The Role of Endogenous Antioxidants in the Treatment of Experimental Arthritis

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Abstract

The pathogenesis of rheumatoid arthritis is poorly understood; however, elevated oxidative stress has been described to be involved. In this chapter, we present experiments with endogenous molecules bearing antioxidative properties. In our studies, we used male Lewis rats, and the arthritis was induced with *Mycobacterium butyricum*. In the first experiment, we tested coenzyme Q_{10} (CoQ_{10}) in the oral daily dose of 100 mg/kg b.w. Markers of inflammation and total antioxidant status were corrected in the group supplemented. CoQ_{10} treatment significantly improved concentrations of the investigated endogenous antioxidants. Further as an important fact, we consider a good bioavailability of used CoQ_{10} formulation which was confirmed by increased CoQ concentrations in plasma, tissue, and mitochondria from skeletal muscles. In the second study, we describe the results with hyaluronic acid (HA) administered in oral daily doses of 0.5 mg and 5 mg/kg b.w. and of different molecular weights (0.43, 0.99, and 1.73 MDa). A notable antioxidative effect of HA was assessed: its administration increased the activities of antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in erythrocytes and total antioxidant capacity of plasma and reduced the marker of oxidative damage to lipids—plasmatic lipid hydroperoxides. HA with the highest molecular weight showed the most significant effect.

**Keywords:** oxidative stress, antioxidants, coenzyme Q_{10}, hyaluronan, arthritis

1. Introduction

1.1 Involvement of oxidative stress in rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease, affecting almost 1–2% of the world's population. Most of patients present rheumatoid factors, which are autoantibodies directed to the Fc fraction of immunoglobulin G and antibodies reacting with citrullinated peptides [1, 2]. The pathogenesis of RA is understood incompletely. Until now, there is a lack of optimal therapy against this disease. The disease is characterized by immunological dysfunction and chronic inflammation which results in synovial joint deformity and destruction. In the course of RA, the synovial membrane of diarthrodial joints is inflamed, and articular tissue is damaged which leads to severe functional

disarrangement of the entire joint. The initial stages of RA synovitis are characterized by proliferation of the microvasculature and secondary edema. Eventually, this process matures into a progressive infiltration of immune cells, including B cells, T cells, and monocytes from the bloodstream. These immune cells are activated in the joint and differentiate and acquire mature phenotypes. The influx of immune cells is also associated with phenotypic changes in synoviocytes, the typical resident cells. Both fibroblast- and monocyte-derived synoviocytes proliferate extensively and participate in inflammatory process. Synovial proliferation, neovascularization, and leukocyte extravasation transform the normal synovium into an invasive tumor-like "pannus." The architecture of the microvasculature is highly dysregulated, and thus efficiency of oxygen supply to the synovium is poor [3]. This, alongside with increased metabolic turnover of the expanding synovial pannus, leads to oxidative stress (OS), altered cellular bioenergetics, and a hypoxic microenvironment, which further promotes synovial invasiveness and abnormal cell function within the joint [4]. Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) have distinct contribution to the destructive, proliferative synovitis of RA and play a prominent role in cell-signaling events (**Figure 1**).

However, few studies had clarified the role of free radicals in the etiopathogenesis of RA. Significant higher serum levels of ROS and RNS in RA patients in comparison with healthy subjects were described. Furthermore, strong positive correlation between ROS, RNS, and the clinical and biochemical markers of RA was observed [5]. In another study glycated, oxidized, and nitrated proteins and amino acids were detected in synovial fluid (SF) and plasma of arthritic patients with characteristic patterns found in early and advanced RA, with respect to healthy control [6]. Combination of estimates of oxidized, nitrated, and glycated amino acids with hydroxyproline and anti-cyclic citrullinated peptide antibody status in plasma provided a biochemical test of relatively high sensitivity and specificity for early-stage diagnosis and typing of arthritic disease. Advanced oxidation protein products (AOPPs) have been confirmed to accumulate in RA patients. A study of Ye et al. [7] demonstrated that AOPPs induce apoptosis of human chondrocyte via ROS-related mitochondrial dysfunction and endoplasmic reticulum stress pathways. These data implicate that AOPPs may represent a novel pathogenic factor that contributes to RA progression. Further it seems that an accurate redox balance is necessary to sustain an immune state that both prevents the development of overt autoimmunity and minimizes collateral tissue damage [8]. The inflamed joint
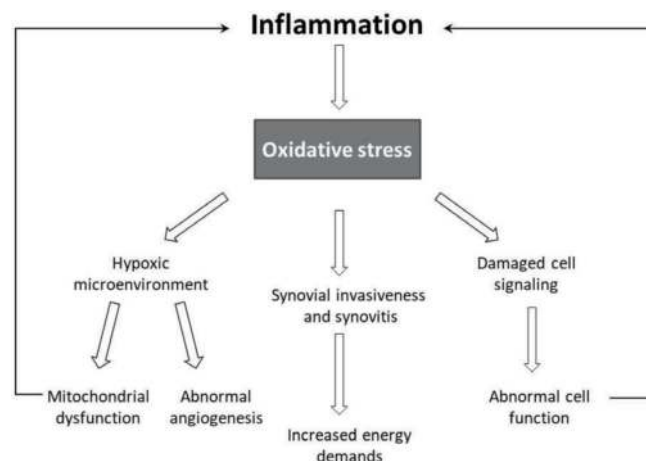
Figure 1.
Pathological changes in arthritic joint induced by oxidative stress.

is profoundly hypoxic, with evidence of oxidative damage and impaired mitochondrial dysfunction, as a result of abnormal angiogenesis and increased energy demands of the expanding synovial pannus [9]. In this hypoxic-inflammatory microenvironment, synovial cells adapt in order to survive through altering their cellular metabolism, which activates complex cross talk of key signaling pathways in the inflamed joint which further exacerbates inflammation. Thus, understanding the underlying mechanisms mediating hypoxia-induced pathways, OS, and subsequent cellular inflammation may provide a basis for novel therapies. It is well documented that ROS can activate different signaling pathways having a vital importance in the pathophysiology of RA [10].

Our chapter is focused on two main aims:

1. To verify the hypothesis that per oral supplementation of CoQ_{10} could affect inflammation in arthritic rats by regulating endogenous antioxidants and OS with detailed analysis performed in plasma and skeletal muscles.

2. To verify the hypothesis that hyaluronan per oral administration can restore the redox balance under the conditions of experimental arthritis. OS has been monitored in erythrocytes and in plasma. The effect of three different molecular weights of polysaccharides has been evaluated.

1.2 Role of antioxidant systems and endogenous antioxidants in remission of rheumatoid arthritis

Epidemiological studies have shown that RA occurs in previously healthy subjects who had low levels of circulating antioxidants [11], implying a pathogenic role of increased OS in the development of RA. Patients with RA have been reported to have lower serum levels of a variety of antioxidants, including vitamin E, vitamin C, β-carotene, selenium, and zinc, in comparison with healthy individuals [12]. In order to prevent the damaging effect of prooxidants, the body has an antioxidant defense system that protects cellular systems from oxidative damage [13]. Some common enzymes that are involved in the neutralization of free radicals are endogenous enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR), and peroxiredoxins. These enzymes neutralize hydrogen peroxide, yielding water (CAT, GPx) and oxygen (CAT) molecules. The nonenzymatic endogenous antioxidants taking part in the first line of defense belong to preventive antioxidants, and in blood plasma they are represented by metal-binding proteins as ceruloplasmin, ferritin, lactoferrin, transferrin, and albumin. These proteins inhibit the formation of ROS by binding with transition metal ions (e.g., iron and copper). Also, metallothionein plays an essential role in the prevention against ROS. The second line of defense against ROS involves nonenzymatic antioxidants that are represented by molecules characterized by the ability to rapidly inactivate radicals and oxidants. The third line of defense consists of repair mechanisms against damage caused by free radicals. This form of protection is provided by enzymatic antioxidants, which can repair damaged DNA and proteins, fight against oxidized lipids, stop chain propagation of peroxyl lipid radicals, and repair damaged cell membranes and molecules [14]. Dietary antioxidants (vitamins C and E, carotenoids, polyphenols, and biogenic elements) can affect the activity of endogenous antioxidants. Endo- and exogenous antioxidants may act synergistically to maintain or re-establish redox homeostasis. The major endogenous nonenzymatic low-molecular-mass antioxidants include glutathione, uric acid, melatonin, coenzyme Q, bilirubin, and polyamines. Considering the mechanism of antioxidant protection, the endogenous substances

can be divided into true scavengers, metal-buffering proteins, and chelators of redox-stable metals [13]. This chapter is focused on evaluating coenzyme Q_{10} and hyaluronan of different molecular weight in experimental arthritis induced in rats.

2. Coenzyme Q_{10} supplementation and its contribution to therapy of arthritis

2.1 The protective properties of coenzyme Q

Chronic inflammation, systemic OS, and mitochondrial dysfunction are the main factors which participate in etiopathogenesis of arthritis. Mitochondria play a central role in ATP formation in the respiratory chain (**Figure 2**) and in maintaining redox homeostasis. OS processes are activated under pathological conditions. Oxidative damage of mitochondria may lead to the dysfunction of the respiratory chain which further increases ROS formation. Thus, mitochondrial dysfunction can contribute to the development of inflammatory human diseases [16].

The therapy of RA is an actual problem in clinical rheumatology due to the toxicity and side effects of antirheumatic drugs; therefore, new treatment options are being sought. Methotrexate (MTX), used in the treatment of RA, can induce hepatocellular injury. In combination with coenzyme Q_{10}, anti-arthritic effect of MTX was potentiated, and hepatotoxicity was suppressed [17]. Preservation of mitochondrial function could reduce OS and may represent a novel therapeutic approach in patients with inflammatory diseases. Progressive muscle atrophy and inflammatory myopathy in RA have been proposed to be mediated by disturbances of myofibrils and mitochondria [18]. Due to the unique properties, coenzyme Q_{10} (CoQ$_{10}$) can serve as a useful adjuvant in the management of arthritis. CoQ$_{10}$
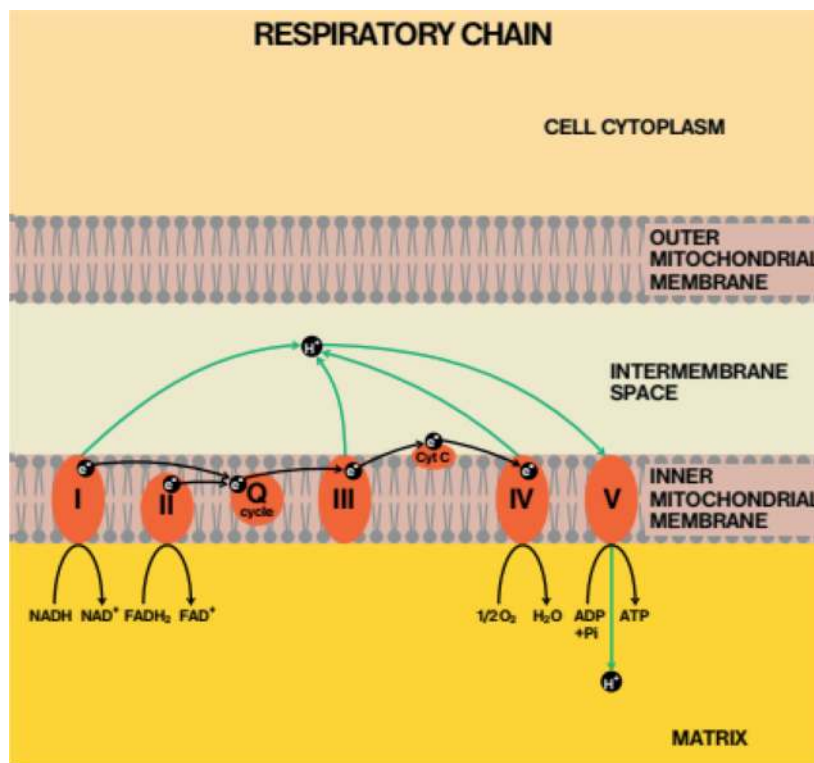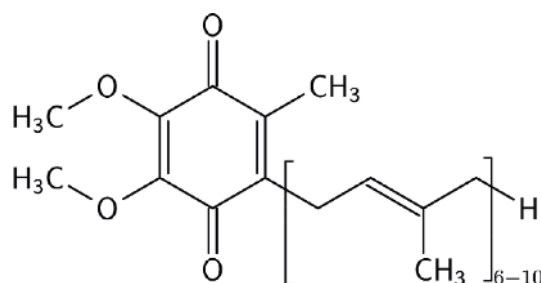

Figure 2.
Function of coenzyme Q in mitochondrial respiratory chain. With permission of [15].

is irreplaceable in mitochondrial bioenergetics; it participates as a cofactor of dehydrogenases in the transport of electrons and protons as well as in ATP production [19]. The respiratory chain located in the inner mitochondrial membrane is organized into five complexes (I, II, III, IV, and V). The transport of electrons from NADH and $FADH_2$ and production of electrochemical potential and proton gradients are necessary for the synthesis of ATP [20, 21]. Electrons are carried out from complexes I and II to complex III by coenzyme Q (CoQ). It has been demonstrated that lipoperoxidation is accompanied by reduced mitochondrial CoQ concentrations concomitantly with the decreased activities of respiratory chain enzymes, such as NADH- and succinate oxidases [22–24]. Increased levels of antioxidants have also been reported including CoQ in tissues as well as activities of antioxidant enzymes in experimental models of diseases associated with increased free radicals' generation such as diabetes mellitus [25–27]. The term "redox signaling" has been introduced to describe a regulatory process in which protective responses against oxidative damage are induced to reset the oxidant-antioxidant balance [28]. CoQ (ubiquinone) is the only lipophilic antioxidant to be biosynthesized; the main form in humans is CoQ_{10}, in rats' coenzyme Q_9 (CoQ_9) (**Figure 3**).

CoQ exerts its antioxidant function either directly on superoxide radicals or indirectly on lipid radicals, both singly and in cooperation with vitamin E [29]. Beneficial antioxidant and anti-inflammatory properties of CoQ_{10} were proved in RA patients [30]. Tocopherols are the subgroup of vitamin E, occurring in isomers α, β, γ, and δ. All tocopherols are potent antioxidants with lipoperoxyl radical-scavenging activities [31]. The main forms of tocopherols in humans are alpha-tocopherol (αT) and gamma-tocopherol (γT) (**Figure 4**).

The most significant difference in metabolism of αT and γT is preferential binding of αT by α-TTP (α-tocopherol transfer protein). The γT is metabolized mainly through cytochrome P450, and formed hydrophilic metabolites are excreted in urine [32]. Isoforms of vitamin E differentially regulate inflammation [33]. In contrast to αT, γT reacts with nitrogen radicals which are formed extensively in inflammatory diseases such as RA. Vitamin E has a potential role in skeletal muscle health, in regulation of OS and inflammation [34]. Low levels of vitamin E and

Figure 3.
Chemical structure of coenzyme Q.
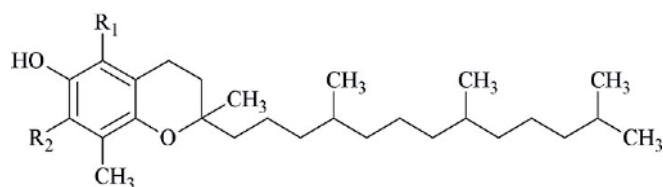
Figure 4.
Chemical structure of tocopherols. α-tocopherol: R1 = CH₃; R2 = CH₃; γ-tocopherol: R1 = H; R2 = CH₃.

other endogenous antioxidants have been considered as a risk factor for the development of RA [35]. We hypothesized that administration of CoQ_{10} could affect inflammation in arthritic rats by regulating the endogenous antioxidants and OS.

2.2 Experimental design of adjuvant arthritis with administration of coenzyme Q_{10}

Adjuvant arthritis (AA) was induced by intradermal injection of *Mycobacterium butyricum* in incomplete Freund's adjuvant to male Lewis rats [36, 37]. The experiment included healthy control animals (HC), arthritic animals (AA), and arthritic animals with administration of CoQ_{10} (liquid liposomal CoQ_{10}—LiQSorb®) in the oral daily dose of 100 mg/kg b.w. (AA-CoQ) by the use of gavage. The analyses were performed 28 days after the arthritis induction and in the beginning of CoQ_{10} supplementation. Concentrations of CoQ_9, CoQ_{10}, αT, and γT were determined by HPLC method with spectrophotometric detection at 275 nm (CoQ) and 295 nm (tocopherols), using external standards [38, 39]. Total CoQ_9 and CoQ_{10} (oxidized and reduced forms) in plasma was measured after oxidation with 1,4-benzoquinone [40]. Mitochondria from hind paw skeletal muscle tissue were isolated by means of differential centrifugation according to slightly modified methods [41, 42]. Mitochondrial proteins were estimated spectrophotometrically [43]. Data were collected and processed using CSW 32 chromatographic station (DataApex Ltd). Concentrations were calculated: in the plasma in μmol/l, in the tissue in nmol/g of wet weight, and in the mitochondria in nmol/mg of proteins. Total antioxidant status (TAS) in plasma was determined using the Randox Total Antioxidant Status kit with colorimetric detection at 600 nm. Markers of inflammation, C-reactive protein (CRP), and monocyte chemotactic protein-1 (MCP-1) were measured by ELISA. Data are expressed as mean ± SEM. Statistical significance between experimental groups was evaluated using Student's t-test, $p < 0.05$, which was considered as a significant result.

2.3 Evaluation of results of administration of coenzyme Q_{10} in experimental arthritis

AA for a period of 28 days significantly increased markers of inflammation—CRP and MCP-1—and decreased TAS (**Table 1**). Concentrations of total CoQ_9 (oxidized and reduced) and γT in plasma of AA rats increased significantly (**Table 2**).

In skeletal muscle tissue and mitochondria of AA rats, concentrations of oxidized form of coenzyme Q_9 (CoQ_{9-OX}) and αT decreased significantly and CoQ_{10-OX} only slightly. Tissue γT increased compared to controls; in mitochondria the increase was marginally significant ($p = 0.077$), (**Tables 3** and **4**). Treatment of arthritic rats with CoQ_{10} (AA-CoQ) for 28 days partially suppressed inflammatory markers and increased TAS, but not statistically significant (**Table 1**). Elevated concentrations of total CoQ_9 and γT in plasma were corrected to control values. Concentration of CoQ_{10} in plasma increased extremely, demonstrating a good bioavailability of CoQ_{10} administered (**Table 2**). In tissue and mitochondria, concentrations of CoQ_9 and CoQ_{10} increased in comparison with AA rats and were comparable to controls. Concentrations of αT in tissue and mitochondria also increased, in the tissue at the limit of significance ($p = 0.071$) and in mitochondria without statistical significance (**Tables 3** and **4**).

Bioenergetic and antioxidant properties of CoQ_{10} are sufficiently described [44]. However, new research findings suggest that CoQ_{10} supplementation has also lowering effects on circulating inflammatory mediators, including CRP,

Plasma	CRP	MCP-1	TAS
	µg/ml	pg/ml	mmol/l
HC	457.4 ± 21.3	1462 ± 159.2	0.673 ± 0.037
AA	602.2 ± 10.7**	2925 ± 389.1**	0.529 ± 0028*
AA-CoQ	563.3 ± 14.5	2539 ± 144.1	0.562 ± 0.033

*$p < 0.05$.
**$p < 0.01$ vs. HC.

Table 1.
Markers of inflammation: C-reactive protein (CRP), monocyte chemotactic protein (MCP-1), and total antioxidant status (TAS) in plasma.

Plasma	CoQ$_{9\text{-TOT}}$	CoQ$_{10\text{-TOT}}$	αT	γT
	µmol/l	µmol/l	µmol/l	µmol/l
HC	0.328 ± 0.023	0.031 ± 0.004	19.9 ± 1.13	0.643 ± 0.051
AA	0.468 ± 0.044**	0.027 ± 0.003	21.6 ± 0.72	0.834 ± 0.060*
AA-CoQ	0.237 ± 0.016++	0.804 ± 0.069++	19.6 ± 1.07	0.678 ± 0.043+

*$p < 0.05$.
**$p < 0.01$ vs. HC.
+$p < 0.05$.
++$p < 0.01$ vs. AA.

Table 2.
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.

Tissue	CoQ$_{9\text{-ox}}$	CoQ$_{10\text{-ox}}$	αT	γT
	nmol/g ww	nmol/g ww	nmol/g ww	nmol/g ww
HC	43.1 ± 3.01	1.90 ± 0.160	23.0 ± 1.23	0.98 ± 0.042
AA	32.7 ± 2.49*	1.63 ± 0.187	18.7 ± 0.829*	1.39 ± 0.155*
AA-CoQ	40.9 ± 4.07	2.43 ± 2.52+	22.2 ± 1.42	1.07 ± 0.084

*$p < 0.05$ vs. HC.
+$p < 0.05$ vs. AA.

Table 3.
Concentrations of oxidized forms of coenzyme Q_9 (CoQ$_{9\text{-OX}}$), coenzyme Q_{10} (CoQ$_{10\text{-OX}}$), α-tocopherol (αT), and γ-tocopherol (γT) in the skeletal muscle tissue.

interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α). Meta-analysis of clinical randomized controlled trials evaluated the effects of CoQ$_{10}$ in some inflammatory diseases but with inconsistent results due to heterogeneity and limited number of studies [45].

In an experimental study, an antiarthritic effect of CoQ$_{10}$ against induced gouty arthritis in rats was found [46]. CoQ$_{10}$ treatment at the dosage 10 mg/kg/body weight for 3 days reduced paw edema, minimized lysosomal enzyme release, boosted antioxidant system, and suppressed lipid peroxidation. Protective mechanism of CoQ$_{10}$ against cartilage degeneration induced by interleukin-1β was studied on isolated rat chondrocytes [47]. The study demonstrated the anticatabolic and cartilage protective potentials of CoQ$_{10}$ by inhibition of overexpression of matrix

Mitochondria	CoQ$_{9\text{-ox}}$	CoQ$_{10\text{-ox}}$	αT	γT
	nmol/mg prt	nmol/mg prt	nmol/mg prt	nmol/mg prt
HC	3.28 ± 0.14	0.144 ± 0.01	0.305 ± 0.02	0.042 ± 0.01
AA	2.67 ± 0.13**	0.126 ± 0.01	0.216 ± 0.02*	0.058 ± 0.01
AA-CoQ	3.16 ± 0.08++	0.149 ± 0.01+	0.289 ± 0.03	0.052 ± 0.01

*$p < 0.05$.
**$p < 0.01$ vs. HC.
+$p < 0.05$.
++$p < 0.01$ vs. AA.

Table 4.
Concentrations of oxidized forms of coenzyme Q$_9$ (CoQ$_{9\text{-OX}}$), coenzyme Q$_{10}$ (CoQ$_{10\text{-OX}}$), α-tocopherol (αT), and γ-tocopherol (γT) in skeletal muscle mitochondria.

metalloproteinases which may represent a new approach in the treatment of patients with osteoarthritis. Administration of CoQ$_{10}$ in the dose 100 mg/kg for 28 days suppressed cartilage degeneration by inhibiting inflammatory mediators and OS in an experimental model of rat osteoarthritis [48]. Beneficial effects of CoQ$_{10}$ supplementation on inflammatory cytokines and OS in RA patients were proved. In the double-blind, randomized controlled clinical trial in patients with RA, CoQ$_{10}$ supplementation with 100 mg/day for 2 months led to a significant decrease of malondialdehyde (MDA) formation and a nonsignificant increase of total antioxidant capacity, indicating beneficial effects on OS. CoQ$_{10}$ also suppressed overexpression of inflammatory cytokines TNF-α significantly and IL-6 nonsignificantly [30].

Our results show that administration of CoQ$_{10}$ to rats with induced adjuvant arthritis in the oral daily dose of 100 mg/kg b.w. for 28 days partially corrected inflammatory markers and TAS but without statistical significance (**Table 1**). CoQ$_{10}$ treatment corrected concentration of CoQ$_9$ in plasma to control value (**Table 2**). In the skeletal muscle tissue and isolated mitochondria, concentrations of CoQ$_9$ and CoQ$_{10}$ increased in comparison with AA rats and were comparable to controls. Concentrations of αT in tissue and mitochondria were also improved, in the tissue marginally significant and in mitochondria without statistical significance (**Tables 3** and **4**). Sufficient concentrations of CoQ together with αT, the main form of vitamin E, may be important in skeletal muscle function, in regulation of OS and inflammation. The role of vitamin E in regulation of diseases has been extensively studied in humans, animal models, and cell systems. It has been reported that isoforms of vitamin E may have opposing regulatory functions during inflammation, when supplementation with αT was anti-inflammatory and γT pro-inflammatory [33]. Different effects of vitamin E isoforms may result from differences in their metabolism, as αT is preferentially bound α-TTP (α-tocopherol transfer protein), while γT is metabolized mainly through cytochrome P450 and its concentrations in plasma and tissues are dependent on cytochrome P450 metabolism in the liver [49]. Our results show elevated concentrations of γT in plasma and skeletal muscle tissue of arthritic rats together with increased markers of inflammation and decreased TAS (**Tables 1–3**). This confirms the previous findings that inflammation and inhibition of the cytochrome P450 can increase γT concentration [50]. Treatment of arthritic animals with CoQ$_{10}$ corrected elevated levels of γT to control values and showed beneficial effect on concentrations of αT, CoQ$_9$, and CoQ$_{10}$ in the skeletal muscle tissue and mitochondria. This can help improve bioenergetic function of the skeletal muscle that is impaired by arthritic inflammatory

process. Elevated concentrations of endogenous antioxidants can contribute to regulation of oxidative stress.

## 3. Hyaluronan in arthritis: regulation of inflammation through antioxidative effects

3.1 The protective properties of hyaluronan

OS is important in the pathogenesis of autoimmune diseases such as RA and in its experimental model-adjuvant arthritis. The control of inflammation and OS in arthritic patients by hyaluronic acid (HA) is one of the approaches to the treatment of RA, concentration of which is reduced in the synovial fluid of patients suffering from arthritis. The most important aspect from a treatment perspective is the fact that HA has been found to be safe and well tolerable. The widespread use of HA also leads to lower use of nonsteroidal anti-inflammatory drugs, which may be an advantage for patients. HA is a high-molecular-weight, ubiquitous glycosamino-glycan (GAG) that naturally occurs within the cartilage and synovial fluid [51]. It is an anionic linear polysaccharide composed of alternating N-acetyl D-glucosamine and D-glucuronic acid residues attached by β(1-4) and β(1-3) glycosidic bonds (**Figure 5**) with molecular mass ranging from 6.5 to 10.9 MDa [52]. It is structurally the simplest compound among GAGs. HA has hydrophilic groups which not only form hydrogen bonds with each other but also interact with water molecules.

In physiological solutions hyaluronan manifests very unusual rheological properties and has exceedingly lubricious and very hydrophilic properties. This is the reason why HA occurs in the salt form, hyaluronate, and is present in every connective tissue and organ such as the skin, synovial fluid, blood vessels, serum, brain, cartilage, heart valves, and the umbilical cord. Synovial fluid in particular has the highest concentration of HA (3–4 mg/ml) compared to anywhere else in the body [53]. HA plays important physiological roles in living organisms which makes it an attractive biomaterial for various medical applications [54, 55]. HA has several diverse physiological functions. Because of its hygroscopic properties, hyaluronan significantly influences hydration and the physical properties of the extracellular matrix. In addition to its function as a passive structural molecule, hyaluronan also acts as a signaling molecule by interacting with cell surface receptors resulting in the activation and modulation of signaling cascades that influence inflammatory processes, including the antioxidant scavenging of the ROS and/or RNS arising from polymorphonuclear nucleosides' respiratory bursts as well as cell migration,
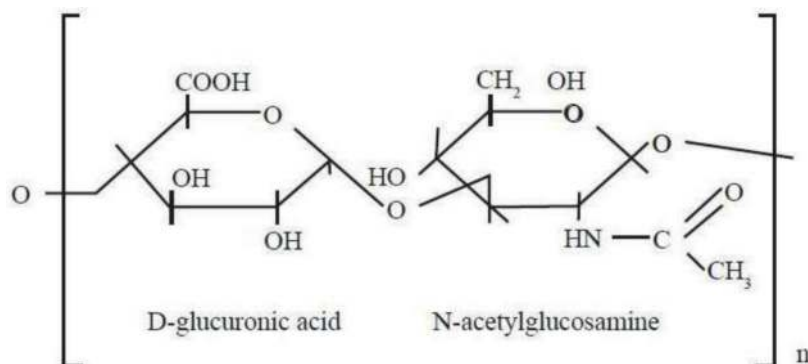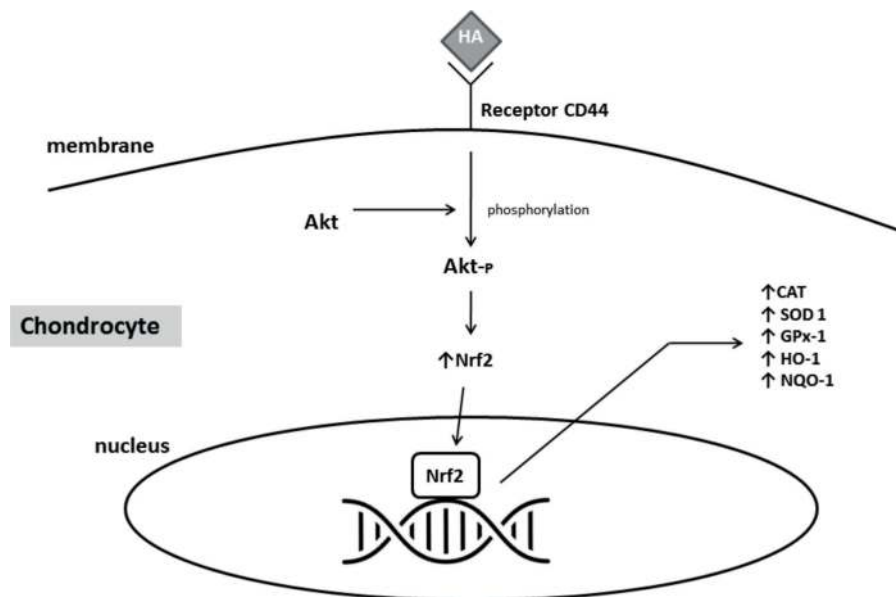

Figure 5.
Chemical structure of hyaluronan.
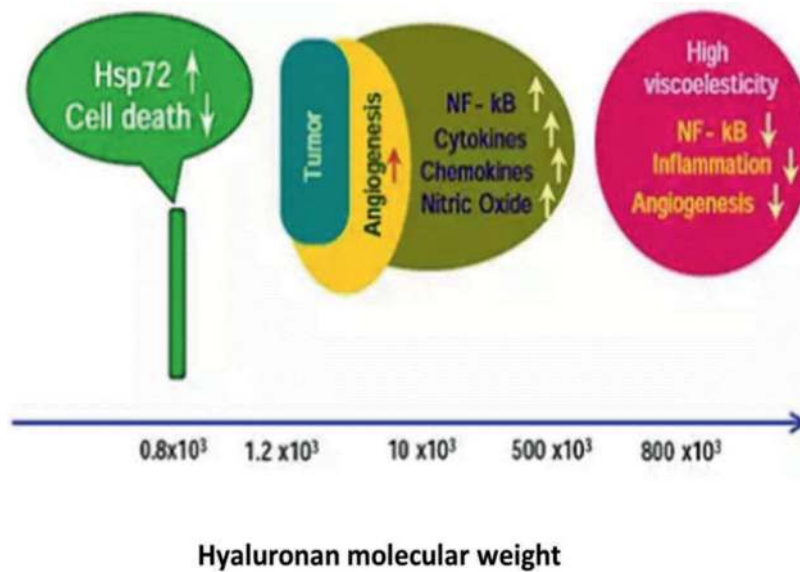
Figure 6.
Biological effects of hyaluronan oligosaccharides depend on their molecular weight.

proliferation, and gene expression [55]. Moreover, there is a brisk metabolism of HA in humans, with approximately one-third (around 5 g) of total HA were degraded and replaced daily predominantly by the reticuloendothelial system [56].

Many physiological effects of HA may be functions of its molecular weight. Already in the year 2000, Camenisch and McDonald [57] published an overview of the effects of HA, dependent on its molecular weight. HA of an average mass of 0.2 MDa prolonged survival of peripheral blood eosinophils in vitro but HA of the mass of 3–6 MDa had a much lower effect. This observation follows from several previous reports suggesting distinct angiogenic and pro-inflammatory biological activities of lower molecular weight HA or HA oligomers. Lower molecular weight HA, but not high-molecular-weight HA, stimulates the production of metalloelastase and expression of inducible nitric oxide synthase in rat liver endothelial and Kupffer cells. In addition, it has been reported that low-molecular-weight degradation products of HA elicit pro-inflammatory responses by modulating the toll-like receptor-4 or by activating the nuclear factor kappa B (NF-kB). In contrast, high-molecular-weight HA manifests an anti-inflammatory effect via CD receptors and by inhibiting NF-kB activation [58] (**Figure 6**). During progression of inflammation and OS in the joints, HA depolymerizes into lower molecular weight compounds (2.7–4.5 MDa) which consequently diminish the mechanical and viscoelastic properties of the synovial fluid [51] as well as activate different signaling pathways. Randomized, double-blinded, placebo-controlled trials have proven the effectiveness of HA (administered by the intra-articular injection or in the form of dietary supplements 48–240 mg/day) for the treatment of symptoms associated with synovitis [53].

Kogan et al. [59] suggest mechanisms, by which HA could exert its therapeutic effect: (i) restoration of elastic and viscous properties of the synovial fluid; (ii) induction of the endogenous synthesis of HA by synovial cells by the effect of exogenous HA, stimulation of chondrocyte proliferation, and inhibition of cartilage degradation; (iii) anti-inflammatory action of HA, since the therapy is associated with decreased inflammatory cell count in synovial fluid, modulation of cytokine expression, and reduction of ROS content; and (iv) analgesic effect. An important feature of HA is its antioxidant properties. The direct radical-scavenging properties of HA have been demonstrated in various experimental models. These results are in accord with the concept that hyaluronic acid mainly acts as a chemical ROS

Figure 7.
Hyaluronan increases enzymatic antioxidant defense through Nrf2 upregulation. HA, hyaluronan; AKT, serine/ threonine protein kinase; Nrf2, factor erythroid 2-related factor 2; CAT, catalase; SOD-1, superoxide dismutase-1; GPx-1, glutathione peroxidase; HO-1, hemeoxygenase-1; NQO-1, NAD(P)H (quinone)dehydrogenase 1.

and/or RNS scavenger in extracellular space [55]. In favor of a direct antiradical activity, there is also the ability of hyaluronic acid (biopolymer) to form a viscous, pericellular meshwork that restricts ROS movement in close proximity to cells and thus interferes with the oxidative cascade [55]. The presence of CD44 hyaluronate receptors on the plasma membrane of granulocytes, which mediate the internalization of the biopolymer via endocytosis, offers another key to interpretation of the HA antioxidant mechanism of action, that is, the reduction in ROS and/or RNS is caused by hyaluronic acid internalization and the intracellular neutralization of the radicals [60]. One important pharmacological function of HA is the reduction of cellular superoxide generation and accumulation through nuclear factor erythroid 2-related factor 2 (Nrf2) regulation, which is a master transcription factor in cellular redox reactions. Antioxidants and phase II detoxifying enzymes such as catalase (CAT), superoxide dismutase (SOD), heme oxygenase-1, glutathione S-transferase, glutathione peroxidase (GPx), and thioredoxin are coordinated at transcription level by Nrf2, so the hyaluronic acid could affect the activity and quantity of these antioxidant enzymes (**Figure** 7) [61].

The aim of this study was to compare the effect of different molecular weights of hyaluronic acid (0.43, 0.99, and 1.73 MDa) applied in two different doses (0.5 and 5 mg/ kg b.w.), on the rat hind paw volume and parameters of OS: activity of antioxidant enzymes in erythrocytes (SOD, GPx, CAT), total antioxidant capacity, and concentration of lipid hydroperoxides (LPx, marker of oxidative damage to lipids) in plasma.

3.2 Experimental design of adjuvant arthritis with administration of hyaluronan

Male Lewis rats were randomly divided into groups according to the treatment they received:

(1) Not treated control groups (HC)

(2) Arthritic animals not treated with HA (AA)

(3) Arthritic animals treated with hyaluronic acid (HA) during 28 days:

  (a) Group NHA (M_w(HA) = 0.43 MDa, in an oral daily dose of
      0.5 mg/kg b.w.)

  (b) Group 5NHA (M_w(HA) = 0.43 MDa, in an oral daily dose of 5 mg/kg b.w.)

  (c) Group SHA (M_w(HA) = 0.99 MDa, in an oral daily dose of
      0.5 mg/kg b.w.)

  (d) Group 5SHA (M_w(HA) = 0.99 MDa, in an oral daily dose of 5 mg/kg b.w.)

  (e) Group VHA (M_w(HA) = 1.79 MDa, in an oral daily dose of 0.5 mg/kg b.w.)

  (f) Group 5VHA (M_w(HA) = 1.79 MDa, in an oral daily dose of
      5 mg/kg b.w.).

Adjuvant arthritis was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* [36, 37]. Blood was collected to obtain plasma and erythrocytes. Total antioxidant capacity and concentration of LPx were determined in plasma. Isolated erythrocytes were washed three times with 0.15 mol/l NaCl solution. After centrifugation ($900 \times g$, 5 min, 4°C), erythrocytes were hemolyzed by adding a triple volume of cold distilled water and stored at −20°C until further analyses. Activities of Cu/Zn-superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and the concentration of hemoglobin (Hb) were determined in the hemolysate of erythrocytes. From clinical parameters hind paw volume (HPV) was evaluated [62]. The activity of SOD was determined using a commercial kit. The results are expressed in U of SOD per mg Hb. The activity of GPx was also determined by a commercial kit. The results are expressed in μkat per g Hb. CAT activity was determined by a modified method according to [63], and the results are expressed in μkat per g Hb. The total antioxidant capacity of plasma was measured using the Trolox equivalent antioxidant capacity (TEAC) assay [64]. Quantification was performed using the dose-response curve for the reference of antioxidant Trolox, a water-soluble form of vitamin E. The results are presented as mmol of Trolox per ml of plasma. The level of LPx in plasma was measured using the method previously described by [64], and the results are presented in nmol per ml of plasma. The experimental data were expressed as the mean ± SEM. Statistical analysis was performed using Student's t-test. The limit for statistical significance was set at $p < 0.05$.

3.3 Evaluation of results of administration of hyaluronans in experimental arthritis

The onset of AA confirmed the increased hind paw volume in arthritic groups (data not shown). HA administration did not cause a significant reduction of HPV in any molecular weight and at any doses used. Parameters of OS are summarized in **Table 5**. Rats with AA had significantly higher activity of SOD and CAT in erythrocytes as well as higher concentration of LPx in comparison to HC group. Activity of GPx was marginally increased ($p = 0.054$) and TEAC was not changed.

The effect of hyaluronic acid on antioxidant enzyme activities, the total antioxidant capacity of plasma, and the effect on LPx concentration are summarized in **Table 6**. We have found significantly higher erythrocyte SOD activity after administration of HA in all molecular forms and doses, whereas GPx activity was significantly higher only after HA administration at the higher dose. At a lower

Parameter	HC	AA	P
SOD (U/mg Hb)	422.65 ± 15.93	546.48 ± 14.25	0.0001
GPx (μkat/g Hb)	51.10 ± 1.45	56.86 ± 2.26	0.054
CAT (μkat/g Hb)	2.61 ± 0.18	3.06 ± 0.09	0.044
TEAC (mmol/l)	4.01 ± 0.07	4.04 ± 0.09	n.s.
LPx (nmol/ml)	20.76 ± 3.55	53.34 ± 5.83	0.003

Control group (HC), arthritis group (AA), statistical significance (P).
Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in erythrocytes, total antioxidant capacity (TEAC), and concentration of lipoperoxides (LPx) in plasma were measured on the 28th day.

Table 5.
Oxidative stress markers in rats with adjuvant arthritis.

dose, we observed a significantly elevated GPx activity only in the SHA group (M_w(HA) = 0.99 MDa). For both enzymes, we have also noticed a significant difference between the effects of the same molecular forms of HA but administered at different doses. The higher HA dose (5 mg/kg b.w.) significantly increased the GPx activity compared to the lower dose (0.5 mg/kg b.w.).

Subanalysis based on the molecular weight of the administered HA revealed the higher SOD activity in the VHA group compared to NHA (p = 0.0013), in the 5SHA and 5VHA groups compared to 5NHA (p = 0.0025, respectively, p = 0.0308), and VHA compared to SHA (p = 0.0107). The higher GPx activity was found in SHA group in comparison to NHA (p = 0.0425), in 5SHA, respectively, 5VHA in comparison to 5NHA (p = 0.0217 respectively p = 0. 0058), and lower activity in VHA group in comparison to SHA (p = 0.0069). A similar trend was observed in the effect of HA on the total antioxidant capacity of plasma. The values of this parameter were increased in all groups but significantly only when HA was administered at a higher concentration.

Also, the higher HA dose significantly increased the total antioxidant capacity compared to the lower dose. Differences in the effect of HA with different molecular weights were seen only in the 5VHA group, whereas TEAC was significantly increased when compared to the 5NHA group (p = 0.0212). On the other hand, HA in all molecular weights and at both monitored doses significantly reduced CAT activity. The effect of different doses was found only in SHA and VHA groups, where the higher dose significantly reduced activity in SHA (p = 0.0004) and significantly increased activity in VHA (p = 0.0192). At higher doses, we found significant reductions of CAT activity in VHA compared to both NHA and SHA (p = 0.0018 and p = 0.0001) and in 5SHA compared to 5NHA (p = 0.0194). Concentration of LPx was significantly reduced in all monitored groups, with no differences in the effect of different molecular weights of HA or in the effect of doses.

Our study, for the first time, evaluated the ability of the HA to affect the activity of erythrocyte antioxidant enzymes, as well as total antioxidant capacity and LPx of rats with AA. We have found increased activities of antioxidant enzymes (SOD, GPx and CAT) in erythrocytes of AA rats with increased plasma LPx concentration. Administration of different molecular weights of HA (0.43, 0.99, and 1.73 MDa) applied in two different doses (0.5 and 5 mg/kg b.w.) resulted in a further increase in activities of these enzymes, but we observed a decreased concentration of plasma LPx.

Inflammatory diseases, including RA, are characterized by sustained overproduction of ROS, accompanied by disruption of the antioxidant defense system resulting in local and systemic OS development in the affected joint-synovial fluid [65], and in addition to the joints, plasma and some organs are affected [66]. The results of the present work showed that in spite of the increased antioxidant enzyme

Parameter	AA	NHA	5NHA	SHA	5SHA	VHA	5VHA
SOD (U/mg Hb)	546.48 ± 14.25	615.26 ± 23.00*	817.37 ± 28.45***	661.41 ± 24.34**	944.73 ± 41.35***	777.17 ± 30.91***	869.42 ± 34.96***
GPx (μkat/g Hb)	56.86 ± 2.26	55.53 ± 3.297	71.17 ± 3.76**	64.95 ± 2.65*	84.89 ± 4.20***	55.44 ± 1.40	93.52 ± 6.83***
CAT (μkat/g Hb)	3.06 ± 0.09	2.61 ± 0.15*	2.40 ± 0.08***	2.81 ± 0.12	2.06 ± 0.10***	2.02 ± 0.05***	2.28 ± 0.09***
TEAC (mmol/l)	4.04 ± 0.09	4.23 ± 0.09	4.57 ± 0.03**	4.07 ± 0.10	4.80 ± 0.24**	4.19 ± 0.10	4.73 ± 0.06**
LPx (nmol/ml)	53.34 ± 5.83	24.35 ± 1.19**	26.84 ± 1.70*	25.3 ± 4.67**	27.98 ± 3.45*	25.13 ± 5.53**	24.49 ± 2.67*

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$ vs. AA.

NHA ($M_w(HA) = 0.43$ MDa, 0.5 mg/kg b.w.); 5NHA ($M_w(HA) = 0.43$ MDa, 5 mg/kg b.w.); SHA ($M_w(HA) = 0.99$ MDa, 0.5 mg/kg b.w.); 5SHA ($M_w(HA) = 0.99$ MDa, 5 mg/kg b.w.); VHA ($M_w(HA) = 1.79$ MDa, 0.5 mg/kg b.w.); 5VHA ($M_w(HA) = 1.79$ MDa, 5 mg/kg b.w.).

Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in erythrocytes, total antioxidant capacity (TEAC), and concentration of lipoperoxides (LPx) in plasma were measured on the 28th day.

Table 6.
Effect of hyaluronic acid on oxidative stress markers.

activities found in erythrocytes of rats with AA, lipid peroxidation in plasma is increased in comparison to control group. Lipid peroxides are generated at the site of tissue injury due to increased ROS production during chronic inflammation and diffuse into blood where they can be estimated [67]. Studies have reported raised levels of MDA, 4-hydroxynonenal, and other markers of oxidative lipid damage in the serum, plasma, and erythrocytes of RA patients [65, 68]. Kumar et al. [68] also found increased plasma SOD activity in patients with RA, similarly to Mazzetti et al. [69]. Similar results were found in early type 2 diabetes patients [70], where increased antioxidant defense in plasma and erythrocytes is explained as a potential mechanism that can overcome oxidative damage induced by ROS overproduction. There are some reports on erythrocyte SOD, CAT, and GPx activities in patients with RA or in rats with AA, but the results are controversial [71, 72]. It is possible that differences between different investigators' results, regarding antioxidant status, are due to differences in the stage of the disease. Chronic inflammation may deplete antioxidant defenses, whereas acute inflammation can upgrade them [73].

In our study, we did not notice a change in total plasma antioxidant capacity in the group of arthritic rats, similarly to Bracht et al. [66] in the mono-arthritic rats. Vijayakumar et al. [74] confirmed susceptibility of erythrocytes to peroxide stress. They have found not only elevated plasma lipid peroxidation but also the excessive lipid peroxidation in erythrocytes and erythrocyte membranes. In addition, they found decreased glutathione levels and GPx activity in plasma but increase in erythrocytes of RA patients as compared to healthy subjects. Superoxide radicals play an important role as a chemical mediator on the inflammatory response to RA. The increased activity of plasma SOD observed in the abovementioned studies as well as increased activity of SOD in erythrocytes observed in our study could therefore be found due to their function in dismutation of superoxide radicals excess. Thus, the activities of antioxidant enzymes in blood cells including erythrocytes could reflect the rate of OS in the affected cells. This could be a suitable approach for assessing the effect of therapy aimed to reduce inflammation and OS.

Our study demonstrated that HA (in all molecular weights and in both doses), orally administered in a rat model of AA, affected all measured markers of OS. Furthermore, erythrocyte antioxidant markers including SOD and GPx, and total antioxidant capacity of plasma, increased significantly during 28 days of supplementation. On the other hand, we have found decreased erythrocyte CAT activity and plasma concentration of LPx. Based on our results, we cannot give a clear explanation how the HA can affect all observed parameters. Numerous studies have confirmed the effect of HA on the activity of these enzymes but in other cells and tissues and not in erythrocytes [75, 76]. It was confirmed that HA can reduce cellular superoxide generation and its accumulation through Nrf2 regulation which can induce transcription of antioxidant enzymes such as SOD, GPx, CAT, and others [61]. Supplementary to its primary role in cytoprotection, Nrf2 is also linked to differentiation, proliferation, growth, and apoptosis, and it is thought that Nrf2 has evolved from an original role in hematopoiesis and the regulation of cell differentiation from early lineages [77].

Based on this, we could assume that during proliferation and differentiation of hematopoietic stem cells, expression of antioxidant enzymes can be induced. In the induction of expression, the CD44 receptor, which binds HA and mediates its role as a signal molecule, could have importance. We could just speculate if the activities of antioxidant enzymes in erythrocytes reflect the effect of administered HA on the activities of these enzymes in other tissues as well. As we observed an increase in both SOD and GPx activities in erythrocytes of AA rats under HA supplementation, we anticipate a similar mechanism of HA action in other cells, e.g., in chondrocytes, where the increase in antioxidant potential could provide antioxidant protection

of synovial fluid and reduction of lipoperoxidation not only in the synovium but also in the plasma as what we have found in our study. Also, the direct antioxidant ability of HA, which has been described, could contribute to the reduction of lipoperoxidation [55]. However, further studies need to be made to confirm these assumptions.

4. Conclusion

New treatment strategies based on blockade of cytokine pathways in late stages of RA are progressing. In spite of their benefits, long-time utilization of these blocking agents has indicated side effects. The antioxidant defense system includes endogenous (enzymatic and nonenzymatic) and exogenous (dietary) antioxidants that interact in establishing redox homeostasis in the body. Therapeutic benefits from antioxidant treatment are primarily bound to reduction of systemic OS.

CoQ_{10} treatment significantly improved concentrations of the investigated endogenous antioxidants (CoQ_9-total, CoQ_{10}-total, and gamma tocopherol in plasma). CoQ_{10} for its bioenergetic, antioxidant, and anti-inflammatory properties might be therapeutically useful for a long-term supplementary administration to patients with inflammatory diseases such as RA.

In our study, we have shown the ability of per orally administered HA to improve the antioxidant defense (SOD, GPx, and total antioxidant capacity of plasma). This therapeutic effect of HA could be a result of direct (intestinal absorption) and indirect (intestinal cell immunomodulatory) anti-inflammatory activities. Recent results have shown that dietary high-molecular-weight HA can be distributed to connective tissues. Dietary HA could be beneficial for joints, knee pain, relief of synovial effusion, or inflammation and improvement of muscular knee strength. HA also binds to toll-like receptor-4 (TLR4) in the luminal surface of the large intestine resulting in the downregulation of systemic proinflammatory cytokines.

However, further research is needed with endogenous antioxidants, mainly human studies, in order to establish an antioxidative treatment approach in inflammatory-based diseases such as RA.

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Conflict of interest

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

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