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Original Article

The Effect of *Taglisodog-eum* Extract on Lipopolysaccharide-induced Otitis media

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Objectives : It has been known that immune reaction plays an important role in the pathogenesis of otitis media (OM). We investigated the change of middle ear mucosal inflammation induced by lipopolysaccharide (LPS) following administration of *Taglisodog-eum* (TSE) in experimental animals.
Materials and Methods : Otitis media was induced by injecting 1 mg/kg of Pseudomonas aeruginosa LPS transtympanically in 60 rats. These rats were divided into two groups, the LPS group (n=30, remained naive after OM elicitation) and the TSE group (n=30, treated with TSE after OM elicitation) and 6 additional rats were kept as a normal control group (n=6, remained naive until tissue collection). The rats were killed at the 1st, 3rd, and 7th days after challenge. The temporal bones in each group were harvested and examined histopathologically by hematoxyline-eosin stain. Middle ear mucosa were taken at the 1st, 3rd, and 7th days after challenge. The levels of spicing variants of TNF-α transcription were evaluated by Realtime-PCR.
Results : TSE suppressed LPS-induced TNF-α mRNA expression and thickness of the submucosal layer and infiltration of inflammatory cells in rat middle ear epithelium.
Conclusion : The results suggest that TSE may be effective in decreasing inflammation with particular application to mucosal metaplasia in OM.

Key Words : Otitis media with Effusion, Endotoxin, *Taglisodog-eum* (TSE)

Introduction

Otitis media (OM) is an inflammatory disease of the middle ear mucosa caused by infection of the middle ear cavity. It is the most common form of ear disease to cause visits to physicians and the most common cause of hearing impairment in children[1]. It has been shown that more than 90% of children under 7 years of age are induced more than one time, especially between 6 months and 4 years old[2]. Most of them heal by themselves within 3 months, but 30-40% of them relapse and 5-10% of them continue for 1 year or more[3].

The OM is caused by infection, dysfunction of the Eustachian tube, allergy, immunological factor, hypertrophy of adenoids, rhinopharyngitis, sinusitis, etc.[4] *nontypeable Haemophilus influenzae* (NTHi) and *Streptococcus pneumonia* (Spn) are chief pathogens of OM. Many antigens of these bacteria cause inflammatory responses. Among these antigens, endotoxin consists of lipo-oligosaccharide complex with outer cellular membrane proteins of NTHi as well as the other Gram-negative bacteria and is a potent inducer of inflammation and immunological responses[5].

Various proinflammatory mediators are known to

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play pivotal roles in the inflammatory response associated with OM. Proinflammatory mediators that have been isolated in relation to middle ear inflammation include lipopolysaccharide (LPS), bacterial components, cytokines, chemokines, and intercellular adhesion molecule-1 (ICAM-1)[6]. These cytokines are considered to be responsible for mucosal damage, bone erosion, and fibrosis. Recently, it has been emphasized that immune reaction plays important roles in the pathogenesis of OM and numerous experiments about cytokine and inflammatory mediators carried out[7,8].

Clinical reports of oriental medicine about OM were about the effects of *Kami-hyunggyeyungyo-tang*[9], *Taglisodog-eum*[10], and *Sonbanghwalmyong-um*[11]. There were also reports about the change of cytokines after *Kami-hyunggyeyungyo-tang* treatment[12,13] and evaluating the effect of herbal medication by using computed tomography (CT) and otoscopic examination[14].

Taglisodog-eum (TSE) have been used in inflammatory diseases; carbuncle, acute suppurative lymphadenitis, suppurative OM, anal fistula, polymyositis, chronic osteomyelitis, etc. There have been experimental studies about anti-allergy effect[15], anti-inflammatory effect, wound healing[16,17], antioncotic effect and immunological reaction[18,19] and clinical studies about OM[10] and prostatitis[20]. However, there are few reports regarding the efficacy of TSE in rats with experimental OM.

We hypothesized that TSE acts to suppress LPS-induced OM in rats by inhibiting the proinflammatory mediator. Accordingly, we investigated the effect of TSE on the proinflammatory mediator, TNF-α and the histological changes of LPS-stimulated middle ear epithelium.

Materials and Methods

1. Materials

A. Reagents

Lipopolysaccharides (LPS) extracted from *Pseudomonas aeruginosa* were purchased from Sigma (L-8643, St Louis, USA). LPS was dissolved in normal saline and used in concentration of 1 mg/㎖.

B. Preparation of TSE

TSE, which contains eleven species of medicinal plants (Table 1), was purchased from Dongguk University International Hospital. TSE (total 68 g) was prepared by decocting the dried prescription of herbs with 500 ㎖ boiling distilled water. The extraction, which was decocted for approximately 3 hours, was then filtered. The filtrate was concentrated in 50 ㎖ by vacuum evaporation using the rotary evaporator, EYELA (Rika-kikai co., Tokyo,

Table 1. Contents of *Taglisodog-eum* (TSE)

Pharmacognostic nomenclature	Dose(g)
Lonicerae Flos	12
Citri Pericarpium	12
Astragali Radix	8
Trichosanthis Radix	8
Ledebouriellae Radix	4
Angelicae gigantis Radix	4
Cnidii Rhizoma	4
Angelicae Dahuricae Radix	4
Magnoliae Cortex	4
Platycodi Radix	4
Gleditsiae Spina	4
Total amount	68

Japan) and was freeze dried. The return rate was 19.14%.

C. Animals

Sixty-six male Sprague-Dawley rats (5 weeks old, 150-180 g, ORIENT BIO Inc., Gyeonggi, Korea) were acclimated to the housing facility (2-3 rats/cage, 23±3℃, 50±10% relative humidity, 12-h light/dark cycle) for 1 week and fed Teklad 8604 rodent chow (Harlan Teklad Inc., IN, USA). The animals were identified as free of middle ear infection by otoscopic examination and were randomly assigned to a normal control group (n=6, remained naive until tissue collection), LPS group (n=30, remained naive after OM elicitation) and TSE group (n=30, TSE treated after OM elicitation). The TSE powder was dissolved in water and administered with oral zondae (1.32 g/kg per day) to the rats of TSE group until sacrifice.

2. Methods

A. Experimental OM model

OM was induced in both ears of rats in the LPS and TSE groups. Under inhalation anesthesia with ethyl ether, LPS solution (50㎕, 1㎎/㎖) was injected into the middle ear.

B. Real time RT-PCR

(Real time reverse transcription polymerase chain reaction)

a. Acquisition of mucosa

At 1st, 3rd and 7th days after LPS injection, 5 rats (10 ears) each from the LPS and TSE groups were killed by cervical dislocation, followed by decapitation. Three rats (6 ears) from the normal control group were killed also. The middle ear mucosa was dissected and collected. Mucosa acquired from every ear was kept at -80℃ until total RNA extraction.

b. Extraction total RNA and synthesization of cDNA

Total RNAs were extracted using RNeasy Mini kit (QIAGEN, Hilden, Germany) from middle ear mucosa. cDNA was synthesized by random priming using SuperScript Ⅲ Reverse Transcriptase (Invitrogen, CA, USA).

c. Real-time RT-PCR

Primer for TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express 2.0 software (Applied Biosystems, CA, USA) (Table 2). Real time RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each reaction mixture contained 12.5 ㎕ 2x SYBR green Master Mix, 6.25 pmol each of sense and antisense primer, and 1 ㎕ of cDNA in a final volume of 25 ㎕. Reaction mixture were incubated at 55℃ for 2 minutes then at 95℃ for 10 minutes to activated Amplitaq Gold DNA polymerase (Applied Biosystems), and then amplified using 40 cycles [15 seconds at 95℃ (denaturation) and 1 minute at 60℃ (annealing)] for TNF-α and GAPDH. Data analysis was performed using Sequence Detection System software (Applied Biosystems). Amounts of TNF-α mRNA were normalized versus endogenous GAPDH mRNA and TNF-α mRNA expressions in experimental groups were calculated relative to the control group.

Table 2. The Primer of TNF-α and GAPDH mRNA

Primer		Primer sequences	Product (bp)
TNF-α	sense	5'-ATCTTCTCGAACCCCGAGTG-3'	51
	antisense	3'-GGGTTTGCTACAACATGGGG-5'	
GAPDH	sense	5'-ATCAACGACCCCTTCATTGACC-3'	204
	antisense	3'-CAGTAGACTCCACGACATACTCAGC-5'	

TNF-α: gene for tumor necrosis factor- α
GAPDH: gene for glyceraldehydes-3-phosphate dehydrogenase

C. Histological examination

a. Acquisition and fixation of middle ear

At 1st, 3rd and 7th days after OM induction, 5 rats (10 ears) each of the LPS and TSE groups were inhalation anesthetized with ethyl ether. The animals were sacrificed by intracardiac perfusion with phosphate buffer saline (PBS. 0.1M, pH 7.2) and then with 4% paraformaldehyde (PFA) for 1st fixing. Their temporal bones were removed bilaterally and fixed with 4% PFA for 24 hours at room temperature (2nd fixing). After 2nd fixing, they were irrigated with PBS and decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, USA) for 12 hours at 4℃. Specimens were embedded in paraffin following a dehydration procedure.

b. Preparation for slide and staining

Samples sectioned to a thickness of 5 ㎛ were fastened to slides and stained with hematoxylin and eosin to evaluate the epithelial thickness and number of inflammatory cells.

3. Result Assessment

A. Change of TNF-α mRNA expression

Data analysis was done using Sequence Detection System software (Applied Biosystems) provided by the manufacturer. Fold changes in TNF-α mRNA expression were determined as 2?Ct, where ?Ct = (Ct TNF-α TSE/LPS ? Ct TNF-α normal control) ? (Ct GAPDH TSE/LPS ? Ct GAPDH normal control). Briefly, the amount of TNF-α mRNA was normalized to the endogenous reference gene (GAPDH) and its expression in TSE/LPS group was calculated relative to a calibrator (normal control group).

B. Histopathologic Changes

a. Change of epithelial thickness of promontory mucosa

Epithelial thickness was evaluated, defined as the maximum thickness measured on the mucosa overlying the promontory under high magnification (×400). The mean epithelial thickness was calculated from 3 sections from each ear in each group.

b. Number of inflammatory cells infiltrated in the epithelium

The numbers of infiltrated leukocytes were counted under high power field (×400).

C. Statistical analysis and Image analysis

The results were expressed as mean ± standard deviation (SD) for the number of experiments. Paired comparisons was performed using the Mann-Whitney U test in SPSS for windows (Ver. 12.0. SPSS Inc., Chicago, IL). Differences were considered significant when p value was less than 0.05. Images were analyzed by Optima 5.2 (Optima Co., USA).

Results

1. Effect of TSE on LPS induced TNF-α gene expression in middle ear mucosa

At the 1st day it was 3.26 ± 0.52 in the LPS group and 1.53 ± 0.18 in the TSE group, and at the 3rd day it was 2.53 ± 0.32 in the LPS group and 1.42 ± 0.25 in the TSE group, and the difference was significant. At the 7th day, relative expression of TNF-α mRNA was 1.55 ± 0.25 in the LPS group and 1.08 ± 0.19 in the TSE group, but the difference was not significant.(Fig. 1.)

2. Effect of TSE on the change of mucosal thickness

Relative mucosal thickness was 4.46 ± 0.57 in the normal control group. At the 1st day it was 52.69 ± 5.96 in the LPS group and 32.09 ± 4.33 in the TSE group, and at the 3rd day it was 38.00 ± 3.43 in the LPS group and 27.30 ± 1.23 in the TSE group, and the difference was significant. At the 7th day, relative mucosal thickness was 29.76 ± 1.83 in the LPS group and 25.62 ± 3.18 in the TSE group, but the difference was not significant. (Table 3, Fig. 2.)

3. Effect of TSE on the number of inflammatory cells in middle ear mucosa

The relative number was 0 in the normal control

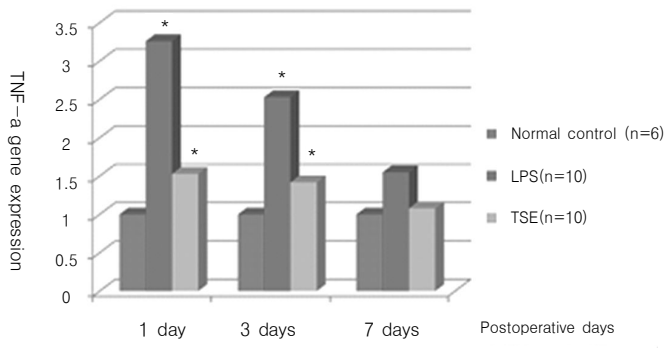

Fig. 1. TNF-α gene expression in middle ear mucosa

TNF-α mRNA expressions were significantly decreased in the TSE group postoperative the on 1st and the 3rd days. However, on the 7th day, the difference between the two groups was not significant.
Normal control: remained naive until tissue collection
LPS: remained naive after OM induction
TSE: TSE treated after OM induction
*: $p<0.05$ (Mann-Whitney U test)
All values are mean ± standard deviation (S.D.)
Probability values of less than 0.05 were considered significant.

group. At the 1st day it was 59.30 ± 7.68 in the LPS group and 30.10 ± 8.02 in the TSE group, and at the 3rd day it was 49.80 ± 6.88 in the LPS group and 30.70 ± 5.33 in the TSE group, and the difference was significant. At the 7th day, the relative number was 18.20 ± 2.12 in the LPS group and 18.40 ± 2.65 in the TSE group, but the difference was insignificant. (Table 4, Fig. 2.)

Table 3. Mean values of mucosal thickness of promontory area

Postoperative days	1 day	3 days	7 days
Normal control (n=6)		4.46 ± 0.57	
LPS (n=10)	52.69 ± 5.96*	38.00 ± 3.43*	29.76 ± 1.83
TSE (n=10)	32.09 ± 4.33*	27.30 ± 1.23*	25.62 ± 3.18

Normal control: remained naive until tissue collection
LPS: remained naive after OM induction
TSE: TSE treated after OM induction
*: $p<0.05$ (Mann-Whitney U test)
All values are mean ± standard deviation (S.D.) rmse

Table 4. Mean values of number of inflammatory cells in the middle ear mucosa

Postoperative days	1 day	3 days	7 days
Normal control (n=6)		0	
LPS (n=10)	59.30 ± 7.68*	49.80 ± 6.88*	18.20 ± 2.12
TSE (n=10)	30.10 ± 8.02*	30.70 ± 5.33*	18.40 ± 2.65

Normal control: remained naive until tissue collection
LPS: remained naive after OM induction
TSE: TSE treated after OM induction
*: $p<0.05$ (Mann-Whitney U test)
All values are mean ± standard deviation (S.D.)

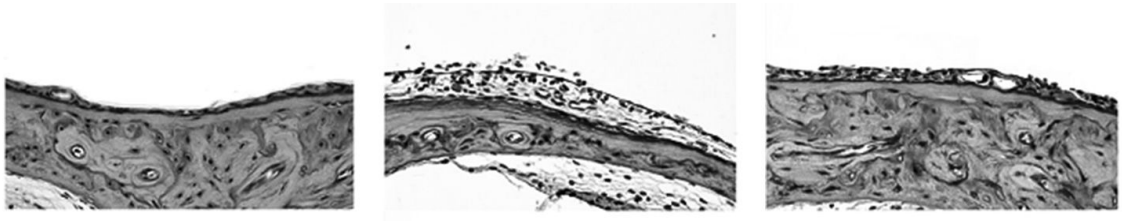

Fig. 2. Photomicrographs of promontory mucosa (hematoxylin and eosin, original magnification ×400)
A) normal control group
B) LPS group at the 3rd day
C) TSE group at the 3rd day
In the normal control group (group A), the epithelial layer is very thin and has few leukocytes. In the LPS group at the 3rd day (group B), the epithelial layer is markedly thickened and infiltrated with many leukocytes. In the TSE group at the 3rd day (group C), the subepithelial thickening and the number of infiltrated leukocytes have decreased.

Discussion

OM is an inflammatory disease of the middle ear mucosa, and the most common disease operated on and second most common disease visited to physicians in childhood. There is controversy about classification; we categorized them into three types: acute otitis media (AOM), otitis media effusion (OME), and chronic otitis media (COM)[21]. OME has effusion in the middle ear without acute infection symptoms, and it has a tendency to increase due to multiple reasons: abuse of antibiotics and increase in antibiotics-resistant bacteria, etc. It incurs serious complications of hearing and language impairment and the other problems.

OM is induced by bacterial infection, dysfunction of the Eustachian tube, allergy, immunological factor, hypertrophy of adenoids, rhinopharyngitis, sinusitis, etc.[4] Children under 7 years of age are commonly induced more than once due to frequent URI, cilia dysfunction, etc.[2] It is known that inflammatory productions induced by infection or tubal obstruction play a very important role in chronic effusion formation. Cell mediated reactions are proved by finding eosinophils, lymphocytes, macrophages, antibodies and cytokines in effusion[22].

OM is diagnosed by history taking, observation of the tympanic membrane with otoscope and video otoscope, especially tympanostomy, tympanometry, or pneumatic otoscope for definite diagnosis of OME and CT and MRI in case of COM suspected erosion of auditory ossicles or cholesteatoma.

Treatment of OM is for recovery of hearing and prevention of complications, and divided into two modes: drug therapy and operation. Drug therapy is carried out first: antibiotics, steroids, antihistamines. When drug therapy has failed or OM has recurred frequently, an operation is carried out, inserting a ventilating tube or adenoidectomy[23].

Drug therapy is known to be effective for most cases, but 10% of AOM patients showed effusion in spite of proper drug therapy for 3 months[24]. Moreover, it is reported that 34% to 66% of COM patients are not detected in bacterial culture test[25]. This means that drug therapy has destroyed the bacteria, but endotoxins from the bacteria and inflammatory products existed, so inflammatory response continued.

Antibiotic-resistant bacteria are increasing gradually, so drug therapy is considered to be limited. It is reported that personal immunologic function or allergic tendency play important roles in an attack of OM[21]. OM is common in children with rhinitis or sinusitis. Importance and interest about immune pathogenesis in the middle ear are increasing, and there are various studies about inflammatory cells

and inflammatory mediators[7,8]. Inflammatory mediators identified in effusion of the middle ear are arachidonic acid metabolites, platelet activating factor (PAF), histamine, kinase, free radical, protease, hydrolytic, lysozyme and cytokine including IL-1, IL-2, IL-6, IL-8, TNF-α and IFN-γ[22,23].

As mentioned above, OM is considered to have begun at bacterial infection, and gone through antibiotics therapy, middle ear's clearance function and host-defense function. Then, LPS remained in effusion to continue to stimulate production of the multiple inflammatory mediator, so a vicious circle of inflammatory response was repeated[24]. Thus, we need new therapies about antibiotic-resistant bacteria, and LPS has remained after drug therapy.

In oriental medicine, TSE has been used in inflammatory diseases: carbuncles, acute suppurative lymphadenitis, suppurative otitis media, anal fistula, polymyositis, chronic osteomyelitis, etc. There have been experimental studies about anti-allergy effect[15], anti-inflammatory effect, wound healing[16,17], antioncotic effect and immunological reaction[18,19] and clinical studies about OME(10) and prostatitis[20]. However, there are few reports regarding the efficacy of the TSE in rats with experimental OM.

We hypothesized that TSE may be suppressing the inflammatory response on OM and carried out a study about rats induced with OM and administered TSE, and investigated TNF-α mRNA expression and change of mucosal damage.

Induction of OM is necessary to study the pathological and therapeutic process of OM. Rat middle ear mucosa is very similar to human, so is appropriate for a human OM experimental model[25]. Induction of OM has been carried out by injection of bacteria[26] and inflammatory mediator[27-29]; also, it was reported that injection of endotoxin or LPS endotoxin induced OM successfully[24,30,31].

Endotoxin consists of lipo-oligosaccharide complex with outer membrane proteins of NTHi as well as the other Gram-negative bacteria. It is detected in effusion of OM in human steadily and effusion that turned out negative on culture test[32]. Like this, endotoxin is considered to be one of the factors that continue and repeat inflammatory response in the middle ear, so secrete effusion in spite of antibiotic therapy. It stimulates macrophages to secrete TNF-α, IL-1β[33] and induces an inflammation response through stimulating diverse inflammatory mediators and immune reaction[7]. In this study, OM models were induced by injecting LPS that stimulated an inflammatory response and immune reaction constantly after antibiotic therapy.

TNF-α is produced in response to stimuli triggered by bacterial LPS and viruses from fibroblasts, T-cells, B-cells, endotheliocytes and epithelial cells[34]. It is a critical proinflammatory mediator leading to inflammation going through the process of activation of polymorphonuclear leukocytes, epithelial cell hyperplasia, genesistasis of endothelial cells and B-cells, sorbefacient of bone and cartilage and chemoattractant of neutrophils, monocytes, macrophages and lymphocytes[35].

TNF-α is believed to be produced mainly by macrophages or mast cells that have infiltrated middle ear mucosa and is associated with tissue damage, fibrosis and bone resorption in the middle ear in OM. In addition, the receptor antagonist of TNF-α was found to have the inhibitory effect on LPS-induced OM in rats. TNF-α has also been detected in bacterial and non-bacterial inflammatory response by investigating TNF-α concentrations in effusion[7]. This means antibiotic therapy destroyed bacteria, but LPS and TNF-α remained after antibiotic therapy, and repeated inflammatory response. Moreover, antibiotic-resistant bacteria are increasing, so sometimes antibiotic therapy can't destroy even bacteria. This is a limitation of antibiotic therapy, so we need another therapy that suppress LPS and TNF-α at the primary inflammatory stage and also after antibiotic therapy.

In this study, TNF-α mRNA expression was increased in rats with LPS-induced OM. After administrating TSE, it decreased significantly at the 1st and the 3rd days. Thickness of promontory mucosa and numbers of inflammatory cells were

increased in LPS-induced rats OM. After administrating TSE, it decreased speedily and significantly at the 1st and the 3rd days. At the 7th day, the TSE group had decreased more than the LPS group, but it was not significant. This may be due to the clearance function of the middle ear, and host-defense function after the lapse of time.

In conclusion, the results of this study demonstrated that TSE inhibits LPS-induced TNF-α mRNA expression and damage of mucosa at the primary stage.

Moreover, from an anatomical view, the middle ear is connected to the upper airway, and intercurrent diseases are common including the common cold, rhinosinusitis, OM and so on. Therefore, we can use TSE not only for OM but also URI with a view to reinforcing treatment and anti-inflammatory activation.

However, because rat middle ear mucosa has immunological function for itself like respirator or digestive system[25,36] and can produce various inflammatory mediators in response to inflammatory stimulation[37], we believe that our results may best reflect the animal model findings.

Conclusion

To study effects of TSE on OM, we experimented with TSE administered to rats induced with OM by LPS. We observed anti-inflammatory effect on the proinflammatory mediator, TNF-α gene exprression and the histological changes of LPS-stimulated middle ear epithelium.

1. LPS increased TNF-α mRNA expression in rat middle ear epithelium.
2. TSE suppressed LPS-induced TNF-α mRNA expression in rat middle ear epithelium.
3. LPS increased thickness of the submucosal layer and infiltration of inflammatory cells in rat middle ear epithelium.
4. TSE suppressed LPS induced thickness of the submucosal layer and infiltration of inflammatory cells in rat middle ear epithelium.

In conclusion, these findings indicate that TSE is an inhibitor of TNF- α mRNA expression and can help recovery of historical damage to the middle ear. Further studies will be required to confirm the efficacy of TSE on morbidity periods, AOM, COM, and dosage and period of TSE for preventing complications.

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