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ORIGINAL CONTRIBUTION

Physical activity and Mediterranean diet based on olive tree phenolic compounds from two different geographical areas have protective effects on early osteoarthritis, muscle atrophy and hepatic steatosis

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Abstract

Purpose Osteoarthritis (OA) leads to progressive loss of articular cartilage, pain and joint disability. An acute injury constitutes an important risk factor for early OA, determining an inflammatory process responsible of cartilage degeneration and muscle atrophy, due to the joint pain and immobility. The study aims to assess the effects of conjugation of physical activity and diet enriched by olive tree compounds [extra virgin olive oil (EVOO) and olive leaf extract (OLE)], on the musculoskeletal system in OA rat model.

Methods OA was induced by anterior cruciate ligament transection and confirmed by Mankin and OARSI scores. Rats were subjected to physical activity on treadmill 5 days a week for 10 min daily and fed with experimental diets (standard diet enriched with Sicilian EVOO, Tunisian EVOO and Tunisian EVOO-OLE) for 12 weeks. Immunohistochemistry was used to evaluate IL-6 and lubricin expression in cartilage tissue and ELISA was used to quantify these proteins in serum at different time points. Histology and histomorphometry analysis were done to valuate liver steatosis, muscle atrophy and cartilage pathological changes.

Results Compared to the OA group, the experimental groups showed general increased lubricin and decreased IL-6 expression, significant muscle hypertrophy and no signs of liver steatosis, suggesting the beneficial effects of physical activity coupled with EVOO-enriched diets on rat articular cartilage. Interestingly, the best result was shown for Sicilian EVOO-enriched diet.

Conclusion In conclusion, the conjugation of physical activity and EVOO-enriched diet determines a significant articular cartilage recovery process in early OA.

Keywords Osteoarthritis · Muscle atrophy · Physical activity · Olive oil · Olive leaf extract · Lubricin · Inflammation

Introduction

Osteoarthritis (OA) is a common degenerative disorder, leading to the progressive loss of articular cartilage, synovial inflammation and changes of subchondral bone structure, resulting in stiffness, pain and total joint disability in the latest stages [1]. The most important risk factors are aging, inflammation, muscle atrophy, acute injury, excessive load and metabolic disorders. Nowadays, there is no resolutory therapy for this complicated disorder. This is due to the multifactorial nature of the relative degenerative process and to the avascular and aneural nature of cartilage tissue [2, 3]. The progressive loss of the joint function is ascribable not only to the articular cartilage degeneration, but also to the impaired ability of skeletal muscle tissue. It has been stated that muscle weakness is responsible for OA onset, especially after joint injury. Indeed, it is followed by the neural inhibition that prevents the central nervous system from fully activating the muscles, a process known as arthrogenic muscle inhibition (AMI) [4–6]. All these data suggest the multifactorial pathogenesis of OA and the relative implication of different tissues of the entire joint, which further complicate the related therapy.

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Innovative approaches such as those based on functional foods have been lately studied for this aim. Olive oil, for example, is the principal source of monounsaturated fatty acids (MUFAs) in the Mediterranean diet, a healthy diet plan associated with a lower incidence of cardiovascular, cancer, degenerative and chronic diseases [7, 8]. Recently, the beneficial effects of products derived from olive tree (*Olea europeae*), such as olive oil (OO) and olive leaf extract (OLE), have been widely demonstrated. Their properties are ascribed to the content of polyphenols, which exert antioxidant, anti-inflammatory and antimicrobial effects [9–11].

Lubricin is a lubricating glycoprotein, recently extensively studied at our laboratory. It has a protective role associated with the preservation of cartilage superficial layer by reducing the coefficient of friction in the diarthrodial joints [12]. It has been shown that after joint injury and during a relative acute inflammatory process, characterized by the flare of proinflammatory cytokines, lubricin synthesis decreases significantly both in cartilage tissue and synovial fluid, predisposing the articular cartilage to degeneration and OA onset [13–15].

Another fundamental and widely studied health-associated factor is represented by regular physical activity. Indeed, many recent studies have been carried out to investigate the beneficial effects of physical activity on the musculoskeletal system and the relative better preservation of articular cartilage and muscle tissues [16–18]. At the molecular and cellular levels, myoblasts and chondrocytes share similar pathological targets and pathways, and close anatomical location, suggesting a possibility of paracrine communication. The protective role of physical activity on both cartilage tissue and skeletal muscle has been widely confirmed. It has been shown that physical exercise reduces inflammation and improves physical function, suggesting its positive role in the prevention of muscle atrophy [19].

All these data confirm the beneficial role of both physical activity and OO with the relative polyphenolic compounds, on different tissues in pathological conditions. However, there is no evidence in literature suggesting that there might be differences associated with the OO origin, olive maturity, OLEs, presenting different polyphenolic complex content and their beneficial or toxic effects on osteoarthritic articular cartilage and muscle atrophy. For this reason, the aim of this study was to assess, for the first time, whether the diet enriched by different olive tree products (OO from different origins and types and OLE), containing diverse percentage of polyphenols, and conjugated with physical activity, could be employed to improve muscle and cartilage preservation/restoration, in mechanically induced OA rat model without signs of liver steatosis.

Methods

Breeding and housing of animals

Forty-eight 3-month-old healthy male Wistar outbred rats (Charles River Laboratories, Milan, Italy), with an average body weight of 300 ± 20 g, were used for this study. Rats were housed in polycarbonate cages (cage dimensions: 10.25″W × 18.75″D × 8″H) at controlled temperature (20–23 °C) and humidity during the whole period of the research, with free access to water and food and photoperiod of 12 h light/dark at the "Center for Advanced Preclinical In Vivo Research (CAPIR)". Surgical procedures for anterior cruciate ligament transection (ACLT) were performed in accordance with the method previously described [20]. After surgery, free cage movement without joint immobilization was permitted for all animals. The pre-operative examinations included physical and photographic examination. The day following the last training (after 12 weeks of experiment), the animals were killed 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, Milan, Italy) at a dose of 50 mg/kg. After euthanasia, both femurs were explanted, cleaned of soft tissues and the samples were used to perform histomorphometric evaluations. Cartilage, skeletal muscle (*tibialis* anterior muscle) and liver tissue were used to perform histological analyses. Moreover, articular cartilage samples from distal epiphysis of the femur (condyles) were used for immunohistochemical evaluation. The blood serum was collected at 2 weeks after ACLT to study acute inflammation, and at 12 weeks after ACLT to study chronic inflammation. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania (Protocol n. 2112015-PR of the 14.01.2015, Italian Ministry of Health). The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

Experimental design

The 48 animals were divided into six groups of eight rats per group, as shown in Table 1.

Diets

The rats were fed ab libitum for 12 weeks. The experimental diets were administered 1 week prior to ACLT. The diets were prepared as follows:

Table 1 Experimental groups

Group	Diet	ACLT	Lifestyle
1	Common	No	Sedentary (healthy control)
2	Common	Yes	Sedentary (experimental control)
3	Common	Yes	Physical activity
4	S-EVOO	Yes	Physical activity
5	T-EVOO	Yes	Physical activity
6	T-enriched-EVOO	Yes	Physical activity

- *Common diet* Standard rat chow (4RF25 GLP) was provided by Mucedola s.r.l. (Settimo Milanese, Milan, Italy). The composition of the common diet has been reported in Table 2.
- *Sicilian extra virgin olive oil* (S-EVOO)-*supplemented diet* S-EVOO was provided by "Oleificio Guccione di Divita Vito e G. SAS". It was produced in the south-east of Sicily, in the town of Chiaramonte Gulfi (RG) (year 2015–2016) and it is a D.O.P. Monti Iblei Sotto-zona Gulfi, derived from the variety of "Tonda Iblea". S-EVOO was obtained by extraction through the "continuous cold cycle and natural decantation" (Alfa Laval) method with particular conditions, especially thermal, not to produce physical alterations of oil. After decantation, S-EVOO was transferred into dark glass bottles and stored. The acidity of S-EVOO was 0,18%. The composition of the S-EVOO-supplemented diet has been reported in Table 2.
- *Tunisian extra virgin olive oil (T-EVOO)-supplemented diet* T-EVOO was provided by the Department of Molecular and Cellular Biology and Plant Physiology at the University of Carthage, Tunisia. It was produced in the northeast of Tunisia, from Béja Province (year 2015–2016), derived from the variety of "Sayali". T-EVOO was obtained by extraction with a laboratory-scale instrument (Abencor analyzer, MC2 Ingenieria y Sistemas, Sevilla, Spain) which includes: a mill, a thermo beater, and an olive pulp centrifuge; olives were crushed according to IOC regulations with a hammer mill, slowly mixed for 30 min at room temperature and the obtained paste was centrifuged without addition of warm water. The oil was separated by decanting, transferred into dark glass bottles, and stored. The acidity of T-EVOO was 0.2%. The composition of the T-EVOO-supplemented diet as been reported in Table 2.
- *Tunisian extra virgin olive oil and leaves extract (T-enriched-EVOO)-supplemented diet*: T-enriched-EVOO belongs to the same variety of T-EVOO, and picked from the same geographic site, to which the same variety of olive leaf extract is added. After extraction, dried and crushed olive leaves were analyzed as previously described [21] and then added to T-EVOO at a concentration of 0.2 g/ml and sonicated for 15 min to increase the polyphenolic content. The composition of the T-enriched EVOO-supplemented diet is reported in Table 2.

Table 2 Composition of diets

Fatty acids	Standard diet	S-EVOO-supplemented diet	T-EVOO-supplemented diet	T-enriched-EVOO-supplemented diet
Water (% m/m)	12.00	11.73	11.73	11.73
Protein (% m/m)	22.00	21.51	21.51	21.51
Fat (% m/m)	3.50	5.62	5.62	5.62
Carbohydrates (crude fibers and nitrogenous extractives) (% m/m)	55.00	53.80	53.80	53.80
Ash (% m/m)	7.50	7.34	7.34	7.34
M.E. [kcal/kg]	2789	3342	3342	3342
Palmitic acid (16:0) (mg/kg)	5762	9002	8572	8572[a]
Palmitoleic acid (16:1) (mg/kg)	289	579	439	439[a]
Stearic acid (18:0) (mg/kg)	1199	1689	1759	1759[a]
Oleic acid (18:1) (mg/kg)	8217	24,047	25,767	25767[a]
Linoleic acid (18:2) (mg/kg)	18,172	20,352	19,082	19082[a]
Linolenic acid (18:3) (mg/kg)	1838	2018	1968	1968[a]
Vitamin E (mg/kg)	68.5	72.167	78.499	78.499[a]
Polyphenols (mg/kg)	–	5.960	10.395	18.250

[a]The content is the same as that of T-EVOO-supplemented diet because the leaf extract is rich mainly in polyphenols

The composition of fatty acids, vitamin E and polyphenols was analyzed by gas chromatography (GC), high-performance liquid chromatography–mass spectrometry (HPLC–MS) (EN ISO 9936:2006) in case of S-EVOO and HPLC–diode array detector-MS (HPLC–DAD-MS) in case of T-EVOO [22]. The composition of fatty acids, vitamin E and polyphenols contained in the experimental diets is reported in Table 3.

To supplement the standard chow with S-EVOO or T-EVOO or T-enriched-EVOO, we added and mixed oil (2.5 ml/100 g of chow corresponding to a quantity of EVOO in grams equal to 2.25 g/100 g of chow), daily at feeding time to minimize oxidation. Body weights and food and drink intake were monitored 3 days per week throughout the experiment.

Treadmill training

Groups 3–6 rats performed physical activity, for the treadmill adaptation, for 1 week prior to ACLT on treadmill (2Biological instrument, Varese, Italy), at a speed of 10/20 m/min (type of exercise: interval training, between mild and moderate) 5 days a week for 10 min daily. This exercise regimen was used to simulate repeated joint flexion and extension. Starting 1 week following surgery, the same groups (3–6) were subjected to treadmill mild exercise for 12 weeks, 5 days a week for 20/30 min daily. The treadmill was inclined at 3° (between 2 and 6 degrees) and set at a speed of 10/30 ms/minute (type of exercise: interval training, between mild and moderate). Exercise procedures were performed in accordance with the method previously described [23, 24].

Histology analysis

Liver, skeletal muscle and cartilage samples were rinsed in phosphate-buffered saline (PBS, Bio-Optica, Milano, Italy), fixed in 10% buffered-formalin (Bio-Optica, Milan, Italy) for 24 h at room temperature. Following an overnight wash, specimens were dehydrated in graded ethanol (Bio-Optica, Milan, Italy), cleared in xylene (Bio-Optica, Milan, Italy) and paraffin embedded (Bio-Optica, Milan, Italy), preserving their anatomical orientation. Sections of 4–5 µm thick were cut from paraffin blocks and stained with hematoxylin and eosin (H&E; Bio-Optica, Milan, Italy) as previously described [25], for general cell identification, for studying the morphological structure of the tissue and for the histomorphometric measurements. The samples were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and a digital camera (AxioCam MRc5, Carl Zeiss) was used to take the pictures.

Table 3 Composition of fatty acids, vitamin E and polyphenols in S-EVOO and T-EVOO

	S-EVOO	T-EVOO	*p*
Palmitic acid (C16:0) (% mass/mass)	14.41 ± 0.09	12.51 ± 0.08	**
Palmitoleic acid (C16:1) (% mass/mass)	1.31 ± 0.03	0.67 ± 0.01	**
Stearic acid (C18:0) (% mass/mass)	2.18 ± 0.04	2.53 ± 0.03	**
Oleic acid (C18:1) (% mass/mass)	70.38 ± 0.31	78.02 ± 0.45	**
Linoleic acid (C18:2) (% mass/mass)	9.69 ± 0.15	4.06 ± 0.42	**
Linolenic acid (C18:3) (% mass/mass)	0.84 ± 0.03	0.61 ± 0.01	**
Vitamin E (mg/kg)	163 ± 1.03	444.4 ± 3.3	**
Polyphenols (mg/kg)	265 ± 1.59	462 ± 2.24	**

*p value < 0.05; **p value < 0.01

Histomorphometric analysis

Articular cartilage samples

The femur explantation procedure and the subsequent cleaning of soft tissues were performed as previously described [26]. Samples from all rats (both medial and lateral femoral condyles of untreated and surgically treated animals) were used for histomorphometric analysis. Histomorphometry was performed with image analysis, Kontron KS 300 software (Kontron Electronics, Eching bei Munchen, Germany) by three blinded investigators (two anatomical morphologists and one histologist). Evaluations were assumed correct if there were no statistically significant differences between the investigators. Seven fields randomly selected from each section were analyzed. The semi-quantitative grading criteria of macroscopic Kraus' modified Mankin score [27] and microscopic Histopathology OARSI system [28] were used. The inter-observer variability among three observers for the Mankin system showed a good intra-class correlation coefficient (ICC > 0.92) as for the OARSI system (ICC > 0.89). Repeat scoring by investigators showed very good agreement (ICC > 0.94).

The Kraus' modified Mankin score provides grades from 0 to 4: Grade 0, normal cartilage; Grade 1, minimal articular damage; Grade 2, articular cartilage damage affecting up to 30% of the articular surface; Grade 3, loss of up to 50% of the articular cartilage; Grade 4, severe loss of cartilage affecting more than 50% of the articular surface.

The Histopathology OARSI system provides grades from 0 to 6: Grade 0, normal articular cartilage; Grade 1, intact surface; Grade 2, surface discontinuity; Grade 3, vertical fissures extending into mid zone; Grade 4, erosion; Grade 5, denudation; Grade 6, deformation.

Skeletal muscle samples

Seven fields, with area of about 600.000 µm^2, randomly selected from each muscle cross section, were subjected to

morphometric analysis. The perimeter of the muscle fibers was considered and calculated using a software for image acquisition (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Negative images were used for a better software performance in the morphometric analysis. Data were expressed as mean ± standard deviation (SD). Statistical significance of results was thus accomplished. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany); 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 case was reconsidered to reach an unanimous agreement.

Immunohistochemistry (IHC) analysis

Articular cartilage samples were processed for immunohistochemical analysis as previously described [29]. After blocking, the sections were incubated overnight at 4 °C with rabbit polyclonal anti-interleukin-6 (IL-6), work dilution in PBS (Bio-Optica) 1:100 (ab6672; Abcam, Cambridge, UK), and with rabbit polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Bio-Optica). The immunoreaction 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, Denmark). The samples were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Goteborg, Sweden) in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA), observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss).

Evaluation of immunohistochemistry

The IL-6 and lubricin-staining status were identified as either negative or positive. As previously described, immunohistochemical staining was defined positive if brown chromogen was detected on the edge of the hematoxylin-stained cell nucleus, within the cytoplasm or in the membrane [30]. Light microscopy was used to evaluate stain intensity and the percentage of immunopositive cells. The intensity of staining (IS) was evaluated on a four-grade scale (0–4) as follows: no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, very strong staining = 4. Three investigators (2 anatomical morphologists and 1 histologist) independently evaluated the percentage of antibody immunopositive cells through the five categories of extent score (ES): < 5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and > 75% (++++), in a blinded manner. Counting was performed under Zeiss Axioplan light microscope at ×200 magnification. If disputes concerning the interpretation occurred, the case was revised to reach an unanimous agreement, as previously described [20]. A digital camera (Canon, Tokyo, Japan) at ×200, ×400 and ×600 magnifications was used to take digital pictures. In this study, positive controls consisted of rat cartilage tissue, and negative control sections, treated with PBS without the primary antibodies, were used to test the specific reaction of primary antibodies used at a protein level. Positive immunolabeling for antibodies were nuclear/cytoplasmic.

Computerized morphometric measurements and image analysis

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 immunolabelling, was used to calculate the percentage area stained with IL-6 and lubricin antibodies in seven fields, randomly selected from each section. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, using objective lens of magnification ×20 i.e., total magnification 400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss). Three blinded investigators (2 anatomical morphologists and 1 histologist) made the evaluations that were assumed to be correct if values did not have statistically significant difference [20]. If disputes concerning interpretation occurred, unanimous agreement was reached after sample re-evaluation.

Biochemical studies: enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-6 and lubricin in blood serum were measured 2 weeks after ACLT and 12 weeks after ACLT using a commercially available ELISA kit (antibodies-online Inc, 11 Dunwoody Park, Suite 145, Atlanta, GA 30338, USA) according to the manufacturer's instructions for the quantitative determination of all samples used (ThermoFisher Scientific, 81 Wyman Street Waltham, MA, USA). Absorbance was measured at 450 nm against 630 nm as reference with an ELISA reader (Dynatec, MR 5000; Dynatech Laboratories, LabX, Canada).

Statistical analysis

Statistical analysis was performed using GraphPad Instat® Biostatistics version 3.0 software (GraphPad Software, Inc. La Jolla, CA, USA). Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Student's *t* test was used for comparisons between two means (immunohistochemical evaluation) and unpaired *t* test with Welch's correction (muscle fiber evaluation), while

analysis of variance (ANOVA) and Bonferroni's test (immunohistochemical evaluation) or Dunnett's test (muscle fiber evaluation) were used for comparison between more than two groups. p values of less than 0.05 ($p < 0.05$) were considered to be statistically significant; p values of less than 0.01 ($p < 0.01$) were considered to be highly statistically significant. Data are presented as the mean ± SD. Cohen's κ was applied to measure the agreement between the two blinded observers and averaged to evaluate overall agreement.

Results

Body weight and calories consumption

Body weights and food and drink consumptions were monitored for all groups, 3 days per week throughout the experiment. In relation to body weight, we observed a physiological increase in all groups and the differences between groups were never significant ($p > 0.05$) (Fig. 1a), as expected. The caloric consumption, instead, showed significant differences between the groups ($p < 0.01$). The results demonstrate that the calorie consumption in group 4 was significantly higher when compared with groups 1, 2 and 3 (**$p < 0.01$). Also, group 5 demonstrated a higher caloric consumption when compared with groups 1 and 2 (*$p < 0.05$). However, the biggest caloric consumption was observed in group 4 (Fig. 1b).

Histology

Liver samples

The H&E staining was used for general cell identification, vitality and to study the morphological structure of the liver in all groups. This investigation was done to demonstrate that the supplementation diet with EVOO does not cause hepatic steatosis. In fact, the liver of rats, in groups 1–3 fed with the common diet, demonstrated no sign of tissue degeneration, with good preservation and absence of lipid droplets, comparable to groups 4–6, fed with EVOO and leaf extract (Fig. 2).

Articular cartilage samples

The H&E staining was used for general cell identification, vitality and to study the morphological structure of the articular cartilage in all groups. Group 1 presented normal articular cartilage; in the superficial zone, cells were flat and small, and in the middle and deep zone, cell were organized in columns; the tidemark was evident (Fig. 3a). In group 2, we found moderate OA cartilage and the structural alterations included a reduction of cartilage thickness of the superficial and the middle zones, demonstrated deep surface clefts, disappearance of cells from the superficial zone, cloning, and a lack of cells in the intermediate and deep zone, which were poorly organized in columns (Fig. 3b). Group 3 presented a slight reduction of the superficial cartilage layer, characterized by less cellular presence and better general tissue preservation compared to group 2 (Fig. 3c). In group 4, the structural alterations were very slight, included a little reduction of cells in the cartilage layers (superficial, intermediate and deep zone) compared to the control, and the cells were organized in columns (Fig. 3d). In group 5, the structural alterations were very slight, in the superficial layer. there was the presence of fibrocartilaginous, scar-like tissue with fibroblast-like cells, and the cells were organized in columns (Fig. 3e). In group 6, structural alterations were present even if in a moderate way. Cell hyperplasia was present and the superficial and intermediate layer was almost replaced by fibrocartilaginous, scar-like tissue with fibroblast-like cells. The cells were organized in columns (Fig. 3f).

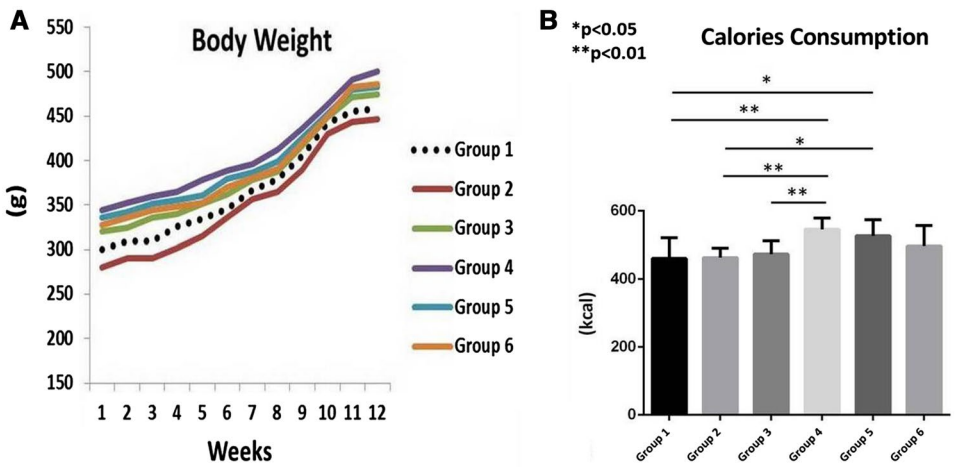


Fig. 1 Graphs: **a** Body weight variations over 12 weeks, showing a physiological increase in all groups, even if the differences between groups were not significant ($p > 0.05$). **b** Calorie consumption expressed as mean + SD in all the groups, showing significant differences between group 4 when compared with groups 1, 2 and 3 (**$p < 0.01$) and between group 5 when compared with groups 1 and 2 (*$p < 0.05$)

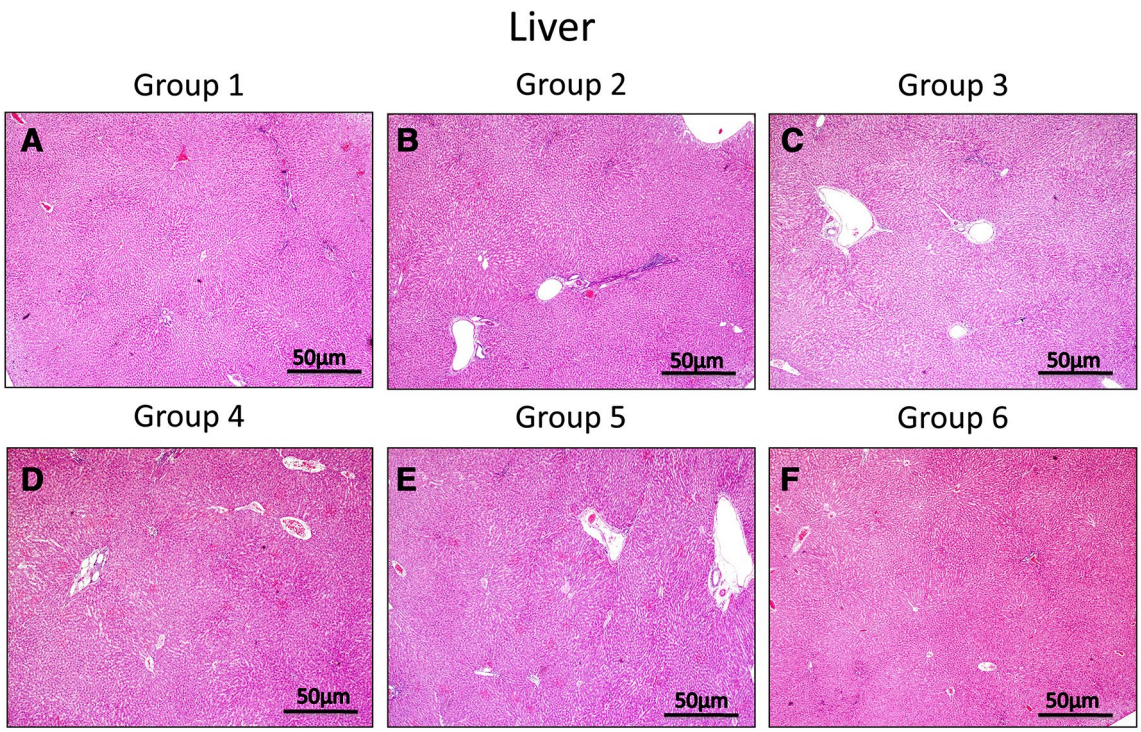


Fig. 2 **a–f** Morphological and histological analysis of liver samples. Explanted liver from all groups demonstrated no sign of hepatic steatosis and no sign of tissue degeneration, with good preservation and absence of lipid droplets. Magnification ×100. Scale bars: 50 µm

Skeletal muscle samples

We performed histological analysis with hematoxylin and eosin to highlight the possible structural alterations in muscle tissue of all experimental groups. Muscle fibers of all groups did not show damaged histological structure. However, we observed muscle fiber hypotrophy in group 2 and hypertrophy in the experimental groups, especially in groups 3, 4 and 5 (Fig. 4), of which we discuss in histomorphometric analysis of muscle fibers.

Histomorphometric analyses

Articular cartilage samples

The histomorphometric parameters used in group 1 confirmed no sign of cartilage degeneration with an intact and normal cartilage structure (Kraus' modified Mankin and Histopathology OARSI system scores were 0). In group 2, more serious pathological changes were evident in the cartilage; in fact, horizontal cleavage tears or flaps and deep lesions were present (Kraus' modified Mankin score of 3 and Histopathology OARSI system score 5). Group 2 confirmed the development of articular degenerative processes compared to group 1 ($p < 0.01$). Group 3 still showed articular degenerative processes compared to group 1 ($p < 0.01$) and to group 2 ($p > 0.05$) (Kraus' modified Mankin score of 2 and Histopathology OARSI system score 3), while groups 4, 5 and 6 showed better cartilage preservation compared to groups 2 ($p < 0.01$) and 3 ($p > 0.05$) (Kraus' modified Mankin score of 1 and Histopathology OARSI system score 2) as confirmed by Kraus' modified Mankin score and Histopathology OARSI system (Fig. 5). The inter-observer variability among five observers for the Mankin system showed a similar good intra-class correlation coefficient (ICC > 0.91) as for the OARSI system (ICC > 0.90). Repeat scoring by three of the five investigators showed very good agreement (ICC > 0.95). Data are presented as the mean ± SD.

Skeletal muscle samples

In our morphometric analysis of the perimeter (µm) (mean ± SD) of the muscle fibers, the comparison between group 1 (health control) and group 2 (experimental control) highlighted a highly significant hypotrophy in group 2 (Figs. 4a, b and 6a). The comparison between group 2 (experimental control) and all experimental groups (3, 4, 5 and 6) showed a highly significant hypertrophy in groups 3, 4 and 5 ($p < 0.01$) and a statistically significant hypertrophy in group 6 ($p < 0.05$) (Figs. 4b–f, 6b). However, when comparing group 1 (health control) and all experimental groups (3, 4, 5 and 6), groups 3 and 4 showed a highly significant hypertrophy ($p < 0.01$) and group 5 showed a significant hypertrophy ($p < 0.05$). On the contrary, group 6 did not

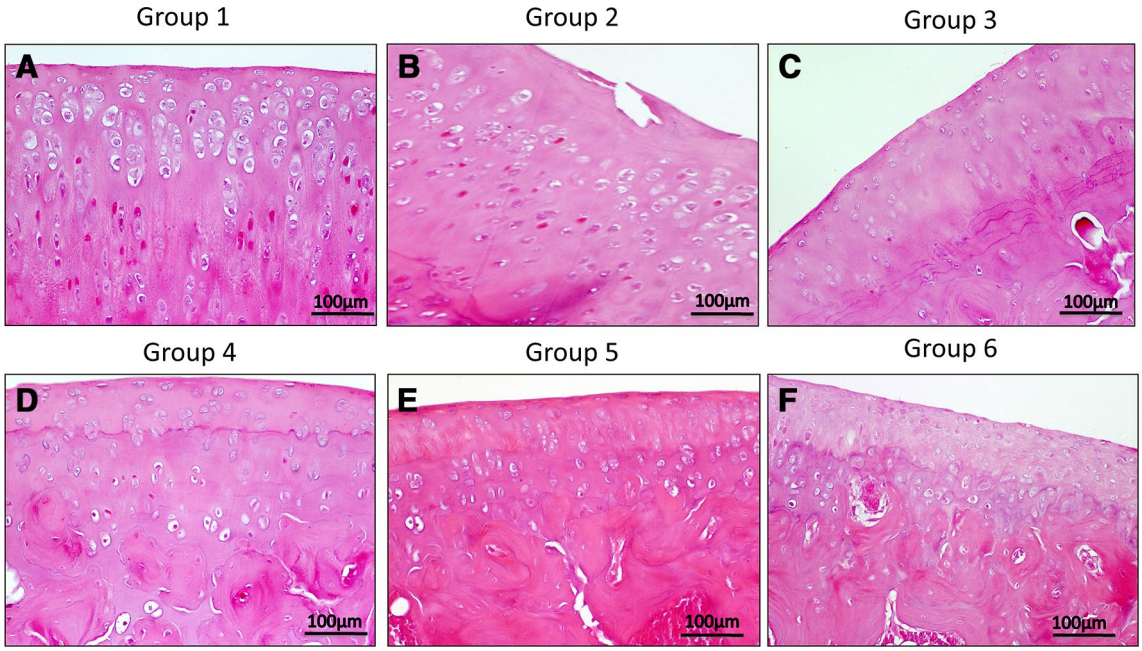


Fig. 3 **a–f** Articular knee cartilage from rat. Hematoxylin and eosin staining. **a** Normal articular knee cartilage from rat. In the superficial zone, cells are flat and small; in the middle and deep zone, cells are organized in columns; the tidemark is evident. **b** Articular knee cartilage from rat at early OA stage after ACLT. Moderate OA cartilage. The structural alterations included a reduction of cartilage thickness of the superficial and the middle zones. Demonstrated deep surface clefts, disappearance of cells from the superficial zone, cloning, and a lack of cells in the intermediate and deep zone, which were poorly organized in columns. The tidemark was no longer intact and the subchondral bone showed fibrillation. **c** Moderate OA cartilage, after ACLT and training treatment. The structural alterations included a reduction of cartilage thickness of the superficial and the middle zones with disappearance of cells from the superficial zone, cloning, and a lack of cells in the intermediate and deep zone, which were poorly organized in columns. **d** Slight OA cartilage, after ACLT, training and Sicilian olive oil diet. The structural alterations were very slight, included a little reduction of cells in the cartilage layers (superficial, intermediate and deep zone) compared to the control and the cells were organized in columns. **e** Slight OA cartilage, after ACLT, training and Tunisian olive oil diet. The structural alterations were very slight, and in the superficial layer there was the presence of fibrocartilaginous, scar-like tissue with fibroblast-like cells. The cell are organized in columns. **f** Slight OA cartilage, after ACLT and training treatment. Structural alterations are present even if in a moderate way, including cell hyperplasia, and the superficial and intermediate layer were almost replaced by fibrocartilaginous, scar-like tissue with fibroblast-like cells. The cell were organized in columns. **a–f** Magnification ×20. Scale bars: 100 µm

show a statistically significant hypertrophy when compared with group 1 (Figs. 4a, c–f and 6c).

Immunohistochemistry (IHC)

IL-6 and lubricin were assessed by immunohistochemical staining in cartilage of all groups. Different patterns of immunopositive cells in the sets of specimens were observed (Table 4). IL-6 immunolabeling was weak/absent (ES = 0; IS = 0) in control group 1 (Fig. 7a). IL-6 was very strong in chondrocytes from OA group 2, mainly in the superficial and middle zones of the cartilage (ES = ++++; IS = 4) (Fig. 7b). IL-6 immunolabeling was moderate in chondrocytes from group 3, mainly in the middle and deep zones of the cartilage rather than the superficial zone (ES = ++; IS = 2) (Fig. 7c). IL-6 immunolabeling was weak/absent (ES = 1; IS = 1) in group 4, (Fig. 7d), 5 (Fig. 7e) and 6 (Fig. 7f), mainly in the middle and deep zones of the cartilage rather than the superficial zone. The negative control treated with PBS without the primary antibody (IL-6) did not show immunostaining (ES = 0; IS = 0) (data not shown). The percentage of IL-6-positive cells and the statistical significance were identified among groups, as shown in Fig. 8a. Inter-observer agreement, measured as Cohen's κ coefficient, was 0.90.

Lubricin immunolabeling was very strong in healthy cartilage from group 1, mainly in chondrocytes from the superficial and middle zones of the cartilage rather than the deep zone (ES = ++++; IS = 4) (Fig. 9a). Lubricin immunolabeling was weak/absent (ES = 1; IS = 1) in cartilage from superficial, middle and deep zones of osteoarthritic cartilage from group 2 (Fig. 9b). Lubricin immunolabeling was moderate (ES = ++; IS = 2) in the superficial and middle zones of the cartilage of group 3 (Fig. 9c). Lubricin immunolabeling was strong in the superficial and middle zone chondrocytes of group 4 (Fig. 9d), group 5 (Fig. 9e) and 6 (Fig. 9f). The negative control treated with PBS without the primary antibody (Lubricin) did not show immunostaining (ES = 0; IS = 0) (data not shown).

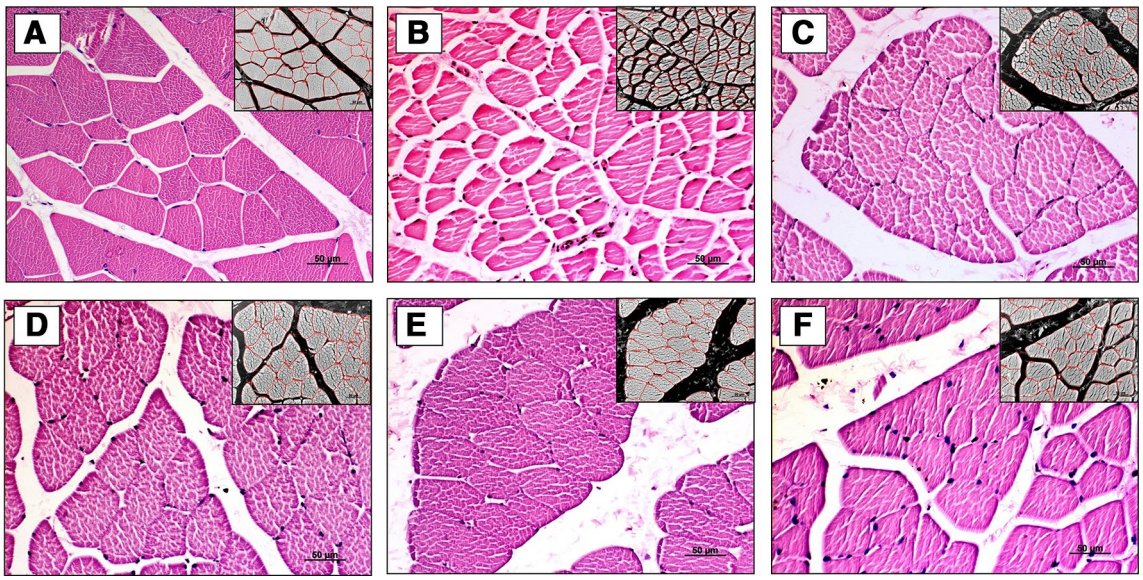


Fig. 4 Hematoxylin and eosin staining and morphometric analysis of the perimeter (µm) (mean ± SD) of the muscle fibers. **a** group 1 and in the inset morphometric analysis by the software; **b** group 2 and in the inset morphometric analysis by the software; **c** group 3 and in the inset morphometric analysis by the software; **d** group 4 and in the inset morphometric analysis by the software; **e** group 5 and in the inset morphometric analysis by the software; **f** group 6 and in the inset morphometric analysis by the software. There was no cytological alteration in the muscle fiber of all groups. However, there was muscle fiber hypotrophy in group 2 and hypertrophy in experimental groups, especially in groups 3, 4 and 5. **a–f**: Lens magnification: ×20. Scale bars: 50 µm

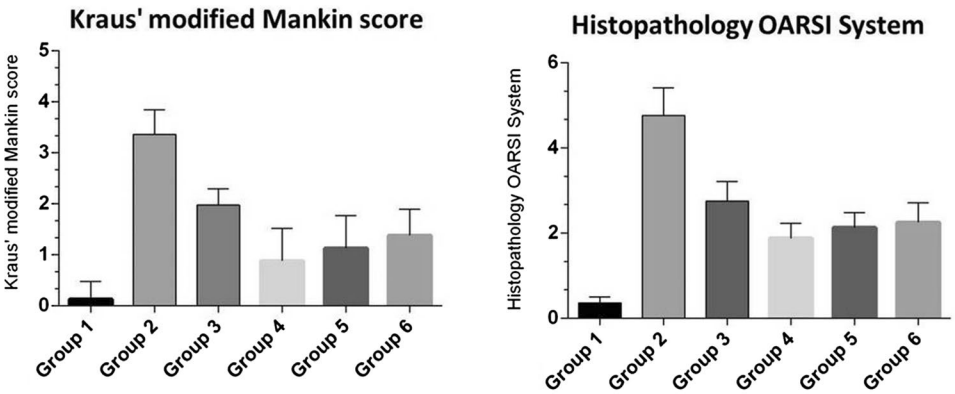


Fig. 5 **a** Kraus' modified Mankin score among groups and **b** Histopathology OARSI system among groups. Results are presented as the mean ± SD. Analysis of variance (ANOVA) was used to evaluate the significance of the results. $*p < 0.05$, $**p < 0.01$, when compared with the control group

COMPARISON BETWEEN GROUPS	*p<0.05 **p<0.01
Group 1 vs. Groups 2 and 3	**
Group 1 vs. Groups 4, 5 and 6	*
Group 2 vs. Group 3	*
Group 2 vs. Groups 4, 5 and 6	**
Group 3 vs. Groups 4, 5 and 6	*

The percentage of Lubricin-positive cells and the statistical significance were identified among groups, as shown in Fig. 8b. Inter-observer agreement, measured as Cohen's κ coefficient, was 0.92.

Biochemical studies

In this study, we examined the serum concentration of lubricin and interleukin-6 at 2 weeks after ACLT (to test

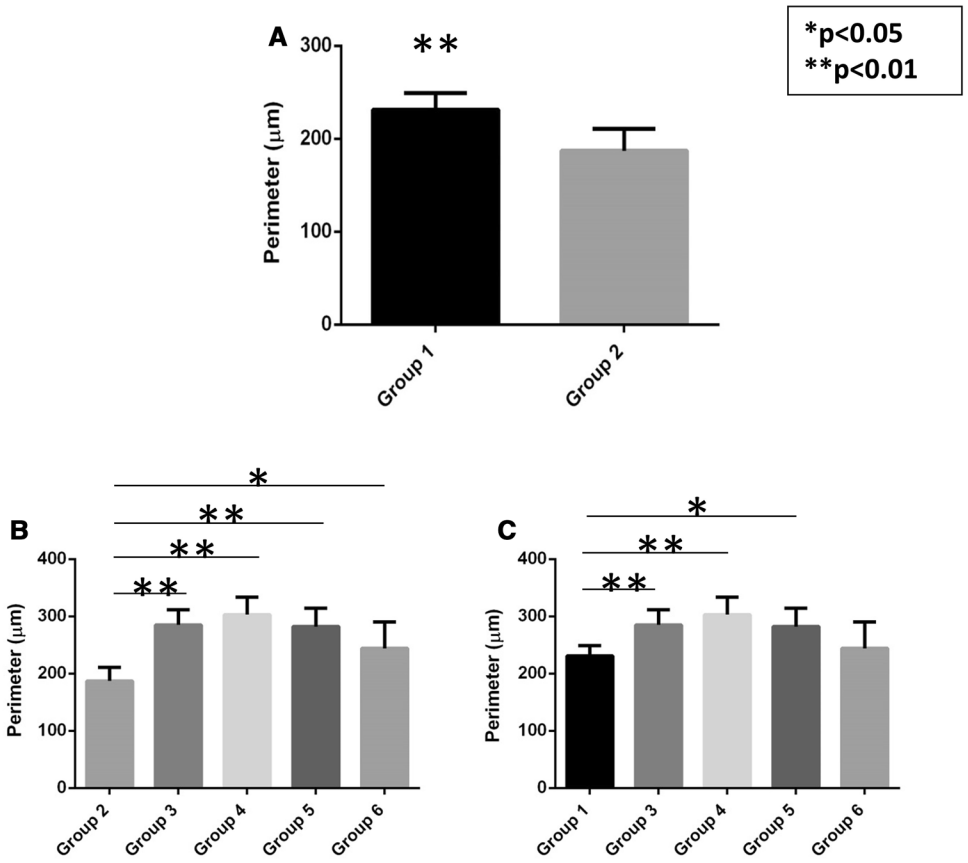


Fig. 6 Graphs with statistical analysis. **a** Comparison between group 1 (health control) and group 2 (experimental control) in which a highly significant hypotrophy in group 2 was highlighted ($p < 0.01$). **b** Comparison between group 2 (experimental control) and all experimental groups (3, 4, 5 and 6) showed a statistically significant ($p < 0.05$) (group 6) and a highly significant ($p < 0.01$) (groups 3, 4 and 5) hypertrophy of the muscle fibers. **c** Comparison between group 1 (health control) and all experimental groups (3, 4, 5 and 6) showed that groups 3 and 4 showed a highly significant ($p < 0.01$) hypertrophy of muscle fibers when compared with group 1, and group 5 showed a significant ($p < 0.05$) hypertrophy of muscle fibers with respect to group 1. On the contrary, group 6 did not show a statistically significant hypertrophy when compared with group 1. The results are presented as the mean ± SD

Table 4 Evaluation of IL-6 and lubricin immunostaining

Group	IL-6	Lubricin
1	Weak/absent immunostaining (ES = 0; IS = 0)	Very strong immunostaining (ES = +++; IS = 4)
2	Very strong immunostaining (ES = +++; IS = 4)	Weak/absent immunostaining (ES = +; IS = 1)
3	Moderate immunostaining (ES = ++; IS = 2)	Moderate immunostaining (ES = ++; IS = 2)
4	Weak/absent immunostaining (ES = +; IS = 1)	Strong immunostaining (ES = +++; IS = 3)
5	Weak/absent immunostaining (ES = +; IS = 1)	Strong immunostaining (ES = +++; IS = 3)
6	Weak/absent (ES = +; IS = 1)	Strong immunostaining (ES = +++; IS = 3)

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 (Extent Score = ES) was independently evaluated by 3 investigators (2 anatomical morphologists and 1 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% (++++). ACLT: anterior cruciate ligament transection

acute inflammation), and at 12 weeks after ACLT (to test chronic inflammation), to evaluate the effects of treadmill training, anti-inflammatory properties of extra virgin olive oil and possible interaction between them. At 2 weeks after ACLT, the serum concentrations of lubricin significantly decreased in groups 2–6 in comparison to group 1 ($p < 0.05$) (Fig. 10a). On the contrary, serum concentrations of IL-6 drastically increased in groups 2–6 in comparison to group 1 ($p < 0.01$) (Fig. 10c). At 12 weeks after ACLT, the serum concentrations of lubricin were highly significantly decreased in groups 2 and 3 compared to group 1 ($p < 0.01$) (Fig. 10b). In group 4, the level of lubricin was almost comparable to group 1. In groups 5 and 6, the levels of lubricin were significantly decreased when compared with group 1 ($p < 0.05$) (Fig. 10b). The serum concentrations of IL-6, although lower compared with the same groups at 2 weeks after ACLT, remained highly significantly increased in groups 2 and 3 in comparison with group 1 ($p > 0.01$) (Fig. 10d). In groups 4 and 5, the levels of IL-6 were similar to group 1. In group 6, the levels of IL-6 were significantly higher than that in group 1 (Fig. 10d).

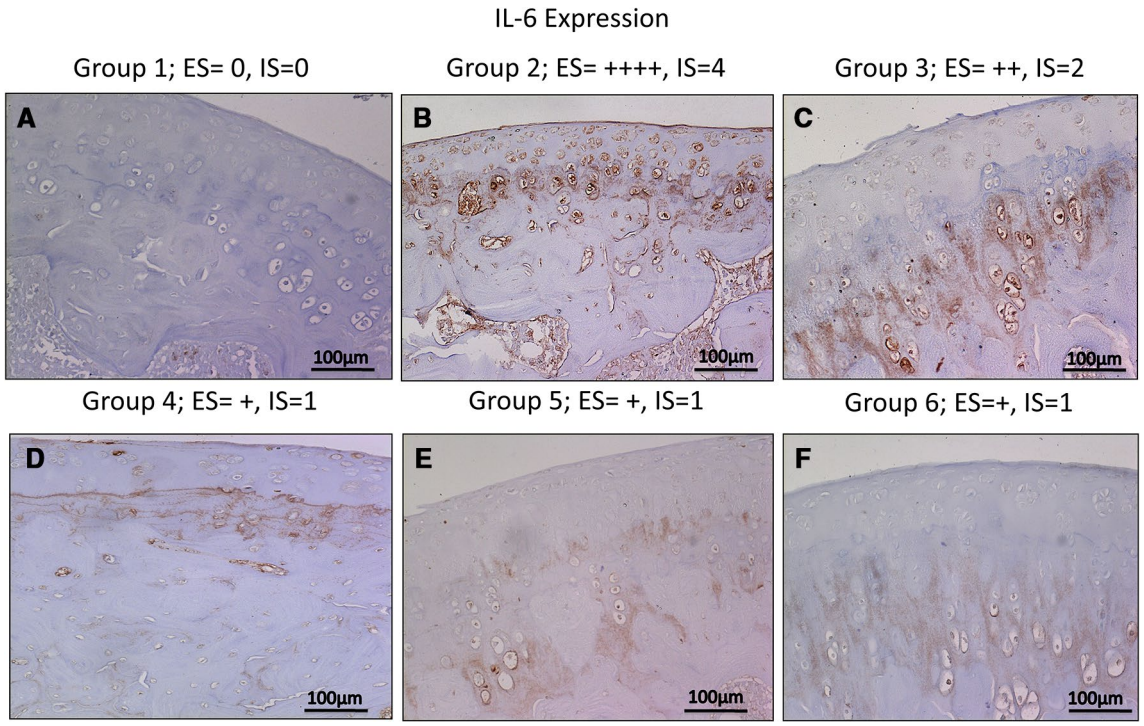
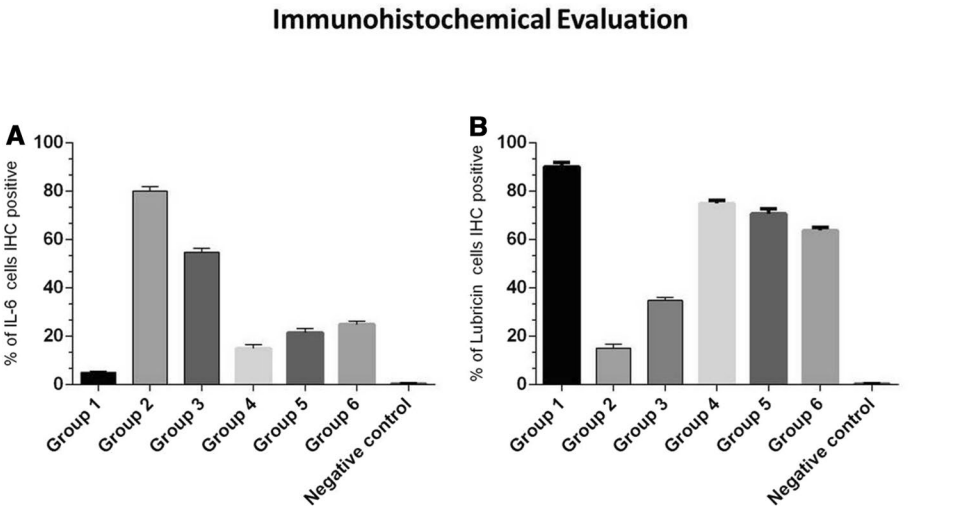


Fig. 7 **a** IL-6 immunolabeling was weak/absent (ES = 0; IS = 0) in group 1. **b** IL-6 was very strong in chondrocytes from group 2 in superficial, middle and deep zones of the cartilage (ES = ++++; IS = 4). IL-6 immunolabeling was moderate in chondrocytes from group 3, mainly in the middle and deep zones of the cartilage rather than the superficial zone (ES = ++; IS = 2). **d–f** IL-6 immunolabeling was weak/absent (ES = 1; IS = 1) in groups 4, 5 and 6. **a–f**: Magnification ×20. Scale bars: 100 µm



IL-6 COMPARISON BETWEEN GROUPS	*p<0.05 **p<0.01
Group 1 vs. Groups 2 and 3	**
Group 1 vs. Groups 4, 5 and 6	*
Group 2 vs. Group 3	*
Group 2 vs. Groups 4, 5 and 6	**
Group 3 vs. Groups 4, 5 and 6	*

LUBRICIN COMPARISON BETWEEN GROUPS	*p<0.05 **p<0.01
Group 1 vs. Groups 2 and 3	**
Group 1 vs. Groups 4, 5 and 6	*
Group 2 vs. Group 3	*
Group 2 vs. Groups 4, 5 and 6	**
Group 3 vs. Groups 4, 5 and 6	*

Fig. 8 **a** The percentage of IL-6-positive cells was identified among groups and ANOVA was used to evaluate the significance of the results. In table comparison between groups, $*p < 0.05$; $**p < 0.01$. Results ae presented as the mean ± SD. **b** The percentage of lubricin-positive cells was identified among groups and ANOVA was used to evaluate the significance of the results. In table comparison between groups, $*p < 0.05$; $**p < 0.01$. Results are presented as the mean ± SD

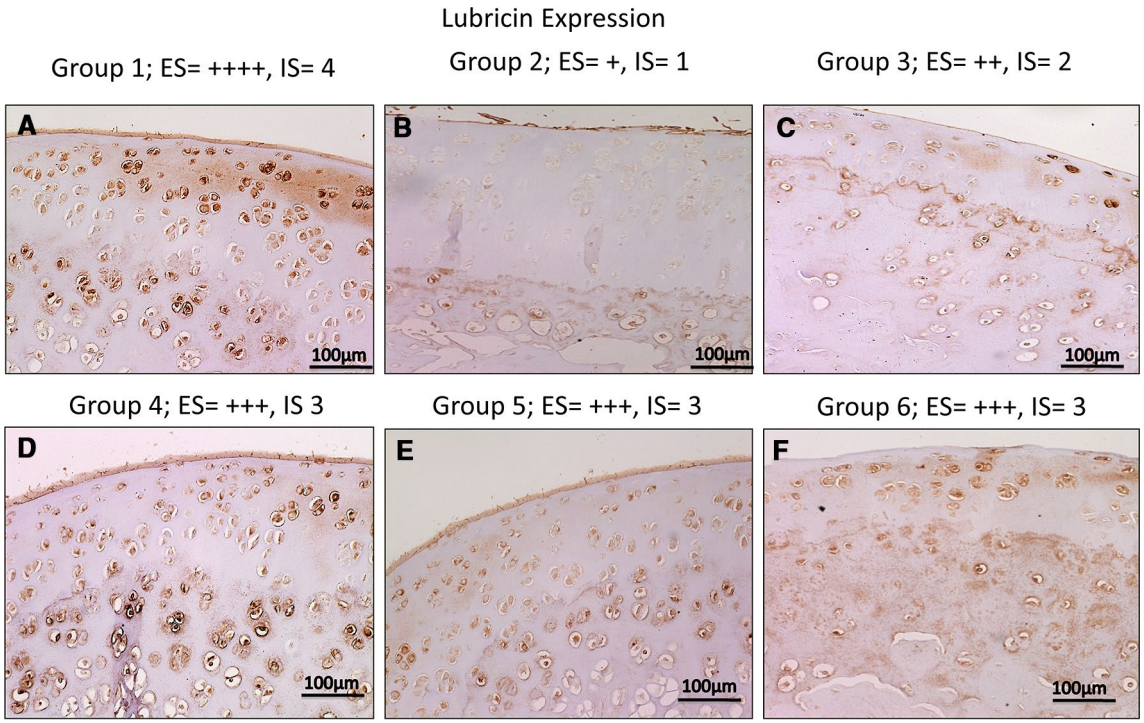


Fig. 9 **a** Lubricin immunolabeling was very strong mainly in chondrocytes from the superficial and middle zone of the healthy cartilage rather than the deep zone (ES = ++++; IS = 4). **b** Lubricin immunolabeling was weak/absent (ES = 1; IS = 1) in group 2. **c** Lubricin immunolabeling was moderate (ES = ++; IS = 2) in group 3. **d–f** Lubricin immunolabeling was strong in chondrocytes from groups 4, 5 and 6. **a–f**: Magnification ×20. Scale bars: 100 µm

Discussion

The aim of this study was to assess the best concentration of phytoactive substances present in olive tree-derived products (OO and OLE), conjugated with regular exercise, to preserve or restore the articular cartilage and skeletal muscle condition, in early stages of OA.

The ACLT process, used to mechanically induce OA in rat knees, determined the significant increase of IL-6 release both in articular cartilage and blood serum, as confirmed by immunohistochemical and biochemical analysis. Indeed, an acute injury of the knee is the initial event responsible for the flare of inflammatory mediators, which can initiate and be responsible or articular pain and the following degenerative process of articular cartilage. Lately, increased expression of IL-6, both in serum and synovial fluid, and in cartilage tissue, has been shown to be correlated with an early onset of OA [31–33]. As expected, the major concentration and expression of IL-6 was found in sedentary rats (group 2) when compared with the control and the other experimental groups (groups 3–6). Moreover, the expression of lubricin decreased drastically, confirming the results found in our previous study [24]. In line with these results, the histological and histomorphometric analysis of articular cartilage and muscle samples of group 2 showed relatively the significant structural alterations of articular cartilage corresponding to moderate OA, reflecting the initiation of degenerative process due to the increased expression of IL-6, decreased expression of lubricin, and a significant atrophy of muscle samples. This reflects the reduced joint mobility following injury due to articular pain and AMI [6]. The condition of cartilage tissue, as well as of the skeletal muscle, as shown by histological and histomorphometric analysis, seems to recover slightly already with physical activity program (group 3) and even more with OO derivatives-supplemented diet (groups 4,5,6). This condition is reflected by the general lower expression of IL-6 and increased expression of lubricin in experimental groups 3, 4, 5 and 6. Immunohistochemical analysis of group 3, when compared with group 2, showed weaker IL-6 expression, especially in the middle zone, and stronger lubricin expression on the cartilage surface, due to the beneficial effect of physical activity. This might be ascribable to the fact that with the movement, major synovial fluid is released into the articular capsule and, consequently, the superficial layer is better nourished by lubricin, which exerts it chondroprotective activity. According to a recent study by Yang et al., it was shown that physical activity, consisting of 60 min treadmill exercise with moderate intensity, was able to reduce the release of pro-inflammatory mediators with subsequent

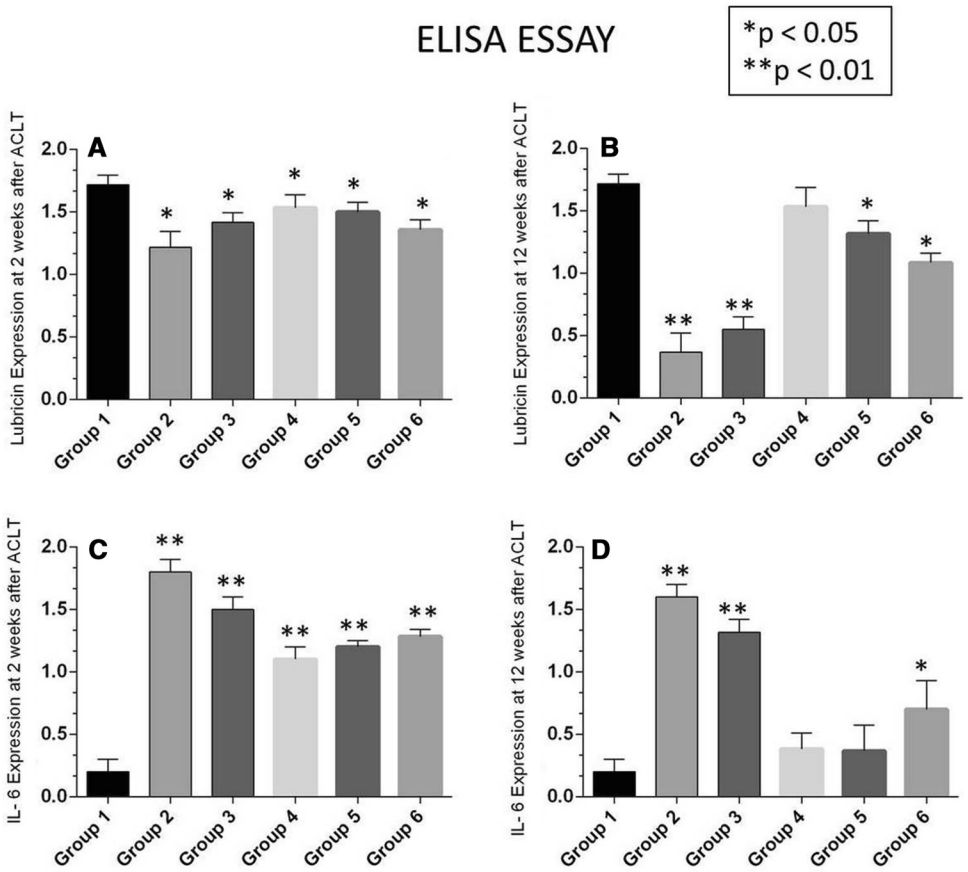


Fig. 10 Lubricin and IL-6 concentrations in the blood serum from all groups of rats were determined by ELISA at 2 (**a** and **c**) and 12 (**b** and **d**) weeks after ACLT. Results are presented as the mean ± SD. ANOVA test was used to evaluate the significance of the results. *$p < 0.05$ and **$p < 0.01$, when compared with group 1

chondroprotective effects [34]. This result has been confirmed by other studies on several experimental OA models and under different exercise conditions [35–37]. In our previous study, we confirmed the protective effects of physical activity in conjunction with S-EVOO-supplemented diet, on the experimental OA rat model, even if the experimental time was less than this study design [38]. In addition, we demonstrated the beneficial role of physical activity on the aged rat model with signs of OA, through the re-established expression of cartilage lubricin when compared with the young controls [23]. The decreased expression of IL-6 is also due to the OO derivatives-supplemented diets. It has been shown that polyphenols contained in the OO has important anti-inflammatory properties. They are particularly effective in the reduction of tumor necrosis factor-alpha (TNF-α) and IL-6, as described in an interesting review by Yarla et al., [39]. The anti-inflammatory effect of OO-associated polyphenols intake was also demonstrated by Medina-Remón et al., in a trial study done in elderly patients through the analysis of total urinary polyphenol excretion and circulating inflammatory biomarkers such as IL-6, suggesting a dose-dependent anti-inflammatory effect of the polyphenols [40]. Many other studies investigated and confirmed the protective effects of OO and the relative polyphenolic compounds on articular cartilage in experimental arthritis [41–45]. This effect has been ascribed to the inhibition of the release of proinflammatory cytokines and chemokines and to the reduction of the leukocyte infiltration in the affected joints [41]. The beneficial effects of Sicilian extra virgin olive oil (S-EVOO) on cartilage tissue and skeletal muscle has been already studied at our laboratory and demonstrated in our previous studies. We reported that the supplementation of diet with S-EVOO in conjunction with physical activity was positively associated with enhanced cartilage preservation in the mechanically-induced OA rat model. The latter was reflected by the decreased expression of interleukin-1 (IL-1) and increased production of lubricin [24]. The beneficial effect of physical activity on cartilage tissue is also reflected by muscle hypertrophy in group 3 in comparison with sedentary groups, and in particular the ACLT procedure (group 2) caused atrophy in the muscle of fibers. Indeed, it has been stated that muscle weakness is a risk factor for OA [46]. Herzog et al. demonstrated that even a short period of muscle weakness could be a risk factor for OA. It was confirmed by a study in which botulinum type-A toxin was injected in the knee extensor muscles of rabbits, determining cartilage degeneration after 4 weeks from injection and suggesting OA onset [47]. However, it has been suggested that also the physiologic age-related decrease of muscle tissue volume constitutes an important risk factor for

OA [48]. Groups 4, 5 and 6 showed a significant recovery of cartilage condition, due to the synchronized effect of physical activity and anti-inflammatory activity of OO derivatives. Il-6 expression decreased even more when compared to group 3. The best effect was shown in group 4, fed with S-EVOO that also had greater calorie consumption when compared with groups 1, 2 and 3 (Fig. 4b). It was interesting to observe that also group 5, fed with T-EVOO, had a greater calorie consumption with respect to groups 1 and 2, but it was a little bit lower when compared with group 4 (not significant); instead in group 6, fed with T-EVOO with OLE, the values were similar to all other groups. In relation to these observations, we could hypothesize that S-EVOO diet had a more appetizing taste determining a greater food intake by the rats of group 4, even if not significant, when compared to groups 5 and 6, fed respectively with T-EVOO and T-EVOO with OLE, characterized by the higher concentration of polyphenols and which determined their bitter taste as already stated by Gutiérrez-Rosales and coauthors [49]. These results are confirmed by the histological analysis of cartilage tissue samples. In addition, the histomorphometric analysis of muscles samples reveals the improved condition of cartilage. Moreover, the morphometric analysis of muscle fiber perimeter showed a highly significant muscle hypertrophy in group 4 and a significant muscle hypertrophy in groups 5 and 6 in comparison with group 2, ascribable, at least in part, to the treadmill training. Instead, comparing all experimental groups with the control, to evaluate if EVOO supplementation in experimental diets could have a role in hypertrophy of muscle fibers, only groups 3, 4 and 5 showed a higher hypertrophy, while group 6 was comparable to the control. These data reflect the behavior of rats, which was noticed to be recurrent during the experiment. Indeed, rats from groups 5 and 6 were less inclined to run on treadmill and got tired more easily. This fact might be explained by the calorie consumption that, as discussed above, could be explained by the lower (but not significant) food intake in group 5 and 6 due to the bitter taste of the T-EVOO and T-enriched-EVOO. Another hypothesis is that the polyphenol intake in these groups could result in toxicity for the rats, determining their general malaise and tiredness. In fact, it was shown that high doses of exogenous antioxidants could be toxic, because at high concentration they start to react with the beneficial concentrations of reactive oxygen species (ROS), which are normally present at physiological conditions and are required for optimal cellular functioning [50]. In this last case, the best beneficial effect, showed for the S-EVOO consumption in group 4, might be ascribable to the synergistic action of a complex of polyphenols and nutrients that it contains in that particular concentration [51]. In our previous study, we demonstrated that dietary S-EVOO supplementation improved the adaptive response of the rat skeletal muscles in conditions of oxidative stress, by increasing its antioxidant capacity [38]. These data have been also confirmed by Wang et al., in the latest study, where the oxidative stress-induced muscle degeneration has been shown to be counteracted by the hydroxytyrosol acetate, an important OO component [52]. The favorable role of olive-associated compounds on skeletal muscle has been also demonstrated in an interesting study by Bronnikov at al., where it was proved that the olive oil-supplemented diet re-establishes the fatty acid composition of the muscle tissue in old rats, restoring the values relative to the young animals [53].

Nevertheless, despite this interesting observation, the analysis of body weight showed a physiological increase in all groups and even if it was a bit higher in groups 4, 5 and 6, the differences between groups were never significant. Indeed, also group 2 showed an initial decrease in body weight, due to ACLT debilitation, but afterwards the values aligned with those of group 1 (health control). Since group 6 calorie consumption was not significantly different from all other groups (in the middle between the higher calories consumption of groups 4 and 5 and the lower of groups 1, 2 and 3), we can postulate that in group 6 the hypertrophy could be solely due to treadmill training, while in groups 4 and 5 hypertrophy could be the result of treadmill in association with S-EVOO- or T-EVOO-supplemented diet.

The present study has some limitations regarding the methodology used to determine the experimental diet compositions obtained just by adding the respective olive oils (S-EVOO, T-EVOO and T-OLE) to the standard diet at each feeding time. This method is based on a simple sum of the respective supplements and the basic composition of the standard diet furnished by Mucedola, as described above. Other limitations are presented by the lack of analysis concerning the exact phenolic profiles, but just the determination of the total polyphenols contained in each experimental diet (Table 3). This was due to the fact that the first aim of this study was to evaluate the general effects and the eventual differences between the consumption of S-EVOO, T-EVOO and T-OLE, on articular cartilage of rats with mechanically induced OA and subjected to physical activity. Therefore, the specific polyphenolic composition of the diets was a bit out of the general aim. Once we obtain interesting results, we would like to proceed with the exact individualization of the specific phytochemical composition in further studies.

In conclusion, given the results of histological, immunohistochemical, histomorphometric and biochemical analysis, we can state that the group of rats fed with S-EVOO and subjected to physical activity (group 4) demonstrated the best articular cartilage recovery process after ACLT when compared with the groups of rats fed with T-EVOO or T-EVOO and OLE, and to the experimental controls (groups 2 and 3). This could be attributable both to the muscle hypertrophy and increased release of lubricin into the articular capsule following physical activity on treadmill, and to the

anti-inflammatory properties of the phytochemical complex contained in S-EVOO. These results confirm our previous study and demonstrate the importance of physical activity in conjugation with S-EVOO-enriched diet in medical therapy aimed to preserve both articular cartilage and muscle tissue and thus to prevent OA development. Moreover, our results clearly underline that S-EVOO contains a rightly balanced concentration of phytoactive substances, able to significantly reduce the OA-related cytokine release (IL-6) and increase lubricin synthesis, exerting its beneficial effect on the entire joint.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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