Effects of chicken type Ⅱ collagen oral spraying on collagen-induced arthritis in mice

WANG Ni-Ping*, SHEN Yu-Xian, WANG Hua, ZHANG Ling-Ling, YUE Li, WEI Wei
(*Institute of Clinical Pharmacology, Anhui Medical University, Hefei* 230032, *China*)

Abstract: AIM To examine whether chicken type Ⅱ collagen (CⅡ) oral spraying has the therapeutic effect on the collagen-induced arthritis (CIA) in mice and investigate its related immunological mechanisms. **METHODS** CIA mouse model was established. The effect of treatment with CⅡ spraying in mice was measured by paw-swelling score. Splenocytes proliferation, activity of interleukin-1 (IL-1) produced by peritoneal macrophages (PMϕ) and interleukin-2 (IL-2) by splenocytes were assayed by MTT method. **RESULTS** CⅡ oral spraying (5, 10, 20 $\mu g \cdot kg^{-1} \cdot d^{-1} \times 10$ d, d 25 – 35 after immunization) decreased the mean of arthritis scores in CIA mice. The effect of CⅡ spraying was comparable with that of ig CⅡ and prednisone. Meanwhile, CⅡ oral spraying (10, 20 $\mu g \cdot kg^{-1} \cdot d^{-1} \times 10$ d) suppressed the ConA-induced splenocyte proliferation and the activity of IL-1 and IL-2 in CIA mice. **CONCLUSION** CⅡ oral spraying suppresses the polyarthritis of CIA mice, which suggests that CⅡ oral spraying have therapeutic effect on CIA mice. Its mechanism maybe related to the modification of the abnormal immunological function of CIA mice.

Key words: collagen; arthritis; collagen diseases; lymphocytes; cell proliferation; interleukin-1; interleukin-2

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Rheumatoid arthritis (RA) is a common chronic disease with synovial inflammation of multiple joints which causes damage to cartilage and bone. As the pathogenic mechanisms underlying RA are not clear, there are no ideal medicine that can optimally suppress the progress of the disease. Mucosal tolerance provides a new and potential immunotherapy for RA, which refers to the systemic immunological unresponsiveness that occurs after absorbing soluble antigens by oral or respiratory mucosal route[1,2]. Type Ⅱ collagen (CⅡ) is a kind of soluble protein derived from the soft sternum cartilage of young chicken or calf. It is a potential autoantigen in RA.

Collagen-induced arthritis (CIA) is an experimental model of autoimmune-mediated polyarthritis that is produced by systemic immunization with CⅡ. The pathogenesis of CIA in many ways resembles RA in humans. It is a useful model to test and develop new drugs and elucidate the mechanisms of tolerance which may be applicable to human disease[3]. In the present study, CIA model was used to evaluate the anti-arthritis effect of CⅡ and its related mechanisms were also investigated and analyzed.

1 MATERIALS AND METHODS

1.1 Animals and reagents

Kunming mice of either sex, weighting 18 – 22 g; and C_{57}BL/6J male mice, weighting 18 – 22 g, were obtained from Experimental Animal Center of Anhui Medical University (AMU), general grade, Certificate №02. In the present study, the incident of CIA is about 60%. All the CIA mice were randomized into 6 groups of 13 – 15 each.

Chicken CⅡ powder was provided by Institute of Bencao Biological Medicine, Shanghai, and dissolved in distilled water at different concentration before use. Prednisone was obtained from Hualian Pharmaceutical Factory, Shanghai, Lot:

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Biographies: WANG Ni-Ping (1977 –), female, native of Anhui Province, postgraduate, major in anti-inflammatory and immunological pharmacology; WEI Wei (1960 –), male, native of Anhui Province, professor, MD, major in anti-inflammatory and immunological pharmacology and clinical pharmacology.

* Corresponding author. Tel: (0551)5161206, Fax: (0551) 5161206, E-mail: wanip122@163.net

991002. The following reagents were also used: concanavalin A (ConA), lipopolysaccharides (LPS) and HEPES (Sigma); RPMI1640 powder (Gibco USA); bacillus calmette guerin (BCG, Shanghai Biological Products Factory, Lot: 200103001); MTT (Merck, USA); isopropanol (Bengbu Chemical Reagents Factory); neonatal bovine serum (Department of Microbiology, AMU, sacrificed before use by that).

1.2 Preparation and assessment of collagen-induced athritis in mice[4]

CⅡ was dissolved in 0.01 mmol·L^{-1} acetic acid and emulsified with an equal volume of complete Freund's adjuvant (CFA) in ice-bath. The final concentration of CⅡ and BCG was both 2 g·L^{-1}. Emulsion 0.1 mL was injected intradermally into the dorsal root of the tail. For second immunization, the same emulsion was injected subcutaneously on d 21. The mice of control group were received the injection of an equal volume of 0.01 mmol·L^{-1} acetic acid at the same location. After the second immunization, the occurrence of the arthritis was observed by the third person every four days. The severity of arthritis was graded according to the paw-swelling scores: 0 = normal; 1 = redness and swelling in part of the paw; 2 = redness and swelling in whole paw; 3 = redness and swelling below the tarsus joints; 4 = redness and swelling including the tarsus joints. The score of each paw was then added, with 16 as maximal score per animal.

Three dosages of CⅡ spraying(5, 10, 20 μg·kg^{-1}) were sprayed into the oral cavities of the mice, from the onset of paw swelling (d 25), once daily for 10 d. During the same time, CⅡ (10 μg·kg^{-1}) and prednisone (2 mg·kg^{-1}) were given intragastrically (ig) to another two groups. Vehicle group and CIA model one were both treated with distilled water spraying.

1.3 Splenocyte proliferation assay[5]

Kunming mice were sacrificed by cervical dislocation on d 57. Spleens were removed in sterile condition and splenocytes were collected. Then the cells were suspended in RPMI1640 medium at a concentration of 1×10^7 L^{-1}. The cell suspension(100 μL) and ConA(100 μL with final concentration of 3 mg·L^{-1}) or LPS(100 μL with final concentration of 4 mg·L^{-1}) were seeded to 96-well culture plate simultaneously. Triplicates were designed. The cultures were incubated at 37℃ in an atmosphere of 5% CO_2 for 48 h. Two hours before the end, 10 μL of MTT(5 g·L^{-1}) were added to each well. The absorbance at 570 nm ($A_{570\ nm}$) was measured on EJ301 ELISA Microwell Reader.

1.4 Production and assay of interleukin-1[5]

PMϕ of Kunming mice were collected in D-Hanks medium by routine method at d 57. Then PMϕ were resuspended in RPMI1640 medium at 1×10^9 L^{-1} and the cell suspension was seeded into 24-well culture plate, by 1 mL per well. After incubation for 2 h at 37℃ in 5% CO_2 atmosphere, supernatants were removed and the adherent cells were washed with Hanks medium which containing 5% neonatal bovine serum for 3 times. Thus the monolayer of PMϕ was obtained. LPS, with final concentration of 4 mg·L^{-1}, was added to each well and RPMI1640 was also added to make final volume per well up to 1 mL. Then the plate was incubated at 37℃ in air with 5% CO_2 for 48 h. After centrifugation ($500 \times g$, 10 min) all the supernatants containing extracellular IL-1 were collected and stored at −20℃ until assay.

IL-1 activity was measured by ConA-induced thymocytes proliferation assay. Suspension of thymocytes(2×10^9 L^{-1}) taken from C_{57}BL/6J mice was distributed over a flat-bottom 96-well plate. Fifty microliters of PMϕ culture supernatants were added to the cell suspension. The cultures were incubated in the presence of ConA (3 mg·L^{-1}) for 48 h, at 37℃ in a 5% CO_2 incubator. Then MTT method was used to assay thymocytes proliferation.

1.5 Production and assay of interleukin-2[5]

Spleens of Kunming mice were removed in a sterile condition at d 57 and splenocytes were collected and suspended in RPMI1640 medium at a concentration of 1×10^{10} L^{-1}. One hundred microliters of suspension and 100 μL of ConA with final concentration of 3 mg·L^{-1} were added to each

well of 24-well culture plate. And RPMI1640 medium was added to each well to make final volume 1 mL.

IL-2 activity was measured by ConA-induced thymocytes proliferation assay with MTT method.

1.6 Statistical analysis

All data were expressed as $\bar{x} \pm s$. Statistical analysis for the paw-swelling scores was performed with Ridit analysis. Difference between groups in the assays of splenocytes proliferation, IL-1 and IL-2 was evaluated by Dunnett's t test.

2 RESULTS

2.1 Effect of chicken type Ⅱ on polyarthritis of collagen-induced arthritis mice

The onset of paw swelling was on d 25 after injection of emulsion. Results showed that the swelling peak appeared on d 37 and then declined (Tab 1). All three dosages of CⅡ spraying were able to reduce the paw-swelling of CIA mice. Prednisone ig and CⅡ ig also suppressed the joints swelling of CIA mice.

2.2 Effects of chicken type Ⅱ on splenocyte proliferation of collagen-induced arthritis mice

The splenocyte proliferation was assayed on d 57. Tab 2 showed that ConA-induced splenocyte proliferation of CIA mice was decreased after given CⅡ spraying (10, 20 $\mu g \cdot kg^{-1}$), CⅡ ig and prednisone ig continuously for 10 d, while LPS-induced proliferation was not changed.

2.3 Effects of chicken type Ⅱ on interleukin-1 and interleukin-2 production of collagen-induced arthritis mice

Tab 3 showed that the production of IL-1 by PMϕ and IL-2 by splenocytes was upregulated significantly in CIA model. Production of IL-1 and IL-2 was decreased after the CⅡ spraying(10, 20 $\mu g \cdot kg^{-1}$) was given to CIA mice for 10 d. Prednisone suppressed both the production of IL-1 and IL-2. CⅡ ig suppressed the production of IL-1 but had no significant suppressive effect on IL-2 production.

3 DISCUSSION

To date, a number of experimental models and clinical trials have shown the efficacy of oral tolerance in preventing or treating systemic inflammation[2]. The mechanisms of oral tolerance have also been extensively studied. In general, there are two different mechanisms which depend on the dose of antigen fed. Low doses of antigen favor the generation of regulatory cells that suppress the specific immune response in the target organ,

Tab 1. Effect of chicken type Ⅱ (CⅡ) collagen spraying on polyarthritis in collagen-induced arthritis (CIA) mice

Group	Dose /$\mu g \cdot kg^{-1}$	Paw-swelling score					
		d 29	d 33	d 37	d 41	d 45	d 49
Vehicle	–	0.2 ± 0.4	0.3 ± 0.5	0.3 ± 0.5	0.4 ± 0.6	0.3 ± 0.6	0.3 ± 0.5
CIA	–	3.0 ± 1.3**	7.4 ± 2.0**	10.0 ± 1.2**	9.5 ± 1.6**	10.2 ± 1.9**	9.2 ± 1.9**
CIA + CⅡ (spraying)	5	2.6 ± 1.5**	5.1 ± 2.0#**	4.8 ± 2.1##**	5.5 ± 1.9##**	5.5 ± 1.8##**	5.5 ± 1.7##**
	10	3.1 ± 0.9**	4.5 ± 2.2##**	4.8 ± 1.9##**	4.6 ± 1.9##**	4.9 ± 2.1##**	4.7 ± 1.2##**
	20	3.1 ± 2.3**	5.3 ± 2.7#**	4.5 ± 2.3##**	4.9 ± 1.7##**	4.7 ± 1.1##**	4.9 ± 1.4##**
CIA + CⅡ (ig)	10	2.8 ± 1.5**	4.8 ± 2.6##**	4.4 ± 2.3##**	4.3 ± 2.0##**	4.1 ± 1.7##**	4.7 ± 2.0##**
CIA + Prednisone (ig)	2000	2.5 ± 1.8**	4.5 ± 2.9##**	4.7 ± 3.3##**	5.0 ± 3.1##**	4.9 ± 2.9##**	5.2 ± 3.1##**

CIA: CⅡ 2 $g \cdot L^{-1}$, dissolved in 0.01 $mmol \cdot L^{-1}$ acetic acid and emulsified with an equal volume of CFA, 0.1 mL emulsion was injected sc into the dorsal root of the tail, respectively, on d 0 and d 21. CⅡ (spraying): 2.5 $mL \cdot kg^{-1}$ spraying into the oral cavities of mice. All drugs were given once daily from d 25 to d 35. $\bar{x} \pm s$, $n = 13 - 15$. * $P < 0.05$, ** $P < 0.01$, compared with vehicle group; ## $P < 0.01$, compared with CIA model group.

Tab 2. Effects of chicken type Ⅱ collagen spraying on splenocyte proliferation in collagen-induced arthritis mice

Group	Dose/$\mu g \cdot kg^{-1}$	$A_{570\ nm}$	
		ConA	LPS
Vehicle	–	0.350 ± 0.034	0.320 ± 0.028
CIA	–	0.460 ± 0.025**	0.310 ± 0.073
CIA + CⅡ (spraying)	5	0.420 ± 0.025*	0.310 ± 0.038
	10	0.370 ± 0.020##	0.300 ± 0.015
	20	0.330 ± 0.048##	0.340 ± 0.024
CIA + CⅡ (ig)	10	0.390 ± 0.042##	0.290 ± 0.038
CIA + Prednisone (ig)	2000	0.290 ± 0.014##*	0.280 ± 0.023

See Tab 1 for experimental procedure. The splenocytes were collected at d 57 and suspended in RPMI1640 medium at a concentration of $1 \times 10^7\ L^{-1}$. Concanavalin A(ConA) 3 $mg \cdot L^{-1}$ or lipopolysaccharides(LPS) 4 $mg \cdot L^{-1}$(final concentration) was added into the 100 μL suspension. The cultures were incubated at 37℃ in air with 5% CO_2 for 48 h, then splenocytes proliferation was measured by MTT method. $\bar{x} \pm s$, $n = 13 - 15$. * $P < 0.05$, ** $P < 0.01$, compared with vehicle group; ## $P < 0.01$, compared with CIA model group.

Tab 3. Effects of chicken type Ⅱ collagen spraying on production of interleukin-1 (IL-1) by peritoneal macrophages and interleukin-2(IL-2) by splenocytes in collagen-induced arthritis mice

Group	Dose /$\mu g \cdot kg^{-1}$	$A_{570\ nm}$	
		IL-1	IL-2
Vehicle	–	0.314 ± 0.018	0.286 ± 0.022
CIA	–	0.495 ± 0.025**	0.433 ± 0.028**
CIA + CⅡ (spraying)	5	0.479 ± 0.019**	0.394 ± 0.013**
	10	0.391 ± 0.021###	0.364 ± 0.028###
	20	0.369 ± 0.022###	0.337 ± 0.037###
CIA + CⅡ (ig)	10	0.396 ± 0.032###	0.414 ± 0.018**
CIA + Prednisone (ig)	2000	0.317 ± 0.023##	0.307 ± 0.014##

See Tab 1 for experimental procedure. The peritoneal macrophages($1 \times 10^9\ L^{-1}$) and splenocytes ($1 \times 10^{10}\ L^{-1}$) were both collected at d 57 and incubated at 37℃ in air with 5% CO_2 for 48 h. The culture supernatants and ConA(3 $mg \cdot L^{-1}$) were added to the thymocyte suspension (with concentration of $2 \times 10^9\ L^{-1}$) and incubated for another 48 h, then MTT method was used to assay thymocyte proliferation. $\bar{x} \pm s$, $n = 13 - 15$. ** $P < 0.01$, compared with vehicle group; ## $P < 0.01$, compared with CIA model group.

whereas high doses of antigen induce an antigen-specific anergic/deletional state in the peripheral immune system[6]. Oral administration and intranasal administration are two important routes to induce mucosal tolerance. Recent studies showed that intranasal administration is more effective than oral one because the result of the latter depends on the functional state of gastrointestinal system which is often damaged by non-steroidal anti-inflammatory drugs[7] or other factors. In the present study, a new approach of administration is adopted which represents simpler to feed animal than intranasal administration. That is spraying CⅡ solution into oral cavity. This new approach is promising because it will be convenient and acceptable for the clinical treatment. Results of our study demonstrated that following administration of CⅡ spraying, polyarthritis of CIA was ameliorated significantly shown by decreased paw-swelling scores. Inasmuch as there are large quantities of lymph tissue in the oral cavity such as tonsil, lymphatic follicles on the back wall of pharynx, which may act as the mucosa associated lymphoid tissue in gut or nasal cavity, CⅡ spraying may

have similar mechanism with oral tolerance and nasal tolerance. But it needs more verification.

As cytokines play an important role in the pathogenesis of CIA, considerable interest has been directed at exploring the role of IL-1 in CIA. The systemic administration of IL-1β increases the incidence and severity of CIA in mice, and accelerates the onset time of arthritis. Suppressing IL-1 as therapeutic approach in treating arthritis is supported by the observation that CIA can be attenuated in mice by using a variety of biologic agents such as IL-1 receptor antagonist and antibodies to IL-1[8]. In our study, IL-1 production by PMϕ was decreased after CIA mice were given by CⅡ spraying(10, 20 $\mu g \cdot kg^{-1}$).

It was reported that during mucosal tolerance of T cell immunity, Th2 cell, induced by antigens in uptake site, emigrates to peripherial tissue such as spleen, lymph node and mediates immune suppression at those sites[6]. In our study, CⅡ inhibited ConA-induced splenocyte proliferation and decreased IL-2 production by splenic T cell and IL-1 by PMϕ in CIA. Prednisone suppressed ConA-induced splenocyte proliferation and IL-1 production but had no effect on IL-2 production. It suggested that the effect of CⅡ be closely related to T cell immunity.

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Ⅱ型胶原口腔喷雾剂对小鼠胶原性关节炎的治疗作用

汪倪萍，沈玉先，王　华，张玲玲，岳　莉，魏　伟

(安徽医科大学临床药理研究所，安徽 合肥　230032)

摘要：目的　观察鸡Ⅱ型胶原(CⅡ)口腔喷雾剂对小鼠胶原性关节炎(CIA)是否具有治疗作用及相关的机理。**方法**　建立小鼠 CIA 模型，观测关节积分变化。MTT 法检测脾淋巴细胞增殖反应，腹腔巨噬细胞产生白介素-1(IL-1)及脾淋巴细胞产生白介素-2(IL-2)的活性。**结果**　CⅡ口腔喷雾剂(5, 10, 20 $\mu g \cdot kg^{-1} \cdot d^{-1} \times 10$ d，免疫后 d 25 ~ d 35)均能降低 CIA 小鼠关节积分，且作用与 ig CⅡ(10 $\mu g \cdot kg^{-1} \cdot d^{-1} \times 10$ d)及泼尼松龙(2 $mg \cdot kg^{-1} \cdot d^{-1} \times 10$ d)相当。口腔喷雾 CⅡ(10, 20 $\mu g \cdot kg^{-1} \cdot d^{-1} \times 10$ d)能抑制 CIA 小鼠刀豆蛋白 A 诱导的脾淋巴细胞增殖反应和 IL-1、IL-2 的产生活性。**结论**　CⅡ口腔喷雾剂能抑制 CIA 小鼠多发性关节炎，提示其对 CIA 小鼠有治疗作用。其机理可能与改善 CIA 小鼠异常的免疫功能有关。

关键词：胶原；关节炎；胶原病；淋巴细胞；细胞增殖；白介素-1；白介素-2

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