Journal of Physiology and Biochemistry (2019) 75:153–162
https://doi.org/10.1007/s13105-019-00666-8

ORIGINAL ARTICLE

Protective effects of atorvastatin on high glucose-induced oxidative stress and mitochondrial apoptotic signaling pathways in cultured chondrocytes

Azam Hosseinzadeh[1] · Kobra Bahrampour Juybari[2] · Tunku Kamarul[3] · Ali Mohammad Sharifi[1,3,4]

Received: 3 August 2018 / Accepted: 3 February 2019 / Published online: 22 February 2019

Abstract

The high glucose concentration is able to disturb chondrocyte homeostasis and contribute to OA pathogenesis. This study was designed to investigate the protective effects of atorvastatin (ATO) on high glucose (HG)-mediated oxidative stress and mitochondrial apoptosis in C28I2 human chondrocytes. The protective effect of ATO (0.01 and 0.1 μM) on HG (75 mM)-induced oxidative stress and apoptosis was evaluated in C28I2 cells. The effects of ATO on HG-induced intracellular ROS production and lipid peroxidation were detected and the protein expression levels of Bax, Bcl-2, caspase-3, total and phosphorylated JNK and P38 MAPKs were analyzed by Western blotting. The mRNA expression levels of antioxidant enzymes including heme oxygenase-1, NAD(P)H quinine oxidoreductase, glutathione S-transferase-P1, catalase, superoxide dismutase-1, glutathione peroxidase-1, -3, -4 were evaluated by reverse transcription-polymerase chain reaction. Pretreatment with ATO remarkably increased the gene expression levels of antioxidant enzymes and reduced HG-induced elevation of ROS, lipid peroxidation, Bax/Bcl-2 ratio, caspase-3 activation, and JNK and P38 phosphorylation. Atorvastatin could considerably reduce HG-induced oxidative stress and mitochondrial apoptosis through increasing the expression of antioxidant enzymes. Atorvastatin may be considered as a promising agent to prevent high glucose-induced cartilage degradation in OA patients.

Keywords Osteoarthritis · Atorvastatin · High glucose · Reactive oxygen species · Apoptosis

Introduction

Diabetes mellitus (DM) is defined as a group of metabolic diseases resulting from carbohydrate metabolism dysfunction characterized by hyperglycemia. Recent epidemiological studies have reported that approximately 50% of patients with DM suffer from some form of arthritis such as osteoarthritis [17]. Osteoarthritis, as a progressive joint disorder, is characterized by irreversible breakdown of articular cartilage. Chondrocytes, the only resident cells in articular cartilage, are responsible for the synthesis and maintenance of extracellular matrix (ECM) molecules, including type II collagen and proteoglycans [11, 13]. Glucose has pivotal role in the chondrocyte homeostasis by producing energy required for synthesizing cartilage matrix molecules. However, high glucose concentration is capable of disturbing chondrocyte homeostasis and contributing to OA pathogenesis [25]. Hyperglycemia induces oxidative stress and inflammatory responses playing pivotal roles in the pathogenesis of OA. Oxidative stress has been identified as a major cause of chondrocyte apoptotic death. Chondrocyte apoptosis is proposed to be a key player in OA pathogenesis via exacerbating cartilage degeneration [12, 17, 25]. According to these findings, excessive generation of ROS induced by high glucose in patients with DM can lead to chondrocyte apoptosis through activation of pro-apoptotic signaling pathways such as caspases and mitogen-activated

✉ Ali Mohammad Sharifi
sharifalim@gmail.com

[1] Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran

[2] Department of Pharmacology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran

[3] Tissue Engineering Group, (NOCERAL), Department of Orthopedics Surgery, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

[4] Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

protein kinases (MAPKs). Inhibition of these cascades has been shown to be effective to reduce the degree of cartilage destruction related to progressive loss of chondrocytes [28]. Chondrocytes possess antioxidant defense, protecting them from ROS detrimental effects. Impaired antioxidant enzyme activities in chondrocytes lead to increased susceptibility to oxidative stress-induced cell death and consequent cartilage degradation. Furthermore, exogenous antioxidants have been reported to protect chondrocytes from ROS-mediated oxidative damage [12]. Atorvastatin (ATO) is a synthetic drug containing penta-substituted pyrrole [23]. Atorvastatin belongs to statins which are known as a group of drugs acting by competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the mevalonate pathway and consequent cholesterol biosynthesis. Statins have been extensively used to lower serum cholesterol levels in cardiovascular diseases. Statins also have other pharmacological properties such as antioxidant, anti-inflammatory, and immunomodulatory, proposing pleotropic effects of statins beyond cholesterol-lowering activity [24]. Molecular studies have demonstrated that ATO inhibits enzymatic source of ROS such as NADPH oxidase leading to reduction of oxidative stress markers [29]. Atorvastatin enhances the activity and the expression of antioxidant enzymes including heme oxygenase-1 (HO-1), NAD(P)H quinine oxidoreductase 1 (NQO1), SOD, and CAT [14, 19, 24]. In addition, ATO alleviates apoptosis via decreasing the expression of apoptosis-related proteins including Bax, cytochrome c, and caspase-3 [6]. The obtained results from our previous studies revealed that antioxidant agents could protect chondrocytes against oxidative damage, which is induced by adding IL-1β to chondrocyte culture [12, 13]. The current study was then designed, first, to identify the role of high glucose (HG) concentration in the induction of oxidative stress and apoptosis, as the major mechanisms of chondrocyte cell death, in C28I2 human chondrocytes and, second, to evaluate the protective effect of ATO on HG-induced chondrocyte injury.

Materials and methods

Reagents and antibodies

All the materials used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA) unless noted. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham, and penicillin–streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). TRIZOL reagent was from Invitrogen (Merelbeke, Belgium) and antibodies were from cell signaling technology (Danvers, MA, USA). Polyvinylidene fluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA, USA). 1× RIPA Lysis buffer and phenyl methane sulfonyl fluoride (PMSF) were purchased from Roche (Applied Science, penzberg, Germany). Chemiluminescence (ECL) kit was obtained from Amersham Biosciences (Buckinghamshire, UK). Oligo (dT) primer and moloney murine leukemia virus reverse transcriptase (M-MLV RT) were from Fermentas (Loughborough, UK). Red-safe was purchased from iNtRON Biotechnology (Seongnam, Korea).

Stock solution of ATO with 10^4 μM concentration was provided in dimethyl sulfoxide (DMSO), and serial dilutions were prepared in DMEM/F12 prior to application. The final concentration of DMSO was less than 0.01% that it was added to untreated control cells.

The concentration of glucose used in this study was based on the findings from previous studies [4, 5]. Although the plasma level of glucose found in diabetic patients is less than that we examined in our study, it is necessary to indicate that our experiment was in short time exposure. Then, the higher concentration of glucose was used compared to plasma level of glucose in diabetic patients to induce oxidative stress and apoptotic signaling pathways in short time.

Cell culture

C28I2 chondrocyte cell line was supplied by Pasteur Institute (Tehran, Iran). Cells were cultured in DMEM/F12 medium supplemented with 10% (*v*/*v*) heat-inactivated FBS and 1% (*v*/*v*) penicillin (100 U/mL)–streptomycin (100 μg/mL) and kept at 37 °C in an incubator with humidified atmosphere of 90% air and 5% CO_2. The culture medium was changed to fresh medium every 48 h and cells were passaged every 2–3 days.

Measurement of intracellular ROS

Intracellular generation of ROS was assessed using 2,7-dichlorofluorescein diacetate (DCF-DA). C28I2 cells were plated into 24-well plates. One day later, the media was removed and cells were incubated with serum-free medium for overnight. Serum-starved cells were pretreated with 0.01 and 0.1 μM of ATO for 6 h prior to the addition of HG (75 mM) for 72 h. After treatment, the medium was discarded and cells were washed with PBS and loaded with DCF-DA for 30 min at 37 °C. Then, cells were rinsed with fresh media and the oxidation products were measured using 485/20 nm excitation and 528/20 nm emission wavelength by a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Determination of malondialdehyde level; TBARS assay

MDA content (as lipid peroxidation index) was measured by a colorimetric method. Briefly, C28I2 cells were cultured into 24-well plates. Twenty-four hours later, the

media was changed to serum-free medium and the cells were incubated for overnight. The cells were pretreated with ATO (0.01 and 0.1 μM) for 6 h before to the addition of HG (75 mM) for 72 h. After treatment, the cells were washed and homogenized. The homogenate was centrifuged and 100 μL of the supernatant was taken and mixed with 1.5 mL of thiobarbituric acid (0.8%), 1.5 mL of acetic acid (20%), and 200 μL of sodium dodecyl sulfate (8%). Each reaction mixture was heated for 60 min at 95 °C. After cooling in room temperature, 5 mL of n-butanol was added and mixture was centrifuged at 3000*g* for 10 min. The absorbance of the supernatants was measured at 532 nm.

Protein extraction/Western blot analysis

Western blot analysis was performed to measure protein concentration. Briefly, serum-starved cells were pretreated with ATO (0.01 and 0.1 μM) for 6 h before the addition of HG (75 mM) for 72 h. To protein extraction, cells were washed twice with ice-cold PBS and lysed via 1× RIPA lysis buffer including protease and phosphatase inhibitor cocktails. Subsequently, total cell lysates were centrifuged at 12,000*g* at 4 °C for 30 min. Bradford assay was used to determine protein concentration. Protein samples in equal amounts were separated by SDS-PAGE and transferred on a PVDF membranes which were incubated with first antibodies (Bax, Bcl-2, cleaved caspase-3, JNK, p38, phospho-JNK, and phospho-p38) at 4 °C with mild shaking, overnight. Then, the membranes were incubated with horseradish peroxidase-labeled secondary antibody. Eventually, the immunocomplexes were visualized via ECL detection kit. Image J software was used to quantify the density of the bands (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-polymerase chain reaction

After incubation with serum-starved medium for overnight, C28I2 cells were pretreated with ATO (0.1 and 0.1 μM) for 6 h before the addition of HG (75 mM) for 72 h. Total RNA was extracted from treated and untreated cells using TRIZOL reagent (Invitrogen Cor., Carlsbad, CA, USA) according to the manufacturer's directions. The quality and concentration of RNA were evaluated using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). In order to synthesize the complementary DNA (cDNA), 3 μg of extracted RNA was employed in a single-round RT reaction containing oligodT (2 μL), deoxyribonucleotide triphosphate (dNTP, 1 μL), M-MLV RT (1 μL), and RNase Inhibitor (0.5 μL) at the total volume of 20 μL. Reverse transcription reaction was carried out at 42 °C for 1 h, followed by incubation at 72 °C for 10 min. Afterwards, cDNA was exposed to PCR utilizing specific primers (Table 1). The standard PCR conditions for *HO-1*, *NQO1*, and *GSTP1* for *SOD1*, *GPx1*, *GPx3*, *GPx4*, and *CAT* primers were as follows: hot start at 94 °C for 3 min, followed by 35 amplification cycles consisting of 94 °C for 30 s, 1 min at 60 °C, 72 °C for 40 s, and a final extension at 72 °C for 5 min and for β-actin (*ACTB* gene) primer was 30 s at 94 °C, 1 min at 56 °C, and 40 s at 72 °C. *ACTB* was used as the

Table 1 Oligonucleotides primers for semi-quantitative RT-PCR analysis

Gene	Primer sequence(5′–3′)	Size (bp)	Gen Bank accession number
HO-1	F: GCTGACCCATGACACCAAG R: GTGTAAGGACCCATCGGAGA	161	NM_002133.2
NQO1	F: ACTGATCGTACTGGCTCACTC R: CCTTCAGTTTACCTGTGATGTCC	167	NM_001286137.1
GSTP1	F: TACACCAACTATGAGGCGGG R: AGGTTGTAGTCAGCGAAGGAG	143	NM_000852.3
CAT	F: TGGCTACTTTGAGGTCACACA R: ACAGTGGAGAACCGAACTGC	102	NM_001752.3
GPx1	F: GTCGGTGTATGCCTTCTCGG R: CAGAGGGACGCCACATTCTC	105	NM_001329503.1
GPx3	F: CACGACATCCGCTGGAACTT R: AGTCCCTCCCCTACATGGTG	197	NM_002084.4
GPx4	F: CAGTGAGGCAAGACCGAAGT R: GCTTCCCGAACTGGTTACAC	109	NM_001039848.2
SOD1	F: GTGAAGGTGTGGGGAAGCAT R: GCAGTCACATTGCCCAAGTC	182	NM_000454.4
ACTB	F: CACCATGGATGATGATATCGC R: AGTCCATCACGATGCCAGTG	467	NM_001101.3

HO-1 heme oxygenase-1, *NQO1* NAD(P)H quinine oxidoreductase 1, *GSTP1* glutathione S-transferase P1, *CAT* catalase, *GPx* glutathione peroxidase, *SOD1* superoxide dismutase1, *ACTB* β-actin

internal control. The PCR products were size-fractionated on agarose gel (2%) and visualized by red-safe staining.

Statistical analysis

The results were expressed as mean ± SD. Statistical significance was analyzed by unpaired student's *t* test for comparisons between two groups and one-way analysis of variance with Tukey's post-test for multiple comparisons. Statistical values of < 0.05 were considered significant.

Results

Effect of ATO on HG-induced elevation of intracellular ROS production and MDA level

The production of intracellular ROS significantly increased after exposure of C28I2 cells to HG ($p < 0.001$ vs. control). Pretreatment of cells with ATO (0.01 and 0.1 μM) for 6 h before addition of HG significantly decreased HG-induced ROS elevation (0.01 μM; $p < 0.05$ and 0.1 μM; $p < 0.001$, Fig. 1a).

MDA level was significantly elevated in HG group compared to the control group ($p < 0.001$). Pretreatment with ATO (0.01 and 0.1 μM) for 6 h prior to addition of HG significantly reduced MDA level when compared to HG group (0.01 μM; $p < 0.05$ and 0.1 μM; $p < 0.001$, Fig. 1b).

Effect of ATO on HG-induced increased Bax/Bcl2 ratio

To determine the influence of ATO on Bax/Bcl-2 ratio in C28I2 cells stimulated by HG, Western blot analysis was performed. High glucose significantly increased Bax/Bcl-2 ratio in comparison with the control group ($p < 0.01$) and ATO pretreatment significantly reduced HG-induced increased Bax/Bcl-2 ratio (0.01 μM; $p < 0.05$ and 0.1 μM; $p < 0.01$, Fig. 2).

Effect of ATO on HG-induced caspase-3 activation

In order to investigate the enzymatic activity of the caspase-3, cleaved form of protein was measured by Western blotting. Our results indicated that HG significantly increased cleaved caspase-3 level compared to the control group ($p < 0.001$). Pretreatment with ATO could significantly attenuate HG-induced increased cleaved caspase-3 level in C28I2 cells (0.01 μM; $p < 0.01$ and 0.1 μM; $p < 0.01$, Fig. 2).

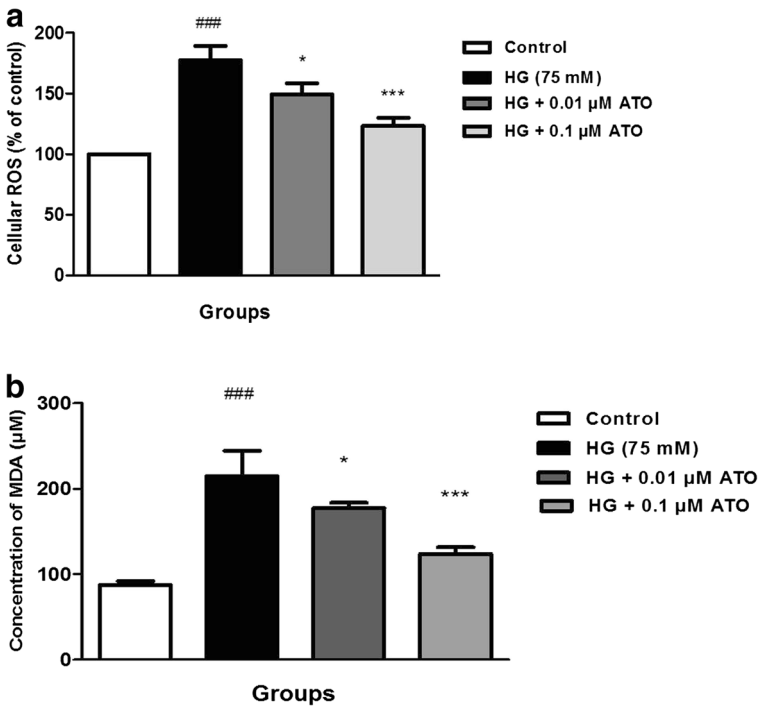

Fig. 1 **a** Effects of ATO (0.01 and 0.1 μM) on HG-induced formation of intracellular ROS in C28I2 cells were detected using DCF-DA. The generation of ROS increased significantly in HG (75 mM)-treated group ($^{###}p < 0.001$) while pretreatment with ATO for 6 h prior to addition of HG significantly reduced HG-induced ROS formation ($^{*}p < 0.05$ and $^{***}p < 0.001$). Results are expressed as percentage mean ± SD ($n = 6$). **b** Effects of ATO (0.01 and 0.1 μM) on HG-induced lipid peroxidation in C28I2 cells were evaluated using TBARS assay. The MDA level significantly increased in HG (75 mM)-treated group ($^{###}p < 0.001$). Pretreatment with ATO significantly decreased HG-induced lipid peroxidation ($^{*}p < 0.05$ and $^{***}p < 0.001$). Results are expressed as percentage mean ± SD ($n = 6$)

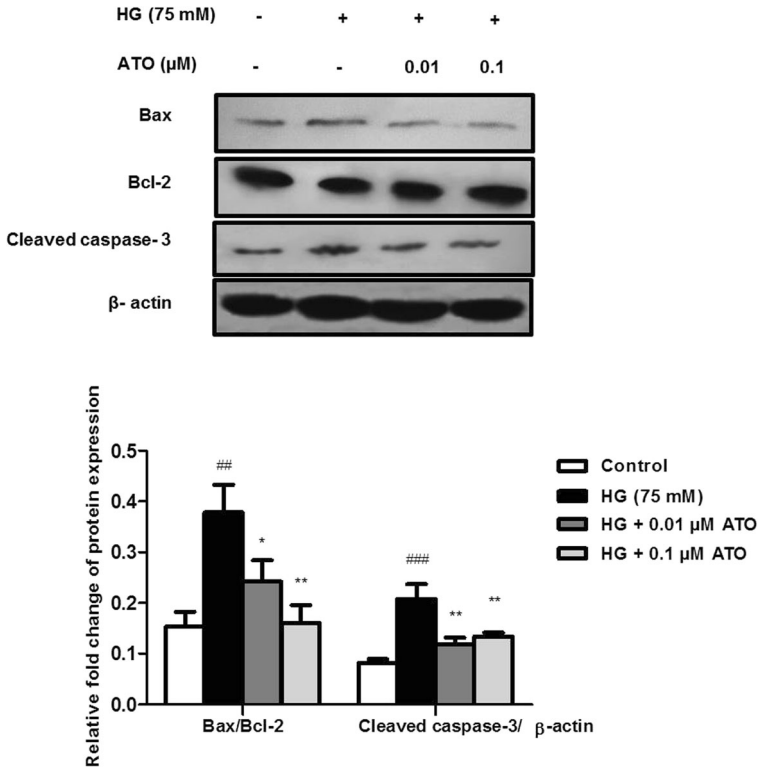

Fig. 2 The effect of ATO (0.01 and 0.1 μM) on caspase-3 activity and Bax/Bcl-2 ratio in C28I2 cells exposed to HG (75 mM) was evaluated using Western blot analysis ($n = 3$). The density of Bax and Bcl-2 bands was analyzed and Bax/Bcl-2 ratio was determined. The ratio of Bax/Bcl-2 significantly increased in HG-treated group ($^{\#\#}p < 0.01$) compared to the control group. Pretreatment with ATO significantly reduced HG-induced elevation of Bax/Bcl-2 ratio ($^{*}p < 0.05$ and $^{**}p < 0.01$). Cleaved caspase-3 band density was determined and cleaved caspase-3/β-actin ratio was analyzed. The ratio of cleaved caspase-3/β-actin significantly elevated in HG-treated group ($^{\#\#\#}p < 0.001$) compared to the control group. Pretreatment with ATO significantly decreased HG-induced elevation of cleaved caspase-3/β-actin ratio ($^{**}p < 0.01$). Data show results from three experiments with the ratios given as a mean ± SD

Effect of ATO on HG-induced JNK and P38 MAPKs phosphorylation

Western blot assay was used to evaluate the effect of ATO on JNK and P38 phosphorylation in C28I2 cells induced by HG for 72 h. High glucose significantly increased JNK1/2 ($p < 0.01$ and $p < 0.05$) and P38 ($p < 0.001$) phosphorylation in comparison with the control group. Pretreatment of cells with ATO (0.01 and 0.1 μM) significantly prevented HG-induced phosphorylation of JNK1 ($p < 0.01$), JNK2 ($p < 0.05$), and P38 (0.01 μM; $p < 0.05$ and 0.1 μM; $p < 0.001$) in C28I2 cells (Fig. 3).

Effect of HG and ATO on gene expression of detoxifying phase II and antioxidant enzymes in RT-PCR

Analysis RT-PCR was performed to examine the effects of HG and ATO on gene expression of antioxidant enzymes including *HO-1*, *NQO1*, *SOD1*, *CAT*, *GPx1*, *GPx3*, and *GPx4* in C28I2 cells. The mRNA expression level of *HO-1* gene exhibited no significant change after treatment with HG for 72 h in comparison with the control group. Moreover, ATO could significantly increase the expression level of *HO-1* gene compared to the control and HG groups ($p < 0.001$, Fig. 4).

Our results also indicated that the expression levels of *NQO1* and *GSTP1* in HG group were significantly less than that in the control group ($p < 0.01$ and $p < 0.001$, respectively). Pretreatment with ATO could significantly enhance the expression levels of *NQO1* ($p < 0.001$) and *GSTP1* ($p < 0.01$) genes compared to that in the HG group. ATO also elevated the expression level of *NQO1* gene compared to the control group ($p < 0.05$, Fig. 4).

The expression levels of *GPx1*, *GPx3*, *GPx4* genes in the HG group were significantly less than control group ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively), while the expression levels of *CAT* and *SOD1* did not significantly change after treatment with HG for 72 h. Pretreatment with ATO significantly increased the expression levels of *GPx1* ($p < 0.01$), *GPx3* ($p < 0.01$), *GPx4* ($p < 0.001$), *CAT* (0.01 μM; $p < 0.05$ and 0.1 μM; $p < 0.001$), and *SOD1* (0.1 μM; $p < 0.01$) genes compared to the HG group. Furthermore, pretreatment with ATO significantly increased the expression levels of *CAT* (0.1 μM; $p < 0.001$), and *SOD1* (0.1 μM; $p < 0.05$) genes compared to the control group (Fig. 5).

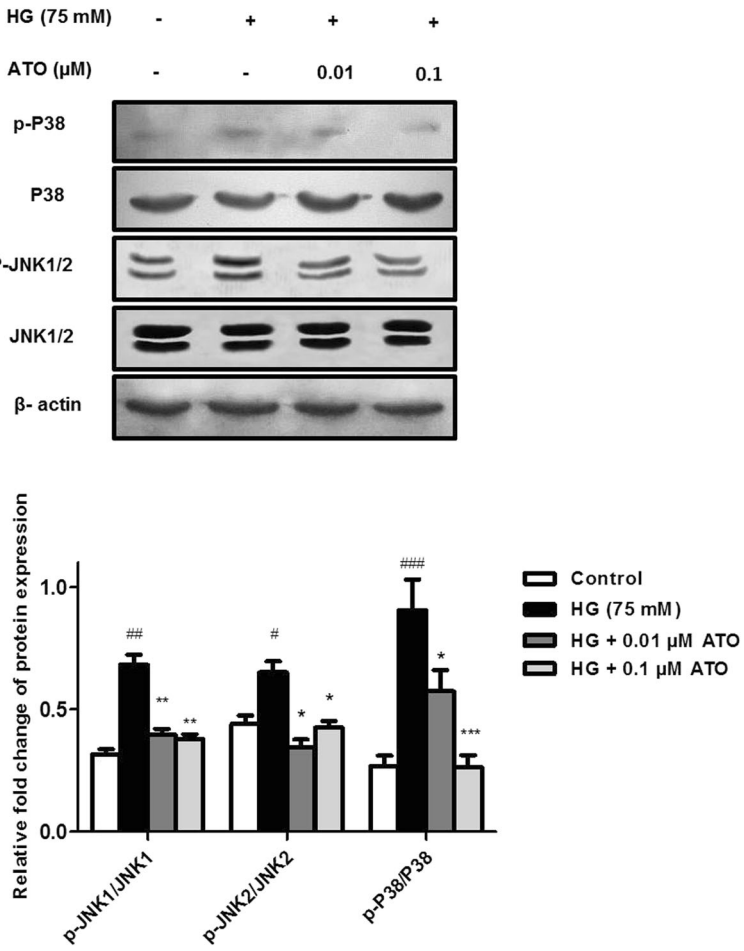

Fig. 3 The effect of ATO (0.01 and 0.1 μM) on HG-induced phosphorylation of JNK1/2 and P38 MAPKs was evaluated in C28I2 cells by Western blotting ($n = 3$). The densities of phosphorylated and total JNK1/2 and P38 MAPKs bands were evaluated and ratios of p-JNKs/JNKs and p-P38/P38 were calculated. High glucose (75 mM) significantly elevated p-JNKs/JNKs ($^{\#\#}p < 0.01$ and $^{\#}p < 0.05$) and p-P38/P38 ($^{\#\#\#}p < 0.001$) ratios in comparison with the control group. Pretreatment with ATO significantly reduced HG-induced elevation of p-JNK1/JNK1 ($^{**}p < 0.01$), p-JNK2/JNK2 ($^{*}p < 0.05$), and p-P38/P38 ($^{*}p < 0.05$ and $^{***}p < 0.001$) ratios. Data show results from three experiments with the ratios given as a mean ± SD

Discussion

Diabetes mellitus, as a chronic metabolic disorder, has been found to induce the initiation and/or the severity of OA [25]. Due to the increase of dyslipidemia and cardiovascular events in diabetic subjects, lipid-lowering therapy is used to reduce the risk of cardiovascular events. Although recent studies have demonstrated that statin-treated individuals are at risk of developing type 2 diabetes, statins are the first-line strategy for reducing low-density lipoprotein cholesterol levels in patients with established type 2 diabetes [31]. Considering that lipid-lowering drugs such as statins are used to modify DM-related dyslipidemia, the present study investigated the effects of ATO on another DM complication, OA, in in vitro.

Oxidative/nitrosative stress is suggested to be one of the main causative factors incriminated in OA initiation and progression [13, 15]. The remodeling of collagen types in ligaments, cartilage, and synovia has been reported to contribute to the ECM degradation and consequent functional limitation of articular joints in streptozotocin-induced diabetic rats [3].

High-glucose conditions directly increase the generation of ROS and NO· by articular chondrocytes via inducing mitochondrial dysfunction and pro-inflammatory cytokine production [20]. Excessive production of ROS and reactive nitrogen species (RNS) contributes to the cartilage degradation through destroying ECM, inhibiting matrix production, activating MMPs, and inducing apoptosis [11]. Recent studies have shown that exogenous antioxidants can protect chondrocytes from oxidative stress-induced damage [1]. Molecular studies have demonstrated that the induction of HO-1 and NQO1 expression inhibits IL-1β and TNF-α-induced chondrocyte apoptosis via decreasing DNA fragmentation, JNK activity, and MMPs and COX2 expression [9, 16]. Considering the role of HG in the induction of pro-inflammatory cytokine production such as TNF-α and IL1-β [17], HG may induce OA through increasing inflammatory responses. Based on results from our previously published studies, inflammatory cytokines such as IL-1β lead to induction of oxidative stress and apoptosis in chondrocytes [12, 13]. We have also conducted experiment which showed that HG increases the activation of nuclear factor-κB (NF-κB), a transcription factor inducing the expression of inflammatory cytokines, in human chondrocytes (data not shown). These findings suggest that HG could induce OA through inducing oxidative stress and inflammation resulting in the apoptotic chondrocyte death.

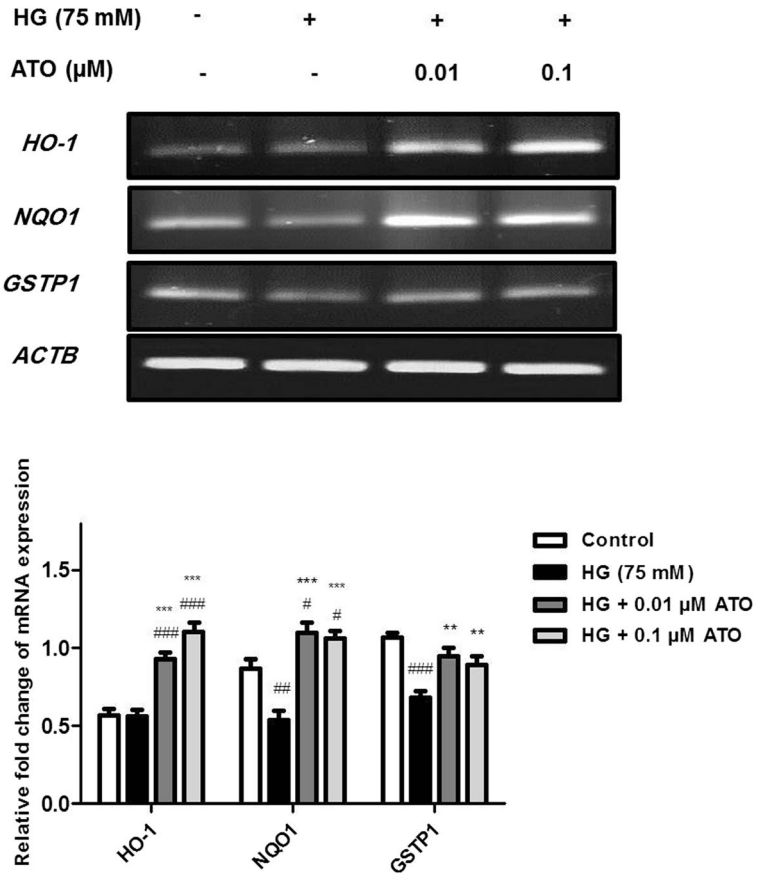

Fig. 4 The expression of *HO-1*, *NQO1*, and *GSTP1* genes was measured using RT-PCR analysis ($n = 3$). The gene expressions were normalized to optical *ACTB* density. The expression of *HO-1* gene was shown no significant change in the HG-treated group compared to the control group. The expression level of *NQO1* and *GSTP1* genes in HG-treated group was significantly less than control group ($^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, respectively). Pretreatment with ATO significantly elevated the expression of *HO-1* and *NQO1* genes compared to the control ($^{\#\#\#}p < 0.001$ and $^{\#}p < 0.05$, respectively) group. ATO significantly increased the expression level of *HO-1*, *NQO1*, and *GSTP1* in comparison with HG-treated group ($^{***}p < 0.001$, $^{*}p < 0.05$, and $^{**}p < 0.01$, respectively). Data show results from three experiments with the ratios given as a mean ± SD

Current results indicated that ATO decreased HG-induced elevated levels of ROS and MDA and enhanced the mRNA expression of antioxidant enzymes including *GPx-1*, *-3*, *-4*, *CAT*, *SOD1*, *GSTP1*, *NQO-1*, and *HO-1* in C28I2 cells. These results are in agreement with previous in vitro and in vivo studies indicating that ATO has strong antioxidant activity. Atorvastatin has been found to reduce the generation of MDA, ROS, and NO·in chronic construction injury-induced neuropathic rats. These effects are attributed to antioxidant property of ATO via enhancing the activity of CAT and GST as well as increasing the levels of reduced glutathione and total thiol [26]. Treatment with ATO can effectively exert protective effects against arsenic-induced hypertension in rats by elevating SOD, catalase, and GPx activity and down-regulating iNOS protein expression, peroxynitrite and ROS generation, and lipid peroxidation [30]. Experimental evidence indicates that ATO enhances the expression of HO-1 and NQO1 through activating the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway [22].

High glucose can activate various signaling pathways such as NF-κB, JNK, and P38 MAPKs [10]. The phosphorylated levels of JNK and p38 MAPKs increase in the articular chondrocytes of OA patients, which are involved in the cartilage destruction [12]. The destructive effects of JNK and p38 MAPKs on articular cartilage can be resulted from induction of apoptosis. Activated JNK and P38 MAPKs regulate apoptotic signaling through increasing the activity and the expression of pro-apoptotic Bcl-2 family proteins. Therefore, inhibition of these MAPKs pathways seems to be a potential therapeutic approach for inhibiting OA development [11].

Current results revealed that ATO significantly attenuated the HG-induced JNK and P38 MAPKs phosphorylation in C28I2 cells. This is in agreement with previous published report indicating that ATO is able to attenuate hyperglycemia-induced nephropathy by inhibiting p38 and/or JNK MAPKs pathways [36].

The main feature of OA is the chondrocyte death. Chondrocyte apoptosis is found to be an important reason for the loss of chondrocytes. In chondrocytes of osteoarthritic cartilage, the expression levels of apoptotic factors such as caspase-3 have been found to be elevated, while anti-apoptotic Bcl2 proteins are expressed at lower levels [35]. Given that HG induces apoptosis in various tissues and cells [2], apoptosis induced by high glucose seems to play an important role in the cartilage destruction in DM patients. Thereby, inhibition of apoptotic signaling pathways may be a potential therapeutic target to prevent cartilage destruction resulted from chondrocytes loss in diabetic patients. Current results indicated that pretreatment with ATO could significantly attenuate the ratio of Bax/Bcl-2 by blocking HG-induced elevation of Bax, as well as reducing the

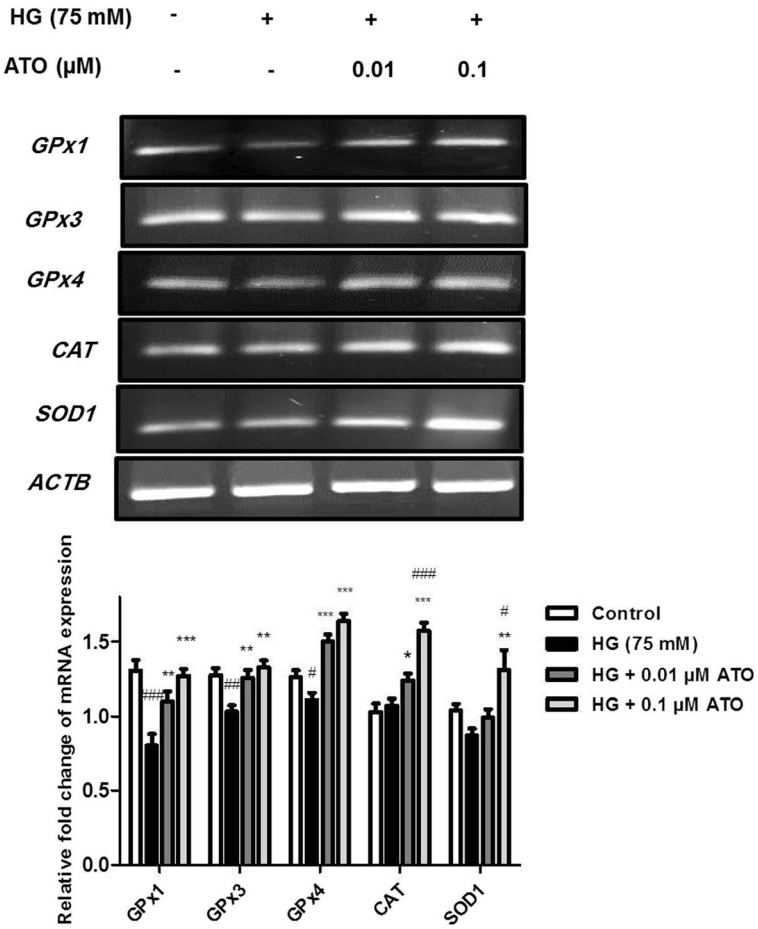

Fig. 5 The effect of ATO (0.01 and 0.1 μM) on the expression of *CAT*, *SOD1*, *GPx1*, *GPx3*, and *GPx4* genes was performed using RT-PCR analysis ($n = 3$). The gene expressions were normalized to optical *ACTB* density. The expression of *GPx1*, *GPx3*, and *GPx4* genes was significantly reduced in the HG-treated group compared to the control group ($^{###}p < 0.001$, $^{##}p < 0.01$, and $^{#}p < 0.05$, respectively). The expression of *CAT* and *SOD1* genes was shown no significant change in the HG-treated group in comparison with the control group. Pretreatment with ATO significantly increased the expression level of *GPx1* ($^{***}p < 0.001$ and $^{**}p < 0.01$), *GPx3* ($^{**}p < 0.01$), *GPx4* ($^{***}p < 0.001$), *CAT* ($^{***}p < 0.001$ and $^{*}p < 0.05$), and *SOD1* ($^{**}p < 0.01$) genes compared to the HG-treated group. Furthermore, ATO (0.1 μM) significantly elevated the expression of *CAT* and *SOD1* genes compared to the control group ($^{###}p < 0.001$ and $^{#}p < 0.05$, respectively). Data show results from three experiments with the ratios given as a mean ± SD

HG-induced elevation of caspase-3 activity in C28I2 cells. This is in line with previous study performed by Cheng et al., shown that ATO could alleviate subarachnoid hemorrhage-induced brain injury through inhibiting caspase cascades [6].

Although statins have been shown to induce apoptosis in cancer cells, these effects may be due to high concentrations of statins or/and variations in the nature of examined cells [21]. The chondroprotective effect of statins has been evaluated in clinical setting and in vitro and in vivo models of OA. Treatment of OA patients with ATO could improve the quality of life and reduce the inflammatory response, the pain syndrome during rest and movement as well as the progression of symptomatic knee OA; these effects of ATO may contribute to the reduction in the need for joint replacement operation [33, 34]. Results from animal studies have shown that ATO suppresses OA development and reduces osteoarthritic pain. Molecular studies have demonstrated that ATO could reduce the serum levels of total cholesterol, TNF-α, IL-6, and C-reactive protein. Furthermore, ATO decreases levels of lipid peroxidation, myeloperoxidase, and MMP2, while increases tissue inhibitor metalloproteinase2, GSH content, and SOD activity in joint tissue samples of animal models [7, 8, 18, 27]. In cultured osteoarthritic chondrocytes, ATO could reduce the expression levels of MMP13 and IL-1β as well as enhance the expression levels of collagen type II and aggrecan; these indicate that ATO may exert chondroprotective effects via decreasing cartilage degradation [32].

Conclusions

The present data revealed that pretreatment of C28I2 chondrocytes with atorvastatin (ATO) could inhibit high glucose (HG)-induced oxidative stress through reducing the elevated level of ROS and lipid peroxidation as well as increasing the gene expression levels of antioxidant enzymes. Furthermore, ATO suppressed HG-induced mitochondrial apoptosis via reducing the ratio of Bax/Bcl-2, the activity of caspase-3, and the phosphorylation of JNK and P38 MAPKs. These findings suggest that protective effects of ATO on HG-induced oxidative stress and subsequent apoptosis may be resulted from its antioxidant properties. Our results demonstrated that ATO may be employed as a promising therapeutic option to impede the progression of cartilage destruction in diabetic patients.

Author's contribution All authors are in agreement with the content of the manuscript: AM sharifi contributed to conception/design; A Hosseinzadeh involved in the collection of data and manuscript writing; K Bahrampour Juybari and T Kamarul analyzed the data and interpreted the results; all authors approved the final version of manuscript.

Funding information This work was supported by the Iran University of Medical Sciences (Grant number 29686).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Afonso V, Champy R, Mitrovic D, Collin P, Lomri A (2007) Reactive oxygen species and superoxide dismutases: role in joint diseases. Joint Bone Spine 74:324–329
2. Aminzadeh A, Dehpour A, Safa M, Mirzamohammadi S, Sharifi A (2014) Investigating the protective effect of lithium against high glucose-induced neurotoxicity in PC12 cells: involvements of ROS, JNK and P38 MAPKs, and apoptotic mitochondria pathway. Cell Mol Neurobiol 34:1143–1150
3. Atayde SA, Yoshinari NH, Nascimento DP, Catanozi S, Andrade PC, Velosa APP, Parra ER, Passarelli M, Nakandakare ER, Capelozzi VL (2012) Experimental diabetes modulates collagen remodelling of joints in rats. Histol Histopathol 27:1471
4. Buranasin P, Mizutani K, Iwasaki K, Mahasarakham CPN, Kido D, Takeda K, Izumi Y (2018) High glucose-induced oxidative stress impairs proliferation and migration of human gingival fibroblasts. PLoS One 13:e0201855
5. Chen M, Zheng H, Wei T, Wang D, Xia H, Zhao L, Ji J, Gao H (2016) High glucose-induced PC12 cell death by increasing glutamate production and decreasing methyl group metabolism. Biomed Res Int 2016
6. Cheng G, Wei L, Zhi-dan S, Shi-guang Z, Xiang-zhen L (2009) Atorvastatin ameliorates cerebral vasospasm and early brain injury after subarachnoid hemorrhage and inhibits caspase-dependent apoptosis pathway. BMC Neurosci 10:7
7. El-Seweidy MM, Sousou I, Elswefy SE, Mashhour MM (2016) Omega 3-fatty acids, atorvastatin as modulators for inflammatory pattern versus diclofenac in osteoarthritis induced in experimental rats. Afr J Pharm Pharmacol 10:472–479
8. Gierman L, Kühnast S, Koudijs A, Pieterman E, Kloppenburg M, van Osch G, Stojanovic-Susulic V, Huizinga T, Princen H, Zuurmond A-M (2014) Osteoarthritis development is induced by increased dietary cholesterol and can be inhibited by atorvastatin in APOE* 3Leiden. CETP mice—a translational model for atherosclerosis. Ann Rheum Dis 73:921–927
9. Healy ZR, Lee NH, Gao X, Goldring MB, Talalay P, Kensler TW, Konstantopoulos K (2005) Divergent responses of chondrocytes and endothelial cells to shear stress: cross-talk among COX-2, the phase 2 response, and apoptosis. Proc Natl Acad Sci U S A 102:14010–14015
10. Ho FM, Lin WW, Chen BC, Chao CM, Yang C-R, Lin LY, Lai CC, Liu SH, Liau CS (2006) High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF-κB and c-Jun NH 2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway. Cell Signal 18:391–399
11. Hosseinzadeh A, Kamrava SK, Joghataei MT, Darabi R, Shakeri-Zadeh A, Shahriari M, Reiter RJ, Ghaznavi H, Mehrzadi S (2016) Apoptosis signaling pathways in osteoarthritis and possible protective role of melatonin. J Pineal Res 61:411–425
12. Hosseinzadeh A, Jafari D, Kamarul T, Bagheri A, Sharifi AM (2017) Evaluating the protective effects and mechanisms of Diallyl disulfide on Interlukin-1β-induced oxidative stress and mitochondrial apoptotic signaling pathways in cultured chondrocytes. J Cell Biochem 118:1879–1888
13. Hosseinzadeh A, Juybari KB, Fatemi MJ, Kamarul T, Bagheri A, Tekiyehmaroof N, Sharifi AM (2017) Protective effect of ginger (Zingiber officinale roscoe) extract against oxidative stress and mitochondrial apoptosis induced by interleukin-1β in cultured chondrocytes. Cells Tissues Organs 204:241–250
14. Jaikumkao K, Pongchaidecha A, Chattipakorn N, Chatsudthipong V, Promsan S, Arjinajarn P, Lungkaphin A (2016) Atorvastatin improves renal organic anion transporter 3 and renal function in gentamicin-induced nephrotoxicity in rats. Exp Physiol 101:743–753
15. Juybari KB, Kamarul T, Najafi M, Jafari D, Sharifi AM (2018) Restoring the IL-1β/NF-κB-induced impaired chondrogenesis by diallyl disulfide in human adipose-derived mesenchymal stem cells via attenuation of reactive oxygen species and elevation of antioxidant enzymes. Cell Tissue Res 1–13
16. Kim HA, Yeo Y, Kim W-U, Kim S (2009) Phase 2 enzyme inducer sulphoraphane blocks matrix metalloproteinase production in articular chondrocytes. Rheumatology 48:932–938
17. King K, Rosenthal A (2015) The adverse effects of diabetes on osteoarthritis: update on clinical evidence and molecular mechanisms. Osteoarthr Cartil 23:841–850
18. Kumar V, Guruprasad B, Wahane V (2010) Atorvastatin exhibits anti-inflammatory and anti-oxidant properties in adjuvant-induced monoarthritis. Inflammopharmacology 18:303–308
19. Kwok S, Samuel SP, Handal J (2012) Atorvastatin activates heme oxygenase-1 at the stress response elements. J Cell Mol Med 16: 394–400
20. Laiguillon M-C, Courties A, Houard X, Auclair M, Sautet A, Capeau J, Fève B, Berenbaum F, Sellam J (2015) Characterization of diabetic osteoarthritic cartilage and role of high glucose environment on chondrocyte activation: toward pathophysiological delineation of diabetes mellitus-related osteoarthritis. Osteoarthr Cartil 23: 1513–1522
21. Liu H, Liang S-L, Kumar S, Weyman CM, Liu W, Zhou A (2009) Statins induce apoptosis in ovarian cancer cells through activation of JNK and enhancement of Bim expression. Cancer Chemother Pharmacol 63:997–1005
22. Liu MW, Liu R, Wu HY, Chen M, Dong MN, Huang YQ, Zhang CH, Wang YZ, Xia J, Shi Y (2017) Atorvastatin has a protective effect in a mouse model of bronchial asthma through regulating tissue transglutaminase and triggering receptor expressed on myeloid cells-1 expression. Exp Ther Med 14:917–930
23. Malinowski JM (1998) Atorvastatin: a hydroxymethylglutaryl-coenzyme a reductase inhibitor. Am J Health Syst Pharm 55: 2253–2267
24. Mehrzadi S, Kamrava SK, Dormanesh B, Motevalian M, Hosseinzadeh A, Hosseini Tabatabaei SMT, Ghaznavi H (2015) Melatonin synergistically enhances protective effect of atorvastatin against gentamicin-induced nephrotoxicity in rat kidney. Can J Physiol Pharmacol 94:265–271
25. Mendes AF, Rosa SC, Rufino AT, Ribeiro M, Judas F (2015) Diabetes-induced osteoarthritis: role of hyperglycemia in joint destruction. BMC Musculoskelet Disord 16:S1
26. Pathak NN, Balaganur V, Lingaraju MC, Kant V, Latief N, More AS, Kumar D, Kumar D, Tandan SK (2014) Atorvastatin attenuates neuropathic pain in rat neuropathy model by down-regulating

oxidative damage at peripheral, spinal and supraspinal levels. Neurochem Int 68:1–9
27. Pathak NN, Balaganur V, Lingaraju MC, Kant V, Kumar D, Kumar D, Sharma AK, Tandan SK (2015) Effect of atorvastatin, a HMG-CoA reductase inhibitor in monosodium iodoacetate-induced osteoarthritic pain: implication for osteoarthritis therapy. Pharmacol Rep 67:513–519
28. Rasheed Z, Haqqi TM (2012) Endoplasmic reticulum stress induces the expression of COX-2 through activation of eIF2α, p38-MAPK and NF-κB in advanced glycation end products stimulated human chondrocytes. Biochim Biophys Acta 1823:2179–2189
29. Rayegan S, Dehpour AR, Sharifi AM (2017) Studying neuroprotective effect of atorvastatin as a small molecule drug on high glucose-induced neurotoxicity in undifferentiated PC12 cells: role of NADPH oxidase. Metab Brain Dis 32:41–49
30. Sarath TS, Waghe P, Gupta P, Choudhury S, Kannan K, Pillai AH, Harikumar SK, Mishra SK, Sarkar SN (2014) Atorvastatin ameliorates arsenic-induced hypertension and enhancement of vascular redox signaling in rats. Toxicol Appl Pharmacol 280:443–454
31. Sattar NA, Ginsberg H, Ray K, Chapman MJ, Arca M, Averna M, Betteridge DJ, Bhatnagar D, Bilianou E, Carmena R (2014) The use of statins in people at risk of developing diabetes mellitus: evidence and guidance for clinical practice. Atheroscler Suppl 15:1–15
32. Simopoulou T, Malizos KN, Poultsides L, Tsezou A (2010) Protective effect of atorvastatin in cultured osteoarthritic chondrocytes. J Orthop Res 28:110–115
33. Vasilyeva L, Lipid-Lowering LD (2015) Pleiotropic effects of statins in patients with osteoarthritis and metabolic syndrome. Biol Med (Aligarh) 7:2
34. Wang Y, Tonkin A, Jones G, Hill C, Ding C, Wluka AE, Forbes A, Cicuttini FM (2015) Does statin use have a disease modifying effect in symptomatic knee osteoarthritis? Study protocol for a randomised controlled trial. Trials 16:584
35. Zhao H, Qiu G, Guan J, Zhao Y, Zhou X (2008) Correlation of apoptosis of articular chondrocytes in osteoarthritis with degree of cartilage destruction and expression of apoptosis-related proteins: surviving, caspase-3, and Bcl-xl. Zhonghua Yi Xue Za Zhi 88: 1339–1341
36. Zuo L, Du Y, Lu M, Gao J, Hu R, Zhang S, Wang Y, Zhu H, Zhou Q, Wei W (2014) Atorvastatin inhibits hyperglycemia-induced expression of osteopontin in the diabetic rat kidney via the p38 MAPK pathway. Mol Biol Rep 41:2551–2558