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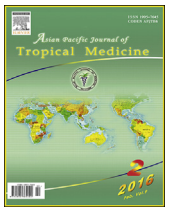

Original research http://dx.doi.org/10.1016/j.apjtm.2016.01.006

Monitoring of renal ischemia reperfusion injury in rabbits by ultrasonic contrast and its relationship with expression of VEGF in renal tissue

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ARTICLE INFO

Article history:
Received 15 Nov 2015
Received in revised form 20 Dec 2015
Accepted 30 Dec 2015
Available online 13 Jan 2016

Keywords:
Ischemia reperfusion injury
Kidney
Ultrasonic contrast
VEGF

ABSTRACT

Objective: To evaluate the renal ischemia reperfusion injury (IRI) in rabbits using the ultrasonic contrast technique and discuss the clinical value of ultrasonic contrast technique in the diagnosis of renal IRI by comparing the time-intensity curve of renal cortex and the expression of vascular endothelial growth factor (VEGF) of renal tissue.

Methods: Twenty 3-month-old New Zealand rabbits were randomly divided into 4 groups, namely Ctrl group, IRI-12 h, IRI-24 h and IRI-48 h groups. The two dimensional gray-scale ultrasonography was employed to determine and mark the position of rabbit kidney. Rabbits were given the intraperitoneal anesthesia with 20% urethane with the dosage of 5 mL/kg. The aseptic operation was performed after the local skin disinfection in the area of both kidneys. The right kidney of animals in the control group was excised without any treatment for the left kidney. After excising the right kidney of animals in groups of IRI-12 h, IRI-24 h and IRI-48 h, the aneurysm clip was used to clip the renal pedicle vessel of left kidney, in order to simulate the ischemia. Because of the tissue ischemia, it could be seen that the color of kidney was changed from bright red to dark red, which indicated the successful modeling of ischemia. The aneurysm clip was released after one hour of maintaining the ischemia. Then the kidney turned out to be bright red from dark red, which indicated that the reperfusion was completed. Taking this moment as the time of ischemia reperfusion, the wound was stitched up. A total of 12, 24 and 36 h after the operation, the two-dimensional and color Doppler flow imaging and ultrasonic contrast were employed for the examination. The dynamic changes of ultrasonic contrast were recorded. The quantitative analysis software (QontraXt) was adopted to analyze the time-intensity curve of echo at different positions of renal cortex. After the ultrasonic contrast testing, rabbits were put to death. The renal cortex tissue was isolated and the tissue RNA and total protein were extracted respectively. Real-time PCR and western blotting were used to detect the VEGF and the Pearson product moment correlation coefficient was used to measure the linear relationship between these two variables.

Results: The ultrasonic contrast could clearly reflect the process of IRI. The results of testing at mRNA and protein level indicated that the expression of VEGF in IRI groups was significantly increased ($P < 0.05$) and the expression of VEGF was also increased by the time of reperfusion.

Conclusions: There is the certain correlation between the expression of VEGF and process of IRI. The correlation coefficient between the ultrasonic contrast parameters of AT and TTP and the relative expression of VEGF is over 0.9, which indicates the relatively high correlation. But there is no significant difference in the change of perfusion peak intensity between groups, which has no correlation with the expression of VEGF.

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Peer review under responsibility of Hainan Medical College.
Foundation project: It was supported by the National Natural Science Foundation of China (Protect No. 81572977).

1. Introduction

The ischemia reperfusion injury (IRI) is the tissue injury caused by the ischemia, namely the tissue injury after the certain duration of tissue ischemia, when the blood supply and perfusion were recovered [1]. According to the previous researches, it was not the ischemia itself that caused the tissue injury, but the excessive oxygen free radical (OFR) which attacked the cells of tissue that regained the blood after restoring the blood supply [2,3], which is thus called as 'tissue IRI'. In the clinical rescue and treatment of ischemic diseases such as the surgical operation, organ transplantation and burns, there would always be the tissue IRI. Because of the special structure and function, the kidney is the organ that has the high perfusion and lacks the collateral circulation and it is quite sensitive to the IRI. During the clinical operations of renal transplantation, partial resection of renal tissue and nephrolithotomy, it is easy to cause the IRI. Meanwhile, the IRI of renal tissue was also one of main causes of acute renal failure [4].

The mechanism of IRI of renal tissue has not been clear yet. According to the previous research, the ATP depletion because of insufficient blood supply, occurrence of OFR after restoring the blood supply, and renal tubular and renal glomerular cell injury mediated by the related genes of cell apoptosis and angiogenesis were all related to the IRI of renal tissue [5]. Besides, such process also involved the neutrophil granulocyte adhering to the vascular endothelial cell to cause the activation and infiltration of neutrophil granulocyte and adhesion and aggregation of leukocytes, which would thus come across the vascular endothelial cells and result in the inflammatory response. Therefore, the IRI of renal tissue should be the complicated process that involves many aspects, while few researches focused on the role of angiogenesis in the IRI of renal tissue. The vascular endothelial growth factor (VEGF) and basic fibroblast growth factor were regarded as two important pro-angiogenesis factors [6,7] and the quantitative indices to reflect the angiogenesis. The study on the correlation between the change in the expression of VEGF and the renal IRI can further specify the occurrence and development of IRI of renal tissue.

The ultrasonic contrast is also known as the acoustic contrast, as the new technique applied in the clinical practice in recent years. By injecting the contrast agent, it was capable to dynamically and clearly show the micro-vessels, significantly improving the differentiation, sensibility and specificity of ultrasound diagnosis [8,9]. As some kind of functional imaging technique, the ultrasonic contrast possesses many advantages that the normal ultrasonography does not have. Relying on the ultrasonic contrast imaging, it is capable to dynamically show the blood supply of kidney. The good correlation between time-intensity curve-related parameters and the blood perfusion of kidney will be of importance to improve the specific diagnosis.

In this study, based on the building of animal model, the ultrasonic contrast was employed to monitor the renal ischemia-reperfusion injury in rabbits and discuss the relationship between the IRI and VEGF of renal tissue at the mRNA and protein level, in order to provide the certain experimental reference for the study on the mechanism of IRI of renal tissue.

2. Materials and methods

2.1. Materials

2.1.1. Laboratory animals and cells

Twenty 3-month-old New Zealand rabbits with the weight of (2.5 ± 0.5) kg, male or female, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. All laboratory animals were given the standard diet and clean water freely during the experiment. The ventilation was good in the feeding room, with the natural lighting day and night. The culture temperature was maintained at 18–25 °C.

2.1.2. Main reagents and instruments

The SonoVue contrast agent was purchased from Bracco (Italy); RNA extraction kit was from Bioteke (China)- RP55011; the reverse transcription kit was from Applied Biosystems (America)-4366597; Real-time PCR fluorescent quantitative PCR was from Bio-Rad Bio-Rad (America)-172-5264; the protein extraction-RIPA lysis buffer was from Wuhan Boster Biological Technology, Ltd. (China); BCA protein kit was from Vazyme Biotech (China); VEGF and glyceraldehyde phosphate dehydrogenase (GAPDH) monoclonal antibody was from Santa Cruz Biotechnology (America) -sc48835 and sc365062; horseradish peroxidase labeled secondary antibody was from Beijing Zhongshan Jinqiao Biotechnology; ECL Chemiluminescent Substrate Reagent Kit was from Life Technologies (America)-WP20005.

DNA/RNA analyzer was Qubit Fluorometer; the fluorescent quantitative PCR system was Bio-Rad -CFX96 Touch; and the color Doppler ultrasonic diagnostic apparatus was Philips HDI5000.

2.2. Methods

2.2.1. Building of animal model

After one week of adaptive feeding, 20 3-month New Zealand rabbits were randomly divided into 4 groups, namely Ctrl group, IRI-12 h, IRI-24 h and IRI-48 h. Rabbits were given the intraperitoneal anesthesia of 20% urethane with the dosage of 5 mL/kg. After 10 min, rabbits were in the period of anesthesia maintenance. After the skin disinfection with 75% alcohol, an incision was made at the center of lower back of animals in the control group to isolate the fascia and fat tissue and excise the right kidney without any treatment for the left one. Afterwards, the ultrasonic testing was performed. The excision of right kidney for rabbits in the groups of ischemia reperfusion was the same. After excising the left kidney, the aneurysm clip was used to clip the renal pedicle vessel of left kidney, in order to simulate the ischemia. Because of the tissue ischemia, it could be seen that the color of kidney was changed from bright red to dark red, which indicated the successful modeling of ischemia. The aneurysm clip was released after one hour of maintaining the ischemia. Then the kidney turned out to be bright red from dark red, which indicated that the reperfusion was successful. Taking such moment as the time of ischemia reperfusion, the wound was stitched up with the intramuscular injection of 400 000 units antibiotic. A total of 12, 24 and 36 h after the operation, the two-dimensional and color Doppler flow imaging and ultrasonic contrast were employed for the examination. The dynamic

changes of ultrasonic contrast were recorded. The quantitative analysis software (QontraXt) was adopted to analyze the time-intensity curve of echo at different positions of renal cortex.

2.2.2. Ultrasonic contrast

After the modeling, the conventional two dimensional ultrasonic testing was performed to detect the rabbit's kidney. The kidney of rabbits in each group had the normal morphology, intact capsule and uniform echo, with the clear boundary between the cortex and medullar substance. The ultrasonic probe 10 L was set as the pulse-inversion harmonic contrast for the examination. A total of 5 mL 0.9% NaCl solution was added in SonoVue lyophilized powder and it was then shaken hard to prepare the sulfur hexafluoride microbubble suspension. The contrast agent (phospholipid microencapsulation of sulfur hexafluoride) was injected in the auricular vein with the dose of 0.15 mL/kg, which was then washed with 1.5 mL 0.9% NaCl. The signaling process of blood flow was observed under the real-time and dynamic grey status and the dynamic contrast images were saved. The mechanical index was regulated to 0.15. The quantitative analysis software (QontraXt) was employed for the analysis of parameters [10,11], while the motion compensation mode for the appropriate compensation to reduce the error. The quantitative indices of ultrasonic contrast included the absolute value of video intensity enhancement during the contrast process (change of perfusion peak intensity, A), arrival time (AT), time-to-peak (TP), area under curve (Area) and curve's peak ascending slope (Grad).

2.2.3. Real-time PCR

The UV spectrophotometer was adopted to detect A260 and concentration of RNA solution, using the ratio of OD260/OD280 to evaluate the purity of RNA. The total RNA was reversely transcripted to cDNA following the instruction manual of reverse transcription kit. The Real-time PCR was employed to detect the expression of related genes. The mRNA sequence of *VEGF* genes could be referred to NCBI database and then the Real-time PCR primers could be designed. All primers were synthesized by SBS Genetech Co., Ltd. The double $\triangle$Ct method was adopted to calculate the relative expression of target gene: the mean of three parallel repeated experiments was regarded as the Ct value of each sample, $\triangle$Ct = Ct (Target Gene) − Ct (reference), $\triangle\triangle$Ct = $\triangle$Ct (sample) -$\triangle$Ct (contro1). Therefore, the relative expression of target gene = $2^{-\triangle\triangle Ct}$ and the relative expression of control group was 2^0 = 1 [12]. Then the correlation between the relative expression of three factors in the renal tissue of animals in each group (Real-time PCR) and the ultrasonic contrast parameters was analyzed to study the correlation between the IRI and angiogenesis, followed by using Minitab15 for correlation analysis. The concentration of primers in PCR system was 325 nM and cDNA template as 100 ng. The reaction conditions included the predenaturation at 96 °C for 10 min, denaturation at 95 °C for 10 s, annealing temperature was set according to the primer Tm for 20 s and extension at 72 °C for 33 s, with 40 cycles in total.

2.2.4. Western blotting

The collected tissues were ground in the liquid nitrogen. Samples were lysed by the protein extraction kit, with the addition of protease inhibitor cocktail. After being put on the ice for 30 min, the probe-type ultrasound was used to produce the short impact with the appropriate frequency on the ice. The lysis mixture was centrifuged at 4 °C and 13 000 r/min for 20 min. The supernatant was transferred to the new centrifuge tube. Protein Assay Kit was employed to detect the protein concentration.

SDS-PAGE electrophoresis was performed on protein samples. The gel was soaked in the transfer buffer for 10 min of equilibrium. It was installed with the transfer 'sandwich' and added with transfer buffer, with 100 V and 45–60 min. After the transfer, PVDF film was washed with TBS for 10–15 min. The film was placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at the room temperature for 1 h. Then the primary antibody with the appropriate degree of dilution was added (diluted with TBST containing 1% (w/v) skimmed milk powder). It was incubated at the room temperature for 2 h and then the film was washed with TBST for 3 times, 5–10 min every time. The film was incubated with the secondary antibody (1:10 000, horseradish peroxidase-labeled) that was diluted with TBST containing 0.05% (w/v) skimmed milk powder. It was incubated at the room temperature for 1 h and then the film was washed with TBST for 3 times, 5–10 min every time. It was exposed and then photographed to save the experimental results. Quantity one v4.62 was used to measure the relative quantitative value of molecular band (target gene/reference gene). The statistical analysis was performed as well.

2.3. Statistical analysis

The experimental data was treated with SPSS16.0. The measurement data was expressed by mean ± SD. The *t* test was employed for the comparison between groups. Pearson correlation coefficient of Minitab was adopted for the correlation analysis. $P < 0.05$ indicated the statistical difference.

3. Results

3.1. Building of animal model

After one week of adaptive feeding, 20 New Zealand rabbits were randomly divided into 4 groups, namely Ctrl group, IRI-12 h, IRI-24 h and IRI-48 h. During 24 h of modeling, there were no rabbits that had the hyperanesthesia or died of infection. The intraperitoneal anesthesia of 20% urethane had the fast working speed and high degree, which could meet the demands of experiment. Up to 36 h after modeling, one rabbit in IRI-48 h had the hematuria with acute renal failure, which died 3 h after having the symptoms. Under the condition of ischemia, it could be seen that the color of kidney was changed from bright red to dark red, which indicated the successful modeling of ischemia. The aneurysm clip was released after one hour of maintaining the ischemia. Then the kidney turned out to be bright red from dark red, which indicated that the reperfusion was completed.

3.2. Ultrasonic contrast testing

With 3–5 s after injecting the contrast agent, it could be seen that the contrast agent was enhanced in the renal artery, arcuate artery, cortex and medullar substance in turn. The signal of contrast agent for animals in IRI groups was obviously delayed, as well as the fading time. As quantitative analysis results shown in Table 1, compared with the control group, AT and TIP were

Table 1
Comparison of quantitative indices of ultrasonic contrast between groups.

Group	A (dB)	AT (s)	TTP (s)	Area (dBs)	Grad (dB/s)
Ctrl	16.92 ± 12.31	7.32 ± 1.05	7.25 ± 0.80	630.62 ± 107.00	4.17 ± 0.29
IRI12	15.07 ± 9.26	11.06 ± 2.35	12.94 ± 1.61*	896.40 ± 63.00*	1.57 ± 0.39*
IRI24	16.39 ± 10.76	12.80 ± 4.08*	18.14 ± 4.06*	971.66 ± 201.00*	1.49 ± 0.28*
IRI48	15.60 ± 16.20	13.49 ± 1.95*	23.69 ± 3.19**	939.00 ± 167.00*	1.19 ± 0.83*

vs. Ctrl $^{*}P < 0.05$; $^{**}P < 0.01$.

all increased in IRI groups and they reached to the peak in IRI48 group, with the value of (13.49 ± 1.95) s and (23.69 ± 3.19) s respectively. The curve's ascending slope (Grad) in IRI groups was decreased compared with that in the control group, showing the negative correlation with the time. There was no significant difference in the area under curve (Area) between IRI groups ($P > 0.05$), but they were all significantly higher than that in the control group ($P < 0.05$).

3.3. Expression of angiogenesis-related factors

3.3.1. Expression of related genes

To study the changes in the expression of *VEGF* during renal IRI, the expression of *VEG*F in the renal tissue of animals in each group was discussed at first. Real-time PCR was employed to detect the difference in the expression of *VEGF* at mRNA level. The Ctrl group was the control and the relative expression of VEGF in Ctrl group was 1 and the expressions of *VEGF* in *IRI12*, *IRI24* and *IRI48* groups were 2.75 ± 0.66, 5.74 ± 1.06 and 8.89 ± 0.75 respectively, which were significantly increased ($P < 0.05$). Besides, the expression of *VEGF* was also increased with the time of reperfusion.

3.3.2. Expression of related factors

After extracting the total protein from the tissue, the expression of VEGF factors was tested at the protein level. The western blotting assay was performed to detect the expression of related molecules in the total protein of renal tissue. Quantity one v4.62 was used to measure the gray value of molecular band, taking VEGF/GAPDH as the reference of relative expression. According to the results, the expression trend of VEGF was in accordance with the findings of Real-time PCR, namely the low expression in the control group and increased expression of VEGF after the reperfusion injury (*vs.* Ctrl $P < 0.05$).

3.4. Correlation analysis

The results showed that the correlation coefficient between three AT and TIP and the relative expression of VEGF was all over 0.9, which indicated the high correlation. But there was little difference in the perfusion peak intensity between groups, showing no correlation with the expression of VEGF.

4. Discussion

After the ischemia in the renal tissue, with the decrease in the blood flow, the tissue may be injured under the state of hypoxia and low ATP. The ischemia reperfusion can recover the function of tissue. However, because of the long period of ischemia and the renal tissue lacks the collateral circulation, the recovery of blood flow may cause the worse injury against the tissue, which is called the IRI. In the clinical practice, the IRI of renal tissue may appear during the treatment of hemorrhagic or toxic shock, renal transplantation and renal lithiasis. The mechanism of IRI of renal tissue has not been clear yet. According to the previous research, the ATP depletion because of insufficient blood supply, occurrence of OFR after restoring the blood supply, and renal tubular and renal glomerular cell injury mediated by the related genes of cell apoptosis and angiogenesis were all related to the IRI of renal tissue [5]. The tissue ischemia reperfusion is always accompanied by the angiogenesis. When the tissue is in the state of ischemia, the changes in the microenvironment of neovascularization will activate a series of signaling pathways to achieve the proliferation, migration and remodeling of vascular endothelial cells based on the original vessel and finally generate the new blood vessels. It was some kind of compensation to cope with the injury caused by ischemia [13,14]. VEGF and bFGF are two important pro-angiogenesis factors. *VEGF* gene consists of 8 exons and 7 introns, which is located in the chromosome 6p21.3. There are different subtypes because of the different exon shearing, where *VEGF121*, *VEGF165* and *VEGF189* are expressed in human being [15,16]. VEGF can be bound with its receptor to change its conformation and then activate the signaling pathway, cause the inflow of sodium ion, regulate the fibrinolysis, induce the expression of endothelial cell integrins and finally promote the angiogenesis and maintain the integrity of new blood vessels [17]. Therefore, VEGF plays a key role in the process of angiogenesis.

The ultrasonic contrast is the new technique applied in the clinical practice in recent years. By injecting the contrast agent, it was capable to dynamically and clearly show the microvessels, significantly improve the differentiation, sensibility and specificity of ultrasound diagnosis [8,9,18,19]. The ultrasonic contrast technique has become the sophisticated tool in the clinical diagnosis and treatment. With the great advances in the ultrasonic contrast technique in recent years, its clinical value has been focused gradually. In this study, based on the building of animal model, the ultrasonic contrast was employed to monitor the renal IRI in rabbits and discuss the relationship between the IRI and VEGF of renal tissue at the mRNA and protein level, in order to provide the certain experimental reference for the study on the mechanism of IRI of renal tissue.

Because IRI process is related to the selection of model animals and tissue properties, IRI process is quite different in the different animal models and tissues. In this study, the rabbit renal model was adopted to study the process of IRI. By completely blocking the artery and maintaining the reperfusion for the certain period, the ultrasonic contrast technique was employed for the observation and detection, which could clearly observe the process of IRI and obtain a series of IRI parameters.

During the modeling, 20 3-month New Zealand rabbits were randomly divided into 4 groups, namely Ctrl group, IRI-12 h, IRI-24 h and IRI-48 h. The appropriate application of anesthetics

is essential in the animal experiment. In this study, animals were given the intraperitoneal anesthesia of 20% urethane with the fast working speed and high degree, which could meet the demands of experiment [20]. Under the condition of ischemia, it could be seen that the color of kidney was changed from bright red to dark red, which indicated the successful modeling of ischemia. The aneurysm clip was released after one hour of maintaining the ischemia. Then the kidney turned out to be bright red from dark red, which indicated that the reperfusion was completed. According to the results of ultrasonic contrast, compared with the control group, AT and TIP were all increased in IRI groups and they reached to the peak in IRI48 group, with the value of (13.49 ± 1.95) s and (23.69 ± 3.19) s respectively. The Grad in IRI groups was decreased compared with that in the control group, showing the negative correlation with the time. There was no significant difference in the area under curve (Area) between IRI groups ($P > 0.05$), but they were all significantly higher than that in the control group ($P < 0.05$). After the ultrasonic contrast testing, the renal tissue was isolated and the expression of *VEGF* in the renal tissue of animals in each group was studied at mRNA level. Real-time PCR was employed to detect the difference in the expression of *VEGF* at mRNA level. As the control, the relative expression of *VEGF* in Ctrl group was 1 and the expression of *VEGF* in IRI groups (IRI12, IRI24 and IRI48) was significantly increased ($P < 0.05$). Besides, the expression of *VEGF* was also increased with the time of reperfusion. Such result indicated that the regulation of expression of *VEGF* could promote the angiogenesis and the compensation was to cope with the injury caused by ischemia. The further testing at the protein level had the same results. To study the correlation between the IRI and angiogenesis, the relative expression of three factors in the renal tissue of animals in each group (Real-time PCR) was analyzed. Besides, Minitab15 was employed for the correlation analysis (with the vertical axis as the relative expression of VEGF and horizontal axis as the mean of ultrasonic contrast parameters). Minitab15 is a simple and practical software that integrates the functions of statistical analysis, mass analysis and correlation analysis. By loading the different functions, it is easy to analyze the statistical data. The correlation between the ultrasonic contrast parameters and the expression of VEGF was analyzed respectively. The results showed that the correlation coefficient between three AT and TIP and the relative expression of VEGF was all over 0.9, which indicated the high correlation. But there was little difference in the perfusion peak intensity between groups, showing no correlation with the expression of VEGF. The specific molecular mechanism for the changes in the expression of VEGF and during the occurrence and development of IRI should be the focus of the further studies.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: pathogenesis and treatment. *World J Transplant* 2015; **5**(2): 52-67.

[2] Erol B, Turker T, Tok A, Bektas S, Mungan G, Ozkanli S, et al. The protective effects of tadalafil on renal damage following ischemia reperfusion injury in rats. *Kaohsiung J Med Sci* 2015; **31**(9): 454-462.

[3] Xu L, Li Y, Fu Q, Ma S. Perillaldehyde attenuates cerebral ischemia-reperfusion injury-triggered overexpression of inflammatory cytokines via modulating Akt/JNK pathway in the rat brain cortex. *Biochem Biophys Res Commun* 2014; **454**(1): 65-70.

[4] Chen JN, Wang CM. Research advances in renal ischemia reperfusion injury. *Med Inform* 2010; **13**(7): 2246-2247.

[5] Sun Y, Pu LY, Lu L, Wang XH, Zhang F, Rao JH. N-acetylcysteine attenuates reactive-oxygen-species-mediated endoplasmic reticulum stress during liver ischemia-reperfusion injury. *World J Gastroenterol* 2014; **20**(41): 15289-15298.

[6] Engels EA, Jennings L, Kemp TJ, Chaturvedi AK, Pinto LA, Pfeiffer RM, et al. Circulating TGF-β1 and VEGF and risk of cancer among liver transplant recipients. *Cancer Med* 2015; **4**(8): 1252-1257.

[7] Wang JH, Liu N, Du HW, Weng JS, Chen RH, Xiao YC, et al. Effects of adipose-derived stem cell transplantation on the angiogenesis and the expression of bFGF and VEGF in the brain post focal cerebral ischemia in rats. *Chin J Cell Mol Immunol* 2008; **24**(10): 958-961.

[8] Joo I. The role of intraoperative ultrasonography in the diagnosis and management of focal hepatic lesions. *Ultrasonography* 2015; **34**(4): 246-257.

[9] Chiorean L, Schreiber-Dietrich D, Braden B, Cui XW, Buchhorn R, Chang JM, et al. Ultrasonographic imaging of inflammatory bowel disease in pediatric patients. *World J Gastroenterol* 2015; **21**(17): 5231-5241.

[10] Yen PL, Wu HK, Tseng HS, Kuo SJ, Huang YL, Chen HT, et al. Vascular morphologic information of three-dimensional power Doppler ultrasound is valuable in the classification of breast lesions. *Clin Imaging* 2012; **36**(4): 267-271.

[11] Kachewar SG, Gandage SG. A classification of patterns of fetal middle cerebral artery velocity waveforms as seen on Doppler ultrasound. *Jpn J Radiol* 2012; **30**(7): 582-588.

[12] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 [-Delta Delta C (T)]. *Methods* 2001; **25**(4): 402-408.

[13] Li H. *Change in expression of HIF-VEGF-Notch signaling pathway-related factors after renal ischemia reperfusion injury* [Master's thesis]. China: Nanchang University; 2012.

[14] Mi H, Haeberle H, Barres BA. Induction of astrocyte differentiation by endothelial cells. *J Neurosci* 2001; **21**(5): 1538-1547.

[15] Patel KR, Vajaria BN, Begum R, Patel JB, Shah FD, Joshi GM, et al. VEGFA isoforms play a vital role in oral cancer progression. *Tumour Biol* 2015; **36**(8): 6321-6332.

[16] Li H, Guo S, Ren Y, Wang D, Yu H, Li W, et al. VEGF189 expression is highly related to adaptation of the plateau pika (*Ochotona curzoniae*) inhabiting high altitudes. *High Alt Med Biol* 2013; **14**(4): 395-404.

[17] Yang J, Chen MQ, Dong J. Progress in anti-tumor angiogenesis research with VEGF/VEGFR2 pathway as a target. *World Chin J Digestol* 2007; **15**(34): 3611-3615.

[18] Yoon JH, Cho A, Lee HS, Kim EK, Moon HJ, Kwak JY. Thyroid incidentalomas detected on 18F-fluorodeoxyglucose-positron emission tomography/computed tomography: thyroid imaging reporting and data system (TIRADS) in the diagnosis and management of patients. *Surgery* 2015; **158**(5): 1314-1322.

[19] Choi JS, Choi Y, Kim EK, Yoon JH, Youk JH, Han KH, et al. A risk-adapted approach using US features and FNA results in the management of thyroid incidentalomas identified by 18F-FDG PET. *Ultraschall Med* 2014; **35**(1): 51-58.

[20] Li XM. *The levels and significance of serum NGAL during the ischemia-reperfusion injury happened in rat kidney* [Master's thesis]. China: Qinghai University; 2012.