




Effect of inhibiting P38MAPK on inflammatory factors and cell apoptosis during flap ischemia-reperfusion injury

Tuo Chen , Rong-Chun Wang, Shi-Bin Lin, Jie-Peng Su, Wei-Feng Guo

Department of Hands and Feet Surgery, Dongguan Changping Hospital in Guangdong Province, Dongguan 523573, China

ARTICLE INFO

Article history:

Received 7 Jul 2016

Received in revised form 17 Jul 2016

Accepted 12 Jul 2016

Available online 24 Jul 2016

Keywords:

Transplanted flap

Ischemia-reperfusion injury

Inflammation

Oxidative stress

Apoptosis

ABSTRACT

Objective: To study the effect of inhibiting P38 mitogen activated protein kinase (P38MAPK) on inflammatory factors and cell apoptosis during flap ischemia-reperfusion injury. **Methods:** Wistar rats were selected as experimental animals and randomly divided into control group, model group and intervention group ($n=12$), control group were made into routine abdominal superficial arteriovenous flap models, model group were made into ischemia-reperfusion flap models and intervention group were made into ischemia-reperfusion flap models and then received SB202190 intervention. 8 d after flap making, tissue was collected to detect the expression of inflammatory factors and apoptosis molecules as well as the levels of oxidative stress indicators. **Results:** NF- κ B, IL-6, TNF- α , Bax and Caspase-3 mRNA expression and protein expression in flap tissue of model group were significantly higher than those of control group ($P<0.05$), ROS, MDA, AOPP and 8-OHdG levels were significantly higher than those of control group, and Bcl-2 mRNA expression and protein expression were significantly lower than those of control group ($P<0.05$); NF- κ B, IL-6, TNF- α , Bax and Caspase-3 mRNA expression and protein expression in flap tissue of intervention group were significantly lower than those of model group ($P<0.05$), ROS, MDA, AOPP and 8-OHdG levels were significantly lower than those of model group ($P<0.05$), and Bcl-2 mRNA expression and protein expression were significantly higher than those of model group ($P<0.05$). **Conclusions:** Inhibiting P38MAPK can reduce the transplanted flap ischemia-reperfusion injury caused by inflammation, oxidative stress and cell apoptosis.

1. Introduction

Flap transplantation is a common method for skin defect repair, and the transplanted flap survival is the main factor determining treatment effect. Flap will experience ischemia and reperfusion in the process of transplantation, and ischemia-reperfusion injury will directly affect the flap survival state[1]. The ischemia-reperfusion injury after flap transplantation mainly occurs in the free flap that

is transplanted from one area to another area, and the inflammation and oxidative stress in the process of ischemia reperfusion are the important pathological processes that cause skin damage[2,3]. Therefore, inhibiting inflammation and oxidative stress is the important target to relieve the transplanted flap ischemia-reperfusion injury and improve the flap survival rate. P38 mitogen activated protein kinase (P38MAPK) signaling pathway is a classic pathway of the MAPK family that has regulatory effect on the secretion of inflammatory factors and the release of oxygen free radicals[4], and SB202190 can target and inhibit P38MAPK activation. In the following study, the effect of inhibiting P38MAPK on inflammatory factors and cell apoptosis in the process flap ischemia-reperfusion injury was analyzed.

Corresponding author: Tuo Chen, Department of Hands and Feet Surgery, Dongguan Changping Hospital in Guangdong Province, Dongguan 523573, China.

Tel: 13712221269

Fund project: Medical Health Project of Dongguan City (No: 200510515035114).

2. Materials and methods

2.1. Research materials

Experimental animals were male Wistar rats purchased from laboratory animal center of Zhongshan University, production license No. was SYXK (Yue) 2011-0029, there were a total of 36 rats and the body mass was 200–300 g; P38MAPK inhibitor SB202190 was purchased from Sigma Company. Enzyme-linked immunosorbent assay kits were from the R&D Company, and the radioimmunoprecipitation kits were from Nanjing Jiancheng Biological Company.

2.2. Model establishment and drug intervention methods

The Wistar rats were randomly divided into control group, model group and intervention group, 12 in each group. The control group were made into conventional abdominal superficial arteriovenous flap models, 3-0 silk was used to flap suture in situ; model group were made into abdominal superficial arteriovenous flap models, then a microscope vascular clamp was used for abdominal superficial arteriovenous clamping, the vascular clamp was removed after six hours and 3-0 silk was used for flap suture in situ; according to the methods of model group, intervention group were made into flap ischemia-reperfusion models, and received intraperitoneal injection of SB202190 2, 4 and 6 d after flap suture in situ respectively, and the dosage was 2 µg/kg.

2.3. Flap tissue collection methods

8 d after flap suture in situ, the rats were executed and anatomized along the suture site to get the flap, which was washed with saline for 2 or 3 times, frozen in liquid nitrogen for 0.5 h, then taken out and placed in a -80 °C refrigerator for long-term preservation.

2.4. Gene expression detection methods

Proper amount of flap tissue was collected, added in Trizol lysis buffer and fully homogenized to extract total RNA, then RT-PCR kits were used to amplify NF-κB, IL-6, IL-8, TNF-α, Bcl-2, Bax and

Caspase-3, and the amplification curve was used to calculate the NF-κB, IL-6, IL-8, TNF-α, Bcl-2, Bax and Caspase-3 mRNA expression of; proper amount of flap was collected, added in RIPA lysis buffer and fully homogenized to extract total protein, then enzyme-linked immunosorbent assay kits were used to determine the NF-κB, IL-6, IL-8, TNF-α, Bcl-2, Bax and Caspase-3 levels, BCA kits were used to determine the total protein content, and then the NF-κB, IL-6, IL-8, TNF-α, Bcl-2, Bax and Caspase-3 levels per unit mass total protein were calculated.

2.5. Oxidative stress index detection methods

Proper amount of flap tissue was collected, added in PBS buffer and then fully grinded, the grinded tissue fluid was centrifuged in the 4 °C centrifuge at 12 000 r/min for 15 min to separate supernatant, radioimmunoprecipitation kits were used to determine ROS, MDA, AOPP and 8-OHdG levels, and BCA kit test results were used to calculate the ROS, MDA, AOPP and 8-OHdG levels per unit mass total protein.

2.6. Statistical analysis

SPSS21.0 software was used to input and analyze data, measurement data comparison among three groups was by variance analysis, pair-wise comparison was by *LSD-t* test and $P < 0.05$ indicated statistical significance in differences.

3. Results

3.1. Inflammatory factor expression in flap tissue

Analysis of inflammatory factors NF-κB, IL-6 and TNF-α expression in flap tissue among three groups of rats is as follows: NF-κB, IL-6 and TNF-α mRNA expression and protein expression in flap tissue of model group were significantly higher than those of control group; NF-κB, IL-6 and TNF-α mRNA expression and protein expression in flap tissue of intervention group were significantly lower than those of model group. Differences in NF-κB, IL-6 and TNF-α mRNA expression and protein expression in flap tissue were statistically significant among three groups of rats ($P < 0.05$) (Table 1).

Table 1

Comparison of inflammatory factor expression in flap tissue among three groups of rats ($n=12$, $\bar{x} \pm s$).

Groups	mRNA expression			Protein expression (ng/mg total protein)		
	NF-κB	IL-6	TNF-α	NF-κB	IL-6	TNF-α
Control group	1.05±0.15	1.02±0.14	0.98±0.12	2.36±0.35	1.89±0.24	4.02±0.56
Model group	2.78±0.36 [*]	2.31±0.34 [*]	2.55±0.31 [*]	5.48±0.71 [*]	4.25±0.52 [*]	9.33±1.02 [*]
Intervention group	1.65±0.22 ^{&}	1.37±0.19 ^{&}	1.65±0.20 ^{&}	3.41±0.48 ^{&}	2.42±0.32 ^{&}	5.67±0.73 ^{&}

^{*}: compared with control group, $P < 0.05$; [&]: compared with model group, $P < 0.05$.

Table 2Comparison of ROS and related oxidation product levels in flap tissue among three groups of rats ($n=12$, $\bar{x}\pm s$).

Groups	ROS (U/mL)	MDA ($\mu\text{mol/L}$)	AOPP ($\mu\text{mol/L}$)	8-OHdG (ng/mL)
Control group	1.05 \pm 0.15	3.58 \pm 0.45	2.68 \pm 0.34	1.98 \pm 0.25
Model group	3.53 \pm 0.48 [*]	9.14 \pm 1.05 [*]	6.51 \pm 0.78 [*]	4.57 \pm 0.57 [*]
Intervention group	1.89 \pm 0.22 [⊗]	4.48 \pm 0.53 [⊗]	3.48 \pm 0.41 [⊗]	2.54 \pm 0.33 [⊗]

* : compared with control group, $P<0.05$; [⊗]: compared with model group, $P<0.05$.**Table 3**Comparison of apoptosis molecule expression in flap tissue among three groups of rats ($n=12$, $\bar{x}\pm s$).

Groups	mRNA expression			Protein expression (ng/mg total protein)		
	Bax	Bcl-2	Caspase-3	Bax	Bcl-2	Caspase-3
Control group	1.03 \pm 0.14	0.98 \pm 0.11	1.05 \pm 0.13	1.87 \pm 0.22	2.57 \pm 0.33	2.24 \pm 0.33
Model group	2.56 \pm 0.36 [*]	0.45 \pm 0.07 [*]	2.37 \pm 0.34 [*]	4.59 \pm 0.57 [*]	1.03 \pm 0.14 [*]	5.15 \pm 0.67 [*]
Intervention group	1.57 \pm 0.22 [⊗]	0.79 \pm 0.09 [⊗]	1.44 \pm 0.18 [⊗]	2.44 \pm 0.34 [⊗]	1.78 \pm 0.22 [⊗]	3.52 \pm 0.44 [⊗]

* : compared with control group, $P<0.05$; [⊗]: compared with model group, $P<0.05$.

3.2. ROS and related oxidation product levels in flap tissue

Analysis of ROS and related oxidation products MDA, AOPP and 8-OHdG levels in flap tissue among three groups of rats is as follows: ROS, MDA, AOPP and 8-OHdG levels in flap tissue of model group were significantly higher than those of control group; ROS, MDA, AOPP and 8-OHdG levels in flap tissue of intervention group were significantly lower than those of model group. Differences in pair-wise comparison of ROS, MDA, AOPP and 8-OHdG levels in flap tissue were statistically significant among three groups of rats ($P<0.05$) (Table 2).

3.3. Apoptosis molecule expression in flap tissue

Analysis of apoptosis molecules Bcl-2, Bax and Caspase-3 expression in flap tissue among three groups of rats is as follows: Bcl-2 mRNA expression and protein expression in flap tissue of model group were significantly lower than those of control group while Bax and Caspase-3 mRNA expression and protein expression were significantly higher than those of control group; Bcl-2 mRNA expression and protein expression in flap tissue of intervention group were significantly higher than those of model group while Bax and Caspase-3 mRNA expression and protein expression were significantly lower than those of model group. Differences in Bcl-2, Bax and Caspase-3 mRNA expression and protein expression in flap tissue were statistically significant among three groups of rats ($P<0.05$) (Table 3).

4. Discussion

Ischemia-reperfusion injury after flap transplantation is an important factor that causes the flap necrosis and transplantation failure, and the inflammation and oxidative stress are considered to be the main pathological links that cause tissue injury during ischemia-reperfusion process. Therefore, antagonizing inflammation and oxidative stress is regarded as the important target to reduce

ischemia-reperfusion injury and improve the flap survival rate. P38MAPK is a member of the MAPK family, is involved in intracellular signal transmission, and mainly regulates intracellular inflammatory and oxidative stress response process[5]. External physical and chemical factor stimulation and ischemia-reperfusion process can significantly activate P38MAPK signaling pathway, and cause the ATF2N-terminal No. 69 and No. 71 threonine phosphorylation to start the expression of a variety of genes[6,7]. In order to define the role of P38MAPK signaling pathway in the flap ischemia-reperfusion injury process, the flap ischemia-reperfusion injury animal models were established and then treated with the specific inhibitor SB202190 of P38MAPK in order to clear the activation of P38MAPK signaling pathway during flap ischemia-reperfusion injury and the protective effect of inhibiting P38MAPK on flap ischemia-reperfusion injury.

IL-6 and TNF- α are the important mediators that mediate the inflammation during transplanted flap ischemia-reperfusion injury, and ischemia-reperfusion stimulus will activate the NF- κ B and start the expression of IL-6 and TNF- α [8]. In the study, analysis of the differences in the above inflammatory cytokine expression in flap tissue between model group and control group showed that NF- κ B, IL-6 and TNF- α mRNA expression and protein expression in flap tissue of model group significantly increased. This shows that ischemia-reperfusion process will activate the inflammatory response in transplanted flap, and increase the expression and release of inflammatory cytokines. P38MAPK is an important signaling pathway mediating inflammatory reaction, NF- κ B is an important transcription factor in the signaling pathway downstream, and the cascade activation of signaling pathways will increase the NF- κ B expression, promote its transfer into the nucleus, and then start the expression of IL-6, TNF- α and other inflammatory mediators[9,10]. In order to further make clear the effect of inhibiting P38MAPK on the inflammatory response during transplanted flap ischemia-reperfusion, the differences in above inflammatory cytokine expression in flap tissue between intervention group and model group were analyzed, and the results showed that NF- κ B, IL-6 and TNF- α mRNA expression and protein expression in flap tissue of intervention group were significantly lower than those of

model group ($P<0.05$). This means that inhibiting P38MAPK can relieve the inflammatory response during transplanted flap ischemia reperfusion.

In the process of transplanted flap ischemia-reperfusion injury, in addition that the activation of the inflammatory response is associated with flap injury, oxidative stress reaction is also a main factor that causes flap damage[11,12]. When transplanted flap ischemia-reperfusion occurs, the production of oxygen free radicals significantly increases, and they lead to antioxidant consumption and continuous accumulation in local area, which causes the oxidation reaction of lipid, protein, nucleic acid and other compositions in cells to lead to cell structure and function damage, and also produce MDA, AOPP, 8-OHdG and other oxidation product[13,14]. In the study, analysis of above oxidative stress product levels in flap tissue between model group and control group showed that ROS, MDA, AOPP and 8-OHdG levels in flap tissue of model group significantly increased ($P<0.05$). This means that ischemia-reperfusion process can increase the production of oxygen free radicals and increase the oxidative stress reaction in the transplanted flaps. Further analysis of the differences in above oxidative stress product levels in flap tissue between intervention group and model group showed that ROS, MDA, AOPP and 8-OHdG levels in flap tissue of intervention group were significantly lower than those of model group ($P<0.05$). This means that inhibiting P38MAPK can relieve the oxidative stress during transplanted flap ischemia reperfusion.

The inflammation and oxidative stress caused by ischemia reperfusion can cause transplanted flap damage, which is specifically characterized by the excessive cell apoptosis in transplanted tissue. Mitochondrial apoptosis pathway is an important mechanism to regulate cell apoptosis, and the inflammation and oxidative stress can cause mitochondrial membrane structure damage and cytochrome C release from the mitochondria into the cytoplasm, and then activate Caspase-3 and induce cell apoptosis through the cascade amplification mediated by Caspase[15]. Bcl-2 and Bax are the key molecules that adjust the mitochondrial membrane transition pore opening and closing, Bcl-2 has inhibitory effect on the opening of mitochondrial membrane transition pore, and can thus reduce the cytochrome C that enters into the cytoplasm and inhibit cell apoptosis process; Bax can form heterodimer with Bcl-2, antagonize the Bcl-2 function, and then promote cell apoptosis process[16]. In the study, analysis of the apoptosis-related molecule expression in transplanted flap showed that Bcl-2 mRNA expression and protein expression in flap tissue of intervention group were significantly higher than those of model group ($P<0.05$) while Bax and Caspase-3 mRNA expression and protein expression were significantly lower than those of model group ($P<0.05$). This means that inhibiting P38MAPK can inhibit the cell apoptosis during transplanted flap ischemia reperfusion.

To sum up, it is believed that inhibiting P38MAPK can reduce the transplanted flap ischemia-reperfusion injury, which is specifically characterized by inhibiting inflammation, oxidative stress and cell apoptosis.

References

- [1] Al-Juboori MJ. Flap designs for implant-related surgical procedures: a review. *Implant Dent* 2016; **25**(6): 845-854.
- [2] Silva JJ, Pompeu DG, Ximenes NC, et al. Effects of kaurenoic acid and arginine on random flap oxidative stress, inflammation, and cytokines in rats. *Aesthetic Plast Surg* 2015; **39**(6): 971-977.
- [3] Joshi YB, Praticò D. The 5-lipoxygenase pathway: oxidative and inflammatory contributions to the Alzheimer's disease phenotype. *Front Cell Neurosci* 2015; **14**(8): 436.
- [4] Ju J, Wu J, Hou R. Role of the p38 mitogen-activated protein kinase signaling pathway in estrogen-mediated protection following flap ischemia-reperfusion injury. *Cell Biochem Funct* 2016; **34**(7): 522-530.
- [5] Han HH, Lim YM, Park SW, et al. Improved skin flap survival in venous ischemia-reperfusion injury with the use of adipose-derived stem cells. *Microsurgery* 2015; **35**(8): 645-652.
- [6] Ozkan O, Ozkan O, Bektas G, et al. The relationship between ischemia time and mucous secretion in vaginal reconstruction with the jejunal free flap: an experimental study on the rat jejunum. *Ann Plast Surg* 2015; **75**(1): 98-101.
- [7] Klarik Z, Tamas R, Toth E, et al. Intra and postoperative evaluations of microcirculation and micro-rheological parameters in a rat model of musculocutaneous flap ischemia-reperfusion. *Acta Cir Bras* 2015; **30**(8): 551-560.
- [8] Wang J, Min P, Grasseti L, et al. Preliminary outcomes of distal IMAP and SEAP flaps for the treatment of unstable keloids subject to recurrent inflammation and infections in the lower sternal and upper abdominal areas. *J Reconstr Microsurg* 2015; **31**(9): 621-630.
- [9] De Toni LG, Menaldo DL, Cintra AC, et al. Inflammatory mediators involved in the paw edema and hyperalgesia induced by Batroxase, a metalloproteinase isolated from *Bothrops atrox* snake venom. *Int Immunopharmacol* 2015; **28**(1): 199-207.
- [10] Du W, Wu PF, Qing LM, et al. Systemic and flap inflammatory response associates with thrombosis in flap venous crisis. *Inflammation* 2015; **38**(1): 298-304.
- [11] Xu M, Lai Y, Torner J, et al. Base excision repair of oxidative DNA damage coupled with removal of a CAG repeat hairpin attenuates trinucleotide repeat expansion. *Nucleic Acids Res* 2014; **42**(6): 3675-3691.
- [12] Bozkurt M, Kapi E, Kulahci Y, et al. Antioxidant support in composite musculo-adipose-fasciocutaneous flap applications: an experimental study. *J Plast Surg Hand Surg* 2014; **48**(1): 44-50.
- [13] Lan L, Nakajima S, Wei L, et al. Novel method for site-specific induction of oxidative DNA damage reveals differences in recruitment of repair proteins to heterochromatin and euchromatin. *Nucleic Acids Res* 2014; **42**(4): 2330-2345.
- [14] Mashima R, Okuyama T. The role of lipoxygenases in pathophysiology; new insights and future perspectives. *Redox Biol* 2015; **6**: 297-310.
- [15] Song K, Zhang M, Hu J, et al. Methane-rich saline attenuates ischemia/reperfusion injury of abdominal skin flaps in rats via regulating apoptosis level. *BMC Surg* 2015; **31**(15): 92.
- [16] Liu YQ, Liu YF, Ma XM, et al. Hydrogen-rich saline attenuates skin ischemia/reperfusion induced apoptosis via regulating Bax/Bcl-2 ratio and ASK-1/JNK pathway. *J Plast Reconstr Aesthet Surg* 2015; **68**(7): e147-156.