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Prophylactic role of acetyl-L-carnitine on knee lesions and associated pain in a rat model of osteoarthritis

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ABSTRACT

Aims: : in the present study, our aim was to validate in vivo the prophylactic role of acetyl-L-carnitine (ALC) using an established knee osteoarthritis (OA) animal model which mimics the pathological changes of OA in humans, targeting cartilage and causing chondrocyte death.
Main methods: : animal model was obtained by an intra-articular injection of monosodium iodoacetate (MIA) into rat femorotibial joint space. Pain was measured in animals submitted to MIA model by paw pressure and compression behavioral tests in the presence or absence of ALC.
Key findings: : morphological analysis of knee-joint from MIA and ALC co-treated rats showed that the total pathological score attributed to histological findings was dramatically lower in rats treated with MIA in the presence of ALC. OA chondrocyte overexpression of pathogenic collagenase matrix-metallopeptidase-13 (MMP13) could be decreased in knee-cartilage from MIA/ALC rats; whereas type II collagen (COL2) expression level could be partially increased to control value.
ALC twice daily treatment was able to attenuate pain in OA rat knee as revealed by mechanical behavioral tests.
Significance: : in our experiments, pain that is usually associated with OA, was correlated with the severity of histopathological findings. Our findings show that there is a place for ALC as chondroprotective agents in cartilage degradation and strongly support the prophylactic and therapeutic potentials of ALC in knee-OA patients.

Introduction

The acetyl ester of L-carnitine isomer (ALC) has been shown to offer a great therapeutic potential. The pharmacological effects of ALC can be attributed to the physiological properties of L-carnitine transport of small-, medium-, and long-chain fatty acids across the mitochondrial membranes. Still, the presence of the acetyl group makes ALC significant to normal mitochondrial function as donor of acetyl-group during high-energy metabolism and in anabolic reactions (Bremer, 1990). In rodent studies, ALC supplementation significantly reversed the age-associated decline in many indices of mitochondrial function and general metabolic activity stabilizing the inner mitochondrial membrane, increasing cardiolipin levels in the heart, and reversing the decline in activity of a number of mitochondrial translocases and of cytochrome *c* oxidase (Paradies et al., 1995). In preclinical studies, ALC improved nerve regeneration and protected neurons from the toxicity of mitochondrial uncouplers or inhibitors, attenuated neurologic damage after brain ischemia suppressing mitochondrial oxidative stress thereby preventing mitochondrial transition induced active cell death (Virmani et al., 1995). These findings could be translated into clinical practice. Particularly, clinical trials with ALC showed some improvements in cognitively impaired alcoholics and in those with Alzheimer's disease or dementia-associated cognitive dysfunction although ALC is more effective in preventing than slowing the progression of this disease (Montgomery et al., 2003). Recent in vitro study performed in cultured human primary chondrocytes showed that L-carnitine was able to affect extracellular matrix synthesis and ameliorate the mitochondrial activity of chondrocytes (Stoppoloni et al., 2013), the only cells present in the cartilage and responsible to produce and maintain the cartilage extracellular matrix. These results prompted us to investigate the role of ALC supplementation on structural pathology and pain-related behaviors in an established animal model of osteoarthritis (OA), a major healthcare affliction and the most prevalent type of arthritis among adults over 65 years that produces loss of joint function, disability and chronic pain. We preferred to use ALC in this in vivo study because it is more widely used than L-carnitine in animal research and clinical trial to gain metabolic benefits to different organs or in condition of disease (Liu et al., 2004). Throughout this study, we used a previously validated animal model (Guingamp et al., 1997) where a knee-OA model is obtained by intra-articular (i.a.)

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injection into rat femorotibial joint space of the metabolic inhibitor monosodium iodoacetate (MIA), which disrupts chondrocyte glycolysis through inhibition of glyceraldehydes-3-phosphate dehydrogenase, leading to eventual cell death finally resulting in histological and morphological changes to the articular cartilage closely resembling those seen in human OA (Guingamp et al., 1997; Guzman et al., 2003; Kobayashi et al., 2003).

Material and methods

Animals

Seven week old male Sprague–Dawley rats, weighting 250–300 g, were obtained from Harlan-Italia (Varese, Italy). Four rats were housed per cage (size 26 × 41 cm) and placed in the experimental room for acclimatization 24 h before the test. The animals were fed with standard laboratory diet and with tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986, 86/609/EEC). Ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; University of Florence assurance number: A5278-01). All studies involving animals are reported in accordance with the ARRIVE guidelines for experiments involving animals (Kilkenny et al., 2010). A total of 149 animals were used in these experiments. In order to reduce as much as possible the number of animals used, sample size criteria as regard to statistical significance were followed as set out in Feinstein (1977). Rats were assigned randomly to the different experimental groups and the experimenter was unaware of the treatment used.

OA model

A single intra-articular (i.a.) injection of 2 mg of MIA (Sigma-Aldrich, Milan, Italy) was performed through the infra-patellar ligament into the joint space of the right knee of lightly anesthetized rats (3% isoflurane in O_2 at 1.5 l/min) in a total volume of 25 μl saline via a Hamilton gas tight syringe (Hamilton, Switzerland).

Experimental groups

A group of rats was treated with MIA as previously described (MIA group). A different group of rats was sham-operated receiving i.a. injection of 0.9% saline (25 μl) alone in the right knee (SHAM group). ALC (Sigma-Tau Pomezia, Italy) was administered intraperitoneally (i.p.) twice daily for 14 consecutive days to rats treated with MIA (MIA/ALC group) starting at the same day of MIA administration. A different group of rats was administered with i.p. saline twice daily for 14 consecutive days (SALINE group) or with i.p. ALC (ALC group). The time of daily administration was scheduled in order to administer the drugs 60 min before the assessment of behavioral tests.

Histological examination

Knee-joint was isolated from MIA treated rats in the presence or absence of ALC at day 14 after MIA administration. Saline intra-articular injected rats were used as controls. Each of the three groups (SHAM, MIA and MIA/ALC) was composed by 10 animals randomly chosen from the corresponding treated group at the end of the behavioral tests. Samples were fixed in Karnovsky's fixative (4% paraformaldehyde, 2.5% glutaraldehyde) in 0.1 M cacodylate buffer, pH 7.35 for 27–29 h at 4 °C and dehydrated in graded ethanol series. Samples were embedded in methacrylate (Tecnovit 7100, Heraeous); 5 μm-thick sections, obtained with a Reichert Jung microtome, were stained with 0.1% toluidine blue and then observed under Carl Zeiss Axioplan 2 imaging microscope. The images were acquired using an AxioCam HR CCD camera and analyzed with quantitative microscopy Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany). We usually examined one section every 25 μm in each knee-joint from each rat for a total length of 900 μm. People grading the sections were blind to the experimental groups.

RNA extraction and RT-PCR

The knee was isolated right after rat termination and the cartilage was detached from the knee-joint, flash frozen in liquid nitrogen and pulverized. For the study of the expression of MMP13 and COL2 mRNA, total RNA from knee joint cartilage was prepared using the RNeasy Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacture's instruction. The study was performed in SALINE, MIA and MIA/ALC independent groups at day 14 after MIA administration. Ten animals were used for each group. The rats belonging to each group were randomly selected from rats previously submitted to the behavioral tests. 5 μg of total RNA was used to synthesize the first strand cDNA with the Superscript II-reverse transcriptase (Invitrogen, Grand Island, NY, USA) and oligo DT under the conditions recommended by the manufacturer. 10% of the first strand cDNA was used as template for the PCR. All samples were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following pairs of primers (5′-3′), based on the previously reported sequences, were used (Hanaoka et al., 2011): 5′-TTGGAGAGAC CATGAACGGC-3′ and 5′-TTAGCGGTGTTGGGAGCC-3′ for COL2; 5′-TGGA GTTATGATGATGCTAACCAGAC-3′ and TGTCGCCAATTCCAGGGA-3′ for MMP13; 5′-CATCACTGCCACCCAGAAGA-3′ and 5′-ATGTTCTGGGCAGCC-3′ for GADPH.

Paw pressure test

The Randall & Sellitto test is based on the determination of the animal threshold response induced in the paw by the application of a uniformly increasing pressure. The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton (Leighton et al., 1988). Briefly, a constantly increasing pressure exerted by a blunt conical probe, controlled by a mechanical device, was applied to a small area of the dorsal surface of the rat's right hind paw. Rats were previously habituated to the test apparatus in order to avoid a startle response. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 20 g or over 75 g during the pre-test were not admitted to the experimental trial. The data were collected by an observer who was blind to the design.

Compression threshold test

According to Barton (Barton et al., 2007) rats were habituated and a baseline measure of mechanical hypersensitivity in knee joint was taken using a digital device (Basile, Varese, Italy) applied to the lateral aspect of the knee, while rats were gently restrained. Gradually increasing pressure was applied to the joint until a move to withdraw the limb was observed (compression threshold). When the animal flinches or vocalizes, the operator releases the joint and the electronic unit will capture the peak force at which the animal vocalized or twitched. The current force applied at each moment of the test was expressed in grams. Rats scoring below 200 g during the pre-test were not admitted to the experimental trial. The data were collected by an observer who was blind to the design.

Radiochemical assay

Acetyl-1-^{13}C-L-carnitine hydrochloride 99 atom%^{13}C (Sigma, St Louis, MO, USA) was dissolved in bicarbonate at a concentration of 30 mg/ml,