Osteoarthritis and Cartilage 21 (2013) 965–972

Osteoarthritis and Cartilage

Gradual strenuous running regimen predisposes to osteoarthritis due to cartilage cell death and altered levels of glycosaminoglycans

C.E.S. Franciozi †*, V.A.F. Tarini †, R.D. Reginato ‡, P.R.S. Gonçalves ‡, V.P. Medeiros §, M. Ferretti †, J.L. Dreyfuss §, H.B. Nader §, F. Faloppa †

† Department of Orthopaedics and Traumatology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil
‡ Department of Morphology and Genetics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil
§ Molecular Biology Program, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 21 December 2012
Accepted 6 April 2013

Keywords:
Articular cartilage
Extracellular matrix
Apoptosis
Running
Chondroitin sulfate
Hyaluronic acid

SUMMARY

Objective: To investigate the hypothesis that strenuous running is a predisposing factor for osteoarthritis.
Design: Wistar rats were divided into two groups: a control group (CG) and a trained group (TG). The TG underwent a strenuous treadmill running training regimen of controlled intensity, exhibiting progressively improvement of fitness over 12 weeks, running at least 55 km during this period and finally performing an ultra-endurance running exercise to exhaustion. After this period, rats from both groups were euthanized and their knees removed. The articular cartilage was dissected and submitted to histomorphometrical, histomorphological, and immunohistochemical analyses evaluating cell death pathway (caspase-3 and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)) and inflammatory cytokines [interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α)]. In addition, the tissues were analyzed regarding the types and the content of glycosaminoglycans.
Results: The TG knee joints exhibited increase in the number of chondrocytes and chondrocyte clusters, as well as significantly increased levels of caspase-3, a protein involved in apoptosis, and of inflammatory cytokines IL-1α and TNF-α. In addition, histologically higher grades of osteoarthritis (Osteoarthritis Research Society International – OARSI grading), and significantly decreased levels of chondroitin sulfate and hyaluronic acid. Knee cartilage thickness and TUNEL did not significantly differ between the two groups.
Conclusions: The articular cartilage of rats subjected to a strenuous running regimen of controlled intensity exhibited molecular and histological characteristics that are present in osteoarthritis.

Introduction

The beneficial effects of a lifestyle that involves physical exercise are well established, and physical inactivity is one of the three leading causes of preventable morbidity, mortality and disability in developed countries[1]. Increasing physical activity levels of the population is a constant objective of government policy and private investment despite the lack of studies evaluating these interventions[2].

Articular cartilage can be affected by physical activity. Its complex composition provides a lubricated surface that absorbs and transfers load to allow joint movement without friction[3]. The development, maintenance, and destruction of cartilage are regulated by mechanical factors throughout life[4]. The microenvironment of the articular cartilage plays important role in its biomechanical function. The properties of this tissue are related to the composition of its extracellular matrix, mainly composed of proteoglycans and hyaluronic acid (HA) entangled in a dense network of collagen fibers that retain large amounts of water. The structures of these molecules play major roles in determining the resilience of the tissue to compression[5–7]. Proteoglycans in articular cartilage form large aggregates that consist of a central HA filament to which multiple monomers are non-covalently attached[5].

Chondrocytes are the only cell type of the articular cartilage. They maintain tissue homeostasis, react to injury and carry out cartilage remodeling. Chondrocyte death and survival are related to cartilage

* Address correspondence and reprint requests to: C.E.S. Franciozi, Department of Orthopaedics and Traumatology, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Borges Lagoa, 783–5°Andar, Vila Clementino, 04038-032 São Paulo, SP, Brazil. Tel: 55-11-5571-6621.
E-mail addresses: cacarlos66@hotmail.com (C.E.S. Franciozi), victor.tarini@gmail.com (V.A.F. Tarini), rejane.dr@uol.com.br (R.D. Reginato), patriciareissgoncalves@yahoo.com.br (P.R.S. Gonçalves), valmedeiros@gmail.com (V.P. Medeiros), marioferretti@uol.com.br (M. Ferretti), jdreyfuss@unifesp.br (J.L. Dreyfuss), hbnader.bioq@epm.br (H.B. Nader), faloppa.dot@gmail.com (F. Faloppa).

1063-4584/$ – see front matter
http://dx.doi.org/10.1016/j.joca.2013.04.007

matrix integrity. Apoptosis and necrosis are mechanistically and morphologically distinct types of cell death[8]. Apoptosis is a form of programmed cell death, and a singular pattern of apoptotic cell death is the activation of caspases. Although not all forms of apoptosis depend on caspases, these proteases are key factors in the initiation and the execution of the process. Pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) induce matrix metalloproteinases and aggrecanases which generate matrix degradation products that can contribute to the activation of catabolic responses in chondrocytes[9,10].

The effects of exercise on articular cartilage remain a subject of debate and speculation, as does the relationship of exercise to the development of osteoarthritis. Moderate exercise benefits this tissue[11], but consensus is lacking regarding deleterious effects of heavy physical activity, elite level competition, and long-distance or marathon running, leaving unanswered the question: is strenuous running a predisposing cause of osteoarthritis?[11–14]

The present study intends to answer that question and the results confirm the hypothesis that strenuous running is a predisposing cause of osteoarthritis.

Methods

Animals

Twenty-eight albino Wistar male rats 15–20 weeks old at the beginning of the experiment were housed at 22°C with a light–dark cycle (7:00 am–7:00 pm). The animals were allowed access to food and water *ad libitum*.

Ultra-endurance exercise training

Ultra-endurance exercise is defined as running a distance of at least 50 km or for more than 4 h[15,16]. To induce this condition in experimental studies, a novel protocol was devised according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines[17,18]. A motorized treadmill (AVS Projetos, São Paulo, SP, Brazil) with six individual lanes and no inclination was used. A shock grid at the back of the treadmill delivered a mild shock (2.0 mA) if the rat's pace fell below the treadmill speed. All rats were adapted to walking on the treadmill for 3 days at 10 m/min for10 min per day. To assess trainability, each animal's performance was rated on a scale of 1–5 according to Dishman *et al.*[19]. Animals with a mean rating of 3 or higher were included in the study and those with a mean rating of 1 or 2 were excluded to avoid differentially stressed animals in the study. Four animals were excluded due to insufficient score.

Animals were divided into two experimental groups of 12 individuals according to Dishman's score: control group (CG) and trained group (TG). The animals from the CG did not perform any exercise after the division into groups. The TG was subjected to a progressive endurance regimen 5 days/week for 12 weeks. The initial velocity of 10 m/min was increased to 15 m/min at the third week, 20 m/min at the fifth week, and at least 25 m/min at the seventh week and thereafter. The duration of the sessions was increased by 10 min every 2 weeks starting at the second week to reach 70 min per session at 12 weeks of training. Top speed tests were performed every 30 days to correct the intensity of the exercise as the animals adapted to the progressive training. The top speed test consisted of running the rats at an initial velocity of 10 m/min. The speed was increased 2 m/min every 3 min until exhaustion. The top speed was designated as that recorded 3 min prior to the point of exhaustion. Since the need to correct and control intensity training, each rat ran at 60% its top speed; this intensity was used throughout the study[17].

After 12 weeks, animals in the TG that had run approximately 55 km underwent an ultra-endurance exercise on a treadmill at an intensity corresponding to 60% top speed until exhaustion. Afterward, the animals of both groups were sacrificed by decapitation under sedation. Femoral condyles from the right and left hind legs were carefully dissected without damaging the cartilage surface.

Histology and histochemistry

The right distal femoral condyles were fixed at room temperature for four days in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate and were decalcified for 30 days in 25% formic acid, pH 2.0. Specimens were dehydrated in graded concentrations of ethanol and embedded in paraffin. Serial 5 μm coronal sections were subjected to histomorphometry, immunohistochemistry, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Sections were stained by hematoxylin and eosin (HE) and safranin O. HE sections were subjected to histomorphometric analysis performed with a semiautomatic image analysis system (AxioVision Rel. 4.6., Carl Zeiss, Oberkochen, Germany). To evaluate the thickness of the articular cartilage, images were captured with a 2.5× objective lens. To count the number of chondrocytes and the clusters of chondrocytes, the images were captured with a 10× objective lens. Three different regions of the articular cartilage at both the weight-bearing and non-weight-bearing areas of the femoral condyles were defined by a rectangle with a standardized area of approximately 10,000 μm^2.

Safranin O sections were evaluated according to the *Osteoarthritis Research Society International* (OARSI) grading system for articular cartilage degeneration[20].

Staining of active caspase-3, IL-1α, TNF-α and HA was performed by indirect immunohistochemical or histochemical analyses using anti-caspase-3, anti-IL-1α, anti-TNF-α (all from Biocare Medical, Concord, CA) as the primary antibodies or biotinylated HA binding protein all diluted 1:100 and incubation on the sections for 1 h. Next, streptavidin-labeled secondary antibodies or streptavidin-horseradish peroxidase (HRP) were applied and then developed with 0.05% diaminobenzidine tetrachloride. HE was used to counterstain. The primary antibodies were replaced with phosphate-buffered saline to prepare negative controls; no detectable staining was evident. Images of chondrocytes in the superficial (SZs) and intermediate (IZs) zones of both the weight and non-weight-bearing areas of the femoral condyles were captured with a 40× objective lens, and those cells within a predetermined rectangular area of 50.56 × 114.78 μm^2 were counted. The proportion of chondrocytes staining for caspase-3, IL-1α, and TNF-α was calculated and expressed as a percentage of the total number of cells within each area. Data were expressed in mean with lower and upper limits of 95% confidence intervals (CIs).

TUNEL assay

Apoptotic cells were detected *in situ* using a TUNEL assay according the manufacturer's instructions (*ApopTag® Peroxidase*, Chemicon, Temecula, CA, USA). TUNEL-positive hypertrophic chondrocytes were observed at the growth plates, serving as an internal positive control for each section. For negative controls, sections were incubated with label solution alone (without terminal transferase or TUNEL reaction mixture) (see Supplementary Data). Positive chondrocytes were counted and the proportion of TUNEL staining was expressed as the percentage of the total cells in each area. Data were expressed as mean with lower and upper limits of 95% CI.