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SHORT COMMUNICATION

L-Carnitine enhances extracellular matrix synthesis in human primary chondrocytes

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Abstract Osteoarthritis (OA) is one of the most common degenerative joint disease for which there is no cure. It is treated mainly with non-steroidal anti-inflammatory drugs to control the symptoms and some supplements, such as glucosamine and chondroitin sulphate in order to obtain structure-modifying effects. Aim of this study is to investigate the effects of L-carnitine, a molecule with a role in cellular energy metabolism, on extracellular matrix synthesis in human primary chondrocytes (HPCs). Dose-dependent effect of L-carnitine on cartilage matrix production, cell proliferation and ATP synthesis was examined by incubating HPCs with various amounts of molecule in monolayer (2D) and in hydromatrix scaffold (3D). L-Carnitine affected extracellular matrix synthesis in 3D in a dose-dependent manner; moreover, L-carnitine was very effective to stimulate cell proliferation and to induce ATP synthesis, mainly in 3D culture condition. In conclusion, L-carnitine enhances cartilage matrix glycosaminoglycan component production and cell proliferation, suggesting that this molecule could be useful in the treatment of pathologies where extracellular matrix is degraded, such as OA. To our knowledge, this is the first study where the effects of L-carnitine are evaluated in HPCs.

Keywords L-Carnitine · Human primary chondrocytes · Glycosaminoglycan synthesis

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Introduction

L-Carnitine is a molecule with a major role in cellular metabolism. It is widely distributed among tissues [1] and is a cofactor for the transport of long-chain acyl CoA through the inner mitochondrial membrane for beta-oxidation [2]. Previous studies have confirmed that L-carnitine affects osteoblastic metabolism both in vitro and in vivo. In particular, in in vitro studies, it has been demonstrated that L-carnitine stimulates protein synthesis in porcine primary osteoblast [3], proliferation and differentiation of human primary osteoblast [4] and protects against apoptosis murine osteoblastic cells [5].

To date, no data have been published about the effect of L-carnitine on chondrocytes, which have same progenitors than osteoblast, mesenchymal stem cells, commonly known as osteochondrogenic cells. Chondrocytes are the only cells present in the cartilage, and they are responsible to produce and maintain the cartilage extracellular matrix, which is composed by collagen fibres, mainly collagen type II, and macromolecular aggregates, such as proteoglycan and hyaluronic acid [6]. Cartilage is continuously remodelled, and an imbalance between synthesis and degradation of cartilage matrix components leads to osteoarthritis (OA). OA is a degenerative joint disease, characterized by changes in joint tissues, mainly in articular cartilage, but also in subchondral bone, synovial membrane and synovial fluid. Cartilage degeneration is triggered by loss of matrix

proteoglycans, changes in collagen-type production, and at later stage, chondrocyte can loss viability due to apoptosis or senescence [7]. Under physiological conditions, chondrocyte metabolism operates at low oxygen tension due, in part, to the absence of a vascular supply and innervation in the tissue. Nevertheless, chondrocytes need ATP to maintain cellular activity, such as active membrane transport systems [8]. In chondrocytes, ATP is synthesized mainly by anaerobic glycolysis pathway and in part by oxidative phosphorylation. During senescence or pathological conditions, such as OA, the low mitochondrial activity in chondrocytes is further reduced. This could explain the high number of apoptotic chondrocytes in OA cartilage resulting in cartilage degeneration.

Despite the large number of active research on drug discovery, in order to identify structure-modifying approaches to inhibit joint destruction, existing therapies are able only to reduce symptoms, such as pain, but none has conclusive efficacy as disease-modifying drug [9].

Aim of this study is to determine whether L-carnitine is able to affect extracellular matrix synthesis of human primary chondrocytes (HPCs) obtained from patients, who underwent a knee or hip replacement surgery.

Materials and methods

Isolation of HPCs

Human primary chondrocytes were isolated from femoral and tibial condyles and from femoral heads, obtained from patients who underwent a total knee and hip replacement surgery. Full ethical consent was obtained from all donors.

Articular cartilages were aseptically dissected from patients with an age range of 59–70 years and osteoarthritis grades I–II (normal or only mild fibrillations according to the modified outerbridge scale). Chondrocytes were isolated as previously described [10] and were grown to 80 % confluence in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with L-glutamine, penicillin/streptomycin (HyClone) plus 10 % foetal bovine serum (FBS). Experiments were performed with first passage cells in DMEM containing 1 % FBS in monolayer or in hydromatrix (3D) culture conditions and were repeated with chondrocytes from at least 3 different donors.

Plating of HPCs on hydromatrix (3D culture)

Hydromatrix, a peptide nanofibre three-dimensional scaffold (Sigma), was dissolved in sterile water to achieve 1 % w/v stock solution (10 mg/ml). A 0.25 % working solution was seeded in plates and incubated at 37 °C for 1 h to allow the gel to form. The medium was changed twice at intervals of 2 h, and then, the cells were seeded on the gel at a density of 4×10^4 cells/ml.

Cell treatment

Cells were left untreated (CTL) or treated, for the required time, with 1 or 2.5 mM L-carnitine (Sigma-Tau). Cells were conveniently processed for Alcian blue staining, sulphated glycosaminoglycans (sGAG) quantification, to assess viability, or harvested and processed for ATP quantification.

Alcian blue staining

The production of sulphated glycosaminoglycans (sGAG) was assessed colorimetrically on HPCs cultured for 7 and 14 days in 24-well plates in hydromatrix by an Alcian blue staining. Alcian blue binds negatively charged sGAG. Briefly, 2×10^4 cells, treated as above reported, were washed in PBS, fixed in 4 % paraformaldehyde for 10 min and washed in PBS. Then, cells were stained at room temperature in 0.1 % Alcian blue (Bio-Rad Laboratories S.r.l., Hercules, California, USA) solution in 0.1 M HCl. After overnight incubation, cells were rinsed with distilled water, and the blue staining of GAG was observed under light microscopy.

sGAG quantification by 1,9-dimethylmethylene blue (DMMB) dye method

Quantitative sGAG production was measured by 1,9-dimethylmethylene blue (DMMB) method [11], which changes absorption spectrum when bound to sGAG. Briefly, 2×10^4 cells, after treatment, were digested with 0.3 mg/ml papain (Sigma) for 24–48 h at 60 °C. An aliquot of 100 µl of each sample digested with papain was added to 2.5 ml of DMMB solution and the absorbance was measured at 525 nm. Shark chondroitin sulphate C (Sigma) was used as standard. DNA content was determined using Hoechst 33258 dye [12].

Assessment of cell viability

To detect potential cytotoxic effects of L-carnitine at concentrations and times used, the survival of the cells treated with this molecule was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide)-based colorimetric assay (Sigma), according to the manufacturer's instructions. Briefly, 5×10^3 cells per well were seeded in a 96-well plate. MTT was added to the well containing untreated and treated cells, 7 and 14 days after treatment. Spectrophotometric absorbance was measured at 570 nm. The background at 690 nm was subtracted.

Quantification of ATP production

Cytosolic ATP concentration was measured using a bioluminescence assay Kit (Adenosine 5'-triphosphate bioluminescent somatic cell assay kit, Sigma-Aldrich). Briefly, 2×10^4 cells were treated as above described for 24, 48 and 72 h. Cells were lysed according to manufacturer's instructions. Light emission was measured in a Thermo Scientific Appliskan (Thermo Fisher Scientific Inc.). A standard curve obtained with diluted ATP solutions was used to calculate ATP concentrations in samples.

Statistics

Each experiment was repeated at least three times. The statistical significance of the differences between mean values was determined by a two-tailed *t* test; $P \leq 0.05$ was considered significant. Where appropriate, results are expressed as the mean ± standard error of the mean (SEM).

Results

Effect of L-carnitine on GAG synthesis

Sulphated glycosaminoglycan (sGAG) is a differentiation marker of chondrocytes and the major components of cartilage tissue. To measure the ability of L-carnitine to induce sGAG production, HPCs were plated both in monolayer and in 3D culture conditions. Then, cells were exposed to 1 and 2.5 mM of L-carnitine for 7 and 14 days. Cells were stained by Alcian blue. Cells in monolayer, after 7 days, were to a complete confluence and the staining was very light (data not shown). Cells in 3D incubated with the above reported concentrations of L-carnitine showed a strong staining compared to cells cultured for 7 days without addition of L-carnitine (Fig. 1a). Cells cultured for 14 days did not show an increase in Alcian blue staining (Fig. 1b).

The production of sGAG was quantitatively measured by DMMB method in 3D culture condition. sGAG synthesis was maximized when cells were cultured in the presence of 2.5 mM L-carnitine, both in cell lysate and in cell culture medium (Fig. 2a, b).

Effect of L-carnitine on cell viability

To understand the dose-dependent and the time-course effect of L-carnitine on cell viability, HPCs, after 3 days culture since initial plating, were exposed to 1 and 2.5 mM of L-carnitine for 7 and 14 days. Cell viability was measured by MTT method. L-Carnitine did not show detrimental effects on cells in monolayer culture conditions, at all investigated concentrations and times (Fig. 3a). On the contrary, cells cultured in 3D showed a high rate of proliferation after 14 days culture in the presence of 2.5 mM of L-carnitine (Fig. 3b).

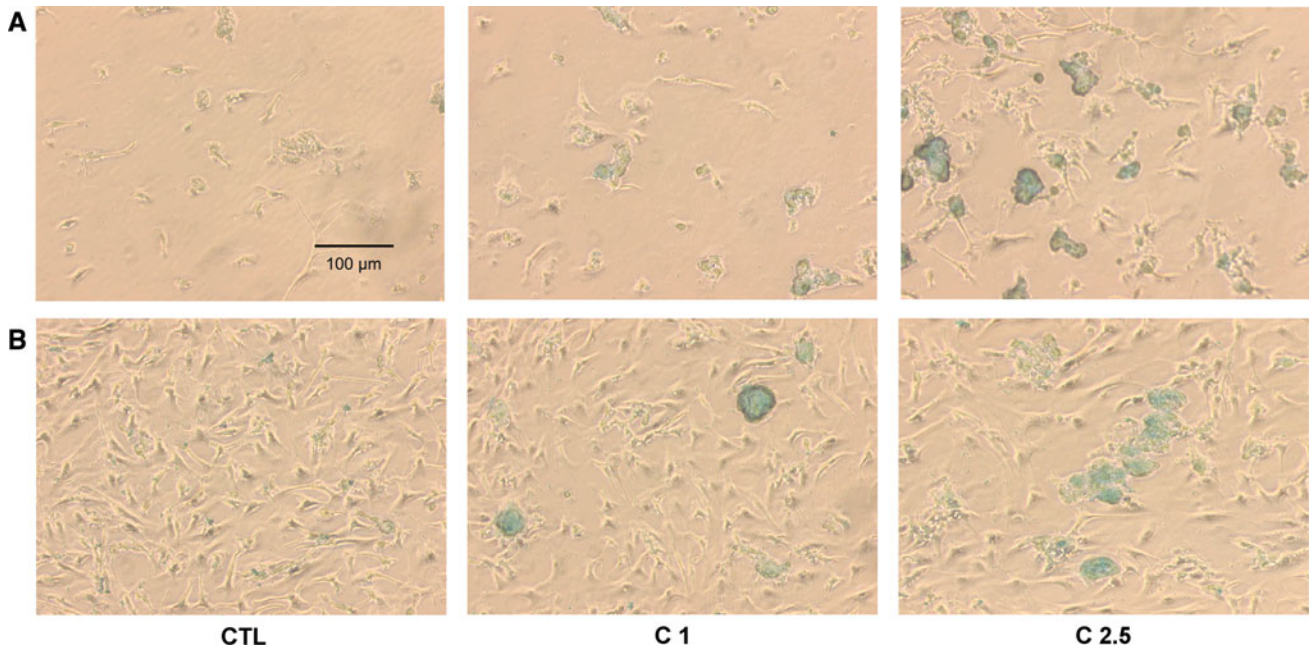

Fig. 1 Alcian blue staining of chondrocytes cultivated within hydromatrix (3D). Chondrocytes were untreated (CTL) or exposed at 1 and 2.5 mM of L-carnitine (C) for 7 days (**a**) and 14 days (**b**). In this figure is reported a representative image of four different experiments

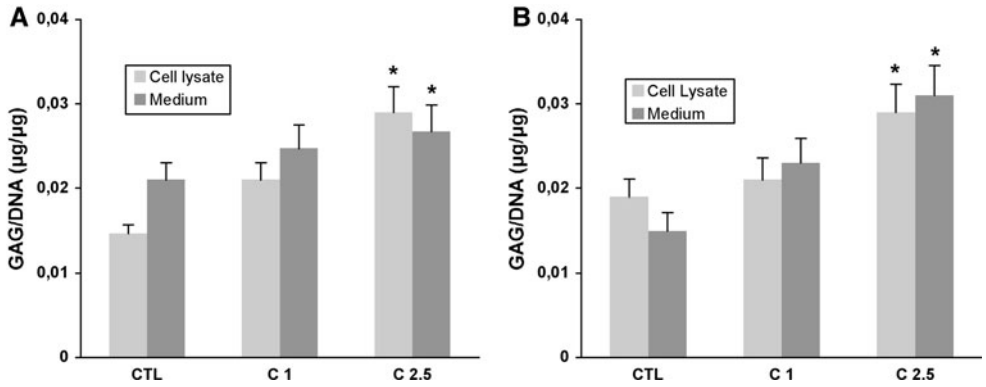

Fig. 2 Quantitative determination of s-GAG synthesis from chondrocytes cultivated within hydromatrix (3D) by dimethylmethylene blue dye method (DMMB). Chondrocytes were untreated (CTL) or exposed at 1 and 2.5 mM of L-carnitine (C) for 7 days (**a**) and 14 days (**b**). Results represent the mean ± SEM of data obtained by four different experiments. *Asterisk* indicates a *P* value ≤0.05

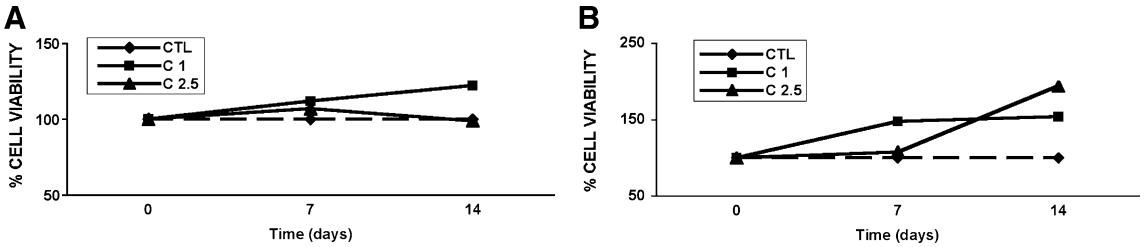

Fig. 3 Effect of L-carnitine on cellular viability. Cellular viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide) method. Chondrocytes were cultivated both in monolayer (2D) (**a**), and in hydromatrix (3D) (**b**), cells were left untreated (CTL) (*dashed line with diamond*) or exposed to 1 mM (*solid line with square*) and 2.5 mM (*solid line with triangle*) L-carnitine (C), for 0, 7 and 14 days

Effect of L-carnitine on ATP production

Since active proliferating chondrocytes are involved in energy production, an important mitochondrial function, we analysed the changes in ATP level after 1 and 2.5 mM L-carnitine treatment for 24, 48 and 72 h. We were interested to evaluate the effect of L-carnitine on ATP synthesis at long-term. We found that both 1 and 2.5 mM were able to induce a statistically significant increase in ATP production at 24 h, and 1 mM was more effective compared to 2.5 mM (Fig. 4).

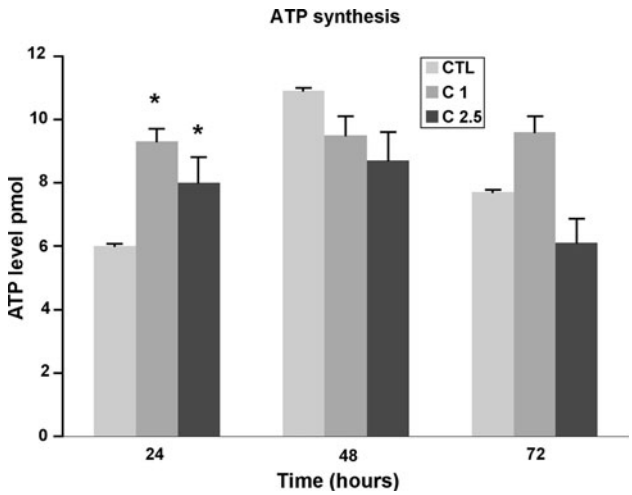

Fig. 4 Effect of L-carnitine on ATP synthesis. Chondrocytes were cultivated both in monolayer (2D) and in hydromatrix (3D); cells were left untreated (CTL) or exposed to 1 and 2.5 mM L-carnitine (C) for 24, 48 and 72 h and collected for whole cell ATP measurement as described in the "Materials and methods" section. Results represent the mean ± SEM of data obtained by four different experiments. *Asterisk* indicates a *P* value ≤0.05

Discussion

In the present study, we analysed the effects of L-carnitine on HPCs obtained from patients, who underwent a total hip or knee replacement surgery. All samples, before treatment, were analysed for collagen type II and aggrecan mRNA expression level, and for the experiments were chosen only samples, with comparable mRNA expression level, in order to try to reduce the individual variability.

We found that proliferation of HPCs was enhanced by L-carnitine. Furthermore, this molecule was able to induce an increase in extracellular matrix production, in particular we analysed, by Alcian blue staining and DMMB method, sulphated GAGs (chondroitin sulphate, dermatan sulphate, eparan sulphate, etc.). These molecules are covalently linked to core proteins to form proteoglycans, which play not only structural functions, but also different role in diverse processes, such as enzyme regulation, cell adhesion, growth, migration and differentiation [13, 14]. These processes are mediated by their abilities to bind and sequester a number of biological active proteins [15]. We can suppose that L-carnitine, by inducing increase in sGAG production, could stimulate cellular proliferation, with long-term effects, since the proliferation is enhanced after 14 days treatment.

Cartilage tissue is poorly vascularized, and as a consequence, it lacks in nutrients and oxygen. Chondrocytes, the only cells found in cartilage, can survive and produce extracellular matrix by maintaining a physiological state similar to that of resting cells with a very reduced cellular synthesis [8]. Mitochondrions are important organelles, because they play a role in energy production necessary for chondrocyte activity. During OA, the low mitochondrial activity is further reduced, the inflammation, which accompanies OA, targets mitochondria resulting in a reduction of ATP synthesis and cellular activity [16]. This decrease in cellular activity can explain the high rate of apoptosis, which is found in osteoarthritic cartilage [17]. L-Carnitine induces proliferation in chondrocytes, which after 14 days culture showed high proliferation rate, and this finding is in agreement with results obtained on human osteoblasts [4]. Moreover, to try to offset the physiological consequences of osteoarthritic processes, the chondrocytes must synthesize matrix proteins, such as proteoglycan, proliferate and repair cartilage destruction. All these activities require ATP; we found that L-carnitine stimulates both glycosaminoglycan production and ATP synthesis. We can explain the increase in ECM synthesis by the ability of L-carnitine to ameliorate the chondrocyte mitochondrial activity as demonstrated by the increase in ATP synthesis and cell viability. The finding that L-carnitine is still effective to induce ATP synthesis, 24 h after administration to cell cultures, suggests that it has a long-term effect, which is a desirable feature for molecules that could be used in the treatment of chronic diseases, such as osteoarthritis. OA can be initially induced by biomechanical insults and then further compromised by pro-inflammatory cytokines long-lasting production. Molecules with short-term effects are scarcely suitable in the treatment of chronic degenerative diseases. To our knowledge, this is the first study where the effects of L-carnitine are evaluated in HPCs. Our findings suggest that this molecule could be useful, in the treatment of osteoarthritis.

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