Effects of Naringenin on experimental rheumatoid arthritis in Wistar rats

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Abstract

Naringenin is one of the most important and abundant known flavonoids found in grapefruit and other citrus fruits. This experimental study aimed to evaluate the clinical effects and immune responses of naringenin in the animal model of rheumatoid arthritis (RA) according to various reports on its anti-inflammatory effects and modulation of the immune system. 40 Wistar rats in the weight range of 160-180 g were randomly divided into four groups (n=10) including healthy, control, naringenin and methotrexate orally treated groups. To induce RA disease, a compound of 200 µl of Freund's adjuvant and collagen type II was injected subcutaneously into the rear footpads of rats. The severity of RA clinical signs was assessed according to a standard scoring
method. The duration of treatment was 3 weeks (days 7-28 after induction). Attained data showed that the levels of C-reactive protein (CRP), myeloperoxidase, nitric oxide, IL-17, and IFN-γ cytokines, significantly increased in the RA rats while the level of their serum antioxidants, significantly reduced compared to the healthy rats. The inflammation of the paws and the level of CRP, decreased similarly in both methotrexate and naringenin treated groups. In naringenin treated group, a further decrease in serum myeloperoxidase, nitric oxide and the total antioxidant capacity occurred compared to the methotrexate treated rats. However, IL-17 and IFN-γ cytokines levels were further decreased in the methotrexate-treated group. As a result, naringenin can be used as a suitable approach to reduce the inflammatory effects and control of RA disease.

**Keywords:** Rheumatoid arthritis, Naringenin, Methotrexate, Wistar rats.

**Introduction:**

Rheumatoid arthritis (RA) is a systemic, chronic, and progressive disease (Dai, Di Zhou et al. 2018) with a prevalence of 0.5-1% in dogs and human population (Heuser 1980). The disease is manifested by formation and deposition of immune complexes in the synovial tissue and other organs. It leads to inflammation, an increase in the thickness of the synovium and finally pain, stiffness, swelling, deformity, displacement and reduced joints mobility and development of immune-mediated inflammatory disease (Okamoto, Koizumi et al. 2007, Pan, Cheng et al. 2017). Dogs with RA also show lethargy, anorexia, and fever (Shaughnessy, Sample et al. 2016). Although the main cause of this disease is unknown, the presence of autoantibodies against citrulline proteins and other joint components makes this disease among the autoimmune diseases (Okada, Eyre et al. 2019). It has been shown that the progression of the disease to areas
outside the joints, can cause complications in coronary arteries and cardiovascular disorders which is one of the leading causes of death in patients with RA (Georgiadis, Voulgari et al. 2008).

In the past, CD4+ T lymphocytes appeared to play a major role in Th-1 conversion and secretion of inflammatory cytokines in the development of RA; however therapeutic measures against it do not reduce the severity of the disease. Since 2005, the prominent role of Th-17 (IL-17) has been considered a potent mediator in RA induction. Th-17- producing cells express the transcription factor RORγt. These cells are differentiated in vitro by TGF-β and IL-6 and proliferated in the presence of IL-23, IL-1, and TNF-α. Due to the regulatory role of Th-1 on Th-17, the lack of cytokines secreted by Th-1 intensifies the differentiation of Th-17. Due to its pleiotropic role on various cells in synovial tissue, IL-17 synergistically increases the production of TNF-α, IL-1, IL-6 by macrophages, and fibroblasts (Inglis, Šimelyte et al. 2008, Kelepouri, Mavropoulos et al. 2018). However various drugs are used to treat RA, including glucocorticoids, nonsteroidal anti-inflammatory drugs, methotrexate, anti-B lymphocyte drugs, anti-cytokines, and CTLA-4 Ig, they are less desirable due to their high side effects or high cost and the responses of many patients are not favorable. Therefore, ongoing research is aimed to find low-risk and low-cost drugs to augment to patients' treatment regimens (Golbahari and Froushani 2019, Ghaffary and Froushani 2020).

4, 5, and 7 trihydroxy flavonoid or naringenin is one of the most important and abundant known flavonoids found in grapefruit and other citrus fruits (Dou, Zhang et al. 2013, Yan, Wen et al. 2016). Flavonoids are important plant pigments with numerous biological and pharmacological effects including strong antioxidant properties (Ravishankar, Rajora et al. 2013). Therefore, it seems that naringenin can modulate acute and chronic inflammatory responses. There have also
been reports of the naringenin effects in controlling sepsis, acute hepatitis, fibrosis, colitis, autoimmune experimental encephalomyelitis, and even cancers (Du, Jin et al. 2009, Alam, Kauter et al. 2013, Jin, Zeng et al. 2017). In addition to having anti-inflammatory effects, naringin also has an effect on lipoprotein metabolism which can be effective in controlling diabetes by decreasing insulin resistance and atherosclerosis. (Mulvihill, Burke et al. 2016). So far, no comprehensive research has been done on the possible effects of naringenin in patients with RA or its experimental model. Therefore, in the current study, it was decided to investigate its role on the course of induced rheumatoid arthritis in rats.

**Materials and methods**

**Analysis method**

All study procedures were approved by the Ethics Committee for Animal Care and Use at Urmia University and Razi Vaccine & Serum Research Institute (Approval No. RVSRI.REC.98.015).

All 40 male Wistar rats (160-180 g), used in this experimental study, were purchased from Razi Vaccine and Serum Research Institute. Rats were kept at 25 °C and light program 12:12 during the study period and had free access to water and food. The straw of cages was sterile and changed every two days. To adapt to environmental conditions, rats were kept in quarantine for three days. Then, they were randomly divided into four equal groups (n=10) as bellows:

- **Healthy rat group:** In this group, RA was not induced and did not receive any treatment except PBS (as a placebo) daily and orally in a volume of 0.5 ml.

- **Control rat group:** In this group, RA was induced and received PBS daily and orally in a volume of 0.5 ml from the seventh day after the onset of the disease (observing swelling in the rats’ paw).
- **Naringenin-treated rat group:** In this group, RA was induced and received naringenin suspension in PBS (40 mg/kg, purchased from Chengdu Kanghui Biotechnology Co., Ltd) daily and orally in a volume of 0.5 ml from days 7-28 after induction.

- **Methotrexate-treated rat group:** In this group, RA was induced and received methotrexate suspension in PBS (1 mg/kg, purchased from Ebew company) daily and orally in a volume of 0.5 ml from days 7-28 after induction.

These doses of naringenin and methotrexate were based on the previous studies (Naji Zavareh and Abtahi Froushani 2019, Salehi, Fokou et al. 2019).

To induce RA, 200 µg of type II collagen (Sigma Aldrich) was dissolved in 100 µl of 0.05 M acetic acid solution, and then 100 µl of Freund's adjuvant (Sigma Aldrich) was added. Mixing was continued until a uniform emulsion obtained. The emulsion (final volume of 200 µl) was injected subcutaneously into the rats’ rear footpads. In the healthy group, the same volume of PBS was injected (Jahan Tigh, Abtahi Froshani et al. 2018). From the 7th day, the clinical signs of induced RA (swelling, redness, and inflammation) were seen, and treatment protocols were begun. In this study, the specific scoring method was used for each paw with a score range of 0-4 as follows:

- **Score 0:** paw without swelling and redness
- **Score 1:** paw with redness and slight swelling
- **Score 2:** paw with moderate edema
- **Score 3:** paw with prominent edema and limited use of the joint
- **Score 4:** paw with severe edema and inability to use the joint.
The injected foot was removed from the scoring process. Therefore, the maximum score per rat could be 12 (Ghaffary and Froushani 2020). Changes in the paw diameter of each rat were recorded on days 7, 14, 21, and 28.

On day 28, the animals were underwent deep anesthesia and bled to get the serum for the following tests to be performed:

Myeloperoxidase (MPO) activity test: 10 µl of serum sample with 80 µl of 0.75 mM hydrogen peroxide and 110 µl of the reaction solution containing 2.9 mmol of TMB in 14.5% dimethyl sulfoxide plus 150 mM of sodium phosphate buffer, pH = 4.5 were mixed. The samples were kept at 37 °C for 15 minutes, then 50 µl of sulfuric acid (2 M solution) was added to stop the reaction. Light absorbance was measured at 450 nm (reference: 620 nm). As a standard for positive control, 10 µl of HRP (Horseradish Peroxidase) (2.5 and 25 mU/ml) was used. Finally, MPO activity was reported based on the difference in absorbance compared to the standard HRP curve in mIU/ml (Abtahi Froushani and Mashhouri 2019).

Nitric oxide measurement in serum samples: In brief, 100 µl of griess solution (0.1% naphthyl ethylene diamide, 3% phosphoric acid and 0.1% sulfanilamide) with 100 µl serum sample were mixed. The resulting mixture was kept in the dark at 25 °C for 10 minutes. Finally, the light absorbance was read at 540 nm. The nitric oxide level was reported compared to the standard curve (Griess method) (Golbahari and Froushani 2019).

Total antioxidant capacity (TAC): The TAC of serum samples was assessed by ferric reducing antioxidant power (FRAP) kit which measures the reducing potential of an antioxidant reaction with a ferric tripyridyltriazine (Fe3+-TPTZ) complex, and producing a colored ferrous tripyridyltriazine (Fe2+-TPTZ)]. For this purpose, 20 µl of serum sample was added to 1 ml of
working solution and vortexed. The optical absorption of the sample was read at 593 nm at zero time and four minutes later, was compared to the control sample (Blank). Then, the amount of light absorbance was entered in the TAC formula to obtain its value. The numerical size of the TAC calculated by using the standard curve (Hadžović-Džuvo, Lepara et al. 2011). Also, the sera levels of C-reactive protein (CRP) were monitored by commercial CRP measuring kits (BD, UK).

In order to evaluate the production of IL-17 and IFN-γ cytokines, on the 28th day after the induction of the disease, the spleens of rats were removed under sterile conditions. Each spleen was then crushed in 5 ml of RPMI 1640 (Sigma) culture medium containing 10% FBS (Gibco) and passed through a wire mesh with a diameter of 20 μm. The resulting cell suspension was centrifuged at 2000 rpm for 10 minutes. 5 ml of RBS lysis buffer was added to the cell sediment and after 5 minutes, it was centrifuged at 3000 rpm for 15 minutes by adding 10 ml of RPMI medium. Finally, the resulting cell sediment was suspended in RPMI 1640 (10% FBS, 0.1 g/l penicillin, 0.1 g/l streptomycin, and 2.5 mg/l amphotericin B). Following cell counting by the trypan Blue method, a suspension containing 10×10^5 cells/ml was prepared and 100 μl of that transferred into each well of the 96-well flat plate. Then, collagen was added to each well at a final concentration of 50 μg/ml and the cells were incubated for 72 h at 37 °C, 5% CO2 concentration and 95% relative humidity (Jahan Tigh, Abtahi Froshani et al. 2018). For each sample, three replications in the presence of collagen and three replications without the presence of collagen were considered. After 72 hours, the plates were centrifuged, and the supernatant was collected. The levels of IL-17 and IFN-γ cytokines were measured as usual by PeproTech ELISA kits.
Non-parametric data related to the arthritis index was analyzed using Kruskal-Wallis test and subsequent Mann Whitney-U evaluation with Bonferroni adjustment. After confirmation of the normal distribution of other data by Kolmogorov-Smirnov test, these findings were evaluated using a one-way analysis of variance with Tukey post hoc test. Data were reported as mean standard deviation and analyzed by MedCalc statistical software (version 18.9.1).

**Results:**

On the 7th day after disease induction, all injected rats showed redness and swelling in the injected paw (Fig. 1). Analysis of the arthritis index (the sum of scores for the carpal and tarsal joints that had not been injected) showed that the administration of methotrexate and naringenin reduced this index. This reduction occurred significantly from day 14 in the methotrexate treated group and day 21 in the naringenin treated group (p <0.05, Fig. 1 A). However, according to Fig. 1 B, on the last day, there was no significant difference in the average of mean arthritis index between rats among two mentioned groups (p = 0.31).

According to Fig. 2 A, the level of myeloperoxidase enzyme in the serum of rats with rheumatoid arthritis compared to healthy rats showed a significant increase (p <0.05). Both methotrexate and naringenin reduced the levels of myeloperoxidase in the serum of treated rats compared with control group (p <0.05). However, the rate of the reduction was higher in the naringenin treated group than in the methotrexate treated group (p <0.05, Fig. 2 A).

Serum nitric oxide levels also showed a significant increase in inducted rats compared to healthy rats (p <0.05, Fig. 2 B). Both Naringenin and Methotrexate treated groups showed a decrease in serum nitric oxide (p <0.05). In the naringenin treated group, the reduction rate of nitric oxide was higher than the methotrexate treated group (p <0.05, Fig. 2 B).
The results obtained from the sera of rats, showed a significant reduction (an average of 61%) in the total serum antioxidant capacity compared to healthy rats (p <0.05, Fig. 2 C). Although both treatment protocols were effective in this case, naringenin treatment resulted in more considerable improvement over methotrexate (p <0.05, Fig. 2 C).

As expected, the induction of rheumatoid arthritis in rats could promote a significant increase in the CRP serum levels of affected rats (Fig. 3 D) which therapeutic regimens were effective in reducing the CRP. Nevertheless, there was no significant distinction in CRP levels between RA rats treated with each of the medicinal treatment regimens (p =0.11, Fig. 3 D).

The results showed that following the stimulation of splenic lymphocytes with collagen, the production and release of inflammatory cytokines such as IFN-γ and IL-17 increased in the supernatant obtained from splenic cell culture (p <0.05, Fig. 3). However, in methotrexate treatment group showed a further decrease in the levels of these two cytokines than naringenin compare to the control group (p <0.05, Fig. 3).

Discussion:

Typically, the treatment of autoimmune diseases such as RA is started after the onset of the disease with the clinical signs and symptoms where we also treated inducted animals after the joint inflammation signs observation. The naringenin treated group had a significant reduction in the severity of edema and swelling of the paw, comparing with the methotrexate treated group. Methotrexate is one of the most effective and well-known drugs commonly used to treat RA which is an immunosuppressive drug (Herrinton, Woodworth et al. 2019). Although
methotrexate is a relatively useful compound, it also has hazardous side effects. The most common complications are pulmonary fibrosis, memory loss, kidney failure, dangerous infections and injuries to the central nervous system due to a weakened immune system along with symptoms such as gastrointestinal disorders, mouth ulcers, skin lesions, fatigue, and headache (Cipriani, Ruscitti et al. 2014, Herrinton, Woodworth et al. 2019). Controlling rampant inflammation plays an essential role in the management of autoimmune diseases. One of the most important causes of tissue damage in inflammatory processes is the occurrence of stress caused by free radicals (Naji Zavareh and Abtahi Froushani 2019). One of the most important justifications for the use of medicinal compounds of natural origin is the role of them in combating the damage caused by the oxygen and nitrogen free radicals. Nowadays, people who eat fewer plant foods are more prone to oxidative stress (Hafidh, Abdulamir et al. 2009).

In this regard, naringenin as an oral flavonoid has been shown to reduce oxidative stress by increasing the antioxidant systems of superoxide dismutase, catalase, and glutathione in chronic diseases such as cardiovascular disorder, neurodegenerative, diabetes, cancer, and nephropathy (Zaidun, Thent et al. 2018). In vitro, the ability of neutrophils to produce oxygen free radicals has also been demonstrated (Cavia-Saiz, Busto et al. 2010). Our research also revealed that from the point of view of improving the total antioxidant capacity of serum, naringenin performed far better than methotrexate due to its potent antioxidant effects. Since there is no report of direct antioxidant effect of methotrexate, slight improvement in treated rats may be due to its anti-inflammatory effects. Myeloperoxidase (MPO) is a well-known lysosomal enzyme that is stored in the primary granules of neutrophils and is depleted when stimulated. This enzyme is one of the main enzymes in the production of active oxygen mediators and oxidative damage in inflammatory sites. It has been clearly shown that there is a logical and positive relationship
between the severity of the RA disease and the level of serum activity of MPO enzyme (Abtahi Froushani 2018). Our findings also indicate that the serum level of this enzyme is significantly increased in rats with RA. Due to the direct antioxidant properties, naringenin treatment was more effective and better than methotrexate in reducing serum myeloperoxidase activity. Decreased MPO enzyme activity in naringenin-treated neutrophils has also been shown in the past (Cavia-Saiz, Busto et al. 2010).

Due to the activation of the nitric oxide synthase (iNOS) pathway, an increase in the serum level of serum nitric oxide has been reported in RA disease. Nitric oxide is one of the most important causes of nitrate damage (Mashhouri, Abtahi Froushani et al. 2020). In this regard, it has been reported that there is a close relationship between serum nitric oxide levels and disease severity in patients with RA (Jahan Tigh, Abtahi Froshani et al. 2018). Our findings also indicate an increase in serum nitric oxide levels in rats with RA. In this study, treatment with naringenin effectively reduced the serum nitric oxide activity in inducted animals. It has been shown that naringenin reduces nitric oxide production in lipopolysaccharide-treated macrophage cells by decreasing the expression of iNOS transcription factors (Chao, Weng et al. 2010). It should be noted that in addition to the direct antioxidant effects, naringenin also has strong direct anti-inflammatory capability, just like common anti-inflammatory drugs, which will be discussed below. For example, naringenin has been shown to reduce PGE2 production in macrophage and glial cells treated with lipopolysaccharide by reducing the expression of COX-2 transcription factors (Chao, Weng et al. 2010).

CRP, an acute-phase protein, could reflect different pathological processes driven by the underlining acute and chronic inflammation. This factor is an important indicator in the RA
tracking, so reducing its level is a good predictor (Benito 2018). Obtained results of this study indicated that both treatment regimens could similarly reduce the CRP level.

Previous studies have shown a decrease in the release of inflammatory mediators such as IL-1, TNF-α, and monocyte chemotactic protein (MCP-1) in lipopolysaccharide-stimulated microglial cells following treatment with naringenin by inhibiting NF-κb transcription factors and MAP kinase (Park, Kim et al. 2012). The results of our study showed a significant reduction in inflammation and arthritis of rats treated with naringenin. Certainly, polarization of T lymphocyte responses and production of pro-inflammatory cytokines contribute to inflammation and joint destruction in RA (Golbahari and Froushani 2019, Ghaffary and Froushani 2020). The inhibitory effects of naringenin on the production of inflammatory cytokines, major actors in RA produced by T lymphocytes and macrophages, have been demonstrated in recent studies. It has been shown that naringenin reduces the production of inflammatory cytokines by macrophages and T lymphocytes, by interfering with the TLR-mediated signaling pathway, altering the stability of cytokines’ mRNA or their translation. Naringenin increases the degradation of intracellular cytokines by increasing lysozyme degradation (Jin, Zeng et al. 2017). In models of autoimmune patients with physiopathological similarities to RA, the effects of naringenin on the polarization of immune responses have been demonstrated. For example, in experimental autoimmune encephalomyelitis (EAE, experimental model of multiple sclerosis), oral administration of naringenin exacerbate symptoms by reducing immune cell infiltration and the rate of demyelination in the spinal cord tissue. According to the results, naringenin decreases the lymphocyte subtypes of Th1, Th9, and Th17 by reducing the expression of the main factors involve in polarization, and instead causes the polarization of lymphocytes to Th2. Decreasing the ratio of immune regulatory lymphocytes (Treg) to the number of inflammatory lymphocytes
is one of the effective factors in creating autoimmunity (Wang, Niu et al. 2018). Research on 25 oral flavonoids has shown that only naringenin can induce Treg cell production by affecting aryl hydrocarbon receptors (Wang, Yeh et al. 2012). In the current study, it was observed that naringin decreased the levels of interferon gamma (Th1 cell index cytokine) and interleukin 17 (Th17 cell index cytokine) by modulating and polarizing the Th1 and Th17 responses to Th2 and Treg. Hence it can be effective in reducing the symptoms of RA in rats (Wang, Niu et al. 2018). It has been shown that naringenin inhibits immunopathological responses by reducing the frequency of Th1 and Th17 cells without changing the frequency of Th2 cells. Overall, the beneficial effects observed by naringenin in this study may be due to its antioxidant properties along with changes in the polarization pathway of immune responses. Indeed, this study was a preliminary report and it is necessary to do more research in the future, especially with histopathological and radiological evaluations and measuring the extent of tissue repair in the affected rat joints.

**Ethics**

All authors emphasize all ethical standards have been respected in preparation of the presented survey.

**Conflict of Interest**

The authors emphasize that they have no conflict of interest.

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Authors’ Contribution

1- Study concept and design: SMAF
2- Acquisition of data: AH
3- Analysis and interpretation of data: SMAF, AAT, SA, and SRBH.
4- Drafting of the manuscript: SMAF and AH.
5- Critical revision of the manuscript for important intellectual content: SMAF, AAT, and SA.
6- Statistical analysis: SMAF and AH
7- Administrative, technical, and material support: SMAF and SRBH.
8- Study supervision: SMAF and AAT.

References:


Figure 1. Assessment of clinical schemas in RA rats. A) mean arthritis index, B) average of mean arthritis. Findings were presented as mean ±S.D. (&p<0.05 versus healthy rats, *p<0.05 versus PBS-treated RA rats). (Naren. Naringenin-treated RA group; Meth., methotrexate-treated RA group).

Figure 2. Changes in the biochemical profiles of sera in RA rats. A) myeloperoxidase activity (MPO), B) Nitric oxide (NO), C) total antioxidant capacity (TAC), and D) C-reactive protein (CRP). Data were reported as mean ±S.D. (&p<0.05 versus healthy rats, *p<0.05 versus PBS-treated RA rats; #p<0.05 versus methotrexate treated RA rats). (Naren. Naringenin-treated RA group; Meth., methotrexate-treated RA group).
Figure 3. The level of inflammatory cytokines in the splenocytes. The values were presented as mean ±S.D. (*p<0.05 versus PBS-treated RA rats; #p<0.05 versus combined-treated RA rats; &p<0.05 versus Thymol-treated RA rats). (&p<0.05 versus healthy rats, *p<0.05 versus PBS-treated RA rats; #p<0.05 versus methotrexate treated RA rats. (Naren. Naringenin-treated RA group; Meth., methotrexate-treated RA group).