

Physical activity ameliorates cartilage degeneration in a rat model of aging: A study on lubricin expression

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Osteoarthritis (OA) is a common musculoskeletal disorder characterized by slow progression and joint tissue degeneration. Aging is one of the most prominent risk factors for the development and progression of OA. OA is not, however, an inevitable consequence of aging and age-related changes in the joint can be distinguished from those that are the result of joint injury or inflammatory disease. The question that remains is whether OA can be prevented by undertaking regular physical activity. Would moderate physical activity in the elderly cartilage (and lubricin expression) comparable to a sedentary healthy adult? In this study we used physical exercise in healthy young, adult, and aged rats to evaluate the

expression of lubricin as a novel biomarker of chondrocyte senescence. Immunohistochemistry and western blotting were used to evaluate the expression of lubricin in articular cartilage, while enzyme-linked immunosorbent assay was used to quantify lubricin in synovial fluid. Morphological evaluation was done by histology to monitor possible tissue alterations. Our data suggest that moderate physical activity and normal mechanical joint loading in elderly rats improve tribology and lubricative properties of articular cartilage, promoting lubricin synthesis and its elevation in synovial fluid, thus preventing cartilage degradation compared with unexercised adult rats.

Osteoarthritis (OA) is an extremely common musculoskeletal disorder characterized by slow, progressive, and degenerative changes in load-bearing synovial joints, most commonly in hand, hip, and knee joints (Loeser, 2010, 2011).

Age is classified as a prominent risk factor for OA disease, which is the most common cause of chronic disability in older adults. It is believed that chondrocyte senescence plays a major role in the development and progression of OA in aging cartilage, since accumulation of senescent cells impairs the intrinsic ability of the tissue to maintain and repair the extracellular matrix (ECM) of cartilage, resulting in a mechanically compromised tissue (Loeser, 2009). However, OA is not an inevitable consequence of aging and aging-related changes in the joint are different from those due to injury or inflammatory disease (Loeser, 2009).

Evidence of cell senescence in tissues from older adults can be obtained by examining for the presence of cellular senescence markers (Loeser, 2009). These markers currently include histological staining for

senescence-associated β -galactosidase (SA- β -gal), senescence-associated heterochromatin (SA heterochromatin), increased p53 (p53), cyclin-dependent kinase inhibitor 1 (p21), and cyclin-dependent kinase inhibitor 2A (p16) and reduced Wnt2 or Irp (int-related protein) (Campisi & d'Adda di Fagagna, 2007). In this study we examined whether the expression of lubricin, also known as proteoglycan 4 (PRG4) or superficial zone protein (SZP), could be used as a marker of chondrocyte senescence and/or OA. Lubricin is a chondroprotective glycoprotein that serves as a critical boundary lubricant between opposing cartilage surfaces (Leonardi et al., 2012a). It has a major protective role in preventing cartilage wear and synovial cell adhesion, proliferation, and in reducing the coefficient of friction of the articular cartilage surface (Leonardi et al., 2012b, c). Several recent studies found that treatment with recombinant lubricin could protect articular cartilage and prevent the process of OA in animal models (Teeple et al., 2011). Lubricin is also upregulated by mechanical compression and shear under homeostatic conditions in cartilage

explant cultures and in whole joints (Nugent-Derfus et al., 2007). Specific stimuli that mimic the kinematics of natural joints, in particular surface motion obtained by bioreactor, may promote the upregulation of lubricin gene expression (Grad et al., 2005). Moreover, dynamic shear stimulation increased lubricin secretion and also the total number of cells expressing lubricin (Nugent et al., 2006).

Physical activity increases lubricin expression and reduces inflammation in articular cartilage damaged by glucocorticoids (Musumeci et al., 2013a). Moreover, a healthy diet, olive oil-based, in conjunction with physical activity, improves the expression of lubricin in the articular cartilage surface, preventing OA (Musumeci et al., 2013b). Furthermore, physical activity increases energy metabolism, contributes to increase bone and skeletal muscle mass, strengthens the joints and can be useful in controlling pain, and improves cardiorespiratory efficiency by promoting psychological well-being (Kragstrup et al., 2011; Pichler et al., 2013). However, immobility, age, malnutrition, and other disabilities can interfere with optimal physical activity for patients with problems (Cordeiro et al., 2014). We studied the expression of lubricin in groups of animals at different ages, with and without physical activity. The overall objective of this work was to subject healthy young, adult, and aged rats to a protocol of physical exercise, adapted to various age groups, for a period of at least 8 weeks to evaluate the expression of lubricin as a new chondrocyte senescence marker. The fundamental questions that we posed at the inception of this study were as follows: could an elderly person undertake regular physical activity that can protect cartilage (by increasing lubricin expression)? Can we use rats as a model to examine the potential benefits of moderate physical activity on articular cartilage, using lubricin as a marker? Thus, the hypothesis that we tested in this study was whether physical activity can modify the natural history of joint degeneration, promoting enhanced lubrication in adult joints, preventing the occurrence of OA.

Materials and methods

Animals

Thirty male albino Wistar rats (Charles River Laboratories, Milan, Italy), five rats for each group, with different body weights and ages, were divided in six groups: (a) young rats, 3 months old, average body weight of 160 ± 20 g, common diet, sedentary; (b) young rats, 3 months old, average body weight of 160 ± 20 g, common diet, subject to physical activity; (c) adult rats, 12 months old, average body weight of 200 ± 20 g, common diet, sedentary; (d) adult rats, 12 months old, average body weight of 200 ± 20 g, common diet, subject to physical activity; (e) older rats, 24 months of age, average body weight of 240 ± 20 g, common diet, sedentary; (f) older rats, 24 months of age, average body weight of 240 ± 20 g, common diet, subject to physical activity. Rats were individually housed in polycarbonate cages (cage dimensions: $10.25''\text{W} \times 18.75''\text{D} \times 8''\text{H}$) during the entire stabling period of the study and were housed at controlled temperature ($20\text{--}23^\circ\text{C}$) with a relative humidity of 40–60%, with free access to water and food and photoperiod of 12 h light and dark. Antibiotic, analgesic,

muscle relaxant, and non-steroidal anti-inflammatory drugs (NSAID) therapy were not administered so as not to alter the results as previously described (Musumeci et al., 2013b). All procedures conformed to the guidelines of the Animal Care and Use Committee of the University of Catania, in accordance with the European Community Council Directive (86/609/EEC) and Italian Animal Protection Law (116/1992).

Experimental design

Common diet: rats were fed *ad libitum*, with standard rat chow containing carbohydrates (40%), proteins containing all essential amino acids (30%), and lipids (30%). Lipids were a mixture of neutral fatty acids, saturated fatty acids, and unsaturated fatty acids. Diets were prepared by Laboratorio Dottori Piccioni, Gessate (Milan), Italy, and were administered for the entire period of the experiment.

Groups 1, 3, and 5 rats were left free without immobilization in their cages.

Groups 2, 4, and 6 rats performed physical activity for 8 weeks. For the first 2 weeks, rats were subjected to exercise on treadmill (Fig. 1) (2Biological instrument, Varese, Italy), at a speed of 10–20 m/min (type of exercise: moderate interval training, depending on the characteristics and on the age of the rats), without any inclination, 5 days a week for 10 min daily. During the next 6 weeks, the same groups (2, 4, and 6) were subjected to treadmill exercise (2Biological instrument), 5 days a week for 20 min daily. The treadmill inclined at 3° (between 2 and 6 degrees) set at a speed of 10–30 m/min (type of exercise: interval training, between moderate and strong depending on the characteristics and on the age of the rats). A minimal electric shock (0.2 mA) forced the rat to walk on the treadmill at a speed and inclination adapted to it. The shock serves to stimulate the rat to walk and to instruct it. Usually the rat learns this activity in the first 2 min of the exercise. This type of exercise is used to stimulate the muscles, joints, and bones in the work of flexion-extension of the limbs. All electric shock data were acquired by data acquisition software (2Biological instrument). Before and after exercise, rats were left free without immobilization in their cages. During the exercise, the possible suffering of the animal was evaluated. The evaluated parameters were based on the clinical aspect of the animals (lucidity of the fur, presence of periocular secretion, weight, food and water consumption). These parameters were

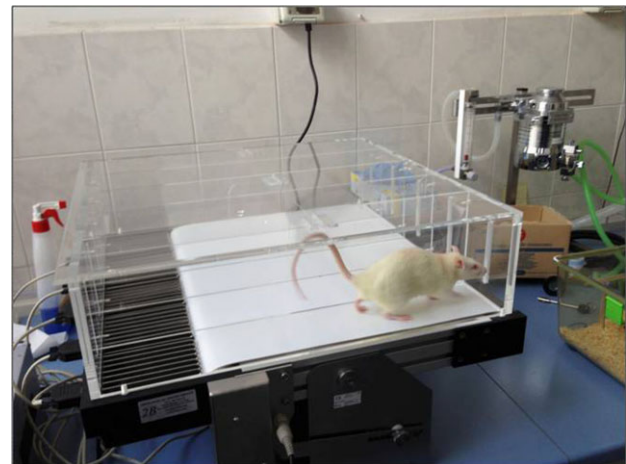


Fig. 1. Rat subjected to physical exercise on a treadmill. Performance of rats subjected to mild exercise on treadmill (2Biological instrument, Varese, Italy) at a speed of 10 m/min without any inclination.

monitored at least once a day. Potential rats discarded from the trial: rats that showed a progressive loss of 10% of their total body weight within 3 days from the start of weight loss; rats that exhibited difficulties in walking; rats that exceeded the number of five electric shocks (0.2 mA) without learning the work to be done on the treadmill. None of the rats presented any of these characteristics and thus no animals were excluded from the study. The day following the last training (after 8 weeks of experiment), the animals were humanely sacrificed by an intravenous lethal injection of anesthetic overdose using a mixture of Zoletil 100 (Virbac, Milan, Italy) at a dose of 80 mg/kg and DEXDOMITOR (Virbac) at a dose of 50 mg/kg. After euthanasia, both femurs were explanted, cleaned of soft tissues. Articular cartilage samples from distal epiphysis of the femur (condyles) were evaluated by histochemical, immunohistochemical, and western blot techniques; synovial fluid was used to quantify lubricin levels by enzyme-linked immunosorbent assay (ELISA).

Histochemistry

Samples from distal epiphysis of the femur (condyles) were rinsed in phosphate buffered saline (PBS) and fixed in 10% buffered formalin. After an overnight wash, specimens were dehydrated in a graded series of ethanol baths, cleared in xylene and paraffin-embedded, preserving their orientation and anatomical ultrastructure. Sections (4–5- μ m in thickness) were cut from paraffin blocks using a microtome, mounted on saline-coated slides, and stored at room temperature. The sections were stained with Alcian Blue pH 2.5 periodic acid Schiff (PAS) to assess the synthesis of sulfated glycosaminoglycans as constituents of proteoglycans (the assessment was made on the intensity of staining). The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photomicrographs were captured using a digital camera (AxioCam MRc5; Carl Zeiss).

Immunohistochemistry

For immunohistochemical analysis, cartilage tissue from distal epiphysis of the femur (condyles) was processed as previously described (Musumeci et al., 2013c). Briefly, the slides were dewaxed in xylene, hydrated using a graded series of ethanol baths, and incubated for 30 min in 0.3% H₂O₂/methanol to quench endogenous peroxidase activity before being rinsed for 20 min with PBS (Bio-Optica, Milan, Italy). The sections were heated (5 min $\times$ 3) in capped polypropylene slide holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica), using a microwave oven (750 W) to unmask antigenic sites as previously described (Musumeci et al., 2013e). The protein-blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA; Sigma, Milan, Italy) in PBS for 1 h in a humidified chamber. BSA was used as a blocking agent to prevent non-specific binding of the primary and secondary antibodies to the tissue sections. Following blocking, the sections were incubated overnight at 4 °C with rabbit polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma) overnight at 4 °C. Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-rabbit was used as secondary antibody) and then detected with peroxidase-labeled streptavidin, both incubated for 10 min at room temperature (LSAB+System-HRP, K0690; Dako, Glostrup, Denmark). Immunoreactivity was visualized by incubating the sections for 2 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in glycerol vinyl alcohol (Zymed Laboratories, San Fran-

cisco, California, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5; Carl Zeiss).

Evaluation of immunohistochemistry

The lubricin-staining status was identified as either negative or positive. Positive immunohistochemical staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described (Musumeci et al., 2013d). Staining intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0–4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of lubricin immunopositive cells [ES (extent score)] was independently evaluated by three investigators (two anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: < 5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and > 75% (++++). Counting was performed at $\times$ 200 magnification. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at the protein level. Positive controls consisted of tissue specimens with known antigenic positivity. Sections treated with PBS without the primary antibody served as negative controls.

Computerized morphometric measurements and image analysis

Fifteen fields from articular cartilage (femoral condyles) randomly selected from each section were analyzed; and the percentage area stained with lubricin antibody was calculated using image analysis software (AxioVision Release 4.8.2 – SP2 Software; Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabeling in each field. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, using objective lens of magnification $\times$ 20, i.e., total magnification $\times$ 400) fitted with a digital camera (AxioCam MRc5; Carl Zeiss); evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement.

Biochemical studies

Joints were washed by injecting 100 μ L of normal saline in the joint capsule, then aspirating 20 μ L of synovial fluid. Concentrations of lubricin in the synovial fluid were measured using a commercially available ELISA kit (Pierce Biotechnology, Rockford, Illinois, USA) as previously described (Ludwig et al., 2012) according to the manufacturer's instructions for the quantitative determination of lubricin. The experiments were repeated in triplicate.

Western blot analysis

Crude extracts were prepared by homogenizing cartilage tissue in a buffer containing 20 mM Tris (pH 7.4), 2 mM ethylenediaminetetraacetic acid, 0.5 mM ethylene glycol tetraacetic acid, 50 mM mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana, USA) using a Teflon glass homogenizer and then sonicated. Protein

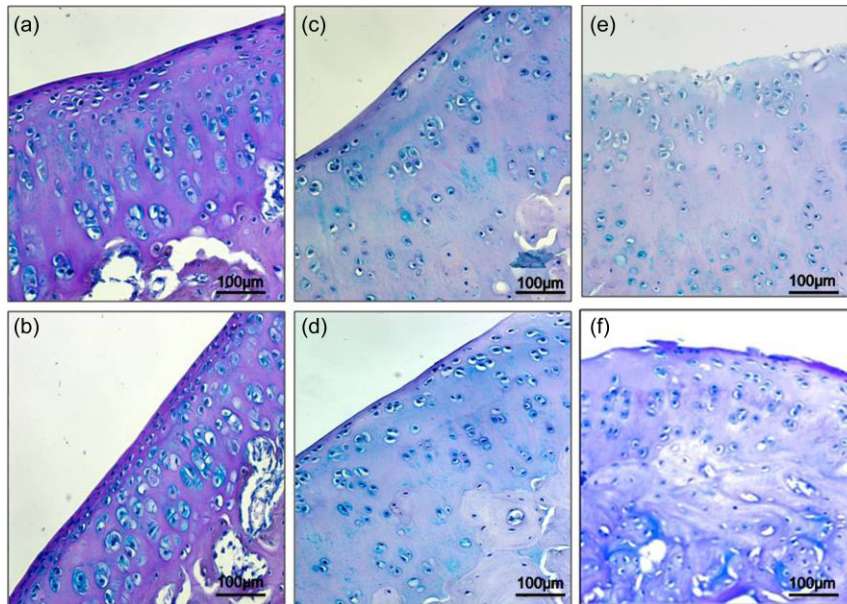


Fig. 2. Histochemical staining. (a–f) Histochemical staining (Alcian Blue pH 2.5 periodic acid Schiff) demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from the first four groups. Cartilage tissue samples from group 5 demonstrated slight structural alterations, including fragmentation and clefts in cartilage tissue, comparable to changes seen in the early stages of osteoarthritis, while group 6 demonstrated absence of structural alterations, due to recovery and improvement after physical activity. (a,b) The chondrocytes from groups 1 and 2 did not show any sign of cellular degeneration demonstrated by intense Alcian Blue staining. (c,d,f) The chondrocytes from groups 3, 4, and 6 showed early signs of cellular degeneration as demonstrated by moderate Alcian Blue staining. (e) The chondrocytes from group 5 showed clear signs of cellular degeneration demonstrated by reduced/weak Alcian Blue staining.

concentrations were determined using the Bradford protein assay (Bradford, 1976), using BSA as a standard. Sample proteins (50 µg) were diluted in 4× sodium dodecyl sulphate (SDS) protein gel loading solution (Invitrogen, Monza, Italy), boiled for 5 min, separated on 4–12% Bis-tris gel (Invitrogen), and electroblotted to nitrocellulose membrane (Invitrogen). Nonspecific binding was blocked for 2 h at 37 °C with 10% nonfat dry milk in Tween-Tris buffered saline (TTBS; Invitrogen). Membranes (Invitrogen), were incubated overnight at 4 °C with the following antibodies: rabbit polyclonal anti-lubricin antibody (ab28484; Abcam) diluted 1:1000, molecular weight 280 kDa, and rabbit anti-β-tubulin (Santa Cruz Biotechnology, California, USA) diluted 1:200, molecular weight 48 kDa, which was used as loading control. HRP-conjugated anti-rabbit secondary antibody (ECL Western Blotting System; GE Healthcare, Milan, Italy) were diluted at 1:10 000 for β-tubulin and 1:7500 for lubricin. All antibodies were diluted in 10% nonfat dry milk solution in TTBS. Blots were developed using the enhanced chemiluminescence technique (GE Healthcare) and relative band densities were quantified using ImageQuantTL 7.0 software (GE Healthcare). No signal was detected when the primary antibody was omitted (data not shown). The experiments were repeated in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS® release 16.0, Chicago, Illinois, USA). Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's *t*-test, while comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. *P*-values of less than 0.05 were considered statistically significant; *P*-values of less than 0.01 were considered highly statistically significant. Data are presented as the

mean ± standard error of the mean (SEM). Cohen's kappa was applied to measure the agreement between the three observers and averaged to evaluate overall agreement as previously described (Musumeci et al., 2013c,d).

Results

Histochemistry

Histochemical staining (Alcian Blue pH 2.5 PAS) demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from the first four groups: (1) young sedentary rats; (2) young rats subject to physical activity; (3) adult sedentary rats; and (4) adult rats subject to physical activity. Clones of chondrocytes were evident. The group 5 (older sedentary rats) demonstrated slight structural alteration with fragmentation and clefts in cartilage tissue, reminiscent of an early stage of OA, and only rare clones of chondrocytes were evident. Conversely, group 6 (older rats subject to physical activity) demonstrated absence of structural alterations and clones of chondrocytes were again apparent.

The chondrocytes from groups 1 and 2 (Fig. 2(a,b)) did not show any sign of cellular degeneration demonstrated by an intense staining, while the chondrocytes from groups 3 and 4 (Fig. 2(c,d)) showed early signs of cellular degeneration demonstrated by a moderate staining. The chondrocytes from group 5 (Fig. 2(e)) showed clear signs of cellular degeneration demonstrated by

reduced/weak staining, although the chondrocytes from group 6 (Fig. 2(f)) showed a moderate staining.

Observations

Lubricin immunohistochemical staining in explanted tissue was observed in the ECM of cells of all groups. Articular cartilage displayed a layer of lubricin on the surface of tissues (superficial zone). However, we observed different patterns of immunopositive cells in the specimens examined. Expression of lubricin was

investigated via immunohistochemistry, *in vivo* explanted tissue (Table 1).

A very strong lubricin staining (IS = 4; ES = +++) was observed in groups 1 and 2, where almost all chondrocytes were immunolabeled in the superficial zone (Fig. 3(a,b)). On the other hand, a moderate lubricin immunoreactivity (IS = 2; ES = ++) was noted in group 3, where less than half of the chondrocytes were immunostained in the superficial zone (Fig. 3(c)). Furthermore, a strong lubricin immunoreactivity (IS = 3; ES = +++) was seen in group 4, where more than half of

Table 1. Evaluation of lubricin immunostaining

Groups (five rats for each group)	Intensity of lubricin immunostaining (IS)	Percentage of lubricin immunopositive cells (ES)
1) Young sedentary rats	Very strong lubricin immunostaining (IS = 4)	Very strong lubricin immunostaining (ES = +++)
2) Young rats subject to physical activity	Very strong lubricin immunostaining (IS = 4)	Very strong lubricin immunostaining (ES = +++)
3) Adult sedentary rats	Moderate lubricin immunostaining (IS = 2)	Moderate lubricin immunostaining (ES = ++)
4) Adult rats subject to physical activity	Strong lubricin immunostaining (IS = 3)	Strong lubricin immunostaining (ES = +++)
5) Older sedentary rats	Weak lubricin immunostaining (IS = 1)	Weak lubricin immunostaining (ES = +)
6) Older rats subject to physical activity	Moderate lubricin immunostaining (IS = 2)	Moderate lubricin immunostaining (ES = ++)

Intensity of staining (IS) was graded on a scale of 0–4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of lubricin immunopositive cells [ES (extent score)] was independently evaluated by three investigators (two anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: < 5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and > 75% (++++).

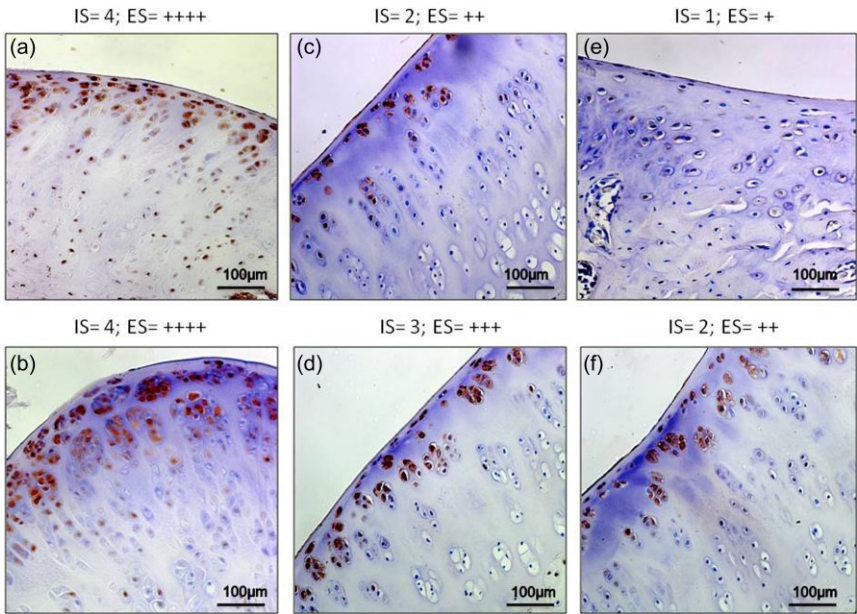


Fig. 3. Immunohistochemical staining. Lubricin immunostaining in cartilage explants was visualized throughout the extracellular matrix in samples from all groups: (a) young sedentary rats; (b) young rats subject to physical activity; (c) adult sedentary rats; (d) adult rats subject to physical activity; (e) older sedentary rats; (f) older rats subject to physical activity. Panels a–f: magnification ×20; scale bars 100 µm. (a,b) A very strong lubricin staining [intensity of staining (IS) = 4; extent score (ES) = +++] was observed in groups 1 and 2, where almost all chondrocytes displayed immunolabeling in the superficial zone. (c) Moderate lubricin immunoreactivity (IS = 2; ES = ++) was noted in group 3, where less than half of the chondrocytes were immunostained in the superficial zone. (d) Strong lubricin immunoreactivity (IS = 3; ES = +++) was detected in group 4, where more than half of the chondrocytes exhibited immunostaining in the superficial zone. (e) Weak lubricin immunoreactivity (IS = 1; ES = +) was observed in group 5, where a few chondrocytes were immunostained in the superficial zone. (f) Moderate lubricin immunoreactivity (IS = 2; ES = ++) was detected in group 6, where less than half of the chondrocytes were immunostained in the superficial zone.

the chondrocytes were immunostained in the superficial zone (Fig. 3(d)). In the other groups 5 and 6, the expression of lubricin significantly decreased compared with groups 1 and 2. In fact, lubricin expression in chondrocytes was weak or absent in group 5 (IS = 1; ES = +), where a few chondrocytes were immunostained in the superficial zone (Fig. 3(e)), while in group 6 the lubricin expression increased comparable to group 3, indeed the expression was moderate (IS = 2; ES = ++) where less than half of the chondrocytes were immunostained in the superficial zone (Fig. 3(f)). No immunoreactivity was observed in the negative control (ES = 0; IS = 0) treated with PBS without the primary antibodies (Fig. 4(a)). The percentage of lubricin-positive cells was observed among groups. Both ES and IS were significantly greater ($P < 0.05$). Statistical analysis showed that the percentage of lubricin-positive cells was statistically significant in groups 3, 4, and 6 ($P < 0.05$), while in group 5 was highly statistically significant ($P < 0.01$), equated to groups 1 and 2, (Fig. 4(b)). Interobserver agreement, measured as Kappa coefficient, was 0.96 (almost perfect).

Biochemical analysis

In order to investigate the effects of physical activity on lubricin levels in the synovial fluid of aging rats, we employed the ELISA method. Data obtained from these analyses confirmed that lubricin levels in rat synovial fluid were strikingly decreased with aging (Fig. 5). As indicated, group 1 showed higher lubricin levels (255 ± 13) when compared with groups 3 (150 ± 7.5) and 5 (100 ± 5). Interestingly, physical activity provoked a relevant enhancement of lubricin concentration as compared with synovial fluid of untrained rats. Such an increase, although less evident in group 2, reaches significant values both in group 4 ($##P < 0.05$ vs group 3) and group 6 ($#P < 0.5$ vs group 5).

Western blot analysis

To evaluate whether physical activity affects lubricin protein expression in cartilage of aging rats, we also performed western blot analysis. The representative western blot bands displayed in Fig. 6 show a high degree of correlation with the ELISA data from synovial

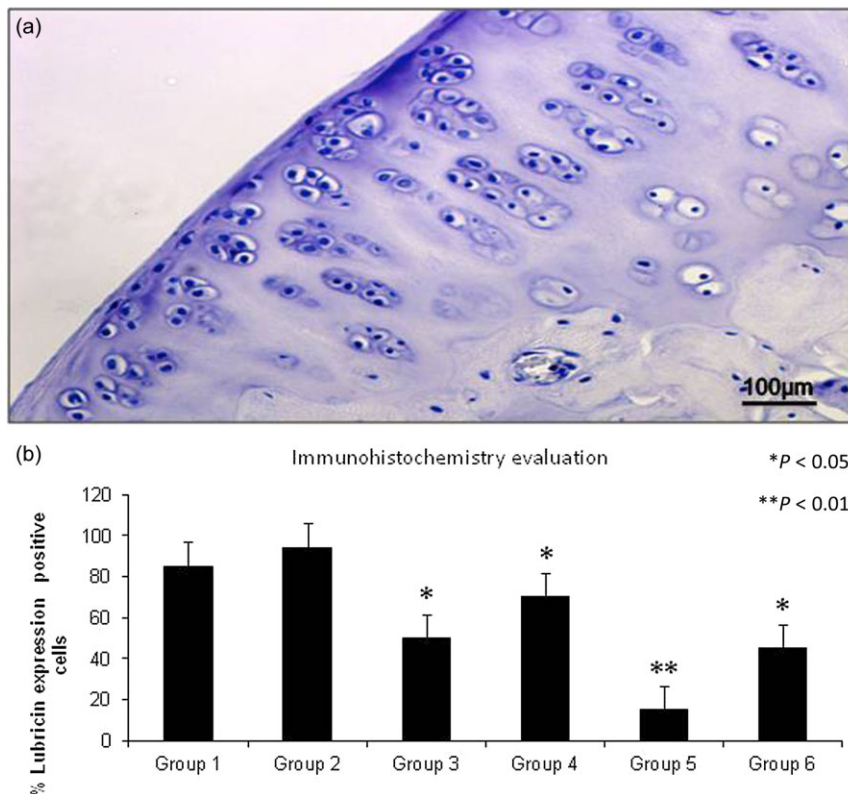


Fig. 4. Immunohistochemical evaluation. (a) No immunoreactivity was observed in negative controls [extent score (ES) = 0; intensity of staining (IS) = 0] treated with phosphate buffered saline in the absence of the primary antibody. Magnification $\times 20$; scale bars 100 μ m. (b) Immunohistochemical evaluation. The percentage of lubricin-positive cells was observed among groups. Both ES and IS were significantly greater ($P < 0.05$). Statistical analysis showed that the percentage of lubricin-positive cells was statistically significant in groups 3, 4, and 6 ($P < 0.05$), while in group 5 was highly statistically significant ($P < 0.01$), equated to groups 1 and 2. Interobserver agreement, measured as Kappa coefficient, was 0.96 (almost perfect). Results were presented as the mean $\pm$ standard error of the mean. Analysis of variance was used to evaluate the significance of the results. * $P < 0.05$ and ** $P < 0.01$, when compared with group 1.

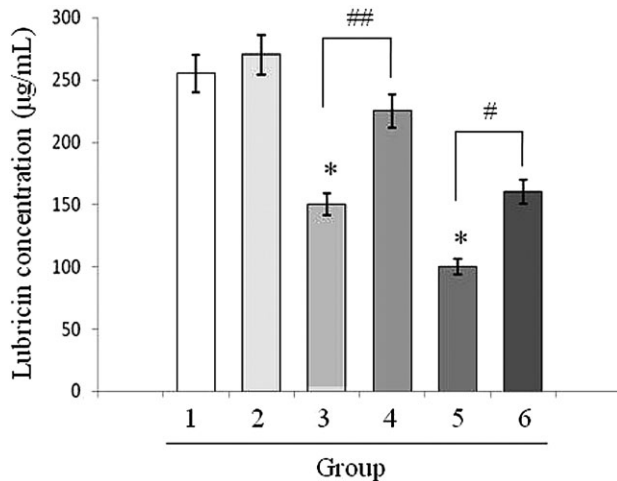


Fig. 5. Enzyme-linked immunosorbent assay (ELISA). Lubricin levels in the synovial fluid from all groups of rats were determined by ELISA. Lubricin levels significantly decreased in groups 3 and 5 (* $P < 0.05$) in comparison to group 1. Lubricin levels significantly increased in groups 4 (## $P < 0.01$) and 6 (# $P < 0.05$) rats in comparison to group 3 and 5, respectively. Results are presented as the mean $\pm$ standard error of the mean. Analysis of variance test was used to evaluate the significance of the results. * $P < 0.05$ vs group 1; # $P < 0.05$ vs group 5; ## $P < 0.01$ vs group 3.

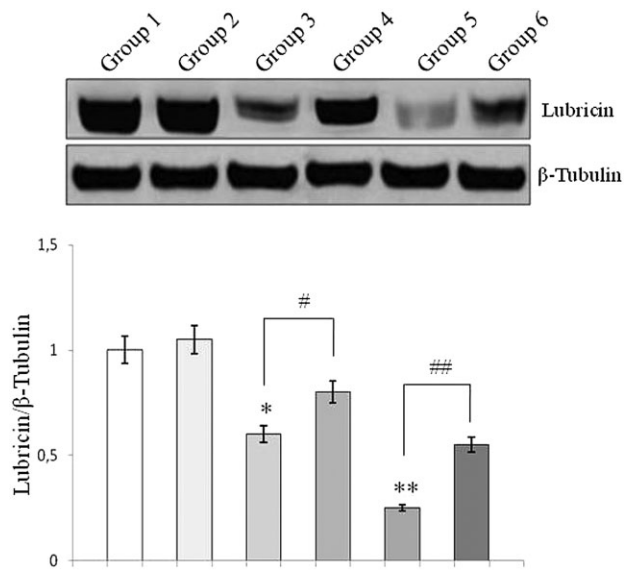


Fig. 6. Western blot analysis. Lubricin protein expression in cartilage from all groups was determined by western blot analysis. In groups 3 and 5, the expression of lubricin decreased compared with group 1. In groups 4 and 6, lubricin expression significantly increased when compared with groups 3 and 5, respectively. Relative band optical densities were quantified using ImageQuantTL software and normalized values were plotted in the histogram. * $P < 0.05$ or ** $P < 0.01$ vs group 1; # $P < 0.05$ vs control group 3 or ## $P < 0.01$ vs group 5. β -Tubulin was used as loading control in each experiment.

fluid. In fact, lubricin protein expression was the strongest in group 1, while in groups 3 and 5 it was the weakest (* $P < 0.01$). In groups 2, 4, and 6 (trained rats), the expression of lubricin increased in comparison to group 1, 3, and 5 (untrained rats), respectively, although significant values were reached only in groups 4 (# $P < 0.5$ vs group 3) and 6 (## $P < 0.05$ vs group 5).

Discussion

Cell senescence may be a mechanism to prevent the replication of cells with damaged DNA and thus tumor formation. OA is probably not a direct consequence of aging, but rather, aging affects the ability of the articular cartilage to maintain homeostasis when stressed. Age-related oxidative stress and damage may play a central role in cartilage aging through modulation of cell signaling pathways that regulate anabolic and catabolic activity.

Lubricin is a boundary lubricant, present in synovial fluid and on the superficial layer of articular cartilage, producing low friction and protecting surfaces from wear (Musumeci et al., 2013a; Chang et al., 2014). This protein is expressed less with aging and during OA (Musumeci et al., 2013b; Waller et al., 2013). Compromising boundary lubrication will cause increased friction, load amplification, and ultimately, greater cartilage damage. Lubricin is involved in several joint diseases and may play a beneficial role against the degradation of articular cartilage (Elsaid et al., 2012; Musumeci et al., 2013c). Lubricin has been noted to be sensitive to mechanical loading, as indeed running induces maximal expression of lubricin in the superficial zone of knee joint articular (Ogawa et al., 2014).

The impact of exercise on cartilage health depends on the extent of injury, the method, and the intensity of exercise (Musumeci et al., 2014a). While a running wheel exercise protocol protects articular cartilage and stimulates cartilage matrix synthesis in an uninjured animal model (Otterness et al., 1998), a forced joint exercise regimen accelerates cartilage degeneration in a rat model (Appelton et al., 2008). In another study, strenuous joint exercise has resulted in cartilage degeneration similar to early OA changes in normal rats (Pap et al., 1998), while an exercise regimen following anterior cruciate ligament transection provided a protective effect on articular cartilage (Galois et al., 2004), as also enhanced by an olive oil-based diet (Musumeci et al., 2013b). In recent studies authors confirmed the beneficial effect of the physical activity in bone (Pichler et al., 2013) and cartilage (Musumeci et al., 2013a) glucocorticoid-induced diseases.

Published data in the literature have showed a link between physical activity and inflammatory biomarkers, although increasing physical activity could be effective for reducing chronic inflammation, especially in individuals with chronic diseases associated with a state of

elevated inflammation (Beavers et al., 2010). In other recent and interesting studies our group demonstrated the effect of the combination of extra virgin olive oil diet and physical activity in skeletal muscle, preventing the oxidative stress from exhaustive exercise in muscles (Musumeci et al., 2014b). Our present study confirms the beneficial effects of physical activity (treadmill training) on articular cartilage. Alcian Blue staining demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from young and adult rats. The group 5 (old sedentary rats) showed a slight structural alteration with fragmentation and clefts in cartilage tissue, and a reduction of clones of chondrocytes comparable to an early stage of OA. Conversely, the group 6 (old rats subject to physical activity) demonstrated absence of structural alterations and increased number of clones of chondrocytes, due to a slight recovery and improvement after physical activity. The chondrocytes from groups 1 and 2 did not show any sign of cellular degeneration demonstrated by an intense staining, while the chondrocytes from groups 3 and 4 presented early signs of cellular degeneration demonstrated by a moderate staining. The chondrocytes from group 5 showed clear signs of cellular degeneration demonstrated by reduced/weak staining, although the chondrocytes from group 6 had moderate staining. This data mirrored the results obtained with immunohistochemistry in the study of lubricin expression. Lubricin immunohistochemical staining in explanted tissue was observed in an overall ECM of cells in all groups. A very strong lubricin staining was observed in groups 1 and 2, moderate lubricin immunoreactivity was observed in group 3, strong lubricin immunoreactivity was noted in group 4, while in the other groups 5 and 6 the expression of lubricin significantly decreased compared with groups 1 and 2. In fact, lubricin expression in chondrocytes was weak or absent in group 5, where a few chondrocytes were immunostained in the superficial zone, while in group 6 the lubricin expression increased comparable to group 3,

indeed the expression was moderate where less than half of the chondrocytes were immunostained in the superficial zone. The western blot and ELISA methods have confirmed these results.

Perspectives

Our findings suggest that moderate physical exercise, normal joint loading, and mechanical stimulation in elderly rats improve lubrication and prevent cartilage degeneration, promoting lubricin synthesis in synovial fluid, compared with unexercised adult rats. Physical activity increases joint mobility, consequently, both synovial fluid and chondrocytes express more lubricin, resulting in enhanced lubrication of articular surfaces. This protective lubrication mechanism help to prevent the onset of OA in aging. Our results also support the hypothesis that physical activity could also be used in aging as a natural therapeutic treatment for cartilage diseases to prevent OA and limit the deleterious effects of chondrocyte senescence. Further longer term *in vitro*, *in vivo*, and clinical studies using athletic cohorts are needed to elucidate the mechanisms that contribute to chondrocyte and cartilage aging. Although this study highlights the benefits of moderate exercise for articular cartilage, more research is needed to understand the precise role of lubricin in the context of cartilage aging. With our results we can also hypothesize that lack of lubricin could be a possible biomarker for the detection of chondrocytes senescence and in chondrocytes affected by OA. This knowledge may help us develop novel, innovative, and preventive strategies to slow or halt the aging process and the development of OA in joint tissues.

Key words: Articular cartilage, chondrocyte, senescence, physical exercise, joint degeneration, lubricin, osteoarthritis.

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