Letter to the Editor

Selenium Supplementation Alleviates Autoimmune Thyroiditis by Regulating Expression of Th1/Th2 Cytokines*

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Selenium (Se) is an essential trace element. Autoimmune thyroid diseases (AITD) are destructive inflammatory or anti-receptor autoimmune diseases characterized by reactivity to self-thyroid antigens. However, the effects of Se on the cytokines in AITD are still unclear. So we researched the role of Selenium (Se) and Th1/Th2 cytokine productions in the pathogenesis of autoimmune thyroid diseases (AITD). The data shown that high Se intake reduced the autoantibody titers and alleviated the pathological changes of thyroids while low Se intake did not alleviate experimental autoimmune thyroiditis (EAT) manifestations. Se treatment dose-dependently inhibited IL-2 expression and affected other cytokines at varying degree. Among all cytokines examinations, the expression of Th1 cytokines increased more significantly than that of Th2 cytokines in EAT rats. It is concluded that Th1 cytokines may play more important roles than Th2 cytokines in the pathogenesis of autoimmune thyroiditis and Se supplementation may alleviate thyroid tissue damages induced by acute inflammation.

AITD are destructive inflammatory or anti-receptor autoimmune diseases characterized by reactivity to self-thyroid antigens. They are the most common autoimmune disorders affecting approximately 2% women and 0.2% men in the population. It is believed that AITD are resulted from a complex interplay between genetic and environmental factors, but mechanisms leading to the disease onset are largely unknown.

Intrathyroidal inflammatory and thyroid follicular cells produce a variety of cytokines, including IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, and IFN-γ. These cytokines have both unique and overlapping functions, forming a complex regulatory network. Many cytokines have been implicated in the induction and effecter phases of the immune responses to inflammation; therefore they play critical roles in the pathogenesis of AITD. T-cells also play an important role in AITD through regulation of systemic inflammatory responses. $CD4^+$ T cells are the primary effecter cells, while $CD8^+$ T cells have an antergic role against $CD4^+$ T cells.

Se is an essential component of several major metabolic pathways including thyroid hormone metabolism, and participates in antioxidant enzyme defense mechanisms[1]. Furthermore, Se influences both the innate and the adaptive immune responses. Se deficiency can reduce lymphocyte proliferation, affecting T-cell number and function, and impairing both the cell-mediated and humoral immunity. Recently, several clinical studies have demonstrated that, in patients with autoimmune thyroiditis, Se supplementation can significantly reduce the titers of thyroid peroxidase antibody (TPOAb), suggesting that Se supplementation might be an effective treatment for AITD[2]. However, The results from other studies suggest that Se has no immunological benefit to autoimmune thyroiditis[3]. Given the implication of cytokines in the disease, we hypothesized that Se deficiency might aggravate AITD, Se supplementation might be beneficial to alleviate AITD, and these benefits could be associated with the regulation of the production of various cytokines.

In this study, Fifty six female Lewis rats purchased from The Vital River (Beijing, China) were used at 6-8 weeks of age and 152-203 g of weight. Randomly selected 36 rats were immunized by s.c. injection with porcine thyroglobulin (pTg, Sigma, US) with complete Freund's adjuvant to induce experimental autoimmune thyroiditis (EAT). Sodium selenite was added into the basic animal fodder (containing less than 0.2 μg/kg of Se) to make the final Se concentration of the fodder at 0.2 μg/kg (normal Se, or NSe) and 2 μg/kg (High Se, or HSe), respectively. EAT model rats were divided into high, normal, and low Se (M+HSe, *n*=8; M+NSe, *n*=20; M+LSe, *n*=8) groups and fed with the fodder containing high, normal, and low level Se, respectively, as described above, for a total of 12 weeks. The rats in control group were fed with the same fodder for M+NSe group (0.2 μg/kg of Se). To confirm the occurring of EAT in the experimental rats,

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12 rats in M+NSe group and 12 in control group were sacrificed one week after the last immunization, Then, the remaining 32 rats were sacrificed 7 weeks after the last immunization (or 12 weeks after feeding with different dose Se). Before the rats were sacrificed, blood samples were taken from them and serum levels of thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TGAb) were detected with radioimmunoassay (RIA), as well as free triiodothyronine (FT3), free thyroxine (FT4), and thyroid stimulating hormone (TSH). After the rats were sacrificed, thyroid tissues were dissected, fixed in 10% formalin and embedded in paraffin for pathological detection and immunohistochemistry examinations for IL-2, IFN-γ, TNF-α, IL-4, IL-6, and IL-10. Total protein of thyroid tissues were extracted and the expressions of IL-2, IFN-γ, TNF-α, IL-4, IL-6, and IL-10 were measured by Western blotting.

Characterized by autoreactive T and B cell responses, EAT can be characterized with lymphocytic infiltration of the thyroid gland and elevated serum levels of TGAb and TPOAb. Compared with control group, the immunized rats in M+NSe group showed significantly higher levels of TGAb and TPOAb, and more severe lymphocytic infiltration in the thyroid (P=0.001). These results confirmed that porcine thyroglobulin mixed with Freund's adjuvant effectively can induce EAT in the rat. which suggests that the method of EAT induction worked efficiently in the inbred Lewis rats.

After 12 weeks of feeding with different dose Se (7 weeks after the last immunization), all EAT rats showed similar serum autoantibody levels (Figure 2) but different grade of thyroiditis. Compared with M+NSe group, M+LSe group showed severe thyroiditis; on the other hand, M+HSe group had significantly milder thyroiditis. The difference in pathologic grade between M+LSe group and M+HSe group was significant, and more serious thyroid lesions occurred in the mice with Se deficiency. More severe cases occurred in the Se deficiency group (M+LSe) than in the Se sufficiency groups (M+Hse+M+NSe). However, no significant difference was seen when M+LSe and M+NSe groups were combined and analyzed against M+HSe group. These data suggest that Se deficiency might aggravate the thyroid lesion in EAT. EAT rats with less Se intake had more serious disease symptoms, and Se supplementation reduced serum levels of TGAb and TPOAb, as well as the severity of thyroiditis. However, even in EAT rats treated with high Se, the autoantibody titers did not return to the normal level and thyroiditis did not completely resolve at the end of the 13 week trial. Therefore, normalizing the autoantibody levels and complete reversal of symptoms may require a longer duration or higher level of Se supplementation. Other studies also showed that Se supplementation for a longer period of time decreased the autoantibody concentrations more efficiently[4]. Despite the lack of statistical significance with Se supply, possibly due to the small sample size, our results showed a beneficial effect of Se supplementation in EAT rats (Figure 1).

Serum level of FT4 was significantly higher in M+NSe group than in control group (P<0.05), and it further elevated in M+LSe group (P<0.05), but did not show significant change in M+HSe group. Serum FT3 levels slightly increased in all the EAT rats over control group and the increase in M+LSe group was statistically significant compared with M+NSe group and M+HSe group (P<0.05). Although TSH showed a

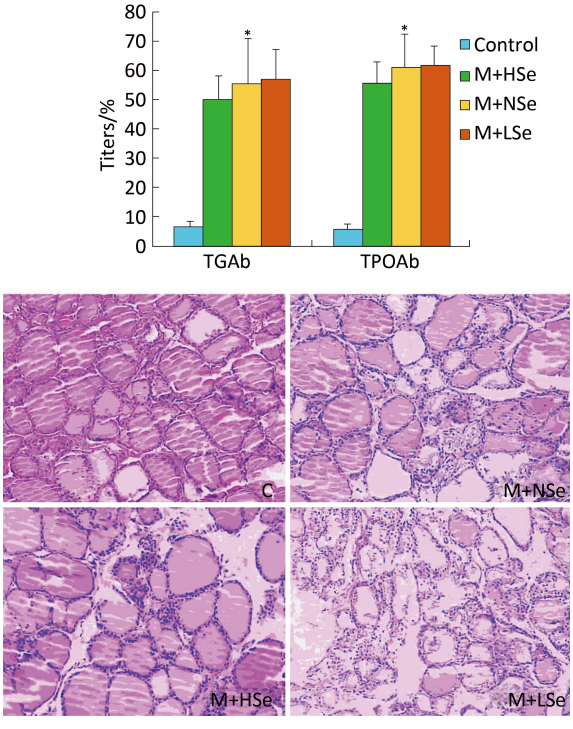

Figure 1. Autoantibody titers and pathology of thyroid tissues from rats fed with different level of Se for 12 weeks. Upper panel: Autoantibody titers; data was presented as means±SD. n=12. $^{*}P$<0.05 *vs.* control. Lower panel: Histology of thyroid tissues from control group (C), EAT groups treated with normal (M+NSe), high (M+HSe), and low (M+LSe) Se. While control tissue shows normal shape of thyroid follicles and plump without lymphatic infiltration, tissue from EAT rats show pathological changes characterized by lymphatic infiltration and early proliferative changes of thyroid follicles (M+HSe and M+NSe), severe destruction of thyroid follicles (M+NSe and M+LSe), and fibrotic changes in some areas (M+LSe and M+HSe).

higher mean values in all the EAT groups, the differences between M+NSe and control group were not significant (P>0.05) due to the big intra-group variation.

These phenomena might be due to an acute release of thyroid hormones in response to acute thyroid tissue damages. Generally, the generation and secretion of thyroid hormone are under the control of Hypothalamus-pituitary-thyroid axis system. However, the process of endocrine is dynamic while the detection on thyroid hormone levels in our study was a cross-section survey. In the previous study, it was demonstrated that the thyroid hormones did not response at the same time[5]. It has a latent time in the reaction of the axis system during the dynamic process. So, there might be a bit of limitation to explain why all the three thyroid hormones in the EAT rats elevated significantly based on the cross-section data. Whatever, it could partially reflect the status of thyroid function with EAT. Although the high Se did not reverse these changes, the higher hormone levels in low Se group seemed to be correlated with the more severe thyroiditis. Interestingly, the previous study showed that, in patients with autoimmune thyroiditis, a significant lower level of Se was correlated with reduced thyroid hormones, and Se therapy could help restore the level of thyroid hormones[6]. The discrepancies between the results obtained from human autoimmune thyroiditis and our EAT rat models might reflect the different thyroid functions under the condition of chronic autoimmune disease in patients and acute thyroiditis induced in the rats.

Cytokines are important immune regulators in the pathogenesis of many diseases. Th1 cytokines can activate macrophages, and produce complement-fixing and -opsonizing antibodies. In general, they are cytotoxic and promote disease progression. On the contrary, Th2 cytokines can inhibit the production of Th1 cytokines[7]. Serum levels of Th1 and Th2 cytokines change dynamically during inflammation and tightly regulate the inflammatory process.

We examined three Th1 cytokines, IL-2, TNF-α and IFN-γ, and three Th2 cytokines, IL-4, IL-10, and IL-6 in EAT rats, among these cytokines, IL-2, TNF-α, and IL-6 showed elevated levels, suggesting their potential roles in the pathogenesis of thyroiditis. For the Th1 cytokines, both IL-2 and IFN-γ had enhanced tissue staining in all the EAT groups. Compared with control group, Western-blotting confirmed the increases in the ranges of 0.2-1.3 fold and 2.2-4.4 fold for these two cytokines, respectively (Figure 2). IL-2 level decreased with Se level dose-dependently among three EAT groups. M+HSe group and M+LSe group had lowest and the highest IL-2 level, respectively (P<0.05 for two groups, Figure 2). IFN-γ level increased in all the EAT groups, especially group M+LSe had significantly elevated IFN-γ among the EAT groups (P<0.05, Figure 2). However, no correlation between Se supplementation and IFN-γ level was identified. TNF-α protein level was not significantly different between rats in the control and M+NSe groups, but among three EAT groups, it decreased significantly in M+LSe group (P<0.05 *vs.* M+Hse, Figure 2).

IL-2 is the only cytokine showed a clear dose-dependent decrease in response to Se treatment, which is consistent with its role in inflammation. Migita K reported that IL-2 significantly augmented the cytotoxicity of mononuclear cells, one of the IL-2 activated killer cells that might contribute to the pathogenesis of autoimmune thyroiditis[8]. The previous observation by Ruka Setoguchi et al.[9] also showed that blocking IL-2 could inhibit proliferation of self-antigen reactive T cells. The decreased IL-2 level in the higher Se groups also supports the role of Se in the protection of EAT manifestation. Two other Th1 cytokines showed less clear pattern of regulation in EAT. TNF-α level was not significantly changed between normal and EAT rats, but up-regulated by high Se diet and down-regulated under Se deficiency. These results are consistent with the previous observations that TNF-α is not essential for development of EAT since anti-TNF-α did not reduce disease severity at the early stage. Another TH1 cytokine IFN-γ increased in EAT rats but it is not regulated by different Se intakes.

The changes in three Th2 cytokines were less dramatic. Among EAT groups, IL-4 and IL-10 both showed a mild increase with the Se level but only the change of IL-10 in group M+HSe was significant (P<0.01, Figure 3). IL-6 levels increased in all the three EAT groups but there was also no significant difference in the IL-6 level among different Se feeding groups (P>0.05, Figure 3). Although Se supplementation increased the two cytokine levels, their roles in the pathogenesis of the disease are unclear under our experimental setting. Previously, Se supplementation has been shown to increase the production of IL-6 and IL-10, and it also protects mice against infection[10]. In our study, Se treatment increased IL-10 level but did not significantly alter IL-6 level. Currently, we are investigating the mechanism of cytokine regulation by Se and the functional consequences.

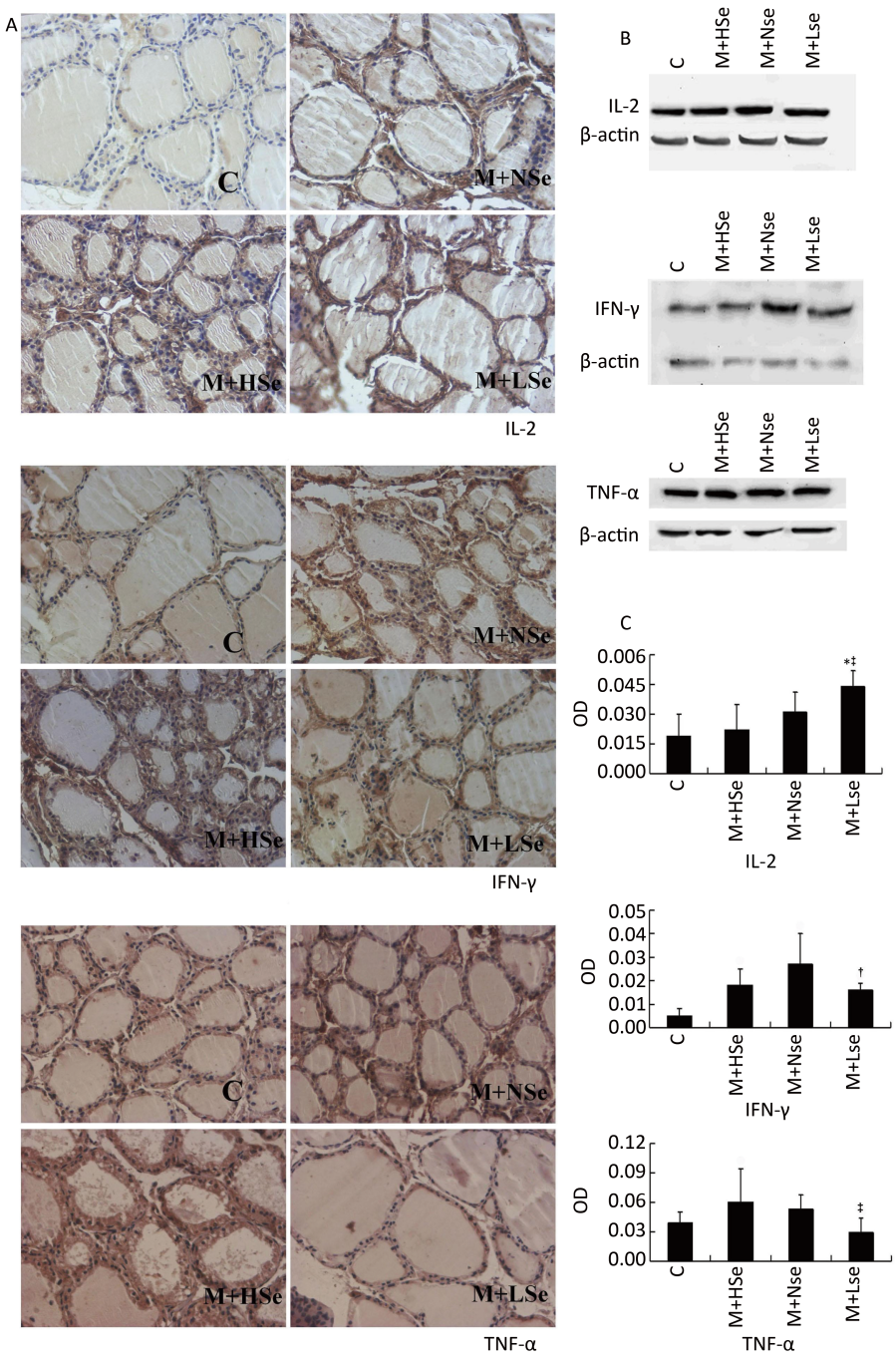

Figure 2. Levels of Th1 cytokines in thyroid tissues from different groups of rats. Three cytokines were shown in three groups of images, as indicated. A: The photograph of IHC stained thyroid tissues; B: Images of Western blotting; C: The protein expression after the values of OD of protein bands were normalized by that of β-actin. $^{*}P<0.05$ *vs.* control. $^{\dagger}P<0.05$ *vs.* M+NSe group; $^{\ddagger}P<0.05$ *vs.* M+HSe group. Data was presented as mean±SD. $n=8$.

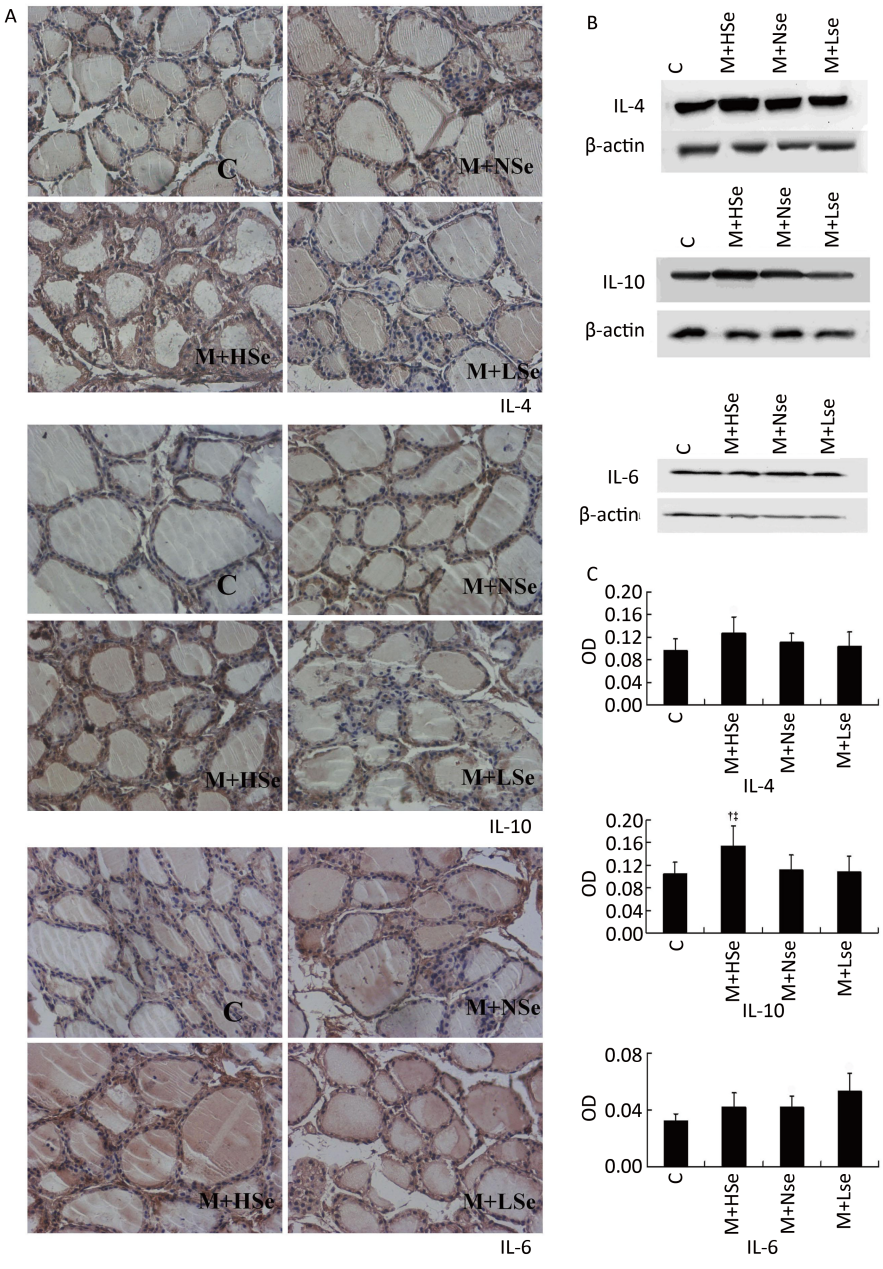

Figure 3. Levels of Th2 cytokines in thyroid tissues from different groups. Three cytokines were shown in three groups of images, as indicated. A: The photograph of IHC stained thyroid tissues; B: Images of Western blotting; C: The protein expression after the values of OD of protein bands were normalized by that of *β*-actin. †*P*<0.05 *vs.* M+NSe group; ‡*P*<0.05 *vs.* M+HSe group. Data was presented as mean±SD. *n*=8.

These results might indicate that Th1 cytokines play more important roles than Th2 cytokines in EAT pathogenesis and Se regulation. Particularly, IL-2 may act as the inflammatory cytokine to induce apoptosis and follicular lesion of thyroid gland. Therefore, Se may exert its action by suppressing IL-2 production. However, the relationship between Se and other cytokines still needs more investigation. Although Se only affected the expression of some of the cytokines in this study, showing more significant effect on Th1 cytokines, there may be more cytokines potentially playing important roles in EAT or being regulated by Se. Se may also exert its protective functions through mechanisms outside the antioxidation and immunological pathways. Future studies should also include a wider dose

range of Se for treatment of a longer time period of time.

In summary, the results of present study confirm the hypothesis that EAT could be aggravated by Se deficiency, and Se supplementation could alleviate inflammatory lesions in the thyroid and reduce TGAb and TPOAb titers in rat model of EAT. Se treatment also dose-dependently inhibited IL-2 expression and affected other cytokines at varying degrees. Although the significance of individual cytokine in the pathogenesis of EAT and the mechanisms of Se regulation have not been clearly elucidated, this study demonstrated the beneficial effect of Se supplementation in improving immune function and protecting thyroid from inflammatory damages.

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