

Chondroprotective Effects of Taurine in Primary Cultures of Human Articular Chondrocytes

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Articular cartilage is characterized by the lack of blood vessels and has a poor self-healing potential. Limited cell numbers and dedifferentiation of chondrocytes when expanded *in vitro* are the major obstacles of autologous chondrocyte implantation. Autologous chondrocyte implantation is a cell-based treatment that can be used as a second-line measure to regenerate chondral or osteochondral defects in younger, active patients. There is an urgent need to find an effective chondrogenic protection agent alleviating or inhibiting chondrocyte dedifferentiation. In this study, we explored the effect of taurine (2-aminoethane sulfonic acid) on proliferation and phenotype maintenance of human articular chondrocytes by analyzing the cell proliferation, morphology, viability, and expression of cartilage specific mRNAs and proteins. Primary chondrocytes were isolated from human articular cartilage tissues. Results showed that taurine effectively promoted chondrocyte growth and enhanced accumulation of glycosaminoglycans and collagens in the conditioned media of chondrocytes. Moreover, taurine exposure caused significant increases in the relative expression levels of mRNAs for cartilage specific markers, including aggrecan, collagen type II and SOX9. Aggrecan is a cartilage-specific proteoglycan, and SOX9 is a chondrogenic transcription factor. In contrast, the mRNA expression of collagen type I, a marker for chondrocyte dedifferentiation, was significantly decreased in cells treated with taurine, indicating that taurine inhibits the chondrocyte dedifferentiation. This study reveals that taurine is effective in proliferation promotion and phenotype maintenance of chondrocytes. Thus, taurine may be a useful pro-chondrogenic agent for autologous chondrocyte implantation in the treatment of cartilage repair.

Keywords: chondrocyte; dedifferentiation; human articular cartilage; pro-chondrogenic agent; taurine
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Introduction

Different from other tissues, articular cartilage is avascular and has a poor healing potential (Carranza-Bencano et al. 2000; Sánchez et al. 2007). Autologous chondrocyte implantation (ACI) has been indicated to regenerate tissues similar in structure to hyaline cartilage through the use of harvested chondrocytes (Brittberg et al. 1994; Chung and Burdick 2008). During the process of ACI, isolation of chondrocytes from the donor tissue and expansion of the cells *in vitro* are necessary. However, this approach is confronted with several problems including limited cell numbers and dedifferentiation of chondrocytes. Dedifferentiated chondrocytes are characterized by a remarkable increase in the expression of collagen type I and by a low level of proteoglycan production. Moreover, the expression of collagen type X also disfavors further implantation,

because its expression precedes the onset of endochondral ossification (Kwan et al. 1997). One solution is the use of growth factors, but the application of growth factors in clinics is limited due to the following reasons: 1) growth factors may induce the formation of osteophyte, resulting in degeneration of articular cartilage (Hsieh et al. 2003); 2) they may lead to tumorigenesis (Waterfield et al. 1983; Josephs et al. 1984; Downward et al. 1984); and 3) they are generally expensive. Therefore, it is of significance to find other substance to substitute for growth factors in the restoration of defect.

Most anti-oxidant and anti-inflammatory agents attenuate free radical toxicity (Takasago et al. 1997) and have the ability to enhance chondrocyte proliferation and matrix secretion (Shang et al. 2009; Wann et al. 2010). A series of antioxidants, such as vitamin C (Tiku et al. 2003), vitamin E (Bhatti et al. 2013), and sodium ferulate (Shang et al.

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2009), have been demonstrated their chondro-protective properties, as well as potentials in reducing apoptosis and senescence. Cartilage specific proteins including collagen type II and aggrecan can also be elevated after treatment with these agents (Bhatti et al. 2013). Furthermore, favorable results were obtained after application in cartilage repair (Omata et al. 2012). These findings suggest that antioxidants are promising candidates to replace growth factors in ACI.

Taurine (2-aminoethane sulfonic acid), a conditional necessary amino acid (Chesney et al. 1998), acts as an antioxidant and protects against toxicity of various substances (Marcinkiewicz and Kontny 2014). It has extensive clinical application not only in the central nervous system and the reproductive system (Lobo et al. 2000; Menzie et al. 2014), but also in the cardiovascular system (Guidotti and Giotti 1970; Satoh and Kang 2009) and the endocrine system (Silaeva and Dokshina 1980). Its cytoprotective role has been reported in various cell types, including hepatocytes, vascular smooth muscle cells, and PC12 rat pheochromocytoma cells (Kearns and Dawson 2000; Li et al. 2004; Heidari et al. 2012). It could also stimulate cell proliferation in neural stem/progenitor cells, neural progenitor cells and osteoblast cells (Jeon et al. 2007; Hernandez-Benitez et al. 2010, 2012). Taurine inhibited serum deprivation-induced cell apoptosis via the taurine transporter and extracellular signal-regulated kinase (ERK) signaling pathway, and increased cell proliferation by ERK 1/2 activation (Jeon et al. 2007; Zhang et al. 2011). It also promoted osteoclastogenesis through the taurine transporter that was down-regulated by calcium blockers in osteoblast cells (Kang 2009; Yuan et al. 2010). However, effect of taurine on cartilage growth and metabolism has seldom been reported.

Taurine is involved in cartilage physiopathology. Taurine was reported to promote the expression of connective tissue growth factor (CTGF) that possesses the ability to repair damaged articular cartilage (Nishida et al. 2004; Yuan et al. 2007). Several researches demonstrated that taurine and its derivatives could inhibit development of rheumatoid arthritis (Kontny et al. 1999; Verdrengh and Tarkowski 2005; Marcinkiewicz and Kontny 2014). On the other side, taurine was found to play an important role in bone mineral density of ovariectomized rats (Kontny et al. 1999; Choi and DiMarco 2009). All lines of evidence suggest that taurine may affect cartilage growth.

The present study was intended to investigate the impact of taurine on chondrocytes *in vitro*. Primary chondrocytes were isolated from human articular cartilage and cultured. We examined the effect of taurine on cell proliferation, morphology, glycosaminoglycan (GAG) production, and cartilage specific mRNA expression in cultured chondrocytes. This study may provide a basis for development of a novel agent for the treatment of articular cartilage defect.

Materials and Methods

Articular chondrocytes culture

Cartilage tissue samples were harvested from amputation patients through permission of the patients or their family and the ethics committee review. Articular cartilage tissue samples from the knee joint were obtained from 6 above-mentioned donors (average age, 50.1 years) undergoing surgery for tibial plateau fracture or total knee arthroplasty. Cartilage slices were dissociated enzymatically with 0.25% trypsin (Solarbio, China) for 30 minutes and then with 2 mg/ml collagenase type II (Gibco, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) for 3 hours. After centrifugation, the chondrocytes were resuspended. Cells were cultured with DMEM containing 20% (v/v) fetal bovine serum (FBS) (Gibco, USA) and 1% (v/v) antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml) (Gibco, USA) in a 5% CO₂ humidified incubator at 37°C with the culture medium replaced every other day after plating. Articular chondrocytes at passage two were used for further studies. The morphology of chondrocytes and collagen type II expression were detected in chondrocytes from different donors. There was little difference among the samples. This study was approved by the institutional ethical committee of Guangxi Medical University (approval no.: 20130221).

Cell treatments

Taurine (2-amino ethane sulfonic acid, purity \geq 98% HPLC) was purchased from Sigma Company (USA). It was prepared as a stock solution in dimethylsulfoxide (DMSO, Sigma, USA) with the concentration of 10 mg/ml and stored at -20°C. The taurine stock solution was diluted with culture medium immediately before treatment. The final concentration of DMSO was less than 0.1 % in all experiments.

Cell cytotoxicity assay

Cytotoxicity of taurine was assessed by counting cell numbers. Articular chondrocytes were cultured in 24-well plates pre-treated with various concentrations of taurine (6.25 to 120 μ g/ml) for 3 days. Cells were digested by 0.25% trypsin and then cell numbers were calculated by hemacytometer.

Cell proliferation assay

Bromodeoxyuridine (BrdU) is incorporated into the newly synthesized DNA strands of actively proliferating cells. We therefore determined cell proliferation using a BrdU cell proliferation detection kit (Nanjing KeyGEN Biotech CO., LTD, China) according to the manufacturer's instructions. Briefly, primary chondrocytes were seeded in a 10-cm diameter culture dish. After 24 hours, cells were treated with different concentrations of taurine for 4 days. Afterwards, cells were collected, suspended to 1×10^6 cells in 500 μ l per tube and fixed with stationary liquid overnight at 4°C. Subsequently, cells were incubated with transparent liquid on the ice for 2 minutes, denatured with DNA working liquid for 30 minutes and then suspended with 195 μ l dyeing buffer. Finally, cells were labeled with 5 μ l FITC-BrdU antibody away from light for 30 minutes at 4°C and then detected by the flow cytometry (BD Bioscience, USA) with excitation and emission settings at 488 nm and 520 nm respectively. Actively proliferating cells were calculated as the ratio of BrdU-positive cells to the total cells. The assays were carried out in triplicate.

Cell viability assay

Viability of chondrocytes was measured using a live-dead viability assay kit (Invitrogen, USA) at day 2, 4 or 6. In brief, 1 μ M calcein-acetoxymethyl (calcein-AM) and 1 μ M propidium iodide (PI) were added to the cell cultures and incubated in the dark for 5 minutes at 37°C. After washed with phosphate buffer saline (PBS, Gibco, USA), the cells were visualized with a laser scanning confocal microscope (Nikon A1, Japan). Viable cells were counted in one image by using the “cell calculating” toolbar in the Nikon A1 software.

Morphological examination

After cultured for 2, 4 or 6 days, cells were washed three times with PBS and fixed in 95% alcohol for 30 minutes. Cells were then washed with PBS again and stained with hematoxylin and eosin (HE) kit (Jiancheng Biotech, China). Finally, cells were observed and photographed utilizing an inverted phase contrast microscope (Zeiss Corporation, Germany).

Biochemical assay

After cultured for 2, 4 or 6 days, cells were digested with proteinase K solution (Sigma, USA) for 16 hours at 60°C. The DNA production was measured by spectrofluorometer using Hoechst 33258 (Sigma, USA) dye at 460 nm with the absorbance value of Hoechst 33258 dye alone as the baseline (Kim et al. 1988). The total secretion of GAGs was quantified by absorbance value employing 1,9-dimethylmethylene blue (DMMB) (Sigma, USA) spectrophotometric assay at 525 nm (Farndale et al. 1986) with chondroitin sulphate as a standard sample. Production of GAGs in each cell was normalized to the total DNA content of the cells, which indicated the biosynthetic activity of the cells in various culture media.

Collagen content

After cultured for 2, 4 or 6 days, the culture media were collected for measurement of total collagen content using a hydroxyproline (Hyp) ELISA kit (Shanghai Westang Bio-Tech CO., LTD, China) according to the manufacturer's instructions. Briefly, 100 μ l of culture media or sample buffer was added in reaction plate for complete mixing at 37°C for 40 minutes. After washed for 4 to 6 times with PBS, the plate was dried. Distilled water (50 μ l) and biotinylated antibody were added in each well except for the control. After 20 minutes of reaction, the plate was rinsed and dried. Subsequently, Enzyme Conjugate (100 μ l) was added in each well, and the plate was left 10 minutes at 37°C for the reaction. The plate was washed, dried, and then placed in the dark for 10 minutes after addition of substrate working solution. Finally, reaction was terminated with 100 μ l of stop solution. The spectrometric absorbance at 450 nm was read using a microplate reader (Thermo Fisher Scientific, UK).

Immunohistochemical staining

Collagen type I and type II were detected immunohistochemically using monoclonal antibody to collagen type I (Boster, China) and collagen type II (Boster, China) according to the instructions. Briefly, to visualize protein, cells were fixed in 4% (w/v) paraformaldehyde and treated with Triton X-100. To eliminate endogenous peroxidase activity, cells were incubated with 3% H₂O₂ for 10 minutes at room temperature. Non-specific staining was blocked with goat serum for 10 minutes at room temperature. After a 1:200 dilution of rat anti-rabbit antibody (collagen type I and II) was added, cells were then incubated with the second antibody and biotin labeled horseradish peroxidase. Subsequently, the antibody binding was visualized with a 3,3'-diaminobenzidine tetrahydrochloride (DAB) kit (Boster, China) before brief counterstaining with hematoxylin. Eventually, cells were gradually dehydrated, sealed with neutral gum, observed and photographed with an inverted phase contrast microscope (Zeiss Corporation, Germany). A percentage of positive (orange-brown) area in total area was calculated from the stained images by the software of Image J (National Institutes of Health, US).

Real-time quantitative PCR (qRT-PCR) analysis

The expression of aggrecan, collagen type II, SOX9, collagen type X and collagen type I mRNAs was analyzed by qRT-PCR analysis. Total RNA was sequentially extracted with an additional purification step employing an RNA isolation kit (Tiangen Biotechnology; Beijing, China) according to the manufacturer's instructions. An equal amount of RNA (300 ng) was used as a template and reverse transcribed into cDNA using reverse transcription kit (Fermentas Company, USA), then amplified using SYBR-Green mix kit (Roche Company, Germany) on a real-time fluorescence quantitative instrument (realplex 4, Eppendorf Corporation, USA). The primers used for PCR are shown (Table 1). The dissociation curve of each primer pair was analyzed to confirm the primer specificity. Marker gene expression of chondrocytes was analyzed by the 2^{- Δ CT} method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each sample was repeated three times for each gene.

Statistical analysis

The data analysis was performed as mean \pm s.d. Statistical significance was determined using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The level of significance was installed to $P < 0.05$.

Results

Cytotoxicity of taurine

The cytotoxicity of taurine on chondrocytes was examined by counting the number of cells. Human articular

Table 1. Primer sequences used in qRT-PCR experiments.

mRNA	Forward primer	Reverse primer
GAPDH	5'-CTATAAATTGAGCCCGCAGC-3'	5'-ACCAAATCCGTTGACTCCG-3'
Aggrecan	5'-CTACACGCTACACCCTCGAC-3'	5'-ACGTCCTCACACCAGGAAAC-3'
collagen type II	5'-AAGCTGGTGAGAAGGGACTG-3'	5'-GGAAACCTCGTTCACCCTTG-3'
SOX9	5'-AAGCTCTGGAGACTTCTGAACG-3'	5'-CGTTCCTCACCGACTTCTCC-3'
collagen type X	5'-CGCTGAACGATACCAAATGCC-3'	5'-TTCCTACAGCTGATGGTCC-3'
collagen type I	5'-GTTTCAGCTTTGTGGACCTCCG-3'	5'-GCAGTTCCTGGTCTCGTCAC-3'

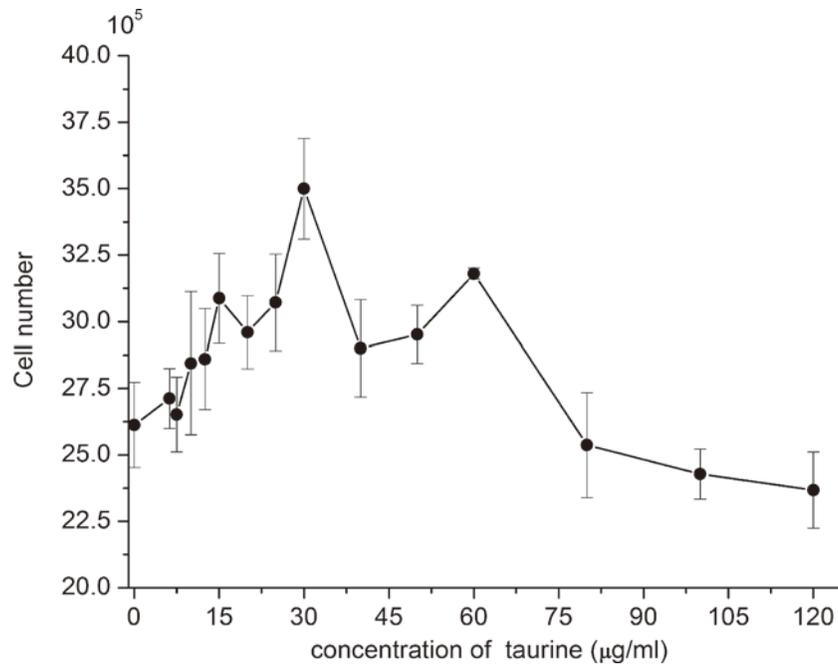


Fig. 1. Effect of taurine on cytotoxicity.

Cytotoxicity analysis of chondrocytes after 3 days of treatment with different concentrations of taurine ranging from 6.25 to 120 µg/ml (mean ± s.d., $n = 4$).

chondrocytes were treated with taurine in increasing concentrations (6.25 to 120 µg/ml). As shown in Fig. 1, taurine concentrations ranging from 6.25 to 60 µg/ml were comparable to the control and therefore nontoxic to cells. However, taurine showed inhibitory effect on chondrocytes at the concentration ranging from 80 to 120 µg/ml. Therefore, concentrations of taurine over the range of 15 to 60 µg/ml which can significantly improve the cell proliferation were used in subsequent assays.

Cell proliferation

Proliferation of chondrocytes cultured with various concentrations of taurine (0, 15, 30 and 60 µg/ml) was detected using BrdU cell proliferation detection kit. Comparatively, the cells cultured with taurine grew faster than those in the control group, as proved by BrdU assay that showed obviously higher cell proliferation than the control at day 4. Furthermore, among all taurine groups, the greatest cell proliferation was achieved at the concentration of 30 µg/ml. The results indicated that taurine facilitated chondrocyte growth, especially at the concentration of 30 µg/ml (Fig. 2).

Cell viability assay

Viable cells and dead cells were determined using calcein-AM/PI staining (Fig. 3). The results demonstrated that taurine exerted a potent effect on chondrocyte survival under identical culture conditions. Calcein-AM/PI staining images showed that survival in taurine groups was higher than those observed in the control. Consistent with the result of cell proliferation, more viable cells were found in

the taurine groups than control, suggesting that taurine could better support cell growth. Among the taurine groups, taurine at the concentration of 30 µg/ml could better support cell growth than 15 µg/ml and 60 µg/ml.

Cell morphology

The morphology of articular chondrocytes after treatment with taurine (0, 15, 30 or 60 µg/ml) was shown in Fig. 4. Cell numbers were counted in one image by using the image analysis program in the software of Nikon A1. There was no obvious difference in cartilaginous morphology among all groups after treatment for 2, 4 or 6 days. Compared with the control, the chondrocytes in the presence of taurine grew better and appeared a higher proliferation tendency that gradually increased with time. In addition, at the concentration of 30 µg/ml, taurine could facilitate the proliferation of chondrocytes compared to that at the other two concentrations.

Biochemical assays

Biochemical assays were used for the quantitative investigation of the production of DNA and GAG after incubation for 2, 4 or 6 days. The DNA content in taurine groups was significantly higher than the control group and achieved the maximum at the concentration of 30 µg/ml (Fig. 5A), which is in agreement with BrdU cell proliferation detection at 4 days (Fig. 2).

The histogram showed the GAG accumulation given as a ratio of GAG to DNA in culture media with different concentrations of taurine (Fig. 5B). The accumulated level of GAG was rapidly increased from 2 days to 6 days in all

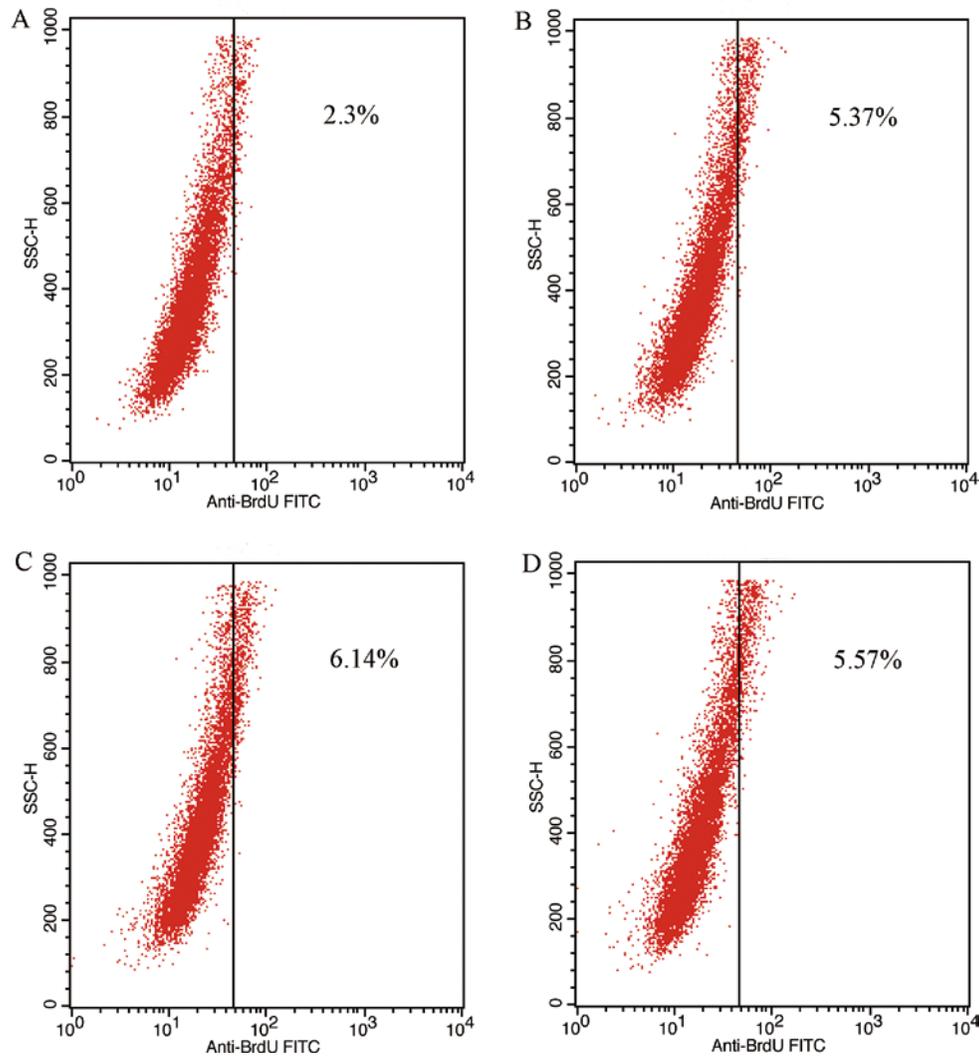


Fig. 2. Effect of taurine on cell proliferation.

Cell proliferation was detected with BrdU cell proliferation detection kit by flow cytometry detecting the cell proliferation rate. The analysis was performed in triplicate. A, B, C and D represent the results of chondrocytes treated for 4 days with taurine at the concentration of 0 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$ and 60 $\mu\text{g/ml}$, respectively. Each number (%) indicates the percentage of BrdU-positive cells in total cells. The assays were performed in triplicate.

groups. Importantly, GAG accumulation in taurine-treated culture media was significantly higher than that in control at the same time point. Especially, taurine at the concentration of 30 $\mu\text{g/ml}$ exhibited the highest accumulation of GAG among the three concentrations.

Collagen content in conditioned media

Similar to GAG accumulation in the conditioned media, collagen content was increased in both dose-dependent and time-dependent manners (Fig. 5C). Collagen accumulation was significantly elevated in taurine groups compared with control. At day 6, the taurine at 30 $\mu\text{g/ml}$ caused the highest accumulation of collagen. The results suggest that taurine might promote collagen production in chondrocytes.

Immunohistochemical staining

To explore whether taurine promotes collagen production, we next detected the expression of collagens type I and II by immunohistochemical staining (Figs. 6 and 7). Only very sparse and light staining was seen for collagen type I in taurine groups (Fig. 6), whereas positive staining with large areas was evident for cartilage-specific collagen type II in taurine groups compared with control groups after incubation for 2, 4 and 6 days (Fig. 7). These results indicate the maintenance of chondrocytic phenotype after treatment with taurine and also suggested that taurine might inhibit the dedifferentiation of chondrocytes cultured *in vitro*.

Expression of ECM-related protein mRNAs

The effect of taurine on chondrocyte ECM synthesis was further investigated through examination of mRNA

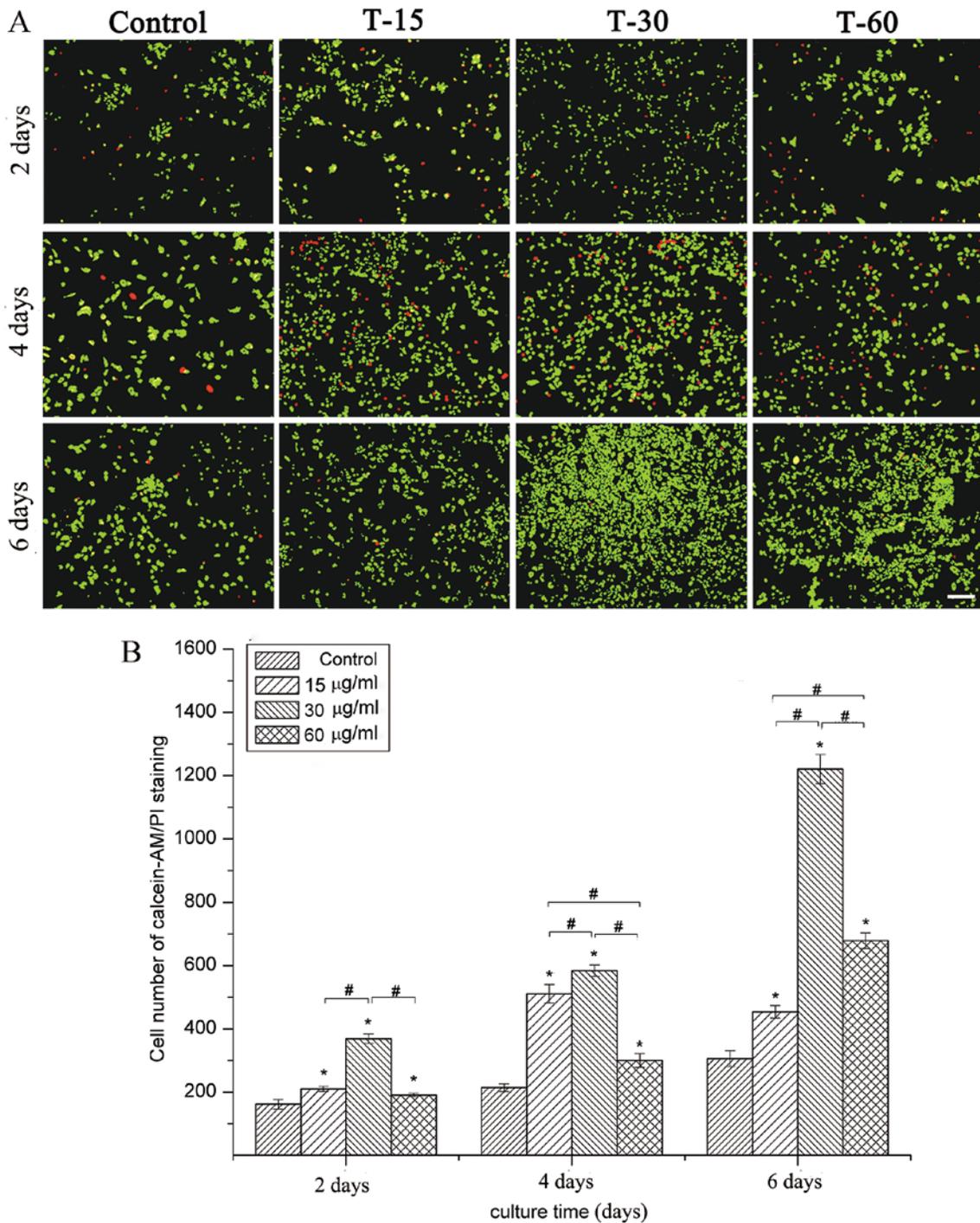


Fig. 3. Effect of taurine on cell viability.

A. Viability of chondrocytes cultured *in vitro* with 15 $\mu\text{g/ml}$ (T-15), 30 $\mu\text{g/ml}$ (T-30) and 60 $\mu\text{g/ml}$ (T-60) taurine for 2, 4 or 6 days. Cell seeding density: $2 \times 10^4/\text{mL}$ (original magnification $\times 100$). Scale bar = 200 μm . B. Cell number calculated and analyzed on the basis of calcein-AM/PI staining images. Cell numbers were counted in one image by using the “cell calculating” toolbar in the Nikon A1 software. The data represent the mean \pm s.d. of three independent culture experiments. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

expression of aggrecan (a cartilage-specific proteoglycan), collagen type II, SOX9, collagen type X and collagen type I after treatment for 2, 4 or 6 days. As shown in Fig. 8, the expression levels of cartilage specific aggrecan, collagen

type II and SOX9 mRNAs were significantly increased by taurine at concentrations ranging from 15 to 60 $\mu\text{g/ml}$. The highest expression levels of aggrecan, collagen type II and SOX9 mRNAs were detected in the taurine group of 30 $\mu\text{g/ml}$.

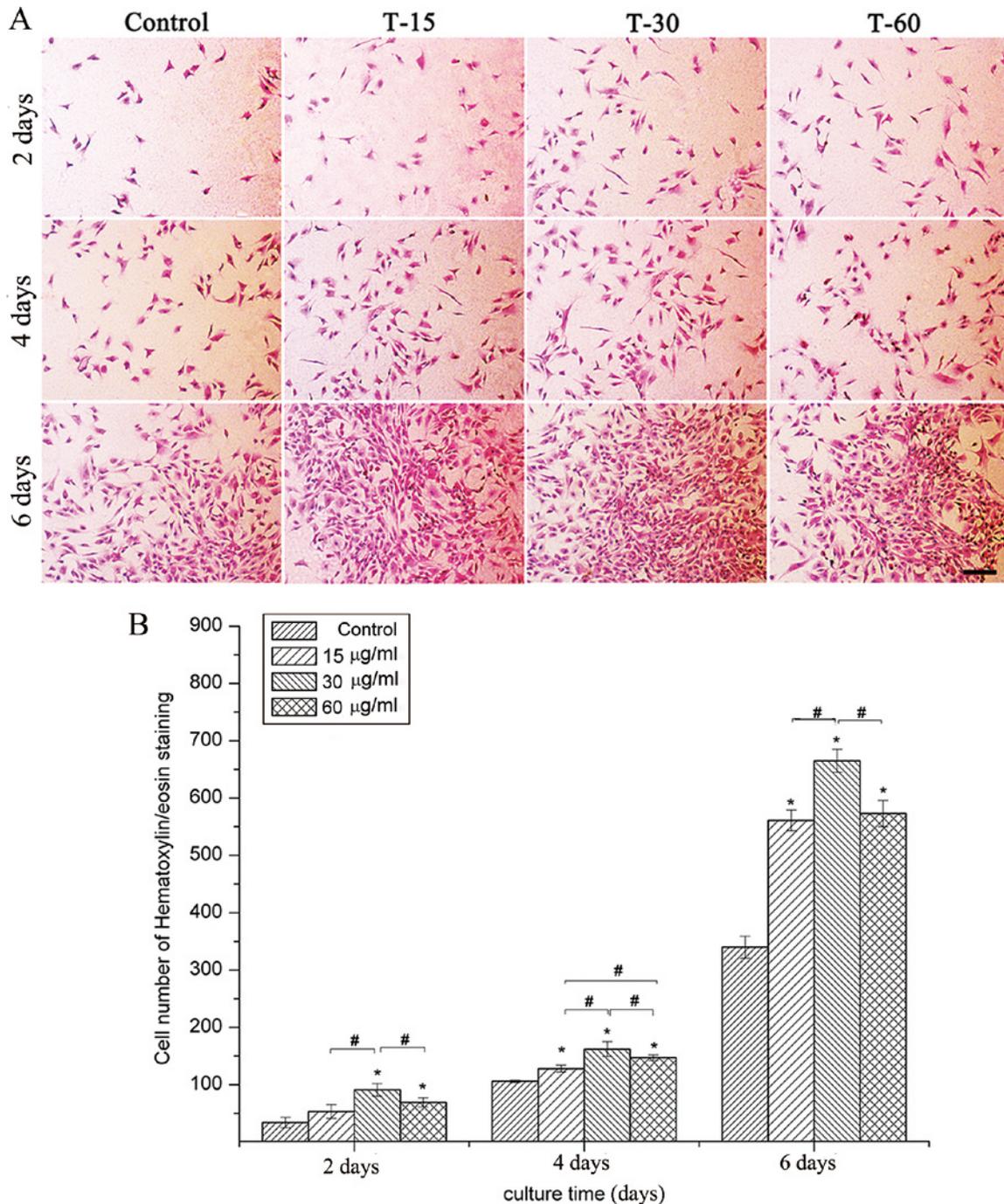


Fig. 4. Effect of taurine on morphology.

A. The morphology of chondrocytes cultured *in vitro* with 15 $\mu\text{g/ml}$ (T-15), 30 $\mu\text{g/ml}$ (T-30) or 60 $\mu\text{g/ml}$ (T-60) taurine for 2, 4 or 6 days. Cell seeding density: $2 \times 10^4/\text{mL}$ (original magnification $\times 100$). Scale bar = 200 μm . B. Cell number calculated and analyzed on the basis of hematoxylin-eosin staining images. Cell numbers were counted in one image by using the "cell calculating" toolbar in the Nikon A1 software. The data represent the mean \pm s.d. of three independent culture experiments. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

ml. Therefore, taurine ranging from 15 to 60 $\mu\text{g/ml}$ might increase the synthesis of cartilage markers. Among all the groups, taurine at the concentration of 30 $\mu\text{g/ml}$ showed the highest expression levels of aggrecan and collagen type II mRNAs, which was in agreement with the results of GAG

production (Figs. 5B and 7).

Importantly, taurine at various concentrations decreased the expression of collagen type I mRNA when compared with the control group after incubation for 2, 4 or 6 days (Fig. 8). In addition, expression of collagen type X

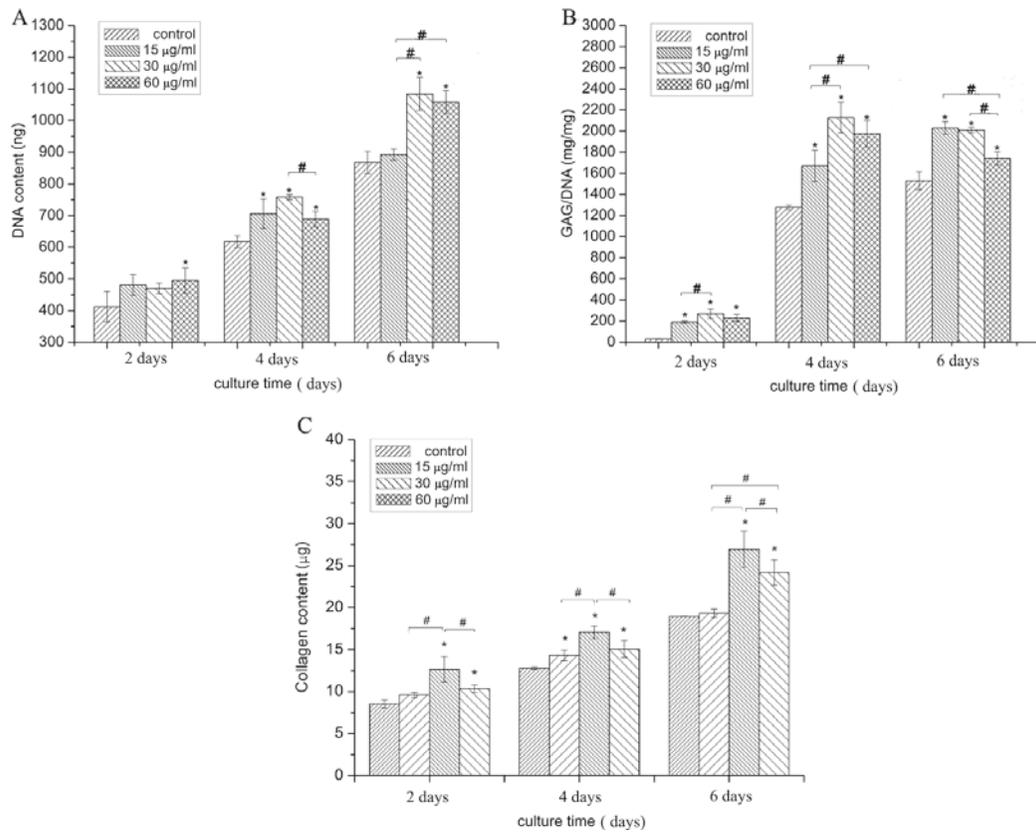


Fig. 5. Effect of taurine on the production of DNA, GAG and collagen.

Quantification of DNA content, GAG production, and collagen content of cells: (A) The DNA content of chondrocytes cultured *in vitro* with 15 µg/ml, 30 µg/ml or 60 µg/ml taurine for 2, 4 or 6 days; (B) GAG (mg) normalized to DNA (mg); (C) The total collagen content (µg). Data from four independent experiments were evaluated, and the mean ± s.d. is shown. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

mRNA was not detectable in all groups (data not shown). Collagen type X is associated with hypertrophic chondrocytes and its expression precedes the onset of endochondral ossification (Kwan et al. 1997). These results suggest that taurine may inhibit the dedifferentiation of chondrocytes.

Discussion

In the present study, we focused on the effect of taurine, a traditional antioxidant, on primary human chondrocytes and have demonstrated taurine as a potential pro-chondrogenic agent that may be useful for cell-based therapy of cartilage repair.

In this study, we showed that taurine could well support the growth of chondrocytes. As demonstrated by cell proliferation assay, morphological examination and cell viability analysis, taurine could significantly promote chondrocyte growth. Previous research reported that taurine could effectively reduce methimazole-induced cytotoxicity in isolated rat hepatocytes (Heidari et al. 2012), which corroborated this study by implying that taurine was non-cytotoxic. Some studies also reported that taurine played a role in promoting cell proliferation on neural stem/progenitor cells and human osteoblast cells (Hernandez-Benitez et al.

2010; Zhang et al. 2011). In addition, taurine could obviously promote GAG deposition in cultured chondrocytes as shown by biochemical assay (Fig. 5B). GAGs constitute a major component of proteoglycans (PGs), which are important components of extracellular matrices of cartilage (Buschmann and Grodzinsky 1995). GAGs and a large number of water molecules generate the expansion pressure and make the cartilage flexible, which plays an important role in maintaining cartilage load-bearing capacity (Robinson et al. 2001; Tew et al. 2005).

In our investigation, taurine significantly enhanced the mRNA expression of cartilage-specific markers, including aggrecan, collagen type II and SOX9. A chondrogenic transcription factor Sox9 plays a major role in an increased level of chondrogenesis in the mouse (Akiyama 2011; Tew and Clegg 2011) in particular by activating co-expression with collagen type II (Ng et al. 1997; Marshall and Harley 2000; Davies et al. 2007). Various gene therapy approaches using viral methods to over-express Sox9 resulted in significant improvements in the production of cartilaginous matrix by articular chondrocytes, bone marrow-derived stem cells and nucleus pulposus cells (Paul et al. 2003; Tsuchiya et al. 2003; Tew et al. 2005). Evidence indicated

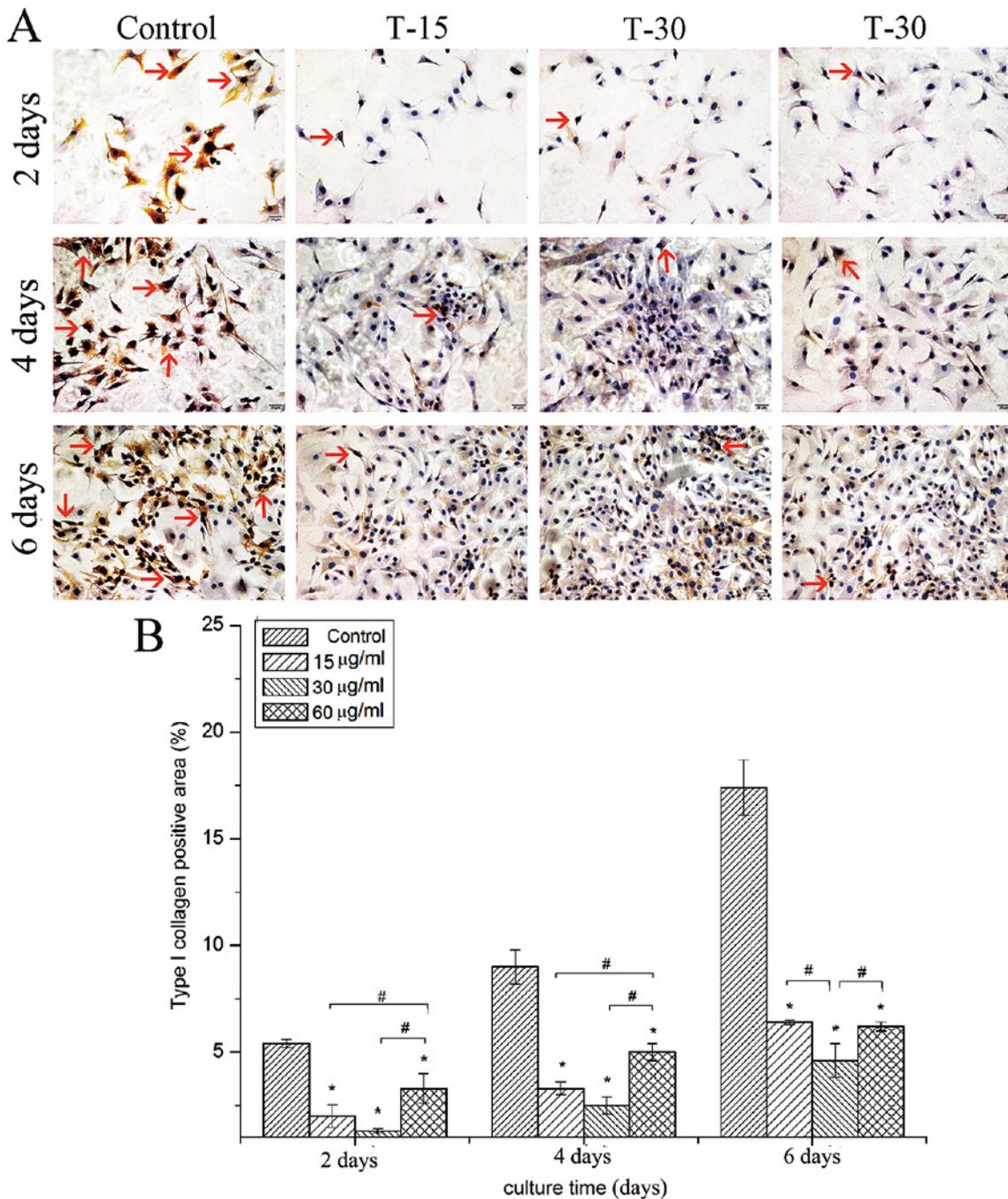


Fig. 6. Effect of taurine on expression of collagen type I.

A. Representative images show the expression of collagen type I. Chondrocytes were cultured with 15 $\mu\text{g/ml}$ (T-15), 30 $\mu\text{g/ml}$ (T-30) or 60 $\mu\text{g/ml}$ (T-60) taurine for 2, 4 or 6 days. Cell seeding density: $2 \times 10^4/\text{mL}$ (original magnification $\times 200$). Scale bar = 200 μm . B. The positive area of type I, calculated as the percentage of positive (orange-brown) area in total area on the basis of immunohistochemical staining images. The data represent the mean \pm s.d. of three independent culture experiments. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

that aggrecan production was also meaningfully up-regulated by the Sox9 gene as an early chondrogenic marker (Bi et al. 1999; Tew et al. 2005). In this study, the increased expression level of SOX9 mRNA was consistent with the increase of GAG production (Fig. 5B) and up-regulation of aggrecan mRNA expression in taurine-treated groups, indi-

cating the regulatory role of taurine in chondrogenesis. The increased collagen type II expression in taurine-treated groups also indicated that taurine could stimulate exuberant cartilage matrix secretion.

Meanwhile, taurine could effectively inhibit the expression of collagen type I, which represents dedifferen-

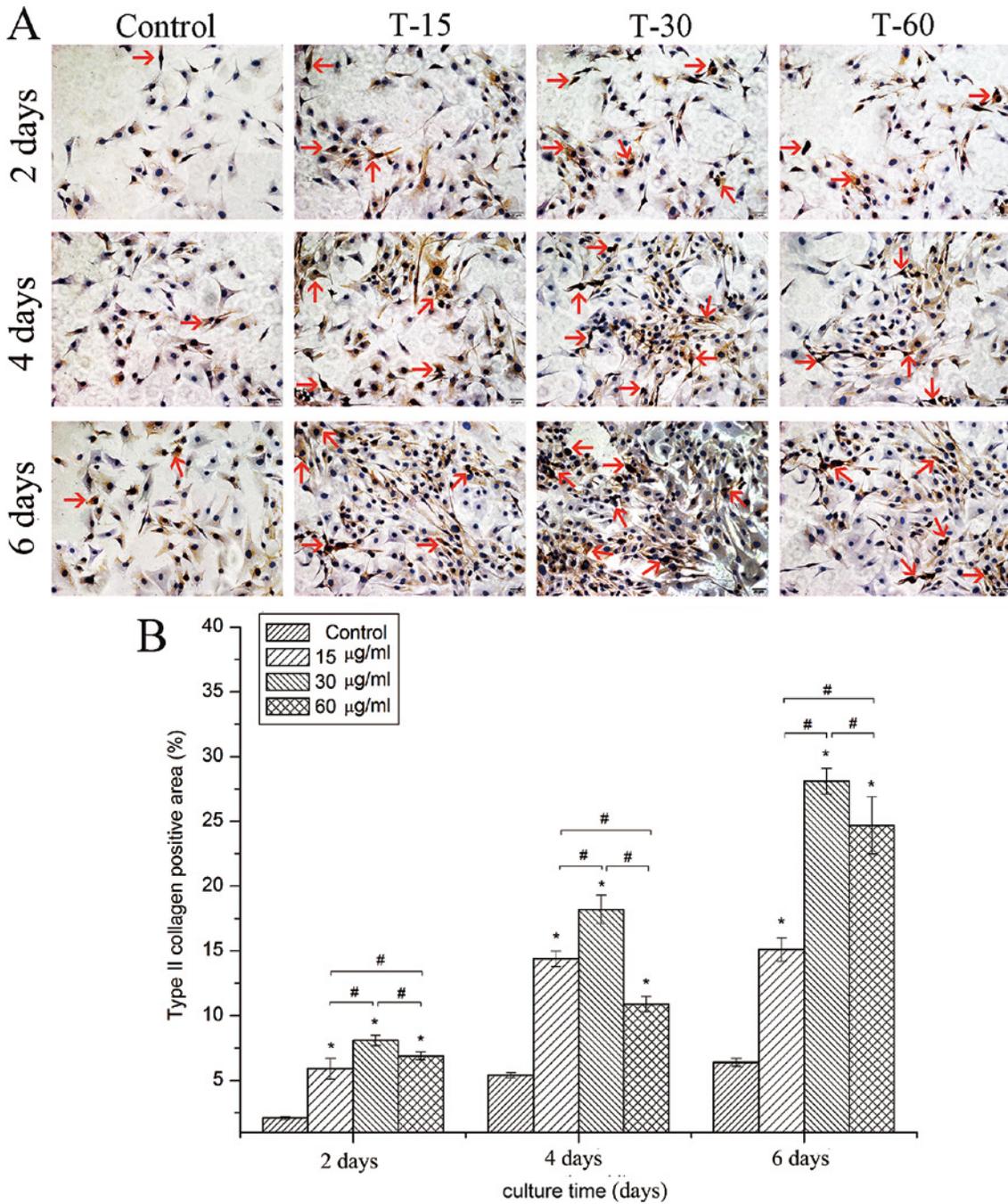


Fig. 7. Effect of taurine on expression of collagen type II. A. Representative images show the expression of collagen type II. Chondrocytes were cultured with 15 $\mu\text{g/ml}$ (T-15), 30 $\mu\text{g/ml}$ (T-30) or 60 $\mu\text{g/ml}$ (T-60) taurine for 2, 4 or 6 days. Cell seeding density: $2 \times 10^4/\text{mL}$ (original magnification $\times 200$). Scale bar = 200 μm . B. The positive area of type II, calculated as the percentage of positive (orange-brown) area in total area on the basis of immunohistochemical staining images. The data represent the mean \pm s.d. of three independent culture experiments. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

tiation of chondrocytes. As is well known, the differentiated phenotype of chondrocytes consists primarily of collagen type II and cartilage-specific proteoglycan, and dedifferentiation occurs when they are lost and replaced by a complex collagen phenotype that consists predominately of collagen type I and exhibits a low level of proteoglycan

synthesis (Benya and Shaffer 1982; Schnabel et al. 2002; Karlsten et al. 2010). Evidenced by PCR, biochemical and immunohistochemical analysis, collagen type I expression was significantly down-regulated by taurine. In agreement with this study, many reports have demonstrated that taurine inhibited the collagen I synthesis of primary fibroblasts

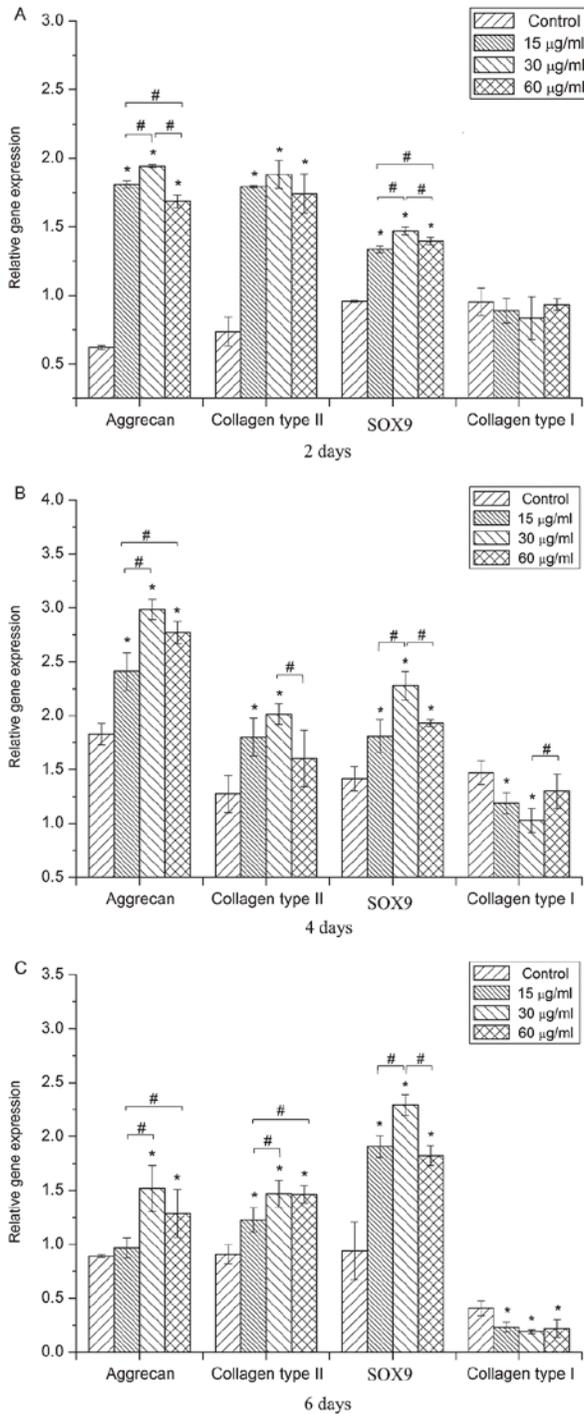


Fig. 8. Effect of taurine on expression of mRNAs for ECM-related proteins.

The expression levels of ECM-related protein mRNAs were analyzed by qRT-PCR. The chondrocytes were cultured with 15 µg/ml, 30 µg/ml or 60 µg/ml taurine for (A) 2 days, (B) 4 days and (C) 6 days ($n = 3$ for each experiment). The relative expression levels of each mRNA in taurine-treated cells relative to the control group were calculated by the $2^{-\Delta\Delta CT}$ method using GAPDH as the internal control. The data represent the mean \pm S.D. of three independent culture experiments. * $P < 0.05$, # $P < 0.05$ (* $P < 0.05$ represents the comparison between the taurine groups and the control group, # $P < 0.05$ represents the comparison among the taurine groups).

and liver fibrosis (Chen et al. 1999; Ren et al. 2008). Besides, collagen type X that is specifically associated with hypertrophic chondrocytes and precedes the onset of endochondral ossification (Kwan et al. 1997) was nearly undetectable in taurine groups, suggesting that hypertrophy of chondrocytes would not be induced by taurine. Therefore, the reduced collagen type I mRNA and the undetectable level of collagen type X mRNA suggested that the dedifferentiation and hypertrophy might be alleviated or prevented by taurine.

As for the recommended dose of taurine, our results showed that the concentration of taurine concerning enhancing chondrocytes proliferation ranged from 15 µg/ml to 60 µg/ml (Fig. 2), which agreed with the earlier reports (Chen et al. 1997; Shivaraj et al. 2012). In their studies, DNA synthesis of human fetal neuron cells was increased in a dose-dependent manner when neurons were cultured in the medium containing taurine at 100 to 6,400 µM (12.515 to 800.96 µg/ml) (Chen et al. 1997). And taurine at appropriate concentrations ranging from 100 µM (12.515 µg/ml) to 500 µM (62.575 µg/ml) stimulated the proliferation of P5 NPCs (neural progenitor cell) for 2 days (Shivaraj et al. 2012). Among the various concentrations, taurine of 30 µg/ml could support the greatest cell proliferation and stimulate the most matrix secretion.

To summarize, we demonstrated that taurine could promote cell growth and maintain phenotype of human articular chondrocytes. In our study, taurine could effectively promote chondrocyte proliferation, enhance secretion and synthesis of cartilage ECM, by up-regulating expression levels of aggrecan, collagen type II and SOX9 mRNAs. In contrast, taurine might prevent chondrocyte dedifferentiation by down-regulating the expression of collagen type I mRNA. A marker for hypertrophy that may lead to chondrocyte ossification was also undetectable in taurine groups. In the taurine groups, the most profound response was observed with 30 µg/ml. Thus, taurine may be a useful pro-chondrogenic agent for ACI in the treatment of cartilage repair. Further studies are needed to elucidate the underlying mechanism of chondrogenesis induced by taurine.

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Conflict of Interest

The authors declare no conflict of interest.

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