was not clear at 12 hours from light microscope. At 24 hours the swelling of cell became more obvious, and part of proximal convoluted tubule cell got swelled heavily and vacuolar degeneration, and part of proximal convoluted tubules were obstructed, and inflammatory cells infiltrated interstitial substance under light microscope. Twenty-four hours after initial verification of BD there were swelled chondriosomes and crista disappeared. During the whole experiment glomeruli of kidney were almost normal, the injury mainly occurred in the proximal convoluted tubules, and all the changes were less severe than those already reported. The reasons maybe the differences of experimental animals, the establishing ways of BD model and the maintaining condition. Nagareda[9] observed the changes of kidney injury in state of BD by kidney biopsy. The changes: there were the circumscribed denatured tubule in the initial phase of BD, and the domain extended over time; these changes of renal tubule

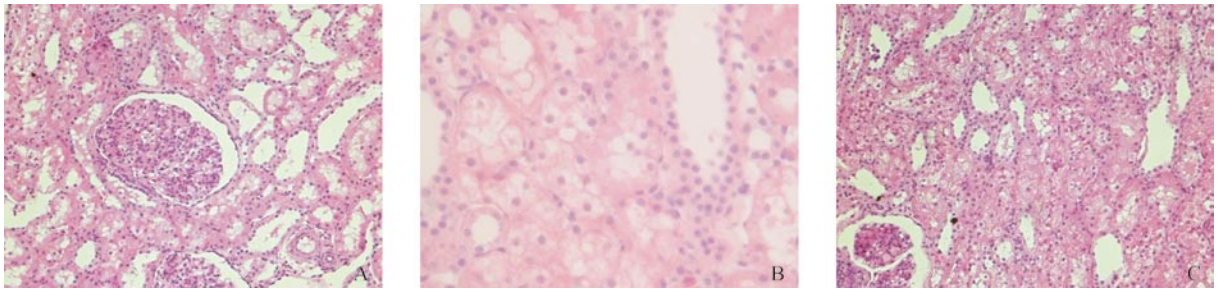

Figure 4. A: Expression by HE strain in group C at 24 hours, × 400; B: Expression by HE strain in group B at 12 hours, × 400; C: Expression by HE strain in group B at 24 hours, × 400.

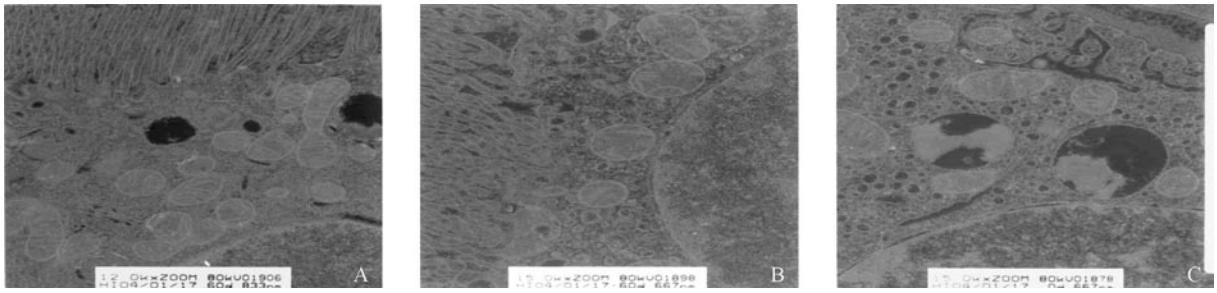

Figure 5. A: Expression in group C at 24 hours, EM × 7000; B: Expression in group B at 12 hours, EM × 10000; C: Expression in group B at 24 hours, EM × 10000.

distal end were extensive than the proximal end, at the same time there were progressive hyperplasy in the endotheliem, engorgement of glomcrulus, glomcrulus circa inflammation, glomcrulus circa fibrosis, and the denature, atrophy, cell necrosis of renal tubular became more obvious after 3 days. The factors such as ethics, morality, religion and law etc. restricted the further research on BD.

In this experiment we established the stable BD model via respiration and circulation sustaining to the stable haemodynamics with Ba-Ma mini pigs to simulate the clinical practice and further research the BD. We observed that there were progressive acute injury changes in the renal tissues during the 24 hours after BD, but there were no glomcrulus circa fibrosis and cell necrosis of renal tubular. That may be the short time we observed, and the injury changes would become worse over time.

The mechanism of the kidney injury changes after BD is unclear at present. Some scientists take that the intensive change of haemodynamics and subsequent hypotension in the state of BD maybe the causes of organs injury[10]. In the condition of stable haemodynamics, at 3 hours after the brain death the levels of serum TNF-α, IL-1β, and IL-6 increased, and persistently increased over time. The levels of BUN and Cr obviously increased 12 hours after BD, which suggested that the inflammatory mediators might induce the kidney injury, and the injury of the BD donor organs had relation with the changes of the immune state.

The research showed that PKC exists in cytoplasm and it played an important role in the IP3-DG signal transduction system. An experiment proved that the PKC activation could induce the synthesis and release of TNF, IL-1, IL-6, IL-8, PAF, NO, etc.; PKC phosphorylated IκB, the inhibitor of NF-κB, thus dissociate NF-κB-IκB from the cytoplasm, as a result, NF-κB were activated to enter the nucleus. NF-κB is considered the chief factor that regulates inflammatory reaction, which can up-regulate varied inflammatory mediators such as TNF-α, IL-1, IL-6, IL-8, and oxygen free radicals[11–13]. In this experiment, 3 hours after brain death, transcriptional level of PKC-α mRNA and NF-κB increased obviously by RT-PCR. The high expression of PKC-α protein could be detected in renal tissues, and the levels of IL-1β, IL-6, and TNF-α increased, too. Both of the increase tendencies were in concordance. We presume that maybe the mechanism of kidney injury is the exterior signal activating PKC-α, and PKC activated NF-κB, then they could induce the synthesis and release of TNF-α, IL-1, and IL-6, etc, therefore induced the non-specificity kidney injury. The station of inflallergize with subsequent cutting of kidney transplantation, warm ischemia, cropping, cold conservation, ischemical reperfusion injury makes the kidney rejection more easy. The pathophsiologic changes of BD are very complicated, and PKC-α induced the mechanism of kidney injury need to be further researched.

5 Conclusion

In this study we began with the PKC-α and NF-κB cell signal pathway to observe the injury changes of renal morphology and function make worse over time. It maybe is one of the risk factors that impact the function of kidney in BD. The progressive injured tendency is similar with the increasing tendency of IL-1β, IL-6 and TNF-α etc, which suggest the inflammatory mediators or the immune state of organs maybe an important factor that induce the renal dysfunction of BD.

References

1. Cantin B, Kwok BW, Chan MC, *et al*. The impact of brain death on survival after heart transplantation: time is of the essence. Transplantation 2003; 76(9): 1275 – 9.
2. Terasaki PI, Cecka JM, Gjertson DW, *et al*. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 1995; 333(6): 333 – 6.
3. Farmer DG, Yersiz H, Ghobrial M, *et al*. Early graft function after pediatric liver transplantation: comparison between *in situ* split liver grafts and living-related liver grafts. Transplantation 2001; 72(11): 1795 – 802.
4. Pratschke J, Wilhelm MJ, Kusaka M, *et al.* A model of gradual onset brain death for transplant-associated studies in rats. Transplantation 2000; 69(3): 427 – 30.
5. Lanza RP, Cooper DK, Novitzky D, *et al*. Survival after cardiac transplantation. S Afr Med J 1983; 64(26): 1007.
6. Zhang SJ, Shi JH, Cui XY, *et al*. Changes of blood pressure and heart rate during the establishment of a Ba-Ma pigs model of brain death by increasing intracranial pressure. Chinese Journal of Experimental Surgery 2005; 22(4): 498 – 500.
7. Du JF, Li DX, Li RB, *et al*. Experimental study of pathologic changes in the state of brain death for the out-brain organs. Journal of Forensic Medicine 1998; 14(2): 67 – 70.
8. Van der Hoeven JA, Ter Host GT, Molema G, *et al*. Effects of brain death and hemodynamic status on function and immunological activation of the potential donor liver in the rat. Ann Surg 2000; 232(6): 804 – 13
9. Nagareda T, Kinoshita Y, Tanaka A, *et al*. Clinicopathology of kidneys from brain-dead patients treated with vasopressin and epinephrine. Kidney Int 1993; 4(6): 1363 – 70.
10. Pratschke J, Wilhelm MJ, Kusaka M, *et al.* Brain death and its influence on donor organ quality and outcome after transplantation. Transplantation. 1999; 67(3): 343 – 8.
11. May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today 1998; 19(2): 80 – 8.
12. Flohe SB, Bruggemann J, Herder C, *et al*. Enhanced proinflammatory response to endotoxin after priming of macrophages with lead ions. J Leukoc Biol 2002; 71(3): 417 – 24.
13. Shishir Shishodia, Anju Shrivastava, Ajit Sodhi. Protein kinase C: a potential pathway of macrophage activation with cisplatin. Immunology Letters 1998; 61: 179 – 86.