Original Research

Possible prevention of cartilage damage in rat knee osteoarthritis by howthorn (aronia) treatment: Histological and immunohistochemical studies

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Abstract: Osteoarthritis is the most common type of joint disease characterized by progressive loss of articular cartilage integrity and other joint changes. We aimed to investigate the possible effects of herbal extract (aronia) on collagenase induced osteoarthritis in albino rats. Twenty four rats were used in this study and were divided into 6 groups. Group 1 served as a control group and received saline only. Groups 2, 3, and 4 received collagenase and the specimens were taken after 10, 15 and 30 days respectively. Groups 5 and 6 received collagenase and aronia daily by oral gavages for 15 and 30 days respectively. Articular cartilage specimens were assessed by hematoxlyin & eosin for general histology, touilidine blue for proteoglycans, and caspase 3 for apoptosis detections. Upon collagenase treatment, rats showed highly destructed chondrocytes with pyknotic nuclei, and no clear lacunae, shrunken cytoplasm could be observed. Toluidine blue stain revealed marked destruction of sulphated proteoglycan in the extracellular matrix and in the cartilage capsules. Caspase 3 immunostaining revealed marked reaction in most of the chondrocytes. On the other hand, rats treated with aronia showed that the chondrocytes were more or less normal in shape, toluidine blue stain showed slight or no loss of proteoglycan, and caspase 3 immunostaining revealed a markedly reduced reaction of chondrocytes. It is concluded that aronia helps to ameliorate the induced osteoarthritis in rats.

Keywords: Knee joint, collagenase, osteoarthritis, apoptosis, howthorn (Aronia), histological and immunohistochemical

Introduction

The spectrum of rheumatic diseases seen in Saudi Arabia appeared to be broadly similar to that seen in the West although interesting differences were noted. Rheumatoid arthritis was the predominant inflammatory joint disease, but was less severe. Osteoarthritis (OA) was characterized by frequent involvement of the knee while the hip was rarely involved. Environmental factors may be responsible for this disease pattern. Regional pain syndromes, associated with obesity, bad posture, and poor physical fitness were also frequent problems [1].

Osteoarthritis is the most common type of joint disease, affecting 80–90% of men and women after age 65, and is characterized by progressive loss of articular cartilage integrity and other joint changes [2]. The poor regenerative capacity of the avascular and aneural articular cartilage prevents the recovery of full function after initial injury [3]. The disease pathogenesis leading to the cartilage degradation remains an important research field [4], as currently available

treatments cannot halt damage such as erosions, surface fibrillation, cell loss, increased osteophyte size, and decreased cartilage thickness. Recent work focuses on catabolic enzymes such as collagenases [5] and degraded cartilage components [6-8] as possible biomarkers to monitor disease progression. Collagen damage is particularly troubling, as the network of fibrils providing the articular cartilage with tensile strength have a slow turnover, often taking years [2]. The collagenases (matrix metalloprotinases (MMP) 1, 8, 13, and MT1-MMP) are the enzymes capable of the initial cleavage in intact collagen necessary to denature the triple helix structure at physiological conditions [9-12]. A C- and N-neoepitope are formed by the cleavage, and antibodies against the C-terminal neoepitope have been evaluated in human and animal model OA tissues. Antibodies against these collagen fragments, also known as type II collagen neoepitopes (TIINE) have become an important tool in the study of OA [6, 7, 13, 14].

Animal models are an important tool for elucidating OA pathogenesis. Numerous surgically and chemically induced models in multiple species have been characterized for proteoglycan matrix, collagen, and bone changes [15]. Desirable models for drug development generate consistent, reproducible articular cartilage lesions in a rapid period of time. Rat surgical models often meet these criteria [4, 14, 16, 17].

Hawthorn belongs to the genus Crataegus, and is deciduous in the Rosaceae family. It is native to the Mediterranean region, North Africa, Europe and Central Asia. There are more than 200 species worldwide, but very few have been tested and used for medicinal purposes, including C. oxycantha, C. laevigata, C. monogyna, C. orientalis and C. pinnatifida [18]. The medicinal use of extracts or tinctures prepared from leaves, flowers and/or fruits dates back to ancient times [19, 20]. They are now officially listed as herbal drugs in pharmacopoeias in countries such as Germany, France, China and England [19-21]. The preparations of Hawthorn are standardized based on their content of flavonoids and oligomeric proanthocyanidins [19, 20] which have significant vasodilator, inotropic, and diuretic effects. Extensive animal as well as human research studies have shown promising benefits of the different Crataegus species in the treatment of congestive heart failure, angina, hypertension, peripheral vascular disease, hyperlipidaemia, and diabetes mellitus [22-25].

C. aronia syn: Azarolus (L), the predominant species populating the mountains of the Mediterranean basin, has not been subjected to adequate scientific research. Several ethnobotanical and ethnopharmacological surveys on the therapeutic use of indigenous plants in Jordan and the Palestinian area revealed the use of Crataegus aronia in Arab traditional medicine to treat cardiovascular diseases, as well as cancer, diabetes and sexual weaknesses [26, 27]. Despite the extensive use of the different species of hawthorns, there are insufficient studies on the effect of C. aronia syn: Azarolus (L) and other species on platelet function.

The objective of this study is to evaluate the effect of herbal medicines in the treatment of osteoarthritis induced in rats.

Materials and methods

Animal experiments

The Institutional Animal Care and Use Committees of the participating institutions approved the study. Three-month- old Sprague Dawley rats, weighing 250–270 gram were used. Twenty four rats were used in this study and were maintained in accordance with the NIH ‘‘Guide for the Care and Use of Laboratory Animals’’. The collagenase type 2 (from Clostridium histolyticum, enzyme activity 321 U/mg, Worthington Biochemical Corporation, Lakewood, NJ, USA) was dissolved in sterile phosphate buffered saline (pH 7.4) to provide the indicated concentrations (50 U/lL). All of the surgical procedures were performed under sterile operating conditions with the rats under intraperitoneal anesthesia (50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride).

They were divided into 6 groups: (1) 4 rats were used as control group and received 2 injections (day 0, 7) of 0.3 ml saline into the right knee joint; (2) 4 rats received 2 injections (day 0, 7) of 50 U/lL of collagenase into the right knee joint and the specimens were taken after 10 days from the last injection; (3) 4 rats received 2 injections (day 0, 7) of 50 U/lL of collagenase the specimens were taken after 15 days from the last injection; (4) 4 rats received 2 injections (day 0, 7) of 50 U/lL of collagenase the specimens were taken after 30 days from the last injection; (5) 4 rats received 2 injections (day 0, 7) of 50 U/lL of collagenase and aronia (200 mg/kg) daily by oral gavages (days 0-15) and (6) 4 rats received 2 injections (day 0, 7) of 50 U/lL of collagenase and aronia (200 mg/kg) daily by oral gavages (days 0-30).

Histology

The knee joints were fixed in 10% neutral buffered formalin for 48 hours and then placed into 5% formic acid for 3-6 days to decalcify. Once decalcified, the knee joints were cut into approximately 2 equal halves in the frontal plane, using the collateral ligament as a landmark. The joints were processed for paraffin embedding and sectioned at 5 µm for toluidine blue staining to evaluate cartilage damage indicating large amounts of GAG accumulation in the matrix surrounding chondrocytes. .

The knee joints of the rats were dissected, and the surrounding soft tissue was removed. The tissues were fixed in 10% buffered formalin, decalcified in hydrochloric acid (CalciClear Rapid; National Diagnostics, Atlanta, GA), and embedded in paraffin. Sections (5µ in thickness) were cut and stained with hematoxylin and eosin (H&E) for routine histological evaluation and toluidine blue to enable evaluation of proteoglycan content [28].

Apoptosis assay

Sections of cartilage 5 µm thick were placed on to poly-L-lysine (Sigma-Aldrich, St Louis, Missouri) coated slides. Detection of apoptosis in situ was performed using the in situ cell death detection kit (Roche, Mannheim, Germany). Briefly, sections were deparaffinised with xylene, dehydrated in a graded series of ethanol, and incubated with proteinase K (20 µg/ml) (Bioneer, Daejeon, Korea) for 25 minutes at 37°C. The specimens were then incubated with 0.3% hydrogen peroxide/methanol for 10 minutes at room temperature. Slides were further incubated with permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for two minutes on ice. A standard indirect immunohistochemistry method was used to stain and detect expression of caspase-3 and performed according to the ABC staining kit (Santa Cruze). Positive immunocytochemistry was visualized by avidin-biotin-complex technique according to the ABC kit. Primary antibody was replaced by PBS as a negative control [29]. Apoptotic cells in the cartilage were observed by high-power field microscopy (Nikon, Garden City, NewYork).

Results

Haematoxylene and eosin stains

In the saline-injected (control) group, the cartilage had never degenerated at any interval studied. The chodnrocytes were lying centrally within its lacunae that arranged in groups. They showed finely granular cytoplasm that contained discrete vacuoles and are surrounded by a cartilage capsule and centrally located nuclei (Figure 1A).

In the second group that was injected with collagenase into knee joint, the specimens were taken after 10 days from the last injection. All rats showed disrupted chondrocytes lying centrally within its lacunae that arranged in groups. The cells showed finely granular cytoplasm that contains discrete vacuoles and are surrounded by cartilage capsule which was faint around some of the chondrocytes (Figure 1B).

In the third group that was injected with collagenase into knee joint, the specimens were taken after 15 days from the last injection. All rats showed disintegrated chondrocytes lying within its lacunae. The cells showed loss of its cytoplasm with disintegration of a cartilage capsule. Note the chonrocytes in some of them showed pyknotic centrally located nuclei (Figure 1C).

In the fourth group that was injected with collagenase, the specimens were taken after 30 days from the last injection. All rats showed highly destructed chondrocytes with pyknotic nuclei without clear lacunae and shrunken cytoplasm Note most of the cells had atrophic nuclei. Extensive cartilage destruction and disappearance of chondrocytes were noticed (Figure 1D).

Histological evaluation of knee cartilage from rats with collagenase–induced osteoarthritis (OA) treated with aronia,

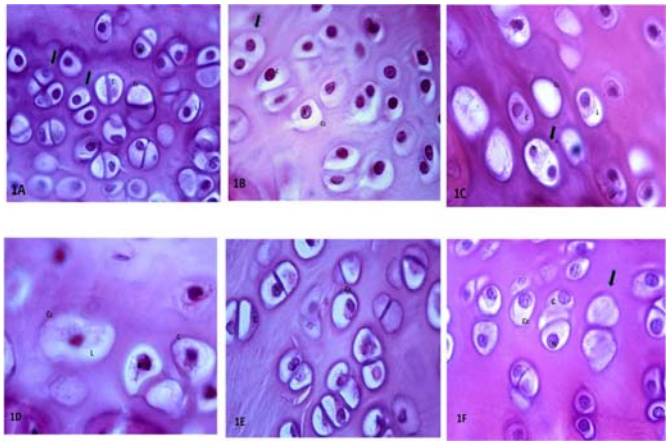

Fig. 1. Photomicrographs of the rat's knee joint stained with haematoxylin and eosin (Original magnification 1000) from:
- Control group [1A] showing chodnrocytes (C) lying centrally within its lacunae (L) that are arranged in groups (arrow). The cells show finely granular cytoplasm that contains discrete vacuoles and are surrounded by a cartilage capsule (Cc) and centrally located nuclei (N).
- Group two that received collagease only for 10 days [1B] showing disrupted chondrocytes (C) lying centrally within its lacunae (L) that are arranged in groups (arrow). The cells show finely granular cytoplasm that contains discrete vacuoles and are surrounded by cartilage cartilage capsule (Cc) which is faint around some of the chondrocytes.
- Group three that received collagease only for 15 days [1C] showing disintegrated chondrocytes (C) lying within its lacunae (L). The cells show loss of its cytoplasm with disintegration of a cartilage capsule (Cc). Note the chonrocytes in some of them (arrow) show pyknotic centrally located nuclei (N).
- Group four that received collagease only for 30 days [1D] showing highly destructed chondrocytes (C) with pyknotic nuclei with no clear lacunae (L), shrunken cytoplasm that contains many vacuoles and surrounded by a faint cartilage capsule (Cc). Note most of the cells have atrophic nuclei (N). Extensive cartilage destruction and disappearance of chondrocytes are noticed.
- Group five that received collagease plus aronia for 15 days [1E] showing some of the chondocytes (C) retain its normal shape but there are some vacuoles inside the cytoplasm with degenerated nuclei (N). Notice loss of cartilage capsule (Cc) in some of the chondrocytes.
- Group six that received collagease plus aronia for 30 days [1F] showing nearly all the chondrocytes (C) are more or less normal in shape with intact nuclei (N) and cartilage capsule (Cc) is very clear.

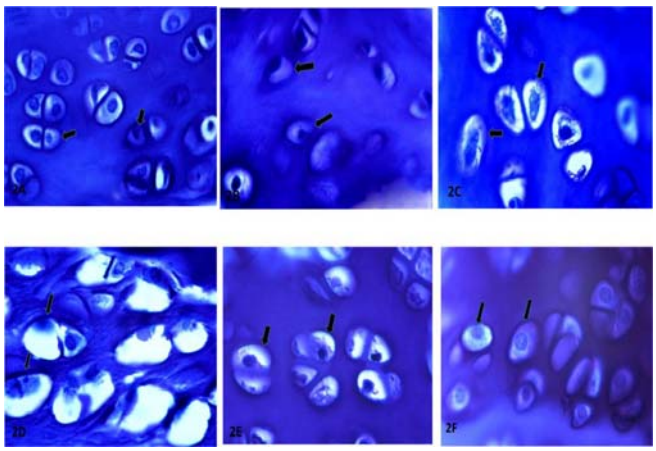

Fig. 2. Photomicrographs of the rat's knee joint stained with Toluidine blue (Original magnification 400) from:
- Control group [2A] showing a significant staining (arrows) for clusters of chondrocytes and their extracellular matrix are noted.
- Group two that received collagease only for 10 days [2B] showing focal cartilage degeneration, characterized by focal chondrocyte and proteoglycan loss (arrows).
- Group three that received collagease only for 15 days [2C] showing slight sulphated proteoglycan deposition in the extracellular matrix and in the cartilage capsules (arrows).
- Group four that received collagease only for 30 days [2D] showing slight deposition of sulphated proteoglycan in the extracellular matrix and in the cartilage capsules in some areas with its loss in others (arrows). Note that the chondrocytes are hypertrophied and degenerated.
- Group five that received collagease plus aronia for 15 days [2E] showing slight sulphated proteoglycan deposition in the extracellular matrix and around the cartilage capsules (arrows). Note that the chondrocytes are degenerated with pyknotic nuclei**.**
- Group six that received collagease plus aronia for 30 days [2F] showing showing no proteoglycan loss (arrows)**.** Note that the chondrocytes are more or less normal with intact nuclei.

in the fifth group that was injected with collagenase and aronia at the same time, the specimens were taken after 15 days from the last injection, all rats showed that some of the chondocytes retained its normal shape but there were some vacuoles inside the cytoplasm with degenerated nuclei. There was a loss of cartilage capsule in some of the chondrocytes (Figure 1E).

In the sixth group that was injected with collagenase and aronia at the same time, the specimens were taken after 30 days from the last injection; all rats showed that nearly all the chondrocytes were more or less normal in shape with intact nuclei and normal cartilage capsule (Figure 1F).

Toluidine blue stain

In the saline-injected (control) group, a significant staining for clusters of chondrocytes and their extracellular matrix were noted, indicating presence of proteoglycan content (Figure 2A). In the second group, focal cartilage degenerated as characterized by chondrocyte and proteoglycan loss (Figure 2B). In the third group, slight deposition of sulphated proteoglycan in the extracellular matrix and in the cartilage capsules were noticed (Figure 2C). Moreover, in the fourth group, very minor deposition of sulphated proteoglycan in the extracellular matrix and in the cartilage capsules was noticed (Figure 2D). Histological evaluation of knee cartilage from rats with collagenase–induced osteoarthritis (OA) treated with aron showed some improvement in proteoglycan deposition in the extracellular matrix and around the cartilage capsules (Figure 2E). In the last group, marked improvement in proteoglycan deposition was noticed (Figure 2F).

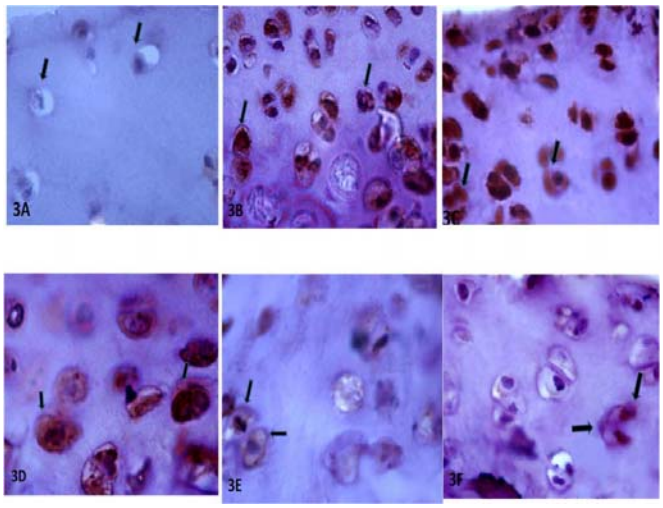

Fig. 3. Photomicrographs of the rat's knee joint stained with caspase 3 immunostaining (Original magnification 1000) from:
- Control group [3A] showing no immunostaining of cartilage sections for caspase-3 activity (arrows).
- Group two that received received collagease only for 10 days [3B] showing immunostaining of cartilage sections for caspase-3 activity in most of the chondrocytes (arrows). Caspase-3 staining is represented by brown-stained cells.
- Group three that received received collagease only for 15 days [3C] showing moderate immunostaining of cartilage sections for caspase-3 activity in all the chondrocytes (arrows).
- Group four that received collagease for 30 days [3D] showing marked immunostaining of cartilage sections for caspase-3 activity in all the chondrocytes (arrows).
- Group five that received collagease plus aronia for 15 days [3E] showing reduced immunostaining of cartilage sections for caspase-3 activity in animals (arrows).
- Group six that received collageaseplus aronia for 30 days [3F] showing markedly reduced immunostaining of cartilage sections for caspase-3 activity in animals (arrows).

Caspase 3 stain

In the saline-injected (control) group, no immunostaining, and normal chondrocytes was observed indicating no apoptotic features were present (Figure 3A). In the second group, positive reaction of most of the chondrocytes was observed (Figure 3B). In the third group, moderate positive reaction of the chondrocytes was observed (Figure 3C). Moreover in the fourth group, marked immunostaining of cartilage sections for caspase-3 activity in all the chondrocytes were observed indicating presence of apoptotic figures (Figure 3D). Histological evaluation of knee cartilage from rats with collagenase–induced osteoarthritis (OA) treated with aroni, reduced immunostaining of cartilage sections for caspase-3 activity in animals (Figure 3E). In the last group, markedly reduced immunostaining of cartilage sections for caspase-3 activity in animals were observed (Figure 3F).

Discussion

The present study demonstrated the therapeutic effect of herbal extract aronia in an OA animal model for the first time. Osteoarthritis (OA) is a multifactorial joint degenerative disease characterized by deep alteration of articular cartilage, changes in subchondral bone, osteophyte formation, and synovial inflammation. It is the most common arthritis, but its etiology is largely unknown. Age, obesity, sex, and previous injury are considered as significant risk factors. Although OA is commonly described as noninflammatory disease, inflammation is recognized as contributing to the symptoms and progression of OA [30, 31]. The pathology of osteoarthritis is characterized by changes in most tissues of the joint, including cartilage, bone, synovium, synovial fluid, ligaments, tendon, and joint capsule [32].

In this study, we demonstrated that intra-articular administration of collagenase induced osteoarthritic changes included highly destructed chondrocytes with pyknotic nuclei with no clear lacunae, shrunken cytoplasm that contained many vacuoles and were surrounded by a faint cartilage capsule. Note most of the cells had atrophic nuclei. Extensive cartilage destruction and disappearance of chondrocytes were seen in collagenase-treated rats. Apoptosis has been identified as one of the mechanisms of cell death, and plays a particularly important role in controlling the number of cells as cells compete for a limited amount of survival factors [33]. Our experiment demonstrates high rates of apoptosis in chondrocytes of OA induced groups observed on caspase 3 immunostaining with marked activity. Apoptosis, a highly regulated process of cell death, is controlled through the expression of specific genes that are largely conserved in a wide variety of organisms, ranging from nematodes to mammals. The bcl-2 family comprises some of the most prominent anti-apoptotic genes. The protein encoded by the bcl-2 gene has pleiotropic antiapoptotic effects, acting within both the mitochondria and the cytosol [34, 35]. Gene transfer of A20, another member of the anti-apoptotic bcl-2 family, inhibits cytokine-induced apoptosis and nuclear factor-κ-B activation in murine and human pancreatic islets [36]. Our earlier study on transfection of human chondrocytes with an anti-apoptotic gene demonstrated that bcl-2 provided cytoprotective action to the articular chondrocytes [36]. As our study demonstrates high rates of apoptosis in chondrocytes transplanted to articular cartilage defects, the use of anti-apoptotic therapy such as arnia may provide cytoprotective effects via antiapoptotic action, and may thus improve OA rat model. By toluidine blue stain, slight, no chondrocyte, or proteoglycan loss was noticed, indicating that large amounts of GAG accumulation in the matrix surrounding chondrocytes were lost. Loss of toluidine blue staining in the articular cartilage (loss of proteoglycan) was observed in this study [37].

Whereas aronia treatment prevented cartilage degeneration, nearly all the chondrocytes were more or less normal in shape with intact nuclei with normal cartilage capsule. Substances in hawthorn berries called anthocyanidins and proanthocyanidins help to stabilize the collagen in cartilage, which reduces joint damage, and hawthorn-flower extracts prevent the formation of thromboxane A2, a hormone involved in inflammatory processes. Hawthorn also stabilizes collagen in the bone itself Hawthorn offers some of the same properties as bilberry. Plants are one of the most important sources of active substances with therapeutic potential to cure a variety of diseases in humans [38]. The evaluation of pharmacological effects can be used as a strategy for discovering new drugs of plant origin [38, 39]. There is an ongoing world-wide revolution which is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs [40]. According to World Health Organization about 80% of the world population relies on traditional medicine for primary health care and more than 30% of the plant species have been used medicinally. However, there is limited scientific evidence regarding the safety and efficacy to support the continued therapeutic application of these medicinal plants. Because of this renewed interest in herbal remedies and the increased use of plants extracts in food, cosmetics and pharmaceutical industries, there is a compelling need for thorough scientific safety evaluation of the medicinal plants [41, 42]. Laboratory animals are sensitive to toxic substances occurring in plants. Hence, the administration of the extracts in increasing amounts enables the evaluation of the acute and sub-acute toxicity limits. Therefore, the test should be carried out for three doses and for both sexes, taking into account other factors such as age, weight, species, diet and environmental conditions [43].

Based on the data presented, aronia appears to alter the course of the osteoarthritis disease and may be suggested to be a disease modifying drug. However, further studies are required in this regard.

Conflict of interest: None declared

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