


# Investigation of the Effects of N-Acetyl Cysteine and Resveratrol to Acute Phase Response in Rats with Experimentally Induced Arthritis <sup>[1]</sup>

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## Summary

The aim of the study was to investigate and compare the effects of N-acetyl cysteine (NAC) and resveratrol on acute phase response in rats with experimentally induced arthritis. In this study, 30 Sprague-Dawley mature rats were inoculated with two doses of Freund's Complete Adjuvant (FCA) by means of intradermal injections into the left hind claws of all rats. Rats were divided into three groups at the 15<sup>th</sup> day of arthritis formation process, 15% ethyl alcohol was applied to group 1 rats via i.p., NAC was applied to group 2 rats via i.p. and resveratrol was applied to group 3 rats via i.p. for 21 days. Following the experimental period, blood was collected from the rats and then they were euthanized, and the joint tissues were collected. No significant differences were found in serum TNF- $\alpha$ , IL-1 $\beta$ , IL-6 levels and ADA activity and a decrease was determined in MDA levels in the resveratrol group. According to histopathological result, decreases in the loss of cellularity and adhesion were determined as  $P<0.05$  and  $P<0.01$ , respectively in group received resveratrol. Compared to arthritis group, the decrease ( $P<0.05$ ) in safranin-O orthochromatosis in group 2 and 3 was found. However the recovery from synovial inflammation ( $P<0.01$ ) was determined both in NAC and resveratrol groups. The best recovery rate was recorded in the resveratrol group. In conclusion, resveratrol may be suggested to use in arthritis therapy alternative to NAC.

**Keywords:** Adjuvant arthritis, N-acetyl cysteine, Resveratrol, Rat

## DeneySEL Olarak Artrit Oluşturulan Ratlarda N-Asetil Sistein ve Resveratrolün Akut Faz Yanıtına Etkisinin Araştırılması

### Özet

Çalışmamızda deneysel olarak artrit oluşturulmuş ratlar üzerinde N-asetil sistein (NAS) ve resveratrolün akut faz üzerine etkileri karşılaştırıldı. Araştırmada Sprague-Dawley ırkı 30 adet yetişkin rat kullanıldı. Freund's Complete Adjuvant (FCA) ile tüm ratların sol arka ayak pençelerine, 0.1 ml iki doz (0. ve 7. gün) intradermal injeksiyon uygulandı. Artrit oluşum sürecinin 15. gününde ratlar üç ayrı gruba ayrıldı; grup 1 ratlara %15 etil alkol i.p. grup 2 ratlara NAS i.p., grup 3 ratlara resveratrol i.p. 21 gün boyunca uygulandı. 21 günlük deneme sürecinin sonunda ratlardan kan alındıktan sonra ötanazi yapılarak eklem dokuları alındı. Biyokimyasal parametrelerden serum TNF- $\alpha$ , IL-1 $\beta$ , IL-6 düzeyleri ve ADA aktivitesinde istatistiksel olarak bir değişiklik meydana gelmezken MDA seviyesinde 3. grupta azalma ( $P<0.05$ ) tespit edildi. Histopatolojik sonuçlarda ise resveratrol verilen grupta kırkırdak selülarite kaybında  $P<0.05$ , adezyonda  $P<0.01$ 'lik bir azalma görüldü. Safranin-O ile ortokromazisinde, her iki grupta da (grup2 ve grup3) artrit grubuna göre azalma  $P<0.05$ , sinovyal inflamasyonda ise NAS ve resveratrol gruplarının her ikisinde de düzelme  $P<0.01$  kaydedilirken, en iyi düzelme resveratrol grubu oluşturdu. Sonuç olarak artrit tedavisinde NAC'a alternatif olarak resveratrolün önerilebileceği düşünülmektedir.

**Anahtar sözcükler:** Adjuvan artrit, N-asetil sistein, Resveratrol, Rat

## INTRODUCTION

Adjuvant arthritis (AA) is an immunologic response developed against an adjuvant as an antigen. This is used

frequently as a rheumatoid arthritis (RA) model because of the similarity of their histopathologic views and formation



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paths (both are results from a cellular immune response)<sup>1,2</sup>. Secondary reactions such as inflammatory lesions at the contralateral claw, articular zones, nose, ears and tail and changes in body weight and organs such as thymus, spleen may be observed after a 10-12 days of latent period following the application<sup>1,3</sup>.

NAC (N-acetyl cysteine), a thiol compound, is a potent antioxidant and anti-inflammatory agent. It is a good glutathione precursor and scavenger free oxygen radicals, hydroxyl radicals and hypochloric acid by its direct effects as the basic reductor<sup>4</sup>. While redox activate the sensitive exchange factors rapidly, inflammation activates the genes encoding the immune and acute phase response<sup>5,6</sup>.

Resveratrol (3,5,4'-*trans*-trihydroxystilbene) is a polyphenol present in grape seed and red vine. It has anti-inflammatory, anticarcinogenic and antioxidative biological activities and prevents lipid peroxidation<sup>7,8</sup>.

The aim of this experimental study is to investigate the effects of resveratrol and NAC, on serum TNF- $\alpha$ , IL-1 $\beta$ , IL-6, ADA and MDA parameters using their anti-inflammatory characteristics, and to determine the histopathologic changes in the articular tissue of AA induced rats.

## MATERIAL and METHODS

### Animal Material

A total of 30, six-month-old male Sprague-Dawley rats of 20-24 weeks weighting 350-500 g were included in this study. Animal were kept in cages at room temperature (22-25°C) with a 12:12 h light:dark cycle. Rats were provided supplied from Ondokuz Mayıs University Medical and Surgical Research Laboratory and were housed at this laboratory during the experimental period. This study was approved by the Ondokuz Mayıs University Ethics Committee. Arthritis in rats was induced according to the methods described previously for RA evaluation<sup>1,9</sup>. Briefly, two 0.1 ml doses of Freund's Complete Adjuvant (FCA) (which is a suspension of heat-killed *Mycobacterium tuberculosis* in mineral oil; Sigma Aldrich, USA.) by intradermal injections (at days 0 and 7) were applied using 22 gauge syringe into the left hind paws of all rats. After the two week period, arthritis formation was evaluated and rats were divided into three groups. Group 1 rats (n=10) were inoculated with 15% ethyl alcohol via i.p. route, Group 2 rats (n=10) were inoculated with 100 mg/kg/day NAC via i.p. (Sigma, Germany) route and Group 3 rats (n=10) were inoculated with 10 mg/kg/day resveratrol solution in 15% ethyl alcohol via i.p. route for 21 days<sup>10,11</sup>. Rats were fed *ad libitum* during whole procedure. Two rats died in Group 2 in the experimental period and procedure was continued with 8 rats in this group. At the end of the period, blood samples were collected and rats were euthanized under high level ether anaesthesia and articular tissues were sampled for histopathologic examination.

### Biochemical Analyses

Serum were obtained from the blood samples and these were assessed for TNF- $\alpha$ , IL-1 $\beta$ , IL-6 using ELISA test kits (BioSource Int. Inc rat kit, California, USA- plate reader digital and analog systems Roma, Italy)

ADA activity was determined according to Giusti<sup>12</sup> method. This is the colorimetric method based on the measurement of the formation of ammonia by Berthelot reaction: ammonia which is produced when ADA acts on excess adenosine forms an intensely blue indophenol with sodium hypochloride and phenol in alkaline solution. Sodium nitroprusside is used as the catalyst of the reaction. The ammonia concentration is directly proportional to the absorbance value of the indophenol at wavelength 628 nm (Genesys 10S UV-Vis spectrophotometer). One unit of ADA is defined as the amount of enzyme required to release 1  $\mu$ mol ammonia per minute from adenosine under standard assay conditions.

Serum MDA levels were determined based on the spectrophotometric measurement of the product generated upon the reaction of MDA with thiobarbituric acid by Yoshioka *et al.*<sup>13</sup>.

### Pathologic Examinations

At the end of the experiment, stifle joints of rats were removed and fixed in 10% formaline. Then, they were decalcified in 10% formic acid prepared with 2% paraformaldehyde for 3 weeks. After decalcification procedure, sagittal sections of stifle joints were taken, washed, dehydrated in degreed ethanol, transparented with xylol and finally blocked into paraffin. Five  $\mu$ m sections were taken from the prepared tissue blocks and stained with Haematoxylin-Eosine and Safranin O stains.

Microscopic examinations were performed by comparing the study groups. Changes formed at the joints were evaluated using a scoring system by modifying the system developed by Salter<sup>14</sup>. Presence of matrix proteoglycan loss in cartilage was examined with Safranin O staining<sup>15,16</sup>.

### Statistical Analyses

Generated linear model analyses method (GENMOD) was used and executed at the logarithmic scale.

## RESULTS

Two intradermal injections of FCA into the left hind paw were resulted in a significant inflammatory reaction in the paw. Following the determination of this formation, in the NAC and resveratrol received groups, a decrease in oedema and local inflammatory reaction symptoms such as regional inflation, heat, pain, function loss was recorded.

### Biochemical Results

Serum TNF- $\alpha$  level was  $45.0 \pm 3.07$  pg/ml in the group 1,

44.8±2.20 pg/ml in group 2 and 42.6±2.10 pg/ml in group 3. Serum IL-1 $\beta$  levels in group 1, group 2 and group 3 were 1.70±2.5 pg/ml, 1.67±1.9 pg/ml and 1.66±2.2 pg/ml, respectively. Serum IL-6 levels in group 1, group 2 and group 3 were 53.8±1.2, 52.2±2.5 and 50.0±2.0, respectively (Table 1). There were no statistical variations (differences) for all three groups. ADA activities were 15.27±4.6 U/L in group 1, 14.43±2.5 U/L in group 2 and 12.77±2.3 U/L in group 3. MDA levels were 11.99±1.3  $\mu$ mol/L in group 1, 11.94±1.6  $\mu$ mol/L in group 2 and 8.2±0.4  $\mu$ mol/L in group 3 (Table 2). While no difference was determined between the groups regarding ADA activity, in MDA level, a decrease was found in group 3 and group 1 compared to group 2 (P<0.05).

**Table 1.** Group 1 serum TNF- $\alpha$ , IL-1 $\beta$ , IL-6 levels  
**Tablo 1.** Grup 1 serum TNF- $\alpha$ , IL-1 $\beta$ , IL-6 düzeyleri

Groups	TNF- $\alpha$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-6 (pg/ml)
11 Group 1 (n=10)	45.0±3.07	1.70±2.5	53.8±1.2
1 Group 2 (n=8)	44.8±2.20	1.67±1.9	52.2±2.5
Group 3 (n=10)	42.6±2.10	1.66±2.2	50.0±2.0

### Pathological Results

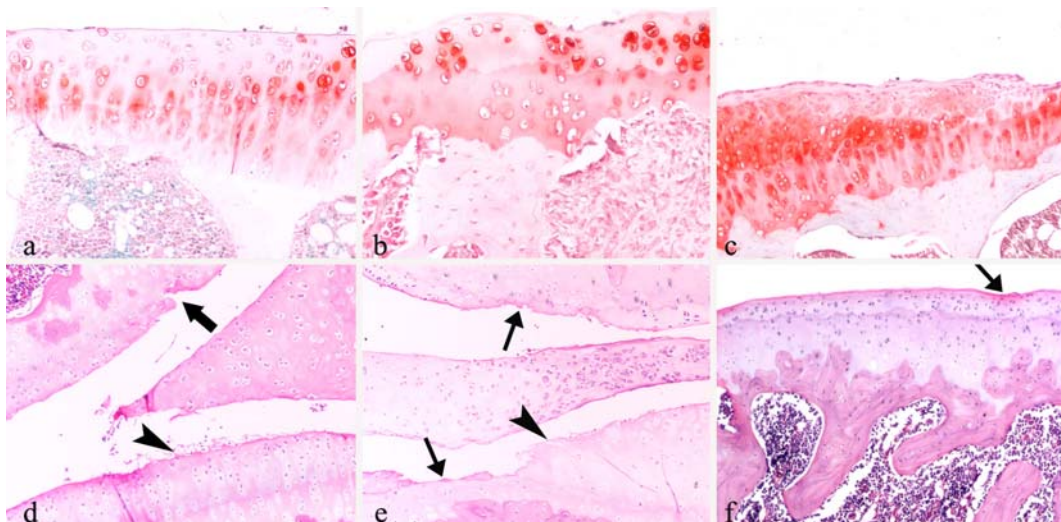
After staining with safranin O performed to detect proteoglycan loss in cartilage, the difference in loss of staining in group 1 was found as statistically important compared to group 1 and group 2 (P<0.05) (Table 3). The structural changes (i.e superficial fibrillation, vertical fissure and cartilage erosion) in the joint cartilage were more severe in group 1 than the other groups. Remarkable chondrocyte loss and pannus formation was observed in group 1 and group 2 compared to group 3. While in major cases in groups 1 and 2, widespread lymphocyte infiltration as well as neutrophil

**Table 2.** Group 2 serum ADA activity and MDA levels  
**Tablo 2.** Grup 2 serum se ADA aktivitesi ve MDA düzeyleri

Groups	ADA (U/L)	MDA ( $\mu$ mol/L)
11. Group 1 (n=10)	15.27±4.6	11.99±1.3 <sup>a</sup>
Group 2 (n=8)	14.43±2.5	11.94±1.6 <sup>a</sup>
Group 3 (n=10)	12.77±2.3	8.2±0.4 <sup>b</sup>
P<0.05		

**Table 3.** Semiquantitative histopathological analysis results of the rat stifle joints  
**Tablo 3.** Grup 1,2,3 rat eklem histopatolojik median ve analiz sonuçları

Groups	Safranin-O Orthochromatosis	Matrix Loss	Loss in Chondrocyte Cellularity	Cloning of Chondrocytes	Synovial Inflammation	Adhesions
Group 1 (n=10)	2.00 <sup>a*</sup>	2.00	1.00 <sup>a*</sup>	0	3.00 <sup>a**</sup>	2.00 <sup>a**</sup>
Group 2 (n=8)	1.00 <sup>b*</sup>	1.50	1.00 <sup>a*</sup>	0	2.00 <sup>b**</sup>	2.00 <sup>a**</sup>
Group 3 (n=10)	1.00 <sup>b*</sup>	1.50	0.50 <sup>b*</sup>	0	1.00 <sup>c**</sup>	1.00 <sup>b**</sup>
*P<0.05, **P<0.01						



**Fig 1.** (a-c) Safranin-O, (d-e) Haematoxyline Eosine, (a) Moderate level proteoglycan loss in cartilage, Group 1, x200, (b) Mild level proteoglycan loss in cartilage, Group 2, x200, (c) Mild level proteoglycan loss in cartilage, Group 3, x400, (d) Vertical fissure in cartilage (*thin arrow*) and superficial fibrillation (*arrow head*), Group 1., x200, (e) Severe erosion (*thin arrows*) and superficial fibrillation (*arrow head*) at the cartilage surface, Group 2, x200, (f) Mild erosion at cartilage surface (*thin arrow*), Group 3, x200

**Şekil 1.** (a-c) Safranin-O, (d-e) Hematoksilin Eozin, (a) Kıkırdakta orta düzeyde proteoglikan kaybı, 1. Grup, x200, (b) Kıkırdakta hafif düzeyde proteoglikan kaybı, 2. Grup, x200, (c) Kıkırdakta hafif düzeyde proteoglikan kaybı, 3. Grup, x400, (d) Kıkırdakta vertikal fissür (*kalın ok*) ve süperfişiyal fibrilasyon (*ok başı*), 1. Grup, x200, (e) Kıkırdak yüzeyinde şiddetli erozyon (*ince oklar*) ve süperfişiyal fibrilasyon (*ok başı*), 2. Grup, x200, (f) Kıkırdak yüzeyinde hafif erozyon (*ince ok*), 3. Grup, x200

leucocytes were occurred from place to place both in synovial membrane and synovial space, in group 3, generally few lymphocytes in synovial membrane and in several cases few neutrophile leucocytes in the synovial space were determined. Thus, it was determined that synovial inflammation was milder in group 3 compared to group 2 and in Group 2 compared to Group 1 ( $P < 0.01$ ).

## DISCUSSION

Arthritis is a dynamic disease process forming as a result of impairment in the normal equilibrium between destruction and restoration events in the joint cartilage and sub-chondrial bone. It is clinically characterised with pain in the joint, local sensitivity, disability in movements, crepitation, sometimes effusion and presence of varying degrees of local inflammation without systemic signs. AA is induced by injection of FCA to rat paw and has become a model frequently used in several studies due to its similarity to RA and Reiter Syndrome in histopathologic views and association with formation of cell-mediated immunity<sup>1,9</sup>.

An increase in Reactive oxygen species (ROS) is also occurred in inflammatory and autoimmune rheumatic diseases<sup>17</sup>. The role of leucocytes in the pathogenesis of inflammatory destruction and their destruction mechanisms are already known<sup>18</sup>. Cytokines take part in the pathophysiological mechanisms by orienting the inflammatory response<sup>19</sup>. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are cytokines that have leucocyte activating effects and the effects of increasing adhesion receptors on astrocytes, endothel cells and leucocytes<sup>20</sup>. It is known that the activation of nuclear factor kappa B (NF- $\kappa$ B) play a role in developing chronic diseases such as rhomatoid arthritis<sup>21</sup>.

NAC clears free radicals and prevents apoptosis by interacting reactive oxygen radicals and prolongs the life of cells by regulating various protein activities<sup>22</sup>. It is used for the prevention of inflammatory joint diseases<sup>23</sup>. TNF- $\alpha$  and IL-1 which increase in rheumatic disease and are gene products controlled by NF- $\kappa$ B increase the production of phagocytic ROS by stimulating the expression of the NADPH oxidase system components. NAC as an antioxidant and a NF- $\kappa$ B inhibitor interferes with this mechanism by abolishing ROS, the primary inflammatory signal molecule<sup>24,25</sup>. It has been stated that low molecular weight antioxidants such as NAC may be effective in the control of injuries resulting from oxidative stress in rheumatic diseases developed by transferring the signal conduction pathways dependent to ROS<sup>26</sup>. Thus, NAC known to decrease inflammation by preventing the production of cytokins and ROS<sup>26,27</sup> was inoculated to an experimental group in this study and it was aimed to compare this group with resveratrol received group.

Kröger et al.<sup>26</sup> reported that ROS production by plasma neutrophil and monocytes was inhibited in the group of mice

with arthritis, which was received 100 mg/kg/day NAC for 6 weeks. Tsuji et al.<sup>27</sup> stated that NAC inhibited plasma TNF- $\alpha$  production more than the inhibitory effect of IL-6 in the study which they have carried out in rat model and they have attributed this to the effect of NAC reducing cytokine production by blocking NF- $\kappa$ B activation. In this study, no statistical differences were recorded in cytokine levels of NAC received group. Similar effect of NAC on cytokine level was also observed in the study performed by Williams et al.<sup>28</sup>. In this study, it was reported that NAC neither had an effect on NF- $\kappa$ B activation pathway nor on cytokine production.

Resveratrol is known to have roles in platelet aggregation, lipid peroxidation, inhibition of reactive oxygen and nitrogen radicals, anticarcinogenic and anti-inflammatory effects<sup>29,30</sup>. Resveratrol also blocks NF- $\kappa$ B activation pathway and inhibits TNF- $\alpha$  and IL-6 production<sup>31</sup>. Gonzales and Orlando<sup>32</sup> reported that resveratrol inhibits NF- $\kappa$ B activation in adipose tissue, therefore cytokine production (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) reduces and resveratrol can be used in the chronic inflammation of lipocytes. On the other hand, in this study, no statistical changes were observed in the cytokine levels (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) of the resveratrol received group.

Resveratrol inhibits lipid peroxidation induced by Fenton's reaction products<sup>33</sup>. NAC is used for the control of injuries resulting from oxidative stress<sup>26</sup>, but in the present study, while no statistical differences were observed in NAC received group in serum MDA level an indicator of lipid peroxidation, a decrease ( $P < 0.05$ ) was determined in MDA levels in the resveratrol group. Banji et al.<sup>34</sup> reported in a similar study that, in arthritis and hepatotoxicity induced by FCA in rats, an elevation was observed in MDA levels, indicator of lipid peroxidation, but curcumin and methotrexate usage decreased this formation. Similarly, Abdin et al.<sup>35</sup> reported regression in lipid peroxidation by atorvastatin and prednisolone in a similarly induced arthritis model. Also, Bauerova et al.<sup>36</sup> observed reduction in lipid peroxidation in arthritis by methotrexate and coenzyme Q<sub>10</sub> in their study.

ADA is an enzyme which shows a correlation with the numbers of lymphocyte, polymorph nuclear cells and macrophage and has a tendency to show increasing in rheumatoid arthritis<sup>37,38</sup>. In the present study, no statistical changes were observed in ADA activity.

Histopathologic results revealed a decrease ( $P < 0.05$ ) in cellularity loss of cartilage and a decrease ( $P < 0.01$ ) in adhesion in resveratrol received group. Regarding orthochromasis with Safranin-O, in both groups (group 2 and group 3) it decreased ( $P < 0.05$ ) compared to arthritis group. Synovial inflammation decreased in both NAC and resveratrol groups ( $P < 0.01$ ). The best recovery was observed in the resveratrol group. Elmali et al.<sup>39</sup> has also obtained similar histopathological findings in their study in which they induced arthritis using lipopolysaccharide. It has been reported that resveratrol reduced the severity of synovial inflammation and decreased orthochromasis. It was

suggested that resveratrol can be alternatively used in inflammatory arthritis. Resveratrol can reduce cartilage erosion and synovial inflammation <sup>16</sup>.

In conclusion resveratrol administration ameliorated MDA level together with histopathological improvement. These data reveals that further studies are required for understanding the effect mechanism of resveratrol and for extending the endication spectrum.

### CONFLICT OF INTEREST

We have no protected, financial, occupational or other personal interests in a product, service and/or a company which could influence the content or opinions presented in the manuscript.

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