Intra-articular Delivery of Kartogenin Conjugated Chitosan Nano/microparticles for Cartilage Repair

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Disclosures: M. Kang: None. J. Ko: None. J. Kim: None. G. Im: None.

Introduction: Intra-articular (IA) drug delivery can be a useful modality in osteoarthritis (OA) treatment, delivering a drug directly to the main focus of the disease. However, the therapeutic effect of IA drug depends mostly on the efficacy of the drug delivery system, due to the short retention time and rapid clearance of soluble drugs from the joint. Kartogenin is a recently characterized material that promotes the selective differentiation of mesenchymal stem cells (MSCs) into chondrocytes, thus stimulating cartilage regeneration. Drug conjugation to a hydrophilic polymer not only enhances the aqueous solubility of hydrophobic drugs, but can also change drug pharmacokinetics in the body. The aim of this study was to (1) synthesis kartogenin conjugated chitosan (CHI-KGN), (2) develop CHI-KGN nano/microparticles, (3) characterize the CHI-KGN particles for sustained release and chondrogenic activity in vitro, (4) evaluate the CHI-KGN particles for IA retention and regeneration of OA joint in vivo.

Methods: Preparation of CHI-KGN nano/microparticles: Kartogenin conjugated low-molecular-weight chitosan (LMWCS-KGN) and kartogenin conjugated medium-molecular-weight chitosan (MMWCS-KGN) were synthesized by carbodiimide chemistry using EDC/NHS catalyst. The covalent conjugations were confirmed by FTIR and $^1$H NMR spectroscopy. CHI KGN nanoparticles (CHI-KGN NPs) and CHI-KGN microparticles (CHI-KGN MPs) were fabricated by an ionic gelation of TPP with the LMWCS-KGN and MMWCS-KGN, respectively. The morphology of the CHI-KGN NPs and CHI-KGN MPs were studied using FE-SEM and the particle size distributions were measured using DLS. In vitro release of kartogenin from the particles was carried out with HPLC.

In vitro chondrogenic differentiation: The hBMMSC (2.5×10^5 cells, passage 3-5) were made by pellets and were cultured in DMEM/F-12 supplemented with BSA (1M), ITS (1% v/v), dexamethasone (10-7 M), ascorbate-2-phosphate (50 μM), L-proline (50 μM), and sodium pyruvate (1 mM). The CHI-KGN NPs and CHI-KGN MPs were added to the pellet culture. After 21 days of in vitro culture, pellets were analyzed for DNA contents, GAG amount, and the expression of chondrogenic markers.

Retention time in OA joint: OA was induced surgically using anterior cruciate ligament transection (ACLT) in rats. The CHI-KGN NPs and CHI-KGN MPs were labeled with fluorescence dye (FCR-675-carboxylic acid. After IA injection of the fluorescence dye-labeled CHI-KGN NPs and CHI-KGN MPs, each fluorescence spectrum in the OA rats was scanned using an IVIS-spectrum measurement system.

In vivo cartilage regeneration: The OA rats were treated with CHI-KGN NPs and CHI-KGN MPs by IA injection at weeks 6 and 9 after OA induction. The distal femora in each group were dissected at 14 weeks after OA induction and evaluated the OA regions by Safranin-O staining and OARSI scoring. Immunohistochemistry of COL2 and aggrecan was also carried out.

Results: Preparation of CHI-KGN nano/microparticles: Successful conjugation of kartogenin and chitosan was confirmed by FTIR and $^1$H NMR spectra. The conjugation efficiency of LMWCS and MMWCS with
Kartogenin was 98.1±1.6% and 97.9±1.9%, indicating almost complete conjugation of kartogenin in the preparation of CHI-KGN conjugates. The particles were spherical in shape, the CHI-KGN NPs had an average size of 150±39 nm with a zeta potential of -11.84±1.2 mV while the CHI-KGN MPs had an average size of 1.8±0.54 µm with a zeta potential of +7.80±1.1 mV.

The CHI-KGN NPs and CHI-KGN MPs showed sustained and continuous release of kartogenin until 7 weeks. A larger amount of kartogenin was released from CHI-KGN MPs (~50%) than from CHI-KGN NPs (~30%) during the test.

**In vitro** chondrogenic differentiation was evaluated in pellet cultures of hBMMSCs (Fig. 1). While DNA levels did not change significantly, GAG per DNA content increased significantly, up to two-fold (p < 0.05), when exposed to CHI-KGN NPs versus no treatment or unconjugated kartogenin. Although the GAG per DNA amount in pellets treated with CHI-KGN MPs was significantly lower than those treated with CHI-KGN NPs (p < 0.05), it was still higher than in those treated with unconjugated kartogenin (p < 0.05). Safranin-O and Alcian blue staining, associated with proteoglycan synthesis, showed the greatest intensity in the pellets treated with CHI-KGN NPs.

The gene expression of COL2A1 and aggrecan increased in hBMMSCs pellets exposed to unconjugated kartogenin and both CHI-KGN particles for 21 days compared with those of untreated hBMMSCs. In particular, hBMMSC pellets treated with CHI-KGN NPs showed significant increases in both genes compared with other pellets treated with either unconjugated kartogenin or CHI-KGN MPs (p < 0.05, p < 0.01).

**Retention time in OA joint** The fluorescence signals from both CHI-KGN particles were observed in the knee joint of OA rat up to 24 days (Fig. 2). In particular, the CHI KGN MPs showed significantly higher fluorescence intensity than CHI-KGN NPs on days 2 (p < 0.01) and 7 (p 0.05).

**In vivo** cartilage regeneration The OARSI scores were significantly lower in CHI-KGN particles-treated rats than those of kartogenin (p < 0.05) or vehicle-treated rats (p 0.05). In the results of immunofluorescence for COL2 and aggrecan, there were notable decreases in both proteins in the cartilage matrix of vehicle- or kartogenin-treated rats while the decrease was less marked in CHI-KGN particle-treated rats.

**Discussion:** CHI-KGN NPs and CHI-KGN MPs were prepared successfully by conjugation of chitosan and kartogenin using EDC/NHS catalysis and different formulations of an ionic gelation method with TPP. They had sustained release of kartogenin for 7 weeks in vitro. CHI-KGN NPs and CHI-KGN MPs induced chondrogenic differentiation of hBMMSCs more effectively than unconjugated kartogenin. In particular, hBMMSCs treated with CHI-KGN NPs exhibited more distinct chondrogenic properties in long-term pellet cultures than those treated with CHI-KGN MPs. The rats in which OA was induced surgically showed much less degenerative changes when treated with CHI-KGN NPs or MPs versus untreated control or rats treated with unconjugated kartogenin.

**Significance:** CHI-KGN NPs and CHI-KGN MPs can be useful polymer-drug conjugates as IA drug delivery systems for OA regeneration.
Figure 2.

A

Day 0  Day 2

Day 7  Day 14  Day 24

L : CHI-KGN NPs, R : CHI-KGN MPs

B

Relative fluorescence intensity (fold to day 0)

Days
Figure 3.

ORS 2015 Annual Meeting
Poster No: 0442