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The effects of genistein and puerarin on the activation of nuclear factor-κB and the production of tumor necrosis factor-α in asthma patients

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Oxidative stress might play an essential role in the pathogenesis of chronic airway inflammatory diseases, indicating that antioxidant therapy may have a potential effect in controlling chronic airway inflammatory diseases. The aim of the present study was to investigate the effect of antioxidants genistein and puerarin on the activation of nuclear factor-κB (NF-κB) and the production of tumor necrosis factor-α (TNF-α) in peripheral blood mononuclear cells (PBMCs) in asthma patients. PBMCs were isolated from blood samples of 32 asthma patients and 31 healthy persons, and randomly divided into four groups, control group, dexamethasone group, genistein group and puerarin group. The expression of NF-κB in nuclei was analysed by immunocytochemical staining. The level of TNF-α was measured by radioimmunoassay. Results showed that the percentage of NF-κB positive cells in PBMCs and the level of TNF-α in PBMCs supernatants were significantly higher in asthma patients 23.1 ± 6.7 %, 2.10 ± 0.38 μg/L than in those of healthy persons 7.2 ± 2.9 %, 0.86 ± 0.53 μg/L (p all < 0.01). There was a positive correlation between the percentage of NF-κB positive cells and the level of TNF-α in asthma patients ($r = 0.709$, $p < 0.01$). The percentages of NF-κB positive cells in PBMCs were significantly decreased in the genistein group 15.2 ± 5.4% and in the puerarin group 16.2 ± 5.1% than those in control groups 23.1 ± 6.7% in asthma patients (p respectively < 0.01, < 0.05). The levels of TNF-α in PBMCs supernatants were remarkably decreased in genistein group 1.08 ± 0.40 μg/L and in puerarin group 1.24 ± 0.29 μg/L than those in control group 2.10 ± 0.38 μg/L in asthma patients (p all < 0.01). There were positive correlations between the percentages of NF-κB positive cells and the levels of TNF-α in genistein group ($r = 0.579$, $p < 0.01$) and in puerarin group ($r = 0.665$, $p < 0.01$) in asthma patients. These results show that the activation of NF-κB and TNF-α pathway of PBMCs may play an important role in the pathogenesis of asthma. Genistein, and puerarin could inhibit the pathway of NF-κB and TNF-α in asthma patients, so it was implicated that asthma patients will benefit from the antioxidants genistein and puerarin.

1. Introduction

A persisting oxidant/antioxidant imbalance is well documented in asthma patients. Several studies have shown that reactive oxygen species (ROS) play a key role in initiation as well as amplification of inflammation in asthma airways. Excessive ROS production in asthma leads to alteration in key enzymatic as well as nonenzymatic antioxidants such as glutathione, vitamins C and E, beta-carotene, uric acid, thioredoxin, superoxide dismutases, catalase, and glutathione peroxidases leading to oxidant-antioxidant imbalance in airways. Oxidant stress leads to pathophysiological effects associated with asthma such as vascular permeability, mucus hypersecretion, smooth muscle contraction, epithelial shedding, and bronchial hyperresponsiveness (Nadeem et al. 2008). The airway inflammation implicated in asthma patients may be amplified by oxidants.

The nuclear factor-kB (NF-κB) is an oxidative stress sensitive transcription factor. It consists of a family of proteins (NF-κB1, NF-κB2, rel-A, rel-B, c-rel) and is present in the cytoplasm of cells where it is attached to an inhibitory protein (IκB). The activation is brought about by phosphorylation and ubiquitination of the IκB subunit followed by its degradation by proteasome, thus allowing NF-κB translocation to the uncleus, where it binds to the promoter regions of various genes particularly those of cytokines and adhesion molecules, thus increase their expression. It has previously been shown that NF-κB is a key regulator of the inflammatory response in asthma. In the mouse model, the inhibition of NF-κB reduces the inflammatory cells infiltration and cytokine expression, and blocks airway inflammation of asthma (Henderson et al. 2002; Choi et al. 2009). Recently there are some reports about the relationship between activation of NF-κB and increased TNF-α in asthma patients (Mehta et al. 2009; Uhl et al. 2009).

Recently, Fitzpatrick et al. (2009) reported that children with severe asthma have increased biomarkers of oxidant stress in the epithelial lining fluid that are associated with increased formation of GSSG and a shift in the GSH redox potential toward the more oxidized state. Further, antioxidants like

Table: Percentage of NF-κB positive cells and the level of TNF-α in asthma patients and healthy persons

Group	n	NF-κB positive cells (%)	TNF-α (μg/L)
Healthy persons	31	7.2 ± 2.9	0.86 ± 0.53
Asthma patients	32	$23.1 \pm 6.7^{**}$	$2.10 \pm 0.38^{**}$

** $p < 0.01$, compared with healthy persons

glutathione-S-transferase (GST) are reduced in asthma patients, and inhalation of mutated GST reduced airway inflammation in mice, and mutated GST can be explored as an adjunct therapy in asthma (Tripathi et al. 2008). Choline administration reduces oxidative stress possibly by modulating the redox status of the cell and inhibits inflammatory response in a mouse model (Mehta et al. 2009). Other antioxidant effects on asthma have been reported (Bede et al. 2008; Hoffmann 2008). Our research group focused on antioxidants effects on some diseases (Zhao et al. 2004; Zhao and Liu 2005; Tang et al. 2005; Liu et al. 2003). Genistein, an isoflavone compound, has been shown to be a broad-spectrum tyrosine kinase inhibitor, and is also one of the antioxidants. Dia et al. (2008) demonstrated that genistein and others possess anti-inflammatory properties and therefore are important in modulating mammalian inflammation pathways which may lead to inhibition of some types of chronic disease. Furthermore, through their interaction they can modulate the inflammatory process Puerarin, the main isoflavone glycoside found in the root of *Pueraria lobata*, has been used for various medicinal purposes in traditional Chinese medicine for thousands of years. Zhang et al. (2008) have reported that puerarin may act as an intracellular ROS scavenger, and its antioxidant properties may protect against beta-amyloid protein 25–35-induced cell injury. Although some antioxidants like genistein and puerarin are useful against oxidative stress, the action of these agents on NF-κB and TNF-α in asthma patients has not been fully elucidated.

2. Investigations and results

2.1. Percentage of NF-κB positive cells and the level of TNF-α in asthma patients

The percentage of NF-κB positive cells in peripheral blood mononuclear cells (PBMCs) $23.1 \pm 6.7\%$ in asthma patients was significantly higher than that in healthy persons $7.2 \pm 2.9\%$ ($p < 0.01$). The level of TNF-α in PBMCs supernatant 2.10 ± 0.38 μg/L in asthma patients was remarkably higher than that in healthy persons 0.86 ± 0.53 μg/L ($p < 0.01$, Table). And a positive correlation between the percentage of NF-κB positive cells in PBMCs and the level of TNF-α in PBMCs supernatant ($r = 0.709$, $p < 0.01$) existed in asthma patients (Fig. 1).

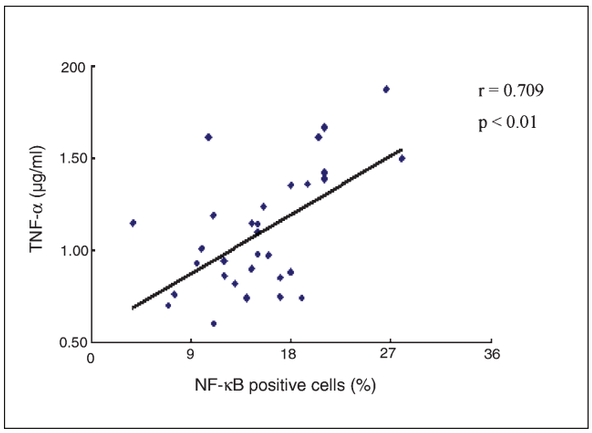


Fig. 1: A positive correlation between the percentage of NF-κB positive cells and the level of TNF-α in asthma patients ($r = 0.709$, $p < 0.01$, $n = 32$)

2.2. Effects of genistein and puerarin on the expression of NF-κB and the secretion of TNF-α in PBMCs in asthma patients

The percentage of NF-κB positive cells in PBMCs decreased with increasing concentrations (12.5–50 μmol/L), and increased with increasing concentrations (100–200 μmol/L) of genistein, the lowest point was at 50 μmol/l. The percentage of NF-κB positive cells in PBMCs gradually decreased with increasing concentrations (0.125–2.0 mg/ml) of puerarin. Genistein (50 μmol/L) and 1.0 mg/ml puerarin were chosen as the optimal concentration points respectively. And the curves of production of TNF-α in PBMCs supernatant were similar (Fig. 2a–d).

The number of NF-κB positive cells in PBMCs in the genistein group $15.2 \pm 5.4\%$, in the puerarin group $16.2 \pm 5.1\%$ was significantly lower than that in the control group $23.1 \pm 6.7\%$ ($p < 0.01$, $p < 0.05$), but still higher than that in the dexamethasone group $8.30 \pm 3.30\%$ ($p < 0.05$, $p < 0.01$) in asthma patients. The levels of TNF-α in PBMCs supernatant in the genistein group 1.08 ± 0.40 μg/L, and in the puerarin group 1.24 ± 0.29 μg/L were remarkably lower than that in the control group 2.10 ± 0.38 μg/L (p all < 0.01), but still higher than that in the dexamethasone group 0.48 ± 0.18 μg/L (p all < 0.01) in asthma patients. There were positive correlations between the percentage of NF-κB positive cells in PBMCs and the level of TNF-α in PBMCs supernatant in the genistein group ($r = 0.579$, $p < 0.01$), and in the puerarin group ($r = 0.665$, $p < 0.01$) in asthma patients (Figs. 3a–b, 4, 5). There were few effects of genistein and puerarin on PBMCs of healthy persons.

3. Discussion

Oxidative stress may play an important role in the pathophysiology of asthma and may be a final common pathway leading to tissue damage (Wood et al. 2003). During an asthma attack, many kinds of inflammatory cells such as eosinophils, mast cells, neutrophils, and monocytes are infiltrated into the airway mucosa. Large amounts of ROS are generated when macrophages and neutrophils phagocytose extraneous materials, extracellular microorganisms, particles and senescent cells. Some studies suggested that oxidative stress mediates activity of NF-κB signaling (Pierce et al. 2009; Jung et al. 2009). NF-κB is known to play a critical role in the regulation of proinflammatory molecules on cellular responses, especially TNF-a, IL-6, and IL-8 (Kim et al. 2008). On phosphorylation, IκB dissociates from the NF-κB-IκB complex, resulting in the translocation of NF-κB from cytoplasm to the nucleus. Activation of NF-κB is regarded as an important initial event in the airway inflammatory response to a variety of infectious agents, toxins, cytokines, growth factors, and oxidant stress (Barnes and Karin 1997). Our data show that the percentage of NF-κB nuclear positive cells in PBMCs is siginificantly increased in asthma patients. This indicates that NF-κB is activated in asthma attacks. TNF-α, a proinflammatory cytokine, is an activator of NF-κB expression and its production is regulated by NF-κB as well. TNF-α has an important amplifying effect in asthma inflammation and potently stimulates airway epithelial cells to produce cytokines (Kim et al. 2008). Our data also showed that the level of TNF-α in PBMCs supernatant was remarkably increased in asthma patients, and a positive correlation existed between the

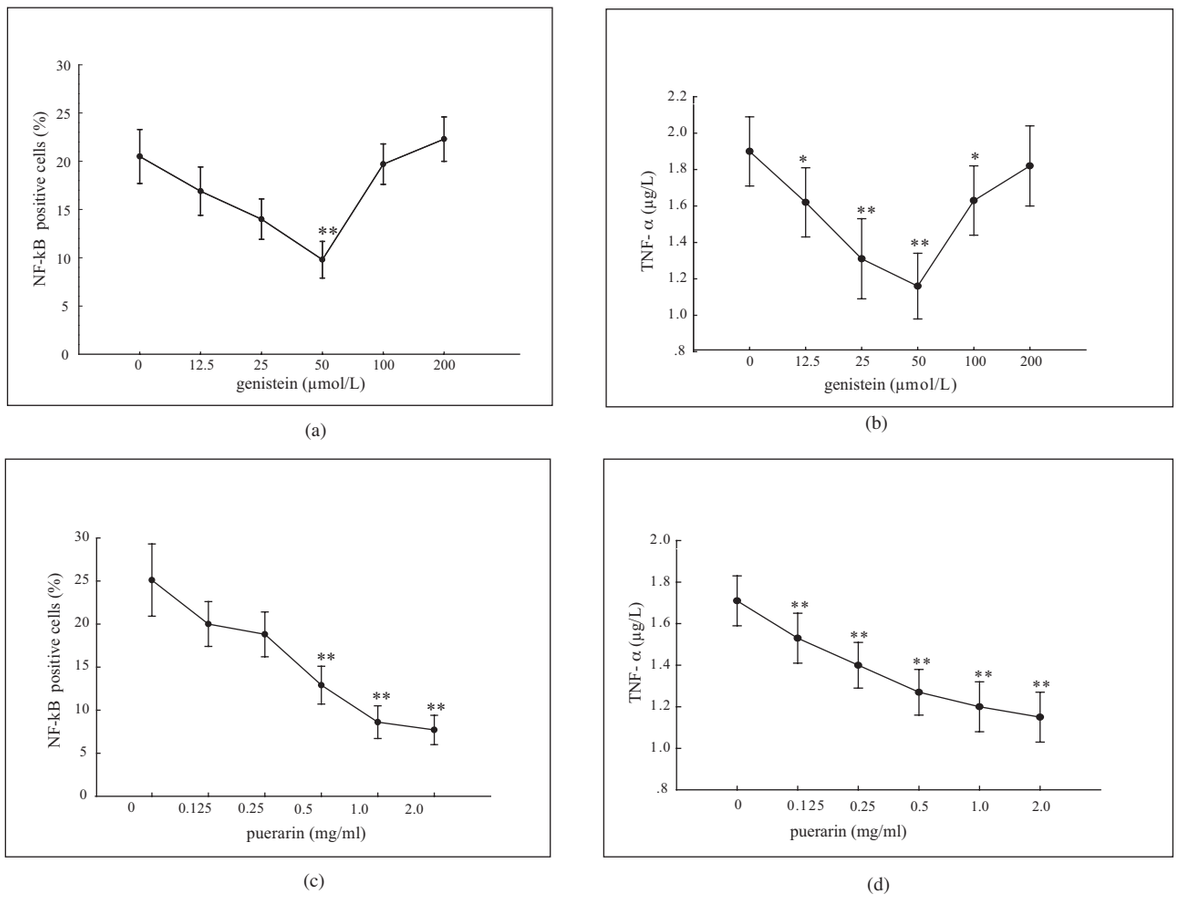
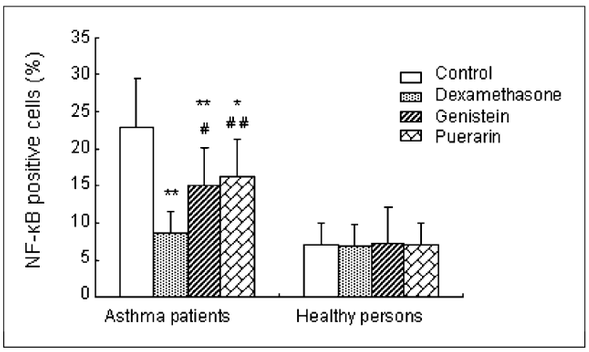


Fig. 2(a–d): The concentration-effect curves of genistein and puerarin on the percentage of NF-κB positive cells and the level of TNF-α in PBMCs of asthma patients (**$p < 0.01$, *$p < 0.05$, compared with 0 concentrations)

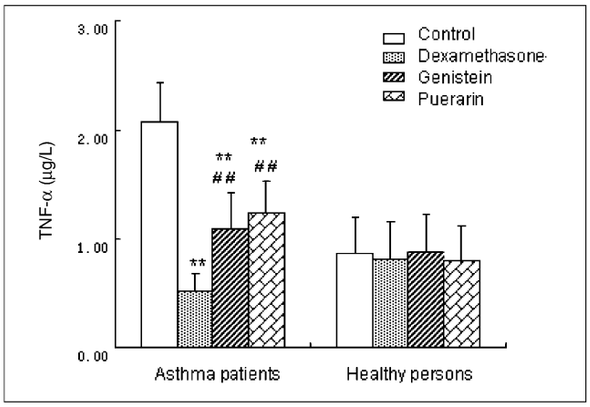
percentage of NF-κB positive cells in PBMCs and the level of TNF-α in PBMCs supernatant in asthma patients.

Zordoky et al. proposed that NF-κB could be one of the links between inflammation and oxidative stress in chronic inflammatory diseases (Zordoky and El-Kadi 2009). The scavenging of ROS by endogenous and exogenous antioxidants has attracted much attention and seems to be a feasible approach to protect asthmatic airways from damage by ROS. Some studies reported that antioxidants attenuate airway inflammation in a murine model of asthma (Kim et al. 2007; Yang et al. 2008; Jung et al. 2008). Genistein, the major isoflavone in soy, scavenges free radicals (Wei et al. 1993), inhibits tyrosine kinase (Akiyama et al. 1987), and exhibits mixed estrogen agonist and antagonist properties, depending on timing, dose, and the tissue examined (Price and Fenwick 1985). Recently some findings revealed that increasing consumption of genistein is associated with better lung function in patients with asthma (Smith et al. 2004), and the inhibition of protein tyrosine kinases by genistein could attenuate ovalbumin induced acute bronchoconstriction, pulmonary eosinophil infiltration, and airway hyperresponsiveness in sensitized guinea pigs (Duan et al. 2003; Assem et al. 2006). The concanavalin A-stimulated TNF-α secreting in rat splenocytes was inhibited by genistein (Lopez-Posadas et al. 2008), and phytohemagglutinin induced TNF-α production in macrophages was also inhibited by genistein, the signals pathway of inhibition from cell surface tyrosine kinases passed to MAP kinases, which further activate the transcription factors (Kesherwani and Sodhi 2007). Our finding exhibited that the activation of NF-κB in PBMCs and the hypersecretion of TNF-α in PBMCs supernatant of asthma patients were remarkably inhibited by genistein, and there was a positive correlation between the percentage of NF-κB positive cells in PBMCs and the level of TNF-α in PBMCs supernatant after genistein treatment in these asthma patients. This indicated that inhibition of genistein on TNF-α hypersecretion in PBMCs of asthma patients was involved partly in activity of NF-κB.

Puerarin, (8-β-D-glucopyranosyl-7 and 4′-dihydroxyisoflavone), a key component of kudzu root, is widely used in Chinese herbal medicine and, amongst diverse actions, is antidipsotropic. Some studies have revealed that puerarin possesses many physiological activities including reduction of fever, pain and symptoms of diabetes. It has long been used to treat cardiovascular disease, including coronary artery diseases, hypertension and arrhythmia (Guo et al. 2004; Zhang et al. 2006) and has antioxidative effects (Wu et al. 2007). Mercer et al. (2005) found that dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures, there were similar antioxidative protective effects on dopamine neurons of puerarin and genistein (Mercer et al. 2005). The antioxidative capacity of puerarin may also block apoptosis between anti- and pro-apoptotic proteins, and attenuates activation of caspase-3 in H_2O_2-induced PC12 cells (Jiang et al. 2003). However, little is reported about the antioxidative effects of puerarin on asthma patients. Our data showed that puerarin could decrease the percentage of NF-κB positive cells and hypersecretion of TNF-α in PBMCs of asthma patients, and the effects of puerarin were dose-dependent. It is possible that the effects of genistein and puerarin on asthma



(a)



(b)

Fig. 3(a–b): The effects of genistein and perarin on PBMCs of asthma patients and healthy persons. (** $P < 0.01$, compare with control, *$P < 0.05$, compare with control. ## $P < 0.01$, compare with dexamethasone, # $P < 0.05$, compare with dexamethasone)

patients may be involved in its antioxidative properties. There had no effects of genistein and puerarin on the activation of NF-κB and secretion of TNF-α of PBMCs in healthy persons. Taken together, the translocation of NF-κB from cytoplasm to the nucleus and hypersecretion of TNF-α existed in PBMCs of asthma patients. Genistein and puerarin can inhibit the translocation of NF-κB and hypersecretion of TNF-α in PBMCs of asthma patients although the effects are weaker than those of dexamethasone. We speculate that the activation of NF-κB and the hypersecretion of TNF-α, in part via stimulation of oxidative stress, play important roles in the pathogenesis of asthma, so asthma patients may benefit from antioxidants of genistein and puerarin.

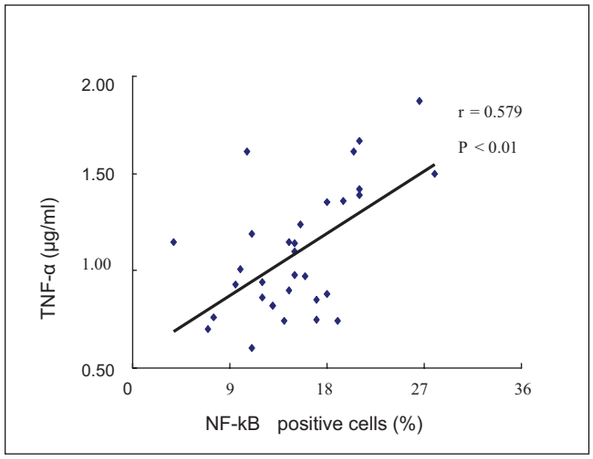


Fig. 4: A positive correlation between the percentage of NF-κB positive cells and the level of TNF-α in genistein group of asthma patients ($r = 0.579$, $P < 0.01$, $n = 32$)

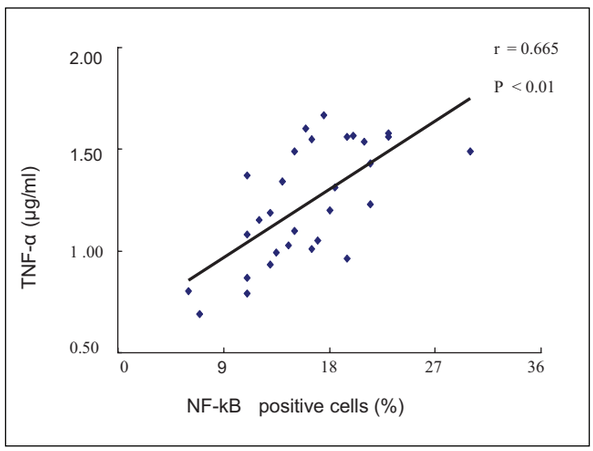


Fig. 5: A positive correlation between the percentage of NF-κB positive cells and the level of TNF-α in puerarin group of asthma patients ($r = 0.665$, $P < 0.01$, $n = 32$)

4. Experimental

4.1. Chemicals and reagents

Genistein was purchased from American Sigma Chemical Co. (St. Louis, MO). RPMI-1640 was bought from GIBCO company. Ficoll-Hypaque was produced by Shanghai Second Reagent Company. Rabbit Anti- NF-κB p^{65} and immunohistological staining kit (named SABC) purchased from BOSTER Biotechnology CO. LTD. TNF-α immunoradioassay kit purchased from Beimian Dongya Biotechniquing institute. All other reagents were analytical purity.

4.2. Subjects

Thirty two patients (20 males and 12 females) with asthma attack were selected, with average age of 48.6 ± 18.9 years. All cases were diagnosed according to the criteria of GINA 2006. None of the subjects had received either any medication for 24 h or steroids for 2 weeks before blood collection. Thirty one healthy persons had age and sex distributions similar to those of the patients (average age 44.1 ± 15.8 years, 18 males and 13 females, $p > 0.05$). According to the ethical guidelines of the Helsinki Declaration, informed consent was obtained from all participants and was monitored by the Local Ethical Committee of the Lanzhou University. Healthy persons were defined on the basis of a lack of a clinical history of allergy or other similar diseases.

4.3. Methods

4.3.1. Preparation and culture of PBMCs

PBMCs were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Hepatiniaed venous blood was layered on Ficoll-Hypaque and centrifuged at $1000 \times g$ for 20 min. PBMCs were collected from the interface, washed with phosphate buffered saline (PBS) twice and suspended in complete RPMI-1640 (10%FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin) at a concentration of 2×10^6 cells/ml. The viability of PBMCs was tested using the trypan blue dye exclusion method. PBMCs were seeded into 24 cell-culture plates and were maintained in a humidified incubator at 37 °C and 95% (vol/vol) air−5% (vol/vol) CO_2. PBMCs from asthma attack patients and healthy persons were randomly divided into four groups, control group, dexamethasone group, genistein group and puerarin group. After 1 h culture 100 μl PBMCs were smeared for NF-κB assay and 48h culture the supernatant was collected for TNF-α assay.

4.3.2. Immunocytochemistry for NF-κB

PBMCs (2×10^6) 100 μl were smeared on slides and fixed in 4% paraformaldehyde for 30 min. Slides were then washed twice for 5 min in PBS and soaked in 3% H_2O_2 (1:50 dilution) for 30 min to inactivate endogenous peroxidase. After three times further 3 min washes in PBS slides were firstly incubated with goat serum (50 μl) for 20 min at room temperature to block non-specific binding. Antibody (50 μl) against the NF-κB p65

(1:100 dilution) was then placed on the cells and incubated overnight at 4 °C. Slides were then washed three times in PBS for 3 min and incubated with biotinylated goat anti-rabbit immunoglobulin G for 20 min at 37 °C. Slides were then washed three times in PBS for 3 min and incubated with 50 μl streptavidin-peroxidase (SABC kit) for 20 min at 37 °C. The slides were washed four times in PBS for 5 min before addition of the peroxidase substrate 100 μl, 3,3′-diamidinobenzidine for 10 min at room temperature. The slides were then washed in distilled water for 3 min. Finally the slides were dried naturally, and then cleared in xylene and mounted in DPX. Negative reagent controls were stained in parallel with the primary p65 antibody, which had been blocked by incubation overnight at 4 °C. with a specific blocking peptide. For each sample, two slides were scored, and at least 500 cells were counted randomly on each slide. NF-κB p65 positive cells were defined as staining brown in cellular nuclear. So the percentage of NF-κB positive cells in PBMCs was calculated.

4.3.3. Assay for TNF-α of supernatant of PBMCs

The level of TNF-α in PBMCs supernatant was measured using radioimmunoassay kit (Beimian Dongya Biotechniquing Institute).

4.3.4. Concentration-effect curves

A kinetic study of the effect of geinstein and puerarin on the expression of NF-κB positive cells and the secretion of TNF-α in PBMCs were made. PBMCs were treated respectively with 0, 12.5, 25, 50, 100, 200 μmol/L genistein, and 0, 0.125, 0.25, 0.5, 1.0, 2.0 mg/ml puerarin 1 h for NF-κB assay, and 48 h for TNF-α assay, then concentration-effect curves of NF-κB and TNF-α were gotten. The optimal concentration points of genistein and puerarin were respectively 50 μmol/L and 1.0 mg/ml.

4.3.5. Statistical methods

The data are presented as means ± S.D. Data were processed and analyzed with software package SPSS 12.0. Statistics analyses are conducted using Student's t test. Differences were considered significant at $p < 0.05$.

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