




Effect of doxycycline intervention on the apoptosis as well as the IL-1, TNF- α and HIF-1 α expression in cornea and aqueous humor in rats with corneal alkali burn

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ABSTRACT

Objective: To study the effect of doxycycline intervention on the apoptosis as well as the IL-1, TNF- α and HIF-1 α expression in cornea and aqueous humor in rats with corneal alkali burn. **Methods:** Male SD rats were selected as experimental animals and randomly divided into control group, alkali burn group and doxycycline group, corneal alkali burn models were established by sodium hydroxide eye drop and then they received doxycycline eye drops intervention. The expression of apoptosis molecules, inflammatory response cytokines and angiogenesis molecules in the cornea as well as the expression of inflammatory response cytokines and angiogenesis molecules in aqueous humor were detected 14 and 28 d after model establishment. **Results:** 14 and 28 d after model establishment, Bcl-2 and PEDF protein expression in cornea tissue of alkali burn group were significantly lower than those of control group while Caspase-3, Caspase-8, Caspase-9, IL-1, TNF- α , HIF-1 α and VEGF protein expression were significantly higher than those of control group; Bcl-2 and PEDF protein expression in cornea tissue of doxycycline group were significantly higher than those of alkali burn group while Caspase-3, Caspase-8, Caspase-9, IL-1, TNF- α , HIF-1 α and VEGF protein expression were significantly lower than those of alkali burn group. **Conclusion:** Doxycycline for corneal alkali burn intervention can inhibit the apoptosis, inflammatory response and angiogenesis.

1. Introduction

Corneal alkali burn is a clinical common type of ocular trauma, and the blindness rate is very high. Apoptosis and inflammatory response activation in early alkaline burn are important factors for corneal damage, and the angiogenesis in late alkaline burn is the important factor affecting the corneal transparency and visual acuity[1,2]. Therefore, inhibition of apoptosis, inflammatory response and angiogenesis is of great value to reduce the corneal injury caused by alkali burn and improve corneal transparency. Doxycycline is a drug with anti-inflammatory and apoptosis-inducing effects[3], which can inhibit airway inflammation when used for the treatment of asthma, and can affect apoptosis and inhibit angiogenesis when used for the treatment of tumor. IL-1 and

TNF- α are cytokines closely associated with corneal inflammation, and HIF-1 α is a transcription factor closely associated with corneal angiogenesis. In the following studies, we analyzed the effect of doxycycline intervention on the apoptosis as well as the IL-1, TNF- α and HIF-1 α expression in cornea and aqueous humor in rats with corneal alkali burn.

2. Experimental materials and methods

2.1 Experimental materials

A total of 36 male SD rats provided by the Institute of Experimental Animals, Sichuan Academy of Medical Sciences were selected as experimental animals, animal license was SCXK (Sichuan) 2004-16, animal experiments passed the hospital ethical review, and the animal experiments and animal processing after death were carried out according to the rules. Surgical instruments were bought in Shanghai Medical Instrument Co., Ltd., ophthalmic microscope was bought in Olympus Company, doxycycline was

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purchased in Sigma Company, and enzyme-linked immunosorbent assay kit was purchased from Shanghai Westang Biotechnology Company.

2.2 Experimental methods

2.2.1 Animal model preparation

SD rats were randomly divided into control group, alkali burn group and doxycycline group. Alkali burn group and doxycycline group were made into corneal alkali burn models according to the following methods: after intraperitoneal injection of chloral hydrate for anesthesia, tetracaine was injected in conjunctival sac for surface anesthesia, 200 μ L 1 mol/L sodium hydroxide was inhaled in 200 μ L spearhead, vertically dropped in the middle of the cornea and maintained there for 30 s, and 20 mL saline was used to flush the cornea with alkali burn. The control group only received intraperitoneal anesthesia and surface anesthesia, which were followed by saline flushing.

2.2.2 Drug intervention

Doxycycline group were given 3 g/L doxycycline eye drops in the eye with alkali burn, 20 μ L each time, 4 times per day after the alkali burn; control group and alkali burn group were given the sterile saline for eye drop, 20 μ L each time, 4 times per day.

2.2.3 Molecule expression detection

14 d and 28 d after the alkali burn, 6 rats were randomly selected from each group and executed to collected corneal tissue and aqueous humor. The corneal tissue was taken to extract the protein with RIPA lysate, and the enzyme-linked immunosorbent assay kit was used to determine the protein expression of Bcl-2, caspase-3, caspase-8, caspase-9, IL-1, TNF- α , HIF-1 α and VEGF; the aqueous humor was taken, and enzyme-linked immunosorbent assay kit was used to directly detect the protein expression of IL-1, TNF- α , HIF-1 α and VEGF.

Table 1.

Apoptosis molecule expression in cornea of three groups of rats.

Groups	Time	n	Bcl-2	Caspase-3	Caspase-8	Caspase-9
Control group	14 d after model establishment	6	2.25 \pm 0.33	0.94 \pm 0.11	93.6 \pm 10.3	132.5 \pm 15.6
	28 d after model establishment	6	2.31 \pm 0.36	0.92 \pm 0.12	95.1 \pm 10.9	133.7 \pm 14.2
Alkali burn group	14 d after model establishment	6	1.12 \pm 0.13*	2.61 \pm 0.33*	315.2 \pm 45.1*	427.2 \pm 51.4*
	28 d after model establishment	6	0.89 \pm 0.11*	3.13 \pm 0.41*	395.3 \pm 48.2*	541.8 \pm 62.6*
Doxycycline group	14 d after model establishment	6	1.62 \pm 0.18#	1.89 \pm 0.23#	203.3 \pm 28.7#	271.3 \pm 34.6#
	28 d after model establishment	6	1.88 \pm 0.24#	1.52 \pm 0.19#	172.4 \pm 20.1#	210.4 \pm 28.9#

Note: * indicated that compared with control group, differences in indexes were statistically significant; # indicated that compared with alkali burn group, differences in indexes were statistically significant.

Table 2.

IL-1 and TNF- α expression in cornea and aqueous humor of three groups of rats.

Groups	Time	n	Cornea		Aqueous humor	
			IL-1	TNF- α	IL-1	TNF- α
Control group	14 d after model establishment	6	11.31 \pm 1.42	8.31 \pm 0.93	6.73 \pm 0.93	4.52 \pm 0.56
	28 d after model establishment	6	11.22 \pm 1.35	8.44 \pm 0.98	6.68 \pm 0.88	4.49 \pm 0.59
Alkali burn group	14 d after model establishment	6	34.51 \pm 4.58*	27.61 \pm 3.67*	27.82 \pm 3.52*	17.68 \pm 2.25*
	28 d after model establishment	6	43.27 \pm 6.24*	34.52 \pm 5.52*	38.12 \pm 4.91*	22.31 \pm 2.94*
Doxycycline group	14 d after model establishment	6	23.41 \pm 3.29#	18.75 \pm 2.04#	16.06 \pm 2.24#	10.32 \pm 1.27#
	28 d after model establishment	6	18.48 \pm 2.05#	14.21 \pm 1.78#	12.31 \pm 1.46#	8.72 \pm 0.93#

Note: * indicated that compared with control group, differences in indexes were statistically significant; # indicated that compared with alkali burn group, differences in indexes were statistically significant.

2.3 Statistical methods

SPSS 21.0 software was used to input and analyze data, differences in data among three groups were by variance analysis and $P < 0.05$ indicated statistical significance in differences.

3. Results

3.1 Apoptosis molecule expression in cornea

14 and 28 d after model establishment, analysis of apoptosis molecules Bcl-2 (ng/mL), Caspase-3 (ng/mL), Caspase-8 (pg/mL) and Caspase-9 (pg/mL) expression in cornea was as follows: Bcl-2 protein expression in cornea tissue of alkali burn group were significantly lower than those of control group while Caspase-3, Caspase-8 and Caspase-9 protein expression were significantly higher than those of control group; Bcl-2 protein expression in corneal tissue of doxycycline group were significantly higher than those of alkali burn group while Caspase-3, Caspase-8 and Caspase-9 protein expression were significantly lower than those of alkali burn group. Differences in pair-wise comparison of Bcl-2, Caspase-3, Caspase-8 and Caspase-9 protein expression in cornea were statistically significant among three groups of rats.

3.2 IL-1 and TNF- α expression in cornea and aqueous humor

14 and 28 d after model establishment, analysis of inflammatory cytokines IL-1 and TNF- α expression in cornea and aqueous humor was as follows: IL-1 and TNF- α protein expression in cornea and aqueous humor of alkali burn group were significantly higher than those of control group; IL-1 and TNF- α protein expression in cornea and aqueous humor of doxycycline group were significantly lower than those of alkali burn group. Differences in pair-wise comparison of IL-1 and TNF- α expression in cornea and aqueous humor were statistically significant among three groups of rats.

Table 3.

Angiogenesis molecule expression in cornea and aqueous humor of three groups of rats (ng/mL).

Groups	Time	n	Cornea			Aqueous humor		
			HIF-1 α	VEGF	PEDF	HIF-1 α	VEGF	PEDF
Control group	14 d after model establishment	6	1.21 \pm 0.18	2.95 \pm 0.35	4.21 \pm 0.55	0.77 \pm 0.10	1.77 \pm 0.20	2.41 \pm 0.31
	28 d after model establishment	6	1.25 \pm 0.15	2.88 \pm 0.31	4.30 \pm 0.57	0.80 \pm 0.09	1.80 \pm 0.23	2.39 \pm 0.33
Alkali burn group	14 d after model establishment	6	2.89 \pm 0.34 [*]	6.51 \pm 0.89 [*]	2.21 \pm 0.27 [*]	2.21 \pm 0.29 [*]	4.21 \pm 0.56 [*]	1.01 \pm 0.13 [*]
	28 d after model establishment	6	3.77 \pm 0.49 [*]	8.02 \pm 0.95 [*]	1.67 \pm 0.20 [*]	3.14 \pm 0.42 [*]	5.19 \pm 0.62 [*]	0.78 \pm 0.09 [*]
Doxycycline group	14 d after model establishment	6	2.01 \pm 0.28 [#]	4.77 \pm 0.52 [#]	3.19 \pm 0.42 [#]	1.52 \pm 0.19 [#]	3.24 \pm 0.42 [#]	1.77 \pm 0.22 [#]
	28 d after model establishment	6	1.77 \pm 0.24 [#]	4.10 \pm 0.46 [#]	3.44 \pm 0.51 [#]	1.14 \pm 0.15 [#]	2.72 \pm 0.32 [#]	2.03 \pm 0.29 [#]

Note: ^{*} indicated that compared with control group, differences in indexes were statistically significant; [#] indicated that compared with alkali burn group, differences in indexes were statistically significant.

3.3 Angiogenesis molecule expression in cornea and aqueous humor

14 and 28 d after model establishment, analysis of angiogenesis molecules HIF-1 α , VEGF and PEDF expression in cornea and aqueous humor was as follows: HIF-1 α and VEGF protein expression in cornea and aqueous humor of alkali burn group were significantly higher than those of control group while PEDF protein expression were significantly lower than those of control group; HIF-1 α and VEGF protein expression in cornea and aqueous humor of doxycycline group were significantly lower than those of alkali burn group while PEDF protein expression were significantly higher than those of alkali burn group. Differences in pair-wise comparison of HIF-1 α , VEGF and PEDF protein expression in cornea tissue and aqueous humor were statistically significant among three groups of rats.

4. Discussion

Corneal alkali burn is a common type of injury in ophthalmology. Alkali in cornea will not only directly cauterize the cornea and cause damage, but also activate apoptosis and cause corneal damage[4,5]. Bcl-2 is the key molecule regulating mitochondrial pathway of apoptosis[6], and it can inhibit the mitochondrial cytochrome C from entering into the cytoplasm, and thereby inhibit the apoptosis mediated by downstream caspase molecule cascade activation[7,8]. In the study, analysis of apoptosis molecule expression in cornea of alkali burn model showed that Bcl-2 protein expression in cornea tissue of alkali burn group were significantly lower than those of control group while Caspase-3, Caspase-8 and Caspase-9 protein expression were significantly higher than those of control group. This indicates that the apoptosis of the mitochondrial pathway is significantly activated during the corneal burn, and the excessive apoptosis is closely related to corneal injury. Doxycycline is a class of tetracycline that has been shown to be able to affect the apoptosis of multiple tumor cells[9,10]. In order to define the doxycycline

effect on apoptosis in the process of corneal alkali burn, differences in apoptosis molecule expression in cornea tissue were compared between doxycycline group and alkali burn group, and the results showed that Bcl-2 protein expression in cornea tissue of doxycycline group were significantly higher than those of alkali burn group while Caspase-3, Caspase-8 and Caspase-9 protein expression were significantly lower than those of alkali burn group. This indicates that doxycycline has inhibitory effect on apoptosis in corneal caustic burn, and can reduce corneal injury by inhibiting apoptosis.

In the process of corneal alkali burn, alkali will activate significantly chemical inflammation, cause the secretion of a variety of inflammatory cytokine, and then cause corneal injury by the biological role of inflammatory cytokines. IL-1 is a cytokine that is synthesized and released early in the inflammatory response, which not only has a chemotactic effect on multiple inflammatory cells, but can also directly affect the cornea and cause tissue damage[11]; TNF- α is a cytokine produced after the activation of mononuclear macrophages, which can act on corneal tissue to cause damage and results in corneal ulcer[12]. In the study, analysis of the expression of inflammatory cytokines in cornea and aqueous humor of corneal alkali burn model showed that IL-1 and TNF- α protein expression in cornea and aqueous humor of alkali burn group were significantly higher than those of control group. This indicates that the inflammatory response is significantly activated in corneal alkali burn, and the excessive expression and secretion of inflammatory cytokines IL-1 and TNF- α are closely related to corneal injury. Doxycycline has definite anti-inflammatory effect, which can significantly inhibit airway inflammatory response and reduce inflammatory cytokine infiltration in the airway when it is used for the treatment of respiratory tract infection and bronchial asthma[13]. In order to define the doxycycline influence on the inflammatory response in the process of corneal alkali burn, differences in the expression of inflammatory cytokines in cornea and aqueous humor were compared between doxycycline group and alkali burn group, and the results showed that IL-1 and TNF- α protein expression in cornea and aqueous humor of doxycycline group were significantly lower than those of alkali burn group. This indicates that doxycycline has inhibitory effect on the inflammatory response in corneal caustic

burn, and can reduce the corneal injury by inhibiting the secretion of inflammatory cytokines.

Apoptosis and inflammatory reaction occur in the early stage of corneal alkali burn, and the angiogenesis process plays a crucial role in the later stage of corneal healing[14]. In the process, moderate angiogenesis is beneficial to the healing of tissue injury, but excessive angiogenesis will occur under the continuous action of inflammatory factors, which causes the corneal scarring and influences corneal transparency. HIF-1 α is the transcription factor adjusting angiogenesis in hypoxia state, and hypoxia stimuli can cause HIF-1 α accumulation in the nucleus and be combined with VEGF gene promoter regions to activate the expression of VEGF and promote angiogenesis through the biological functions of VEGF[15]. PEDF is an important anti-angiogenesis molecule in the local cornea, which can antagonize the biological function of VEGF and maintain the avascular state within the cornea[16]. In the study, analysis of the expression of angiogenesis molecules in the cornea and aqueous humor of corneal alkali burn model showed that HIF-1 α and VEGF protein expression in cornea and aqueous humor of alkali burn group were significantly higher than those of control group while PEDF protein expression were significantly lower than those of control group. This indicates that there is a pathologic state of hyperactivation of angiogenesis in corneal alkaline burn process, and the excessively generated new blood vessels can affect the healing of corneal injury. Further analysis of the doxycycline effect on angiogenesis in the corneal alkali burn process indicated that HIF-1 α and VEGF protein expression in cornea and aqueous humor of doxycycline group were significantly lower than those of alkali burn group while PEDF protein expression were significantly higher than those of alkali burn group. This indicates that doxycycline has inhibitory effect on the angiogenesis during corneal alkali burn.

Doxycycline for corneal alkali burn intervention can inhibit mitochondrial pathway of apoptosis, reduce inflammatory response and reduce the secretion of the corresponding inflammatory cytokine, and it can also inhibit angiogenesis process.

References

- [1] Paschalis EI, Zhou C, Lei F, Scott N, Kapoulea V, Robert MC, et al. Mechanisms of retinal damage after ocular alkali burns. *Am J Pathol* 2017; **187**(6): 1327-1342.
- [2] Bai JQ, Qin HF, Zhao SH. Research on mouse model of grade II corneal alkali burn. *Int J Ophthalmol* 2016; **9**(4): 487-490.
- [3] Bian F, Pelegriano FS, Henriksson JT, Pflugfelder SC, Volpe EA, Li DQ, et al. Differential effects of dexamethasone and doxycycline on inflammation and mmp production in murine alkali-burned corneas associated with dry eye. *Ocul Surf* 2016; **14**(2): 242-254.
- [4] Li WJ, Hu YK, Song H, Gao XW, Zhao XD, Dong J, et al. Observation on ultrastructure and histopathology of cornea following femtosecond laser-assisted deep lamellar keratoplasty for acute corneal alkaline burns. *Int J Ophthalmol* 2016; **9**(4): 481-486.
- [5] Welling JD, Pike EC, Mauger TF. Alkali burn of the ocular surface associated with a commonly used antifog agent for eyewear: two cases and a review of previous reports. *Cornea* 2016; **35**(2): 289-291.
- [6] Shan M, Fan TJ. Cytotoxicity of carteolol to human corneal epithelial cells by inducing apoptosis via triggering the Bcl-2 family protein-mediated mitochondrial pro-apoptotic pathway. *Toxicol In Vitro* 2016; **35**: 36-42.
- [7] Liu C, Vojnovic D, Kochevar IE, Jurkunas UV. UV-A irradiation activates nrf2-regulated antioxidant defense and induces p53/caspase3-dependent apoptosis in corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2016; **57**(4): 2319-2327.
- [8] Song X, Stachon T, Wang J, Langenbacher A, Seitz B, Szentmáry N. Viability, apoptosis, proliferation, activation, and cytokine secretion of human keratoconus keratocytes after cross-linking. *Biomed Res Int* 2015; **2015**: 254237.
- [9] Zhang L, Xu L, Zhang F, Vlashi E. Doxycycline inhibits the cancer stem cell phenotype and epithelial-to-mesenchymal transition in breast cancer. *Cell Cycle* 2017; **16**(8): 737-745.
- [10] Zhao Y, Wang X, Li L, Li C. Doxycycline inhibits proliferation and induces apoptosis of both human papillomavirus positive and negative cervical cancer cell lines. *Can J Physiol Pharmacol* 2016; **94**(5): 526-533.
- [11] Yan C, Gao N, Sun H, Yin J, Lee P, Zhou L, et al. Targeting imbalance between il-1 β and il-1 receptor antagonist ameliorates delayed epithelium wound healing in diabetic mouse corneas. *Am J Pathol* 2016; **186**(6): 1466-1480.
- [12] Launay PS, Reboussin E, Liang H, Kessal K, Godefroy D, Rostene W, et al. Ocular inflammation induces trigeminal pain, peripheral and central neuroinflammatory mechanisms. *Neurobiol Dis* 2016; **88**: 16-28.
- [13] Lanza GM, Jenkins J, Schmieder AH, Moldobaeva A, Cui G, Zhang H, et al. Anti-angiogenic nanotherapy inhibits airway remodeling and hyper-responsiveness of dust mite triggered asthma in the brown norway rat. *Theranostics* 2017; **7**(2): 377-389.
- [14] Wilkerson JL, Mandal NA. Angiogenesis model of cornea to understand the role of sphingosine 1-phosphate. *Methods Mol Biol* 2017; **1609**: 267-276.
- [15] Ashki N, Chan AM, Qin Y, Wang W, Kiyohara M, Lin L, et al. Peroxynitrite upregulates angiogenic factors VEGF-A, BFGF, and HIF-1 in human corneal limbal epithelial cells. *Invest Ophthalmol Vis Sci* 2014; **55**(3): 1637-1646.
- [16] He J, Cortina MS, Kakazu A, Bazan HE. The PEDF neuroprotective domain plus dha induces corneal nerve regeneration after experimental surgery. *Invest Ophthalmol Vis Sci* 2015; **56**(6): 3505-3513.