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ORIGINAL PAPER

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Histological changes following the administration of two different chondroitin sulfate products in experimental osteoarthritis models in rats

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ABSTRACT

Introduction. Osteoarthritis (OA) is generally a progressive disease that affects synovial joints, resulting in abnormalities to articular cartilage subchondral bone, synovium, and adjacent soft tissues.

Aim. The purpose of this work was to examine the histological changes in knee cartilage and bone following the administration of two different chondroitin sulfate products in two experimental OA models in rats.

Material and methods. OA was induced in rats by either a single injection of mono-iodoacetate or four once-weekly injections of dexamethasone. 70 adult rats were included: 30 received mono-iodoacetate, 30 received dexamethasone and the 10 remaining controls received no injection. Samples of knee bone and cartilage were then analyzed histologically.

Results. Animals with OA that received CS had significantly less inflammation, improved motor activity, and better analgesia compared with those that did not receive CS, with little difference between products. Histologically, both products reduced the signs of OA and resulted in the activation of regenerative processes of cartilage and bone and stimulation of proliferation and formation of amorphous material.

Conclusion. These results substantiate the importance of using high-quality pharmaceutical-grade CS to ensure optimal efficacy and safety of the final product in patients with OA.

Keywords. chondroitin sulfate, chondroprotection, osteoarthritis

Introduction

Osteoarthritis (OA) is generally a progressive disease that affects synovial joints, resulting in abnormalities to articular cartilage, subchondral bone, synovium, and adjacent soft tissues.[1,2] It has been estimated to affect over 40 million people in Europe, resulting in reduced quality of life and significant healthcare costs.[3] Treatment options include non-pharmacological interventions (e.g. exercise, weight loss) and pharmacological treatments.[4,5] Guidelines from the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) for patients with knee osteoarthritis suggest the

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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following – escalating – pharmaceutical options: Symptomatic Slow-Acting Drugs in Osteoarthritis (SYSADOAs), e.g. glucosamine sulfate or chondroitin sulfate (CS); paracetamol; non-steroidal anti-inflammatory drugs; intra articular injections hyaluronic acid and/or corticosteroids; and opioids.[5]

CS, which is a component of cartilage and bone, has been widely tested as a treatment for osteoarthritis.[2] As recently reviewed by Hochberg et al., various *in vitro* and *in vivo* animal studies have shown that CS has anti-inflammatory and anti-apoptotic effects; exerts a beneficial effect on the metabolism of chondrocytes and subchondral bone cells; and reduces cartilage destruction.[2] Meta-analyses of clinical studies have also shown that CS can result in a reduction in joint space width decline and pain.[6,7] However, as recently reviewed by Martel-Pelletier et al., not all CS products are equivalent. CS is a complex, heterogeneous polysaccharide that is extracted from the cartilage of various animals using a variety of extraction processes.[8] As such, different CS products can have different CS content, structure, and molecular weight.[9] This variability could compromise the efficacy and safety of the final product. For this reason, it is very important that patients use a high-quality, pharmaceutical-grade CS as has been used in clinical trials in patients with osteoarthritis, and which has been demonstrated to be effective and safe.[8,10,11]

Aim

The purpose of this work was to examine the histological changes in knee cartilage and bone following the administration of two different CS products in two experimental osteoarthritis models in rats.

Material and methods

Design

A total of 70 healthy adult mongrel white rats (aged 10-12 weeks; weight 180-230 g) that passed acclimatization for 10 days were randomized (on Day 0) into seven groups of 10 animals (5 male and 5 female). There was one control group and six experimental groups, as listed in Fig. 1.

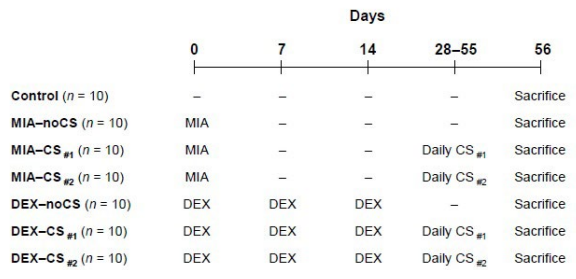

Fig. 1. Study design – $CS_{\#1}$ chondroitin sulfate (Artedja Injections), $CS_{\#2}$ chondroitin sulfate (Mukosat neo), *DEX* dexamethasone, *MIA* mono-iodoacetate

Ethics approval

The Committee on Bioethics of the SI "Dnipropetrovsk Medical Academy of the Ministry of Health of Ukraine" approved the study protocol and all procedures related to the maintenance of the animals, their humane treatment, and their use in the experiments. These also complied with Good Laboratory Practice requirements and the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes.

Osteoarthritis models

Three groups of rats had osteoarthritis induced by mono-iodoacetate (MIA), and three groups using systemic dexamethasone (DEX) suppression (Fig. 1). The MIA osteoarthritis model involved a single MIA injection (3 mg in 50 µL of sterile saline) into the right hind leg knee joint, as described by Guingamp et al. on Day 0.[12,13] The DEX suppression osteoarthritis model involved three intramuscular injections of DEX solution (7 mg/kg) into the femoral muscle on Days 0, 7 and 14 (Fig. 1).

Chondroitin sulfate administration

$CS_{\#1}$ was Artedja Injections (PRJSC "Fitofarm", Ukraine), whose raw material is CS produced by Bioibérica S.A.U. (Barcelona, Spain). $CS_{\#2}$ was Mukosat neo (RUE Belmedpreparaty, Republic of Belarus). Both products contain chondroitin-4-sulfate and chondroitin-6-sulfate of bovine origin. $CS_{\#1}$ is highly purified (99.9%) and has an average molecular weight of 15.1 kDa. This product has been approved as a prescription treatment for OA in many European countries. $CS_{\#2}$ has a purity of 99.4% and a lower molecular weight (10.3 kDa). Characteristics of both raw materials (for the specific batches used in this study) are detailed in Table 1.

Table 1. Chondroitin sulfate characteristics

Characteristic	$CS_{\#1}$	$CS_{\#2}$
Species	Bovine	Bovine[a]
CS content (%)	99.9	99.4
Molecular weight (kDa)	15.1	10.3
Intrinsic viscosity (m^3/kg)	0.040	0.051
Chlorides (%)	0.34	0.0167
Free sulfates (%)	0.14	0.035
Oxalate (%)	0.01	0.0040
ΔDisaccharide 0-S (%)	5.7	5.5
ΔDisaccharide 4-S (%)	62.8	58.4
ΔDisaccharide 6-S (%)	31.5	28.8

CS chondroitin sulfate, $CS_{\#1}$ chondroitin sulfate (Artedja Injections), $CS_{\#2}$ chondroitin sulfate (Mukosat neo).
[a] Source assumed to be bovine based on disaccharide composition.

Both products were solutions for injection in 2 mL ampoules containing 200 mg CS (100 mg/mL). Animals in the relevant groups (see Fig. 1) were injected intramuscularly with one of the CS products (35 mg/kg/day) during Days 28-56 (Fig. 1). This dose was based on the experience of the team and recommendations from the Center for Drug Evaluation and Research Ministry of Health of Ukraine.[14-18]

Physical parameters

Four rats per group were assessed for the influences of $CS_{\#1}$ and $CS_{\#2}$ on knee size (MIA model only). The knees were measured (largest circumference) at baseline and Days 28 and 56 using micrometer engineering. Four rats per group were assessed for the influences of $CS_{\#1}$ and $CS_{\#2}$ on activity and their analgesic effect (MIA and DEX models). Activity was assessed on Days 28 and 56 by placing the rats into a 1 m×1 m area that had been divided into 16 squares, each with a 3-cm diameter hole. The following parameters were assessed during 2 minutes: the number of borders crossed (horizontal motor activity), the number of hind-leg rises (vertical motor activity), the number of burrows (i.e. looks into the holes; research activity), the number of defecation acts (emotional activity), and the number of grooming acts. Analgesia was assessed on Day 56 by immersing the tails 3 cm into hot water (50°C) and measuring the time to tail flick.

Sectioning and Histology

All animals were killed accordind to Ethical Approvement on Day 57 by intraperitoneal administration of a thiopental sodium solution (40 mg/kg body weight) and samples of bone and cartilage from the right knees were taken. Tissue samples were fixated using 10 % neutral formalin for 5-7 days. They were then decalcified using 10 % nitric acid and embedded in celloidin-paraffin. A microtome was used to prepare thin slices (6-8 μm), which were stained using hematoxylin–eosin. Microphotography was performed using a Ulab XY-B2T microscope.

Statistical analysis

Physical parameters are reported as means ± errors. Depending on the normality of the distribution (as assessed using the Shapiro-Wilk test) and the groups being compared, the Student's t-test, the paired t-test, the Mann-Whitney U-test, or the paired Wilcoxon test were generally used. For knee circumference, a one-way dispersion analysis and Duncan's test were used. The level for significance was taken to be $P<0.05$. Statistical processing was performed using STATISTICA 6.1 software product provided (StatSoft Inc., serial No AGAR909E-415822FA).

Results

Physical parameters

Mean knee circumference increased by 37% from baseline to Day 28 in the MIA–noCS group (P<0.05). On Day 56, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had smaller knees than those in the MIA–noCS group (–22% and –18%, respectively; both P<0.05) (Table 2), showing an anti-inflammatory effect of both CS products.

On Days 28 and 56, motor activity was reduced in the MIA–noCS and DEX–noCS groups compared to Control rats (–18 to –55%; P<0.05) (Table 2). On Day 56, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had better motor activity than those in the MIA–noCS group (25–35% improvement; P<0.05); those in the DEX–$CS_{\#1}$ and DEX–$CS_{\#2}$ groups had only slightly bet-

Table 2. Knee circumference, motor activity (squares visited and hind-leg stands), research activity (burrows), emotional activity (defecation acts), grooming acts, and analgesia (time to tail flick after immersion in hot water) on Day 56

	Control 1 (n = 4)	MIA–noCS (n = 4)	MIA–$CS_{\#1}$ (n = 4)	MIA–$CS_{\#2}$ (n = 4)	Control 2 (n = 4)	DEX–noCS (n = 4)	DEX–$CS_{\#1}$ (n = 4)	DEX–$CS_{\#2}$ (n = 4)
Knee circumference (mm)	23.8±0.4[a]	34.5±0.8*	26.8±0.6**	28.2±1.1**	NA	NA	NA	NA
Borders crossed (n)	14.9±0.5	9.6±0.5*	12.9±0.6**	12.7±0.6**	19.2±0.3	14.3±0.5†	15.9±0.8	15.8±0.8
Hind-leg stands (n)	6.5±0.5	2.9±0.3*	3.7±0.2**	3.9±0.4**	7.9±0.4	6.2±0.6†	6.9±0.4	7.0±0.5
Burrows (n)	1.1±0.9	1.0±0.7	1.1±0.8	1.2±1.0	1.3±1.1	1.1±0.7	1.2±0.9	1.2±1.1
Defecation acts (n)	1.8±1.2	1.4±1.0	1.7±1.1	1.6±1.2	2.3±1.3	1.9±1.4	2.2±1.6	2.1±1.2
Grooming acts (n)	12.4±3.9	11.3±3.6	12.6±4.2	13.1±3.8	12.4±3.9	11.3±3.6	12.6±4.2	13.1±3.8
Time to tail flick (s)	113.5±0.6	91.7±0.5*	106.0±1.5**	105.0±1.5**	102.9±0.7	87.6±1.0†	102.0±0.7††	105.4±1.2††

Data are mean ± error. *$CS_{\#1}$* chondroitin sulfate (Artedja Injections), *$CS_{\#2}$* chondroitin sulfate (Mukosat neo), *MIA* monoiodoacetate, *NA* not available, *noCS* no chondroitin sulfate administered

[a]This value was a baseline measurement in the MIA–noCS group; all other values in the Control 1 column are at Day 56 in the Control 1 group.

*P<0.05 versus Control 1 (or baseline MIA–noCS for knee circumference)

**P<0.05 versus MIA–noCS

† P<0.05 versus Control 2

†† P<0.05 versus DEX–noCS

ter motor activity than those in the DEX–noCS group (8–11% improvement; NS) (Table 2). There were no significant differences in research activity, emotional activity, or grooming between groups at either time point (Table 2).

At Day 56, time to tail flick after hot water immersion was significantly reduced in the MIA–noCS and DEX–noCS groups compared to Control rats (–15% to –19%; P<0.05). Rats in the MIA–$CS_{\#1}$, MIA–$CS_{\#2}$, DEX–$CS_{\#1}$, and DEX–$CS_{\#2}$ had significantly longer times compared to the MIA–noCS and DEX–noCS groups, respectively (15–17% improvement; P<0.05) (Table 2). This indicates that both CS products had an analgesic effect.[17,18]

Histological results

Control group

In histological slides of Control rat knees, the perichondrium, cartilage, and subchondral bone are well visualized (Fig. 2).

The perichondrium, which is moderately oxyphilic, consists of two layers – superficial and deep (cellular and fibrous) – which together form a thin layer around the cartilage. In the deep layer of the perichondrium, there are small, nuclear-type, moderately basophilic cells. The surface layer of the cartilage contains many cells, often arranged in pairs, with intensely basophilic nuclei. The cartilage has is an even, weakly oxyphilic color without areas of hyperchromia. The deeper cartilage is mainly composed of an amorphous substance, with widely spaced groups of cells that have weakly basophilic nuclei, similar in color to that of the amorphous substance. The cartilage contains small groups of 4-10 cells (Fig. 2a), some of which have intensely basophilic nuclei. The boundary between cartilage and bone tissue is clearly visible. The bone trabeculae are moderately oxyphilic and plates of bone tissue have orderly architecture. The osteocytes in the bone tissue have basophilic nuclei. Bone marrow sites and vessels are visible between the trabeculae.

Mono-iodoacetate osteoarthritis model

No chondroitin sulfate

Among rats in the MIA–noCS group, the synovium was thickened, the structure was loose and heterogeneous, and there were areas of degradation (Fig. 3a).

The subchondral bone tissue had altered chromophility, with an increased degree of basophility of some trabeculae. In some parts of the surface, there were visible areas of bone destruction (arrow in Fig. 3a). The surface of the perichondrium had an uneven edge, sometimes vacuolated (* in Fig. 3b), and some layers of the perichondrium had been destroyed due to swelling. The amorphous substances of the deep zones have a hyperchromatic basophilic color. The deep zones also have areas of cell destruction, which are hyperchromic, their structure is not clearly visible (arrows in Fig. 3b). Superficial areas of the basic substance are lighter in color due to swelling of the amorphous substance.

Compared with Control rats, MIA–noCS rats had signs of inflammation of the synovial membrane, disruption of the structure of the perichondrium with swelling, disturbance of trophism of the deep layers of cartilage, destruction of a number of chondrocytes, and changes of the histochemical properties of amorphous materials. Marked destruction of bone tissue was observed and there were isolated pockets of violation of the architecture of the bone trabeculae.[17]

Chondroitin sulfate #1

In the MIA–$CS_{\#1}$ group, the outer contour of the perichondrium was uneven, but without areas of vacuolation (Fig. 3c). No signs of tissue swelling or opening were observed. The density of cells in the germ layer was comparable to that in Control animals. The cartilage contained groups of 4–8 slightly hypertrophied cells,

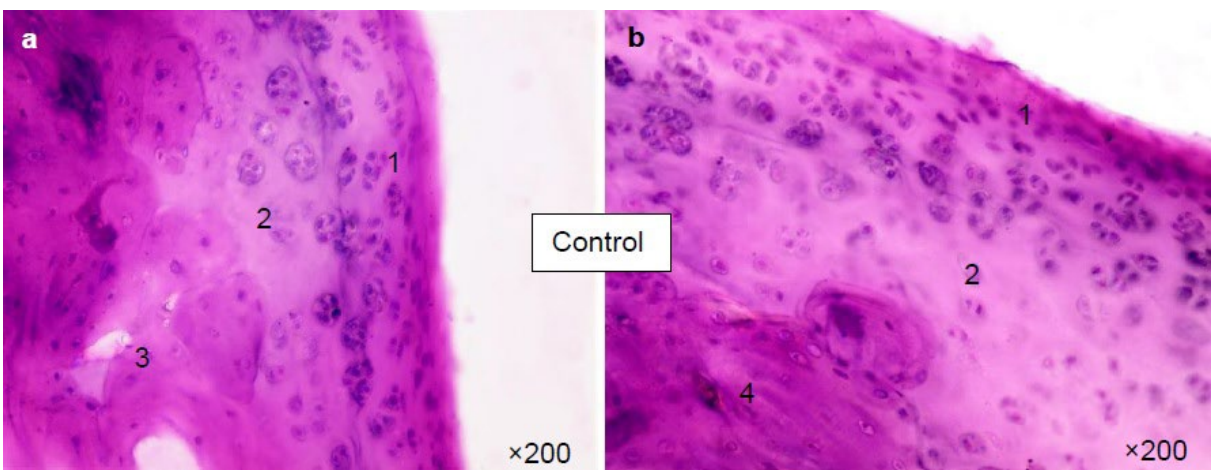

Fig. 2. Knee articular cartilage from Control animals. These images are representative of all Control animals. *1* perichondrium, *2* cartilage, *3* bone trabecula, *4* bone tissue

and the density of the cells in the surface layer was ≥1.5 times higher than in the MIA–noCS group. Unlike in MIA–noCS rats, hyperchromic areas were not observed in MIA–$CS_{\#1}$ rats. The transition zone in the subchondral bone had an irregular contour. There were visible sites of implantation of the bone tissue into the cartilage, indicating active bone regeneration. There were also visible areas of neovasculogenesis (arrows in Fig. 3d). The synovium had a structure comparable to that of Control animals, except for residual signs of inflammation in the joint capsule. Areas of subchondral bone bordering the cartilage had no pronounced structure, indicating that this was newly formed tissue.

Thus, while rats in the MIA–noCS group expressed signs of osteoarthritis, those in the MIA–$CS_{\#1}$ group did not. Cartilage and bone tissue samples from the MIA–$CS_{\#1}$ group were largely comparable to those from the Control group, but with active regeneration of cartilage and bone tissue, with areas of neovascularity. However, $CS_{\#1}$ did not result in recovery of the pronounced inflammation of the joint capsule induced by MIA.

Chondroitin sulfate #2

Cartilage from the knees of rats in the MIA–$CS_{\#1}$ group had a deformed contour (arrows in Fig. 3e). The perichondrium was not expressed and the distribution of layers (fibrous and deep, germ) was violated. Zones of active proliferation were observed in the cartilage and perichon-

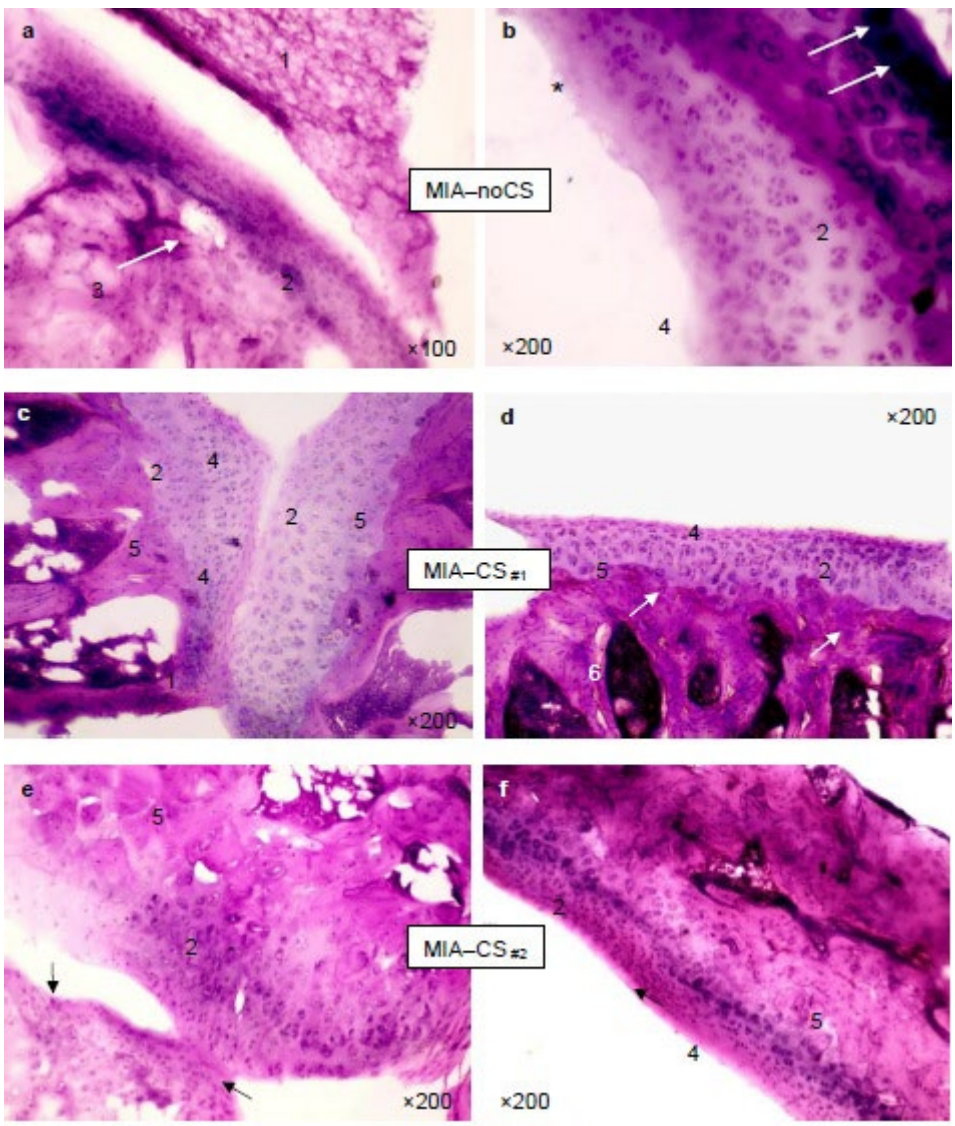

Fig. 3. Articular cartilage of the knee joints of rats in the **a** and **b** MIA–noCS, **c** and **d** MIA–$CS_{\#1}$, and **e** and **f** MIA–$CS_{\#2}$ groups. These images are representative of all animals in the respective groups. *1* synovial capsule, *2* cartilage, *3* bone trabeculae, *4* perichondrium, *5* subchondral bone, *6* bone marrow, * vacuolated perichondrium, *MIA–$CS_{\#1}$* rats with mono-iodoacetate-induced osteoarthritis given chondroitin sulfate (Artedja Injections), *MIA–$CS_{\#2}$* rats with mono- iodoacetate-induced osteoarthritis given chondroitin sulfate (Mukosat neo), *MIA–noCS* rats with mono-iodoacetate-induced osteoarthritis given no chondroitin sulfate. Arrows show: **a**, **b** bone destruction, **d** neovasculogenesis, **e** deformed contour, **f** active proliferation.

drium, defined by a high cell density (arrow in Fig. 3f). However, the chromophilic properties of the amorphous substance and cells were dissimilar to those in Control rats. The structure of the connective tissue (forming the joint capsule) was loose, with signs of swelling. The trabeculae were moderately oxyphilic and consisted of unstructured amorphous material and randomly distributed osteocytes, suggesting that it was "young" tissue. The boundary between cartilage tissue and bone had become blurred.

Compared with the MIA–noCS group, those in the MIA–$CS_{\#2}$ group had stimulated proliferation and formation of amorphous substance in both cartilage and bone tissue. However, samples were not fully comparable with Control animals – there were residual effects of osteoarthritis in the form of modified histochemical properties of amorphous substances and swelling of the connective tissue in the joint capsule. Like $CS_{\#1}$, $CS_{\#2}$ also did not result in recovery of the pronounced inflammation of the joint capsule induced by MIA.

Dexamethasone osteoarthritis model

No chondroitin sulfate

The perichondrium from rats in the DEX–noCS group had a reduced oxyphilic color and consisted of two layers – deep germ and superficial fibrous. The perichondrium was, on average, four times thicker than in animals from the Control group due to swelling (Fig. 4a and 4b).

The cells in the cartilage were randomly placed and

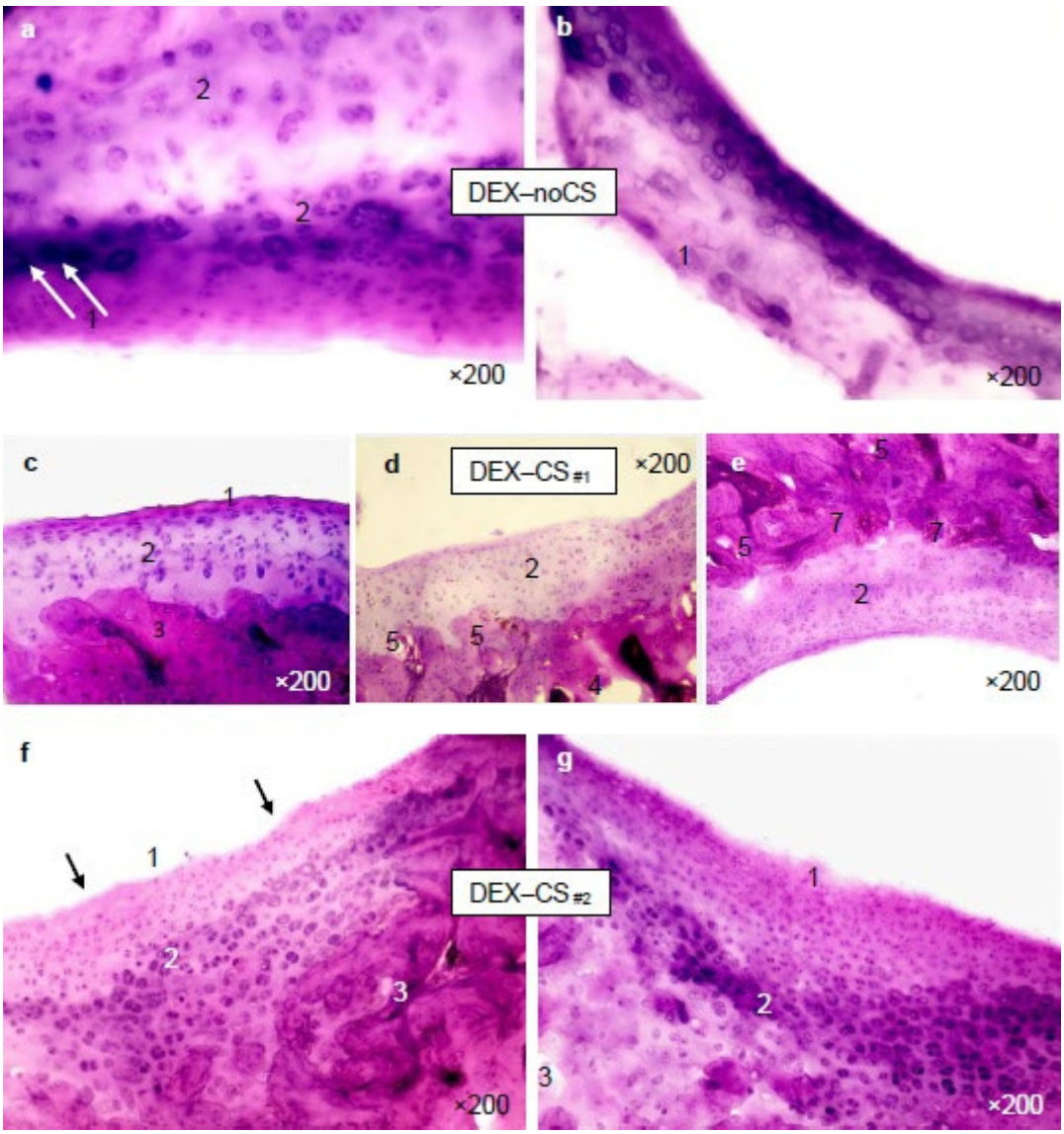

Fig. 4. Articular cartilage of the knee joints of rats in the a and b DEX–noCS, c–e DEX–$CS_{\#1}$, and f and g DEX–$CS_{\#2}$ groups. These images are representative of all animals in the respective groups. *1* perichondrium, *2* cartilage, *3* subchondral bone, *4* area of destroyed bone, *5* zone of active osteogenesis, *6* bony trabeculae, *7* vessels, *DEX–$CS_{\#1}$* rats with dexamethasone-induced osteoarthritis given chondroitin sulfate (Artedja Injections), *DEX–$CS_{\#2}$* rats with dexamethasone-induced osteoarthritis given chondroitin sulfate (Mukosat neo), *DEX–noCS* rats with dexamethasone-induced osteoarthritis given no chondroitin sulfate. Arrows show: a apoptosis, f tissue swelling.

had a weakly basophilic cytoplasm and intensely colored nuclei. One third of the cells had a hyperchromatic nucleus. In the area of cartilage close to the perichondrium, there were hyperchromatic cells undergoing apoptosis (arrows in Fig. 4a). The outer contour of the cells had an intense basophilic color: some were fully "defined" in this way, others, only partly, which we believe represents a different stage of apoptosis. In deeper sites, there were other hyperchromatic cells, but in smaller amounts than in the surface layers (ratio 1:10). Bone tissue showed areas of thinning of the bone trabeculae and areas of violated integrity (destruction), with small cavities inside the trabeculae.

Compared with Control rats, those in the DEX–noCS group had signs of inflammation (swelling) of the cartilage, activated cell death, and changes in the histochemical properties of the amorphous substances. In addition, the bone had signs of destruction. These signs indicate that the DEX induced osteoarthritis in the rats.

Chondroitin sulfate #1

The cartilage from rats in the DEX–$CS_{\#1}$ group had a thin perichondrium with an intensive oxyphilic color and chondroblasts in the deep layer (Fig. 4c). The density of chondroblasts was higher than in Control rats (Fig. 4c vs Fig. 2b). While rats in the DEX–noCS group had hyperchromic areas, these were not observed in DEX–$CS_{\#1}$ animals. The main substance of the cartilage was generally poorly basophilic (Fig. 4d) and consisted of an amorphous substance and groups of cells. The density of the cells in the deep cartilage was slightly higher than in the DEX–noCS group. The contact zone of the cartilage and bone tissue was well visualized. The bone trabeculae had a large area and were moderately oxyphilic, sometimes basophilic. There were signs of active regenerative processes: no areas of destruction in the newly formed bone, a disordered architecture of the trabeculae, and active vasculogenesis on the cartilage–bone border (Figs. 4d and 4e).

Compared to DEX–noCS rats, those in the DEX–$CS_{\#1}$ group had no signs of inflammation or destruction of bone tissue (i.e. osteoarthritis). An active recovery process (regeneration) with the activation of proliferative potential of cartilage and bone tissue had taken place.

Chondroitin sulfate #2

In DEX–$CS_{\#2}$ rats, the perichondrium had a moderately oxyphilic color and a clear boundary with the cartilage (Fig. 4f). The density of cells in the perichondrium was higher than in Control animals and it was several times thicker. The outer contour of the cartilage was uneven and there were small areas of tissue swelling (arrows in Fig. 4f). The cartilage was characterized by high-density groups of chondrocytes, some with an intensively basophilic color. Areas of hyperchromia were observed (Fig. 4g), as were seen in DEX–noCS rats. The boundary between cartilage and subchondral bone was blurred. There were signs of regeneration of bone tissue, but without obvious neovascularity. The amorphous substance of the bone tissue was intensively oxyphilic and the bone trabeculae were not ordered.

Compared with DEX–noCS rats, those in the DEX–$CS_{\#2}$ group had active regeneration of cartilage and bone tissue. However, there were still residual signs of osteoarthritis.

Discussion

Physically, rats in the MIA–$CS_{\#1}$ and MIA–$CS_{\#2}$ groups had significantly reduced knee swelling, improved motor activity, and analgesia compared with those in the MIA–noCS group after 4 weeks of CS injections. Histologically, animals in the MIA–$CS_{\#1}$ and DEX–$CS_{\#1}$ groups had regeneration of bone and cartilage, resulting in tissue structures similar to those in Control rats. Although $CS_{\#2}$ stimulated regeneration of bone and cartilage tissue, it was less effective than $CS_{\#1}$, and some morphological parameters were different from the Control group. Overall, in both the MIA and DEX osteoarthritis models, $CS_{\#1}$ had a more pronounced beneficial effect than $CS_{\#2}$.

These differences could be at least partly explained by the varying properties of the two CS products. Further, the source and structure of CS can result in differences in bioavailability and pharmacokinetic variables.[19] The number and positions of sulfate groups generally differ in CS extracted from different animal sources.[9] In this study, as indicated in Table 1, both CS products are from bovine origin according to the disaccharide composition identified. However, one parameter that is very different between the two compounds is the percentage of free sulfates (0.14 for $CS_{\#1}$; 0.035 for $CS_{\#2}$). Thus, although further studies are required to establish a definite correlation between chemical structure and activity, the difference in free sulfates might contribute to the differential effects of the two tested CS products.

Such discrepancies between CS products is not a new phenomenon. A review by Martel-Pelletier et al. highlighted the differences in purity, composition, chemical properties, and *in vitro* effects between different CS products.[8] Recently, Li et al. tested 15 different CS products: three commercially available CSs (from shark [Yantai Dongcheng Co., Ltd., Yantai, China], porcine [Huamao Shuanghui Co., Ltd., Luohe, China], and bovine [Shandong Kangping Bio Technology Co., Ltd., Linyi, China] cartilage) and 12 low-molecular-weight CSs that they had produced by degradation (four different methods) of the three CS products.[20] *In vitro* testing was used to ascertain which CSs had the best and worst anti-complement activity. These CSs were then given orally at doses of 50, 150, or 450 mg/kg ("best

CS") or 150 mg/kg ("worst CS") to mice in which osteoarthritis had been induced by surgical destabilization of the medial meniscus. The two highest doses of the "best CS" significantly attenuated articular cartilage erosion, while the "worst CS" had a small, insignificant effect.[20] These and our results highlight the differences in effects between CS products and the importance of using high-quality CS.

A number of other histological studies have also examined the effects of CS, but CS sources, doses, durations and routes of administration, animals, and osteoarthritis models have varied widely (Table 3).[20-28]

We chose a dose of 35 mg/kg/day for 4 weeks, but other studies have used doses as low as 500 mg/kg/month or as high as 450 mg/kg/day for durations ranging from just 12 days to nearly 1.5 years.[20,22,24,25] Two of the studies showed that higher doses of CS were more beneficial – Li et al., (as discussed above) and Campo et al.[20,21] The latter induced arthritis in mice via an intradermal injection of bovine type II collagen in complete Freund's adjuvant at the tail base and then administered intraperitoneal CS (Sigma–Aldrich Srl, Milan, Italy) at doses of 30, 60, and 120 mg/kg for 25 days.[21] They found that CS dose-dependently reduced cartilage erosion, proteoglycan depletion, and inflammation; as well as the incidence and severity of arthritis. Regarding length of treatment, Taniguchi et al.[22] studied the effects of CS or glucosamine in Hartley guinea pigs (bred to develop spontaneous osteoarthritis). Oral CS (Seikagaku Co., Tokyo, Japan) 200 mg/kg/day – administered from age 3 weeks to 8, 12, or 18 months – reduced cartilage degeneration at each time point, with better results at 12 and 18 versus 8 months.

Another important factor to consider is the route of administration. In the current study, both products were administrated via intramuscular injection, ensuring higher bioavailability compared to oral treatment. The other studies detailed in Table 2 administered CS orallyor by injection (intraperitoneal, subcutaneous or into the knee joint).[20-28] Although the kinetics of CS are still not well understood, studies performed by Conte et al. suggest that the absolute bioavailability of orally administered CS is 13.2% in humans.[29] Therefore, parenteral intramuscular administration could be a useful approach for CS therapy. In animal *in vivo* experiments, it has been demonstrated that CS administered to rats by intramuscular injection results in very rapid increases in plasma concentrations, with distribution to the liver, cartilage and kidneys.[30] Since the absolute bioavailability of orally administered CS is 13.2%, the bioavailability by intramuscular injection is more than in seven times that of oral administration.[29-31] Hence, the intramuscular route becomes an interesting choice for patients with osteoarthritis.

We studied two different osteoarthritis models – MIA and DEX – both in rats. MIA, at the dose used in this study, has been shown to have a destructive effect on the osteochondral structures of the knee joint, quick-

Table 3. Summary of histological studies that included a "CS alone" arm in animal models of osteoarthritis

Study	CS source(s)	Dose	Route	Duration (weeks)	Animal	Osteoarthritis model	Histological outcome
Current study	PRJSC Fitofarm ($CS_{#1}$) and RUE Belmedpreparaty ($CS_{#2}$)	35 mg/kg/day	IM	4	Rats	MIA or DEX	$CS_{#1}$ more chondroprotective than $CS_{#2}$
Li et al.[20]	Various[a]	50, 150, 450 mg/kg/day	PO	12	Mice	Surgical	"Best CS" attenuated osteoarthritis via the complement system
Campo et al.[21]	Sigma–Aldrich Srl	30, 60, 120 mg/kg/day	IP	4	Mice	Bovine type II collagen	Dose-dependent inflammation; cartilage erosion; apoptosis activation inhibited
Taniguchi et al.[22]	Seikagaku Co.	200 mg/kg/day	PO	32, 49, 75	Hartley guinea pigs	Spontaneous	Cartilage degeneration
Largo et al.[23]	Bioibérica S.A.U.	100 mg/kg/day	IP	5	Rabbits	Ovalbumin	Inflammation; synovial lesions
Xiao et al.[24]	NR	500 mg/kg/month	PO	4, 9, 13, 17, 22	Hartley guinea pigs	Spontaneous	Pathological lesions delayed
Caraglia et al.[25]	NR	0.3 mg/day	PO	2	C57 Black 6N mice	Spontaneous	Histological features of chondrodegeneration; apoptosis
Permuy et al.[26]	Bioibérica S.A.U.	11.5 mL/kg/day	IP	8	Rabbits	Surgery	Cartilage swelling; no effect on cartilage surface, synovial membrane, subchondral bone
Torelli et al.[27]	NR	1 mL of 12%/week	SC	12	Rabbits	Immobilization	Not effective
Chen et al.[28]	DongCheng Biochemicals Co., Ltd.	0.3 mL/week	Knee injections	5	Rabbits	Papain	Degenerative changes not significantly improved vs control

CS chondroitin sulfate; *DEX* dexamethasone, *IM* intramuscular, *IP* intraperitoneal; *MIA* mono-iodoacetate, *PO* per os, *NR* not reported
[a]Yantai Dongcheng Co., Ltd., Huamao Shuanghui Co., Ltd., Shandong Kangping Bio Technology Co., Ltd., and 12 degradation products

ly resulting in osteoarthritis-like lesions and function impairment.[12] Chronic exposure (once per week for 3 weeks) of high-dose DEX (7 mg/kg) has been shown to result in the apoptotic death of 50–70% of rat articular cartilage cells.[15] Other studies, however, have used different osteoarthritis models – spontaneous or induced surgically; by immobilization; or using ovalbumin, papain, or bovine type II collagen in guinea pigs, mice, or rabbits (Table 3).[20-28]

Seven of the nine studies listed in Table 3 reported at least some beneficial effect of CS on histological parameters, three of which have been discussed above.[20-22] In addition, Largo et al. induced inflammatory arthritis and atherosclerosis in rabbits by intraarticular injections of ovalbumin and a hypercholesterolemic diet.[23] Compared with control rabbits, intraperitoneal CS (Bioibérica S.A.U.) 100 mg/kg/day for 5 weeks reduced signs of synovitis and partially prevented inflammatory cell infiltration and intimal layer proliferation in the synovial membrane. Xiao et al. studied the effects of glucosamine (1000 mg/kg) and/or CS (500 mg/kg) monthly for 5 months in Hartley guinea pigs.[24] Pathological lesions developed in the articular cartilage after 1 month in untreated animals, but not until after 3 or 4 months in glucosamine- or CS-treated animals, respectively. Guinea pigs given glucosamine plus CS had virtually no pathological changes by study end.[24] Caraglia et al. tested CS (0.3 mg/day for 12 days) and/or "earth elements" mud therapy (once daily for 12 days) in a spontaneous osteoarthritis mouse model.[25] They found that CS had a beneficial effect on apoptosis and chondrodegeneration, which was further improved by the addition of mud therapy.[25] Permuy et al. tested CS against a range of other SYSADOAs in rabbits with surgically induced osteoarthritis.[26] The SYSADOAs were administered for 8 weeks, starting 3 weeks after surgery. Although intraperitoneal CS (Bioibérica S.A.U.) 11.5 mL/kg/day prevented cartilage swelling, similarly to the other SYSADOAs tested, it had no effect on the cartilage surface, synovial membrane, or subchondral bone. However, the dose of CS in this study is unclear as the strength of the CS solution was not reported.

Two of the studies in Table 3 reported a lack of effect of CS. Torelli et al. induced osteoarthritis in rabbits by immobilization of one knee for 12 weeks. Subcutaneous CS (1 mL of a 12% solution), administered weekly for 12 weeks, did not reduce the histological changes induced by this osteoarthritis model.[27] However, the source of CS was not reported, and CS was only administered once each week, which may explain the lack of efficacy. Chen et al. induced osteoarthritis in rabbits by injecting papain into both knees.[28] CS (prepared from CS [DongCheng Biochemicals Co., Ltd., Yantai, Shandong, China] that had been boiled for 30 min and filtered) and/or hyaluronic acid were injected into the knees once weekly for 5 weeks. Histological studies showed that intra articular hyaluronic acid had a chondroprotective effect, but that oral CS alone had no significant benefit over control, and hyaluronic acid plus CS introduced intra articular was not significantly better than hyaluronic acid alone.[32-34] However, it is unclear what dose of CS was given, or what effect boiling the CS could have had.

Overall, the current study and prior histological animal studies indicate that CS is likely to have a chondroprotective effect, but that factors such as the CS product used and its dose, route of administration, and duration of dosing can affect efficacy.

Conclusions

In two rat osteoarthritis models – in which osteoarthritis was induced by MIA or DEX – both CS products tested resulted in activation of the regenerative processes in cartilage and bone tissue. However, $CS_{\#1}$ had a stronger chondroprotective effect on cartilaginous tissue than $CS_{\#2}$ in both models. These results, along with those from various other studies, highlight the importance of using a high-quality pharmaceutical-grade active principle ingredient of CS in order to ensure optimal efficacy and safety of the final product in patients with osteoarthritis.

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