Chapter

Impact of Oxidative Stress on Inflammation in Rheumatoid and Adjuvant Arthritis: Damage to Lipids, Proteins, and Enzymatic Antioxidant Defense in Plasma and Different Tissues

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Abstract

Animal models of rheumatoid arthritis (RA) are widely used for testing potential new therapies for RA. The most commonly used models of human RA are adjuvant-induced arthritis (AIA) and collagen-induced arthritis in rats and mice. In this chapter, we will focus on inflammatory and oxidative stress (OS) processes during the development of AIA. OS is a result of increased production of reactive oxygen species (ROS) or a reduction in the body's endogenous antioxidant defense system. ROS and reactive nitrogen species (RNS) can contribute to the pathogenesis of RA by the induction of membrane oxidation, irreversible damage to proteins and DNA, cartilage damage, and induction of bone resorption. ROS/RNS can also modulate a variety of signaling events that control gene expression and affect cellular processes that participate in chronic inflammation. Our research team has been studying the course of OS during the development of rat AIA for more than a decade. We have analyzed the course of OS using markers of lipid peroxidation (malondialdehyde, 4-hydroxy-2-nonenal, and F-2 isoprostanes), protein carbonyls, antioxidant enzymes (heme oxygenase and gamma-glutamyl transferase), and levels of endogenous antioxidants (coenzyme Q_{10} and Q_9, gamma-tocopherol) in plasma and different tissues (joint, liver, lung, skeletal muscle, and spleen).

Keywords: animal models, arthritis, redox signaling, cachexia, antioxidants

1. Introduction

Research on animal models is necessary to better understand the etiopathology of rheumatoid arthritis (RA) and has enabled successful new strategies for innovative drug research. Recently the discovery of novel biomarkers of presymptomatic and emerging stages of human RA focused the attention on interventions that underlie different disease variants. This development in the field underlying RA pathogenesis has also led to the increased need of new animal models. Integration of

the knowledge on human and animal models will allow to create a comprehensive "pathogenesis map" to the subset of disease they mimic [1].

Rheumatoid arthritis occurs due to the continuous deterioration of cells and tissues that ultimately affects major organs. Both oxidative stress (OS) and inflammation are considered major role players in the pathogenesis of RA [2]. Even if there is a lot of evidence from animal models of RA and human RA, about that OS plays an important role in tissue damage and also promotes cardiovascular diseases in patients with RA [3]; until now, a therapeutic strategy to reduce OS in RA has not yet been established. Thus, understanding how the OS is influencing the development of animal and human RA is of great importance.

In this chapter, we will discuss the importance of OS in the pathogenesis of human RA and its experimental model, rat adjuvant arthritis (AIA).

2. Pathogenesis of rheumatoid arthritis

RA is a chronic, progressive, inflammatory autoimmune disease associated with articular, extra-articular, and systemic effects. It has been reported that RA affects multiple comorbidities [4]. Mortality rates are more than twice as high in patients with RA as in the general population (Wolfe et al. [5]). Although the exact cause of RA remains unknown [5, 6], several findings suggest a genetic basis for disease development. More than 80% of patients carry the epitope of the HLA-DRB1*04 cluster [7], and patients expressing two HLA-DRB1*04 alleles are at elevated risk for major organ involvement and surgery related to joint destruction [8]. Environmental factors, such as smoking and infection, may also influence the development, rate of progression, and severity of RA [9, 10]. In addition to joint symptoms, many patients experience extra-articular or systemic manifestations or both. Extra-articular manifestations include rheumatoid nodules, vasculitis, pericarditis, uveitis, and rheumatoid lung [11]. Systemic manifestations include often anemia, cardiovascular disease, osteoporosis, fatigue, and depression [12, 13]. The earliest event in RA pathogenesis is the activation of the innate immune response that includes the activation of dendritic cells by exogenous material and autologous antigens. Antigen-presenting cells, including dendritic cells, macrophages, and activated B cells, present arthritis-associated antigens to T cells. T-cell activation and B-cell activation result in increased production of cytokines and chemokines. In addition to antigen presentation, macrophages are involved in osteoclastogenesis and are a major source of cytokines, including TNF-α, IL-1, and IL-6 [6, 7]. Within the synovial membrane, there is a great increase in activated fibroblast-like synoviocytes, which also produce inflammatory cytokines, prostaglandins, and matrix metalloproteinases (MMPs). Synoviocytes contribute to the destruction of cartilage and bone by secreting MMPs into the synovial fluid (SF) and by direct invasion into these tissues [7]. Pro-inflammatory cytokines are involved in the pathogenesis of RA [2, 14]. TNF-α and IL-6 play dominant roles in the pathobiology of RA; however, IL-1, vascular endothelial growth factor (VEGF), and IL-17 have also a significant impact on the disease process. These cytokines activate genes associated with inflammatory responses, including additional cytokines and MMPs involved in tissue degradation [6]. Th-17 lymphocytes have a critical role in synovitis in RA patients [15]. TNF-α, IL-6, and IL-1 are key mediators of cell migration and inflammation in RA [7]. IL-6 acts directly on neutrophils through membrane IL-6 receptors that contribute to inflammation and joint destruction by secreting proteolytic enzymes and reactive oxygen intermediates [12]. Furthermore, an in vitro study with fibroblasts from patients with RA demonstrated the role of IL-6 in promoting neutrophil recruitment by activated fibroblasts [16]. The principal

cause of bone erosion is the pannus that is found at the interface with the cartilage and bone. Angiogenesis is a key process in the formation and maintenance of pannus because invasion of cartilage and bone requires increased blood supply. In patients with RA, many pro-angiogenic factors are expressed in synovium, among them, VEGF plays the central role in new blood vessel development [17]. Cartilage degradation in RA occurs when TNF-α, IL-1, and IL-6 activate synoviocytes, resulting in the secretion of MMPs into the SF [6, 7]. Cytokines also activate chondrocytes (**Figure 1**), leading to the direct release of additional MMPs into the cartilage [7]. ROS have been produced mainly during oxidative phosphorylation and by activated phagocytic cells during oxidative burst. It has been known that ROS can function as a second messenger to activate nuclear factor kappa-B (NF-κB) which orchestrates the expression of a spectrum of genes involved in the inflammatory response. Several cytokines, including TNF-α and IL-1β, are known initiators of NF-κB activation cascade [18] and are under its transcriptional control.

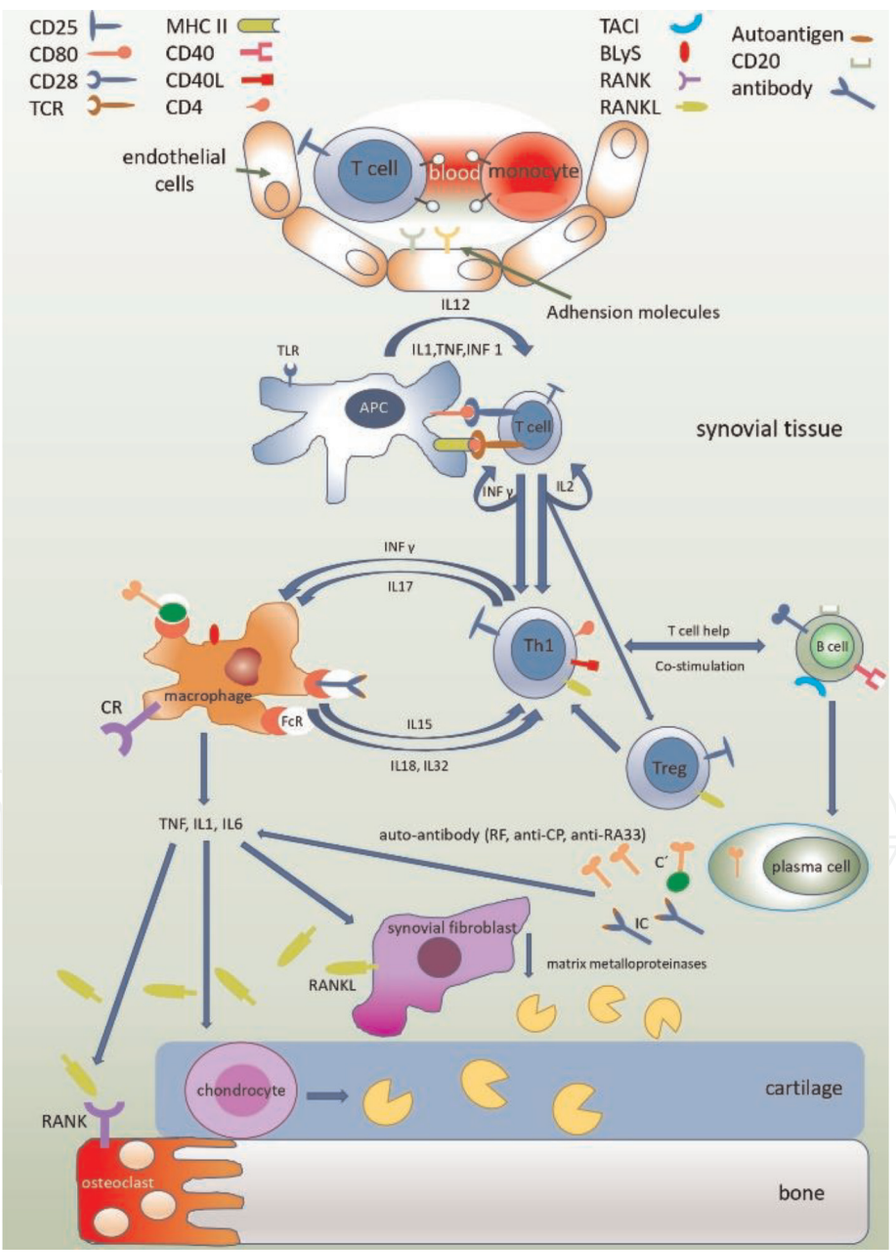

Figure 1.
Pathogenesis of cartilage and bone damage in rheumatoid arthritis. MHC, major histocompatibility complex; TCR, T-cell receptor; TACI, transmembrane activator and CAML interactor; BLyS, B-lymphocyte stimulator; RANK, receptor activator of nuclear factor κ B; RANKL, receptor activator of nuclear factor κ B ligand; TNF, tumor necrosis factor; INF, interferon; IL, interleukin; CR, complement receptor; RF, rheumatoid factor.

TNF-α participates positively in the phosphorylation of kinase kappa inhibitor, allowing NF-κB dimers (p50 and p65 portions) to migrate to the nucleus and then bind to promoters of pro-inflammatory genes [19] and stimulate the NADPH oxidase activation. Increased cytokine production driven by NF-κB can enhance expression of vascular adhesion molecules that attract leucocytes into the joint as well as MMPs.

3. Rat adjuvant arthritis

Animal models of arthritis play an important role in unraveling mechanisms of chronic inflammation in rheumatoid synovial tissue. They are used extensively to study new treatment strategies for RA. AA can be induced by intradermal or footpad injection of heat-killed mycobacterial species, preferably in a fine suspension in a mineral or vegetable oil (CFA). The disease is restricted to susceptible rodents, mostly certain rat strains, such as Lewis, Buffalo, Sprague-Dawley, and Wistar rats [20]. Following AA induction with CFA, rats not only develop arthritis but also systemic features of inflammation, such as uveitis, inflammation of the gastrointestinal tract, and a loss in body weight that starts 24–48 h before the clinical onset of arthritis. AA is a symmetric polyarthritis, affecting primarily the peripheral joints. The affected joints are red, swollen, and painful. The onset of overt clinical arthritis is seen 10–14 days following the induction of AA with CFA (**Figures 2** and **3**). The first histopathological signs of arthritis, an accumulation of mononuclear cells in connective tissues adjacent to periosteal surfaces, are already manifested 6 days after disease induction. Approximately 10 days after disease induction, the first radiological signs of inflammation become visible: localized osteoporosis, with erosive lesions, and periosteal reaction. The synovial infiltrate leads to pannus formation, resulting in cartilage deformation, and severe destruction of the joint [21]. An important component of the disease process is the trafficking of arthritogenic leukocytes into the target organ. The synovial cellular infiltrate during the initial phase of inflammation in AA consists primarily of mononuclear cells (mostly monocytes, macrophages, and T cells) and relatively fewer neutrophils [22]. The arthritogenic T cells migrate into the synovium before the appearance of clinical signs of the disease [23]. Data in AA suggesting that

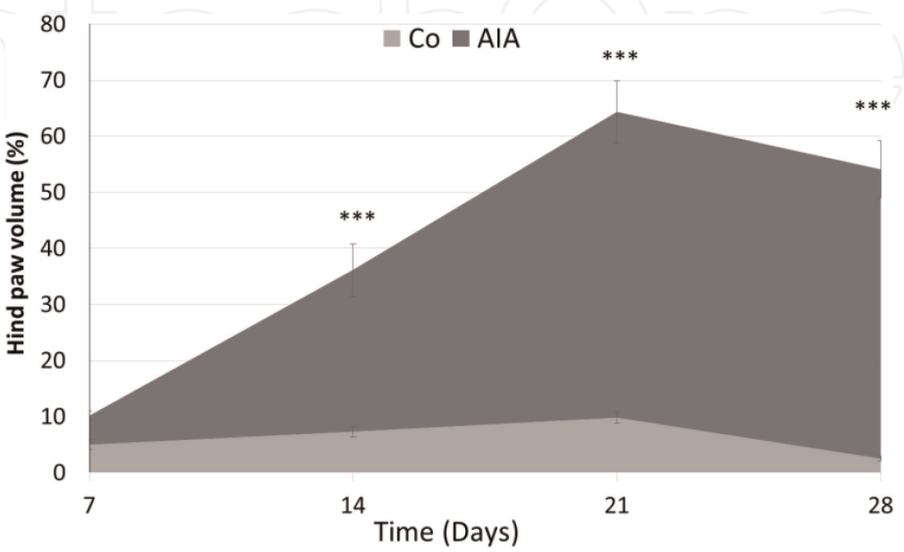

Figure 2.
Changes in hind paw volume during development of adjuvant-induced arthritis. Co, control healthy rats; AIA, adjuvant-induced arthritic rats.

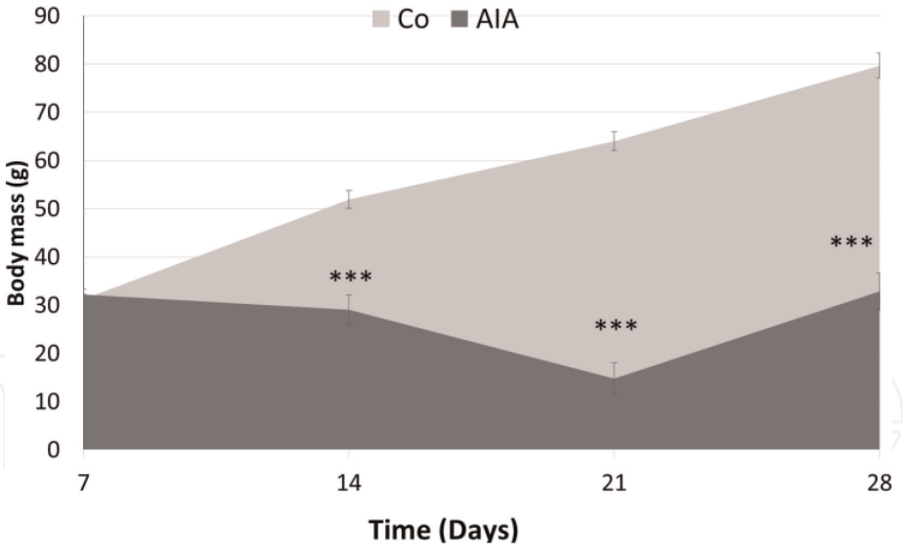

Figure 3.
Changes in body mass during development of adjuvant-induced arthritis. Co, control healthy rats; AIA, adjuvant-induced arthritic rats.

immune-stimulatory DNA sequences (ISS) may be a critical factor contributing to the chronicity of inflammation in chronic autoimmune arthritis. ISS can stimulate the expression of co-stimulatory molecules and the production of cytokines such as IL-12, TNF-α, and interferons by macrophages, dendritic cells, B cells, and NK cells [24] and are capable of skewing an immune response toward a strong and prolonged Th1 type of immunity [25]. AA has been used in the evaluation of nonsteroidal inflammatory drugs, such as phenylbutazone and aspirin during the early 1960s, and later in cyclooxygenase-2 inhibitors such as celecoxib. AA in rats shares many features with human arthritis, including genetic linkage, synovial CD4 + cells, and T-cell dependence [26].

4. Oxidative stress and inflammation

Inflammation is a natural defense mechanism against pathogens. It occurs in many pathogenic diseases (microbial and viral infections, exposure to allergens, radiation and toxic chemicals, autoimmune diseases, etc.). Chronic diseases linked with higher production of ROS result in OS and variety of protein oxidations [27]. Furthermore, some oxidized proteins trigger a release of inflammatory signal molecules, and peroxiredoxin 2 (PRDX2), which has been recognized as an inflammatory signal [28]. Relationship between OS and inflammation has been documented by many authors. Evidences indicated that OS plays a pathogenic role in chronic inflammatory diseases. Damage of OS such as oxidized proteins, glycated products, and lipid peroxidation results in neuron degenerations mostly reported in brain disorders [29]. ROS generated in brain tissues can modulate synaptic and non-synaptic communication between neurons that result in neuro-inflammation and cell death and then in neurodegeneration and memory loss [29]. Tripeptide glutathione (GSH) is an intracellular thiol antioxidant; lower level of this GSH causes higher ROS production, which results in imbalanced immune response, inflammation, and susceptibility to infection [30]. A study was conducted on the role of GSH and its oxidized form and their regulatory function and gene expressions beyond free radical scavenging activities linked with GSH. GSH is involved in the redox regulation of immune system [31] through disulfide bounds between protein

cysteines and GSH. This process is called as glutathionylation, which regulates signaling proteins and transcription factors [32]. Inflammatory stimuli induce the release of PRDX2, a ubiquitous redox-active intracellular enzyme. PRDX2 is a redox-dependent inflammatory mediator, which activates macrophages to produce and release TNF-α. During intracellular oxidative stress GSH binds with PRDX2 and this protein glutathionylation occurs before or during PRDX2 release, and glutathionylated PRDX2 regulates immunity. PRDX2 is a part of inflammatory cascade and is able to induce TNF-α release. This study showed that PRDX2 and thioredoxin from macrophages could alter the redox balance of cell surface receptors and enable the induction of inflammatory process [28].

4.1 Oxidative stress in rheumatoid arthritis

RA is one of the conditions that induces OS. A fivefold increase in mitochondrial ROS production in whole blood and monocytes of RA patients–compared with healthy subjects–suggests that OS is a pathogenic hallmark in RA. Free radicals are indirectly implicated in joint damage because they also play a role as secondary messengers in inflammatory and immune cellular response in RA. T-cell exposure to increased OS becomes refractory to several stimuli including those for growth and death and may perpetuate the abnormal immune response [33]. On the other hand, free radicals can degrade directly the joint cartilage, attacking its proteoglycan and inhibiting its synthesis [34]. Oxidative damage of hyaluronic acid and lipoperoxidation products and oxidation of low-density lipoproteins and carbonyl increment resulting from protein oxidation have been demonstrated in RA. Increased levels of 4-HNE have been assessed in serum (or plasma) and synovial fluid of patients with RA [35, 36]. Peroxidative damage induced by free radicals has been demonstrated to play a role in the pathogenesis not only of RA but also of systemic lupus erythematosus, progressive systemic sclerosis, diabetes mellitus type 1, and myasthenia gravis. Increased OS has been associated with increased lipid peroxidation in these patients. Lipid peroxidation occurs as a result of increased OS stemming from deranged pro-oxidant/antioxidant balance and represents an important pathogenic process in the oxygen toxicity. As a result of lipid peroxidation increases in the levels of conjugated dienes, isoprostanes, 4-HNE, and malondialdehyde have been demonstrated [37]. Study of Basu et al. [38] has shown that blood and synovial fluid from patients with various rheumatic diseases have high levels of both free radical-mediated F2-isoprostanes and the cyclooxygenase-derived PGF2 metabolite. This suggests that both oxidative injury and inflammation play a part to various degrees in these chronic inflammatory diseases. The measuring of arachidonic acid metabolites in body fluids opens unique opportunities for studying the role of lipid peroxidation [38]. ROS-induced genotoxic events have also been linked to mutation of p53 in RA-derived fibroblast-like synoviocytes [39]. Furthermore, it has been suggested that antioxidants systems, either enzymatic or not, are impaired in RA. Low levels of glutathione [40], tocopherols, β-carotene, and retinols and low activities of glutathione reductase and superoxide dismutase have been observed in patients with RA [41]. In a recent study, RA patients were, as usually, sub-grouped according to the presence or absence of rheumatoid factor, disease activity score, and disease duration. In addition, RA patients and healthy controls were evaluated for the oxidant-antioxidant status by monitoring ROS production, biomarkers of lipid peroxidation, protein oxidation, and DNA damage. The endogenous levels of enzymatic and nonenzymatic antioxidants were also measured. RA patients showed a marked increase in ROS formation, lipid peroxidation, protein oxidation, DNA damage, and decrease in the activity of antioxidant defense system leading to OS, which obviously contributes to tissue damage and to the

chronicity of the disease [42]. Oxidative modification of proteins has been shown to elicit antibodies in a variety of diseases including systemic lupus erythematosus (SLE), alcoholic liver disease, diabetes mellitus, and finally RA. Oxidative stress processes enhance the reactivity of the adaptive response. Oxidation of carbohydrates increased the antibody response to coadministered coantigens. In addition, the use of the Schiff base-forming agent Tucaresol during immunization with protein antigen increased T-cell-dependent immune response. Direct modification of protein antigen has been shown to be required for the enhancement of the immune response [43]. In SLE, oxidatively modified DNA and low-density lipoproteins (LDL) are present and induce a premature atherosclerosis. In an animal model of SLE, immunization with 4-hydroxy-2-nonenal (HNE)-modified autoantigens accelerated epitope spreading. Pentosidine, an advanced glycation end product (AGE), and AGE-modified IgG have correlated with RA disease activity. Oxidatively modified glutamic acid decarboxylase is important in type 1 diabetes mellitus. Oxidative modification induced fragmentation of scleroderma-specific autoantigens and seems to be responsible for the production of autoantibodies. Growing evidence for the involvement of oxidative damage in autoimmunity is pointing to the administration of antioxidants could be a viable untried alternative for preventing or ameliorating autoimmune disease [37]. OS occurring during inflammation can cause proteins to become nonenzymatically damaged by glyoxidation. This process results in the generation of AGE. The immunoglobulin molecule can also undergo similar glyoxidation to generate AGE-IgG. In inflammatory arthritis, they have shown that antibodies to AGE-IgG are specifically associated with RA, whereas the actual formation of AGE-IgG is related to the intensity of the systemic inflammatory response [44].

Studies focusing on direct detection of ROS and RNS found all these biomarkers elevated in RA patients suggesting an active OS. The redox status of neutrophils sourced from SF was measured by flow cytometry in terms of total ROS and hydroxyl radicals. Neutrophils a major cellular component of the SF of RA patients and their levels of ROS correlated strongly with protein carbonylation and lipid peroxidation. In patients with RA, the strong correlation between DAS28 score, levels of ROS, and markers of oxidative damage suggests that measurement of OS could serve as a marker for monitoring disease severity [45]. In another study, RA patients had significantly higher levels of ROS (O_2-, H_2O_2) than controls. Significant differences where monitored in serum levels of NO in patients with high activity of disease. More intensive response in samples with higher disease activity suggests that oxidative/nitrosative stress markers may be valuable in evaluating the RA progression and helpful in elucidating the mechanisms of disease pathogenesis [46]. The chronic OS in the RA synovium increases ROS production in the cellular oxidative phosphorylation and induces repetitive cycles of hypoxia/reoxygenation. The hypoxia in RA joints whose origin is a consequence of the rapid cellular proliferation induced by the inflammatory response, however, precedes inflammation at least in an animal arthritis model [47]. From the "danger model," in which the synoviocyte is an impaired cell, this sequence of events could be happening in the human disease [48]. Activated phagocytic cells can also enhance this OS during oxidative burst. Kundu et al. [49] showed neutrophils as most important phagocytes responsible for elevating OS in synovial infiltrates and peripheral blood of RA patients: The basal levels of total ROS, superoxide, and hydroxyl radicals were significantly increased in neutrophils from peripheral blood and synovial infiltrate. Furthermore, raised levels of superoxide in neutrophils of synovial infiltrate showed a positive correlation with NADPH oxidase activity in synovial fluid. However, there was no major increase in the RNS generated in monocytes from both sources.

4.2 Oxidative stress in adjuvant arthritis

In the development of AIA, not only immunological and inflammatory pathological changes are involved, but also the redox homeostasis is shifted toward increased production of ROS and RNS. Overproduction of ROS and RNS damages lipids, proteins, and DNA (also exhausts the natural enzymatic and nonenzymatic antioxidant defense), which is possible to detect with different markers of oxidation in biological structures. In human RA OS-mediated damage to lipids, proteins, and DNA and changes in enzymatic and nonenzymatic antioxidant defense are extensively studied. AIA in animals resembles the OS caused damage in human rheumatic diseases; therefore, it is a very useful tool to study process of OS during autoimmune diseases. Since there has been no standard therapy to reduce OS damage in diseases established yet, AIA could be a promising candidate for developing this type of therapy.

4.2.1 Peroxidation of lipids

4.2.1.1 4-HNE and MDA

The 4-HNE is one of the aldehydes specific to lipid peroxidation. 4-HNE is believed to be predominantly responsible from the cytopathologic effects seen during OS. Any factor compatible with stress or activity of antioxidant enzymes may trigger potentially dangerous metabolic pathway of peroxidative damage [50]. Our results showed that the level of HNE protein adducts was significantly increased on day 14 in rat AA [51]. The level of malondialdehyde (MDA) in the plasma of arthritic animals was also elevated [52–54] (**Table 1**). He et al. demonstrated an increased level of MDA in serum of AIA rats, which was significantly decreased by the administration of anthocyanins from cherries [53]. AA induced in male Sprague-Dawley rats increased plasma MDA levels, levels of glutathione, enzyme activities of SOD and GPx were decreased [55]. Also, Wang et al. demonstrated a significant increase of MDA and moreover nitrites in plasma of AIA rats [56]. Levels of anti-type II collagen antibody, nitrite/nitrate, and lipid peroxidation (levels of 4-HNE and MDA) were determined in the serum, joints, and brain. CIA elevated levels of nitrite/nitrate and 4-HNE and MDA levels in serum and the brain [57]. We also measured an increased levels of 4-HNE and MDA in plasma and the brain of AIA rats (**Tables 1** and **2**) [58].

4.2.1.2 Isoprostanes

Isoprostanes are a complex family of compounds produced from arachidonic acid via a free radical-catalyzed mechanism. They are reliable markers of lipid peroxidation. A strong link between lipid peroxidation and diseases associated with ischemia-reperfusion, atherosclerosis, and inflammation has been suggested by

Oxidative stress in plasma	MDA (μg/mL)	HNE (ng/mL)	Protein carbonyls (nmol/mL)
CO	2.4 ± 0.39	1.54 ± 0.16	391.2 ± 14.34
AIA	5.79 ± 0.44***	2.5 ± 0.19***	457.72 ± 11.09**

*Values are expressed as average ± standard error of mean, statistical significance (ANOVA-Tukey-Kramer post hoc test): **$p < 0.01$, ***$p < 0.01$ vs. CO.*

Table 1.
Markers of oxidative stress (malondialdehyde (MDA), 4-hydroxynonenal (HNE), and protein carbonyls) in plasma of arthritic animals measured on day 28.

Oxidative stress in brain	MDA (μg/g tissue)	HNE (ng/g tissue)
CO	5.38 ± 0.73	3.26 ± 0.17
AIA	10.12 ± 1.01***	4.78 ± 0.5**

*Values are expressed as average ± standard error of mean, statistical significance (ANOVA-Tukey-Kramer post hoc test): **$p < 0.01$, ***$p < 0.01$ vs. CO.*

Table 2.
Markers of oxidative stress (malondialdehyde (MDA) and 4-hydroxynonenal (HNE) in the brain of arthritic animals measured on day 28).

elevated levels of F2-isoprostanes observed in such diseases. Quantification of F2-isoprostanes as pathophysiological markers is suitable for the investigation of lipid peroxidation in human diseases and provides an interesting biomarker of antioxidant efficacy in diseases where OS might be involved [59]. There are only few evidences about F2-isoprostanes in animal models of RA. In one of our previous experiments, we have measured an elevated level of F2-isoprostanes in plasma of AIA rats, which were significantly increased compared to control healthy animals [60]. In a CIA model, authors investigated the ability of grape seed proanthocyanidin extract (GSPE) to reduce the development of mice arthritis. They have found that CIA significantly increased the level of 8-isoprostane in plasma. Plasma levels of 8-isoprostane and serum level of collagen type II-specific IgG2a in GSPE-treated mice were significantly decreased than those in the control mice [61]. Authors demonstrated that F2-isoprostanes are increased also in the urine of CIA mice [62]. F2-isoprostanes as an important marker of lipid peroxidation should be more extensively studied in AIA animal models, to obtain a better picture about the similarity with human RA.

4.2.2 Oxidation of proteins

Protein carbonyls (aldehydes and ketones) are produced directly by oxidation or via reactions with other molecules generated by the oxidation process. Autoimmune attack, resulting from abrogation of self-tolerance, is involved in many human diseases. Autoimmune disease may be either organ specific (type 1 diabetes, thyroiditis, myasthenia gravis, and primary biliary cirrhosis) or systemic (RA, progressive systemic sclerosis, and systemic lupus erythematosus). Nearly all these diseases have autoantibodies. Autoantibodies are typically present several years prior to diagnosis of SLE and serve as markers for future disease. Inflammation, infection, drugs, ROS, and environmental factors induce formation of neo-antigens [63]. The protein thiol groups were 59% diminished by AIA. The protein carbonyls content, an indicative of protein damage, was increased by arthritis (41%). Protein damage in both liver and brain was estimated as the tissue content of protein carbonyl groups. Corroborating previous results, arthritis increased protein damage in both tissues, 55% in the liver and 51% in the brain [64]. Authors Hemshekhar et al. [65] also showed a significant decrease in total protein thiol content with reference to saline-fed rats up to 51 and 36.05%, respectively, in liver and spleen homogenates of arthritic rats [65]. In a study about protective effects of green tea extract in AIA rats, authors detected a significant OS-caused damage to proteins and lipids in the liver, brain, and plasma [66]. The antioxidant defense, reduced in arthritis, is improved by the green tea treatment, as shown in the restoration of the GSH and protein thiol levels and by the tendency for normalizing the activities of the antioxidant enzymes. In arthritis rats, we found a significant increase of protein carbonyls in plasma [66–69] (**Table 1**). This finding emphasizes the role of OS in

inflammatory diseases such as AIA, not only in tissues directly affected by the disease (cartilage, bone, and skeletal muscle) (**Table 2**).

4.2.3 Production of reactive oxygen species by neutrophils

Recent evidence from animal models of RA emphasized the importance of neutrophils in the initiation and progression of AIA [70]. Progressive erosion of articular cartilage is a prominent feature of this disease. Not surprisingly, immunosuppressive approaches such as blockade of CD4+ lymphocytes effectively reduce the intensity of damage and the progression of AIA. The report of Santos et al. [71] convincingly demonstrates a requirement not only for CD4+ lymphocytes but also for neutrophils, the latter determined by the protective effects of neutrophil depletion. The sequence of events showed that CD4+ cells are necessary for the establishment of the immune response, which leads to the recruitment of neutrophils, with the involvement of cytokines (TNF-α, IL-1) and the IL-8 family of chemokines. The combination of products (oxidants, proteinases, and cytokines) from stimulated neutrophils, synovial macrophages, and lymphocytes is important to set the stage for acute and progressive polyarthritis [72]. We assessed ROS production in stimulated neutrophils of arthritic rats, and it was found to be increased, with a maximum on day 14 and 21 of AIA. Neutrophils in the whole blood of AIA animals reacted excessively to stimulation and produced 6–9 times more ROS [73]. We also demonstrated oxidative damage of tissues in AIA: ROS levels in the joint and the spleen were significantly elevated [74] (**Table 3**).

4.2.4 Levels of endogenous antioxidants

The mammal organism has several mechanisms to counteract with OS by producing antioxidants, which are either produced in situ or externally supplied with foods or supplements. The nonenzymatic antioxidants are distinguished as metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants referred also as endogenous antioxidants such as glutathione, lipoid acid, L-arginine, melatonin, coenzyme Q_{10}, uric acid, bilirubin, metal-chelating proteins, and transferrin are produced by metabolic processes, while nutrient antioxidants are compounds that cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, and omega-3 and omega-6 fatty acids [75]. Decreased levels of nonenzymatic antioxidant glutathione and vitamin C were observed in the liver of AIA rats compared to the normal rats [76]. Antioxidant state showed that plasma vitamin E, vitamin C, vitamin A, and β-carotene were significantly lower in arthritic control rats than normal rats [77]. Reduction of plasmatic antioxidants is indicating reduced antioxidant capacity and elevation of oxidative stress during adjuvant arthritis which is similar to rheumatoid arthritis in human [78].

Chemiluminescence (RLU*s)	Spontaneous	PMA stimulated	Neutrophil count in 1 μL of blood
CO	41,802 ± 2452	150,789 ± 9159	12,174 ± 747
AIA	168,203 ± 12815***	1,165,603 ± 94470***	40,260 ± 3325***

*RLU*s, relative light units; PMA, phorbol-12-myristate-13-acetate; values are expressed as average ± standard error of mean, statistical significance (ANOVA-Tukey-Kramer post hoc test): ***$p < 0.001$ vs. CO.*

Table 3.
Spontaneous and stimulated chemiluminescence and neutrophil count in whole blood of arthritic rats.

CoQ_{10} plays a central role in the electron transport chain and as a radical-scavenging antioxidant; therefore we studied its level in plasma during AA. In our experiments the arthritis process increased significantly the level of CoQ_{10} in comparison with healthy control rats. The arthritic processes also stimulated the synthesis of CoQ_9 (dominant form of CoQ in rats) and its transport to plasma [79] (**Table 4**). In the skeletal muscle mitochondria, we have measured significant changes in levels of α- and γ-tocopherol (**Table 5**).

Similarly in AIA, also in patients with RA, a depletion of endogenous antioxidants was measured. The plasma concentration of beta-carotene and vitamin E, hemoglobin, and hematocrit were significantly lower in patients with RA than in controls. These results provide evidence for a potential role of raised lipid peroxidation and lowered enzymic and nonenzymic antioxidants in RA because of its inflammatory character. These results suggested that OS plays a very important role in the pathogenesis of RA [80, 78].

4.2.5 Changes in antioxidant enzymes

In order to protect tissues from oxidative injuries, the body possesses enzymatic antioxidant enzymatic systems such as superoxide dismutases and catalase enzymes. It has been reported that AA decreases serum or synovial SOD and catalase activities together with other endogenous antioxidant systems [81]. Ramos-Romero et al. [82] showed a decrease in splenic catalase activity and, paradoxically, an increase in splenic total and mitochondrial SOD in AIA. The decreased catalase activity could be associated with the consumption of catalase in neutralizing the H_2O_2. On the other hand, increased splenic SOD activities could reflect the response of the body to increased ROS concentrations, and/or it could be due to the fact that arthritis was in its recovery phase 1 month after its induction. Moreover, SOD increase could also be explained by the increase in the oxidative stress found in arthritic rats and by the increased TNF-α secretion present in arthritis [82]. Both OS and TNF-α are shown to induce SOD synthesis [83]. It should be added that a similar increase in SOD activity was found in the plasma of RA patients [84] and in

Plasma	CoQ9TOT (μmol/L)	CoQ10TOT (μmol/L)	αT (μmol/L)	γT (μmol/L)
CO	0.328 ± 0.023	0.031 ± 0.004	19.9 ± 1.13	0.643 ± 0.051
AIA	0.468 ± 0.044**	0.027 ± 0.003	21.6 ± 0.72	0.834 ± 0.060*

*Values are expressed as average ± standard error of mean, statistical significance (ANOVA-Tukey-Kramer post hoc test): *$p < 0.05$, **$p < 0.01$ vs. CO.*

Table 4.
Concentrations of total coenzyme Q_9 ($CoQ_{9\text{-}TOT}$), total coenzyme Q_{10} ($CoQ_{10\text{-}TOT}$), α-tocopherol (αT), and γ-tocopherol (γT) in plasma.

Skeletal muscle mitochondria	CoQ9TOT (μmol/L)	CoQ10TOT (μmol/L)	αT (μmol/L)	γT (μmol/L)
CO	43.1 ± 3.01	1.90 ± 0.160	23.0 ± 1.23	0.98 ± 0.042
AIA	32.7 ± 2.49*	1.63 ± 0.187	18.7 ± 0.829*	1.39 ± 0.155*

*Values are expressed as average ± standard error of mean, statistical significance (ANOVA-Tukey–Kramer post hoc test): *$p < 0.05$ vs. CO.*

Table 5.
Concentrations of total coenzyme Q_9 ($CoQ_{9\text{-}TOT}$), total coenzyme Q_{10} ($CoQ_{10\text{-}TOT}$), α-tocopherol (αT), and γ-tocopherol (γT) in skeletal muscle mitochondria.

the synovial membrane of mice with collagen-induced arthritis [85]. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen, thus preventing the oxidation of biological structures by hydrogen peroxide. Authors demonstrated the elevated and LPO activity and NO level and decreased GSH, SOD, and catalase activities in AIA rats [86]. OS in AIA model is depleting antioxidant enzymes, which is in good agreement with human RA studies.

Activity of glutathione peroxidase (GPx) in blood serum and muscles of rats with AIA increased and activity of glutathione reductase (GR) in these tissues increased in comparison with the control. Probably, changes in enzyme activity are a defense response of the body to ROS generation in RA and can be a result of ROS activation or stimulation of their synthesis [87]. Similarly in the study of Sahu et al. [88], CIA increased antioxidant enzyme GPx and GR activities in joints, liver, kidney, and spleen tissues of rats.

Several pathologic factors have been suggested to be involved in the overexpression of heme oxygenase-1 HO-1 in RA lesions. In addition to superoxides and pro-inflammatory cytokines, hypoxia may play an important role in HO-1 expression in the lesions [89, 90]. AIA is an experimental model widely used to evaluate etiopathogenetic mechanisms in chronic inflammation. Devesa et al. [91] have examined the participation of HO-1 in AIA. They have found an increased nitric oxide (NO) production in the paw preceded the upregulation of HO-1, whereas selective inhibition of inducible NO synthase (iNOS) after the onset of arthritis lowered HO-1 expression, suggesting that this enzyme may depend on NO produced by iNOS. Administration of the HO-1 inhibitor protoporphyrin IX ameliorated the symptoms of arthritis. This compound significantly decreased leukocyte infiltration, erosion of articular cartilage, and osteolysis, as well as the production of inflammatory mediators. In this model, HO-1 can be involved in vascular endothelial growth factor production and angiogenesis. These results support a role for HO-1 in mediating the progression of the disease in this model of chronic arthritis [91]. Our research group showed that extra-articular manifestations of AIA are present also in lung, where the expression of heme oxygenase-1 was reduced during AIA [60].

5. Potential role of free radicals in rheumatoid cachexia

Cachexia is one of the major causes of progressive weight loss and affects up to 20% of RA patients [92]. Unlike sarcopenia, which is a normal physiological process of body mass reduction affected by aging, cachexia appears to be a secondary manifestation of an already ongoing disease [93]. Cachexia associated with RA can occur in two forms. The first form is cachectic RA or rheumatoid cachectic obesity, which is manifested by severe muscle wasting, with little or no fat mass loss. It is a less threatening form of cachexia mainly because the energy demands of muscles can be compensated by lipid metabolism [94]. The second form is rheumatoid cachexia, which is manifested by severe muscle wasting as well as fat loss.

Rheumatoid cachexia (RC) is a progressive form of RA, which is primarily thought to be caused by the abnormal production of the pro-inflammatory cytokines produced by the immune cells localized in the synovial tissue of the affected joints. Excessive concentrations of several cytokines, especially TNF-α, IL-1β, IL-6, and INF-γ, could potentially affect the intracellular mechanisms of muscle fibers, leading to severe muscle atrophy and weakness [95]. The most dominant cytokine in RA and RC pathogenesis appears to be TNF-α which acts synergically with IL-1β. When bound to their specific receptors, these cytokines cause activation of NF-κB signaling cascade. A study by Cai et al. [96] suggests that muscle atrophy is

predominantly promoted by the NF-κB pathway via the activation of MuRF1 transcription factor which ultimately induces immoderate proteolysis of muscle proteins by activating the ubiquitin-proteasome system. Moreover, Castillero et al. [97] observed overexpression of MuRF1 as well as several other myogenic factors, such as atrogin-1/MAFbx ubiquitin ligases in adjuvant arthritis.

Another important pathogenic factor of RC is reduced physical activity, which appears to be the result of either poor pain management of inflamed and swollen joints, metabolic changes, or merely general caution for physical activity. Lower physical activity leads to reduced muscle fiber stimulation, which significantly disrupts the cycle of muscle proteolysis and proteosynthesis in favor of proteolysis [98]. One of the possible triggers of RC could also be increased free radical concentration and onset of OS.

As mentioned in the previous text, ROS and RNS concentrations have been reported to be elevated in the joint area as well as plasma. This may indicate that an increase in free radicals levels could also be found in skeletal muscle tissue. There are several sites of free radical production in muscles including mitochondria, sarcoplasmic reticulum, and sarcolemma [99]. As metabolically highly active organs, muscles dramatically increase their oxygen consumption during physical activity in order to compensate various energy-dependent processes. Concurrently excessive amounts of oxidants are produced, which then serve as messenger molecules in multiple intracellular cascades. The main site of free radical generation is mitochondria during aerobic metabolism and oxidative phosphorylation. It has been shown that complexes I and III and more recently complex II of mitochondrial electron transport chain are key producers of ROS in muscle fibers [100]. Several authors suggest that the major ROS produced in muscle cells is superoxide anion ($O2^{\bullet-}$), which is a very unstable radical and rapidly undergoes reduction resulting in dismutation into hydrogen peroxide (H_2O_2) [101]. Even though H2O2 is quite a stable nonradical molecule, excessive concentrations of H_2O_2 could ultimately result in increased generation of hydroxyl radical (•OH)–a highly reactive ROS which could potentially damage various cellular molecules and disrupt many intracellular mechanisms. Free radicals are also regularly produced by several enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) family as well as xanthine oxidase (XO) [99]. In skeletal muscles only Nox 2 and Nox 4 isoforms have been found, and it is believed that both of these isoforms are localized primarily in mitochondria [102]. However, the precise mechanism by which the increased activity of these enzymes is promoted is to date poorly understood. Under physiological conditions, excessive concentrations of free radicals are regularly scavenged and converted into non-radical molecules by antioxidant defense system molecules. However, several studies have observed low concentrations of some nonenzymatic antioxidants such as GSH [41] as well as low activity of enzymatic antioxidants such as SOD and glutathione peroxidase (GPX) in RA, which could potentially affect muscle tissue [103]. It has been proposed that decreased physical activity in RC patients could play a major role in oxidative damage of muscle cells since lower muscle stimulation reduces antioxidant capacity thus causing impaired balance in oxidant-antioxidant ratio [104].

Several long-term studies have reported a number of negative effects of free radicals in muscles at the molecular level. Oxidative damage of lipids, particularly in cell membranes [105], as well as nucleic acids in the DNA [106] is of great importance to normal cellular functioning, lately there has been a great deal of emphasis on protein modifications caused by ROS in multiple diseases.

Proteins as functional units of the cell can cause great damage to the cell itself if its space conformation is disrupted. Perhaps the most common protein modification caused by imbalance of oxidative status is carbonylation of side chains of multiple

amino acids such as arginine, lysine, threonine, and proline [107]. Moreover, carbonylation of proteins that are part of the contractile apparatus could be crucial in RC muscle dysfunction. Fedorova et al. [108] showed that carbonylation of actin could very much affect actomyosin ATPase activity, thus promoting subsequent muscle atrophy. Taken together, action of pro-inflammatory cytokines, mitochondrial dysfunction, and enhanced activity NADPH oxidase and xanthine oxidase contribute to the overproduction of ROS/RNS.

Decreased physical activity results in downregulation of antioxidants. Combination of these factors consequently leads to imbalance in protein synthesis and degradation resulting in muscle wasting (**Figure 4**).

5.1 Animal models in rheumatoid cachexia

Rheumatoid cachexia still remains a poorly investigated disease, and many scientists are trying to understand the exact mechanism by which the disease takes place. Several animal models of RA are used in the study of this condition. The best studied animal model to date has been CIA, which, by its characteristics, offers the most accurate comparison with humans, as the onset of this affection is relatively slow and the immune mechanisms driving the onset of cachexia are closest to rheumatoid cachexia in people [109].

Recently, Albarse et al. [110] have been investigating the development of cachexia in CIA in DBA1/J mice. In their study, they have observed significant increase in free exploratory locomotion as well as grip strength and endurance exercise performance. Additionally, they registered reduction of muscle weight in several muscles, which could indicate that mechanisms, which led to the onset of arthritis, could subsequently promote muscle atrophy and weakness.

Another model of rheumatoid arthritis and adjuvant arthritis was also used to investigate muscle wasting in male and female Lewis rats in the study of Roubenoff et al. [111]. It was shown that adjuvant-induced rats also manifested severe muscle loss when compared to control as well as pair-fed groups. This makes adjuvant arthritis suitable model for study of cachexia in chronic inflammatory diseases. Even though there have been multiple authors dedicated to unraveling the true cause of rheumatoid-induced cachexia, much more study is needed in order to

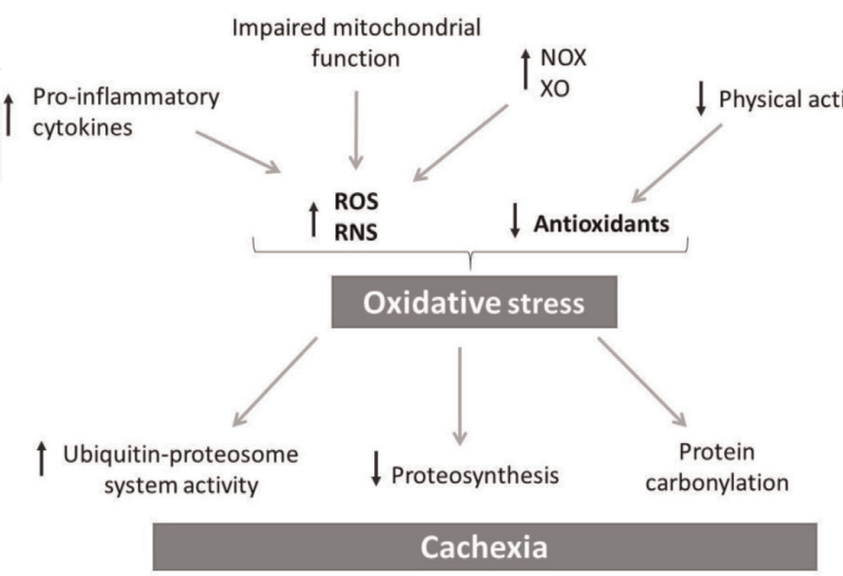

Figure 4.
Mechanism of the effect of oxidative stress on the onset of cachexia in rheumatoid arthritis. NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; XO, xanthine oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species.

sufficiently understand precise mechanism by which this serious condition occurs. This could greatly improve quality of RA patients and thus contribute to modern medicine.

6. Conclusion

The animal model adjuvant arthritis gives a broad spectrum of possibilities to study different pathological mechanism of rheumatoid arthritis. One important pathological pathway is the connection between inflammation and oxidative stress, which is studied on both systemic and local levels. From our original results as well as from results reported by other authors, it is evident that treatment with compounds possessing redox balance modulating properties might be of great relevance for new strategies for therapy of rheumatoid arthritis. For this purpose, adjuvant arthritis seems to be an ideal animal model. Moreover, this animal model has also a good potential in the research of inflammatory cachexia and its pharmacological intervention.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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