Chapter

Inflammation in the Pathogenesis of Rheumatoid Arthritis and in Experimental Arthritis: Evaluation of Combinations of Carnosic Acid and Extract of *Rhodiola rosea* L. with Methotrexate

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Abstract

The host immune response generates the pro-inflammatory immune response as a protective measure against invading pathogens, allergens, and/or trauma. However, dysregulated and chronic inflammation may result in secondary damage to tissues and immune pathology to the host. Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease which primarily involves synovial inflammation, joint pain, immobility, and stiffness. Increased infiltration of inflammatory immune cells and fibroblast-like synoviocytes into joints, form pannus and small blood vessels that lead to synovium and cartilage destruction. In this chapter we will focus on the role of inflammatory cytokines (IL-1β, IL-6 and IL-17), chemokine monocyte chemotactic protein-1 and matrix metalloproteinase-9 in the pathogenesis of experimental arthritis in animals and in human RA. Further, we will be discussing about methotrexate's (cornerstone of anti-rheumatic therapy) immune suppressing activity, anti-inflammatory properties of carnosic acid and extract of *Rhodiola rosea* L., and their innovative combination treatments with methotrexate in rat adjuvant arthritis.

Keywords: arthritis, IL-1β, IL-6, IL-17, monocyte chemotactic protein-1, matrix metalloproteinase-9, carnosic acid, *Rhodiola rosea* L

1. Introduction

Inflammation is an inherent defensive mechanism against damage of tissues, infection and is quickly stopped in physiological state of organism. In chronic diseases, the inflammation continues and is able to cause substantial organ and tissue damage. A lot of evidence showed that pathological inflammatory response is closely related with different chronic diseases, particularly autoimmune ones, such as systemic lupus erythematosus, rheumatoid arthritis (RA), inflammatory bowel disease, diabetes, and gout [1–3]. Although the key feature of inflammatory dysregulation in many chronic diseases has been supported by plenty of studies,

the pathogenesis of this dysregulation in the autoimmune diseases is not well understood yet. Knowledge about the signaling and mechanism of regulation of inflammation will bring noticeable clinical benefits for the therapy of autoimmune disease.

In this chapter we will present our preliminary results from new original combination treatments of methotrexate with carnosic acid and with extract of *Rhodiola rosea* L and discuss about the role of IL-1β, IL-6 and IL-17, chemokine monocyte chemotactic protein-1 and matrix metalloproteinase-9 in the pathogenesis of experimental arthritis in animals and in human RA.

2. Cytokines involved in rheumatoid arthritis

To fully understand a complex disease like a RA, animal models are indispensable due to their ability to mimic the conditions and demonstrate the similarity to the human RA. Rodent models are essential for further knowledge of the pathogenic processes of RA in humans and therefore are important in the process of testing new and already existing drugs for their efficiency and safety. There are many animal models used for the research of RA, but each model varies in the similarities to the human RA. The most frequently used animal models are collagen-induced arthritis and adjuvant-induced arthritis models. Less often are used animal models with proteoglycan-induced arthritis and streptococcal cell wall-induced arthritis [4].

The adjuvant-induced arthritis (AIA) model has been used widely for testing novel drugs for inflammatory arthritis and for studies of the disease pathogenesis. After administering an injection with complete adjuvant, it was possible to induce polyarthritis [5]. AA is inducible in susceptible rat strains, for example, Lewis rat strain, by a single subcutaneous injection of heat-killed *Mycobacterium tuberculosis* H37Ra in oil. Following the induction, the inflammation begins in 8–10 days, the symptoms are the most apparent on the 15th or 16th day, and then undergo spontaneous recovery. Autoimmune inflammation of the paws starts with the infiltration of mononuclear cells, mostly lymphocytes, macrophages, and monocytes [6]. The severity of the RA could lead to chronic malformation of affected joints, together with ankylosis. Adjuvant-induced arthritis exhibit similar symptoms to human RA, such as joint swelling, invasion of lymphocytes, and destruction of cartilage [4].

The difference between AIA in rats and human RA seems to be in the rapid onset of the erosive polyarthritis in the AIA model, Rheumatoid Factor is not present, the disease seems to have a monophasic course. There is also an involvement of axial skeleton seen in the model of AIA, affected gastrointestinal, genitourinary tract and skin, periostitis, ankylosis, and extra-articular manifestations not typical of RA [7]. Inflamed joints of rats with AIA contain activated T-cells. T-cells infiltrating joints originate from several compartments, such as the spleen, Peyer's patches, lymph nodes, and T-cell pool that recirculates [8]. Specific antigen heat shock protein (Hsp65) has been shown to activate the immune response, with peptide 180–186 being the responsible epitope [9]. The cytokines that are expressed in the joint during the early stages of inflammation include IL-17, IFN, and TNF-α, as well as cytokines implicated in macrophage stimulation. Increased levels of IL-4, IL-6, monocyte chemotactic protein 1 (MCP-1), and TGF-β can be observed as inflammation progresses in the joint. TNF-α, IL-1β, IL-21, and IL-17 all contribute to the pathology of this disorder [8]. The main source of the irreversible tissue damage is in an area rich in macrophages, called the pannus, which is located at the junction of the synovium lining of the joint capsule together with the cartilage and a bone. Pannus cells migrate over the cartilage and into the subchondral bone, subsequently

causing the erosion of these tissues [10]. The activity of matrix metalloproteinases (MMPs) seems to be the reason for the irreversible destruction of the cartilage seen in RA. MMPs are enzymes produced as a response to proinflammatory cytokines as IL-1 and TNFα by activated macrophages and fibroblasts [11]. MMPs can be further divided into three main groups. Collagenase MMP-1 (interstitial) and MMP-8 (neutrophil), whose major substrates are collagen forms I, II, and III, belong to the first group. The second group consists of the gelatinase/type IV collagenases such as MMP-2, the 72kD gelatinase A, and 92-kD gelatinase B (MMP-9). The main function of these matrix metalloproteinases from the second group is to degrade gelatin and collagen type IV in the basement membrane. Group 3 consists of the stromelysins, stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), and pump-1 (MMP-7). These stromelysins have activity against a range spectrum of substrates, mainly proteoglycans, fibronectin, laminin, and some collagens [11]. During arthritis, especially MMP-1 and MMP-3 play an important role in the pathophysiology of the disease, and what is worse, the destruction of the connective tissue they cause is largely irreversible [12–14]. Fibroblasts from a healthy organism produce very low levels of both enzymes [12–14]. On the other hand, during RA and osteoarthritis levels of these enzymes rapidly increase in response to various stimuli [12–14]. Potent inducers of collagenases and stromelysins could be cytokines such as IL-1α and IL-1β, epidermal growth factor (EGF), platelet-derived growth factor, and tumor necrosis factor α. Inducers of these two enzymes could also be crystals of monosodium urate monohydrate, debris phagocytosis, and formulation of multinucleated giant cells. In an environment of stimulated synovial fibroblast cells, which resembles proliferating rheumatoid synovial tissue, collagenase and stromelysin becomes major gene product of these synovial fibroblasts [14]. Patients with RA and OA also have higher levels of collagenase and stromelysin in cartilage and the synovial fluid, especially patients with RA [15, 16]. The level of enzymatic activity is increased concordantly with the severity of the disease [17]. Apart from MMPs, there are other enzymes synthesized by cells within cartilage and bone as well as infiltrating inflammatory cells. These enzymes include aspartic, serine, and cysteine endopeptidases such as cathepsin B, which are capable of cleaving and therefore destructing the main components of cartilage and bone (such as proteoglycan and collagen type I, II, IX, X, and XI) [18].

2.1 Interleukin-1β

Interleukin-1β (IL-1β) is a cytokine belonging to the same family of cytokines as IL-1α, yet they show different features and are produced by two different genes [19]. IL-1β is mainly produced by macrophages as an inactive precursor (pro-IL-1β) and then cleaved by cysteine protease caspase-I into its mature form (IL-1β) [20]. The major distinction between IL-1β and IL-1α is that pro-IL-1β is biologically inactive, while pro-IL-1α and mature IL-1α can bind to their receptors and therefore stimulate cellular responses. Most IL-1α also stays coupled with the plasma membrane and stimulates cells by direct cell–cell interaction, which can induce its functions [21]. IL-1β is produced by blood monocytes, tissue macrophages, and dendritic cells by direct cellular contact with stimulated T-lymphocytes, a mechanism related to chronic inflammation [22]. IL-1β mRNA requires an extra signal for synthesis so transcription of IL-1β is a rate-limiting step of its synthesis. The extra signal to induce the production of IL-1β can be a microbial product or cytokines as TNF-α, IL-1α, IL-18, or IL-1β itself [23]. By binding to the same receptors as IL-1α and IL-1β, yet not inducing any consequent cellular responses, IL-1 receptor antagonist (IL-I Ra) acts as a naturally occurring inhibitor [24]. IL-1β seems to be not present in healthy individuals, or its levels

are hard to detect by standard assays. Such low levels are needed to be maintained due to the potency of IL-1β to induce inflammatory responses [25]. During RA, serum levels of IL-1β are higher in patients with RA compared to healthy individuals, and the concentrations of IL-1β increase during the acute phase of the disease [26].

2.2 Interleukin-6

IL-6 has been suggested to be a major player in the pathological changes during RA because of the broad spectrum of activities IL-6 participates in. Il-6 is recognized as an endogenous pyrogen [27], and also as an inducer of acute phase response genes [28]. IL-6 stimulates B- and T-cells activity and promotes proliferation of plasmablast into mature immunoglobulin-producing plasma cells [29]. IL-6 acts stimulatory on the immune system's cells, vascular endothelial cells, synovial fibroblasts, and osteoclasts upon coupling with its soluble IL-6 receptor (sIL-6Rα). Activated sIL-6Rα complex stimulates the production of a subset of chemokines by endothelial cells and subsequently upregulates the expression of adhesion molecules, resulting in direct recruitment of leukocytes to the sites of inflammation [30]. Apart from that, by having stimulatory effects on synovial fibroblast and osteoclast activation, IL-6 contributes to the formation of synovial pannus and bone resorption in inflamed joints [31, 32]. Interestingly, patients with various forms of arthritis have high levels of IL-6 in serum and synovial fluids, but on the other hand, their structural cells from joints (chondrocytes, fibroblasts, synoviocytes, and endothelial cells) lack expression of IL-6R [33]. These cells are also not responsive to IL-6 itself. The complex of IL-6 bound to its receptor might, therefore, represents the mechanism behind the action of IL-6 during arthritis. In a synovial fluid of RA patients, it has been shown that an increase in sIL-6Rα correlates with the extent of the joint destruction which coincides with more advanced stages of RA [32].

2.3 Interleukin-17

IL-17 is another cytokine possibly contributing to the pathogenesis of RA. IL-17 is produced by CD4+ CD45RO+ memory T cells in synovium during RA, upon activation with phorbolmyristate acetate/ionomycin or CD3/CD28 Abs [34, 35]. IL-17A is relatively homologous to IL-17F (~50%) with which it can form heterodimers (IL-17A/F). Activated human CD4+ T cells produce IL-17A/F heterodimers along with IL-17A and IL-17F homodimers [36]. The signaling is based on the coupling of IL-1A and IL-1F to a multimeric receptor composed of two subunits IL-17RA and IL-17RC [37]. Cytokines from the IL-17 family activate pro-inflammatory pathways through activating NF-κB or inducing signaling through MAPK and the C/EBP transcription factors. It seems IL-17A signaling intends to activate a gene expression of an innate-type inflammatory effector program that mediates potent inflammation and plays a critical role in a defense of a host [38]. It has been shown that IL-17 can trigger the production of IL-6, IL-8, GM-CSF, and also prostaglandin E2 (PGE2), a strong mediator of inflammation, in human synoviocytes [34, 35, 39]. Additionally, IL-17 showed stimulating effect on granulopoiesis in a murine model [40], on osteoclastogenesis [41], up-regulated synthesis of NO in cultured human cartilage [42], stimulated the synthesis of proinflammatory mediators as TNF-α, IL-1β, IL-10, IL-12, stromelysin, and IL-1Ra in human peripheral blood macrophages [43]. Furthermore, levels of IL-17 in synovial fluid and serum from RA patients are high in contrast to OA patients [44].

2.4 Monocyte chemoattractant protein-1

The rheumatoid synovial environment suggests a possible role for leukocyte chemoattractant molecules such as chemokines. Chemokines form a superfamily consisting of low molecular weight peptides (7–15 kDa) with conserved four-cysteine motif and consist of at least two subfamilies: first are the C-X-C (α) chemokines which all majorly attract neutrophils. Here belong IL-8, melanoma growth stimulating activity, and epithelial neutrophil-activating peptide 78. Secondly, C-C (β) chemokines are RANTES (regulated upon activation normal T cell expressed and secreted), monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α), which chiefly recruit T cells and monocytes [45]. Many of the cells present in RA joints, such as endothelial cells, macrophages, fibroblasts, and lymphocytes can release chemokines. In the pathogenesis of RA, members of both subclasses of chemokines have been implicated. The production of MCP-1 is enhanced in human RA patients compared to osteoarthritis patients [46]. In the murine model of collagen-induced arthritis the earliest detectable levels of MIP-1α, MCP-1, and MIP-2 expression were observed 4 weeks after the initial collagen challenge [47].

2.5 Matrix metalloproteinase 9

Degradation of articular cartilage is important feature of RA and is caused by elevated activity of proteolytic enzymes [48]. In RA, synovial fibroblasts are extensively producing the matrix-degrading enzymes [49] known as matrix metalloproteinases (MMPs). MMPs are a zinc-dependent peptidases, which are degrading the components of extracellular matrix. MMPs are the key proteases associated with the degradation and invasion through anatomical barriers [50]. The MMP-9 (gelatinase B) and MMP-2 (gelatinase A), are very important in the degradation of collagen by cleaving the denatured collagen, produced by collagenases. Moreover, these MMPs degrade other substrates, such as collagen I and II [51] and aggrecan, which is abundant in cartilage [50].

MMP-9 has a posttranscriptional regulation on multiple levels. Its activity is inhibited in tissues by inhibitors of metalloproteinase (TIMP-1 to TIMP-4) with strongest binding between TIMP-1 and MMP-9 [52]. MMPs (including MMP-9) are produced and secreted in latent soluble form of enzyme, which needs activation extra-celullarly. In tissues the mast cell-derived tryptase and chymase are effective activators of MMPs [53, 54]. Regulation of MMPs is situated at the level of their transcription. Expression of MMPs is modulated by different stimuli including also cytokines [55] and growth factors [56].

MMP-9 was first discovered in neutrophils [57]. MMP-9 is also present in other leukocytes including T cells, macrophages, and eosinophils [58]. MMP-9 cleaves IL-8 and increases its activity as a chemoattractant for neutrophil more than 10-fold according to acute and chronic inflammatory processes [59]. The evidence is now growing that along with the storage of serine proteases, mast cells are secreting significant amount of MMPs such as MMP-9 [60, 61]. Although there is limited evidence for the expression of MMP-9 in mast cells in rheumatoid synovium [62], its regulation in RA is poorly understood. MMP-9 expression in rheumatoid synovial mast cells is via its regulation by TNF-α and IFN-γ in cord blood-derived human mast cell and the human mast cell line-1 (HMC-1). MMP-9 is not a product which is permanently stored in mast cells, but this enzyme is secreted under inflammatory conditions. MMP-9 may help in the migration of mast cell progenitors to inflammatory sites and could also promote the local damage of tissues [63]. In RA, MMP-9 is markedly elevated in serum and joint synovial fluid and positively correlates

with disease progression and severity [64]. MMP-9 knockout mice show decreased severity of antibody-induced arthritis [65].

3. Innovative combination treatments of methotrexate with natural compounds in experimental arthritis

Current drugs for rheumatoid arthritis (RA) are: corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and biological response modifiers [66]. However, these antirheumatics have several adverse effects. NSAIDs are dangerous to patients due to the adverse effects such as bleeding of upper gastrointestinal tract, liver, and kidney adverse reactions [67]. Moreover, cognitive disorders, headaches, allergic reactions often force the patients to stop the treatment. This behavior is greatly limiting the use of NSAIDs. The long-term administration of corticosteroids can induce hypersplenism, hypertension, infection, osteoporosis and fractures [68]. DMARDs often cause diarrhea, rashes, vomiting, decreased white blood cell levels, and impaired kidney and liver functions [69]. Biological agents with high target specificity and less side effects are the new agents for therapy of RA [70]. However, these biological agents are expensive and not available for many patients [71]. Thus, development of novel anti-rheumatic drugs and strategies for RA therapy is a high priority. The combination treatments of low-dose methotrexate (MTX) with natural substances, which have the potential to improve the efficacy and to reduce adverse side effects of drugs, could be one possible direction in these strategies for RA therapy. Extract or phytochemical selected for combination therapy with MTX is expected to have anti-inflammatory and antioxidant activity to treat the inflammation and oxidative stress, occurring during RA development. Many chronic diseases with inflammatory pathology are abundant in elderly population. The widely administered anti-inflammatory drugs have many side effects and are expensive (biologic drugs). Alternative option are natural extracts and substances used in traditional medicine. These natural products offer a possibility to identify the bioactive compounds and for the development of new inflammatory drugs. Traditional remedies and phytochemicals are being used for the treatment of inflammatory and other disorders since ancient times [72] and with proper scientific research background can be more extensively used for treatment also in the present.

3.1 Methotrexate

MTX is still for decades a primary antirheumatic drug and the cornerstone of the RA treatment. MTX has an acceptable safety profile, efficacy, and low cost as well as many years of clinical experience make it the gold standard of RA treatment and the key drug for combination with different biological drugs [73]. MTX is usually effective in RA treatment and patients are usually administered for several years with MTX, thus information about long-term safety is very important. However, administration of MTX is in some cases limited because of its toxic adverse effects. During long treatment period by MTX, often adverse reactions occur such as mucous ulceration, cytopenia, nausea, liver damage and serious infections. Some studies showed that due to toxic manifestations, the interruption of MTX treatment in RA patients is in the range from 10–37% [74].

Despite the introduction of numerous biologic agents for the treatment of RA, low-dose MTX therapy remains still the gold standard in the RA therapy. MTX is generally the first-line drug for the treatment of RA, psoriatic arthritis, and it enhances the effect of most biologic agents in RA. Methotrexate inhibits

polyglutamates inhibit aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC), leading to intracellular accumulation of AICAR and increased adenosine release; adenosine binds to cell surface receptors and suppresses many inflammatory and immune reactions [75].

The activity of MTX has also been studied in monocyte cell lines. Different from fibroblast like synoviocytes and T-lymphocytes, monocytes trigger apoptosis as a response to MTX treatment. Moreover, MTX activates a dose-dependent elevation in the expression of inflammatory cytokines, such as TNF, IL-1 and IL-6, in monocytic cell lines [76]. Adenosine (AS) via its receptors regulates monocyte activity, and hence MTX may influence monocytes indirectly by increasing AS release by other immune cells. AS binds to its A_1 receptor on peripheral blood monocytes and activates the formation of giant cells with multiple nuclei [77]. Moreover, the binding of AS to A_{2a} receptors and A_3 receptors on monocytes decreases the production and release of IL-6 and TNF and initiates the transformation of inflammatory M1 phenotype of monocytes to anti-inflammatory M2 phenotype.

Macrophages with M2 phenotype have are responsible for termination of inflammation, clearing the apoptotic cells and support wound healing by secreting profibrotic and angiogenic cytokines. Adenosine, binding on A_{2a} receptors, inhibits the production of inflammatory cytokines and promotes the expression of anti-inflammatory mediators such as vascular endothelial growth factor and IL-10 [78]. A_{2a} receptor stimulation triggers a switching from an M1 (pro-inflammatory phenotype) to a modified macrophage M2 phenotype [79]. One way by which A_{2a} receptor binding affects macrophage function is by stimulating the expression of the NR4A - orphan nuclear receptor, which is inhibiting the activation of NFκB-dependent nuclear gene expression [80]. A_{2b} receptor also induces the switching from a M1 macrophage phenotype to a M2 phenotype [81]. Cultivating synovial fibroblasts and T cells from RA patients triggered T cell TNF-α, IL-17, and IFNγ expression, which resulted in increased fibroblast IL-6, IL8 and IL-15 expression [82]. Methotrexate inhibited the upregulation of IL-6, IL8 and IL-15 by stimulated RA synovial fibroblasts. MTX also decreased IFNγ and IL-17 expression in T cells co-cultured with RA synovial fibroblasts (**Table 1**).

3.2 Combination of methotrexate and carnosic acid

In our previous study, we have selected the carnosic acid for combination with methotrexate for its anti-inflammatory and antioxidative properties, to reduce the development of rat adjuvant arthritis.

3.2.1 Carnosic acid

Carnosic acid (CA) was discovered first by Linde in *Salvia officinalis* L. [83]. Carnosic acid ($C_{20}H_{28}O_4$, **Figure 1**), is a phenolic diterpene that belongs to the terpene class of secondary metabolites [84], is localized in rosemary leaves, more precisely in chloroplasts of trichome cells. CA and carnosol have been reported to display beneficial effects against acute and chronic inflammation, cardiovascular diseases, obesity, and cancer [85, 86], inhibition of prostaglandin synthesis [87], skin inflammation [88], inhibition of NF-κB [89], inhibition of 5-lipoxygenase [90] and antioxidant activity *in vivo* [91].

CA prevented cartilage degeneration though induction of hemeoxygenase-1 (HO-1) in cell culture with human chondrocytes. The results showed that CA increased enzyme levels in a dose-dependent manner. Moreover, it was able to restore HO-1 levels under IL-1β treatment, which specifically inhibits the antioxidant effects of this enzyme. CA induced HO-1 and miR-140 expression in human

Cell type	Methotrexate action
Monocyte	Inhibition of IL-1β, IL-6, and TNF-α production; downregulation of receptors FcγRI and IIa; increases ROS synthesis and apoptosis
Macrophage	Inhibition of IL-1β, IL-6, and TNF-α production;
Th-1 lymphocyte	Decreases IL-2, IFN-γ and IL-17 gene expression; increases ROS synthesis and apoptosis
Th-2 lymphocyte	Increases IL-4 and IL-10 gene expression
Neutrophil	Increases ROS synthesis
Synovial fibroblast	Inhibition of IL-15, IL-6, and IL-8 expression; inhibitory effect on prostaglandin E2 production; inhibition of COX-2 and MMP expression

ROS, reactive oxygen species; COX-2, cyclooxygenase 2; MMP, synovial matrix metalloproteinase.

Table 1.
Immune regulatory action of low dose MTX in the RA synovial tissue (according to Miranda-Carús et al. [82]).

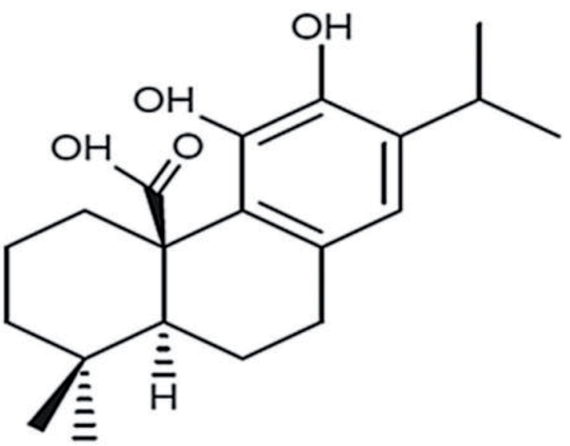

Figure 1.
Chemical structure of carnosic acid.

articular chondrocytes, thus cartilage degeneration was attenuated by CA treatment [92]. The activation of macrophages triggered by exogenous infection or endogenous stress stimuli is thought to be implicated in the pathogenesis of various inflammatory diseases. In a study of Wang et al. [93], authors applied an integrated approach based on unbiased proteomics and bioinformatics analysis to elucidate the anti-inflammatory property of CA. CA significantly inhibited the increase of NO and TNF-α, downregulated cyclooxygenase-2 (COX-2) protein expression and decreased the transcriptional level of inflammatory genes including NOS-2, TNF-α, COX-2, in LPS-stimulated RAW264.7 macrophages. The liquid chromatography-based assessment showed CA negatively regulated 217 proteins elicited by lipopolysaccharide (LPS), which are responsible for multiple inflammatory pathways including nuclear factor (NF)-κB, MAPK and FoxO signaling. A following analysis showed that CA effectively inhibited ERK/JNK/p38 MAPKs, IKKβ/IκB-α/NF-κB and FoxO1/3 signaling. These results illustrate the ability of CA to regulate the inflammatory signaling triggered by LPS [93].

In another study by de Oliveira [94] authors have found that activation of cell antioxidant defense is mediated via transcription factor nuclear factor erythroid 2-related factor (Nrf2). Therefore, authors investigated whether CA is able to block paraquat (PQ)-induced inflammatory alterations in SH-SY5Y neuroblastoma cells. CA reduced the PQ-induced changes on the levels of TNF-α, IL-1β, and COX-2 via

signaling responsible for the activation of the Nrf2/HO-1 pathway. Furthermore, they observed a crosstalk between the Nrf2/HO-1 signaling pathway and the activation of the nuclear factor-κB [94]. Two Rosemary extracts and their main components - CA and carnosol affected the cell migration. Monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) were determined by Western blot and gelatin zymography, respectively, in RAW 264.7 macrophages and vascular smooth muscle cells (VSMCs). MMP-9 and MCP-1 levels were significantly diminished with methanol extract (RM), n-hexane fraction (RH), and CA in RAW 264.7 macrophages. RM, RH, CA, and carnosol suppressed TNF-α induced VSMC migration by inhibiting MMP-9 expression. Rosemary, especially its CA component, has potential anti-atherosclerotic effects related to cell migration [95].

Liu and colleagues [96] studied the anti-inflammatory activity of CA on destruction of osteoclasts, fibroblast-like synoviocytes in the collagen-induced arthritis model. Abovementioned *in vitro* and *in vivo* experiments showed that CA inhibited the expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-17, IL-8 and MMP-3, and suppressed the secretion of RANKL. Moreover, authors determined that CA reduced osteoclastogenesis and resorption of the bone *in vitro* and had therapeutic protective activity against joint damage *in vivo*. Further results showed that CA inhibited RANKL-induced activations of MAPKs (JNK and p38) and NF-κB resulting in the suppressing of NFATc1 [96].

3.2.2 Effect of the combination therapy of methotrexate and carnosic acid in rat adjuvant arthritis

In this section we will present our preliminary results from combination therapy of methotrexate (MTX) and carnosic acid in rat adjuvant arthritis.

Hind paw volume (HPV) was significantly increased on days 14, 21 and 28 during the development of AA. CA in monotherapy was without a significant effect on this parameter. The administration of methotrexate in sub-therapeutic dose markedly reduced HPV on days 14 and 21, but not on day 28. The combination of MTX and CA was more effective in decreasing the HPV on days 14, 21 and 28 than MTX in monotherapy. The most effective reduction of HPV was on day 21 (**Table 2**).

MCP-1 is responsible for recruiting monocytes on the sites of inflammation, and it is involved in the pathogenesis of human [46] and also in experimental arthritis [47]. AA caused a significant increase in the levels of MCP-1 on days 14, 21 and 28. Neither CA nor MTX administered in monotherapy were able to significantly reduce the elevated MCP-1 levels on days 14, 21 and 28. On day 21, only the combination of MTX and CA significantly decreased the level of MCP-1 in plasma of AA animals (**Table 3**).

3.3 Combination of methotrexate and ethanol extract of *Rhodiola rosea*

Rhodiola rosea L. is known as an adaptogen and has been confirmed to possess protective effects against inflammatory diseases, including cardiovascular diseases, neurodegenerative diseases, diabetes, sepsis, and cancer [97]. Less is known about the anti-inflammatory activity of Rhodiola extract in the experimental arthritis, thus we decided to select this extract for our study in monotherapy and in combination with methotrexate.

*3.3.1 **Rhodiola rosea** L.*

In this section we will focus on the anti-inflammatory effect of *Rhodiola rosea* L. (RhR). RhR has been found to possess anti-inflammatory properties in diseases

Changes in hind paw volume (%)	Day 7	Day 14	Day 21	Day 28
CO	4.66 ± 1.83	8.14 ± 3.23	9.79 ± 2.27	12.35 ± 1.95
AA	6.82 ± 2.13	35.90 ± 5.40*	71.79 ± 5.45**	54.81 ± 5.56***
AA-CA	4.73 ± 1.56	43.59 ± 9.70	72.63 ± 4.80	55.79 ± 5.11
AA-MTX	8.26 ± 1.85	11.63 ± 2.58^{+}	30.47 ± 7.85^{+++}	34.40 ± 9.74
AA-CA-MTX	3.84 ± 1.30	7.41 ± 1.53^{++}	8.43 ± 0.81$^{+++/\#}$	12.33 ± 1.90^{+++}

CO: healthy control animals, AA: untreated arthritic animals, AA-CA: arthritic animals treated with carnosic acid, AA-MTX: arthritic animals treated with methotrexate, AA-CA-MTX: arthritic animals treated combination of methotrexate and carnosic acid.
Values are expressed as average ± standard error of mean, statistical significance was calculated using ANOVA-Tukey–Kramer post hoc test.
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$ *vs. CO.*
$^{+}p < 0.05$.
$^{++}p < 0.01$.
$^{+++}p < 0.001$ *vs. AA.*
$^{\#}p < 0.05$ *vs AA-MTX.*

Table 2.
Effect of carnosic acid, methotrexate and their combination on hind paw swelling.

MCP-1 (pg/mL)	Day 14	Day 21	Day 28
CO	306.43 ± 7.91	337.27 ± 17.06	137.36 ± 20.61
AA	395.68 ± 19.20**	516.31 ± 22.00***	183.96 ± 12.48*
AA-CA	431.30 ± 21.14	510.00 ± 21.92	174.75 ± 18.45
AA-MTX	410.44 ± 9.75	491.74 ± 20.25	181.87 ± 25.07
AA-CA-MTX	411.82 ± 17.71	429.94 ± 13.38^{+}	165.21 ± 13.95

CO: healthy control animals, AA: untreated arthritic animals, AA-CA: arthritic animals treated with carnosic acid, AA-MTX: arthritic animals treated with methotrexate, AA-CA-MTX: arthritic animals treated combination of methotrexate and carnosic acid.
Values are expressed as average ± standard error of mean, statistical significance was calculated using ANOVA-Tukey–Kramer post hoc test.
$^{*}p < 0.05$.
$^{**}p < 0.01$.
$^{***}p < 0.001$ *vs. CO.*
$^{+}p < 0.05$ *vs AA.*

Table 3.
Effect of carnosic acid, methotrexate and their combination on levels of monocyte chemotactic protein-1 in blood plasma.

such as sepsis, endotoxemia, asthma *in vivo* and *in vitro*. Pu et al. [97] have found that seven compounds (Ferulic acid, Kaempferol, Salidroside, Tyrosol, Catechin, Gallic acid and Caffeic acid phenethyl ester) isolated from RhR showed protective activity against LPS-induced sepsis in mice via decreasing TNF-α, nitric oxide and lactate dehydrogenase [97]. By many scientists, salidroside (SAL) was reported to possess protective ability in many disease models through particularly regulating different inflammatory mediators.

SAL decreased the inflammatory injury via reducing inflammatory cytokines (IL-1β, TNFα, IL-6), small molecules (mainly nitric oxide), chemokines (monocyte chemo-attractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α) and COX-2 in animal models, such as LPS induced endotoxemia in mice [98], LPS induced murine acute lung injury [99], ovalbumin induced asthma in

mice [100], and ethanol triggered acute gastric ulceration [101]. Further *in vitro* experiment confirmed the protective effects of SAL in neuro-inflammation. In murine microglial BV2 cells treated by LPS, Lee et al. showed that the main compounds of RhR (salidroside and rosarin) reduced the production of nitric oxide and inflammatory cytokines such as IL-6, IL-1β, and TNF-α via the NF-κB and MAPK signaling pathways [102]. Another *in vitro* study showed that SAL may inhibit the synthesis of inflammatory mediators. Authors found that in mice macrophages (J774.1 and RAW264.7) activated by LPS, SAL pre-treatment can reduce the levels of IL-1β, TNFα, IL-6, NO and MCP-1 via NF-κB pathway [103]. Further experiment showed that the mechanism might also be associated with down regulation of STAT3 and JAK2, and with translocation of STAT3 in nucleus [99]. STAT3 belongs to STAT (Signal Transducers and Activators of Transcription) family and has a key role in inflammatory processes. Many cytokines bind to GP130, which is a IL-6-type cytokines receptor, and activate Janus kinases (JAKs), what leads to the phosphorylation of STAT3. The phosphorylated STAT3 is translocated into the nucleus and regulates the expression of different target genes including also pro-inflammatory mediators [104].

Osteoarthritis (OA) is the most common disease, which seriously affects the daily life of the elderly. Currently, no drug therapy has been shown to explicitly block the progression of OA. The study by Gao et al. [105] showed that salidroside could significantly promote the proliferation of chondrocytes in OA rats induced by an anterior cruciate ligament transection and renew the OA-induced changes of cartilage. Salidroside increased the levels of aggrecan and collagen II and reduced the MMP-13 level. Moreover, salidroside reduced Th-17 cells and the levels of IKBα and p65, and IL-17, while elevated the count of CD4 + IL-10+ cells and IL-10. The reduction of IL-17 levels further diminished the dissociation of IKBα to p65, what resulted in the reduction of the release of VCAM-1 and TNF-α. Salidroside decreases the cartilage degradation via promoting proliferation of chondrocytes, reducing collagen fibrosis, and regulating the inflammatory processes and immune responses through NF-κB pathway in anterior cruciate ligament transection-induced OA in rats [105]. Another study involving chondrocytes by Wu et al. [106] showed that salidroside suppressed IL-1β-induced apoptosis in chondrocytes. Salidroside stimulated proliferation of chondrocytes, reduced IL-1ß-triggered inflammation and apoptosis, and scavenged NO and reactive oxygen species generated by chondrocytes. Salidroside upregulated the level of B-cell lymphoma 2 protein and downregulated the level of apoptosis regulator Bax. Salidroside also inhibited the production of caspase 3/9 and suppressed the phosphorylation of phosphoinositide-3-kinases (PI3K) and protein kinase B (AKT). These results indicate that salidroside prevents osteoarthritis by its anti-inflammatory, anti-apoptotic and pro-proliferating activities by suppressing the PI3K/AKT pathway [106].

3.3.2 Effect of the combination therapy of methotrexate and extract of Rhodiola rosea *in rat adjuvant arthritis*

Hind paw volume (HPV) was significantly increased on days 14 and day 21 during the development of AA. Administration of *Rhodiola rosea* ethanol extract (RS) in monotherapy markedly decreased HPV on day 14, but it had no effect on HPV on day 21. MTX and the combination of MTX with RS administered in monotherapy significantly decreased the HPV on days 14 and 21 (**Table 4**).

AA caused significant increase in the levels of IL-6 on days 14 and 21. Administration of MTX in monotherapy significantly decreased the plasmatic level of IL-6 only on day 14. Administration of RS in monotherapy had no effect on

Changes in hind paw volume (%)	Day 7	Day 14	Day 21
CO	0.55 ± 1.05	7.14 ± 1.33	11.99 ± 1.01
AA	3.16 ± 1.63	21.34 ± 3.70***	55.38 ± 2.76***
AA-MTX	3.95 ± 0.91	5.40 ± 0.86^{+++}	14.79 ± 2.66^{+++}
AA-RS	3.79 ± 1.88	8.35 ± 2.12^{++}	48.62 ± 5.34
AA-RS-MTX	6.13 ± 1.66	7.77 ± 2.49^{++}	12.10 ± 4.24^{+++}

CO: healthy control animals, AA: untreated arthritic animals, AA-RS: arthritic animals treated with extract of Rhodiola rosea, AA-MTX: arthritic animals treated with methotrexate, AA-RS-MTX: arthritic animals treated combination of methotrexate and extract of Rhodiola rosea.
Values are expressed as average ± standard error of mean, statistical significance was calculated using ANOVA-Tukey–Kramer post hoc test
$^{***}p < 0.001$ *vs. CO.*
$^{++}p < 0.01$.
$^{+++}p < 0.001$ *vs. AA.*

Table 4.
Effect of Rhodiola rosea *ethanol extract, methotrexate and their combination on hind paw swelling.*

IL-6 (pg/mL)	Day 14	Day 21
CO	62,67 ± 4,30	51,50 ± 4,77
AA	141,45 ± 14,66*	88,33 ± 5,74*
AA-MTX	82,10 ± 18,95^{+}	70,19 ± 7,12
AA-RS	148,92 ± 10,44	77,99 ± 5,44
AA-RS-MTX	70,05 ± 6,84^{+}	43,13 ± 3,05^{+}

CO: healthy control animals, AA: untreated arthritic animals, AA-RS: arthritic animals treated with extract of Rhodiola rosea, AA-MTX: arthritic animals treated with methotrexate, AA-RS-MTX: arthritic animals treated combination of methotrexate and extract of Rhodiola rosea.
Values are expressed as average ± standard error of mean, statistical significance was calculated using ANOVA-Tukey–Kramer post hoc test.
$^{*}p < 0.05$ *vs. CO.*
$^{+}p < 0.05$, *vs. AA.*

Table 5.
Effect of Rhodiola rosea *ethanol extract, methotrexate and their combination on levels of IL-6 in blood plasma.*

levels of IL-6. However, the combination treatment of MTX and RS significantly decreased the levels of IL-6 on both measured days (**Table 5**).

4. Conclusions

Animal models of rheumatoid arthritis (RA) are used widely in research on pathogenesis of inflammatory arthritis and in the testing of potential anti-arthritic agents. In this chapter we highlighted the importance of inflammatory mediators IL-1β, IL-6, IL-17, MCP-1 and MMP-9 in experimental arthritis and RA. We have demonstrated, that MTX is a therapeutic standard for human arthritis as well as for adjuvant arthritis in rats, which make this model suitable for studying the pharmacotherapy of RA. Our preliminary results with combination treatments of MTX with carnosic acid and *Rhodiola rosea* ethanol extract showed, that these combinations are more effective in reducing hind paw volume, and the levels of MCP-1 and IL-6 than MTX in monotherapy. Thus, natural compounds with anti-inflammatory activities could be also a perspective candidate for combination treatments with MTX to treat human autoimmune diseases.

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Conflict of interest

Authors have no conflict of interests.

Notes/thanks/other declarations

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