Acta Histochemica 117 (2015) 566–575

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Effects of high molecular weight hyaluronic acid on induced arthritis of the temporomandibular joint in rats

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ARTICLE INFO

Article history:
Received 22 February 2015
Received in revised form 10 May 2015
Accepted 12 May 2015

Keywords:
Temporomandibular joint disorders
Arthritis
Hyaluronic acid

ABSTRACT

High molecular weight hyaluronic acid (HMWHA) has been used to treat temporomandibular joint (TMJ) disorders, but controversial results have been described. This study aimed to characterize the morphological and biochemical actions of HMWHA on induced arthritis of the TMJ. Twenty-four male Wistar rats were used, and arthritis of the TMJ was induced through an intra-articular injection of Complete Freund's Adjuvant (CFA) (50 μl). One week after arthritis induction, the animals were treated with HMWHA (once per week for three weeks). Histological analyses were performed using sections stained with hematoxylin-eosin, toluidine blue and Picrosirius. Were also performed histomorphometric analysis and birefringence of collagenous fibers (polarization microscopy). Biochemical analyses of TMJ tissues were carried out through measurements of sulfated glycosaminoglycans and zymography for evaluation of metalloproteinase-2 and -9 (MMP-2 and -9). Data were analyzed using paired *t*-test and unpaired *t*-test, with a 5% significance level. HMWHA reduced histologic changes and thickness of the articular disc, led to a greater arrangement of collagenous fibers, lower concentration of sulfated glycosaminoglycans and lower activity in all isoforms of MMP-2 and -9 in TMJs with induced arthritis. These findings suggest that HMWHA may exert a protective effect on the TMJ.

Introduction

Hyaluronic acid (HA) is a natural polysaccharide belonging to the family of glycosaminoglycans that is responsible for maintaining the viscosity of synovial fluid and the lubricating and damping properties of articular cartilages such as shock absorption and better load distribution (De Leeuw, 2010).

Intra-articular injections of HA have been used to treat various joint disorders including osteoarthritis, and significant efficacy of this procedure was previously documented (Tang et al., 2008; Huang et al., 2010; Campo et al., 2011).

In this respect, HA may have mechanical, metabolic or biological actions. The mechanical concept is that HA maintains lubrication and therefore minimizes wear of articular surfaces, whereas it plays an important metabolic role in nutrition of the articular disc and cartilage (Neo et al., 1997). Regarding the biological effect, studies *in vitro* and *in vivo* demonstrated significant action of HA in blocking of different inflammatory mediators such as TNF-α, IL-1β, IL-17, PGE2 and inducer of nitric oxide synthase (iNOS), as well as inhibiting expression of enzymes that degrade the extracellular matrix such as matrix metalloproteinases (MMPs) 1, 3, 2, 9 and 13 (Wang et al., 2006; Hsieh et al., 2008; Hashizume et al., 2010; Shimizu et al., 2010; Campo et al., 2011).

With regard to TMJ disorders, treatment with HA has shown promising results in the reduction of the degenerative changes in animals with induced osteoarthritis in the TMJ (Xinmin and Jian, 2005; El-Hakim and Elyamani, 2011). In humans, Guarda Nardini et al. (2004) showed significant improvement in symptoms related to osteoarthritis of TMJ in volunteers followed up for a period of up to six months. A similar outcome was described by Yeung et al. (2006) in patients with disc displacement without reduction, monitored for a period of one year.

Abbreviations: AG, animal group with induced arthritis of the TMJ; BSA, bovine serum albumin; CEMIB, Multidisciplinary Center for Biological Research; CEUA, Ethics Committee on Animal Use; CFA, Complete Freund's Adjuvant; CG, control group; DMMB, dimethylmethylene blue; ECM, Extracellular Matrix; GAGs, sulfated glycosaminoglycans; HA, hyaluronic acid; HE, hematoxylin-eosin; HG, Group of animals with induced arthritis of the TMJ treated with high molecular weight hyaluronic acid; HMWHA, high molecular weight hyaluronic acid; MMP, matrix metalloproteinase; PGs, proteoglycans; TB, toluidine blue; TMD, temporomandibular disorder; TMJ, temporomandibular joint.

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http://dx.doi.org/10.1016/j.acthis.2015.05.003
0065-1281/

Nevertheless, a systematic review of the literature on the use of HA in the treatment of temporomandibular disorders (TMD) found unclear results, emphasizing the need for further evidence on the effectiveness of HA in treating this type of disorder (Shi et al., 2003). Furthermore, studies on other joints with osteoarthritis have not shown reduced signs and symptoms after intra-articular injection of HA (Altman et al., 2004; Jørgensen et al., 2010; Teeple et al., 2011).

In relation to the molecular weight of HA, studies in humans or in animal models have demonstrated greater efficacy of the high molecular weight hyaluronic acid (HMWHA) due to its longer half-life and potential to increase the amount of endogenous HA (Campo et al., 2010; Boettger et al., 2011; Duygu et al., 2011). Campo et al. (2010) also pointed out that low molecular weight HA can be associated with the development of a proinflammatory response, while HMWHA exhibits anti-inflammatory activity. However, the action of HMWHA in TMJ inflammatory disorders is not fully understood, and studies are needed to better understanding of their mechanisms of action and influence about the repair process in this joint.

Thus, the aim of this study was to characterize the morphological and biochemical actions of HMWHA on induced arthritis in the TMJ of rats.

Material and methods

A total of 24 male Wistar rats, with a mean age of 60 days, weighing between 300 and 350 g, were used. The animals were kept in standard plastic cages (two per cage) with water and food *ad libitum*, under standard lighting conditions (light/dark cycle of 12 h) and temperature of 22 °C. This study was approved by the Ethics Committee on Animal Use (CEUA), State University of Campinas, under protocol number 3020-1.

Experimental groups

The animals were allocated into the following groups:

AG—animals with TMJ arthritis induced by CFA ($N = 12$);
HG—animals with TMJ arthritis induced by CFA and treated with HMWHA ($N = 12$).

All experiments were performed in the left TMJ, in order to not compromise the chewing function of the animals. The right TMJs in the AG group were used as non-injected control (CG). Contralateral TMJs in the HG group were not used, since the data from this group were compared only with the AG, in order to evaluate the effects of HMWHA on induced arthritis.

Treated animals (AG and HG) received 875 mg/kg sodium dipyrone, by oral route, administered in the drinking water, after intra-articular CFA injection, in order to prevent painful symptoms resulting from the induced inflammatory process (Nakagaki and Camilli, 2012). Each animal received the same amount of water containing dipyrone, as previously described dose. Water was freely consumed over a week.

The animals were treated one week after injection of CFA, since there may be morphological changes in the TMJ in this period, such as osteophytes and erosion of the condylar surface, according to Kuroki et al. (2011).

Induction of arthritis in the TMJ

The animals were anesthetized by intraperitoneal injection of a solution of Ketamine (70 mg/kg) and Xylazine (10 mg/kg). Each animal was injected in the left TMJ with 50 μl of Complete Freund's Adjuvant (CFA) (5881; Sigma-Aldrich, USA), diluted 1:1 (oil/saline) (Spears et al., 2005; Kramer et al., 2010; Kameoka et al., 2009; Kuroki et al., 2011). Before the injection of CFA, we carried out a trichotomy in the TMJ region for better visualization of anatomical structures and facilitating the penetration of the injection needle. The bristle was carefully removed using a scalpel.

Verification of the TMJ injection site and evidence of an inflammatory response have been previously reported (Zhou et al., 1999, Spears et al., 2005; Kramer et al., 2010; Kuroki et al., 2011, Wang et al., 2012). CFA was injected using a 30-gauge needle (G1/2) coupled to a 1 ml plastic syringe (Spears et al., 2005; Kuroki et al., 2011). The zygomatic arch and the mandibular condyle were fingered, and the needle was then inserted immediately below the posterior inferior border of the zygomatic arch and advanced anteriorly to contact the posterior-lateral edge of the mandibular condyle (Zhou et al., 1999). The animals were sacrificed 28 days after induction of arthritis in the TMJ.

Treatment with High Molecular Weight Hyaluronic Acid (HMWHA)

Treatment with HMWHA was started one week after intra-articular injection of CFA into the left TMJ. The animals were anesthetized by intraperitoneal injection of a solution of Ketamine (70 mg/kg) and Xylazine (10 mg/kg), and then received an intra-articular injection of 50 μl of HMWHA (hylan G-F 20; Genzyme, USA) into their left TMJs, once a week for a total of three injections at the same time of administration (Duygu et al., 2011).

A 30-gauge needle (G1/2) coupled to a 1 ml plastic syringe was used for intra-articular injection of the drug, based on the technique described by Zhou et al. (1999). The animals were euthanized one week after the last injection of HMWHA (El-Hakim and Elyamani, 2011), i.e., 28 days after induction of arthritis in their TMJ.

Tissue harvest

The animals were euthanized by anesthesia overdose (Ketamine and Xylazine). After euthanasia, the TMJs were dissected and removed en bloc for morphological analysis. The articular disc, capsule and retrodiscal tissue were harvested for biochemical assays. For each technique (morphological analysis, concentration of sulfated glycosaminoglycans and Zymography) four animals were used per group. The sample size was calculated using the formula $n = 1 + [x2C(s/d)2]$, where, $C = (z\alpha + z\beta)\,2$, being established a statistical power of 90%, significance level (α) of 0.05, maximum standard deviation (s) of 20% and minimum difference (d) of 50%. For a 90% statistical power, the $z\beta$ value is 1.282 and $z\alpha$ corresponds to $\alpha/2$ (Zar, 2010; Sokal and Rohlf, 2012). An approximate result of four animals for each technique was obtained. The animals were randomly selected.

Morphological analysis

Histological processing

After dissection, the TMJs were fixed in 4% formaldehyde in Millonig's buffer (0:13 M sodium phosphate, 0.1 M NaOH – pH 7.4) for 24 h at 22 °C ($N = 4$). Then the samples were washed in water for 12 h and decalcified in 5% EDTA solution (ethylenediaminetetraacetic acid) in 0.1 M sodium phosphate buffer. The proper decalcification of the samples was checked when the tissue was pierced by a needle without offering resistance, which occurred after two months of the beginning of the experiment. After decalcification, the samples were washed in running water for 12 h, dehydrated in alcohol, cleared with xylene and embedded in paraffin. Seven-micrometer thick sagittal sectioning of the TMJ was performed, including the