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RESEARCH PAPER

Nano-selenium supplementation upregulate TLR-7, MyD88, NF-kB, and TRAF6 genes in thymus of Wistar rats following treatment with cyclosporine A

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ABSTRACT

Objective(s): Selenium Nanoparticles can modulate the function of the immune system and improve immunity. We investigate the expression of toll-like receptor-7 (TLR-7), myeloid differentiation primary response 88 (MyD88), Nuclear factor kappa B (NF-κB), and TNF receptor associated factor 6 (TRAF6) genes in thymus of Wistar rats following treatment with cyclosporine A (CsA) and Nano-selenium (Nano-Se) supplementation.

Materials and Methods: Twenty-four male Wistar rats (200-220 grams) were divided into 3 groups of control (n=8), CsA (n=8), and CsA+Nano-Se (n=8). Rats in CsA and CsA+Nano-Se group's received cyclosporine A and olive oil solution by subcutaneous injection for 10 days at a dose of 5 mg/kg/day. Nano-Se with a dose of 2.5 mg/kg of body weight was gavaged to the CsA+Nano-Se group once a day and 3 times a week. Real-time PCR were used for gene expression of TLR-7, MyD88, NF-kB, and TRAF6 at thymus.

Results: The result of this study show that CsA significantly decreased expressions of TLR-7, MyD88, NF-kB, and TRAF6 at thymus compared to control group (P<0.05). However, expressions of TLR-7, MyD88, NF-kB, and TRAF6 at thymus in CsA+ Nano-Se group was significantly increased compared to CsA group (P<0.05).

Conclusion: Nano-Se supplementation significantly regulated the expression of TLR-7, MyD88, NF-kB and TRAF6 genes in the thymus of rats treated with cyclosporine A. Therefore, Nano-Se supplementation can be recommended to boost immune function after using immunosuppressive drugs. However, more research is needed in the future.

Keywords: *Cyclosporine A, Immune system, Nanoparticle, Selenium, Thymus*

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INTRODUCTION

Selenium and Nano-selenium (Nano-Se) or (Se-NPs) have been used in maintaining human health [1]. Nano-Se can be used in biomedicine and drug delivery [2] food supplements, therapeutic agents [3] and nano-pharmaceutical applications [4]. The nano-form of selenium attracts even more attention, thanks to its high bioavailability and lower toxicity than inorganic and organic forms, [5, 6] where inorganic compounds are more toxic than organic ones [7]. The biological properties of selenium nanoparticles depend on their size: smaller particles have a greater activity [8]. Particle size affects the cellular uptake of NPs; for example, *in vitro* uptake of 0.1 µm particles was found to be 2.5 and 6 times higher compared to 1 and 10 µm particles, respectively. Concerns about the toxicity of selenium has limited the doses used in chemoprevention. Based on previous studies, intakes of 400 µg/day and plasma selenium of 1000 ng/ml [9]. In rats, the median lethal dose (LD50) of intraperitoneal selenomethionine (SeMeth) was determined to be 4.25 mg Se/kg [10]. Standard dose of selenium is important for immune initiation. In addition, it has been stated that Selenium plays a role in regulating excessive

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immune responses and chronic inflammation. The use of Nano technology on selenium, multiplies its delivery and absorption and leaves better effects. Mahdavi et al. (2017) showed that oral administration of Nano-Se induced a robust Th1 cytokine pattern after a hepatitis B surface antigen vaccination in an animal model [11]. Hooshangi et al (2022) showed that Nano-selenium consumption with exercise training regulated IL-4 and IFN-γ gene expression in thymus of rats which were exposed to dexamethasone [12].

Cyclosporine A (CsA) is a lipophilic and cyclic endopeptidase [13, 14]. CsA was mainly metabolized with liver tissue and the terminal half-life of non-modified formulation is 19 h and the terminal half-life of modified formulation is 8.4 hr. Less than 1% of it appears in urine or feces. It has been stated that CsA has several adverse effects, the most notable is acute and chronic renal toxicity, but also its other effects include immunosuppression, hypertension, hyperlipidemia, neurotoxicity, hypomagnesemia, hyperuricemia, and thrombotic microangiopathy [13]. The effect of CsA on T cell maturation and selection in the thymus was shown before [15]. Differentiation, selection, and proliferation of T lymphocytes occurs in the thymus [16]. It has been stated that the number of mature T lymphocytes correlates with immune function at cellular levels [17, 18]. Many environmental pollutants and pharmaceuticals, especially CsA, can affect the normal function of the thymus. Hu et al. (2019) demonstrated that atmospheric hydrogen sulfide (H2S) triggers the TLR-7/MyD88/NF-kB pathway to promote activation of NOD-like receptor protein 3 (NLRP3) inflammasome [19]. Consequently, H2S activates the TLR-7/MyD88/NF-kB signaling and the NLRP3 inflammasome to promote the inflammation, which then causes tissue damage in the thymus of the animal model (broiler chickens) [19]. However, the effect of CsA on this pathway, especially in the thymus tissue, which is highly important in the immune system, has not been investigated. In addition to the mentioned factors, it has been stated that TNF receptor-associated factor 6 (TRAF6) a member of the TNF receptor-associated factor family. TRAF6 can activate several cellular signal such as NF-κB [20]. TRAF6 also plays an important role in the regulation of innate and adaptive immunity, tissue homeostasis, and bone metabolism. It has been shown that TRAF6 have a critical role in development, homeostasis, and activation of various immune cells [21]. Therefore, regulation of this factor in thymus tissue can have a therapeutic role.

Since limited studies have investigated the role of Nano-Se in the immune system, especially the TLR-7/MyD88/NF-kB and TRAF6 pathway in the immune system, the aim of the present study is also investigating the expression of TLR-7, MYD88, NF-κB, and TRAF in thymus of rats induced by CsA (as an immunosuppressive drug) and Nano-Se supplementation.

MATERIALS AND METHODS

Animals

Twenty-four male Wistar rats (200-220 grams) were purchased from Pasteur Laboratory Animal Breeding Center (Tehran, Iran). Then the rats were transferred to the animal center laboratory. Animals had free access to water and food. Rats were kept in standard conditions with light (12/12 hr), temperature (24), humidity (55%) [22]. Experiments were performed according to NIH Guidelines for animal study on *Wistar* rats (ethical code: IR.IAU.PIAU.REC.1401.004). The rats were divided into 3 groups: control, cyclosporine A (CsA), and Nano-selenium (Nano-Se) + cyclosporine A (CsA) by simple randomization.

Cyclosporine A

One week after familiarization with the environment, cyclosporine A (CsA) and olive oil solution were administered subcutaneously for 10 days at a dose of 5 mg/kg/day (CsA and CsA + Nano-Se group's). Each capsule of CsA (25 mg) was dissolved in 5 ml of olive oil and 200 µl of this mixture was injected into each animal.

Nano selenium

In this study, a Nano-selenium manufactured by ARMINANO company was utilized (Armina Engineering Co, Tehran, Iran). To prepare the mixture, firstly based on the company description, the aqueous extract of ginger obtained was utilized as a precursor for the synthesis of nano-selenium. Ginger extract (2 ml) was added dropwise into the 20 ml solution of SeO_3 (10 mM), with vigorous stirring. The mixture was incubated by placing the solution onto a rotatory orbital shaker operating

at 200 rpm, 30 °C for 72 hr in dark conditions. The reduction of selenium ions was monitored by sampling an aliquot (3 ml) of the mixture at intervals of 24 hr, followed by measurement of maximum absorption. Maximum absorption was determined by measuring the optical density of the content from wavelength 350 to 700 nm using UV–Vis spectrophotometer [23-25]. SeNPs at a dose (of 2.5 mg/kg b.w) were given to the Nano-Se group by oral gavage once a day for 3 times/ week [26].

Gene expression

Real Time PCR technique was used to investigate the expression of TLR7, NF-κβ, MyoD88 and TRAF6 genes in the thymus tissue of different groups. First, primer design was done and then total RNA was extracted from thymus tissue and converted into cDNA (Table 1). Then, the cDNA was amplified by PCR and analyzed for the expression of the mentioned genes [27].

For molecular investigations at the level of gene expression, RNA extraction from the tissues in all investigated groups was done according to the manufacturer's protocol (Qiagen, Germany). First, 200-300 of Kyazol was added and it was kept at -80 for 24 hr. After 24 hr, the plaque in the cryotube was crushed by the head sampler in a semi-frozen state, then it was pipetted. Then, about 100 chloroform was added to the sample to lyse the cells. This solution should be in contact with the cells for about 1 min. After 1 min, the solution was centrifuged at 12000 rpm for 10 min. After centrifugation, the solution was divided into three phases:

The clear liquid from the top of the tube containing the RNA was gently removed and placed in a DEPC microtube. Then, 1 cc of isopropanol was poured on the clear RNA and stirred by hand for 1 min. Isopropranol is clear and RNA is also clear, but when these two are mixed together, they produce a cloudy liquid. It is better to keep this solution overnight at -80 temperature. After adding isopropanol, the samples were centrifuged at 12,000 rpm for 10 min. After removing from the centrifuge, the supernatant liquid was drained and 1 cc of alcohol 70 was added to it. After vortexing, the mixture was centrifuged for 10 min at 7500 rpm. Then the supernatant was drained with a sampler and then the plaque was dried inside the microtube. In order to dissolve RNA, 200 µL of distilled water at 60 degrees was poured on the plate inside the microtube. Then, it was placed on a plate for 5 min with a pipette head sampler. The extracted RNA was kept at -80 temperature until use.

After extracting RNA with high purity and concentration from all studied samples, cDNA synthesis steps were performed according to the manufacturer's protocol (Fermentas, USA) and then the synthesized cDNA was used to perform the reverse transcription reaction. First, all the designed primers related to all the genes were examined, and then the expression of the genes was examined using the quantitative q-RT PCR method. The expression ratio of the studied genes in this study was evaluated by the comparative method of Threshold Cycle (CT). By putting data into a formula:

$$R = 2^{-(\Delta\Delta CT)}$$

$$\Delta\Delta CT = (CT_{target} - CT_{refence})_{Time\ X} - (CT_{target} - CT_{refence})_{Time\ 0}$$

Table 1. Primer pattern used

	Genes name	Primer sequences	Product lenght	Accession number
1	Rattus norvegicus toll-like receptor 7 (TLR7)	Forward: GCTTCCCAGAAAACGTCCTC Reverse: CCACCAGACAAACCACACAG	95 nt	NM_001097582.1
2	Rattus norvegicus RELA proto-oncogene, NF-kB subunit (Rela)	Forward: ACGCAAAAGGACCTACGAGA Reverse: ATGGTGCTGAGGGATGTTGA	171 nt	NM_199267.2
3	myeloid differentiation primary response gene 88 (Myd88)	Forward: ACCTGTGTCTGGTCCATTGCCA Reverse: GCTGAGTGCAAACTTGGTCTGG		NM_010851
4	TNF receptor-associated factor 6 (Traf6)	Forward: TTTCCCTGACGGTAAAGTGCCC Reverse: ACCTGGCACTTCTGGAAAGGAC		NM_009424.3
5	Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward: CAAGTTCAACGGCACAGTCA Reverse: CCCCATTTGATGTTAGCGGG	102 nt	NM_017008.4

The specific standard curve of each gene was drawn using at least 5 logarithmic concentrations in diluting order from the positive control of each gene.

The expression level of the target gene was normalized with the reference gene and the expression of the genes of the healthy group was considered as a calibrator [28].

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}}}{(E_{\text{reference}})^{\Delta CT_{\text{reference}}}}$$

$$(\Delta Ct_{\text{refrence}} = Ct_{\text{control}}\text{-}Ct_{\text{treatment}} \qquad \Delta Ct_{\text{target}} = Ct_{\text{control}}\text{-}Ct_{\text{treatment}})$$

Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) along with Tukey's *post hoc* test was used to compare the groups (P≥0.05). All statistical studies were done using SPSS version 23 software.

RESULTS

Changes in NF-kB gene expression in the thymus tissue of different study groups are shown in Fig. 1. CsA caused a significant decrease in NF-kB gene expression in thymus tissue compared to the control group (P<0.001). Meanwhile, the use of Nano-Se supplement after CsA significantly increased the expression of NF-kB gene in thymus tissue compared to CsA group (P<0.001) (Fig. 1).

Changes in MyoD-88 gene expression in the thymus tissue of different study groups are shown in Fig. 2. As it is shown, CsA caused a significant decrease in the expression of MyoD-88 gene in the thymus tissue compared to the control group (P=0.001). Meanwhile, the use of Nano-Se supplement after CsA significantly increased the expression of MyoD-88 gene in thymus tissue compared to CsA group (P=0.012) (Fig. 2).

Changes in TLR-7 gene expression in the thymus tissue of different study groups are shown in Fig. 3. As can be seen, CsA caused a significant decrease in TLR-7 gene expression in thymus tissue compared to the control group (P<0.001). Meanwhile, the use of Nano-Se supplement after CsA significantly increased the expression of TLR-7 gene in thymus tissue compared to CsA group (P=0.001) (Fig. 3).

Changes in TARF-6 gene expression in the thymus tissue of different study groups are shown in Fig. 4. The figure shows that CsA caused a significant decrease in TARF-6 gene expression in thymus tissue compared to the control group (P<0.001). Meanwhile, the use of Nano-Se supplement after CsA significantly increased

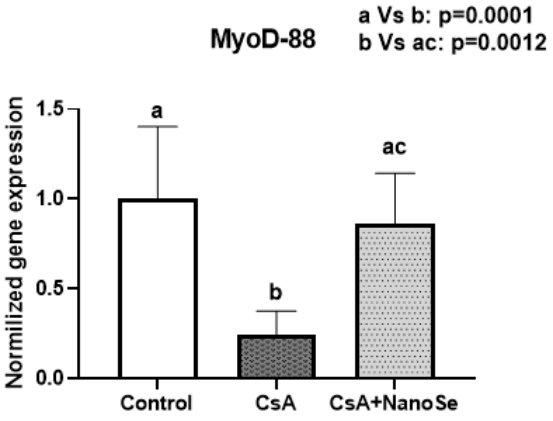

Fig. 2. MyoD-88 gene expression in different study groups. Data are shown as mean ± standard deviation. A significance level of P<0.05 is considered. CsA: Cyclosporine A, Nano Se: nano-selenium

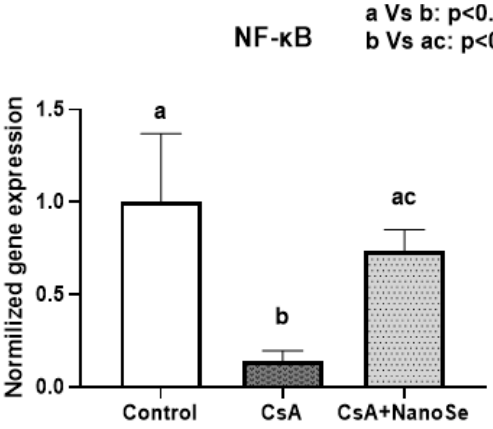

Fig. 1. NF-kB gene expression in different study groups. Data are shown as mean ± standard deviation. A significance level of P<0.05 is considered. CsA: Cyclosporine A, Nano Se: nano-selenium

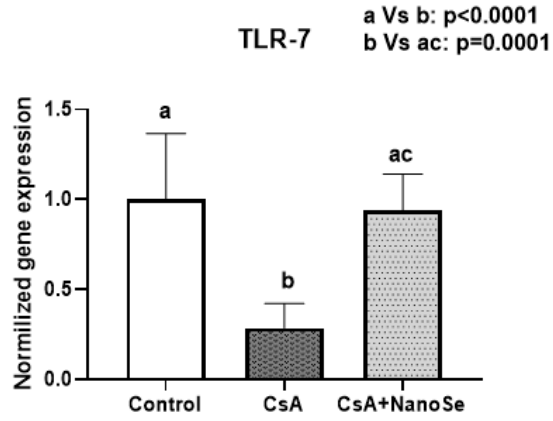

Fig. 3. TLR-7 gene expression in different study groups. Data are shown as mean ± standard deviation. A significance level of P<0.05 is considered. CsA: Cyclosporine A, Nano Se: nano-selenium

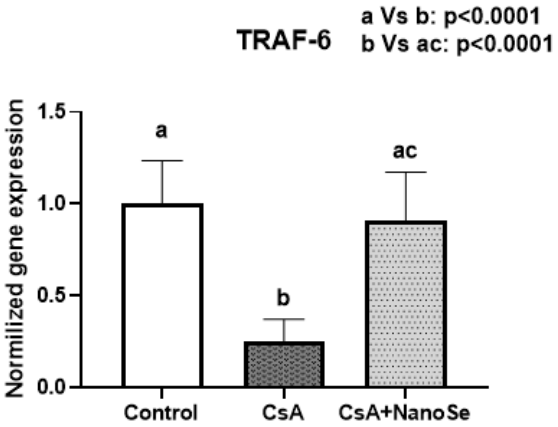

Fig. 4. TRAF-6 gene expression in different study groups. Data are shown as mean ± standard deviation. A significance level of p<0.05 is considered. CsA: Cyclosporine A, Nano Se: nano-selenium

the expression of TARF-6 gene in thymus tissue compared to CsA group (P<0.001) (Fig. 4).

DISCUSSION

Selenium is an essential trace element for human health [29]. The insufficient amount of this element leads to an increased risk of many diseases related to the immune system and chronic degenerative diseases. Also, studies have shown that treatment with excess selenium leads to toxicity and increases lipid peroxidation due to strong oxidative stress caused by high doses of selenium and has a negative effect on health [30]. Using nanotechnology, it has been confirmed that selenium nanoparticles (Se NPs) have stronger biological activity and less toxicity than traditional selenium compounds [31, 32]. Therefore, the purpose of this study was to investigate the expression of TLR-7, MyD88, NF-κB, and TRAF-6 in thymus of rats induced by CsA and Nano-Se supplementation.

The results of the present study showed that CsA injection caused a significant decrease in NF-kB gene expression in thymus tissue, while Nano-selenium supplementation prevented a significant decrease in this gene. Jin et al. (2017) showed that CsA injection in models with kidney damage with Klotho overexpression led to an increase PDLIM2, a decrease in the NF-κB p65 and inhibition of the iNOS and inflammatory cytokines (TNFα, IL-6, IL-12). These researchers stated that Klotho appeared to increase PDLIM2 expression and decrease NF-κB p65 expression, while PDLIM2 siRNA could block the inhibitory effects of Klotho on NF-κB p65 expression [33]. In this study, although Klotho values were not evaluated and the target tissue was the thymus, it seems that in the present study, CsA factors such as Klotho were effective in inhibiting NF-kB. Contrary to these results, Sarhan et al. (2020) also showed that the oral dose of cyclosporine A (25 mg/kg of body weight) for 21 days caused a significant decrease in Bcl-2 and an increase in NF-kB, PAI- 1, Caspase-3 and p53 in kidney tissue of treated mice [34]. Meanwhile, the present study aimed to investigate the immune system as well as lower doses of CsA. As stated, the changes of NF-κB reduction in the CsA group were lower than the healthy control group. Aberrant T cell development is a central risk factor for autoimmune disease. It has been stated that the reduction or negative regulation of NF-κB or IKKα-inducing kinase in mice leads to severe T-cell-mediated inflammation [35], damage and fibrosis in the liver and lung, which increases the mortality rate [36]. Therefore, the increase of NF-kB to reach the optimal level with the control group can also prevent these damages in the immune system, which in the current study of the Nano-Se consuming group, these changes were confirmed and thus reduced the destructive effects of CsA on the thymus tissue. It has been shown that in the hearts of rats poisoned with cadmium, the use of Nano-Se modulated the inflammatory response caused by Cadmium through the NF-kB/IkB pathway [37], although in the present study, the changes of this factor were evaluated in the thymus tissue, but it seems that Nano-Se can be effective in regulating NF-kB and modulating the immune system.

The results of MyoD-88 and TLR-7 gene expression in thymus tissue also showed that CsA decreased these genes expression and NanoSe modulated them. TLRs have an important role in innate immune system and recognize molecules that are widely shared by pathogens. Therefore, excessive reduction or increase of this factor can cause disorders, especially in the immune system. In the present study, CsA decreased this factor compared to the healthy control group. Resiquimod (a TLR-7 agonist) has been shown to activate NF-κB and release cytokines (IFN-α and IL-6) through a TLR-7/MyD88-dependent pathway [38]. Furthermore, MyD88 is a critical component leading to the increase inflammatory cytokines [39]. Although the level of inflammatory cytokines was not investigated in this study, it seems that the abnormal secreted cytokines may

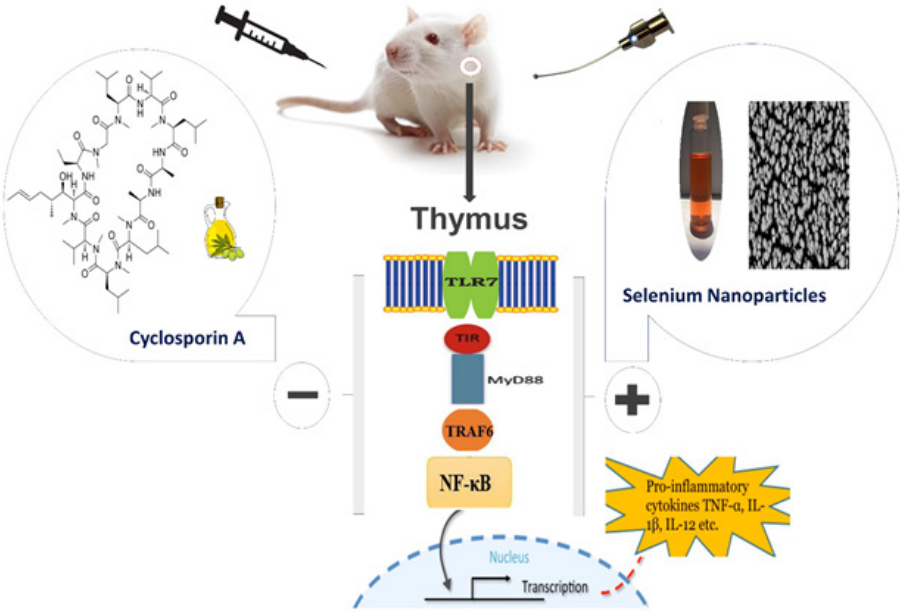

Fig. 5. The protective effects of Nano-Se supplementation on TLR-7/MyD88/TRAF-6/NF-κB pathway in thymus of rats after immunosuppression caused by Cyclosporine A

be regulated by the TLR-7/MyD88 pathway in the thymus. The data of the present study showed that in the thymus tissue, the expression of TLR-7, MyD88 and NF-kB genes were significantly upregulated by Nano-selenium supplementation after CsA, which is consistent with the hypothesis of immune regulation. Bai et al. (2022) showed that selenium deficiency causes inflammatory damage by activating the TLR signaling pathway in an animal model. These researchers stated that selenium deficiency increases several pro-inflammatory cytokines and it seems that it is due to the activation of TLR/MyD88/NF-kB pathway, which causes inflammatory damage in the bursa of Fabricius of broiler chickens [40]. Meanwhile, in the present study, the rat animal model was used with CsA induction and Nano-selenium supplement was used for treatment, and therefore, in the present study, the effect of Nano-selenium on immune modulation through the TLR/MyD88/NF-kB pathway was confirmed. In this study, in addition to the mentioned factors, TRAF-6 gene was also investigated in the thymus tissue and like other variables, it was decreased with CsA injection and increased with NanoSe consumption. Cho et al (2007) demonstrated that TLR-2 stimulation engages MyD88-IRAK4-TRAF6 pathways, leading to NF-κB activation [41]. Although TLR-2 changes were not investigated in the present study, MyoD88 changes were consistent with TRAF-6. In immune system, TRAF6-mediated signaling is important for the development, homeostasis, and/or activation of B, T, and myeloid cells. This factor is also necessary for organogenesis of thymus and secondary lymphoid tissues [42]. In multiple cellular contexts, TRAF6 function is essential not only for proper activation of the immune system, but also for maintaining immune tolerance. Therefore, the modulation of this cellular pathway by the expression of TLR-7, MyD88, NF-kB and TRAF6 genes with nano-selenium supplementation after immunosuppression caused by CsA can confirm its protective role in the immune response (Fig. 5).

CONCLUSION

Based on the results of the present study, the use of nano-selenium supplements in reducing immune function, especially in CsA-induced immunosuppressive models, can play an immune protective role by modulating the TLR-7/MyD88/TRAF-6/NF-κB pathway in the thymus. However, future studies on human samples are suggested.

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ETHICAL APPROVAL

Experiments were performed according to NIH Guidelines for animal study on Wistar rats (ethical code: IR.IAU.PIAU.REC.1401.004).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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