Evaluate *Toxocara canis* excretory-secretory antigens in experimental allergic encephalomyelitis (EAE)

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Key words:
disability score, EAE gene expression, experimental allergic encephalomyelitis, immune system in EAE *Toxocara canis* excretory-secretory antigens

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Abstract:

BACKGROUND: *Toxocara canis* is the most prevalent intestinal roundworm of canid species. **OBJECTIVES:** This study aims to evaluate the effects of *Toxocara canis* excretory-secretory antigens (TcES Ag) on modulating the immune system in Experimental Allergic Encephalomyelitis (EAE) model. **METHODS:** Adult worms of *T.canis* were collected from dogs to obtain excretory-secretory antigens. Female C57BL/6 mice were divided to four groups (5 mice in each) including: group 1 (MOG +TcES Ag), 2 (MOG), 3 (normal) and 4 (TcES Ag). EAE was induced in groups 1 and 2 using myelin oligodendrocyte glycoprotein peptide. Before EAE induction, TcES Ag was injected in group 1. Twenty-nine after EAE induction, mice spleens were removed. Mononuclear cells were cultured and used for RNA extraction.Real Time PCR was performed to evaluate RNA expression levels of T-bet (Th1 lineage-specific transcription factor), GATA-3 (Th2 transcription factor), and FOXP3 (Treg transcription factor). **RESULTS:** Our results indicated the clinical signs (disability score) of mice in group 1 were decreased significantly as compared to the control group. Gene expression of T-bet in the TcES Ag treatment group were noticeably diminished compared to MOG and normal group. The expression of GATA-3 gene in group 1 was lower than that in group 2. **CONCLUSIONS:** It seems that the TcES Ag may reduce disability score in multiple sclerosis EAE model, and other recombinant antigens should be examined.

Introduction

According to the "hygiene hypothesis", lack of serious infection in children leads to defects in the immune system. It has been indicated that exposure to infectious agents such as bacteria, viruses, and parasites, especially in childhood, can reduce the risk of autoimmune diseases (Osada and Kanazawa 2010). A number of previous investigations support the hygiene hypothesis, assuming that infections can have a protective role rather than inducing or accelerating autoimmune diseases such as MS (Bach 2002). Worms are known as the most important factors in modulation of the immune response (Maizels, et al., 2004). In vitro models have shown that worms and their products inter-

fere with the proliferation of lymphocytes and decreases in B lymphocytes, resulting in IgE production and activation of macrophages type 2 modulators.

Chronic infection of worms can modulate the immune response to the Th2, T reg, and Th2-related cytokine, including IL4, IL5, and IL13. Moreover, safety regulation in response to chronic infection is caused by worms and leads to reduction of the incidence of autoimmune diseases and allergies (Chow et al., 2000; Harnett 2006; Smits et al., 2010; Daniłowicz-Luebert, O'Regan et al., 2011; Cooke 2012; Elliott Weinstock 2012). Based on the theory, it has been demonstrated that worms' products have potential therapeutic effects for treatment of allergies as well as inflammatory and autoimmune diseases (Van Riet et al., 2007).

The prevalence of allergic diseases and autoimmune diseases such as multiple sclerosis is rising in developed and developing countries. Multiple sclerosis is an inflammatory disease of the central nervous system (a related-myelin membrane destruction) (Goldenberg 2012; Hafler 2012). In mouse models of autoimmune diseases, the presence of helminthes infections has been reported to be associated with disease resistance, and the infection by eggs and adult worm of *Schistosoma mansoni* has been indicated to inhibit the development of type 1 diabetes(Cooke, et al., 1999; El-Wakil, et al., 2002; Zaccone, et al., 2003).

Moreover, the previous studies have shown therapeutic effects of parasitic worms including nematodes (*Heligmosomoides polygyrus* and *Trichinella spiralis*), trematodes (*Schistosoma mansoni*, *Schistosoma japonicom*, and *Fasciola hepatica*), and cestodes (*Taenia crassiceps*) in the Experimental Allergic Encephalomyelitis Model (Sewell et al., 2003; Zheng et al., 2008; Walsh et al., 2009; Gruden-Movsesijan et al., 2010; Wilson et al., 2010; Kuijk et al., 2012; Zhu et al., 2012).

In developed and developing countries allergic and autoimmune diseases like multiple sclerosis are increasing due to reduced exposure of infectious agents.

Recently, it has been shown that some products of parasites can be effective in modulating immune response. Therefore, evidence reveals that helminthes infections are associated with reduced severity of autoimmune disease in animal models. It has been suggested that helminthes infection or products play an effective role in the course of autoimmune pathology in both spontaneous and induced models of human autoimmune diseases (Khan et al., 2002;La Flamme et al., 2003;Walsh, Brady et al., 2009;Wilson, Taylor et al., 2010). In the present study, the effects of TcES Ag on experimental models of autoimmune diseases are evaluated to understand the therapeutic effects of this antigen.

Materials and Methods

Sample collection and antigen preparation: *T.canis* adult worms were collected from dogs, rinsed with salt, and cultured in RPMI-1640 which contained 100u/ml penicillin, 100u/ml streptomycin, and 0.25µg amphotericin B (Page et al., 1991).

T.canis was kept in the medium for 5 days at 30°C and 5% Co2, and the culture medium was collected every 24 hours as a source of excretory-secretory antigens.

Protein measurement and concentration

TcES Ag were concentrated using dialysis bags (cut off 12) and were measured by Bradford method (Bradford 1976).

Injection of the antigen to rabbits: TcES Ag (500 µl RPMI-1640 containing 30 µg Ag) were subcutaneously injected with complete adjuvant (1:1) to the rabbit (10 weeks-old and male). Two boosters (250 µl RPMI-1640 containing 10 µg Ag) with incomplete adjuvant were also injected (1:1) 10 and 20 days after the first injection. Furthermore, the sera were separated 2 days after the third injection. Western blot was performed to determine immunogenicity of antigens.

TcES Ag injection into C57BL/6 mice: The female C57Bl/6 mice (10 weeks old) were classified into four groups as follows (5 mice in each group).

In the first group, excretory-secretory antigens of *T.canis* with adjuvants were subcutaneously injected (6µg antigen) to mice, and booster injection with incomplete adjuvant was performed after 7 days (3µg antigen). In the second group, the distilled water was injected to mice. In the third group, mice were used without injection of TcES Ag, but distilled water was injected (normal group). Seven days later, injection with distilled water was repeated. In the fourth group, mice were assigned as the control group and 250 ml of excretory-secretory antigens of *T.canis* with complete adjuvant (6µg antigen) were subcutaneously injected, and 125 ml antigen incomplete adjuvant was used for injection after 7 days (3µg antigen).

EAE induction and scoring: In the first and second groups, mice were injected subcutaneously with 300µg myelin oligodendrocyte glycoprotein peptide35-55 (MOG35-55). Two hours later, 100 ng of Pertussis Toxin were intra-peritoneally injected, which was repeated 24 hours later according to the manufacturer's protocol.

All animals were checked daily for clinical signs, and the weighting and scoring were as follows: grade 0, no abnormality; grade 0.5, tip of tail is limp; grade 1, limp tail; grade 1.5, limp tail and hind leg inhibition; grade 2.0, limp tail and weakness of hind legs; grade 2.5, limp tail and dragging of hind legs; grade 3.0, limp tail and complete paralysis of hind legs; grade 3.5, limp tail and complete paralysis of hind legs and mouse is moving around the cage, but when placed on its side, is unable to right itself; grade 4.0, limp tail, complete hind leg and partial front leg paralysis; grade 4.5, complete hind and partial front leg paralysis, no movement around the cage; grade 5.0, the mouse is spontaneously rolling in the cage or death (scoring was performed according to the manufacturer's protocol).

Cell culture: Twenty-nine days after induction of EAE, mice spleens were removed.

Mononuclear cells in culture medium RPMI-1640 (containing 10%FBS, 100 units of penicillin, streptomycin, 2ml Glutamine, and 25 ml Hepes) to 3×10^6 cells/ml were cultured in each well. 25µg of antigens of *T.canis* were added to the medium, and the culture was collected after 72 hours of incubation at 37°C and 5% CO2 and subsequently centrifuged at 10,000rpm. Furthermore, and the precipitate was separated and stored at -70°C until use.

Real Time PCR: RNA was extracted from cultured mononuclear cells by the DNA binding column, and 8µl of RNA was used for reverse transcriptase. Eva green jumpstart Taq Ready mix (5 X HOT FIREPol®EvaGreen®qPCR Mix Plus) in a final volume of 20µl each sample was submitted to 36 cycles in a real time thermal cycler (ABI) according to the manufacturer's pro-

tocol.

Sequences for our target genes were βActin (Forward: ATGCTCCCCGGGCTGTAT Reverse: CATAGGAGTCCTTCTGACCCATTC), T-bet (Forward: GCCAGGGAACCGCTTATATG Reverse: AACTTCCTGGCGCATCCA), GATA-3 (Forward: CAGAACCGGCCCCTTATCA Reverse: CATTAGGAGAGGTGTGAAAGC), and FOXP3 (Forward: GCAGGGCAGCTAGGTATCTGTAG Reverse: TCGGGAGATCCCCTTTGTCTATC). Gene expression was normalized to the expression of the constitutively expressed gene βActin.

Results

Western blot is a rapid sensitive technique that uses antibody for the specific detection of proteins. This method showed that our T.cES antigen extracted from adult worm had high quality.

Western blot of TcES antigens with anti-TcES rabbit: The western blot using anti-TcES rabbit serum is shown in Fig. 1. The recognized immunogenic bands of the T.c ES antigen were 57, 35, 80, 60, and 180 KDa.

Scoring in EAE: Our results indicated that the clinical signs (disability score) in the treatment group (MOG +TcES) of female C57BL/6 mice significantly decreased when compared with the control group (Fig. 2). In addition, the average weight was lower in the control group (MOG) as compared with TcES Ag treatment group(MOG +TcES) (Fig. 3).

Immune response results: Real time PCR results showed that the gene expression T-bet in the treatment group 1 noticeably decreased compared to MOG and normal groups.

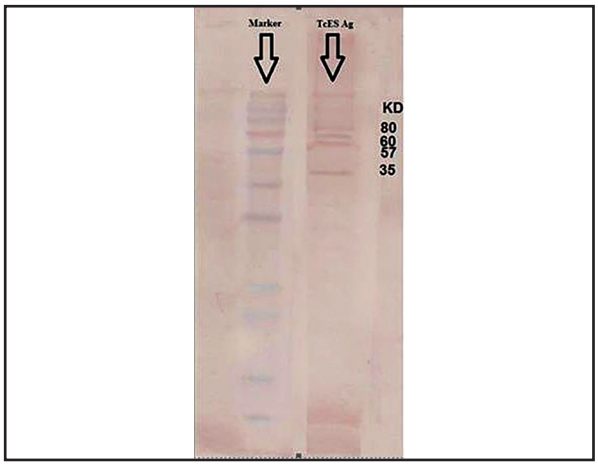

Figure 1. Western blot analysis of TcES antigens with anti-TcES rabbit.

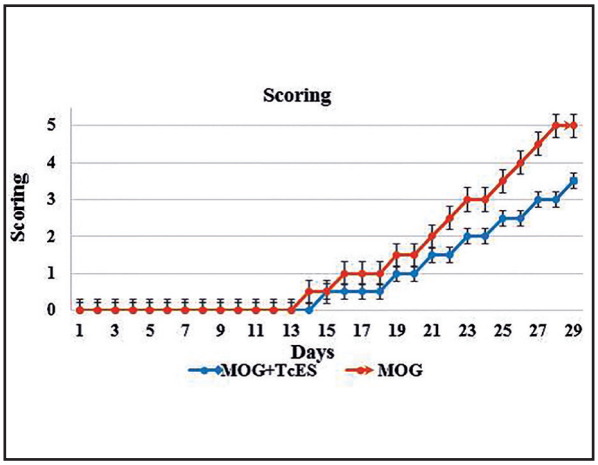

Figure 2. Average score disability of group1 (MOG +TcES Ag) and group2 (MOG) in EAE model: disability score of treatment group (MOG +TcES Ag) markedly decreased when compared with the MOG group.

The gene expression of FOXP3 and GATA-3 in TcES Ag challenge group (group 1) was not significantly different from those of the other groups (Fig. 4). Furthermore, the expression of GATA-3 gene in group 1 was lower than that in group 2.

Discussion

Helminthes are known as the most crucial factor in modulating immune system (Maizels et al., 2004). It has been demonstrated that helminthes can modify the host

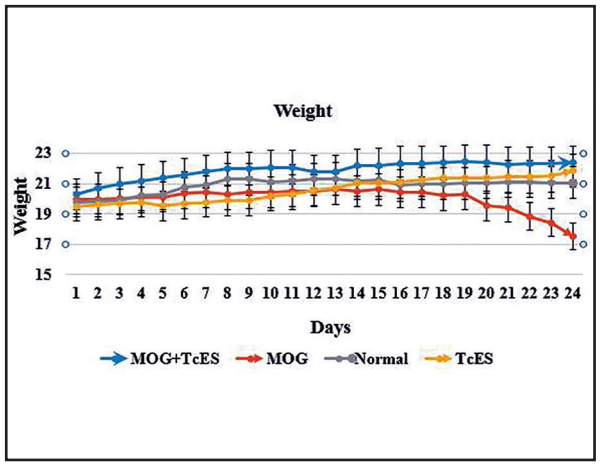

Figure 3. Clinical signs of group 1 (MOG +TcES Ag) and group 2 (MOG) in EAE model: the average weight declined in the control group (MOG) in comparison withTcES Ag treatment group (MOG +TcES Ag).

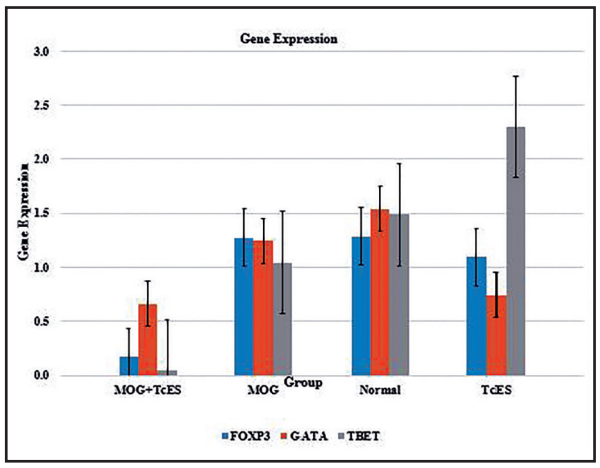

Figure 4. Real time PCR findings showed that the gene expression of TcES Ag T-bet in the treatment group 1 strongly diminished compared with MOG and normal groups.

immune system. At least 20 immune modulatory mechanisms are expressed by worms, including the production of Treg cytokine (Fleming et al., 2011).

Parasitic helminthes is considered as a potential treatment for a wide range of diseases like inflammatory bowel disease, autoimmune diseases, and asthma. Many experimental studies have shown that infection by worms has a protective effect against autoimmune diseases in animal models (colitis, arthritis, and diabetes) and allergies in human (Osada 2010).

In experimental mouse models with diabetes, it has been indicated that immunization with *Taenia crasiseps* metasestod can reduce the inflammation and lead to significant inhibition of improving clinical score. Therefore, soluble worm products (soluble egg antigen and soluble worm) followed by homogenized and live worm are used as therapeutic agents for a wide variety of diseases such as inflammatory bowel disorders, autoimmune diseases, and asthma (Kuijk et al., 2012).

Moreover, treatment with live worms is not desirable for most people. It is reported that worms' soluble products (*Schistosoma mansoni* eggs injected i.p) play an important role in regulating the immune system (Pearce 2002; Harn et al., 2009; Andersen et al., 2009; Everts et al., 2010; Van Die 2010).

Several researches have confirmed that the expression of FOXP3 gene has increased in infected mice with the gastrointestinal nematode parasite (Finney et al., 2007; McSorley et al., 2008; Rausch, et al., 2008).

Previous studies indicated that the soluble products of intestinal helminthes suppress Th1 and Th17, which are important in the pathogenesis of autoimmune and inflammatory diseases (Kuijk et al., 2012).

In vitro studies have shown that *Trichuris Suis* and *Trichinella spiralis* products can suppress the secretion of proinflammatory cytokines such as TNF (Van Vliet et al. 2007).

In the present study, TcES Ag was injected into mice and then EAE was induced.

Our study showed that the expression of T-bet gene decreased in the group 1, reducing the number of Th1 cells and the disability score.

In this study, the expression of GATA-3 gene diminished in the group 1, suggesting

that the number of Th2 cells was reduced. The minimum number of parasite antigens should be used for regulation of the immunemodulatory system, because increase of Th2 cells and their cytokines led to adverse reactions (allergic disease).

Jose et al. reported that expression of T reg cells declined in EAE model (Reyes et al., 2011). In this research, expression of FOXP3 gene did not increase, suggesting that the number of T reg cells did not increase either.

Optimization of the antigen in the worm therapy is necessary and can solve the limitation of using helminthes antigens for helminthes therapy.In conclusion, it seems that the TcES Ag may reduce disability score in EAE model, and other recombinant antigens can be examined independently.

Ethics committee: The present study was confirmed by the local ethical board in Faculty of Veterinary Medicine, Tehran University.

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ارزیابی بالینی و ایمنولوژیک اثر آنتی ژن‌های دفعی ترشحی تو کسو کارا کنیس در مدل تجربی EAE

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چکیده

زمینه مطالعه: تو کسو کارا کنیس شایع‌ترین انگل روده ای سگ می‌باشد. **هدف:** هدف از این مطالعه بررسی آنتی ژن‌های دفعی ترشحی تو کسو کارا کنیس در تعدیل سیستم ایمنی موش‌های مدل تجربی آلرژیک آنسفالومیلیت است. **روش کار:** پس از کشت دادن کرم بالغ آنتی ژن دفعی ترشحی تو کسو کارا کنیس جمع آوری شد. موش‌های C۵۷ به چهار گروه (هر گروه پنج موش) گروه اول (پپتید گلیکوپروتئین الیگودندروسیت به اضافه آنتی ژن تو کسو کارا کنیس)، گروه دوم (پپتید گلیکوپروتئین الیگودندروسیت)، گروه سوم (نرمال) و گروه چهارم (آنتی ژن دفعی ترشحی تو کسو کارا کنیس) تقسیم بندی شدند. بیست و پنج روز بعد از القاء، علائم بالینی مورد بررسی قرار گرفت و سپس طحال موش‌ها جداشد و RNA سلول های تک هسته ای استخراج شد و با استفاده از Real Time PCR بیان ژن‌های FOXP۳T, GATA۳ و Tbet مورد ارزیابی قرار گرفت. **نتایج:** علائم بالینی در گروه اول نسبت به گروه کنترل بطور قابل توجهی کاهش نشان داد و همچنین بیان ژن GATA۳ در گروه اول کمتر از گروه دوم بود. **نتیجه‌گیری‌نهایی:** بنظر می‌رسد که آنتی ژن دفعی ترشحی تو کسو کارا کنیس قادر است نمره ناتوانی موش را در مدل تجربی آنسفالومیلیت کاهش دهد و همچنین دیگر آنتی ژن‌های نوترکیب می‌تواند بطور جداگانه در درمان بیماری‌های اتوایمیون مورد بررسی قرار گیرند.

واژه های کلیدی: نمره ناتوانی، بیان ژن در مدل تجربی ام اس، مدل تجربی آنسفالیت خودایمن، سیستم ایمنی در مدل تجربی آنسفالیت خودایمن، آنتی ژن دفعی ترشحی توکسوکارا کنیس

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