**Cellular & Molecular Biology**
*Cell. Mol. Biol.* 2015; 61 (7): 1-5
Published online November 8, 2015 (http://www.cellmolbiol.com)
Received on September 25, 2015, Accepted on October 20, 2015.
doi : 10.14715/cmb/2015.61.7.1

# Protective effect of Esculin in adjuvant-induced arthritic (AIA) rats via attenuating pro-inflammatory cytokines and oxidative stress

L. Zheng[1], L. Yang[1]✉, Z. Wang[1], C. Chen[1] and Y. Su[2]

[1] Department of orthopedics, the Second Affiliated Hospital of Dalian Medical University, Liaoning 116027,China
[2] Dalian Medical University Graduate School, Dalian 116027 China

**Corresponding author:** Liang Yang, Department of Orthopedics, the Second Affiliated Hospital of Dalian Medical University , No.467, Zhongshan Road, Dalian City, Liaoning Province 116027, China. E-mail: yangliangyang@126.com, yangliang956@hotmail.com

**Abstract**

The present study was intended to exemplify the protective effect of Esculin (ES; 6,7-dihydroxycoumarin-6-o-glucoside) on the adjuvant induced arthritis in adult female Sprague Dawley rats. It has been found that, treatment of ES has significantly improved the body weight of rats accompanied with a reduction of paw volume in comparison to arthritic control. In addition, ES exhibit inhibitory effect on various pro-inflammatory cytokines, for instance, IL-1β and TNF-α. The level of oxidative stress markers, i.e., nitric oxide and peroxide was also found suppressed after treatment. The treatment of ES prevents the tissue injury mediated via oxidative stress via up-regulating the level of endogenous GSH in a dose dependent manner. Thus, it has been corroborated that, ES exerts potent anti-arthritic activity via attenuating pro-inflammatory cytokines and oxidative stress.

***Key words:*** Anti-arthritic activity, Esculin, cytokine, IL-1β and TNF-α.

## Introduction

Rheumatoid arthritis (RA) is regarded as an autoimmune ailment characterized by inflammation of synovial and permanent obliteration joints of bone. It results in a persistent, systemic disorder that causes discomfort, inflammation and ultimately leads to disability (1, 2). It has affected approximately 1% population across the globe; reported frequently in female than male and its frequency depends upon age. The etiology of this disease is still unknown, but the skeletal problems of RA have been started with focal erosion of cartilage afterwards insignificant and sub–chondral bone loss. Whereas, the extensive joint destruction with ankylosis and generalized bone loss are characteristic for late complications (3,4). Numerous studies suggest the role of different pro-inflammatory mediators in the progression of this disorder, such as, reactive oxygen species (ROS), prostaglandins (PGs), leukotrines and cytokines released by macrophages (5,6). The conventional therapies to treat this disorder based on treatment with steroids and non-steroidal anti–inflammatory drugs (NSAIDs), but their action is short-lived and often associated with severe side effects which includes gastrointestinal distress and cardiovascular toxicity (7-10). Consequently, there is a need to develop new long acting anti–inflammatory agents with minimum side effects (11-15).

Coumarins chemically classified as fused benzene and α-pyrone ring system found in diverse plants (16). It falls under the category of natural phenolic compounds that scavenge the reactive oxygen species (ROS) and suppresses the damage mediated from free radical (17-18). Among the derivatives of coumarins, Esculin (6,7-dihydroxycoumarin-6-*o*-glucoside) obtained from Aesculus hippocastanum L. (Horse-chestnut) (19) exhibit various pharmacological activities, such as, 5- and 12-lipoxygenase inhibitor (20), antioxidant and anti-cancer activity (21-23) including dopamine-induced caspase-3 cleavage in dopamine-induced cytotoxicity (24) and anti-inflammatory activity in mice (25). Despite of high antioxidant activity of Esculin, till now, no study has been reported, its effect on various pro-inflammatory mediators in arthritis (21). Thus, the present study was undertaken to elucidate the role of Esculin on inflammation-related cytokines and oxidative stress in adjuvant-induced arthritic (AIA) rats.

## Materials and methods

### *Animals*

The female Sprague Dawley (SD) rats (215–230 g) used in this study was housed at standard laboratory condition with free access to food and water, *ad libitum*. The study was performed in accordance with the institutional ethical committee and duly approved by the Second Affiliated Hospital of Dalian Medical University, China. Experimental Rats were randomly distributed into each treatment group and each group receives twelve animals.

### *Generation of arthritis*

For induction of arthritis, Lyophilized H37Ra strain of *Mycobacterium tuberculosis* was obtained from DIFCO Laboratories, USA) was used. The adjuvant was prepared freshly on the same day of experimentation. For this, a volume of 0.1 ml of a 1 mg suspension of MT H37Ra was injected intradermally in the tails of the rats in the dose of 20 mg/kg/5mg/kg.

### *Drugs*

The standard indomethacine and Esculin were obtained from Sigma (USA) and required test dose were

injected intraperitoneally. Whereas, the saline administered rats serve as a control group.

### *Assessment of adjuvant induce arthritis*

Using a macroscopic scoring system experimental Rat was evaluated on alternate days for the arthritis as per the standard protocol given elsewhere (27).

### *Measurement of TNF-α and IL-1β*

The ELISA assay kits (Cayman Chemicals, USA) were used for the quantitative determination of pro-inflammatory cytokines IL-1β and TNF-α in serum of the experimental rats according to the standard protocol. The experiment was conducted in duplicate and the average data were taken.

### *Determination of oxidative stress parameters*

Near to 16–18 days of the treatment, experimental animals were sacrificed and by puncturing the heart, the whole blood was collected. The un-coagulated blood samples were then centrifuged and plasma was collected. It was then used for the determination of oxidative stress parameters, such as, nitric oxide (NO), peroxide and glutathione (GSH) activity in the plasma samples of with or/ without treatment using the Quantichrome™ Assay kit, U.S.A.

### *Statistical analysis*

The results were expressed as mean ± SEM. The data were analyzed using ANOVA, while the Bonferroni's post hoc test was used for the determination of significant value among the tested group.

## Results

### Effect of ES on Body weight and paw volume

Initially, the effect of ES was determined by the change in the body weights and paw volume. As shown in figure 1, the rats in the arthritic control group showed signs of inflammation in both of the hind paws. It was noteworthy to mention that, after 14 days of the induction, the paw volume was observed considerably higher compared to the normal control. On the progression of the disease to interphalangeal and metatarsal joints, an insignificant upsurge was observed in the arthritic groups treated with ES (5mg/kg) and indomethacine (5mg/kg). However, an increased concentration of the ES (10mg/kg), the paw volume was found considerably lower in comparison with indomethacin. Results suggest that, the mode of action of the ES was similar to that of indomethacin and exhibit a concentration dependent reduction of adjuvant-induced arthritis.

The effect of ES was further determined on the body weights of the experimental animals. As depicted in fig 2, initially, up to 4 days of the treatment, the body weights of the animals was found stable. However, as the disease progressed, a significant change in the body weight was observed. The animals in the arthritic group showed the marked decline in the body weight. Whereas, on starting the treatment with ES the body weight has been improved significantly in comparison to the arthritic control. Whereas, the animal treated with indomethacin showed much improved activity and the body weights of the animal were close to the normal group.

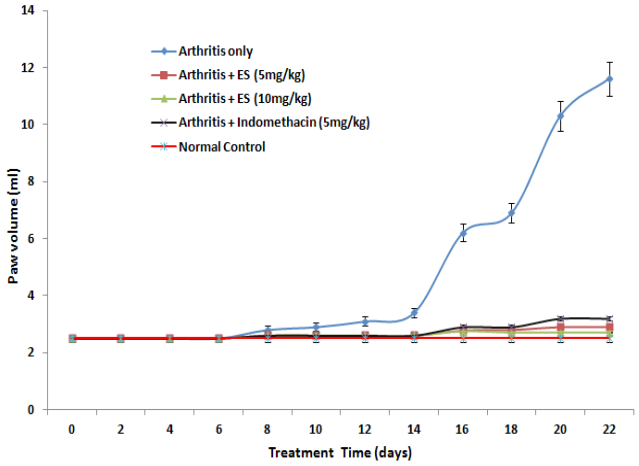


**Figure 1.** Effect of ES in various concentrations on the inflammation. Values are given as means ± SE.

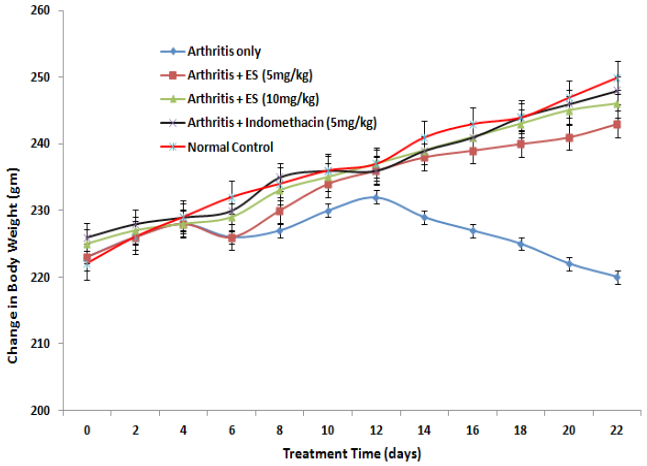


**Figure 2.** Effect of ES on the body weight in experimental rats over the duration of twenty-two days. Values are given as means±SEM.

### *Effect of ES on pro-inflammatory cytokines*

The effect of ES was investigated on the level of IL-1β and TNF-α. It has been evident from the figure 3 that, the level of IL-1β ($p > 0.005$) and TNF-α ($p > 0.001$) was highly over-expressed in arthritic rats than normal control group. The level of these cytokines was significantly decreased on treatment of ES ($p > 0.009$, and $p > 0.005$ for 5 mg/kg and 10 mg/kg dose of ES, respectively. Moreover, the standard indomethacin also showed considerable inhibition of IL-1β and TNF-α as compared to arthritic control rats.

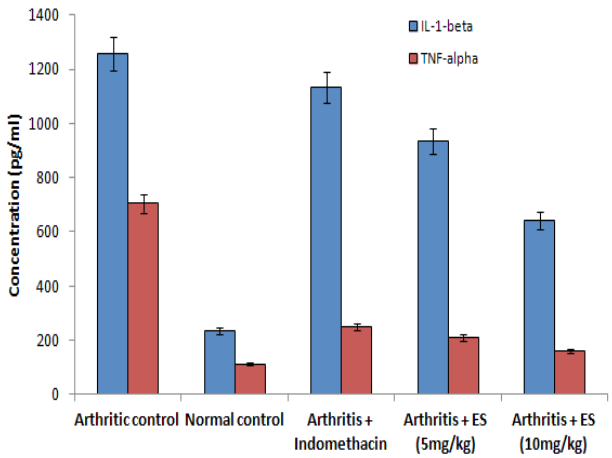


**Figure 3.** The effect of ES on the level of TNF α and IL-1β (pg/ml) measured in the serum of experimental rats. The values are expressed means ± SEM.

### *Effect of ES on oxidative stress*

The effect of ES was assessed on the level of various parameters related with oxidative stress in the experimental animals after the induction of arthritis. The results have been shown in fig. 4-6.

The level of studied parameters was found elevated in the case of arthritic control animals than normal control. Particularly, in the case of NO, the treatment of indomethacin significantly reduces its level (*p > 0.06) than control group. In similar fashion, the treatment of ES also reduces the level of NO and peroxide in dose dependent manner. The treatment of ES significantly increases the level of GSH (**p > 0.04 for and ***p > 0.006 for 5 mg/kg and 10 mg/kg, respectively) in comparison to arthritic control group. The treatment of indomethacin also reduces the level of NO and peroxide accompanied with elevated level of GSH than arthritic control.

## Discussion

Rheumatoid arthritis is usually rereferred as an inflammatory disorder often summarized by inflammation of synovial cells of the joints, which leads to restricted movements (26). For quantification assessment of the role of drugs for the treatment of RA, Adjuvant-induced arthritis has been extensively used as model of the disease (27). It efficiently mimics the clinical and immunological features similar to the human arthritis. As an initial symptom of adjuvant induced arthritis, the paw volume of the experimental rats was significantly elevated due to swelling. However, upon starting the treatment with ES, the paw volume was reduced considerably in comparison to arthritic control. As the arthritis progressed in the experimental rats, the results showed that, body weight was reduced prominently. While the body weights of the affected rats were improved significantly upon treatment with ES in a dose-dependent manner.

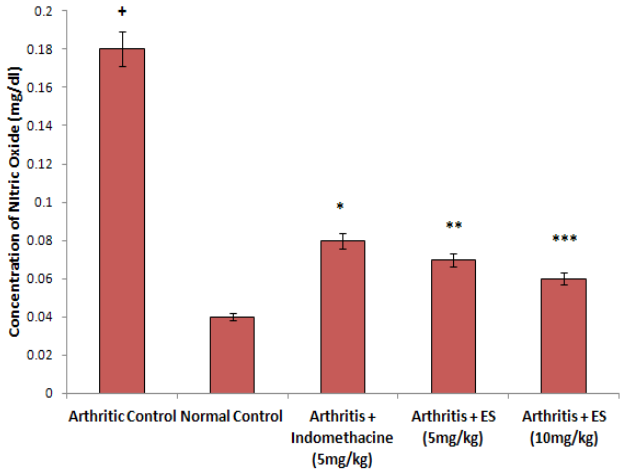


**Figure 4.** Effect of ES on the level of nitric oxide (mg/dl) in the plasma of the experimental rats. Results were expressed as means ± SEM for 12 rats in each group.

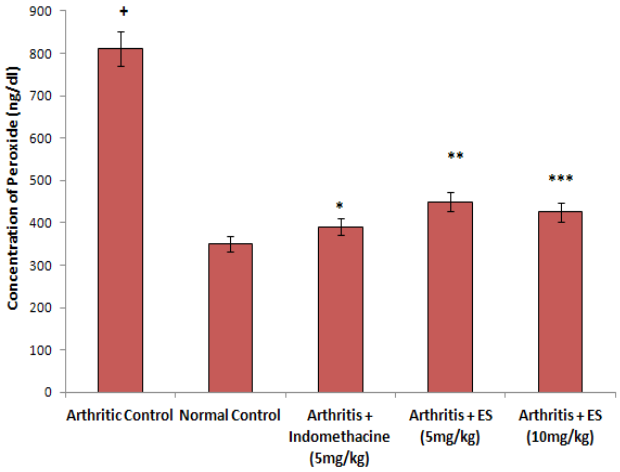


**Figure 5.** Effect of ES on the level of peroxide (ng/dl) in plasma. Each bar represents the means ± SEM.

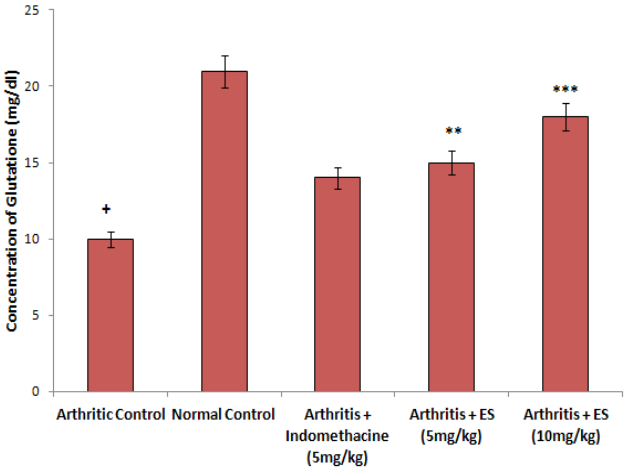


**Figure 6.** Effect of ES on the level of glutathione (mg/dl) in the plasma of experimental rats. Values are expressed as means ± SEM. The glutathione level ($^{+}p>0.035$) in arthritic group showed a significant decrease in comparison to normal control group. Whereas, the treatment of ES elevated the level of glutathione compared to the untreated (**p > 0.03, and ***p>0.004 for ES (5mg/kg) and ES (10 mg/kg), respectively).

Various studies have indicated the role of macrophages, T-lymphocytes and reproducible synovial cells in the pathogenesis of RA (28-30). These components secrete a range of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α which contribute to the progression of RA and acts a specific biomarker for the disease (31). The level of these cytokines was found elevated in patients affected with RA (5,6). Thus, to provide relief, one of the most popular approaches is to regulate the level of these cytokines. In connection with the previous studies, as shown, ES in a significantly reduces the level of these cytokines and offer relief from the progression of inflammatory condition. The mode of action of the ES was found majorly dependent upon the concentration, and as the concentration rises, the inhibitory activity was enhanced.

Oxidative stress generated from the ROS act as an important mediator of the damage of the tissue by activation of the infiltration of the leukocyte into the inflamed tissue (32-34). This results in secretion of various pro-inflammatory mediators for the progression of RA. Therefore, to counteract these ROS, various endogenous antioxidants were generated in response of that, such as superoxide dismutase (SOD) or GSH (35). Nonetheless, the activities of these enzymes were compromised in RA; which in turn leads to the loss of the viscosity of synovial fluid in the joints by de-polymerization of hyaluronic acid. It has been found that administration of ES inhibit the excess level of nitric oxide and peroxide.

Apart from it, ES treated group showed significant upsurge in the level of GSH needed to scavenge the toxic free radical.

In the present study, for the first time we have evaluated the protective role of Esculin in adjuvant-induced arthritic (AIA) rats via inhibition of inflammation-related cytokines and ROS. Esculin was found to exert its effect in a dose-dependent manner via targeting pro-inflammatory cytokines and oxidative stress. However, further studies are needed to get insight about the molecular mechanism of Esculin for its mechanism of action. Our studies are in progress and reported in future communications.

## References


1. Grassi, W., De Angelis, R., Lamanna, G. and Cervini, C., The clinical features of rheumatoid arthritis. *Eur J Radiol*. 1998,**1**: S18-24. doi: 10.1016/S0720-048X(98)00038-2

2. Firestein, G.S., Etiology and pathogenesis of rheumatoid arthritis. In: Firestein GS, Budd RC, Harris ED Jr, et al, eds. *Kelley's Textbook of Rheumatology*, 8th ed. Philadelphia, PA: Saunders Elsevier; 2008, 1035–1086.

3. Persselin, J.E., Diagnosis of rheumatoid arthritis. Medical and laboratory aspects. *Clin Orthop Relat Res*. 1991, **265**: 73-82.

4. Harris, E.D. and Jr, Firestein, G.S., Clinical features of rheumatoid arthritis. In: Firestein GS, Budd RC, Harris ED Jr, et al, eds. Kelley's Textbook of Rheumatology, 8th ed. Philadelphia, PA: Saunders Elsevier; 2008, 1087–1118.

5. Smolen, J.S., Redlich, K., Zwerina, J., Aletaha, D., Steiner, G. and Schett, G., Pro-inflammatory cytokines in rheumatoid arthritis: pathogenetic and therapeutic aspects. *Clin Rev Allergy Immunol*. 2005, **28**: 239-48.

6. Burska, A., Boissinot, M. and Ponchel, F., "Cytokines as Biomarkers in Rheumatoid Arthritis," *Mediators of Inflammation*, 2014, **24**: doi:10.1155/2014/545493.

7. Smolen, J.S., Aletaha, D. and Machold, K.P., Therapeutic strategies in early rheumatoid arthritis. *Best Pract Res Clin Rheumatol.* 2005,**19**: 163-77. doi: 10.1016/j.berh.2004.08.009

8. Smolen, J.S. and Aletaha, D., What should be our treatment goal in rheumatoid arthritis today? *Clin Exp Rheumatol*. 2006, **24**: S-7-13.

9. Smolen, J. and Aletaha, D., The burden of rheumatoid arthritis and access to treatment: a medical overview. *Eur J Health Econ*. 2008, **8**: S39-47. doi: 10.1007/s10198-007-0087-9

10. Singh, G., Ramey, D.R., Morfeld, D., Shi, H., Hatoum, H.T. and Fries, J.F., Gastrointestinal tract complications of nonsteroidal anti-inflammatory drug treatment in rheumatoid arthritis. A prospective observational cohort study. *Arch Intern Med*. 1996, **156**: 1530-6.

11. Riese, R.J., Krishnaswami, S. and Kremer, J., Inhibition of JAK kinases in patients with rheumatoid arthritis: Scientific rationale and clinical outcomes. *Best Pract Res Clin Rheum.* 2010, **24**: 513-526. doi: 10.1016/j.berh.2010.02.003

12. Bonilla-Hernán, M.G., Miranda-Carús, M.E. and Martin-Mola, E., New drugs beyond biologics in rheumatoid arthritis: The kinase inhibitors. *Rheumatology*. 2011, **50**: 1542–1550. Doi: 10.1093/rheumatology/ker192

13. Okamoto, H. and Kobayashi, A., Tyrosine kinases in rheumatoid arthritis. *J Inflammation*. 2011, **8**: 21. doi: 10.1186/1476-9255-8-21

14. Weinblatt, M.E., Kavanaugh, A., Genovese, M.C., Musser, T.K., Grossbard, E.B. and Magilavy, D.B., An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. *N Engl J Med*. 2010, **363**: 1303–1312. doi: 10.1056/NEJMoa1000500.

15. Olsen, N.J. and Stein, C.M., New drugs for rheumatoid arthritis. *N Engl J Med*. 2004, **350**: 2167-79.

16. Venugopala, K. N., Rashmi, V. and Odhav, B., Review on Natural Coumarin Lead Compounds for Their Pharmacological Activity, *BioMed Research International*, 2013, **14**: doi:10.1155/2013/963248

17. Kostova, I., Bhatia, S., Grigorov, P., Balkansky, S., Parmar, V.S., Prasad, A.K. and Saso, L., Coumarins as antioxidants. *Curr Med Chem*. 2011, **18**: 3929-51. doi: 10.2174/092986711803414395

18. Kostova, I., Synthetic and natural coumarins as antioxidants. *Mini Rev Med Chem*. 2006, **6**: 365-74. Doi: 10.2174/138955706776361457

19. Stanic, G., Jurisic B. and Brkic, D., HPLC Analysis of Esculin and Fraxin in Horse-Chestnut Bark (Aesculus hippocastanum L.). *Croatica Chemical Acta*. 1999, **72**: 827-834.

20. Kaneko, T., Tahara, S. and Takabayashi, F., Suppression of lipid hydro peroxide induced oxidative damage to cellular DNA by esculetin, *Biol. Pharm. Bull*. 2003, **26**: 840–844.

21. Lee, B.C., Lee, S.Y., Lee, H.J., Sim, G.S., Kim, J.H., Kim, J.H., Cho, Y.H., Lee, D.H., Pyo, H.B., Choe, T.B., Moon, D.C., Yun, Y.P. and Hong, J.T., Antioxidative and photo-protective effects of coumarins isolated from Fraxinus chinensis. *Arch Pharm Res*. 2007, **30**: 1293–1301.

22. Park, C., Jin, C. Y., Kim, G. Y., Choi, I. W., Kwon, T. K., Choi, B. T., Lee, S. J., Lee, W. H. and Choi, Y. H., Induction of apoptosis by esculetin in human leukemia U937 cells through activation of JNK and ERK. *Toxicol. Appl. Pharmacol*. 2008, **227**: 219−228. doi: 10.1016/j.taap.2007.10.003

23. Wang, C. J., Hsieh, Y. J., Chu, C. Y., Lin, Y. L. and Tseng, T. H., Inhibition of cell cycle progression in human leukemia HL-60 cells by esculetin. *Cancer Lett*. 2002, **183**: 163−168. doi: 10.1016/S0304-3835(02)00031-9

24. Zhao, D.L., Zou, L.B., Lin, S., Shi, J.G. and Zhu, H.B., Anti-apoptotic effect of esculin on dopamine-induced cytotoxicity in the human neuroblastoma SH-SY5Y cell line. *Neuropharmacology*. 2007, **53**: 724-32. doi: 10.1016/S0304-3835(02)00031-9

25. Stefanova, Z., Neychev, H., Ivanovska, N. and Kostova, I., Effect of a total extract from Fraxinus ornus stem bark and esculin on zymosan- and carrageenan-induced paw oedema in mice. *J Ethnopharmacol*. 1995, **46**: 101-6. doi: 10.1016/0378-8741(95)01233-4

26. McInnes, I.B. and Schett, G., The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011, **365**: 2205-19. doi: 10.1056/NEJMra1004965.

27. Whiteley, P. E. and Dalrymple, S. A., Models of Inflammation: Adjuvant-Induced Arthritis in the Rat. *Current Protocols in Pharmacology*. 2001, **13**: Chapter 5, Unit 5.5. doi: 10.1002/0471141755.ph0505s13

28. Goronzy, J.J. and Weyand, C.M., Thymic function and peripheral T-cell homeostasis in rheumatoid arthritis. *Trends Immunol* 2001, **22**: 251–5. doi: 10.1016/S1471-4906(00)01841-X

29. Kohem, C.L., Brezinschek, R.I., Wisbey, H., Tortorella, C. and Lipsky, P.E., Oppenheimer-Marks, N. Enrichment of differentiated CD45RBdim, CD27 memory T cells in the peripheral blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis. *Arthritis Rheum* 1996, **39**: 844–54.

30. Falgarone, G., Semerano, L., Rullé, S. and Boissier, M.C., Targeting lymphocyte activation to treat rheumatoid arthritis. *Joint Bone Spine* 2009, **76**: 327–32. doi: 10.1016/j.jbspin.2008.12.007.

31. Hata. H., Sakaguchi, N., Yoshitomi, H., Iwakura, Y., Sekikawa, K., Azuma, Y., Kanai, C, Moriizumi E, Nomura T, Nakamura T, Sakaguchi S. Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J Clin Invest.* 2004, **114**, 582-588. doi:10.1172/JCI21795.

32. Wruck, C.J., Fragoulis, A., Gurzynski, A., Brandenburg, L.O., Kan, Y.W., Chan, K., Hassenpflug, J., Freitag-Wolf, S., Varoga, D., Lippross, S. And Pufe, T., Role of oxidative stress in rheumatoid arthritis: insights from the Nrf2-knockout mice. *Ann Rheum Dis*.

2011, **70**: 844-50. doi: 10.1136/ard.2010.132720.
33. Ostrakhovitch, E.A. and Afanas'ev, I.B., Oxidative stress in rheumatoid arthritis leukocytes: suppression by rutin and other antioxidants and chelators. *Biochem Pharmacol*. 2001, **62**: 743-6. doi: 10.1016/S0006-2952(01)00707-9
34. García-González, A., Gaxiola-Robles, R. and Zenteno-Savín, T., Oxidative stress in patients with rheumatoid arthritis. *Rev Invest Clin*. 2015, **67**: 46-53.
35. Afonso, V., Champy, R., Mitrovic, D., Collin, P. and Lomri, A., Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine.* 2007, **74**: 324-9. doi:10.1016/j.jbspin.2007.02.002