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## Combined methotrexate and coenzyme Q<sub>10</sub> therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress\*

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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 Q<sub>10</sub> (CoQ<sub>10</sub>) 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 CoQ<sub>10</sub>, with methotrexate, and with a combination of CoQ<sub>10</sub> and methotrexate. The two latter groups received a daily oral dose of 20 mg/kg b.w. of CoQ<sub>10</sub>, either alone or with methotrexate in an oral dose of 0.3 mg/kg b.w. twice a week. We found that CoQ<sub>10</sub> potentiated 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 CoQ<sub>9</sub> and IL-1 $\alpha$ , and partially also for  $\gamma$ -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 summary, combined administration of CoQ<sub>10</sub> 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

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### 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). TNF $\alpha$  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

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**Abbreviations:** AA, adjuvant arthritis; AA-CoQ<sub>10</sub>, arthritic animals treated with coenzyme Q<sub>10</sub>; AA-MTX, arthritic animals treated with methotrexate; AA-MTX+CoQ<sub>10</sub>, arthritic animals treated with combination of CoQ<sub>10</sub> and methotrexate; ACK, ammonium-chloride-potassium chloride; b.w., body weight; BSA, bovine serum albumin; CoQ, coenzyme Q; CoQ<sub>9</sub>, coenzyme Q<sub>9</sub>; CoQ<sub>10</sub>, coenzyme Q<sub>10</sub>; DNPH, dinitrophenylhydrazine; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; GGT, gammaglutamyl transferase; GSH, glutathione; HC, healthy animals; HE, hydroxyethidine; HNE, 4-hydroxy-2-nonenal; HPLC, high-performance liquid chromatography; HPV, hind paw volume; HRP, horse radish peroxidase; IgG, immunoglobulin; IL-1, interleukin-1; IL-1 $\alpha$ , interleukin-1 alpha; 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; TNF $\alpha$ , tumor necrosis factor-alpha;  $\gamma$ -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; Gvozdjakova *et al.*, 2004; Jancinova *et al.*, 2009; Kogan *et al.*, 2005; Nosal *et al.*, 2007; Rovensky *et al.*, 2008; 2009a; Sotnikova *et al.*, 2008; 2009).

Based on our results with mitochondrial energetics modification and the observed anti-inflammatory and antioxidant effects (Gvozdjakova *et al.*, 2004; Bauerova *et al.*, 2005a; 2008a; Ponist *et al.*, 2007), we chose CoQ<sub>10</sub> as a candidate for combinatory 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 *et al.*, 1996). Maneiro *et al.* (2003) found inhibition of functions of complex II and III of the respiratory chain and higher frequency of energetically “exhausted” mitochondria in chondrocytes of patients with osteoarthritis compared to healthy donors. Following those findings, we decided to support the impaired mitochondrial functions by CoQ<sub>10</sub> supplementation and also reduce the increased oxidative stress in AA. CoQ<sub>10</sub>, a lipophilic antioxidant, has many protective functions which could be helpful in arthritic diseases: membrane stabilizing properties, protection of DNA against free radical damage, and ability to regenerate other important antioxidants as tocopherol and ascorbate (Crane, 2001). Some evidence from the literature showed that anti-rheumatic therapies which increased the level of CoQ<sub>10</sub> were able 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 (Gvozdjakova *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 CoQ<sub>10</sub> 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 Q<sub>10</sub> (AA-CoQ<sub>10</sub>), arthritic animals treated with methotrexate (AA-MTX), and arthritic animals treated with the combination of CoQ<sub>10</sub> and methotrexate (AA-MTX+CoQ<sub>10</sub>). The two latter groups received a daily oral dose of 20 mg/kg b.w. of CoQ<sub>10</sub> either alone (AA-MTX) or with methotrexate in the oral dose of 0.3 mg/kg b.w. twice a week (AA-MTX+CoQ<sub>10</sub>). AA-MTX was performed as a reference treatment. Methotrexat<sup>®</sup> Lachema 50 sol. inj. was used. CoQ<sub>10</sub> in the form of Li-Q-Sorb<sup>®</sup> 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/xylazine 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/xylazine 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 multiwell plates (Nunc Immunosorp 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*-phenyldiamine. 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).

**Determination of lipid peroxidation.** The presence of oxidative damage was assessed in plasma by determining the fluorescent adducts formed between peroxidation-derived aldehydes and plasma proteins. In particular, two types of protein-aldehyde adducts were determined: HNE-protein adducts (355 nm excitation; 460 nm emission) and MDA-protein adducts (390 nm excitation; 460 nm emission). Preparation of samples and fluorimetric measurements were performed according to Tsuchida *et al.* (1985) and Biasi *et al.* (1995). Briefly, 150  $\mu$ l of plasma was suspended in 6 ml ethanol/ether (3:1, v:v); after 10 min incubation in ice, the samples were centrifuged at 1500  $\times$ g, the supernatant discarded and the pellets washed twice with ethanol/ether (3:1, v:v). After the last centrifugation the pellets were dissolved in 3 ml of 0.1% sodium dodecyl sulfate (SDS) and immediately used for spectrofluorimetry (Kontron SFM 25). In parallel, standard curves were prepared with decreasing amounts of HNE or MDA dissolved in BSA (1 mg/ml); the adducts aldehydes with BSA were allowed to form by incubating the solutions at 37°C for 40 min, after which the samples were immediately read for fluorescence. Data were expressed as arbitrary units/mg protein. Protein detection was done with the Peterson method (Peterson *et al.*, 1977).

**Coenzyme Q<sub>9</sub> determination.** Concentration of oxidized coenzyme Q<sub>9</sub> was determined by isocratic high-performance liquid chromatography (HPLC, LKB, Sweden) according to Lang *et al.* (1986) with some modifications (Kucharska *et al.*, 1998). Plasma samples (500  $\mu$ l) were vortexed twice for 5 min with 2 ml of a mixture of hexane/ethanol (5:2, v/v; Merck, Germany). Collected organic layers were evaporated under nitrogen; the residue was taken up in ethanol and injected into a Separon SGX C18 7  $\mu$ m 3 mm  $\times$  150 mm column (Tessek, Czech Republic). Elution was performed with methanol/acetonitrile/ethanol (6:2:2, by vol.; Merck, Germany). The concentration of coenzyme Q<sub>9</sub> was determined spectrophotometrically at 275 nm, using external standards (Sigma, Germany). Data were collected and processed using a CSW 32 chromatographic station (DataApex Ltd, Czech Republic). Concentrations of compounds in the plasma were calculated in  $\mu$ mol  $\cdot$  l<sup>-1</sup>.

**Rat IL-1 $\alpha$  assay.** For determination of IL-1 $\alpha$  in plasma an ELISA kit from Bender MedSystems was used as described in the product manual Rat IL-1 $\alpha$  ELISA BMS627 and BMS627/TEN. The rat IL-1 ELISA is an assay for quantitative detection of rat IL-1. Rat IL-1 present in the samples binds to anti-rat IL-1 antibodies adsorbed to the microwells. The reaction of a secondary biotin-conjugated anti-rat IL-1 antibody is evaluated by Streptavidin-HRP. Tetramethylbenzidine oxidation with HRP bound to the immune complex was measured at 490 nm against reference wavelength of 620 nm. The results were calculated from a standard calibration curve obtained for internal standards.

**Tissue activity of cellular  $\gamma$ -glutamyltransferase.** The activity of cellular  $\gamma$ -glutamyltransferase (GGT) in hind paw joint tissue and in spleen tissue homogenates was measured by the method of Orłowski and Meister (1970) as modified by Ondrejckova *et al.* (1993). Samples were homogenized in a buffer at 1:9 (w/v) (buffer composition: 2.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 15 mM EDTA, 68 mM NaCl; pH 8.1) by Ultra Turax TP 18/10 (Janke & Kunkel, Germany) for 1 min at 0°C. Substrates (8.7 mM  $\gamma$ -glutamyl-*p*-nitroanilide ( $\gamma$ -GPN); 44 mM methionine) were added in 65% isopropylalcohol to final concentrations of 2.5 mM and 12.6 mM, respec-

tively. After incubation for 60 min at 37°C, the reaction was stopped with 2.3 ml cold methanol and the tubes were centrifuged for 20 min at 5000 rpm. Absorbance of supernatant was measured in a Hewlett Packard Vectra 286/12 spectrophotometer in 0.5 cm cuvette at 406 nm. Reaction mixtures in the absence of either the substrate or acceptor were used as reference samples.

**Simultaneous phagocytosis and oxidative burst.** Throughout phagocytosis accompanied by respiratory burst of rat granulocytes, the double fluorescence of fluorescein isothiocyanate (FITC)-labeled ingested *Staphylococcus aureus* cells and that of hydroxyethidine (HE) oxidized to ethidium bromide were evaluated by flow cytometry (Beckman-Coulter FC 500 flow cytometer running under CXP software). The previously reported method (Kronek *et al.*, 2010) was modified for whole rat blood. For each blood sample, a fluorescence histogram of 5000 cells was generated and analyzed. Gates were set around granulocytes to exclude debris. Measurement of phagocytosis, i.e. the ingestion of bacteria, took place under controlled conditions, using fluorescein-labeled opsonized *Staphylococcus aureus* (SPA-FITC) (Invitrogen Molecular Probes, USA). Metabolic activity was determined as the oxidative burst causing transformation of the originally non-fluorescent hydroxyethidine (Invitrogen Molecular Probes, USA) into ethidium following SPA-FITC ingestion. Aliquots of rat peripheral blood in lithium-heparin were incubated with HE (15.75 mg in 5 ml of dimethylformamide) for 15 min at 37°C. Following treatment with SPA-FITC for the next 15 min at 37°C, the reaction was stopped by placing the samples on ice. The subsequent erythrocyte lysis was performed for 15 min with an ice-cold ammonium-chloride-potassium chloride (ACK) lysis buffer (200 ml deionized water, 1.658 g NH<sub>4</sub>Cl; 0.2 g KHCO<sub>3</sub>; 7.4 mg Na<sub>2</sub>EDTA, pH 7.2–7.4) (Lachema, Czech Republic). Blood specimens were obtained by retroorbital sinus puncture under light anesthesia (ketamine/xylazine) of animals. Whole blood samples were collected into lithium heparin (Sarstedt Multivette) blood sample collectors.

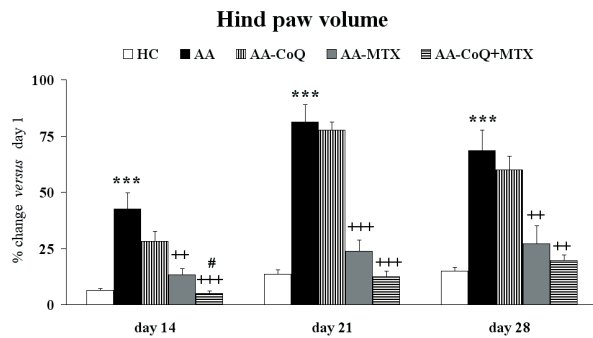
**Statistics.** The data were expressed as arithmetic mean  $\pm$  S.E.M. Arthritis group untreated was compared with healthy control animals (\*), treated arthritis groups were compared with untreated arthritis animals (+), and arthritis groups with combination therapy were compared with the reference treatment (#). For significance calculations unpaired Student's *t*-test (two sample, unequal variance) was used with the following significance designations: extremely significant ( $P < 0.001$ ); highly significant ( $P < 0.01$ ); significant ( $P < 0.05$ ); not significant ( $P > 0.05$ ).

## 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. CoQ<sub>10</sub> supplementation to arthritis animals slightly decreased the HPV on all experimental days. In the

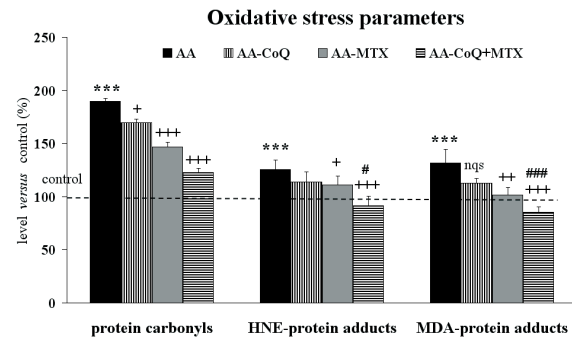




**Figure 1. Time profile of hind paw volume**

Effects of CoQ<sub>10</sub> and methotrexate in monotherapy and combined therapy on the progression of adjuvant arthritis. Change in hind paw swelling in all experimental groups was recorded on days 1, 14, 21, and 28 of AA and calculated as percentage of increase: [(Day n) / (Day 1)] × 100 = [%]. 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, and \*\*\**P* < 0.001 with respect to untreated arthritic animals; #*P* < 0.05 for comparison of methotrexate monotherapy with combined therapy.

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.*, 2009c). 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 combinatory therapy of MTX is recommended with the aim to eliminate or minimize these limitations. Besides the classical antirheumatics given in the combination schedule with a low dose of MTX, new candidates with antioxidative properties are being studied (Rovensky *et al.*, 2002; 2008; 2009a; 2009b; Cuzzocrea *et al.*, 2005; Dadhania *et al.*, 2010; Kogure *et al.*, 2010). As the pathogenesis of arthritis is associated predominantly with the formation of free radicals at the site of inflammation, we chose CoQ<sub>10</sub> as an appropriate candidate. Antirheumatic treatment affecting the level of CoQ<sub>10</sub> 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 CoQ<sub>10</sub> depended on the severity of the induced adjuvant arthritis (AA) in rats. The results with solubilized CoQ<sub>10</sub> (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 CoQ<sub>10</sub> 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 CoQ<sub>10</sub> 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



**Figure 2. Comparison of oxidative stress parameters assessed in plasma on day 28 of adjuvant arthritis**

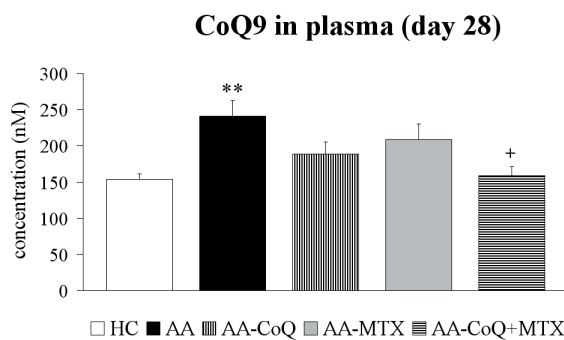
Effects of CoQ<sub>10</sub> and methotrexate in monotherapy and combined therapy on oxidative stress occurring in adjuvant arthritis. Oxidative stress parameters were measured in plasma by using ELISA and fluorimetric methods. Changes in all groups with arthritis were calculated with respect to the control value assessed for healthy control animals on experimental day 28. The dashed line represents the value of control as 100%. 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* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 with respect to untreated arthritic animals; #*P* < 0.05 and \*\*\**P* < 0.001 for comparison of methotrexate monotherapy with combined therapy.

all experimental days selected. Moreover, for day 14, we found a statistically significant difference between MTX monotherapy and its combination with CoQ<sub>10</sub>.

#### 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 CoQ<sub>10</sub> alone, MTX alone, combination of CoQ<sub>10</sub> and MTX. The most pronounced effect found for the combination of MTX and CoQ<sub>10</sub> 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 arthritic damaged animals the same level of significance as found for protein carbonyls (Fig. 2).

Significant changes in the levels of CoQ<sub>9</sub> and/or CoQ<sub>10</sub> have been noted in a wide variety of diseases in both animal and human studies. These changes may be caused by impairment in CoQ biosynthesis or excessive utilization of CoQ by the body, or any combination of these processes (Littarru *et al.*, 1991; Bauerova *et al.*, 2008a). In this experiment, we focused on evaluating the CoQ<sub>9</sub> plasmatic levels as the dominant form of CoQ in rats. Its concentration is about 10 times higher than the concentration of CoQ<sub>10</sub> (Dallner & Sindelar, 2000).



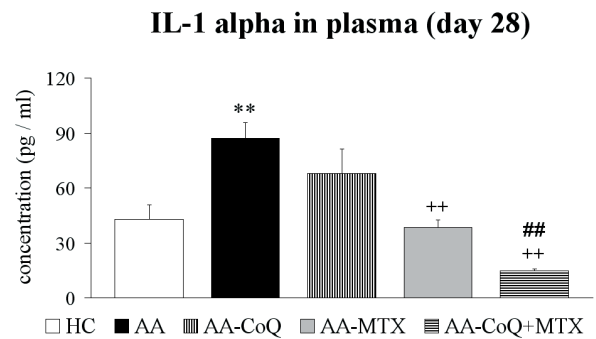
**Figure 3. Levels of CoQ<sub>9</sub> in plasma determined in the model of adjuvant arthritis on day 28**

Effects of CoQ<sub>10</sub> and methotrexate in monotherapy and combined therapy on plasma concentration of CoQ<sub>9</sub>. The data were expressed as arithmetic mean  $\pm$  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*<0.05 with respect to untreated arthritic animals.

As shown in Fig. 3, the arthritis process increases significantly the level of CoQ<sub>9</sub> in comparison with HC. The effect of therapy on this phenomenon unveils 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 was on the level of HC. Evidently, the arthritic processes stimulate the synthesis of CoQ 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 CoQ<sub>9</sub> in plasma may indicate adaptive changes in diseases associated with oxidative stress (Kucharska *et al.*, 2000; Kuzelova *et al.*, 2008). Supplementation with CoQ<sub>10</sub> is not sufficient to inhibit these processes, while the combination of CoQ<sub>10</sub> with MTX proved successful in returning the increased plasmatic levels of CoQ<sub>9</sub> to control levels (Fig. 3).

#### Changes in plasma pro-inflammatory cytokine IL-1 levels

For further evaluation of the efficacy of the administration of CoQ<sub>10</sub>+MTX, and with the aim to support the obtained clinical and biochemical data with immunological measurements, we performed an analysis of IL-1 $\alpha$  in plasma, which is one of the most important pro-inflammatory cytokines. IL-1 $\alpha$  is secreted by monocytes/macrophages activated *via* TNF $\alpha$  and/or bacterial endotoxin. Furthermore, IL-1 $\alpha$  markedly potentiates the toxic effect of TNF $\alpha$  in animal experiments (Waage *et al.*, 1991). Rheumatoid arthritis is associated with elevated levels of IL-1 in the synovium. IL-1 is closely related to inflammation and articular damage in several arthritis models and it is therefore generally accepted that IL-1 has a pivotal role in the pathophysiology of rheumatoid arthritis. In particular, IL-1 is a potent stimulator of synoviocytes, chondrocytes and osteoblasts. Moreover, IL-1 is a key mediator of synovial inflammation and pannus formation (Dinarello & Moldawer, 2002). In the AA model used in our experiments, IL-1 $\alpha$  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-1 $\alpha$  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 CoQ<sub>10</sub>, MTX and



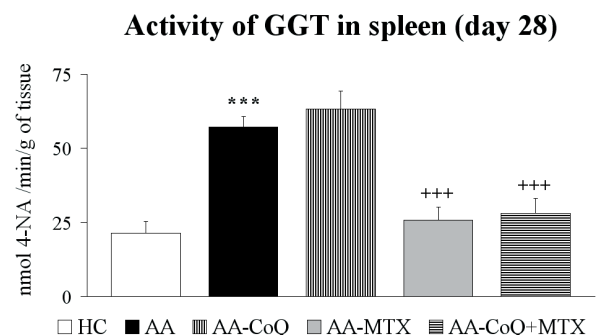
**Figure 4. Levels of IL-1 $\alpha$  in plasma determined in the model of adjuvant arthritis on day 28.**

Effects of CoQ<sub>10</sub> and methotrexate in monotherapy and combined therapy on plasma concentration of IL-1 $\alpha$ . The data were expressed as arithmetic mean  $\pm$  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*<0.01 with respect to untreated arthritic animals; ##*P*<0.01 for comparison of methotrexate monotherapy with combined therapy.

CoQ<sub>10</sub>+MTX. Furthermore, a statistically significant difference was found between MTX monotherapy and its combination with CoQ<sub>10</sub>.

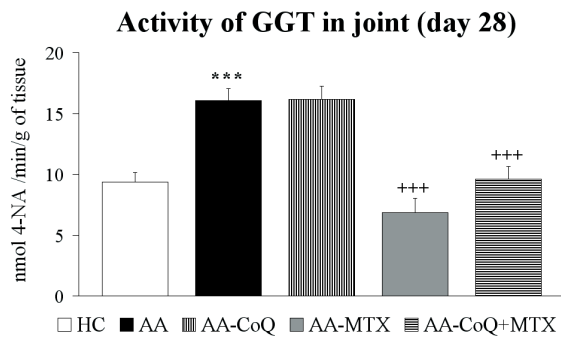
#### Activity of GGT in selected tissues

As the only enzyme of the  $\gamma$ -glutamyl cycle located on the outer surface of the plasma membrane,  $\gamma$ -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-



**Figure 5. GGT activities in spleen homogenates of rats 28 days after arthritis induction**

Effects of CoQ<sub>10</sub> and methotrexate in monotherapy and combined therapy on the activities of GGT in the spleen. 4-NA: 4-nitroaniline. The data were expressed as arithmetic mean  $\pm$  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*<0.001 with respect to untreated arthritic animals.



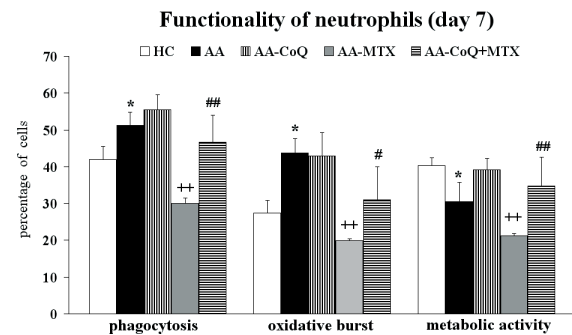
**Figure 6. Activities of GGT in hind paw joint homogenate determined in the model of adjuvant arthritis on day 28**

Effects of CoQ<sub>10</sub> 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  $\pm$  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*<0.001 with respect to untreated arthritic animals.

ment both at the systemic and local level (Bauerova *et al.*, 2006). Figure 5 shows the change in GGT activity in spleen homogenate, which could be described as a systemic inflammatory marker. Arthritis increases GGT activity, which was found to be three times higher than in HC. MTX decreased the increased activity back to the control value. Although monotherapy with CoQ<sub>10</sub> was without effect on this parameter, the combination therapy returned the GGT activity to control level of healthy animals. For the local inflammatory parameter — the activity of GGT in joint homogenate — an approximately double increase was recorded on comparing arthritic animals with HC (Fig. 6). The treated groups presented the same results as shown in Fig. 4. These findings suggest that the cycles of GGT and CoQ are not directly coupled.

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

Neutrophils, also known as polymorphonuclear leucocytes (PMNs) or phagocytosing 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 (Cascão *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



**Figure 7. Functionality of peripheral blood neutrophils determined on day 7 of adjuvant arthritis**

Effects of CoQ<sub>10</sub> 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  $\pm$  S.E.M. Each group contained 8–10 animals. Statistical significance was evaluated applying Student's *t*-test for independent variables: \*\*\**P*<0.05 with respect to control healthy animals; ++*P*<0.01 with respect to untreated arthritic animals. #*P*<0.05 and ##*P*<0.01 for comparison of methotrexate monotherapy with combined therapy.

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 CoQ<sub>10</sub> to MTX modulated all processes back to the level of HC. The observed immunoenhancing activity of CoQ<sub>10</sub> 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 CoQ<sub>10</sub> 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 CoQ<sub>9</sub> and IL-1 $\alpha$ , partially also for  $\gamma$ -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 CoQ<sub>10</sub> 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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