Polymerized-Type I Collagen Induces a High Quality Cartilage Repair in a Rat Model of Osteoarthritis

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Introduction

Osteoarthritis (OA) is a degenerative joint disease that is classically described as the result of both mechanical and biochemical events that increase the degradation of articular cartilage and subchondral bone. It is characterized by morphological, biochemical, molecular and biomechanical changes producing softening, fibrillation, ulceration and loss of articular cartilage, sclerosis and eburnation of the subchondral bone, osteophytes and subchondral cysts. Patients afflicted with OA may experience joint pain and pressure, crepititation, movement limitation, stroke and varying degrees of local inflammation with deleterious effects on patient activity level and lifestyle habits [1, 2].

Knee OA is the most common of the rheumatic diseases affecting approximately 3.8% (95% CI: 3.6-4.1%) of the global population. Moreover, OA is regarded as a prevalent cause of morbidity and disability worldwide. Prevalence is higher in females than males. OA of the hip and knee contributes the most to OA burden, often resulting in joint replacement surgery [3].
However, despite the prevalence and severity of the disease, relatively little is known about its exact etio- and pathophysiology. Recent compelling investigations have attributed the onset of OA to age, female sex, genetic factors, adiposity, obesity, and diet and joint-level factors such as injury, malalignment, and joint biomechanics [2, 4]. Latest studies suggest that patients exhibit inflammatory infiltration of synovial tissue membranes [5-8].

Moreover, the factors involved in joint destruction in OA including a) chondrocyte and synovial tissue cells that synthesize proinflammatory cytokines (interleukin (IL)-1β, IL-6, IL-15, IL-18, oncostatin M and tumor necrosis factor (TNF)-α), adipo-
kines (leptin, visfatin, adipin), and/or chemokines (IL-8, CC-
chemokine ligand 5 (CCL5) and CCL19); b) the inflammatory mediators, such as prostaglandin E2 (PGE2), nitric oxide (NO), re-
active oxygen species (ROS) and complement; c) the depletion of extracellular matrix proteins through degradation by metallopro-
teinase (MMP)1, MMP3, MMP8, MMP13, aggrecanases, a disinteg-
rin and metalloproteinase with thrombospondin motifs (AD-
AMTS4, ADAMTS5 and cathepsins; d) the cell-derived and/or matrix-derived products in regions of matrix depletion, such as alarmin (e.g. S100), fibronectin fragments, hyaluronic acid frag-
ments, type II collagen cleavage epitopes, proteoglycan fragments and high-mobility group box; and e) the increased of autophagy and chondrocytes [2, 4, 9-11].

Current therapeutic strategies for OA are targeted primarily at symptomatic relief, have limited effects on the underlying cascade of joint obliteration and have high incidence of side effects [12, 13]. Nonetheless, no proven structure-modifying therapy is available as yet. Local drug delivery strategies may provide for the development of more successful OA treatment outcomes that have potential to reduce local joint inflammation, reduce joint prote-
olysis, offer pain relief, and restore activities of daily living levels and diarthrodial joint function. For this reason, we have evalu-
ated the effect of a γ-irradiated mixture of a telopeptidic por-
cine type I collagen and polyvinylpyrrolidone (polymerized-type I collagen), which has anti-inflammatory properties [14, 15]. One percent polymerized-type I collagen addition to synovial tissue cultures from non-rheumatoid arthritis (RA) and RA does not in-
duce any change in DNA concentration or metabolism. However, polymerized collagen modifies the histological and biochemical pattern of fibrosis, without altering the total collagen content. Polymerized-type I collagen induces the recovery of type III col-
lagen at similar levels to those detected in normal synovial tissue. This biodrug diminishes the accumulation of dense and tightly packed type I collagen fibers and contributes to establish normal tissue architecture. Moreover, polymerized-type I collagen down-regulates proinflammatory cytokines (IL-1β, and TNF-α); adhesion molecule expression [endothelial leukocyte adhesion molecule (ELAM)-1, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1], cyclooxygenase (Cox)-1 as well as collagenolytic activity and up-regulates tissue inhibitor of metalloproteinase (TIMP)-1 production [16, 17]. Likewise, 1% polymerized-type I collagen addition to cartilage and syno-
vial tissue co-cultures obtained from total knee replacement of patients with OA up-regulates chondrocyte proliferation (Ki-67 antigen), the synthesis of highly sulphated proteoglycans, COMP and type II collagen, modifying extracellular matrix turnover in cartilage and restoring normal tissue architecture [18]. Furthermore, down-regulation of TNF-α and IL-1β seems to stimulate selectively activated synovial cell death via apoptosis in synovium cultures [16-18]. Finally, polymerized-collagen has demonstrated to be clinically safe and efficient for the treatment of RA [19, 20]. Thus, we performed this study to evaluate the effect IA ad-
ministration of polymerized-type I collagen on cartilage repair in partial meniscectomized rat model of OA.

Rationale

The rationale to inject IA polymerized-type I collagen was based on the encouragement from previous experience related to the biodrug effects (anti-inflammatory and tissue regenerator effects) observed during the course of the treatment of hypertrophic scars, scleroderma (SSc) skin lesions and RA patients [14-21].

Animals and Methods

Rats

Thirty Wistar male rats (130-150 g) were kept under specific path-
genre-free conditions. All animals were set aside under standard conditions in 12 h day/night rhythm with access to food and fresh water ad libitum. All animal procedures and experiments were car-
ried according to international guidelines (ICLAS-WHO) and Mexican official regulatory guideline NOM 062-ZOO-1999. The protocol of this study was approved by the Animal Care Research Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (CINVA:1093).

Induction of OA

Rats were anesthetized with intraperitoneal injection of ketamine (50 mg/kg PISA) 0.09 ml and xylazine (5mg/kg) 0.02 ml. The right back leg was shaved. Under sterile surgical conditions an anteromedial incision was done in the knee’s internal region, par-
allel to the leg long axis. The soft tissues were dissected parallel to the bones until the internal ligaments were viewed. The liga-
ments were sectioned perpendicular to the long axis and the joint capsule was excised to expose the medial compartment of the knee. The meniscus was clearly seen after a 90-degree flexion of the knee. It was carefully separated from the internal condyles and tibia plateau with a pointed knife scalpel. Special precautions were taken to avoid damaging the cartilage. A blunt curved need-
dle was used to pass a surgical thread through the meniscus, and then it was gently pulled upwards and held with fine pointed pliers. Then it was sectioned with a sickle-shaped scalpel in order to remove 30-50% of the meniscus (Figure 1). Tissues were closed with number 4.0 silk suture. Postoperative casts were not used. Animals were maintained within their cages where they moved freely. Ambulation started the day after surgery, with gentle and then with progressive intensity according to the possibility for us-
ing the injured joint. After three days, the animals used both legs entirely and they were subjected to high impact exercise for 15 minutes each day during 21 days. Five animals were placed in a box of 33 cm wide X 60 cm high X 33cm high deep with a bed of sawdust. During the first week 3 cycles of daily high impact exer-
cise were performed, as follows: 1) the box with rats was balanced back and front for 2 min (approximately 100 displacements per animal), 2) then each rat was grasped and released from a height of 30 cm (approximately 15 times/rat); and lastly 3) the box was shaken for one minute (approximately 80 continuous jumps up to 10 cm in height). During the second week, rats were grasped and
released from the total height of the box. This exercise stimulated the muscles, joints and bones by working flexion and extension and compression of the limbs [9, 22-24].

The inflammatory progression in each animal was assessed every week post training by measuring the thickness of the ankles (vernier), temperature, and weight. At 9 (early disease) or 13 (long-term disease) weeks rats were sacrificed. To test the effect of Polymerized-type I collagen in rats with OA, 50 μL was injected via intra articular (IA) once a week for 4 weeks, previous sedation with sevoflurane (sevoflurane, ABBOT).

Disease Models

Training control group: Six rats without partial meniscectomy and without treatment were subjected to high impact exercise for 15 minutes each day during 3 weeks and were sacrificed at the 9th (n=3) and 13th (n=3).

Sham/surgery control group: Six rats in which the capsule and synovial membrane were cut but the meniscus was not removed and without treatment were subjected to high impact exercise for 15 minutes each day during 3 weeks and sacrificed at the 9th (n=3) and 13th (n=3) post incisional wound on the right back leg.

Toxicity group: Six rats without partial meniscectomy were subjected to high impact exercise for 15 minutes each day during 3 weeks and were treated by 4 IA administrations, once per week during 4 weeks, with 50 μL of citric/citrate buffer (vehicle control). Three rats were sacrificed at the 9th (early disease) and three rats at the 13th post-meniscectomy (long-term disease).

OA under treatment with Polymerized-type I collagen: Six rats with partial meniscectomy were subjected to high impact exercise for 15 minutes each day during 3 weeks and were treated by 4 IA administrations, once per week during 4 weeks, with 50 μL of polymerized-type I collagen. Three rats were sacrificed at the 9th (early disease) and three rats at the 13th post-meniscectomy (long-term disease).

Radiographic Evaluation

After the sacrifice, a radiographic AP and lateral record was made in order to evaluate joint space, the presence of osteophytes, sclerosis and the integrity of hyaline cartilage (Tryumph X-ray apparatus). Measurement of bony features of knee was rated on a scale of 0 (absent) to 3 (highly advanced), according to the consensus of two blinded readers [25].

Safranin O Staining

Cartilage proteoglycans content was detected by the Safranin O staining method, according to standard protocols [26, 27]. Briefly, cryosections were stained with Weigert’s iron hematoxylin for 10 min, then with fast green (Sigma, St. Louis, MO) for 5 min, rinsed with 1% acetic acid solution for 10 seconds and stained with 0.1% Safranin O (Sigma) for 5 min. Finally, sections were dehydrated, mounted in Poly-Mount and evaluated by optical microscopy.
**Immunofluorescence**

Cartilage samples from normal and OA knee of male rats were obtained at the 9th and 13th post-meniscectomy. Cartilages of all joints were obtained from the weight bearing areas of the condyles. All samples were immediately fixed in 4% PBS-paraformaldehyde during 24 hours at room temperature [22-24]. Fixed samples were cryosectioned (Leica CM 1100; Heerbrugg, Switzerland) and mounted on gelatin coated slides. Tissues were processed for IF as has been described elsewhere [28]. In order to identify type I Collagen, type II Collagen and MMP13 in chondrocytes, independent samples were incubated with rabbit (Abcam pel, CA, UK). Nuclei were counterstained with DAPI (Sigma) and cover-slipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). As secondary antibody FITC-tagged donkey anti-rabbit (Jackson Immuno Research Laboratories, Inc, WestGrove) was used. Then, all samples were observed through a Leica confocal microscope (TCS-SP5-DMI6000B with Plan Neor).

To quantify the immunolabeling, Leica LAS AL light confocal program was used, in which fluorescence intensities were measured in each zone by the number of pixels per area (µm²). To perform the microscopy analysis, each zone was selected according to the morphological characteristics expressed in each area has been described elsewhere [10, 28]. As an internal control, sham samples were also compared with normal cartilage; negative controls were included, omitting the primary antibody.

**Statistical Analysis**

It was performed using the SigmaStat 11 program (Aspire Software International, Leesburg, VA, USA) by one way analysis of variance on Ranks and by Holm-Sidak method for all pairwise multiple comparison procedures, as well as Tukey-Kramer multiple comparison test. Data were expressed as the mean ± SD. The P-values smaller than or equal to 0.05 were considered as significant.

**Results**

**Demographic and Clinical Characteristics**

Except for the group of trained rats with partial meniscectomy and OA, the other groups of rats presented a significant increase in weight in 8 week. No statistically significant differences were determined between the group of rats with early and long-term disease treated by 4 IA administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen when compared with training or sham/operated control groups (Figure 2A).

In regards to temperature, only the group of rats with early and long-term OA treated with placebo had an increase of temperature at 8 week when compared with the groups of toxicity, sham, training and OA treated with polymerized-type I collagen. No statistically significant differences were found between the sham/operated control group, the polymerized-type I collagen toxicity assessment and the group of early and long-term OA treated with the polymerized-type I collagen (Figure 2B).

The back leg knee thickness of long-term OA treated with placebo had significant increase at 10 week as compared with the groups of toxicity, sham, exercise and OA treated with polymerized-type I collagen. No statistically significant differences were found between the sham/operated control group, the polymerized-type I collagen toxicity assessment group and the group of early and long-term OA treated with (Figure 2C).

**Macroscopic Findings**

Knee cartilage from training control (data not shown), the polymerized-type I Collagen toxicity assessment (data not shown) and sham/operated control displayed a white, smooth, glossy surface (Figure 3A, left panel). OA cartilage in early disease showed a less glossy, more opalescent, and a depression was frequently observed in the weight-bearing region of the condyles. OA cartilage in long-term disease in trained rats was softened, irregular and displayed a fibrillated surface and ulcerations, all animals showed a thinner, irregular and in many cases absent cartilage. Frequently the subchondral bone was exposed to the surface (Figure 3A, middle panel). The group of early and long-term OA treated by 4 IA administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen exhibited a cartilage of similar characteristics to those observed in the control groups (Figure 3A, right panel).

**Radiographic Findings**

Based on the radiographic assessment the index was grade 0 regarding to OA severity in training control group, sham/operated control (Figure 3B, left panel), and the polymerized-type I collagen toxicity assessment groups (data not shown). The group of early and long-term OA treated with 50 µL of polymerized-type I collagen exhibited grade 0 and grade 1 regarding to OA severity. The OA in early and long-term disease in trained rats exhibited grade 3 in OA severity index. OA was accompanied by increases in the volume, thickness and contour of the cortical plate, alterations in the state of bone mineralization and material properties, changes in the subchondral trabecular bone architecture and bone mass, the formation of bone cysts, the appearance of osteophytes and joint space narrowing. Tilting with bone contact was also observed (Figure 3B, middle panel). Meanwhile, rats with OA treated with polymerized-type I collagen showed joints similar to control groups, avoiding the joint narrowing and preserving the articular structure (Figure 3B, right panel).

**Histological Findings**

Knee cartilage from control groups (training, sham/operated and the toxicity assessment of polymerized-type I Collagen) showed the typical chondrocytes from the three main zones of the tissue, flattened chondrocytes from the superficial zone, the rounded secretory chondrocytes from the middle zone and hypertrophic chondrocytes from the deep zone. Expression of proteoglycans was observed homogeneous, covering the three zones of the cartilage (Figure 4A).

OA cartilage in early disease in trained animals was thickened. Some chondrocytes from the superficial and upper-middle zones of the cartilage had started to contract, they had increased in number and were frequently organized in clusters. Changes in OA cartilage in long-term disease were more evident. The surface of
Figure 2. Effects of polymerized-type I collagen in early and long-term rat model of OA. Fifty microliters of polymerized-type I collagen (toxicity model and OA/polymerized-type I collagen treatment) or citric/citrate buffer (OA/placebo model) were administered intraarticular once a week during 4 weeks. (A) Weight of the rats throughout the study. (B) Temperature of the rats during the study. (C) Right back knee thickness in early (9th week) and long-term OA (13th week). Data represent mean ± SEM (each group, n = 6). *P<0.05.

Figure 3. Macroscopic and radiographic characteristics of OA rat model. (A) Cartilage from training control group shows condyles with a white, smooth and glossy surface (left panel). Long-term OA cartilage display opalescent, opaque and rough surface condyles. Cartilage loss is also observed in some regions (black circle; middle panel). Long-term OA cartilage treated by 4 intraarticular administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen (right panel). (B) Radiography of the right back knee showing the joint space (white arrow; left and right panel) and the characteristic narrowing of the joint space (white arrow; middle panel) chondrocalcinosis (orange arrow; middle panel).
the cartilage was observed with deep fibrillation and the superficial zone was lost in some regions of the cartilage. The number of fibroblasts and fibers tended to increase. Also, the number of chondrocytes diminished, whereas hypertrophic, densely contracted chondrocytes and empty lacunae were increased. Furthermore, a progressive and severe reduction of proteoglycans content was observed. The extracellular matrix displayed a disorganized architecture and fibrillated surface. The tidemark was observed as a fuzzy region and in many cases it was not visible (Figure 4A).

The group of early and long-term OA treated with 50 µL of polymerized-type I collagen exhibited a cartilage of similar histological characteristics to those observed in the control groups (Figure 4A).

It is important to mention that contralateral leg of these trained rats did not show OA degeneration (data not shown).

**Effect of Polymerized-Type I Collagen on Chondrocytes**

**Type I collagen-expressing chondrocytes:** The content of type I collagen in cartilage from control groups (training, sham/operated and the toxicity assessment of polymerized-type I collagen) was scarce, as it was observed in immunofluorescence. Nonetheless, cartilage from rats with OA had a conspicuous number of type I collagen-expressing chondrocytes (Figure 4B and C). The group of early and long-term OA treated by 4 IA administrations of 50 µL of polymerized-type I collagen showed similar number of type I collagen-expressing chondrocytes to those observed in the control groups (Figure 4B and C).

**Type II collagen-expressing chondrocytes:** Type II collagen-expressing chondrocytes in cartilage from control groups (exercise, surgery/sham and the toxicity assessment of polymerized-type I Collagen) were higher and statistically significant when compared with cartilage from rats with OA (Figure 5A and B). The group of early and long-term OA treated by 4 IA administrations of 50 µL of polymerized-type I collagen showed similar number of type II collagen-expressing chondrocytes to those observed in the control groups (Figure 5A and B).

**MMP13 expressing chondrocytes:** MMP13-expressing chondrocytes in cartilage from control groups (training, sham/operated and the toxicity assessment of polymerized-type I Collagen) were limited. Nonetheless, cartilage from rats with OA had a higher number of MMP13-expressing chondrocytes when compared with OA treated with polymerized-type I collagen (Figure 6A and B). The group of early and long-term OA treated with 50 µL of polymerized-type I collagen showed similar number of MMP13-expressing chondrocytes to those observed in the control groups (Figure 6A and B).

**Adverse Events**

There were no evidence of adverse events after the injection of placebo or polymerized-type I collagen with lingering effects still absent out to 13 weeks. Adverse events evaluation included determination of pseudoseptic reaction, crystalline arthritis, swelling,

Figure 4. (A) Proteoglycan content in the middle zone of rat cartilage processed for safranin-O fast green staining. Arrow shows fibrillated surface of the tissue, arrowhead depicts chondrocyte cluster and star indicates loss of proteoglycan. (B) Type I collagen expression in rat cartilage. Type I collagen was labeled with FITC (green) and nuclei were counterstained with DAPI. (C). Graphic shows a statistically significant increase of type I collagen in cartilage from OA rats treated with placebo (50µL of citric/citrate buffer) vs. training control, sham/operated control, toxicity model or the group of early and long-term OA treated by 4 intraarticular administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen (*p<0.05). Data from three independent experiments (n=6). Results are expressed as mean ± SD.
Figure 5. (A) Type II collagen expression in rat cartilage. Type II collagen was labeled with FITC (green) and nuclei were counterstained with DAPI. (B) Graphic shows a statistically significant decrease of type II collagen in cartilage from OA rats treated with placebo (50µL of citric/citrate buffer) vs. training control, sham/operated control, toxicity model or the group of early and long-term OA treated by 4 intraarticular administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen (*p<0.05). Data from three independent experiments (n=6). Results are expressed as mean ± SD.

Figure 6. (A) MMP13-expression in rat cartilage. MMP13 was labeled with FITC (green) and nuclei were counterstained with DAPI. (B). Graphic shows a statistically significant increase of MMP13 in cartilage from OA rats treated with placebo (50µL of citric/citrate buffer) vs. training control, sham/operated control, toxicity model or the group of early and long-term OA treated by 4 intraarticular administrations, once a week for 4 weeks, with 50 µL of polymerized-type I collagen (*p<0.05). Data from three independent experiments (n=6). Results are expressed as mean ± SD.
local skin reactions, etc. The only adverse event observed was pain lasting less than 15 min in the injection site.

Discussion

At the present time is widely accepted that OA results from an admixture of changes occurring throughout the joint tissues, including cartilage, synovium, ligament, and bone and that inflammation is a key player in the progression of cartilage destruction and joint disease [29, 30]. Increased levels of proinflammatory cytokines are frequently present in the synovial fluid and tissues of OA-affected joints, particularly, IL-1β and TNF-α directly contribute to reduced anabolic and enhanced catabolic activities in OA-affected joints through regulation of proteases expression that acts to degrade cartilage [2, 24, 31]. Hence, a chronic cycle of joint destruction ensues with inflammation, increasing proteolytic activity that changes local tissue mechanics and generates extracellular matrix fragment release, factors that further promote inflammation. Therapeutic agents aiming to interrupt or modify this local proinflammatory, catabolic joint environment are of great interest for the potential to alter the progression of joint destruction and reduce the functional and symptomatic consequences of OA [32].

Numerous disease-modifying OA drugs have been proposed. They are focus on antagonizing the production or activity of inflammatory mediators, such as IL-1β and TNF-α and proteolytic enzymes [33]. The goal of these compounds is to block or modify the local proinflammatory, such as MMP inhibitors, catabolic environment found in the OA affected joint [34, 35]. Although several advances have been made in the area of arthritis drug development, only a very small fraction of these have been investigated for local drug delivery applications in the treatment of osteoarthritis. Therefore, the present study outlines efficacy of IA injections of Polymerized-type I collagen in a rat model of early and long-term knee OA at clinical, radiological, histological, and molecular level. Four intraarticular administrations of the biologic were an effective treatment modifying the progression of OA, inducing chondroprotection and a high quality cartilage repair (hyaline cartilage). Biodrug was innocuous as determined in the toxicity model. Polymerized-type I collagen was well tolerated during therapy and adverse events were not detected, except pain lasting less than 15 min in the injection site [36].

Clinical evaluation such as weight, joint thickness and temperature showed significant differences between the group with early and long-term OA treated with polymerized-type I collagen compared with the group with OA under placebo treatment. The weight was higher in the group of trained rats that underwent meniscectomy and were treated with polymerized-type I collagen. The increase in the weight of rats treated with the biodrug may be due to the reduction of the inflammatory process that is reflected in the animal’s ability to move, to feed and in the general well-being. This is reinforced by the fact that the groups of treated rats had a lower thickness in the joint compared with the group of rats with OA. Furthermore, based on its role as inducer of tissue repair, polymerized-type I collagen reduced degenerative changes in the cartilage structure. Articular surface did show neither fibrillation nor irregularity. Additionally, less MMP13 and type I collagen content and the increase of immunoreactive cell percentage for type II collagen and proteoglycans in trained rats with OA under treatment with polymerized-type I collagen indicates a high quality cartilage repair (hyaline cartilage) [37]. In contrast, matrix degradation was found with regards to MMP13 immunolabeling increased in the superficial and the middle zone of OA cartilage [37]. The considerable reduction of proteoglycans in cartilage of trained rats with OA under placebo treatment was accompanied by presence of type I collagen inducing the substitution of hyaline cartilage by fibrohyaline cartilage.

Conclusion

Summing up, our data suggest that the biological role of polymerized-type I collagen is not only restricted to providing protection, lubrication and mechanical stability to the collagen network, cells and joint surfaces, but also to up-regulate the synthesis of highly sulphated proteoglycans and type II collagen and to down-regulate type I collagen and collagenolytic activity.

Polymerized-type I collagen could be a successful treatment for OA, is a safe and effective for the treatment of early and long-term disease and that the improvement was sustained during follow up. It is a chondroprotective biodrug with disease-modifying effects. Polymerized-type I collagen induces high quality cartilage repair maintaining proteoglycan content, increasing type II collagen synthesis and decreasing MMP13 expression. Adverse events (pseudoseptic reaction, crystalline arthritis, swelling, local skin reactions, etc.) were not detected, except pain lasting less than 15 min in the injection site.

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References
