

# Modern Pharmacological Approaches to Therapies: Substances Tested in Animal Models of Rheumatoid Arthritis

Katarina Bauerova<sup>1</sup>, Silvester Poništ<sup>1</sup>, Radomir Nosaľ<sup>1</sup>,  
Maria Stančíkova<sup>2</sup> and Jozef Rovensky<sup>2</sup>

<sup>1</sup>*Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences,*  
<sup>2</sup>*National Institute of Rheumatic Diseases*  
*Slovakia*

## 1. Introduction

Preclinical research on animal models of rheumatoid arthritis (RA) is very important for alerting the healthcare and scientific community and pharmaceutical companies of the existence of new or “forgotten” molecules. Most antirheumatics have side-effects when used in higher doses and/or within long-term dosage. Combinatory therapy is expected to have a higher efficacy without increased toxicity. Methotrexate (MTX) has become the main immunosuppressive substance used in the treatment of patients with RA. However, the use of MTX has to be limited due to its toxic manifestations, e.g. abdominal disorder, alopecia, oral ulcers, and cytopenia (Alarcon et al., 1989). Ineffectiveness of treatment can be also observed. In the survey of McKendry and Dale (McKendry & Dale, 1993), due to the risk of treatment, termination was substantiated in 75% of patients with RA taking MTX for 60 months. An adverse drug effect proved to be a more common reason for treatment termination (53%) compared to loss/lack of beneficial effect (22%), other reasons (16%), or lost of follow-up (9%). On the other hand, the therapeutic efficacy of MTX can be increased by combination with other synthetic drugs or inhibitors of TNF- $\alpha$  (Smolen et al., 2010). Application of biological therapy (antibodies or soluble receptors of TNF- $\alpha$ , IL-1 and IL-6) represents a great progress in the therapy of RA. Yet biological treatment is also frequently combined with MTX (Maini et al., 1998; Weinblatt et al., 1999). There are countless possibilities for combinations with MTX. Many substances were neglected when they failed to show good efficacy in monotherapy compared to standard antirheumatics. They would not get a second chance if the expected reduction of clinical parameters (mainly edema of joints) did not materialize, despite the fact that they improved many biochemical disease markers. Our research in the last years was focused on evaluation of new therapeutics for the combinations of the classical immunosuppressive treatment with immunomodulators and compounds affecting reduction/oxidation homeostasis.

Development of drugs for the therapy of RA has been very intensive in recent years. Biological therapy targeted on neutralizing the effect of antiinflammatory cytokines, particularly TNF- $\alpha$ , IL-1 and IL-6, by using antibodies or soluble receptors provided a great progress in RA therapy. However, not even these expensive drugs are able to cure RA

definitely, although they remarkably inhibit the development of arthritis and improve the life quality of patients. Following treatment interruption, a fast development of RA occurs. Biological therapy also has adverse effects, such as development of resistance and secondary infections. For these reasons, the search for new drugs which could avoid these infections or suppress them is still an up-to-date problem. As already mentioned, the most frequently applied conventional drug for RA has been MTX. Its application is usually additional to the biological therapy or it is used in combination with other conventional drugs. Intensive immunosuppressive treatment with MTX or biological therapy adversely affects immunological homeostasis of the organism and increases the risk of infections. For these reasons, there is a search for alternative immunomodulatory approaches, which could minimize side effects of immunosuppressive therapy on cellular and humoral immunity. One possibility is represented by the combination of immunosuppressive and immunostimulatory substances or compounds regulating redox balance of the organism. Their application can establish immunological and redox homeostasis and increase resistance of the organism to infections.

The focus of this chapter is mostly on substances of natural origin possessing antiinflammatory, antioxidative or immunomodulating properties along with minimal side effects when administered to animals. The safety of long-term therapy of RA is very important, because patients with RA are usually treated for two or more decades. We describe our results obtained in adjuvant arthritis (AA) with endogenous antioxidants as carnosine and coenzyme Q<sub>10</sub>, glucomannan and Immunoglukan®, as well as selected extracts and compounds from plants. These results will be confronted with results of other authors from preclinical and clinical studies. The aim is to present an overview of the potential of new compounds for the therapy of RA with the focus on approving their ability for combination therapy with methotrexate.

## **2. Adjuvant arthritis: An animal model for preclinical evaluation of new antirheumatics**

In AA the injection of rats with complete or incomplete adjuvant induces polyarthritis, possibly by way of a mechanism involving heat shock proteins. Arthritis induced in rats with intradermal injection of adjuvants containing mycobacteria is an animal model often used for evaluation of potential antirheumatic drugs. This model is also a good methodological tool for investigation of pathological mechanisms in RA. An intradermal injection, into the base of the tail, with heat-killed *Mycobacterium tuberculosis* in incomplete Freund's adjuvant (IFA) results in destructive arthritis within 14 days in susceptible Dark Agouti or Lewis inbred rat strains. AA can also be induced with cell walls from other bacterial types in IFA, although the arthrogenicity varies (Joe et al., 1999). In many experiments, the induction of arthritis is achieved also with *Mycobacterium butyricum* in IFA (Akiyama et al., 2005; Bauerova et al., 2006, 2009). Basic clinical manifestations of AA are paw swelling, infiltration of mononuclear and polymorphonuclear phagocytes into joints, formation of pannus, periostitis and erosions of cartilage and bone (Williams et al., 1998). The intensity of AA development is described by so-called "basic clinical parameters", i.e. arthritic score, hind paw edema, which are usually measured once a week over the whole duration of the experiment. Increased synthesis of TNF- $\alpha$ , IL-1 and IL-6 is detected as early as day four after adjuvant injection. The disease progresses rapidly over several weeks in what appears clinically to be a monophasic process. Granulocytes and autoreactive CD41

cells play major roles in the disease. Humoral immune mechanisms appear not to contribute to the disease process (Joe et al., 1999).

AA has been extensively used for pharmaceutical testing, and therefore much data exists for comparison in humans. While this model does not mimic perfectly the condition of human arthritis, it is easily reproducible, well defined and has proven useful for the development of new therapies for arthritis, as exemplified also by cytokine blockade therapies (Bendele et al., 1999). AA has been used in the evaluation of nonsteroidal inflammatory drugs, such as phenylbutazone and aspirin during the early 1960s, and later COX-2 inhibitors such as celecoxib were studied. AA in rats shares many features with human arthritis, including genetic linkage, synovial CD4<sup>+</sup> cells and T cell dependence. On the contrary, one of the major differences between the AA model and human arthritis is simply that the inciting agent is known in the model, though the need for any specific antigen is controversial.

### 2.1 Markers applied in adjuvant arthritis

The parameters characterizing efficacy of the substances tested on the immunological, oxidative and inflammatory processes will be reported in this chapter. The spectrum of these parameters is suitable for the description of AA development and provides optimal possibilities for studying pharmacological influence of the substances tested as well as for the elucidation of the mechanisms of their action. In our laboratory, plasma and blood samples were collected regularly from animals in light anesthesia. Tissue samples were collected at the end of the experiment after sacrificing the anesthetised animals. The end of the experiment was mostly day 28 after injection of *Mycobacterium butyricum*. All characteristics monitored could be divided into three groups: clinical, biochemical and immunological parameters. For data interpretation and statistical analysis the data were expressed in terms of arithmetic mean  $\pm$  S.E.M. In all cases the untreated arthritis group was compared with healthy control animals (\*-symbol), the treated arthritis groups were compared with the untreated arthritis animals (+-symbol). For significance calculations the unpaired Student's t-test (two samples 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 ( $p > 0.05$ ).

#### 2.1.1 Clinical markers

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. The 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). In one of our previous experiments, we confirmed that this clinical parameter became significantly modified starting around day 14 and its significant increase in comparison with healthy controls is maintained until the end of the experiment (Bauerova et al., 2007).

#### 2.1.2 Biochemical markers

Numerous studies have suggested an important role of oxidative stress (OS) in the pathogenesis of RA (Bauerova & Bezek, 1999; Bohanec et al., 2009). Several clinical studies as well as preclinical studies using animal models of RA have documented an imbalance in the body's redox homeostasis to a more pro-oxidative environment, suggesting that therapies

which restore the redox balance may have beneficial effects on the disease process. The role of reactive oxygen species (ROS) in the etiology of RA is supported by numerous studies documenting that not only the damaging effects of ROS but also the role that ROS play in regulating various inflammatory processes contributes to the pathogenesis of the disease (Kunsch et al., 2005). The net effect of redox signaling is highly specific changes in gene expression and in the cellular phenotype. Therefore, by serving as second messengers, ROS/reactive nitrogen species are not only toxic agents but also mediators of physiological function (Giustarini et al., 2004; Poli et al., 2004). Considering these facts, we monitored parameters of OS together with the inflammatory marker plasma C-reactive protein (CRP). For the determination of rat plasma CRP concentration, the ELISA kit from Immunology Consultant Laboratories, Inc. (ICL) was used. CRP comparable to HPV was already significantly increased in arthritic rats on day 14 (Bauerova et al., 2010).

$\gamma$ -Glutamyltransferase (GGT), as a non-specific marker of inflammation and OS, has been assessed in different cells and tissues of the lymphatic system – T-lymphocytes, macrophages, spleen and thymus tissue (Koner et al., 1997). The ectoenzyme form of GGT is not present on non-active peripheral T-lymphocytes, but its expression rises after activation of native T-lymphocytes. In other tissues, GGT is essential for "scavenging" glutathione metabolites (mostly  $\gamma$ -glutamyl) and their transport into cells, where GSH is synthesized *de novo* (Carlisle et al., 2003). GGT is an important component of inflammatory processes since its activity is closely connected with the overall antioxidant status of the organism. In our experiments, GGT in the joint from the hind paw (cartilage and soft tissue without bone) and in spleen tissue was determined at the end of the experiment, on day 28. The activity of GGT was measured by the method of (Orlowski & Meister, 1970), modified by (Ondrejickova et al., 1993). We found that the activity of GGT was approximately 3–6 times higher in AA animals than in healthy controls in the spleen and 1.4–2.3 times higher in the joint (Bauerova et al., 2006, 2008b, 2009; Sotnikova et al., 2009). We assume that the increased activity of GGT in AA is a result of elevated systemic OS. The finding that the GGT activity is also elevated in peripheral joint tissue is in good agreement with clinical studies of patients with RA who had increased levels of GGT not only in the serum and urine but also in synovial fluid (Rambabu et al., 1990). In one of our studies we showed a good correlation between GGT activity in joint tissue and the HPV of arthritic animals (Bauerova et al., 2006). GGT expression has been detected in active lymphocytes that accumulate at the inflammation region, as observed in RA. Ishizuka et al. (2007) found that neutralizing antibodies against GGT had a therapeutic effect on joint destruction in collagen-induced arthritis in mice. Elevated expression and activity of GGT in joint tissue is a good marker for synovial inflammation and bone resorption. Substances able to reduce the activity and/or expression of GGT could be important for RA therapy. OS, a consequence of chronic inflammatory processes occurring in AA, increased after the experimental day 14, which was also the onset of clinical manifestations of the disease. OS increased the consumption of endogenous antioxidants in plasma and thus caused a lowering of the plasma antioxidant capacity, measured as the total antioxidant status with RANDOX® TAS kit (Bauerova et al., 2009, Mihalova et al., 2007). A frequently used marker of lipid peroxidation is malondialdehyde (MDA), assessed as an adduct with thiobarbituric acid (TBA). We used the substances reacting with thiobarbituric acid measured in plasma at 535nm (Brown & Kelly, 1996). Clinical studies have shown increased plasmatic levels of MDA in patients with RA (Baskol et al., 2005, 2006; Sarban et al., 2005). The level of MDA in the plasma of arthritic animals was also elevated (Bauerova et al., 2008b, 2009; He et al., 2006; Sotnikova et al., 2009;

Strosova et al., 2008, 2009; Tastekin et al., 2007). In recent years, methods based on chemiluminescence and fluorescence measurements have been widely used for determination of ROS, RNS and lipid peroxidation metabolites. Fluorescent protein adducts are derivatives formed by reaction of secondary metabolites of lipid peroxidation (especially HNE and MDA) with free amino groups of proteins (Aldini et al., 2007; Requena et al., 1996). In murine and human serum, albumin protein fraction was identified as the most fluorescent fraction of proteins (Tsuchida et al., 1985). Several authors have suggested that protein adducts with secondary metabolites of lipid peroxidation also have immunogenic properties and may play a significant role in the pathogenesis of auto-immune diseases including RA (Kurien et al., 2006; Tuma, 2002). This highlights the importance of monitoring these adducts in auto-immune, chronic inflammatory diseases. The presence of oxidative damage in AA plasma was evaluated in our experiments also by measurement of fluorescence adducts in plasma. Two types of aldehydes were measured: HNE and MDA adducts of proteins (Biasi et al., 1995; Tsuchida et al., 1985). In rat AA, we were the first to monitor the HNE and MDA protein adducts in a time profile (Ponist et al., 2010). The level of HNE adducts was slightly increased already on day 7. The maximal level of HNE adducts was reached on day 14, and then it slowly decreased to the control level (day 28). Similar changes were recorded in the level of MDA adducts during the experiment, except that they were still significantly elevated on day 21 (Ponist et al., 2010). The time course of lipid peroxidation measured by the MDA protein adducts resembles the temporal pattern of ROS production by phorbol myristate acetate (PMA) - stimulated neutrophils measured by chemiluminescence of whole blood of arthritic animals, with maximum on day 14 and 21 (Nosal et al., 2007). Recent evidence from animal models of RA emphasizes the importance of neutrophils in the initiation and progression of AA (Cross et al., 2006). There are multiple experimental studies dedicated to neutrophil-generated chemiluminescence of whole blood (Arnhold et al., 1994; Cedergren et al., 2007; Miesel et al., 1996) and of synovial fluid (Arnhold et al., 1994; Cedergren et al., 2007), depending on the disease severity in patients with RA. An increase in whole blood chemiluminescence (2-8 fold) was shown in RA patients compared with healthy volunteers (Miesel et al., 1996). The results published by Nosal (Nosal et al., 2007) are in good agreement with this finding. Arthritic animals had significantly elevated spontaneous chemiluminescence from the seventh experimental day until the end of experiment (day 28). Neutrophils in whole blood of AA animals reacted excessively to stimulation with PMA and produced 6-9 times more ROS. The development of AA in rats was also accompanied with an increase in blood neutrophil count when compared with control animals (Nosal et al., 2007). Oxidative damage to the tissues in AA was demonstrated - ROS levels in the joint and the spleen were analysed by chemiluminescence assessment (Drabikova et al., 2009). Measurements in the joint were completed by spectrophotometric analysis of myeloperoxidase activity in an experiment focused on evaluation of therapeutic effects of two stilbenoids in AA (Macickova et al., 2010). In this study, myeloperoxidase (MPO) activity in the hind paw joint homogenate of arthritic rats was approx. 3-times higher than in healthy controls. This finding is of importance as MPO is the most abundant enzyme in neutrophils. It is a marker of OS and ROS generated by MPO can deplete the NO level in vascular endothelium (Brennan & Hazen, 2003). MPO enhances the binding of leukocytes, including monocytes and neutrophils, to the endothelium (Johansson et al., 1997). Vascular endothelial cells are also capable of secreting various cytokines, which are potent chemoattractants for neutrophils. Both MPO and cytokines participate in the recruitment of cells into the area of

inflammation. Lefkowitz et al. (1999) reported that MPO may be an important mediator in the inflammatory response.

Moreover, in the scale of systemic OS parameters, phagocytosis, oxidative burst and metabolic activity of rat granulocytes isolated from peripheral blood were monitored. Flow cytometric analysis was used for these measurements, according to the method published by Kronek et al. (2010) and modified for the model of AA (Bauerova et al., 2010a). Interestingly, increased production of ROS by neutrophils recorded by whole blood chemiluminescence measurements emerged already in an early phase of disease, on the day 7. We therefore 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. Due to arthritis, both phagocytosis and oxidative burst were already significantly increased on experimental day 7. Metabolic activity of neutrophils as the percentage of double positive cells (simultaneously phagocytotic and positive for oxidative burst) was decreased. This finding could be explained by an increased number of “arthritic” neutrophils, which are positive only for oxidative burst and therefore are not counted as double positive cells (Bauerova et al., 2010a). Further we analyzed in plasma the level of one of the most important endogenous antioxidants in rats – coenzyme Q<sub>9</sub> (CoQ<sub>9</sub>). 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 (Bauerova et al., 2008a; Littarru et al., 1991). 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). In AA the arthritis process increases significantly the level of CoQ<sub>9</sub> in comparison with healthy controls. Evidently, the arthritic processes stimulate the synthesis of CoQ<sub>9</sub> and its transport to plasma. In addition to monitoring lipid peroxidation, also protein oxidation was followed up in AA. Arthritis, similarly to many other diseases, is accompanied by oxidative damage of plasma proteins induced by the action of free radicals. Protein carbonyls (aldehydes and ketones) are produced directly by oxidation or *via* reactions with other molecules generated by the oxidation process. The assay of protein carbonyls as biomarkers of OS in various diseases is with advantage used in diagnostics because of the relatively early formation and relative stability of carbonylated proteins (Dalle-Donne et al., 2003). The ability of certain compounds to reduce the amount of carbonyls is considered as one of the indirect proofs of their antioxidant activity. In our AA experiments, enzyme linked immunosorbent assay (ELISA) was used most frequently for quantitative determination of protein carbonyls in plasma (Buss et al., 1997). The first measurement of protein carbonyls in our experiments with AA was performed in a study with carboxymethyl (1/3)- $\beta$ -D-glucan isolated from *Saccharomyces cerevisiae* administered to arthritic rats (Kogan et al., 2005). In this study, the content of carbonyls in the arthritic animals increased significantly in comparison with healthy controls and protein carbonyl determination in plasma was performed according to the method described by (Levine et al., 1990) and modified in agreement with the previously applied experimental conditions (Bauerova et al., 2002). Also in the following experiments with AA we found significant damage of proteins caused by oxidative stress accompanying arthritis (Bauerova et al., 2005b; Strosova et al., 2009). In addition to determination of protein carbonyls in plasma, we performed an assay of carbonyls in brain tissue and applied it as a marker of antioxidative properties of carnosine evaluated for monotherapy of AA (Ponist et al., 2011). Protein carbonyls in brain tissue homogenates were significantly elevated,

comparable to protein carbonyls in plasma. This novel finding emphasizes the systemic role of OS in chronic inflammatory diseases such as AA, with oxidatively modified proteins, not only in directly affected tissues (cartilage, bone and skeletal muscle).

### 2.1.3 Immunological markers

RA 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 RA. 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). It has a severe impact on different cell populations and exerts biological effects, e.g. increased synthesis of acute phase reactants. 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). 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; Bauerova et al., 2010a). The course of plasma levels of both pro-inflammatory cytokines IL-1 $\alpha$  and TNF- $\alpha$  in AA was very similar, with the maximum on day 14 and with decreasing levels on days 21 and 28 in comparison to day 14 (Bauerova et al., 2009). These results are of importance as TNF- $\alpha$  controls the gene expression of various cytokines and chemokines in different cell types engaged in the host immune response to infection and triggers the cascade of cytokines acting in the inflammatory response. The efficient biological activities of TNF- $\alpha$  include direct activation of T- and B-lymphocytes, macrophages, and natural killer cells, release of acute-phase proteins, and endothelial cell activation. The activated monocyte or macrophage represents the primary source for TNF- $\alpha$ , especially after IFN- $\gamma$  priming. TNF- $\alpha$  is a key regulator of other pro-inflammatory cytokines such as IL-1 $\alpha$ , IL-6, and IL-8. Further, we followed the course of monocyte chemoattractant protein 1 (MCP-1) (Bauerova et al., 2009). This chemokine is mainly expressed by macrophages in response to a wide range of cytokines, e.g. TNF- $\alpha$  and IL-1.

In this experiment, the significant maximum of MCP-1 plasma level measured on day 21 and the following decrease is in close association with kinetics of both TNF- $\alpha$  and IL-1 $\alpha$ . According to the target cell specificity, MCP-1 was postulated to play a pathognomonic role in various diseases with monocyte cell infiltration. MCP-1 is a member of the CC chemokine subfamily that modulates monocyte chemotaxis both *in vitro* (Oppenheim et al., 1991) and *in vivo* (Rollins, 1996; Volejnikova et al., 1997). MCP-1 displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils. Expression of MCP-1 has been detected in a number of pathologic conditions associated with monocyte aggregation, including atherosclerosis, arthritis, and glomerulonephritis (Rollins, 1996). The synovial fluid (SF) and serum MCP-1 concentrations are significantly higher in RA patients. This suggests that MCP-1 is mainly produced locally by activated cells, where it may exacerbate and sustain inflammation by attracting proinflammatory leukocytes, predominantly monocytes (Stankovic et al., 2009). Substances that can suppress the production of MCP-1 have shown beneficial effects in animal models of arthritis (Guglielmotti et al., 2002; Inoue et al., 2001). A completely different picture was revealed for IL-4. The level of this anti-inflammatory cytokine was increasing with time with the maximum observed on day 28 in AA animals (Bauerova et al., 2009). IL-4 is a pleiotropic cytokine produced by mature Th2 cells and mastocyte- and/or basophil-derived cells. IL-4 has marked inhibitory effects on the expression and release of monocyte-derived pro-inflammatory cytokines, e.g. IL-1, TNF- $\alpha$ ,

IL-6, IL-8, and MIP-1 $\alpha$ . It was shown to suppress macrophage cytotoxic activity, parasite killing, and macrophage-derived nitric oxide production (Vannier, et al., 1992). In our experiments, detection of plasma IL-1 $\alpha$ , IL-4, TNF- $\alpha$ , and MCP-1 was done by the flowcytometric (Cytomics FC 500, Beckman Coulter Inc. Fullerton, USA) fluorescent bead-based multiplex assay Rat Cytokine Flow Cytomix Multiplex (Bender Med System, GmbH., Austria). Additionally for determination of IL-1 $\alpha$  in plasma an ELISA kit from Bender MedSystems or from R&D Systems Quantikine<sup>®</sup> was used and to assess MCP in plasma by Instant ELISA kit from eBioscience<sup>®</sup>.

### 3. Newer experimental therapies with antioxidants evaluated in AA

Oxygen metabolism has an important role in the pathogenesis of RA. ROS produced in the course of cellular oxidative phosphorylation and by activated phagocytic cells during oxidative bursts exceed the physiological buffering capacity and result in OS. The excessive production of ROS can damage proteins, lipids, nucleic acids, and matrix components. They also serve as important intracellular signaling molecules that amplify the synovial inflammatory-proliferative response. Repetitive cycles of hypoxia and reoxygenation associated with changes in synovial perfusion are postulated to activate hypoxia-inducible factor-1 $\alpha$  and nuclear factor- $\kappa$ B, two key transcription factors that are regulated by changes in cellular oxygenation and cytokine stimulation, and that in turn orchestrate the expression of a spectrum of genes critical to the persistence of synovitis. Understanding of the complex interactions involved in these pathways might allow the development of novel therapeutic strategies for RA (Hitchon & El-Gabalawy, 2004). Free radicals from oxygen metabolism destroy antioxidant systems (Harris, 2003). Researchers such as Heliovaara et al. (1994) have suggested that enzymatic and/or nonenzymatic antioxidant systems are impaired in RA. RA patients are therefore exposed to oxidant stress (Harris, 2003). Consequently, a number of different activities of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase have been reported to be effective in treating RA (Blake et al., 1981; Mazetti et al., 1996; Shah & Vohora, 2002). Some other researchers found that RA patients were more inclined to lipid peroxidation because of the reduced antioxidant defense system (Rowley et al., 1984; Gambhir et al., 1997). The synovial fluids from patients with RA were found to present high levels of antioxidant damage (Miyata et al., 1998; Chapman et al., 1999). According to Babior (2000) reactive oxidants are essential tools for the pathogenesis of RA.

There is widespread availability and interest in the use of antioxidant supplementation by patients with inflammatory arthritis, although proof of efficacy is modest. A traditional Mediterranean diet relatively high in antioxidants improved RA disease and functional status after 3 months compared with a standard 'Western' diet, although clinical improvement was not associated with any significant change in plasma levels of antioxidants (Heliovaara et al., 1994; Skoldstam et al., 2003). In a separate study of patients with RA, supplementation with the antioxidants vitamin A, E, and C increased plasma antioxidant levels with a corresponding decrease in MDA, a key marker of OS; however, a clinical response was not reported (Jaswal et al., 2003). Specific supplementation of oral vitamin E, the major lipid-soluble antioxidant in human plasma, erythrocytes, and tissue, had no effect on RA disease activity or indices of inflammation but did improve pain, suggesting a role in central analgesia mechanisms (Edmonds et al., 1997). Various forms of antioxidant therapy have demonstrated promising results in experimental arthritis models (Bandt et al., 2002; Cuzzocrea et al., 2000; Venkatraman & Chu, 1999).



As resulted also from our previous experiments (Bauerova et al., 2005a, 2005b, 2008a, 2008b, 2009, Drabikova et al., 2009; Drafi et al., 2010; Jancinova et al., 2009; Kogan et al., 2005; Macickova et al., 2010; Ponist et al., 2010; Sotnikova et al., 2009; Strosova et al., 2008, 2009), all performed in the AA model, substances with antioxidant properties have a high potency to be used in therapy of RA. They decreased the progression of AA when administered to rats with AA over the period of 28 days. For our experiments, we chose substances with antioxidative properties and low toxicity. These antioxidative substances were synthetic antioxidants, as pyridoinol derivatives, and natural substances possessing antioxidative properties. We chose compounds and extracts isolated from plants, polysaccharides isolated from yeast and mushrooms and finally also endogenous antioxidants. An overview of some selected results is given below along with new unpublished data to provide complex information about administration of antioxidants in AA.

### 3.1 Natural substances isolated from plants

The world of plants is an unlimited source of compounds with healing effects, including antiinflammatory, antioxidative and immunomodulating properties. We chose some of them for our experiments with AA. Figure 1 compares all these plant ingredients concerning their effect on the basic clinical parameter – change of hind paw volume (HPV), together with selected parameters of OS as plasmatic TBARS and GGT assessed in spleen and joint tissue homogenates. We compared the effect of *Boswellia serrata* extract (Bo), *Arctostaphylos uva-ursi* extract (UV), *Zingiber officinale* extract (Zg) in combination with two previous extracts (Bo-UV-Zg), sesame oil in combination with *Arctostaphylos uva-ursi* extract (Bo-So), arbutin (Ar), curcumin (CU), Pycnogenol® (PYC) and two stilbenoids – pinosylvin (PIN) and pterostilbene (PTE). The compounds and extracts were all given per os in a single dose immediately after induction of AA and were administered daily until the end of the experiment – experimental day 28. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary and Food Administration. AA was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) to male Lewis rats. The injection was performed near the tail base. All experiments included healthy animals (HC), arthritic animals not treated (AA), arthritic animals treated with the compounds/extracts studied. The oral daily doses used were 30 mg/kg b.w for AA-PIN and AA-PTE, 10 mg /kg b.w for A-PYC, 50 mg/kg b.w for AA-UV, AA-Bo, AA-Bo-So, AA-Ar and AA-CU, 50+25+25 mg/kg b.w for the mixture AA-Bo-UV-Zg and 0.1 ml/kg b.w. for sesame oil. For statistical analysis of the obtained data the same procedure was applied in all experimental cases. The data for all parameters are expressed as arithmetic mean  $\pm$  S.E.M. For significance calculations unpaired Student's *t*-test was used with \* $p$ <0.05 (significant), \*\* $p$ <0.01 (very significant), \*\*\* $p$ <0.001 (extremely significant). The arthritis group was compared with healthy control animals (\*-symbol). The treated arthritis groups were compared with untreated arthritis (+-symbol). In each experimental group 8–10 animals were used. In Figure 1 the reduction of HPV and OS parameters is illustrated in relation to untreated arthritic rats (100% represented by dot-and-dash line). The situation for AA is complicated due to the dominant involvement of Th 1-driven autoimmune etiopathology. OS in this animal model occurs as a reaction to autoimmune processes. Under these conditions, control of OS is of secondary importance, although it could enhance immunomodulatory therapy of RA (Bauerova et al., 2011). Figure 1 clearly shows that plant-related treatment is not enough for successful improvement of

HPV (excluding pinosylvin) although some of the compounds and extracts tested (e.g. UV, Ar, CU, Bo-So or Bo-UV-Zg) achieved a biochemical improvement in the body redox state expressed as reduction of plasmatic TBARS and GGT activity assessed in joint and spleen. Moreover, CU was found to be a potent inhibitor of neutrophil functions in experimental arthritis. AA was accompanied by an increased number of neutrophils in blood and by a more pronounced spontaneous as well as PMA stimulated chemiluminescence. Whereas the arthritis-related alterations in neutrophil count and in spontaneous chemiluminescence were not modified by CU, the increased reactivity of neutrophils to PMA was less evident in CU-treated animals. The effects of CU were comparable with those of methotrexate. CU was found to be a potent inhibitor of neutrophil functions (Jancinova et al., 2009). As neutrophils are considered to be cells with the greatest capacity to inflict damage within diseased joints, the observed effects could support the beneficial use of CU in RA treatment. Further the beneficial effect of sesame oil (So) administered alone on markers of OS accompanying AA was demonstrated not only by decrease of plasma TBARS, decrease of GGT activity in the joint and spleen tissues, but also the level of protein carbonyls, TAC in plasma and activity of NAGA in serum and in the kidney were improved, yet not significantly. In HPV the maximal increase was found on day 28 of AA, and at the same time we observed a

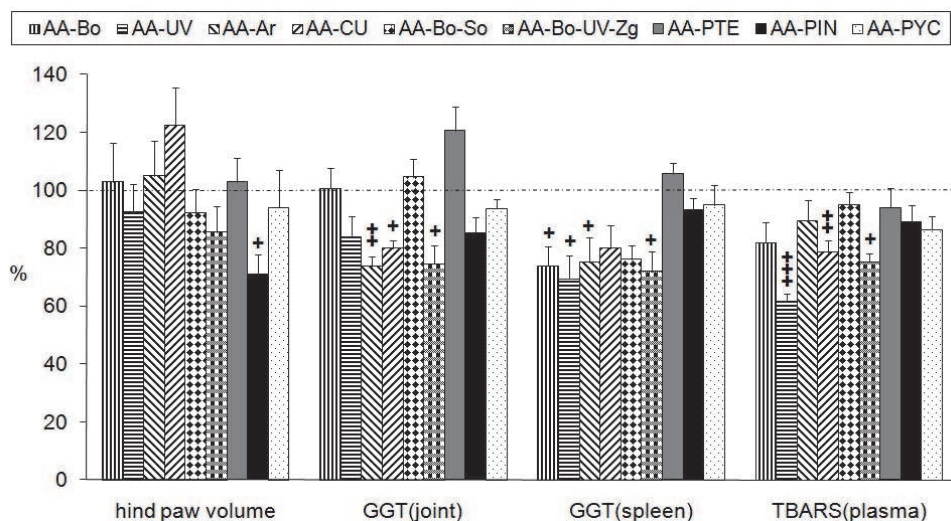


Fig. 1. Comparison of the effect of different plant treatments in adjuvant arthritis (AA) on reduction of hind paw volume (HPV) and on GGT ( $\gamma$ -glutamyltransferase) activity in spleen and joint and level of TBARS (thiobarbituric acid reactive substances) in plasma measured on experimental day 28. Changes in parameters are illustrated in relation to untreated arthritic rats (100% represented by dot-and-dash line). The data were expressed as arithmetic mean  $\pm$  S.E.M. Statistical significance was evaluated applying Student's t-test for independent variables: +P < 0.05, ++P < 0.01, and +++P < 0.001 compared to untreated arthritic animals. *Boswellia serrata* extract (Bo), *Arctostaphylos uva-ursi* extract (UV), *Zingiber officinale* extract (Zg), combination of three previous extracts (Bo-UV-Zg), sesame oil in combination with *Arctostaphylos uva-ursi* extract (Bo-So), arbutin (Ar), curcumin (CU), Pycnogenol® (PYC), pinosylvin (PIN) and pterostilbene (PTE).

significant decrease of aortic endothelium-dependent relaxation. Administration of So resulted in mild, non-significant decrease of hind paw swelling and in significantly increased acetylcholine-evoked relaxation of aorta (Sotnikova et al., 2009).

### 3.1.1 Pinosylvin and methotrexate combination in AA

PIN [3',5'-dihydroxystilbene] and PTE [3,5-dimethoxy-4'-hydroxystilbene] used in our experiments were synthesized and purified by Šmidrkal et al. (2010) and Harmatha et al. (2011). PIN and PTE are natural substances from the stilbenoid group, wide-spread in a variety of plants. They are chemically related to resveratrol. Both substances studied inhibited significantly the chemiluminescence (CL) of whole human blood and the CL of isolated human neutrophils (Perečko et al. 2008). The new information on the inhibitory effect of PIN and PTE on HPV, production of ROS and MPO activity suggests that the protective effect of PIN may be beneficial in controlling inflammation in experimental AA (Macickova et al., 2010). PIN was also the most effective in reducing HPV on day 28, when administered in the dose of 30 mg/kg b.w. per os (Fig. 1). According to these findings we chose PIN as a suitable candidate for combination therapy with methotrexate (MTX). In the combination AA-PIN+MTX, arthritic animals were treated twice a week with MTX in the oral dose of 0.4 mg/kg b.w. and daily with PIN in the oral dose of 50 mg/kg b.w. Monotherapy was performed with the same doses. In addition to the routine statistical analysis the combination treatment was compared to individual MTX treatment (#-symbol). In arthritic rats, PIN potentiated the antiarthritic effect of MTX on days 14 and 21, evaluated as decrease of HPV (Table 1). Activity of GGT in spleen homogenate (Table 2), plasma levels of MCP-1 and CRP (Table 3) were not improved by addition of PIN to MTX, due to the prominent effect of MTX alone on these parameters. Arthritic animals showed an increase in OS, evaluated as plasma levels of TBARS. PIN enhanced the antioxidant effect of MTX (Table 2) (Bauerova et al., 2010b).

| HPV (%)    | Day 14                  | Day 21                 | Day 28              |
|------------|-------------------------|------------------------|---------------------|
| CO         | 8.48 ±0.69              | 12.98 ±0.97            | 17.05 ±0.83         |
| AA         | 33.68 ±5.22<br>***      | 87.24 ±7.50<br>***     | 81.62 ±7.20<br>***  |
| AA-PIN     | 26.30 ±2.61             | 86.02 ±4.56            | 84.03 ±5.51         |
| AA-MTX     | 7.57 ± 0.87<br>+++      | 13.14 ± 2.22<br>+++    | 16.22 ± 2.10<br>+++ |
| AA-PIN-MTX | 4.34 ± 0.79<br>+++ / ## | 7.49 ± 1.14<br>+++ / # | 11.44 ± 1.16<br>+++ |

Table 1. Hind paw volume (HPV) changes in an experiment with methotrexate (MTX) and pinosylvine (PIN) in monotherapy and in combination therapy PIN+MTX measured in time profile. The data were expressed as arithmetic mean ± S.E.M. Statistical significance was evaluated applying Student's t-test for independent variables: \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 compared to control healthy animals (CO); +P < 0.05, ++P < 0.01, and +++P < 0.001 compared to untreated arthritic animals (AA); #P < 0.05, ##P < 0.01, and ###P < 0.001 compared to methotrexate monotherapy (AA-MTX).

| Parameters                           | CO           | AA                | AA-PIN     | AA-MTX            | AA-PIN-MTX          |
|--------------------------------------|--------------|-------------------|------------|-------------------|---------------------|
| <b>TBARS (nmol/ml)</b>               | 10.59±0.23   | 19.10±0.53<br>*** | 17.89±0.47 | 14.33±0.95<br>+++ | 11.78±0.47<br>+++/# |
| <b>Activity of GGT in spleen (†)</b> | 19.977±1.843 | 90.45±4.52<br>*** | 85.90±4.07 | 37.38±2.95<br>+++ | 35.47±1.89<br>+++   |

† - nmol 4-nitroaniline / min / g tissue

Table 2. Plasmatic level of TBARS (thiobarbituric acid reactive substances) and activity of GGT ( $\gamma$ -glutamyltransferase) in spleen measured on experimental day 28 in an experiment with methotrexate (MTX) and pinosylvine (PIN) in monotherapy and in combination therapy PIN+MTX. For statistical analysis of data see table 1.

| Parameters                          | CO          | AA                  | AA-PIN            | AA-MTX            | AA-PIN-MTX        |
|-------------------------------------|-------------|---------------------|-------------------|-------------------|-------------------|
| <b>MCP-1 (<math>\mu</math>g/ml)</b> | 6.896±0.438 | 14.089±1.159<br>*** | 9.470±0.603<br>++ | 8.446±0.616<br>++ | 8.316±0.257<br>++ |
| <b>CRP (<math>\mu</math>g/ml)</b>   | 337.4±14.8  | 722.6±49.3<br>***   | 662.7±19.6        | 385.4±27.4<br>+++ | 366.2±8.8<br>+++  |

Table 3. Plasmatic level of MCP-1 (monocyte chemotactic protein-1) and CRP (C-reactive protein) measured on experimental day 14 in an experiment with methotrexate (MTX) and pinosylvine (PIN) in monotherapy and in combination therapy PIN+MTX. For statistical analysis of data see table 1.

Effect of PIN and MTX, applied separately or in combination, was further studied on spontaneous and stimulated chemiluminescence and neutrophil count in blood of arthritic rats. In rats treated with MTX, all the arthritis-induced changes were significantly reduced and this inhibition became more pronounced when MTX was applied along with PIN. MTX alone decreased neutrophil count, spontaneous and stimulated chemiluminescence by 28%, 41% and 43%, respectively, whereas in combination with PIN, it inhibited these parameters by 59%, 69% and 63%, respectively. Monotherapy with PIN failed to induce any detectable changes either in the number of neutrophils or in oxidant concentration (Jancinova et al., 2010).

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 disease modifying anti-rheumatic drugs (DMARDs) often have side effects at high doses and/or during long-term administration, increased efficacy without increased toxicity is expected for combination therapy of RA. 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 effects of MTX in vivo may be mediated by reducing cell proliferation, increasing the rate of

apoptosis of T cells, increasing endogenous adenosine release, altering the expression of cellular adhesion molecules, influencing production of cytokines, humoral responses and bone formation (Wessels et al., 2008). 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 this case a lowering of the dose could be beneficial and this could be achieved by combination therapy, for which we could recommend PIN, as indicated by the results of our studies.

### 3.2 Natural substances isolated from yeast and mushrooms

The control of inflammation in RA patients by natural and synthetic substances with anti-inflammatory and/or antioxidant and immunomodulatory effects, which are safe also during long-term administration, could become a relevant part of RA therapy. Modulation of OS accompanying RA can offer a new approach and crucially modify treatment of this disease. The key goal of this proposal will be the investigation of the combination of immunosuppressive therapy of MTX with immunomodulators-antioxidants with the aim to achieve enhancement of its efficacy in RA treatment, which would enable dosage reduction in clinical conditions and, consequently, decrease the frequency of occurrence of its dose-dependent side effects. Thus new ways of supplementary or combinatory RA therapy are of great importance. The aim is to find an alternative or additive to classical RA therapy with natural molecules without side effects possessing immunomodulatory, antiinflammatory, and antioxidative properties.

In recent decades, polysaccharides isolated from botanical sources (mushrooms, algae, lichens, and higher plants) have attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (Tzianabos, 2000). Plant and mushroom polysaccharides reveal immunomodulatory effects that depend on polysaccharide structure and molecular weight (low molecular weight – inhibition, high molecular weight – activation) (Schepetkin & Quinn, 2006). Prokopova (Prokopova et al., 1993) were the first to describe a therapeutic effect of simple carbohydrates on AA. We were first to report on the protective antioxidant and antiinflammatory activities of carboxylated (1-3)-beta-D-glucan isolated from *Saccharomyces cerevisiae* in Lewis rats with AA (Kogan et al., 2005). Glucomannans (GM) from *Candida utilis* were evaluated in the same model. The antiarthritic activity for cell-wall GM was associated with antioxidant activity *in vivo* (Bauerova et al., 2006; Mihalova et al., 2007). In the following experiment, the beneficial action of GM was revealed mainly in HPV decrease. Further a decrease of the activity of GGT in the spleen, hind paw joint and muscle tissue homogenates, decrease of the plasmatic activity of N-acetyl-beta-D-glucosaminidase (NAGA), and finally suppression of lysozyme and peroxidase activity assessed in peritoneal macrophages were observed in arthritic animals treated with GM. All these findings speak in favor of the antiinflammatory activity of GM. Moreover, a significant improvement of the arthritis induced suppression of total antioxidant status and decrease of the level of the arthritis-associated protein carbonyls in plasma were detected. In this experiment two doses of GM – 5 and 7.5 mg/kg b.w. were evaluated successfully. Peroral and intraperitoneal ways of administration were also compared (Bauerova et al., 2008b). In the following study, we tested the effect of GM in a higher dose of 15 mg/kg b.w. administered per os. On day 28 after *Mycobacterium butyricum* induced AA, GM was found to reduce HPV. Neutrophil count in whole blood was significantly increased on day 28 after induction of AA, yet GM in the dose of 15 mg/kg b.w. did not change it significantly. The spontaneous and PMA-induced CL was significantly increased in whole blood of rats with AA in comparison with healthy

controls. GM 15 mg/kg b.w. decreased spontaneous as well as PMA-stimulated CL. CL of spleen and joint in rats with AA was significantly increased in comparison with controls ( $3.38 \pm 1.07$  mV/1mg wet weight *vs.*  $1.33 \pm 0.16$  mV/1mg wet weight, and  $6.63 \pm 1.34$  mV/100mg wet weight *vs.*  $1.11 \pm 0.11$  mV/100mg wet weight). GM significantly decreased CL of joints, while CL of the spleen was not affected by GM. The obtained results showed that GM reduced ROS generation in arthritic rats. The predominant decrease of extracellular ROS production suggests a protective effect of GM against tissue damage, especially in the hind paw joint of arthritic rats (Drabikova et al., 2009).

Further we decided to compare the effect of GM to Imunoglukan<sup>®</sup>, a beta-(1,3/1,6)-D-glucan (IMG), which was isolated from *Pleurotus ostreatus*. Figure 2 shows a comparison of the effect of GM and IMG on HPV as well as on OS parameters. GM was tested in two doses: 7.5 (GM1) and 15 mg/kg b.w (GM2). IMG was evaluated also in two doses: 1 (IMG1) and 2 mg/kg b.w. (IMG2). The experimental and statistical design was the same as described in section 3.1. of this chapter. Both GM as IMG were effective in reducing HPV and improving the oxidative status. As no clear dose dependency was shown, we chose for the next experiment higher doses with the aim to detect the more effective immunomodulator for combinatory therapy with MTX. The experiment included healthy intact animals as reference controls (CO), arthritic animals without any drug administration (AA), and arthritic animals with the administration of GM (AA-GM) in the oral daily dose of 15 mg/kg b.w. and of IMG (AA-IMG) in the oral daily dose of 2 mg/kg b.w. Table 4 shows that as to the capability of lowering the HPV no differences between GM and IMG were found. However, as to the antioxidant potential expressed as a more prominent decrease of

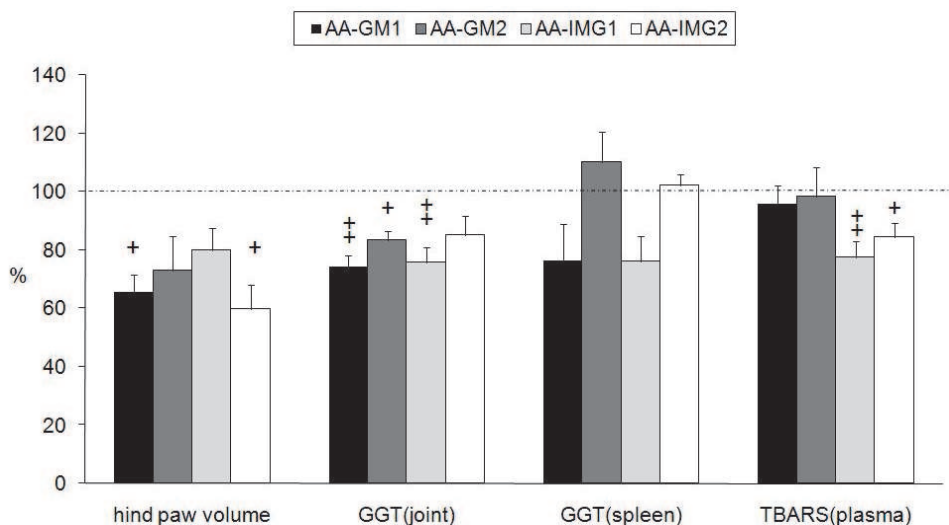


Fig. 2. Comparison of the effect of different glucomannan (GM) and Imunoglukan<sup>®</sup> (IMG) doses used as monotherapy in adjuvant arthritis (AA) on hind paw volume (HPV), GGT ( $\gamma$ -glutamyltransferase) activity in spleen and joint and level of TBARS (thiobarbituric acid reactive substances) in plasma measured on experimental day 28. Changes in parameters are illustrated in relation to untreated arthritic rats (100% represented by dot-and-dash line). For statistical analysis of data see Fig. 1.

TBARS accompanied with a more pronounced increase of TAS measured in plasma on day 28, IMG was more effective than GM.

| Parameters      | CO         | AA                 | AA-GM           | AA-IMG          |
|-----------------|------------|--------------------|-----------------|-----------------|
| HPV (%)         | 17.20±1.81 | 91.45±10.61<br>*** | 66.57±3.84<br>+ | 54.50±7.77<br>+ |
| TAS (mmol/l)    | 1.02±0.21  | 0.52±0.20<br>*     | 0.89±0.18       | 1.16±0.13<br>+  |
| TBARS (nmol/ml) | 3.50±0.20  | 5.90±0.20<br>***   | 5.70±0.30       | 4.70±0.30<br>++ |

Table 4. Changes in hind paw volume (HPV), plasmatic TAS (total antioxidant status) and plasmatic TBARS (thiobarbituric acid reactive substances) in an experiment with glucomannan (GM) and Imunoglukan® (IMG) measured on experimental day 28. For statistical analysis of data see table 1.

The good antioxidative and antiinflammatory effect of IMG stimulated us to study in a more complex way its effect on the course of the main cytokines/chemokines in AA. Daily administration of IMG suppressed significantly the levels of pro-inflammatory cytokines IL-1α (Table 5) and TNF-α (Table 6) on all days monitored. Moreover, the observed

| IL-1α (pg/ml) | Day 14               | Day 21            | Day 28             |
|---------------|----------------------|-------------------|--------------------|
| CO            | 186.44±77.42         | N.M.              | N.M.               |
| AA            | 732.90±128.56<br>*** | 588.58±272.50     | 633.06±117.40<br>* |
| AA-IMG        | 339.80±117.40<br>+   | 168.88±38.98<br>+ | 184.40±18.44<br>+  |

N.M. - not measured

Table 5. Plasma level of IL-1 α (interleukin-1α) in an experiment with Imunoglukan® (IMG) analyzed in time profile. For statistical analysis of data see table 1.

| TNF-α (pg/ml) | Day 14              | Day 21          | Day 28          |
|---------------|---------------------|-----------------|-----------------|
| CO            | 59.64±13.58         | N.M.            | N.M.            |
| AA            | 202.10±26.23<br>*** | 100.15±28.35    | 100.65±42.85    |
| AA-IMG        | 98.58±27.43<br>++   | 49.50±4.30<br>+ | 29.45±2.90<br>+ |

N.M. - not measured

Table 6. Plasma level of TNF-α (tumor necrosis factor-α) in an experiment with Imunoglukan® (IMG) analyzed in time profile. For statistical analysis of data see table 1.

inhibitory effect of IMG became stronger with time. After IMG treatment, the MCP-1 level was also decreasing significantly on days 14 and 21 (Table 7). The time course of the MCP-1 level was found to be comparable for treated and untreated arthritis animals. The level of cytokine IL-4 was increasing with time – the maximum was observed on day 28 in AA animals. IMG exerted probably an indirect time dependent inhibitory effect on this cytokine (Table 8).

| MCP-1 (pg/ml) | Day 14             | Day 21              | Day 28            |
|---------------|--------------------|---------------------|-------------------|
| CO            | 229.44±4.87        | N.M.                | N.M.              |
| AA            | 360.62±51.26<br>** | 559.52±98.22<br>*** | 363.28±89.57<br>* |
| AA-IMG        | 187.42±28.06<br>++ | 363.28±89.57<br>+   | 287.42±108.02     |

N.M. - not measured

Table 7. Plasma level of MCP-1 (monocyte chemotactic protein-1) in an experiment with Imunoglukan® (IMG) analyzed in time profile. For statistical analysis of data see table 1.

| IL-4 (pg/ml) | Day 14      | Day 21           | Day 28            |
|--------------|-------------|------------------|-------------------|
| CO           | 10.50±4.90  | N.M.             | N.M.              |
| AA           | 25.00±10.75 | 31.78±11.26<br>* | 40.43±4.58<br>*** |
| AA-IMG       | 23.05±4.50  | 10.15±4.15<br>+  | 7.70±3.85<br>+++  |

N.M. - not measured

Table 8. Plasma level of IL-4 (interleukin-4) in an experiment with Imunoglukan® (IMG) analyzed in time profile. For statistical analysis of data see table 1.

On the basis of all the obtained results with GM and IMG, we finally chose IMG for combinatory treatment of AA with MTX.

### 3.2.1 Imunoglukan® and methotrexate combination in AA

The study of Rovensky et al (Rovensky et al., 2011) was focused on the effect of IMG on inflammatory and arthritic markers in rats with AA during basal treatment with MTX. The treatment was prophylactic, which means that the animals were treated immediately after administration of the adjuvant, with the same design as used in our previous experiments. The results of our investigation confirmed the already reported effect of MTX treatment in rats with AA (Connolly et al., 1988; Welles et al., 1985). MTX at a dose of 1 mg/kg/week (0.5 mg/kg twice a week) suppressed but did not prevent arthritis development. In our study, MTX significantly suppressed the hind paw swelling and decreased arthrogram scores. IMG alone decreased both the hind paw swelling and the arthrogram on days 21 and 28. The remarkable finding was that IMG potentiated the beneficial effect of MTX; reduction of hind paw swelling and arthrogram scores on days 21 and 28 were more significant compared to



the rats treated with MTX alone. Hetland et al. (1998) showed that  $\beta$ -glucan reduced growth of *Mycobacterium tuberculosis* in macrophage cultures and had a protective effect against *Mycobacterium bovis*, BCG infection in BALB/c mice (Hetland & Sandven, 2002). Certain microbes, fungi and viruses led to the generation and activation of autoimmune T cells resulting in the development of a particular autoimmune disease in genetically susceptible individuals. Thus IMG, an effective activator of the immune system may also be beneficial in humans in preventing or eliminating bacterial infections, which are known to induce reactive arthritis. In our studies, we tested the pure  $\beta$ -glucan - Imunoglukan® isolated from *Pleurotus ostreatus*. This  $\beta$ -glucan decreased arthritis development in rats and had an additional and beneficial effect to that of MTX treatment.

### 3.3 Endogenous antioxidants

Inflammation is one of the leading causes of mortality in the western world. Much evidence suggests a major role for dysregulation of the immune response to toxic stress (Itoh et al., 2003; Lynn & Golenbock, 1992). The intensive production of ROS associated with inflammation generally results in OS (Macdonald et al., 2003). Under conditions of high OS, the abilities of cells to eliminate ROS become exhausted, and dietary sources of antioxidants are required (Novoselova et al., 2009). We studied two important endogenous antioxidants - coenzyme Q<sub>10</sub> (CoQ) and carnosine (CARN) as supplementary therapy in AA with the aim to contribute to the alternatives for dietary complementary healing of RA. In Figure 3, two dose of CoQ - 20 (CoQ1) and 200 mg/kg b.w. (CoQ2) are compared with one dose of CARN - 150 mg/kg b.w. The experimental and statistical design was the same as described in section 3.1.

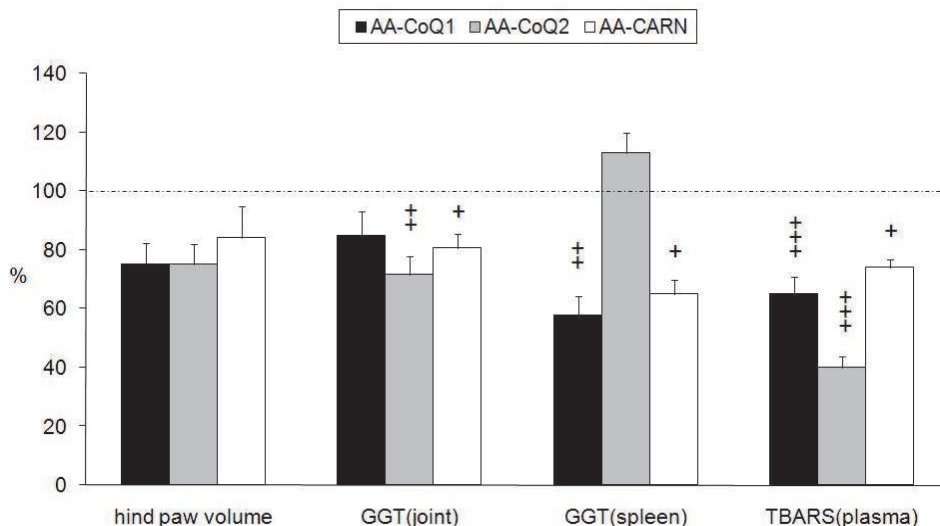


Fig. 3. Comparison of the effect of carnosine (CARN) and two different doses of coenzyme Q<sub>10</sub> (CoQ) used as monotherapy in adjuvant arthritis (AA) on hind paw volume (HPV), GGT ( $\gamma$ -glutamyltransferase) activity in spleen and joint and level of TBARS (thiobarbituric acid reactive substances) in plasma measured on experimental day 28. Changes in parameters are illustrated in relation to untreated arthritic rats (100% represented by dot-and-dash line). For statistical analysis of data see Fig. 1.

Both antioxidants tended to improve HPV (not significantly) and significantly corrected the parameters of OS. No dose dependency was shown with exception of CoQ influence on GGT in spleen. In the next experiments we evaluated CARN in monotherapy of AA and CoQ in the lower dose for combinatory therapy with MTX in AA. The obtained results are described below.

### 3.3.1 Carnosine in monotherapy of AA

Carnosine (CARN) is a dipeptide consisting of  $\beta$ -alanine and L-histidine. It was shown to be a specific constituent of excitable tissues of all vertebrates accumulating in amounts exceeding that of ATP (Boldyrev & Severin, 1990). The antioxidant capacity of this compound is well documented, as well as its pH buffering, osmoregulating, and metal-chelating abilities (Boldyrev, 1990). A potentially useful characteristic of CARN is its ability to act as an anti-glycating agent (Boldyrev, 2002; Boldyrev, 2005; Hipkiss et al., 1998; Hipkiss & Brownson, 2000), to quench superoxide anion and hydroxide radical (Pavlov, et al. 1993; Rubtsov et al., 1991) and to neutralize 4-hydroxy-nonenal (HNE) and other toxic aldehydes (Aldini et al., 2002, 2011, Liu et al., 2003). In order to study the efficiency of carnosine as geroprotector, senescence accelerated mice (SAM), which have increased levels of ROS and deficiency of antioxidant capacity, was used (Boldyrev et al., 2001; Yuneva et al., 2002). CARN decreased the content of protein carbonyls and lipid peroxides in their blood, demonstrating normalization of oxidative metabolism in SAM tissues as a cause of increased life span. Oxygen metabolism has an important role in the pathogenesis of RA. ROS produced in excessive amounts under some pathological states, exceed the physiological ROS buffering capacity and result in OS. Excessive production of ROS can damage proteins, lipids, nucleic acids, and matrix components (Bauerova & Bezek, 1999). With respect to this fact we evaluated CARN in AA. The aim of this study was to assess whether administration of CARN in AA would ameliorate inflammation and disease progression. CARN beneficially affected the clinical parameter HPV in the model of AA measured in time profile (days 14, 21 and 28), significantly on day 14 (Table 9).

| HPV (%) | Day 14            | Day 21            | Day 28            |
|---------|-------------------|-------------------|-------------------|
| CO      | 8.81±1.91         | 11.67±2.54        | 13.09±2.61        |
| AA      | 40.84±4.53<br>*** | 91.32±6.00<br>*** | 88.42±5.44<br>*** |
| AA-CARN | 23.58±4.88<br>+   | 72.76±12.91       | 74.19±9.60        |

Table 9. Hind paw volume (HPV) changes in an experiment with carnosine (CARN) evaluated as monotherapy in time profile. For statistical analysis of data see table 1.

Activity of GGT in joint was significantly reduced by CARN administration (Table 10). Markers of redox imbalance in plasma (TBARS, and protein carbonyls) were significantly decreased (Table 10). Protein carbonyls in brain tissue homogenates were significantly elevated and were decreased by CARN to control values (Table 10). The reduction of immunological markers of inflammation (IL-1 $\alpha$  and MCP-1) in plasma by CARN is a result supporting its anti-inflammatory activity (Table 11).

In the present experiment, the GGT activity was elevated in peripheral joint tissue. This finding is in good agreement with clinical studies of patients with RA who had increased levels of GGT not only in serum and urine but also in synovial fluid (Rambabu et al., 1990). CARN effectively reduced the activity of GGT in joint. Administration of CARN lowered the level of secondary products of lipid peroxidation in plasma measured as TBARS. Cheng (Cheng et al., 2011) showed that CARN, but not other conventional antioxidants, could protect neurons against MDA-induced injury through decomposition of protein cross-linking and may serve as a novel agent in the treatment of neurodegenerative diseases. The “anti-carbonyl” effect of CARN administration was also evidenced by other authors (Aldini et al., 2010).

| Parameters  | CO         | AA                | AA-CARN         |
|---|------------|-------------------|-----------------|
| Activity of GGT in joint (†)                        | 11.03±1.81 | 26.30±2.04<br>*** | 21.22±1.28<br>+ |
| Protein carbonyls in plasma (nmol/mg protein)       | 0.15±0.01  | 0.20±0.01<br>**   | 0.14±0.02<br>+  |
| Protein carbonyls in brain tissue (nmol/mg protein) | 10.68±0.78 | 17.57±0.96<br>*** | 12.98±0.53<br>+ |
| TBARS (nmol/ml)                                     | 1.60±0.12  | 2.38±0.10<br>**   | 1.44±0.12<br>++ |

† - nmol 4-nitroaniline /min /g tissue

Table 10. Activity of GGT( $\gamma$ -glutamyltransferase) in joint, plasmatic TBARS (thiobarbituric acid reactive substances), and protein carbonyls in plasma and brain measured on experimental day 28 in an experiment with carnosine (CARN) evaluated as monotherapy. For statistical analysis of data see table 1.

We found that CARN was effective in decreasing protein carbonyls in plasma as well as in brain tissue homogenate of arthritic rats. These findings might provide, at least partially, an explanation for the antiinflammatory activity of CARN in chronic autoimmune disease, such as RA. The action of CARN resulted in decreased systemic inflammation in AA, monitored by plasmatic level of proinflammatory cytokine IL-1 $\alpha$  and chemokine MCP-1. CARN was also effective in reducing the MCP-1 level in plasma in our experiment, suggesting that it may have a good potential in the treatment of chronic inflammatory diseases including RA where IL-1 and MCP-1 are involved.

| Parameters            | CO                   | AA                        | AA-CARN                   |
|-----------------------|----------------------|---------------------------|---------------------------|
| IL-1 $\alpha$ (pg/ml) | 47.55 $\pm$ 3.63     | 70.5 $\pm$ 5.94<br>*      | 49.87 $\pm$ 4.13<br>+     |
| MCP-1 (pg/ml)         | 5345.70 $\pm$ 734.30 | 7946.50 $\pm$ 878.70<br>* | 5422.00 $\pm$ 658.50<br>+ |

Table 11. IL-1 $\alpha$  (interleukin-1 $\alpha$ ) and MCP-1 (monocyte chemotactic protein-1) measured on experimental day 28 in an experiment with carnosine (CARN) evaluated as monotherapy. For statistical analysis of data see table 1.

### 3.3.2 Coenzyme Q<sub>10</sub> in combination with MTX in AA

Based on our results with mitochondrial energetics modification and the observed anti-inflammatory and antioxidant effects (Bauerova et al., 2005a, 2008a; Gvozdjakova et al., 2004; 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 the development of myopathies. OS-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. In light of these findings, we decided to support the impaired mitochondrial functions by CoQ<sub>10</sub> supplementation and thus to reduce the increased OS in AA. Some evidence from the literature showed that antirheumatic 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; Kucharska et al., 2005). The 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 also expect impairment in myocardial mitochondrial functions. 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). The results with solubilized CoQ<sub>10</sub> (water-soluble form) indicated its therapeutic effect in the experimental model of AA (Bauerova et al., 2005a, 2008a; Gvozdjakova et al., 2004; Ponist et al., 2007). These findings are of potential significance in the treatment of patients with RA.

The aim of the present study was to examine the combined effect of CoQ<sub>10</sub> and MTX on the progression of AA. For this purpose, we used monitoring of HPV along with evaluation of OS and inflammation markers assessed in plasma and tissues. The experiments included healthy animals (CO), arthritic animals not treated (AA), arthritic animals treated with coenzyme Q<sub>10</sub> (AA-CoQ), arthritic animals treated with methotrexate (AA-MTX), and arthritic animals treated with the combination of CoQ<sub>10</sub> and methotrexate (AA-MTX+CoQ). 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 the oral dose of 0.3 mg/kg b.w. twice a week. AA-MTX was performed as a reference treatment. CoQ<sub>10</sub> supplementation to arthritis animals slightly decreased the HPV on all experimental days (Table 12). In the present

study, the decreasing effect of MTX monotherapy on hind paw swelling was evident on all monitored days (Table 12). The significance of this effect was a confirmation of its well known antiarthritic effect, which we proved also previously on the AA model (Jurcovicova et al., 2009; Nosal et al., 2007; Rovensky et al., 2009). As shown in Table 12, 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 CoQ<sub>10</sub>. These promising clinical results were further completed by measurements of HNE- and MDA-protein adducts and protein carbonyls in plasma (Table 13). 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%. We obtained a good agreement of HPV with the parameters of OS: 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 OS 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 (Table 13).

| HPV (%)    | Day 14                 | Day 21              | Day 28              |
|------------|------------------------|---------------------|---------------------|
| CO         | 6.228±0.942            | 13.64±1.891         | 14.874±1.744        |
| AA         | 42.411±7.411<br>***    | 81.083±7.901<br>*** | 68.629±8.952<br>*** |
| AA-CoQ     | 28.094±4.515           | 77.635±3.599        | 59.949±6.039        |
| AA-MTX     | 13.307±2.673<br>++     | 23.793±4.946<br>+++ | 26.996±8.201<br>++  |
| AA-CoQ+MTX | 4.965±1.026<br>+++ / # | 12.552±2.328<br>+++ | 19.571±2.426<br>++  |

Table 12. Hind paw volume (HPV) changes in an experiment with coenzyme Q<sub>10</sub> (CoQ) and methotrexate (MTX) evaluated as monotherapy and combination therapy (CoQ+MTX) in time profile. For statistical analysis of data see table 1.

As shown in Table 14, the arthritis process increases significantly the level of CoQ<sub>9</sub> in comparison with healthy controls. The effect of therapy on this phenomenon unveils a picture comparable to that found for other OS parameters (Table 13). The combination therapy was again the most effective and significant in comparison to the untreated arthritis group and the improvement was on the level of CO (Table 14). Table 14 shows also that the effects of the given treatments on the AA-increased IL-1 $\alpha$  levels are very close to the effects illustrated in table 13. The improving effect on the increased cytokine plasmatic levels is raising in the order CoQ<sub>10</sub>, MTX and CoQ<sub>10</sub>+MTX. Furthermore, a statistically significant difference was found between MTX monotherapy and its combination with CoQ<sub>10</sub>. For the local inflammatory parameter – the activity of GGT in joint homogenate – an approximately double increase was recorded on comparing arthritic animals with CO (Table 14). The treated groups presented similar results as already described for IL-1 $\alpha$ . All these findings suggest that the cycles of GGT and CoQ are not directly coupled.

| Oxidative stress parameters (%) | Protein carbonyls   | HNE-protein adducts    | MDA-protein adducts         |
|---------------------------------|---------------------|------------------------|-----------------------------|
|                                 | AA                  | 189.49±3.19<br>***     | 125.80±8.74<br>***          |
| AA-CoQ                          | 169.74±3.19<br>+    | 114.03±9.41            | 112.92±4.49                 |
| AA-MTX                          | 146.50±4.78<br>+++  | 111.31±7.85<br>+       | 101.49±7.45<br>++           |
| AA-CoQ+MTX                      | 122.93±3.886<br>+++ | 91.66±8.571<br>+++ / # | 85.376±5.129<br>+++ / # # # |

Table 13. Protein carbonyls, HNE (4-hydroxynonenal) and MDA (malondialdehyde)-protein adducts levels in plasma measured on experimental day 28 in an experiment with coenzyme Q<sub>10</sub> (CoQ) and methotrexate (MTX) evaluated as monotherapy and combination therapy (CoQ+MTX). Changes in all groups with arthritis were calculated compared to the control value assessed for healthy control animals on experimental day 28. For statistical analysis of data see table 1.

| Parameters                          | CO             | AA                | AA-CoQ      | AA-MTX           | AA-CoQ +MTX            |
|-------------------------------------|----------------|-------------------|-------------|------------------|------------------------|
| IL-1 $\alpha$ (pg/ml)               | 42.95±7.88     | 87.11±8.52<br>**  | 67.76±13.59 | 38.27±4.21<br>++ | 14.83±0.90<br>++ / # # |
| Activity of GGT in joint (†)        | 9.38±0.76      | 16.08±0.96<br>*** | 16.16±1.07  | 6.83±1.21<br>+++ | 9.60±1.05<br>+++       |
| CoQ <sub>9</sub> in plasma (nmol/l) | 154±7.70<br>** | 241±21.28         | 189±16.51   | 209±21.75        | 158±12.65<br>+         |

† - nmol 4-nitroaniline / min / g tissue

Table 14. GGT ( $\gamma$ -glutamyltransferase) activity in joint, IL-1 $\alpha$  (interleukin-1 $\alpha$ ) and CoQ<sub>9</sub> (coenzyme Q<sub>9</sub>) levels in plasma measured on experimental day 28 in an experiment with coenzyme Q<sub>10</sub> (CoQ) and methotrexate (MTX) evaluated as monotherapy and combination therapy (CoQ+MTX). For statistical analysis of data see table 1.

The functionality of peripheral blood neutrophils in AA was evaluated by phagocytosis, oxidative burst and metabolic activity (Table 15).

Both phagocytosis and oxidative burst were increased due to arthritis. The immunosuppressive effect of MTX was demonstrated in lowering all characteristics of the functionality of peripheral blood neutrophils, not only in comparison with arthritis but also with CO. The addition of CoQ<sub>10</sub> to MTX modulated all processes back to the level of CO. 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 experimental model on rats.

| Parameters             | CO         | AA              | AA-CoQ     | AA-MTX           | AA-CoQ<br>+MTX   |
|------------------------|------------|-----------------|------------|------------------|------------------|
| Phagocytosis (%)       | 41.90±3.56 | 51.28±3.51<br>* | 55.48±4.07 | 30.07±1.44<br>++ | 46.60±7.38<br>## |
| Oxidative burst (%)    | 27.36±3.44 | 43.70±3.97<br>* | 42.95±6.29 | 19.97±0.47<br>++ | 31.07±8.98       |
| Metabolic activity (%) | 40.38±2.04 | 30.56±5.10<br>* | 39.13±3.07 | 21.20±0.61<br>++ | 34.79±7.81<br>## |

Table 15. Functional parameters of neutrophils measured on experimental day 7 in an experiment with coenzyme Q<sub>10</sub> (CoQ) and methotrexate (MTX) evaluated as monotherapy and combination therapy (CoQ+MTX) of AA. For statistical analysis of data see table 1.

In summary, combined administration of CoQ<sub>10</sub> and MTX suppressed arthritic progression in rats more effectively than did MTX alone. This finding may become a beneficial contribution to the treatment of RA. Restoration of redox homeostasis in chronic inflammatory diseases may be of significant importance in new therapeutic strategies.

#### 4. Conclusion

In the past our research team, using the AA model, has monitored OS and inflammation in time course using different clinical and biochemical/immunological markers, and at the same time we have assessed the efficacy of the administered experimental substances with regard to their ability to reduce OS and inflammatory processes. In our experiments on AA rats we observed a beneficial effect of administration of low molecular weight antioxidants (coenzyme Q and carnosine), high molecular weight immunomodulators/antioxidants (glucomannan, Imunoglukan®) and compounds related to plants (curcumin, arbutin, pinosylvin, sesame oil, and extracts from *Boswellia serrata*, *Arctostaphylos uva-ursi* and *Zingiber officinale*).

In light of these results, we proceeded in the search for the most suitable therapeutic substance (an antioxidant/immunomodulator) with the ability to improve the therapy of RA with MTX. The aim was to find a potential enhancement of the antirheumatic effect of MTX in particular combinations compared to monotherapy. Carnosine, coenzyme Q, pinosylvin and Imunoglukan® were selected for assessment of a combinatory therapy with MTX. The already performed experiments on arthritic rats with pinosylvin, Imunoglukan® and coenzyme Q confirmed the hypothesis about the beneficial effect of adding a suitable immunomodulator/antioxidant to the therapy with MTX. Final safety and efficacy of these approaches calls for further more detailed research not only in preclinical but also in clinical conditions.

#### 5. Acknowledgment

The authors wish to thank Dr. Magda Kouřilova, PhD. for correction of the English language. Experimental work was supported by grants VEGA 2/0045/11; VEGA 2/0090/08; VEGA 2/0003/10; APVV-51-017905; APVV-3015-07, APVV-21-055205 and

APVV-0052-10. Study with Coenzyme Q<sub>10</sub> was performed in the frame of CNR/SAS bilateral project 2010-2012: "In vitro and in vivo models of arthritic processes for studying the mechanisms of inflammation and oxidative stress link-up. New perspectives for arthritis". Study with carnosine was performed in the frame of RAMS/SAS bilateral project 2010-2012: "Regulation of cytokine synthesis during inflammation development in brain and other tissues"

Imunoglukan® was donated from Pleuran s.r.o. company (Bratislava, Slovak Republic). Glucomannan was isolated by Martin Pajtinka (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic). Pinosylvin and pterostilbene were prepared by Prof. Jan Šmidrkal (Institute of Chemical Technology, Prague, Czech Republic) and Ing. Juraj Harmatha, PhD (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of Czech Republic). Coenzyme Q<sub>10</sub> in the form of Li-Q-Sorb® was purchased from Tishcon Corp., USA and carnosine was purchased from Hamary Chemicals Ltd., Japan. Pycnogenol® was obtained from VULM, s.r.o., Modra, Slovak Republic. All other used plant-related compounds and extracts were provided by assoc. Prof. Daniela Kostalova (Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic).

## 6. References

- Akiyama, T., Mori, S., Mashiba, T., Miyamoto, K., Komatsubara, S., Cao, Y., Manabe, T., Norimatsu, H., Dobashi, H. & Tokuda, M. (2005) Incadronate disodium inhibits joint destruction and periarticular bone loss only in the early phase of rat adjuvant-induced arthritis. *Journal of Bone and Mineral Metabolism*, Vol. 23, No. 4, (December 2004) pp. 295-301, ISSN 1435-5604
- Alarcon, G. S., Tracy, I. C. & Blackburn, W.D. Jr. (1989) Methotrexate in rheumatoid arthritis. Toxic effects as the major factor in limiting long-term treatment. *Arthritis & Rheumatism*, Vol. 32, No.6, (Jun 1989) pp. 671-676, ISSN 1529-0131
- Aldini, G., Carini, M., Beretta, G., Bradamante, S. & Facino, R.M. (2002) Carnosine as a quencher of 4-hydroxynonenal: through what mechanism of reaction? *Biochemical and Biophysical Research Communications*, Vol. 298, No. 5, (November 2002) pp. 699-706, ISSN 0006-291X
- Aldini, G., Dalle-Donne, I., Facino, R.M., Milzani, A. & Carini, M. (2007) Intervention strategies to inhibit protein carbonylation by lipoxidation derived reactive carbonyls. *Medical Care Research and Review*, Vol. 27, No. 6, (November 2007) pp. 817-868, ISSN 1552-6801
- Aldini, G., Orioli, M., Rossoni, G., Savi, F., Braidotti, P., Vistoli, G., Yeum, K.J., Negrisoli, G. & Carini, M. (2010) The carbonyl scavenger carnosine ameliorates dyslipidemia and renal function in Zucker obese rats. *Journal of Cellular and Molecular Medicine*, Vol. 15, No. 6, (Jun 2010) pp. 1339-54, ISSN 1582-4934
- Arnhold, J., Sonntag, K., Sauer, H., Häntzschel, H. & Arnold, K. (1994) Increased native chemiluminescence in granulocytes isolated from synovial fluid and peripheral blood of patients with rheumatoid arthritis. *Journal of Bioluminescence and Chemiluminescence*, Vol. 9, No. 2, (March - April 1994) pp. 79-86, ISSN 1099-1271
- Babior, B.M. (2000) Phagocytes and oxidative stress. *American Journal of Medicine*, Vol. 109, No. 1, (Jul 2000) pp. 33-44, ISSN 0002-9343
- Bandt, M.D., Grossin, M., Driss, F., Pincemail, J., Babin-Chevaye, C. & Pasquier, C. (2002) Vitamin E uncouples joint destruction and clinical inflammation in a transgenic



- mouse model of rheumatoid arthritis. *Arthritis Rheumatism*, Vol. 46, No. 2, (February 2002) pp. 522-532, ISSN 1529-0131
- Baskol, G., Demir, H., Baskol, M., Kilic, E., Ates, F., Kocer, D. & Muhtaroglu, S. (2005) Assessment of paraoxonase 1 activity and malondialdehyde levels in patients with rheumatoid arthritis. *Clinical Biochemistry*, Vol. 38, No. 10, (October 2005) pp. 951-955, ISSN 0009-9120
- Baskol, G., Demir, H., Baskol, M., Kilic, E., Ates, F., Karakukcu, C. & Ustidal, M. (2006) Investigation of protein oxidation and lipid peroxidation in patients with rheumatoid arthritis. *Cell Biochemistry and Function*, Vol. 24, No. 4, (Jul-August 2006) pp. 307-311, ISSN 1099-0844
- Bauerova, K. & Bezek, S. (1999) Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis. *General Physiology and Biophysics*, Vol. 18, (October 1999) pp. 15-20, ISSN 0231-5882
- Bauerova, K., Neradova, B., Zitnanova, I., Horakova, L. & Stefek, M. (2002). Oxidative protein damage in rats with adjuvant arthritis. Comparison of carbonyl assay performed by ELISA and a spectrophotometric method. In J. Timko, J. Turna, & J. Pastorek (Eds.), Abstracts of the XVIII. Biochemical congress held in Vysoke Tatry, Stara Lesna, Slovakia, 10-13 September 2002, VEDA-Publishing House of Slovak Academy of Sciences, p. 256, Bratislava
- Bauerova, K., Kucharska, J., Mihalova, D., Navarova, J., Gvozdjakova, A. & Sumbalova, Z. (2005a). Effect of coenzyme Q(10) supplementation in the rat model of adjuvant arthritis. *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czech Republic*, Vol. 149, No. 2, (December 2005) pp. 501-503, ISSN 1213-8118
- Bauerova, K., Valentova, J., Ponist, S., Navarova, J., Komendova, D., Mihalova, D. (2005b) Effect of copper complexes on the development of adjuvant arthritis: Therapeutic and toxicological aspects. *Biologia*, Vol. 60, No. 17, (2005) pp. 65-68, ISSN 0006-3088
- Bauerova, K., Ponist, S., Ondrejickova, O., Komendova, D. & Mihalova, D. (2006) Association between tissue gamma-glutamyl-transferase and clinical markers of adjuvant arthritis in Lewis rats. *Neuroendocrinology letters*, Vol. 27, No. 2, (December 2006) pp. 172-175, ISSN 0172-780X
- Bauerova, K., Paulovicova, E., Mihalova, D., Komendova, D. & Ponist, S. (2007) Monitoring of pro-inflammatory cytokines in the development of adjuvant arthritis. *Chemické listy*, Vol. 101, (2007) pp. 166-167, ISSN 1213-7103
- Bauerova, K., Kucharska, J., Ponist, S. & Gvozdjakova, A. (2008a) Coenzyme Q10 supplementation in adjuvant arthritis (pre-clinical study). In: *Mitochondrial medicine: mitochondrial metabolism, diseases, diagnosis and therapy*. Gvozdjakova, A., (eds), pp. 340-342, Springer, ISBN 9781402067143, Netherlands.
- Bauerova, K., Ponist, S., Navarova, J., Dubnickova, M., Paulovicova, E., Pajtinka, M., Kogan, G. & Mihalova, D. (2008b) Glucosaminan in prevention of oxidative stress and inflammation occurring in adjuvant arthritis. *Neuroendocrinology letters*, Vol. 29, No. 5, (October 2008) pp. 691-696, ISSN 0172-780X
- Bauerova, K., Paulovicova, E., Mihalova, D., Svik, K. & Ponist, S. (2009) Study of new ways of supplementary and combinatory therapy of rheumatoid arthritis with immunomodulators. Glucosaminan and Imunoglukan in adjuvant arthritis. *Toxicology and Industrial Health*, Vol. 25, No 4-5, (May-Jun 2009) pp. 329-335, ISSN 1477-0393

- Bauerova, K., Paulovicova, E., Mihalova, D., Drafi, F., Strosova, M., Mascia, C., Biasi, F., Rovensky, J., Kucharska, J., Gvozdjakova, A. & Ponist, S. (2010a) Combined methotrexate and coenzyme Q<sub>10</sub> therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress. *Acta Biochimica Polonica*, Vol. 57, No. 3, (September 2010) pp. 347-354, ISSN 0001-527X
- Bauerova, K., Ponist, S., Drafi, F., Mihalova, D., Paulovicova, E., Jancinova, V. & Nosal, R. (2010b) Study of combination of pinosylvin and methotrexate in the model of adjuvant arthritis: beneficial effect on clinical and non-clinical parameters. *Interdisciplinary Toxicology*, Vol. 3, No. 3, (2010) pp. 32-33, ISSN 1337-9569
- Bauerova, K., Ponist, S., Mihalova, D., Drafi, F. & Kuncirova, V. (2011) Utilization of adjuvant arthritis model for evaluation of new approaches in rheumatoid arthritis therapy focused on regulation of immune processes and oxidative stress. *Interdisciplinary Toxicology*, Vol. 4, No. 1, (March 2011) pp. 33-39, ISSN 1337-9569
- Bendele, A., McComb, J., Gould, T., McAbee, T., Sennello, G., Chlipala, E. & Guy, M. (1999) Animal models of arthritis: relevance to human disease. *Toxicologic Pathology*, Vol. 27, No. 1, (January - February 1999) pp.134-142, ISSN 1533-1601
- Biasi, F., Bosco, M., Lanfranco, G. & Poli G (1995) Cytolysis does not per se induce lipid peroxidation: Evidence in man. *Free Radical Biology and Medicine*, Vol. 18, No. 5, (May 1995) pp. 909-912, ISSN 0891-5849
- Blake, D.R., Hall, N.D., Treby, D.A., Halliwell, B. & Gutteridge, J.M. (1981) Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid patients. *Clinical Science*, Vol. 61, No. 4, (October 1981) pp. 483-486, ISSN 1470-8736
- Bohanec, G.P., Logar, D., Tomsic, M., Rozman, B. & Dolzan, V. (2009) Genetic polymorphisms modifying oxidative stress are associated with disease activity in rheumatoid arthritis patients. *Disease Markers*, Vol. 26, No. 1, (March 2009) pp. 41-48, ISSN 0278-0240
- Boldyrev, A.A. (1990) Retrospectives and perspectives on the biological activity of histidine-containing dipeptides. *International Journal of Biochemistry*, Vol. 22, No. 2, (1990) pp.129-132, ISSN 0020-711X.
- Boldyrev, A. A. & Severin, S.E. (1990) The histidine-containing dipeptides, carnosine, and anserine: distribution, properties, and biological significance. *Advances in Enzyme Regulation*, Vol. 30, (1990) pp. 175-193, ISSN 0065-2571
- Boldyrev, A.A., Yuneva, M.O., Sorokina, E.V., Kramarenko, G.G., Fedorova, T.N., Konovalova, G.G. & Lankin, V.Z. (2001) Antioxidant systems in tissues of senescence accelerated mice (SAM). *Biochemistry Moscow*, Vol. 66, No. 10, (October, 2001) pp.1157-1163, ISSN 1608-3040
- Boldyrev, A. (2002) Carnosine as natural antioxidant and neuroprotector: biological functions and possible clinical use. In: *Free Radicals, Nitric Oxide, and Inflammation: Molecular, Biochemical, and Clinical Aspects*. Tomasi, A., Ozben, T. & Skulachev, V. (Eds), 202-217. IOS Press, ISBN 1-58603-198-8, Amsterdam
- Boldyrev, A. (2005) Protection of Proteins from Oxidative Stress A New Illusion or a Novel Strategy? *Annals of the New York Academy of Sciences*, Vol.1057, (December 2005) pp. 193-205, ISSN 1749-6632
- Brennan, M.L. & Hazen, S.L. (2003) Emerging role of myeloperoxidase and oxidant stress markers in cardiovascular risk assessment. *Current Opinion in Lipidology*, Vol. 14, No. 4, (August 2003) pp. 353-359, ISSN 1473-6535

- Brown, R. K. & Kelly, F.J. (1996) Peroxides and other products, In: *Free Radicals, a Practical Approach*. PUNCHARD, N.A. & Kelly, F.J., (eds) pp. 119-131, Oxford University Press, ISBN 0-19-963559-5, Oxford
- Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M. & Winterbourn, C.C. (1997) Protein carbonyl measurement by a sensitive ELISA method. *Free Radical Biology and Medicine*, Vol. 23, No. 3, (1997) pp. 361-366, ISSN 0891-5849
- Cardoso, S.M., Pereira, C. & Oliveira, C.R. (1999) Mitochondrial function is differently affected upon oxidative stress. *Free Radical Biology and Medicine*, Vol. 26, No. 1-2, (January 1999) pp. 3-13, ISSN 0891-5849
- Carlisle, M.L., King, M.R. & Karp, D.R. (2003) Gamma-glutamyl transpeptidase activity alters the T cell response to oxidative stress and Fas-induced apoptosis. *International Immunology*, Vol. 15, No. 1, (January 2003) pp. 17-27, ISSN 1460-2377
- Cedergren, J., Forslund, T., Sundqvist, T. & Skogh, T. (2007) Intracellular oxidative activation in synovial fluid neutrophils from patients with rheumatoid arthritis but not from other arthritis patients. *The Journal of Rheumatology*, Vol. 34, No. 11, (November 2007) pp. 2162-2170, ISSN 1499-2752
- Chapman, A.P., Antoniow, P., Spitali, M., West, S., Stephens, S. & King, D. J. (1999) Therapeutic antibody fragments with prolonged in vivo half-lives. *Nature Biotechnology*, Vol 17, No. 8, (August 1999) pp. 780-783, ISSN 1087-0156
- Cheng, J., Wang, F., Yu, D.F., Wu, P.F. & Chen, J.G. (2011) The cytotoxic mechanism of malondialdehyde and protective effect of carnosine via protein cross-linking/mitochondrial dysfunction/reactive oxygen species/MAPK pathway in neurons. *European Journal of Pharmacology*, Vol. 650, No. 1, (January 2011) pp. 184-94, ISSN 0014-2999
- Comstock, G.W., Burke, A.E., Hoffman, S.C., Helzlsouer, K.J., Bendich, A., Masi, A.T., Norkus, E.P., Malament, R.L., & Gershwin, M.E. (1997) Serum concentrations of alpha-tocopherol, beta-carotene, and retinol preceding the diagnosis of rheumatoid arthritis and systemic lupus erythematosus. *Annals of Rheumatic Disease*, Vol. 56, No. 5, (May 1997) pp. 323-325, ISSN 1468-2060
- Connolly, K.M., Stecher, V.J., Danis, E., Pruden, D.J. & LaBrie, T. (1988) Alteration of interleukin-1 production and the acute phase response following medication of adjuvant arthritic rats with cyclosporin-A or methotrexate. *International Immunopharmacology*, Vol. 10, No. 6, (1988) pp. 717-728, ISSN 1878-1705
- Cross, A., Barnes, T., Bucknall, R.C., Edwards, S.W. & Moots, R.J. (2006) Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *Journal of Leukocyte Biology*, Vol. 80, No. 3, (September 2006) pp. 521-528, ISSN 0741-5400
- Cuzzocrea, S., McDonald, M.C., Mota-Filipe, H., Mazzon, E., Costantino, G., Britti, D., Mazzullo, G., Caputi, A.P. & Thiemermann, C. (2000) Beneficial effects of tempol, a membrane-permeable radical scavenger, in a rodent model of collagen-induced arthritis. *Arthritis Rheumatism*, Vol. 43, No. 2, (February 2000) pp. 320-328, ISSN 1529-0131
- Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta*, Vol. 329, No. 1-2, (March 2003) pp. 23-38, ISSN 0009-8981

- Dallner, G. & Sindelar, P.J. (2000) Regulation of ubiquinone metabolism. *Free Radical Biology and Medicine*, Vol. 29, No. 3-4, (August 2000) pp. 285–294, ISSN 0891-5849
- De Palma, L., Chillemi, C., Albanelli, S., Rapali, S. & Bertoni-Freddari, C. (2000) Muscle involvement in rheumatoid arthritis: an ultrastructural study. *Ultrastructural Pathology*, Vol. 24, No. 3, (May-Jun 2000) pp. 151–156, ISSN 1521-0758
- Dinarello, C.A. & Moldawer, L.L. (2002) *Pro-inflammatory and anti-inflammatory cytokines in rheumatoid arthritis: a primer for clinicians*. 3rd ed., pp 351, Thousand Oaks, CA: Amgen
- Drabikova, K., Perecko, T., Nosal, R., Bauerova, K., Ponist, S., Mihalova, D., Kogan, G. & Jancinova, V. (2009) Glucosaminan reduces neutrophil free radical production in vitro and in rats with adjuvant arthritis. *Pharmacological Research*, Vol. 59, No. 6, (Jun 2009) pp. 399–403, ISSN 1096-1186
- Drafi, F., Bauerova, K., Valachova, K., Poništ, S., Mihalova, D., Juranek, I., Boldyrev, A., Hrabarova, E. & Soltes, L. (2010). Carnosine inhibits degradation of hyaluronan induced by free radical processes in vitro and improves the redox imbalance in adjuvant arthritis in vivo. *Neuroendocrinology letters*, Vol. 31, No. 2, (2010) pp. 96–100, ISSN 0172-780X
- Edmonds, S.E., Winyard, P.G., Guo, R., Kidd, B., Merry, P., Langrish-Smith, A., Hansen, C., Ramm, S. & Blake, D.R. (1997) Putative analgesic activity of repeated oral doses of vitamin E in the treatment of rheumatoid arthritis. Results of a prospective placebo controlled double blind trial. *Annals of the Rheumatic Diseases*, Vol. 56, No. 11, (November 1997) pp. 649–655, ISSN 1468-2060
- Gambhir, J.K., Lali, P. & Jain, A.K. (1997) Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis. *Clinical Biochemistry*, Vol. 30, No. 4, (Jun 1997) pp. 351–355, ISSN 0009-9120
- Genestier, L., Paillot, R., Quemeneur, L., Izeradjene, K. & Revillard, J.P. (2000). Mechanisms of action of methotrexate. *Immunopharmacology*, Vol. 47, No. 2-3, (May 2000) pp. 247– 257, ISSN 0162-3109
- Giustarini, D., Rossi, R., Milzani, A., Colombo, R. & Dalle-Donne, I. (2004) S-Glutathionylation: from redox regulation of protein functions to human diseases. *Journal of Cellular and Molecular Medicine*, Vol. 8, No. 2, (April-June 2004) pp. 201–212, ISSN 1582-4934
- Guglielmotti, A., D'Onofrio, E., Coletta, I., Aquilini, L., Milanese, C. & Pinza, M. (2002) Amelioration of rat adjuvant arthritis by therapeutic treatment with bindarit, an inhibitor of MCP-1 and TNF-alpha production. *Inflammation Research*, Vol. 51, No. 5, (May 2002) pp. 252–258, ISSN 1420-908X.
- Gvozdzjakova, A., Kucharska, J., Tanaka, S., Neradova, B. & Bauerova, K. (2004) Coenzyme Q 10 supplementation differently modulated heart and skeletal muscle mitochondrial function induced by adjuvant arthritis. *Mitochondrion*, Vol. 4, (2004) pp. 20–21, ISSN 1567-7249
- Gvozdzjaková, A., Kucharska, J., Ponist, S. & Bauerova, K. (2007) Coenzyme Q 10 supplementation in an experimental model of adjuvant arthritis. Kobe, Japan, November 9–12, The fifth conference of the International Coenzyme Q 10 Association, p.180
- Hagfors, L., Leanderson, P., Skoldstam, L., Andersson, J. & Johansson, G. (2003) Antioxidant intake, plasma antioxidants and oxidative stress in a randomized,

- controlled, parallel, Mediterranean dietary intervention study on patients with rheumatoid arthritis. *Nutrition Journal*, Vol. 2, No. 5, (Jul 2003) p. 5, ISSN 1475-2891
- Harmatha, J., Zidek, Z., Kmoníčková, E. & Šmidrkal, J. (2011) Immunobiological properties of selected natural and chemically modified phenylpropanoids. *Interdisc. Toxicol.*, Vol. 4, No. 1, p. 5-10, ISSN 1337-6853
- Harris, E.D. (1993). Etiology and pathogenesis of rheumatoid arthritis. In: *Textbook of rheumatology*, Kelley, W.N., Harris, E.D., Ruddy, S., Sledge, C.B. (Ed.), pp. 833-873, WB Saunders, ISBN 9780721631554, Philadelphia
- He, Y.H., Zhou J., Wang, Y.S., Xiao, C., Tong, Y., Tang, J.C., Chan, A.S., Lu, A.P. (2006) Anti-inflammatory and anti-oxidative effects of cherries on Freund's adjuvant-induced arthritis in rats. *Scandinavian Journal of Rheumatology*, Vol. 35, No. 5, (September-October 2006) pp. 356-358, ISSN 1502-7732
- Heliovaara, M., Knekt, P., Aho, K., Aaran, R.K., Alftan, G. & Aromaa, A. (1994) Serum antioxidant and risk of rheumatoid arthritis. *Annals of the Rheumatic Diseases*, Vol. 53, No. 1, (January 1994) pp. 51-53, ISBN 1468-2060
- Hetland, G., Lovik, M. & Wiker, H.G. (1998) Protective effect of beta-glucan against mycobacterium bovis, BCG infection in BALB/c mice. *Scandinavian Journal of Immunology*, Vol. 47, No. 6, (Jun 1998) pp. 548-553, ISSN 1365-3083.
- Hetland, G. & Sandven, P. (2002) Beta-1,3-glucan reduces growth of Mycobacterium tuberculosis in macrophage cultures. *FEMS Immunology and Medical Microbiology*, Vol. 33, No. 1, (March 2002) pp. 41-45, ISSN 1574-695X.
- Hipkiss, A.R., Worthington, V.C., Himsworth, D.T. & Herwig, W. (1998) Protective effect of carnosine against protein modification mediated by malondialdehyde and hypochlorite. *Biochimica et Biophysica Acta*, Vol. 1380, No. 1, (March 1998) pp. 46-54, ISSN 0005-2736
- Hipkiss, A.R. & Brownson, C. (2000) Carnosine reacts with protein carbonyl groups: another possible role for the anti-ageing peptide? *Biogerontology*, Vol. 1, No. 3, (2000) pp. 217-223, ISSN 1573-6768
- Hitchon, C.A. & El-Gabalawy, H.S. (2004) Oxidation in rheumatoid arthritis. *Arthritis Research and Therapy*, Vol. 6, No. 6, (October 2004) pp. 265-278, ISSN 1478-6362
- Inoue, T., Yamashita, M. & Higaki, M. (2001) The new antirheumatic drug KE-298 suppresses monocyte chemoattractant protein (MCP)-1 and RANTES production in rats with adjuvant-induced arthritis and in IL-1beta-stimulated synoviocytes of patients with rheumatoid arthritis. *Rheumatology International*, Vol 20, No. 4, (May 2001) pp. 149-153, ISSN 1437-160X
- Ishizuka, Y., Moriwaki, S., Kawahara-Hanaoka, M., Uemura, Y., Serizawa, I., Miyauchi, M., Shibata, S., Kanaya, T., Takata, T., Taniguchi, N. & Niida, S. (2007) Treatment with anti-gamma-glutamyl transpeptidase antibody attenuates osteolysis in collagen-induced arthritis mice. *Journal of Bone and Mineral Research*, Vol. 22, No. 12, (December 2007) pp. 1933-1942, ISSN 0884-0431
- Itoh, K., Udagawa, N., Kobayashi, K., Suda, K., Li, X., Takami, M., Okahashi, N., Nishihara, T. & Takahashi, N. (2003) Lipopolysaccharide promotes the survival of osteoclasts via Toll-Like Receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. *Journal of Immunology*, Vol. 170, No. 7, (April 2003) pp. 3688-3695, ISSN 1550-6606

- Jancinova, V., Perecko, T., Nosal, R., Kostalova, D., Bauerova, K. & Drabikova, K. (2009) Decreased activity of neutrophils in the presence of diferuloylmethane (curcumin) involves protein kinase C inhibition. *European Journal of Pharmacology*, Vol. 612, No. 1-3, (Jun 2009) pp. 161-6, ISSN 0014-2999
- Jancinova, V., Nosal, R., Lojek, A., Ciz, M., Ambrozova, G., Mihalova, D., Bauerova, K., Harmatha, J. & Perecko, T. (2010). Formation of reactive oxygen and nitrogen species in the presence of pinosylvin - an analogue of resveratrol. *Neuroendocrinology letters*, Vol. 31, No. 2, (2010) pp. 79-83, ISSN 0172-780X
- Jaswal, S., Mehta, H.C., Sood, A.K. & Kaur, J. (2003) Antioxidant status in rheumatoid arthritis and role of antioxidant therapy. *Clinica Chimica Acta*, Vol. 338, No. 1-2, (December 2003) pp. 123-129, ISSN 0009-898
- Joe, B., Griffiths, M.M., Remmers, E.F. & Wilder, R.L. (1999) Animal models of rheumatoid arthritis and related inflammation. *Current Rheumatology Reports*, Vol. 1, No. 2, (December 1999) pp. 139-148, ISSN 1534-6307
- Johansson, M.W., Patarroyo, M., Oberg, F., Siegbahn, A. & Nilsson, K. (1997) Myeloperoxidase mediates cell adhesion via the alpha M beta 2 integrin (Mac-1, CD11b/CD18). *Journal of Cell Science*, Vol. 110, No. 9, (May 1997) pp. 1133-1139, ISSN 0021-9533
- Jurcovicova, J., Svik, K., Scsukova, S., Bauerova, K., Rovensky, J. & Stancikova, M. (2009) Methotrexate treatment ameliorated testicular suppression and anorexia related leptin reduction in rats with adjuvant arthritis. *Rheumatology International*, Vol. 29, No. 10, (August 2009) pp. 1187-1191, ISSN 1437-160X
- Kalpakioglu, B. & Senel, K. (2008) The interrelation of glutathione reductase, catalase, glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate in the pathogenesis of rheumatoid arthritis. *Clinical Reumatology*, Vol. 27, No. 2, (February 2008) pp.141-145, ISSN 1434-9949
- Knekt, P., Heliovaara, M., Aho, K., Alftan, G., Marniemi, J. & Aromaa, A. (2000) Serum selenium, serum alpha-tocopherol, and the risk of rheumatoid arthritis. *Epidemiology*, Vol. 11, No. 4, (Jul 2000) pp. 402-405, ISSN 1531-5487
- Kogan, G., Staško, A., Bauerova, K., Polovka, M., Šoltés, L., Brezova, V., Navarova, J., Mihalova, D. (2005). Antioxidant properties of yeast (1/3)-β-D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in adjuvant arthritis. *Carbohydrate Polymers*, Vol. 61, (2005) pp.18-28, ISSN 0144-8617.
- Koner, B.C., Banerjee, B.D. & Ray, A. (1997) Effects of stress on gamma glutamyl transpeptidase (GGT) activity in lymphoid system of rats: modulation by drugs. *Indian Journal of Experimental Biology*, Vol. 35, No. 3, (March 1997) pp. 222-224, ISSN 0019-5189
- Kronek, J., Luston, J., Kronekova, Z., Paulovicova, E., Farkas, .P, Petrencikova, N., Paulovicova, L. & Janigov, I. (2010) Synthesis and bioimmunological efficiency of poly(2-oxazolines) containing a free amino group. *Journal of Materials Science: Materials in Medicine*. Vol 21, No. 3, (March 2009) pp. 879-86, ISSN 1573-4838
- Kucharska, J., Gvozdjakova, A., Sumbalova, Z., Mihalova, D. & Bauerova, K. (2005) Can coenzyme Q10 supplementation protect heart and skeletal muscle mitochondrial function and antioxidants dysbalance in adjuvant arthritis? Los Angeles USA, Fourth Conference of the International Coenzyme Q10 Association, 2005, Abstr. p. 125

- Kunsch, C.H., Sikorski, J.A. & Sundell, C.L. (2005) Oxidative stress and the use of antioxidants for the treatment of rheumatoid arthritis. *Current Medicinal Chemistry - Immunology, Endocrine & Metabolic Agents*, Vol. 5, No. 3, (June 2005) pp. 249-258, ISSN 1568-0134
- Kurien, B.T., Hensley, K., Bachmann, M. & Scofield, R.H. (2006) Oxidatively modified autoantigens in autoimmune diseases. *Free Radical Biology and Medicine*, Vol. 41, No. 4, (August 2006) pp. 549-556, ISSN 0891-5849
- Lefkowitz, D.L., Gelderman, M.P., Fuhrmann, S.R., Graham, S., Starnes, J.D. 3rd, Lefkowitz, S.S., Bollen, A. & Moguelevsky, N. (1999). Neutrophilic myeloperoxidase-macrophage interactions perpetuate chronic inflammation associated with experimental arthritis. *Clinical Immunology*, Vol. 91, No. 2, (May 1999) pp. 145-155, ISSN 1521-6616
- Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B.W., Shaltiel, S. & Stadtman, E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, Vol. 186, (1990) pp. 464-478, ISSN 0076-6879
- Littarru, G.P., Lippa, S., Oradei, A., Fiorni, R.M. & Mazzanti, L. (1991) Metabolic and diagnostic implications of blood CoQ10 levels. In: *Biomedical and clinical aspects of coenzyme Q*. Folkers, K., Yamagami, T. & Littarru, G.P.,(eds), pp. 167-178. Elsevier Science, ISBN 0444808019, Amsterdam.
- Liu, Y., Xu, G. & Sayre, L.M. (2003) Carnosine inhibits (E)-4-hydroxy-2-nonenal-induced protein cross-linking: structural characterization of carnosine-HNE adducts. *Chemical Research in Toxicology*, Vol. 16, No. 12, (December 2003) pp. 1589-1597, ISSN 1520-5010
- Luft, R. (1995) The development of mitochondrial medicine. *Biochimica et Biophysica Acta*, Vol. 1271, No. 1, (May 1995) pp. 1-6, ISSN 0005-2736
- Lynn, W.A. & Golenbock, D.T. (1992) Lipopolysaccharide antagonists. *Immunology Today*, Vol. 13, No. 7, (Jul 1992) pp. 271-282, ISSN 0167-5699
- Macdonald, J., Galley, H.F. & Webster, N.R. (2003) Oxidative stress and gene expression in sepsis. *British Journal of Anaesthesia*, Vol. 90, No. 2, (February 2003) pp. 221-232, ISSN 1471-6771.
- Macickova, T., Drabikova, K., Nosal, R., Bauerova, K., Mihalova, D., Harmatha, J. & Pecivova, J. (2010) In vivo effect of pinosylvic acid and pterostilbene in the animal model of adjuvant arthritis. *Neuroendocrinology letters*, Vol. 31, No. 2, (2010) pp. 91-95, ISSN 0172-780X
- Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., Antoni, C., Leeb, B., Elliott, M.J., Woody, J.N., Schaible, T.F. & Feldmann M. (1998) Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis & Rheumatism*, Vol. 41, No. 9, (September 1998) pp. 1552-1563, ISSN 1529-0131
- Maneiro, E., Martin, M.A., deAndres, M.C., Lopez-Armada, M.J., Fernandez-Sneiro, J.L., delHoyo, P., Galdo, F., Arenas, J. & Blanco, J. (2003) Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. *Arthritis Rheumatism*, Vol. 48, No. 3, (March 2003) pp. 700-708, ISSN 1529-0130

- Mazetti, I., Grigolo, B., Borzi, R.M., Meliconi, R. & Facchini, A. (1996) Serum copper/zinc superoxide dismutase levels in patients with rheumatoid arthritis. *International Journal of Clinical and Laboratory Research*, Vol. 26, No. 4, (1996) pp. 245–249, ISSN 1434-4467
- McKendry, R.J. & Dale, P. (1993) Adverse effects of low dose methotrexate therapy in rheumatoid arthritis. *The Journal of Rheumatology*, Vol. 20, No. 11, (November 1993), pp. 1850-1856, ISSN 1499-2752
- Miesel, R., Hartung, R. & Kroeger, H. (1996) Priming of NADPH oxidase by tumor necrosis factor alpha in patients with inflammatory and autoimmune rheumatic diseases. *Inflammation*, Vol. 20, No. 4, (August 1996) pp. 427–438, ISSN 1573-2576
- Miesel, R., Murphy, M.P. & Kroger H (1996) Enhanced mitochondrial radical production in patients which rheumatoid arthritis correlates with elevated levels of tumor necrosis factor alpha in plasma. *Free Radical Research*, Vol. 25, No. 2, (August 1996) pp. 161–169, ISSN 1029-2470
- Mihalova, D., Ponist, S., Kucharska, J., Komendova, D. & Bauerova, K. (2007) Total antioxidant status-systemic marker of oxidative stress in adjuvant arthritis. *Chemická listy*, Vol. 101, No. 14, (2007) pp. 225-226, ISSN 1215-7103
- Miyata, T., Ishiguro, N., Yasuda, Y., Ito, T., Nangaku, M., Iwata, H. & Kurokawa, K. (1998) Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. *Biochemical and Biophysical Research Communications*, Vol. 244, No. 1, (March 1998) pp. 45–49, ISSN 1090-2104
- Moreland, L.W., Heck, L.W. Jr & Koopman, W.J. (1997). Biologic agents for treating rheumatoid arthritis: concepts and progress. *Arthritis Rheumatism*, Vol. 40, No. 3, (March 1997) pp. 397–409, ISSN 1529-0131
- Nosal, R., Jancinova, V., Petrikova, M., Ponist, S. & Bauerova, K. (2007) Suppression of oxidative burst of neutrophils with methotrexate in rat adjuvant arthritis. *Chemická Listy*, Vol. 101, (2007) pp. 243–244, ISSN 1215-7103
- Novoselova, E.G., Lunin, S.M., Novoselova, T.V., Khrenov, M.O., Glushkova, O.V., Avkhacheva, N.V., Safronova, V.G. & Fesenko, E.E. (2009) Naturally occurring antioxidant nutrients reduce inflammatory response in mice. *European Journal of Pharmacology*, Vol. 615, No. 1-3, (August 2009) pp. 234-240, ISSN: 0014-2999
- Ondrejickova, O., Ziegelhoeffer, A., Gabauer, I., Sotnikova, R., Styk, J., Gibala, P., Sedlak, J. & Horakova, L. (1993) Evaluation of ischemia-reperfusion injury by malondialdehyde, glutathione and gamma-glutamyl transpeptidase: lack of specific local effects in diverse parts of the dog heart following acute coronary occlusion. *Cardioscience*, Vol. 4, No. 4, (December 1993) pp. 225–230, ISSN 1015-5007
- Oppenheim, J.J., Zachariae, C.O., Mukaida, N. & Matsushima, K. (1991) Properties of the novel proinflammatory supergene “intercrine” cytokine family. *Annual Review of Immunology*, Vol. 9, (1991) pp. 617–48, ISSN 0732-0582.
- Orlowski, M. & Meister, A. (1970) The  $\gamma$ -glutamyl cycle: a possible transport system for amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 67, No. 3, (November 1970) pp. 1248–1255, ISSN 1091-6490
- Pavlov, A.R., Revina, A.A., Dupin, A.M., Boldyrev, A.A. & Yaropolov, A.I. (1993) The mechanism of interaction of carnosine with superoxide radicals in water solutions.

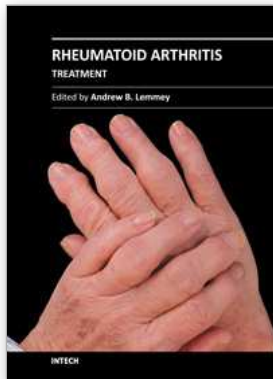


- Biochimica et Biophysica Acta*, Vol. 1157, No. 3, (Jun 1993) pp. 304– 312, ISSN 0006-3002
- Perecko, T., Jancinova, V., Drabikova, K., Nosal, R. & Harmatha, J. (2008). Structure efficiency relationship in derivatives of stilbene. Comparison of resveratrol, pinosilvin and pterostilbene. *Neuroendocrinology letters*, Vol. 29, No. 5, (October 2008) pp. 802–805, ISSN 0172-780X
- Poli, G., Leonarduzzi, G., Biasi, F. & Chiarotto, E. (2004) Oxidative stress and cell signalling. *Current Medicinal Chemistry*, Vol. 11, No. 9, (May 2004) pp. 1163–1182, ISSN 0929-8673
- Ponist, S., Kucharska, J., Gvozdjakova, A., Komendova, D., Mihalova, D. & Bauerova, K. (2007) Mitochondrial bioenergetics of skeletal muscle studied in adjuvant arthritis. *Chemické Listy*, Vol. 101, (2007) pp. 256–257, ISSN 1213-7103
- Ponist, S., Mihalova, D., Jancinova, V., Snirc, V., Ondrejickova, O., Mascia, C., Poli, G., Stancikova, M., Nosal, R. & Bauerová, K. (2010) Reduction of oxidative stress in adjuvant arthritis. Comparison of efficacy of two pyridoindoles: stobadine dipalmitate and SME1.2HCl. *Acta Biochimica Polonica*, Vol. 57, No. 2, (Jun 2010) pp. 223-228, ISSN 0001-527X
- Ponist, S., Drafi, F., Kuncirova, V., Mihalova, D., Ondrejickova, O., Trunova, O., Fedorova, T., Boldyrev, A., Bauerova, K. (2011) Anti-inflammatory effect of carnosine on rat adjuvant arthritis. International Congress on Carnosine in Exercise and Disease, July 10-12, 2011 Ghent (Belgium), Programme and abstract book p. 41
- Prokopova, A., Kery, V., Stancikova, M., Grimova J., Capek, P., Sandula, J. & Orvisky E. (1993) Methyl- $\alpha$ -D-mannopyranoside, mannoooligosaccharides and yeast mannans inhibit development of rat adjuvant arthritis. *Journal of Rheumatology*, Vol. 20, No. 4, (April 1993) pp. 673–677, ISSN 1499-2752
- Rambabu, K., Ansari, A.A., Shaafe, I.A., Chelvam A.P., Ziu, M.M. (1990) Gamma-glutamyl transpeptidase in synovial fluid, serum, and urine of patients with rheumatoid arthritis. *Biochemical Medicine and Metabolic Biology*, Vol. 43, No. 3, (Jun 1990) pp. 183–192, ISSN 0885-4505
- Requena, J.R., Fu, M.X., Ahmed, M.U., Jenkins, A.J., Lyons, T.J. & Thorpe, S.R. (1996) Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrology Dialysis Transplantation*, Vol. 11, No. 5, (1996) pp. 48–53, ISSN 1460-2385
- Rollins, B.J. (1996) Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. *Molecular Medicine Today*, Vol. 12, No. 5, (May 1996) pp. 198–204, ISSN 1357-4310
- Rovensky, J., Stancikova, M., Svik, K., Uteseny, J., Bauerova, K. & Jurcovicova, J. (2009) Treatment of adjuvant-induced arthritis with the combination of methotrexate and probiotic bacteria *Escherichia coli* O83 (Colinfant). *Folia Microbiologica*, Vol. 54, No. 4, (2009) pp. 359–363, ISSN 1874-9356
- Rovensky, J., Stancikova, M., Svik, K., Bauerova, K. & Jurcovicova, J. (2011) The effects of  $\beta$ -glucan isolated from *Pleurotus ostreatus* on methotrexate treatment in rats with adjuvant arthritis. *Rheumatology International*, Vol. 31, No. 4, (April 2011) pp.507-11, ISSN 1437-160X
- Rowley, D., Gutteridge, M.C., Blake, D., Farr, M. & Halliwell, B. (1984) Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in

- synovial fluid from rheumatoid patients. *Clinical Science*, Vol. 66, No. 6, (Jun 1984) pp. 691-695, ISSN 1470-8736
- Rubtsov, A.M., Schara, M., Sentjurc, M. & Boldyrev, A. (1991) Hydroxyl radical-scavenging activity of carnosine: a spin trapping study. *Acta pharmaceutica Jugoslavica*. Vol. 41, (1991) pp. 401-407, ISSN 0001-6667
- Sarban, A., Kocyigit, A., Yazar, M. & Isikan, U.E. (2005) Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. *Clinical Biochemistry*, Vol. 38, No. 11, (November 2005) pp. 981-986, ISSN 0009-9120
- Schepetkin, I.A. & Quinn, M.T. (2006) Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, Vol. 6, No. 3, (March 2006) pp. 317-333, ISSN 1878-1705
- Shah, Z.A. & Vohora, S.B. (2002) Antioxidant/restorative effects of calcined gold preparations used in indian systems of medicine against global and focal models of ischaemia. *Pharmacology & Toxicology*, Vol. 90, No. 5, (May 2002) pp. 254-259, ISSN 1600-0773
- Skoldstam, L., Hagfors, L. & Johansson, G. (2003) An experimental study of a Mediterranean diet intervention for patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases*, Vol. 62, No. 3, (March 2003) pp. 208-214, ISSN 1468-2060
- Smolen, J.S., Aletaha D., Bijlsma, J.W., Breedveld, F.C., Boumpas, D., Burmester, G., Combe, B., Cutolo, M., de Wit, M., Dougados, M., Emery, P., Gibofsky, A., Gomez-Reino, J.J., Haraoui, B., Kalden, J., Keystone, E.C., Kvien, T.K., McInnes, I., Martin-Mola, E., Montecucco, C., Schoels, M. & van der Heijde, D. (2010) Treating rheumatoid arthritis to target: recommendations of an international task force. *Annals of the Rheumatic Diseases*, Vol. 69, No. 4, (April 2010) pp. 631-637, ISSN 1468-2060
- Sotnikova, R., Ponist S., Navarova, J., Mihalova, D., Tomekova, V., Strosova, M., & Bauerova, K. (2009) Effects of sesame oil in the model of adjuvant arthritis. *Neuroendocrinology letters*, Vol. 30, No. 1, (2009) pp. 22-24, ISSN 0172-780X
- Stankovic, A., Slavic, V., Stamenkovic, B., Kamenov, B., Bojanovic, M. & Mitrovic, D.R. (2009) Serum and synovial fluid concentrations of CCL2 (MCP-1) chemokine in patients suffering rheumatoid arthritis and osteoarthritis reflect disease activity. *Bratislavské Lekárske Listy*, Vol. 110, No. 10, (2009) pp. 641-6, ISSN 1336-0345
- Strosova, M., Tomaskova, I., Ponist, S., Bauerova, K., Karlovska J., Spickett, C.M. & Horakova, L. (2008) Oxidative impairment of plasma and skeletal muscle sarcoplasmic reticulum in rats with adjuvant arthritis - effects of pyridoinole antioxidants. *Neuroendocrinology letters*, Vol. 29, No. 5, (October 2008) pp. 706-711, ISSN 0172-780X
- Strosova, M., Karlovska, J., Spickett, C. M., Orszaghova, Z., Ponist, S., Bauerova, K., Mihalova, D. & Horakova, L. (2009) Modulation of SERCA in the chronic phase of adjuvant arthritis as a possible adaptation mechanism of redox imbalance. *Free Radical Research*, Vol. 43, No. 9, (September 2009) pp. 852-864, ISSN 1029-2470
- Smidrkal, J., Harmatha, J., Budesinsky, M., Vokac, K., Zidek, Z., Kmonickova, E., Merkl, R. & Filip, V. (2010) Modified approach for preparing (E)-stilbens related to resveratrol, and evaluation of their potential immunobiological effects. *Collection of Czechoslovak Chemical Communications*, Vol. 75, No. 2, p. 175-186, ISSN 0010-0765

- Tastekin, N., Aydogdu, N., Dokmeci, D., Usta, U., Birtane, M., Erbas, H. & Ture, M. (2007) Protective effects of L-carnitine and alpha-lipoic acid in rats with adjuvant arthritis. *Pharmacological Research*, Vol. 56, No. 4, (October 2007) pp. 303–310, ISSN 1096-1186
- Tsuchida, M., Miura, T., Mizutani, K. & Aibara, K. (1985) Fluorescent substances in mouse and human sera as a parameter of in vivo lipid peroxidation. *Biochimica et Biophysica Acta*, Vol. 834, No. 2, (April 1985) pp. 196–204, ISSN 0006-3002
- Tuma, D.J. (2002) Role of malondialdehyde-acetaldehyde adducts in liver injury. *Free Radical Biology and Medicine*, Vol. 32, No. 4, (February 2002) pp. 303–308, ISSN 0891-5849
- Tzianabos, A.O. (2000) Polysaccharide immunomodulators as therapeutic agents: structural aspects and biological function. *Clinical Microbiology Reviews*, Vol. 13, No. 4, (October 2000) pp. 523–533, ISSN 1098-6618
- Vannier, E., Miller, M.C. & Dinarello, C.A. (1992) Coordinated anti-inflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up regulates gene expression and synthesis of interleukin 1 receptor antagonist. *Proc Natl Acad Sci United States Am*, Vol. 89, No. 9, (May 1992) pp. 4076–4080, ISSN 1091-6490
- Venkatraman, J.T. & Chu, W.C. (1999) Effects of dietary omega-3 and omega-6 lipids and vitamin E on serum cytokines, lipid mediators and anti-DNA antibodies in a mouse model for rheumatoid arthritis. *Journal of the American College of Nutrition*, Vol. 18, No 6, (December 1999) pp. 602–613, ISSN 1541-1087
- Volejnikova, S., Laskari, M., Marks, S.C. Jr. & Graves, D.T. (1997) Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. *American Journal of Pathology*, Vol. 150, No. 5, (May 1997) pp. 1711–21, ISSN 1525-2191
- Waage, A., Brandtzaeg, P., Espevik, T. & Halstensen, A. (1991) Current understanding of the pathogenesis of gram-negative shock. *Infectious Disease Clinics of North America*, Vol. 5, No. 4, (1991) pp. 781–791, ISSN 0891-5520
- Weinblatt, M.E., Kremer, J.M., Bankhurst, A.D., Bulpitt, K.J., Fleischmann, R.M., Fox, R.I., Jackson, C.G., Lange, M. & Burge, D.J. (1999) A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *The New England Journal of Medicine*, Vol. 340, No. 4, (January 1999) pp. 253–259, ISSN 1533-4406
- Welles, W.L., Silkworth, J., Oronsky, A.L., Kerwar, S.S. & Galivan, J. (1985) Studies on the effect of low dose methotrexate in adjuvant arthritis. *Journal of Rheumatology*, Vol. 12, No. 5, (October 1985) pp. 904–906, ISSN 1499-2752
- Wessels, J.A., Huizinga T.W., Guchelaar H.J. (2008) Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. *Rheumatology (Oxford)*, Vol. 47, No. 3, pp. 249–55, ISSN 1462-0324.
- Williams, H.J., Willkens, R.F., Samuelson, C.O. Jr., Alarcon, G.S., Guttadauria, M., Yarboro, C., Polisson, R.P., Weiner, S.R., Luggen, M.E., Billingsley, L.M., Dahl, S.L., Egger, M.J., Reading, J.C. & Ward, J.R. (1985). Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis: a controlled clinical trial. *Arthritis Rheumatism*, Vol. 28, No. 7, (Jul 1985) pp. 721–730, ISSN 1529-0131

- Williams, R.O., Mauri, C., Mason, L.J., Marinova-Mutafchieva, L., Ross, S.E., Feldmann, M. & Maini, R.N. (1998) Therapeutic actions of cyclosporine and anti-tumor necrosis factor alpha in collagen-induced arthritis and the effect of combination therapy. *Arthritis & Rheumatism*, Vol. 41, No. 10, (October 1998) pp.1806-1812, ISSN 1529-0131
- Yuneva, A.O., Kramarenko, G.G., Vetreshchak, T.V., Gallant, S. & Boldyrev, A.A. (2002) Effect of carnosine on *Drosophila melanogaster* lifespan. *Bulletin of Experimental Biology and Medicine*, Vol. 133, No. 6, (Jun 2002) pp. 559-561, ISSN 1573-8221



## **Rheumatoid Arthritis - Treatment**

Edited by Dr. Andrew Lemmey

ISBN 978-953-307-850-2

Hard cover, 366 pages

**Publisher** InTech

**Published online** 18, January, 2012

**Published in print edition** January, 2012

The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 17 chapters, with contributions from numerous countries (e.g. UK, USA, Canada, Japan, Sweden, Turkey, Bosnia and Herzegovina, Slovakia), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Treatment will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Katarina Bauerova, Silvester Poništ, Radomir Nosaļ, Maria Stančikova and Jozef Rovensky (2012). Modern Pharmacological Approaches to Therapies: Substances Tested in Animal Models of Rheumatoid Arthritis, Rheumatoid Arthritis - Treatment, Dr. Andrew Lemmey (Ed.), ISBN: 978-953-307-850-2, InTech, Available from: <http://www.intechopen.com/books/rheumatoid-arthritis-treatment/modern-pharmacological-approaches-to-therapies-substances-tested-in-animal-models-of-rheumatoid-arth>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.