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This is the author's manuscript

Original Citation:
Combined methotrexate and coenzyme Q therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress / Bauerova K; Paulovicova E; Mihalova D; Drafi F; Strosova M; Mascia C; Biasi F; Rovensky J; Kucharska J; Gvozdjakova A; Ponist S. - In: ACTA BIOCHIMICA POLONICA. - ISSN 0001-527X. - 57(2010), pp. 347-354.

Availability:
This version is available http://hdl.handle.net/2318/75839 since

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Combined methotrexate and coenzyme Q₁₀ therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress*

Katarina Bauerova¹², Ema Paulovicova², Danica Mihalova¹, Frantisek Drafi¹, Miriam Strosova¹, Cinzia Mascia³, Fiorella Biasi³, Jozef Rovensky¹, Jarmila Kucharska⁵, Anna Gvozdjakova⁵ and Silvester Ponist¹²

1Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovakia; 2Center of Excellence GLYCOMED, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; 3Department of Clinical and Biological Sciences, University of Turin, Italy; 4National Institute for Rheumatic Diseases, Piestany, Slovakia; 5School of Medicine, Comenius University, Bratislava, Slovakia

Rheumatoid arthritis is a common severe joint disease that affects all age groups, it is thus of great importance to develop new strategies for its treatment. The aim of the present study was to examine the combined effect of coenzyme Q₉ (CoQ₉) and methotrexate (MTX) on the progression of adjuvant-induced arthritis in rats. Adjuvant arthritis (AA) was induced by a single intradermal injection of heat-inactivated Mycobacterium butyricum in incomplete Freund’s adjuvant. The experiments included healthy animals, arthritic animals not treated, arthritic animals treated with CoQ₁₀ with methotrexate, and with a combination of CoQ₁₀ and methotrexate. The two latter groups received a daily oral dose of 20 mg/kg b.w. of CoQ₁₀ either alone or with methotrexate in an oral dose of 0.3 mg/kg b.w. twice a week. We found that CoQ₁₀ potentiates both the antiarthritic (decrease of hind paw volume) and the antioxidant effect of methotrexate on the level of oxidation of proteins (suppression of protein carbonyl level in plasma) as well as lipoperoxidation (suppression of levels of HNE-adducts and MDA-adducts to plasma proteins). The same effect was observed for plasmatic levels of CoQ₉ and IL-1α, and partially also for γ-glutamyltransferase activity assessed in joints and spleen. Moreover, the combination therapy improved the functionality of peripheral blood neutrophils in AA, with a balancing effect on the immunosuppressive caused by MTX monotherapy. In summary, combined administration of CoQ₁₀ and methotrexate suppressed arthritic progression in rats more effectively than did MTX alone. This finding may help improve treatment of rheumatoid arthritis.

Keywords: combined therapy, methotrexate, coenzyme Q, arthritis, inflammation, oxidative stress

Received: 28 February, 2010; revised: 27 June, 2010; accepted: 23 August, 2010; available on-line: 09 September, 2010

INTRODUCTION

Rheumatoid arthritis (RA) is a common severe joint disease affecting all age groups. It is thus of great importance to develop new strategies for its treatment. As a number of disease-modifying anti-rheumatic drugs (DMARDs) often have side effects at high doses and/or during long-term administration, increased efficacy without increased toxicity are expected for combination therapy of RA. Methotrexate (MTX), a folic acid antagonist, has become the predominant immunosuppressive agent used in the treatment of patients with RA (Williams et al., 1985). MTX acts mainly on actively proliferating cells during the S-phase of proliferation, suppresses macrophage function, modulates interleukin-1 (IL-1) and superoxide anion production, and inhibits neutrophil chemotaxis (Moreland et al., 1997). Furthermore, MTX treatment was shown to decrease synovial collagenase gene expression in patients with RA (Genestier et al., 2000). The use of MTX has been limited by some of its toxic manifestations, such as abdominal discomfort, alopecia, oral ulcerations, and cytopenia (Alarcon et al., 1989). In clinical studies, infliximab or etanercept have been used in combination with methotrexate to produce greater efficacy of the treatment of RA (Maini et al., 1998; Weinblatt et al., 1999). TNFα blockers may be used alternatively with other candidates for RA combination therapy. The lack of thorough understanding of the pathogenesis of RA is a major problem in the introduction of new therapies. Several clinical studies as well as preclinical animal models of RA have documented an imbalance in the body redox homeostasis to a more pro-oxidative environment, suggesting that therapies that restore the redox balance may have beneficial effects (Kunsch et al., 2005). Bauerova and Bezek (1999) and Jaswal et al. (2003) described oxidative stress as a primary factor in the pathogenesis of RA. A preliminary report on the same subject was presented as poster at the European meeting of SFRR in Rome, Italy, 26-29 August 2009.

Abbreviations: AA, adjuvant arthritis; AA-CoQ₁₀, arthritic animals treated with coenzyme Q₁₀; AA-MTX, arthritic animals treated with methotrexate; AA-MTX-CoQ₁₀, arthritic animals treated with combination of CoQ₁₀ and methotrexate; ACK, acetylcholinesterase; MB, Mycobacterium butyricum; MDA, malondialdehyde; MTX, methotrexate; PMNs, polymorphonuclear leukocytes; RA, rheumatoid arthritis; SEM, standard error of the mean; sol. inj., solution for injection; SPA-FITC, fluorescein-labeled opsonized S. aureus; TBARS, thiobarbituric acid reacting substances; TNFα, tumor necrosis factor-alpha; γ-GPN, gamma-glutamyl-para-nitroanilide
involved in the pathogenetic changes during rheumatoid arthritis. In our studies, synthetic and natural substances with antioxidant activity were evaluated by using adjuvant arthritis (AA) — an animal model of RA which allows monitoring the disease processes in the acute (days 14–21) and subchronic phase (after day 28). The advantage of this model is its great similarity to RA, such as symmetrical joint involvement, persistent joint inflammation, synovial hyperplasia, and a good response to most therapies effective in RA (Bina & Wilder, 1999). Many of the substances with antioxidant properties tested in monotherapy proved to be effective in suppressing the progression of AA (Bauerova et al., 2005a; 2005b; 2008a; 2008b; 2009; Drabikova et al., 2009; Gvozďáková et al., 2004; Jancinova et al., 2009; Kogan et al., 2005; Nosal et al., 2007; Rovensky et al., 2008; 2009a; Sotníková et al., 2008; 2009).

Based on our results with mitochondrial energy metabolism and the observed anti-inflammatory and antioxidant effects (Gvozďáková et al., 2004; Bauerova et al., 2005a; 2008a; Ponist et al., 2007), we chose CoQ10 as a candidate for combinatorial therapy of RA. Patients with RA often suffer muscle weakness and atrophy. It is assumed that progressive muscle atrophy in RA patients is caused by damaged myofibrils and impaired mitochondria (De Palma et al., 2000). Disruption of mitochondrial bioenergetics caused by free radicals is involved in development of myopathies. Oxidative stress—caused alteration of mitochondrial functions can manifest in different manners (Cardoso et al., 1999). Leakage of free radicals from the respiratory chain leads to damaged mitochondrial membrane, proteins, DNA and inhibits oxidative phosphorylation (Luft, 1995; Miesel dam and impaired mitochondrial membrane, proteins, DNA and inhibits oxidative phosphorylation (Luft, 1995; Miesel).

To slow down RA progression (Comstock et al., 1997; Knekt et al., 2000; Kucharská et al., 2005), hind paw muscle of arthritic animals lies very close to the inflamed joint and could be also sensitive to joint inflammation (Ponist et al., 2007). Moreover, AA is a systemic inflammatory disease and we might expect also impairment in myocardial mitochondria functions, which we indeed demonstrated in our previous experiments. We found that the reactions of skeletal muscle and myocardium muscle on CoQ supplementation in AA were different, which was not so surprising in view of their different structure and functions in the organism (Gvozďáková et al., 2007). Our experiments confirmed CoQ as a good antioxidant in AA showing no side effects even at the high dose of 200 mg/kg b.w. Thus CoQ administration could be an appropriate supplement to a basal anti-rheumatic therapeutic regimen. The aim of the present study was to examine the combined effect of CoQ10 and methotrexate on the progression of adjuvant-induced arthritis in the rat. For this purpose, we used monitoring of hind paw volume (HPV) — a basic clinical parameter — along with evaluation of oxidative stress and inflammation markers assessed in plasma and tissues.

**MATERIAL AND METHODS**

**Animals, experimental design and treatments.** Male Lewis rats weighing 160–180 g were obtained from the Breeding Farm Dobra Voda (Slovakia). The rats had free access to standard pelleted diet and tap water. The animal facilities comply with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary Committee of Animal Experimentation. Adjuvant arthritis (AA) was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* (MB) in incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). The injection was performed near the tail base. The experiments included healthy animals (HC), arthritic animals not treated (AA), arthritic animals treated with coenzyme Q10 (AA-CoQ), arthritic animals treated with methotrexate (AA-MTX), and arthritic animals treated with the combination of AA-MTX and methotrexate (AA-MTX+CoQ). The two latter groups received a daily oral dose of 20 mg/kg b.w. of CoQ10, either alone (AA-MTX) or with methotrexate in the oral dose of 0.3 mg/kg b.w. twice a week (AA-MTX+CoQ). AA-MTX was performed as a reference treatment. Methotrexate® Lachema 50 sol. inj. was used. CoQ10 in the form of Li-Q-Sorb® was purchased from Tishcon Corp. (USA). In each experimental group, 8–10 animals were used. The duration of the experiment was 28 days. Blood was collected under light ketamin/xylasine anesthesia from the retro-orbital plexus on day 7 and immediately flow cytometric measurements were performed. After the animals had been sacrificed under deep ketamin/xylasine anesthesia, blood for plasma preparation and tissues for spleen and for hind paw joint homogenate preparation were taken on day 28. Plasma was stored at −70°C until biochemical and immunological analysis.

**Clinical parameter evaluated: hind paw volume.** We monitored one basic clinical parameter: the hind paw volume (HPV). The HPV increase was calculated as the percentage increase in the HPV on a given experimental day relative to the HPV at the beginning of the experiment. Hind paw volume was recorded on days 1, 14, 21, and 28 with the use of an electronic water plethysmometer (UGO BASILE, Comerio-Varese, Italy).

**Biochemical and immunological analysis.** **Protein carbonyl assay.** Enzyme linked immunosorbent assay (ELISA) was used for quantitative determination of protein carbonyls in plasma (Buss et al., 1997). Protein samples were derivatized with dinitrophenylhydrazine (DNPH) and adsorbed in multwell plates (Nunc Immunosorb plates, Roskilde, Denmark). A biotin-conjugated anti-dinitrophenyl rabbit IgG (Sigma, USA) was used as the primary antibody and a peroxidase conjugated monoclonal anti-rabbit-IgG antibody (Sigma, USA) as the secondary antibody. The development was performed with *ortho*-phenylenediamine. Absorbance was determined at 492 nm. The method was calibrated using oxidized bovine serum albumin (BSA). Oxidized and reduced BSA was prepared according to the method of Buss et al. (1997).
**RESULTS AND DISCUSSION**

**Clinical manifestation of adjuvant arthritis and effects of therapy**

We monitored the hind paw volume change after clinical development of arthritis on experimental days 14, 21 and 28. In our previous experiments, we confirmed that clinical parameters, such as hind paw volume and body weight, became significantly modified starting around day 14 (Bauerova et al., 2007). As illustrated in Fig. 1, the HPV is significantly increased for the arthritis group in comparison with healthy control already on day 14 and this increase is maintained until the end of the experiment. CoQ10 supplementation to arthritis animals slightly decreased the HPV on all experimental days. In the
treatment of RA, methotrexate (MTX) is the most commonly prescribed disease-modifying anti-rheumatic drug. It has suppressive effects on inflammation in AA, first described by Welles et al. (1985). In the present study, the decreasing effect of MTX monotherapy on hind paw swelling was evident on all monitored days (Fig. 1). The significance of this effect was a confirmation of its well-known antiarthritic effect, which we proved also previously on the adjuvant arthritis model (Nosal et al., 2007; Jurcovicova et al., 2009; Rovensky et al., 2009e). Due to the adverse effects of MTX accompanying its administration to arthritic patients, which are factors often limiting the acceptable dose, duration and safety of the therapy with MTX (Visser & van der Heijde, 2009), a combinatorial therapy of MTX is recommended with the aim to eliminate or minimalize these limitations. Besides the classical antiarthritics given in the combination schedule with a low dose of MTX, new candidates with antioxidative properties are being studied (Rovensky et al., 2009c). Due to the pathogenesis of arthritis is associated predominantly with the formation of free radicals at the site of inflammation, we chose CoQ10 as an appropriate candidate. Antithrombosis treatment affecting the level of CoQ10 was found to slow down the progression of the disease in arthritic patients (Comstock et al., 1997; Knekt et al., 2000). Mitochondrial function in the heart and skeletal muscle and efficacy of supplementation with CoQ10 depended on the severity in the induced adjuvant arthritis (AA) in rats. The results with solubilized CoQ10 (water-soluble form) indicated its therapeutic effect in the experimental model of AA (Gvozdjakova et al., 2004; Bauerova et al., 2005a; 2008a; Ponist et al., 2007). These findings are of potential significance in the treatment of patients with rheumatoid arthritis. On the basis of the results achieved with CoQ10 monotherapy, we selected this endogenous antioxidant with the aim to establish its suitability for the combination with MTX. The selected oral doses (for MTX 0.3 mg/kg of b.w. twice a week and for CoQ10 20 mg/kg of b.w. daily over one month) were established previously (Bauerova et al., 2005a; Jurcovicova et al., 2009). As shown in Fig. 1, the combination therapy was the most effective in decreasing the HPV of arthritic animals on all experimental days selected. Moreover, for day 14, we found a statistically significant difference between MTX monotherapy and its combination with CoQ10.

**Different parameters of oxidative stress monitored in adjuvant arthritis and effect of treatment**

These promising clinical results were further completed by measurements of HNE- and MDA-protein adducts and protein carbonyls in plasma (Fig. 2). We obtained a good agreement of HPV with the parameters of oxidative stress: the effect was increasing in the order CoQ10 alone, MTX alone, combination of CoQ10 and MTX. The most pronounced effect found for the combination of MTX and CoQ10 was significant for all oxidative stress parameters compared with non-treated arthritic animals. Moreover, the combination decreased all parameters close to the control group values, being more effective than the individual substances (Fig. 2). On using measurements of plasmatic protein carbonyls, we found damage of proteins caused by oxidative stress accompanying arthritis in past experiments (Bauerova et al., 2005b; Kogan et al., 2005; Strosova et al., 2009). Progression of lipid peroxidation in AA was previously described by analysis of TBARS plasmatic levels (Bauerova et al., 2005a; 2008b; 2009; Bauerova & Bezek, 2009; Strosova et al., 2008; 2009). Although the percentage increase was not so high for adducts as for protein carbonyls and TBARS levels in plasma, advanced measurements of HNE- and MDA-protein adducts showed for non-treated arthritis damaged animals the same level of significance as found for protein carbonyls (Fig. 2).
As shown in Fig. 3, the arthritis process increases significantly the level of CoQ9 in comparison with HC. The effect of therapy on this phenomenon reveals a picture comparable to that found for other oxidative stress parameters (Fig. 2). The combination therapy was again the most effective and significant in comparison to the untreated arthritis group (Fig. 3). The improvement is on the level of HC. Evidently, the arthritic processes stimulate the synthesis of CoQ9 and its transport to plasma. These results are in good agreement with the results obtained on rat experimental diabetes models, in which increased concentrations of CoQ9 in plasma may indicate adaptive changes in diseases associated with oxidative stress (Kucharska et al., 2000; Kuzelova et al., 2008). Supplementation with CoQ9 is not sufficient to inhibit these processes, while the combination of CoQ9 with MTX proved successful in returning the increased plasmaic levels of CoQ9 to control levels (Fig. 3).

Changes in plasma pro-inflammatory cytokine IL-1 levels

For further evaluation of the efficacy of the administration of CoQ9+MTX, and with the aim to support the obtained clinical and biochemical data with immunological measurements, we performed an analysis of IL-1α in plasma, which is one of the most important pro-inflammatory cytokines. IL-1α is secreted by monocytes/macrophages activated via TNFα and/or bacterial endotoxin. Furthermore, IL-1α markedly potentiates the toxic effect of TNFα in animal experiments (Waage et al., 1991). Rheumatoid arthritis is associated with elevated levels of IL-1α in the synovium. IL-1α is closely related to inflammation and articular damage in several arthritis models and it is therefore generally accepted that IL-1α has a pivotal role in the pathophysiology of rheumatoid arthritis. In particular, IL-1α is a potent stimulator of synoviocytes, chondrocytes and osteoblasts. Moreover, IL-1α is a key mediator of synovial inflammation and pannus formation (Dinarello & Moldawer, 2002). In the AA model used in our experiments, IL-1α was significantly increased in plasma on day 14 and also on day 28 (Bauerova et al., 2007; 2009). Figure 4 shows that the effects of the given treatments on the AA-increased IL-1α levels are very close to the effects illustrated in Figs. 1 and 2. The improving effect on the increased cytokine plasmatic levels is rising in the order CoQ9+MTX and CoQ9+MTX. Furthermore, a statistically significant difference was found between MTX monotherapy and its combination with CoQ9.

Activity of GGT in selected tissues

As the only enzyme of the γ-glutamyl cycle located on the outer surface of the plasma membrane, γ-glutamyltransferase (GGT) plays a key role in glutathione (GSH) homeostasis by breaking down extracellular GSH and providing cysteine, the rate limiting substrate for intracellular de novo synthesis of GSH (Zhang et al., 2005). Moreover, GGT is a multifunctional protein. There is evidence that cellular GGT plays an important role in the antioxidant defense system (Kugelman et al., 1994; Lieberman et al., 1996). Ectoplasmic GGT favors the cellular supply of GSH, the most important non-protein antioxidant of the cell. Some epidemiological studies have suggested that serum GGT within its normal range might be an early marker of oxidative stress (Lee & Jacobs, 2005) as well as of the activation of systemic inflammation (Yamada et al., 2006). GGT activity in organs such as spleen and joint could be a simple and inexpensive marker of AA and RA develop-
Neutrophils, also known as polymorphonuclear leukocytes (PMNs) or phagocytizing cells, play a vital role within the immune system. The processes of phagocytosis and the subsequent production of reactive oxygen intermediates during oxidative burst are crucial components of the host defense system. Apparently, neutrophils exhibit many of the properties of phagocytotic cells of the monocyte/macrophage lineage. Neutrophils are present in a number of chronic inflammatory diseases, such as arthritis, and this is indicative of their important role in chronic inflammation. Their rapid secretion of different factors, released also by macrophages, lymphocytes and fibroblasts, indicates that neutrophils can play similar roles in chronic inflammation. Many of these factors are pivotal in causing tissue destruction, either directly or indirectly (Edwards & Hallett, 1997; Haynes, 2007).

Figure 6. Activities of GGT in hind paw joint homogenate determined on day 28 after adjuvant arthritis. Effects of CoQ10 and methotrexate in monotherapy and combined therapy on the activities of GGT in the joint. 4-NA: 4-nitroaniline. The data were expressed as arithmetic mean ± S.E.M. Each group contained 8–10 animals. Statistical significance was evaluated applying Student’s t-test for independent variables: ***P<0.001 with respect to control healthy animals; **P<0.01 with respect to untreated arthritic animals.

Figure 7. Functionality of peripheral blood neutrophils determined on day 7 of adjuvant arthritis. Effects of CoQ10 and methotrexate in monotherapy and combined therapy on phagocytosis, oxidative burst and metabolic activity of peripheral blood neutrophils. Phagocytic activity, oxidative burst and metabolic activity were expressed as percentage of neutrophils actually undergoing these processes in population of 5000 neutrophils. The data were expressed as arithmetic mean ± S.E.M. Each group contained 8–10 animals. Statistical significance was evaluated applying Student’s t-test for independent variables: **P<0.01 with respect to control healthy animals; ***P<0.001 with respect to untreated arthritic animals. +P<0.05 and **P<0.01 for comparison of methotrexate monotherapy with combined therapy.

The functionality of peripheral blood neutrophils in adjuvant arthritis affected by evaluated treatment

Neutrophils, also known as polymorphonuclear leukocytes (PMNs) or phagocytizing cells, play a vital role within the immune system. The processes of phagocytosis and the subsequent production of reactive oxygen intermediates during oxidative burst are crucial components of the host defense system. Apparently, neutrophils exhibit many of the properties of phagocytotic cells of the monocyte/macrophage lineage. Neutrophils are present in a number of chronic inflammatory diseases, such as arthritis, and this is indicative of their important role in chronic inflammation. Their rapid secretion of different factors, released also by macrophages, lymphocytes and fibroblasts, indicates that neutrophils can play similar roles in chronic inflammation. Many of these factors are pivotal in causing tissue destruction, either directly or indirectly (Edwards & Hallett, 1997; Haynes, 2007). Recent data have revealed that RA-derived peripheral and synovial fluid neutrophils develop an intense respiratory burst (Cascao et al., 2010). In the model of AA, we observed already on experimental day 7 that AA was accompanied by an increased number of neutrophils in blood and by a more pronounced spontaneous as well as phorbol myristate acetate-stimulated chemiluminescence (Nosal et al., 2007). Interestingly, increased production of ROS by neutrophils emerged already in an early phase of disease, therefore we decided to investigate this finding more precisely using flow cytometry. Another reason was that the changes in neutrophils occur before the clinical parameter HPV starts to be increased. The functionality of peripheral blood neutrophils in AA was evaluated by phagocytosis, oxidative burst and metabolic activity (Fig. 7). Both phagocytosis and oxidative burst were increased due to arthritis. Metabolic activity of neutrophils is the percentage of double positive cells — simultaneously phagocytotic and positive for oxidative burst. Decreased metabolic activity could be explained with increased number of “arthritic” neutrophils, which are positive only for oxidative burst and therefore are not counted as double positive cells. The immunosuppressive effect of MTX was demonstrated in lowering all parameters, not only in comparison with arthritis but also with HC. The addition of CoQ10 to MTX modulated all processes back to the level of HC. The observed immunoenhancing activity of CoQ10 may prove beneficial in MTX routine treatment. In this experiment, flow cytometric determination of the functionality of neutrophils was first applied for an adjuvant arthritis experimental model on rats.

In summary, we found that CoQ10 could potentiate both the antiarthritic (decrease of hind paw volume) and the antioxidant effect of methotrexate on the level of oxidation of proteins (suppression of levels of protein carbonyls in plasma) as well as lipoperoxidation (suppression of levels of HNE adducts and MDA adducts to plasma proteins). Further, the same effect was observed for plasmatic levels of CoQ9 and II-1α, partially also for γ-glutamyltransferase activity assessed in joints and spleen. Moreover, the combination therapy improved the functionality of peripheral blood neutrophils in AA, with a balancing effect on the immunosuppression caused by MTX monotherapy.

In conclusion, combined administration of CoQ10 and methotrexate suppressed arthritic progression in rats more effectively than did MTX alone. This finding may become a beneficial contribution to the treatment of rheumatoid arthritis. Restoration of redox imbalance in chronic inflammatory diseases may be of significant importance in new therapeutic strategies.
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Acknowledgements

The authors wish to thank Mrs. Denis Komendova and Dr. Veronika Tomekova for their excellent technical assistance.

This work was supported by grants APVV-51-017905, APVV-21-055205, VEGA 02/0900/08, COST B35.

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