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BIOCHEMICAL EFFECT EVALUATION OF MICROBIAL CHONDROITIN SULPHATE IN EXPERIMENTAL KNEE OSTEOARTHRITIS MODEL

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ABSTRACT

Osteoarthritis (OA) is the most common chronic joint disease, primarily due to aging. Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) commonly used to treat osteoarthritis. CS can delay the progression of pathology or reverse morphological changes in joint structure. Traditionally CS is produced from animal sources. However, due to different reasons such as contamination, ecological risk, and the possibility of infectious diseases, the trend towards microbial sources has increased because of its advantages such as purer, more antiallergic, and lower Molecular Weight (MW) than animal sources. Biochemical analysis of Microbial CS (MCS), which is a new and significant alternative as a source of CS in the OA healing process, has not been evaluated in the literature yet. This study was designed to analyze the biochemical effects of MCS produced by our team from a microbial source, with an MW value of 269 Daltons, on the osteoarthritis healing process compared to the commercial form. We aim to reach data that MCS has a higher antioxidant effect than animal-sourced CS, and in this way, it is a more suitable production for the treatment of osteoarthritis. In this study, knee osteoarthritis was surgically induced in experimental rabbits; and TGF-1β, CAT, MPO, TOS, and OSI parameters measured in blood samples before the operation and after the healing period were analyzed comparatively. After the surgical application, the rabbits were randomly divided into three groups: control, animal-sourced CS, and *E. coli* sourced MCS. The standard rabbit diet was administered daily to 10 rabbits in Group 1 (control), and CS and MCS were applied daily to the other groups as 17 mg/kg for 12 weeks. Blood samples were taken from rabbits at the 12th week after surgery, and TGF-1β, CAT, MPO, TOS, and OSI parameters were biochemically evaluated. This study has confirmed that the antioxidant properties of MCS and data on its effectiveness in controlling oxidative stress compared to animal-sourced CS. Based on these results, it can be concluded that MCS has a significant potency of nutraceutical and therapeutic agents for OA treatment.

KEYWORDS:
Chondroitin sulfate, Microbial chondroitin sulfate, Osteoarthritis

INTRODUCTION

OA, known as degenerative joint disease, is characterized by a progressive loss of articular cartilage in the synovial joints. [1]. Pharmacological treatment of OA is aimed at preventing pain and improving joint function. Standard treatments mainly include a combination of pharmacological and non-pharmacological methods. The first option is pharmacological therapy with analgesics or Non-steroidal anti-inflammatory drugs (NSAIDs) to control pain and improve motility [2]. However, various studies have reported that the long-term use of NSAIDs can cause adverse effects on the gastrointestinal and cardiovascular systems. Therefore, considering the prolongation of the patient treatment period over the years, the need for new therapeutic agents for OA treatment that can relieve pain and potentially reduce damage has developed. The natural extractive compound CS has been adopted as an alternative to NSAIDs. [3, 4]. CS is an effective substance in delaying joint damage, reducing pain, and improving joint mobility in OA patients, and it is in the class of Symptomatic Slow-Acting Drugs (SySADOAs) and Disease-Modifying Osteoarthritis Drugs (DMOADs) for OA.

CS is an essential component of the extracellular matrix. It is found in the extracellular matrix of many tissues such as cartilage, bone, skin, and tendons [5]. It is widely used in medical applications as cell and tissue regenerative. Previous studies have demonstrated that the differences in the

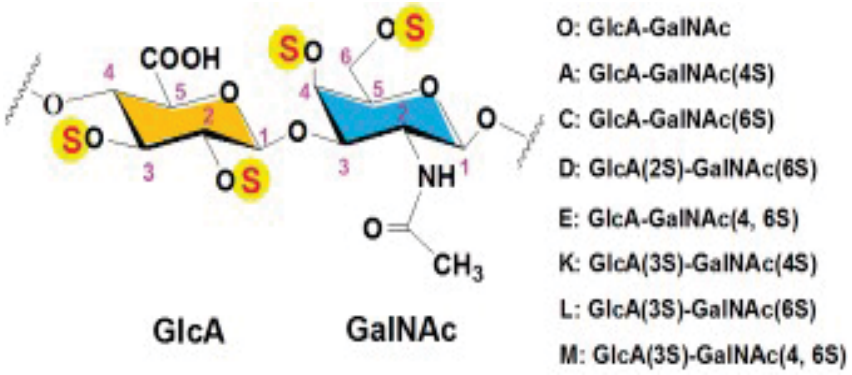


FIGURE 1
The structural formula of CS and its types according to the position of sulfate groups [9]

sources from which CS is obtained and the CS production methods affect the degree of CS purity, and consequently, its effectiveness in treatment may change [6, 7]. Furthermore, depending on the source of CS, different bioavailability and pharmacokinetic properties have been determined [8]. The molecular weight of animal-sourced CS varies from species to species and is approximately 50-100 kDa on average. Microbial CS has all the functions of animal-sourced CS and has a lower molecular weight (10-30 kDa) than its animal-derived counterparts. For this reason, it provides a higher absorption after oral intake and is more effective in the treatment process [8]. CS is a sulfated glycosaminoglycan (GAG) consisting of a long unbranched polysaccharide chain formed by repeating the disaccharide structure consisting of N-acetyl galactosamine and Glucuronic acid (Figure 1)

Different types of CS are formed by changing the number of sulfate groups attached to chondroitin and the position to which these groups are attached. Animal-sourced CS mostly shows CS-A and CS-C structures, and double sulfation is not encountered, while marine-sourced CS is found in CS-A, CS-C, and CS-E forms. The MCS that we produce is in CS-A and CS-E forms like animal-sourced CS (Figure 2). These structural properties were verified by Nuclear Magnetic Resonance (NMR) and High Performance Liquid Chromatography (HPLC) analysis.

CS is absorbed by 70% when intake orally [10], decreases leukocyte elastase and hyaluronidase activity, and increases the hyaluronic acid ratio [11]. The anti-inflammatory, antioxidant, antimicrobial, and antiapoptotic properties of CS make it effective in medical applications. In these usances, animal-sourced CS is mainly preferred. Prionic, viral and ecological risks, which are frequently attributed to animal sources in the current literature, have led to the need for alternative resources in this area. MCS obtained from microbial sources has high purity, low MW values, therefore higher kinetic activity. Moreover, It does not carry prionic or viral risks and is an essential alternative to animal sources with its unique, antiallergic, and biocompatible, non-toxic properties. In the European League against Rheumatism (EULAR) recommendation on OA, CS has been awarded 1A and A ratings, expressing both the highest degree of evidence and the highest recommendation strength [12].

CS is one of the SYSADOAs. The initial effects of SYSADOA treatments other than analgesics and NSAIDs are noticeable after 2 to 3 weeks of regular intake and have a prolonged effect that persists for several

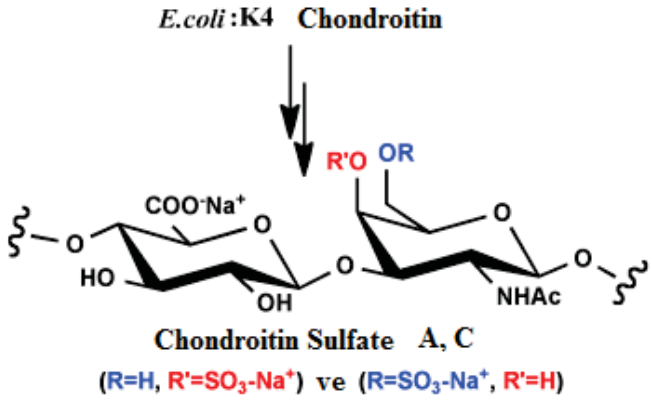


FIGURE 2
The structural formula of MCS produced from *E. coli* K4 strain [7]

months. CS affects OA symptoms such as pain and inflammation and acts as a structure-modifying OA drug (SMOAD). CS enhances hyaluronan production in human synovial cells, which has a beneficial effect on maintaining synovial fluid viscosity [13]. CS stimulates chondrocyte metabolism and leads to collagen and proteoglycan synthesis, the essential components of new cartilage. In addition, CS inhibits high concentrations of leukocyte elastase and hyaluronidase enzymes in the synovial fluid of patients with rheumatic disease. CS increases the production of hyaluronic acid by synovial cells, which then improves viscosity and synovial fluid levels. In general, CS inhibits cartilage destruction processes and stimulates the anabolic processes involved in forming new cartilage [14]. MCS has the advantage of fulfilling these effects more quickly and effectively because of its MW value of 269 Daltons. MCS, our team has produced by biotechnological methods, was also verified by HPLC and NMR analysis (Figure 3 and 4) [15]. It is in the vegan product category as it is produced from a microbial source.

In CS in natural products, the sulfate group in position 4 is never present on its own but is only found bound to the sulfate group at position 6. Generally, commercial chondroitin sulfate contains 40% sulfate at position 4 and 60% sulfate at position 6. The sulfonation process was carried out on the microbial chondroitin obtained at position 6.

In the NMR analysis, the standard structure and the MCS spectrum structure produced were identical. In both spectra (Figure 4), the CS-based H4 proton at 4.74' and H6 proton at 4.21' are clearly observed. At 4.15', the NH amide proton can be clearly observed. In addition, aliphatic methyl hydrogen at 1.98' and ether-based CH peaks at 3.61' can be seen.

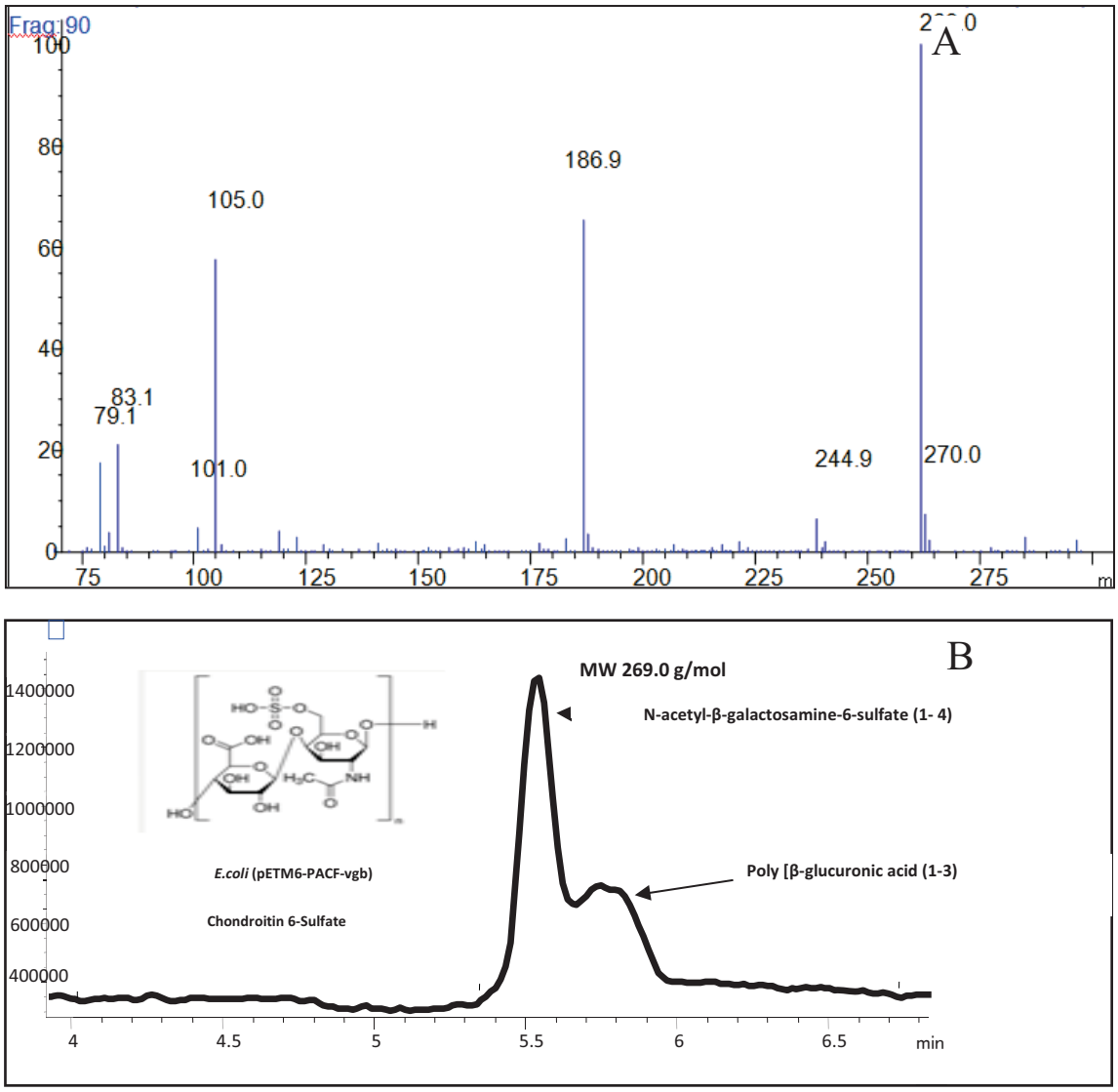


FIGURE 3
A and B: HPLC result verifying the *E. coli* (*kfo-vgb*) strain derived CS production [15].

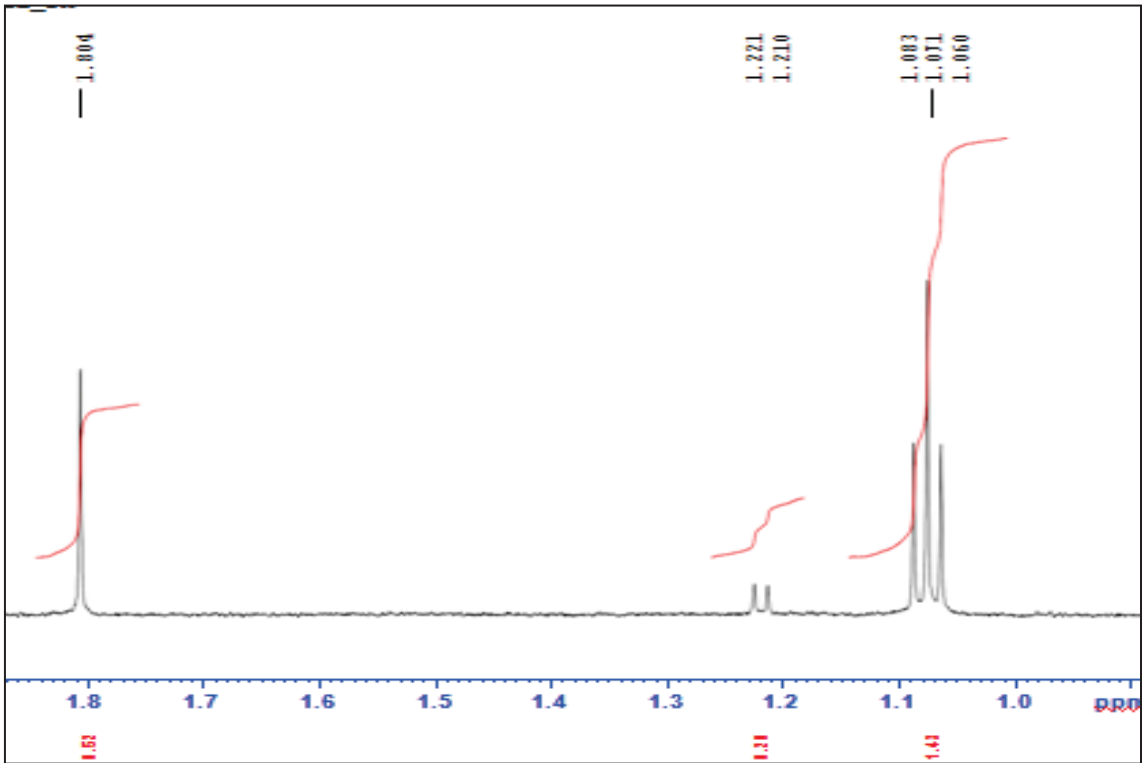


FIGURE 4
NMR result confirming MCS production in *E. coli* (kfo-vgb) strain [15].

Arthropathies such as osteoarthritis and rheumatoid arthritis are associated with increased free radical formation [16-18]. Reactive oxygen species (ROS), commonly expressed in OA [19-22], plays an important role in matrix and cartilage degeneration, inhibition of matrix synthesis, cell death, and apoptosis of chondrocytes. Specific mediators produced in OA may act directly on cartilage and multiple signaling pathways that activate other key inflammatory molecules. The varying effectiveness of these pathways is associated with the pathophysiology of OA. It is crucial to evaluate the effectiveness of CS in the pathophysiological process through biochemical parameters. In vitro experiments confirmed that mechanical shear stress increases oxidant production in cartilage explants. [23]. For this reason, the primary motivation of our study is to obtain data on the microbial-sourced CS with low MW, which has been proven to be effective in the treatment of OA due to its antioxidant effect, compared to animal-sourced CS, and maybe more advantageous for osteoarthritis treatment. In this study, MCS produced by biotechnological methods in recombinant strains created by our team was used [15,24]. In the experimental osteoarthritis model created using Himalayan rabbits, the effects of CS produced in *E. coli* (C2987) strain on the osteoarthritis healing process were evaluated using biochemical parameters compared to animal-sourced CS.

MATERIALS AND METHODS

In this study, 40 adult New Zealand rabbits, weighing between 3000–4000 g were used for the anterior cruciate ligament dissection model to create experimental osteoarthritis. At the beginning of the study, sufficient blood samples were taken for biochemical analysis and kept until the end of the study. For anesthesia, 0.1 ccs/kg 2% xylazine hydrochloride and 20 mg/kg ketamine hydrochloride were administered intramuscularly, and then the right knee joints were reached with an anterior longitudinal incision. After medial parapatellar arthrotomy, the patella was dislocated laterally, and the anterior cruciate ligament was cut. Whether the cruciate ligament was cut completely or not was evaluated with the anterior drawer test, and the experimental animals were left to normal cage activity in the postoperative period. Rabbits were randomly divided into 4 groups after 4 weeks following surgical intervention. 10 rabbits in Group1 [Control] have fed with the standard rabbit diet daily for 12 weeks. 10 rabbits in Group2 [TCS] have fed with 17 mg/kg/day of animal-sourced CS with a standard diet. CS used in this group was obtained from cattle trachea, and supplied in packaged lyophilized powder form. This product has been chosen from the SIGMA catalog, among the alternatives, as it is the most cost-effective and relatively easy to obtain. The other 10 rabbits in Group3 [ECS] have fed with 17mg/kg/day of recombinant *E. coli*-derived microbial CS produced

in our project with the standard diet. The microbial CS types used in this study were produced by our team [15], and Sigma Aldrich C9819 product was used as commercial-animal CS. At the end of 12 weeks, blood samples were retaken from the rabbits, and biochemical analyses were performed.

At the beginning and end of the experimental animal study process, a sufficient amount (approximately 10 ccs) of blood samples were taken from rabbits' ears. TGF-1β (Transforming growth factor-beta 1), Catalase (CAT), Miyeloperoksidaz (MPO), Total antioxidant status (TAS), Total oxidant status (TOS), and Oxidative Stress Index (OSI) parameters were analyzed for biochemical analysis. TAS and TOS levels were determined using colorimetric kits (Rel Assay, Turkey) based on measuring color changes caused by oxidants and antioxidants in the samples at 660 nm wavelength. OSI values were calculated according to the following formula OSI = TOS/TAS. TGF-1β levels were measured using ELISA (Enzyme-Linked Immunosorbent Assay) method (Boster Biological Technology, California, USA). Rabbit TGF-1β present in the sample bound to antibodies adsorbed to the microwells. Captured TGF-1β in the samples was stabilized by a biotin-conjugated anti-rabbit TGF-1B antibody. Finally, a colored absorbance of the product was measured at 450 nm wavelength. CAT activity was measured by the Aebi method [25]. The principle of this method was based on the hydrolyzation of H2O2 and decreasing absorbance at 240 nm. MPO activity was evaluated according to Bradley et al.'s method [26].

RESULTS

There was no statistical difference between Control, ECS, and TCS groups in TGF-1β, CAT, MPO, TAS, TOS and OSI parameters measured in serum samples taken at the beginning of the study ($p>0,05$). The data of the measurements at the beginning of the study are given in Table 1. The measurement results of the serum samples taken at the end of the study are given in Table 2. Statistically significant decreases were observed between groups in TGF-1β, CAT, MPO, TOS, and OSI parameters ($p<0,05$). It was observed that the decreases in these parameters were higher in ECS groups compared to the control and TCS groups, and it was statistically significant ($p <0.05$). TAS results were statistically significantly higher in the ECS and TCS groups compared to the control group ($p<0.05$). In the comparison between the ECS and TCS groups, it was observed that the TAS results of the ECS group were higher than those of the TCS group ($p<0.05$).

TAS, TOS, and OSI are markers associated with the general oxidative stress of the organism. When oxidative stress increases, TAS decreases with an increase in TOS and OSI. In this study, besides the increase in TAS in the ECS group, the decrease in TOS and OSI shows that MCS has a strong antioxidant activity. CAT and MPO parameters are antioxidant enzymes that increase, especially in

TABLE 1
The values of TGF-1β, CAT, MPO, TAS, TOS, and OSI parameters at the beginning of the study

	TGF-1β (pg/ml) Median (Min-Max)	**TAS (mmol/L)** Median (Min-Max)	**TOS (µmol/L)** Median (Min-Max)	**CAT (U/L)** Median (Min-Max)	**MPO (U/L)** Median (Min-Max)	**OSI** Median (Min-Max)
CNT*	751,24[a] (712,96-805,63)	1,2495 [a] (0,80-1,53)	7,58 [a] (3,65-9,75)	38,05 [a] (30,70-46,50)	125,00 [a] (112,72-142,94)	6,07 [a] (4,56-7,69)
ECS	746,23 [a] (713,65-785,00)	1,22 [a] (1,13-1,31)	7,96 [a] (5,57-8,25)	38,90 [a] (36,60-48,90)	125,89 [a] (120,79-136,52)	6,69 [a] (4,46-6,93)
TCS	774,23 [a] (675,01-789,63)	1,24 [a] (1,09-1,26)	6,98 [a] (6,05-9,93)	42,90 [a] (40,80-45,70)	132,68 [a] (119,78-135,96)	5,57 [a] (4,89-7,88)

*CNT refers to Control

TABLE 2
The values of TGF-1β, CAT, MPO, TAS, TOS, and OSI parameters at the end of the study

9	**TGF-1β (pg/ml)** Median (Min-Max)	**TAS (mmol/L)** Median (Min-Max)	**TOS (µmol/L)** Median (Min-Max)	**CAT (U/L)** Median (Min-Max)	**MPO (U/L)** Median (Min-Max)	**OSI** Median (Min-Max)
CNT*	1273,85 [a] (1026,00-1417,55)	1,39 [c] (1,11-1,69)	16,28 [a] (15,39-17,58)	80,40 [a] (72,50-85,10)	202,00 [a] (195,00-223,00)	11,45 [a] (9,46-15,89)
ECS	812,36 [c] (805,36-838,00)	2,83 [a] (2,74-2,98)	8,17 [c] (8,05-8,99)	57,50 [c] (48,60-60,50)	162,00 [c] (160,00-174,23)	2,93 [c] (2,82-3,18)
TCS	963,25[b] (942,00-1000,00)	2,27 [b] (2,21-2,38)	13,23 [b] (12,56-13,79)	65,10 [b] (62,70-67,30)	185,00 [b] (181,00-194,56)	5,81 [b] (5,56-6,07)

*CNT refers to Control

inflammation events. The reduction of these two enzymes, such as TOS, indicates that MCS supports the antioxidant system more than the animal-sourced CS and calms inflammatory events. TGF-1β is a cytokine that increases in inflammation and bone resorption events. In the study, the increase in TGF-1β in the control group indicates increased bone destruction and inflammation. Decreases in ECS and TCS groups decreased destruction and inflammatory events, and a higher decrease in the ECS group indicates higher efficiency of MCS.

DISCUSSION

In a study, serum samples from 29 patients with Knee OA and 26 healthy controls were examined for their oxidative status, and total antioxidant capacity (TAC) and OSI were determined as antioxidant parameters [19]. Compared to healthy controls, OSI values were significantly higher, while all markers for antioxidant activity were lower in OA patients. Furthermore, Prolidase activity (a marker of collagen synthesis) was found to be significantly lower in OA patients, and enzyme activity was reported to be positively correlated with TAC and negatively correlated with oxidative stress (OS). According to these results, the higher the antioxidant concentration, the stronger the cartilage metabolism. Conversely, oxidative stress has been associated with impaired cartilage metabolism [19].

In another study, it has been reported that the concentration of antioxidants (vitamins C and E) is significantly lower, and oxidative stress is high in OA patients. Oxidative stress was measured based on malondialdehyde (MDA) concentration [20]. In addition to regeneration and anti-inflammatory processes in OA treatment, it is necessary to reduce oxidative stress in patients. As a result of this study, MCS is a significant therapeutic potency in this regard.

Chondroprotectors are essential components of cartilage metabolism and stimulate important cartilage regeneration processes, thereby adjust the balance of catabolic and anabolic processes in osteoarthritis. The chondroprotective effect of CS is known. In this study, it has been revealed that MCS can be a powerful substance for treatment with its higher antioxidant effect and higher chondroprotective effect compared to its traditional animal-derived counterpart. Current studies indicate that inflammation and oxidative stress are symptoms of all stages of OA. Chondroprotectors can block many of these symptoms. They defend chondrocytes against oxidative stress-induced apoptosis, reduce inflammatory mediator-induced articular cartilage degeneration, and reactivate the anabolic processes of the extracellular matrix components. In studies conducted with CS types, it was determined that the source and MW size significantly changed the effect of this molecule. MCS produced by our team has a low MW value. It is unique in terms of its microbial origin and advantageous in terms of its functions. Furthermore, it will be a new and powerful alternative in medical applications with the data that will be supported by new studies to be planned.

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