Lactoferrin Inhibits IL-1β-Induced Chondrocyte Apoptosis Through AKT1-Induced CREB1 Activation

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Key Words
Lactoferrin • Osteoarthritis • Chondrocyte • Apoptosis

Abstract
Background/Aims: Chondrocyte apoptosis is largely responsible for cartilage degeneration in osteoarthritis (OA). Interleukin-1 beta (IL-1β) is widely used as a chondrocyte apoptosis-inducing agent, while lactoferrin (LF) is an anabolic reagent which has the potential to inhibit chondrocyte apoptosis. We assessed the effects of LF on cartilage degeneration in IL-1β-induced chondrocytes and in a rat model of OA, and explored the potential molecular mechanisms involved. Methods: Human articular chondrocytes (HACs) were treated with IL-1β alone or in combination with LF. MTT and flow cytometric assays were used to detect changes after treatment with LF. Western blotting was used to examine the relevant molecules regulating apoptosis. Results: We found that IL-1β reduced the viability of HACs, whereas 200 μg/mL of LF significantly counteracted the inhibitory effect of IL-1β. LF significantly inhibited IL-1β-induced HAC apoptosis. The protein expression of the apoptotic markers Caspase-3 and PARP was also significantly reduced in the LF treatment group when analyzed by western blotting. Furthermore, we found that LF triggered CREB1 phosphorylation in IL-1β-induced HAC apoptosis through AKT1 signaling. In addition, LF promoted the repair of articular cartilage damage in a rat OA model with elevated p-CREB levels. Conclusions: These studies suggest that LF has an anti-apoptotic effect on IL-1β-induced chondrocytes, and thus may be a promising novel therapeutic agent for OA.

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Introduction

Osteoarthritis (OA) is a common age-associated disease characterized by breakdown of the cartilage matrix, chondrocyte hypertrophy and ectopic growth of bony structures in the joints [1, 2]. With our population ageing, the prevalence of OA in the developed world is expected to increase and it is anticipated that OA will become the fourth leading cause of disability in the coming decades [3]. To date, pharmacological treatments of OA are only focused on the alleviation of pain and inflammation by using nonsteroidal anti-inflammatory drugs (NSAIDs) or other agents [4]. Therefore, there is an urgent need for effective, widely available measures to aid OA patients.

Chondrocyte apoptosis has been shown to be involved in the pathogenesis of OA. The occurrence of OA cartilage damage is always accompanied by increased apoptosis of articular chondrocytes and its inhibition attenuates the severity of OA [5-7]. Thus chondrocyte apoptosis has gradually become one of the potential therapeutic targets for OA [8]. Proinflammatory cytokines secreted by chondrocytes, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-α), play an important role in the progression of OA [9]. Specifically, IL-1β can trigger large scale chondrocyte apoptosis and is widely used as a chondrocyte apoptosis-inducing agent [10]. Thus, chondrocytes treated with IL-1β provide a useful model of OA chondrocytes.

Lactoferrin (LF), an iron-binding glycoprotein of the transferrin family, is abundant in milk and most biological fluids. Previous reports have shown that LF is a multifunctional factor with significant antimicrobial, anti-oxidative, antitumor, and immunomodulatory activities [11]. Recently, it has been found that LF acts as an anabolic reagent mainly through its powerful proliferative and anti-apoptotic actions [12-15]. In human articular cartilage, Yan et al. revealed that LF attenuated the effects of IL-1β on the expression of cartilage-degrading enzymes, destructive cytokines, and inflammatory mediators. LF specifically activated ERK MAPK and Akt signaling pathways, which may account for its anti-inflammatory activity [16, 17]. However, the effect of LF on chondrocyte apoptosis in OA and the molecular mechanism of action of LF remain unclear.

The anti-apoptotic properties of LF have made it an attractive intervention in attenuating IL-1β-induced chondrocyte apoptosis. In view of this, the present study aimed to investigate the potential protective effect of LF on IL-1β-stimulated human articular chondrocytes (HACs), as well as articular cartilage in a rat OA model, and to clarify the underlying mechanisms.

Materials and Methods

Reagents

Recombinant human lactoferrin (LF) was purchased from Sigma–Aldrich (St Louis, MO, USA). Recombinant rat IL-1β was obtained from PeproTech (Rocky Hill, NJ, USA). IL-1β (1 mg/mL) was dissolved in double distilled water and rhLF powder (1 mg/mL) was dissolved in phosphate-buffered saline (PBS). Antibodies against AKT, p-AKT (Ser473), CREB, p-CREB (Ser133), Caspase-3, cleaved-Caspase-3, PARP, cleaved-PARP, Col II, GAPDH, and AKT inhibitor (triciribine) were from Cell Signaling (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Human articular chondrocyte culture

Following full ethical approval from the local research ethics committee (LREC), eight OA cartilage specimens were collected from patients (aged 61 ± 4 years) undergoing total knee joint replacement surgery at Yangpu District Central Hospital (Shanghai, China). Written informed consent was obtained from each patient before the operation. HACs were isolated from undamaged femoral condyle and intercondylar fossa of the knee joint as described previously [18]. Human OA articular cartilage was maintained in DMEM/F12 with 10% FBS. When the HACs reached 80% confluence, they were passaged and chondrocytes between passages 1 and 3 were used for subsequent experiments.
Optimizing LF dose
After reaching 80% confluence, the HACs were treated with DMEM/F12 with 1% FBS containing IL-1β (10 ng/mL) for 2 h. Then, cells were treated with LF at 0, 25, 50, 100, and 200 μg/mL for a further 24 h before use for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The optical absorbance of the resulting colored solution was quantified at 570 nm using a microplate reader (BMG Labtech, Aylesbury, UK). The optimal concentration of LF (200 μg/mL) was chosen for subsequent experiments.

Inhibition of AKT phosphorylation
HACs were treated with the AKT inhibitor triciribine at 30 μM for 1 h prior to treatment with IL-1β (10 ng/mL) for 2 h, and then treated with LF for 24 h. Western blot and flow cytometry assays were conducted 24 h after LF treatment.

RNA interference
siRNAs specific to CREB1 and AKT1 were purchased from Santa Cruz Biotechnology and transfected into HACs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. A scramble sequence was used as the control siRNA. After transfection, the cells were maintained in DMEM/F12 with 10% FBS until they reached 80% confluence, at which point they were used for subsequent experiments.

Flow cytometric analysis
The apoptotic rate was evaluated using an Annexin V-FITC apoptosis detection kit (eBioscience, San Diego, CA, USA) according to the manufacturer’s instructions provided. Briefly, after exposing them to different treatments, cells were washed with PBS and harvested using trypsin-EDTA solution (Invitrogen). Then, the cells were incubated in buffer containing FITC-annexin V and PI for 5 min at room temperature in the dark. Apoptotic cells were analyzed on a BD flow cytometer (Becton Dickinson, Oxford, UK).

Western blotting
After exposing the cells to different treatments, they were collected and lysed in lysis buffer. The protein concentrations were measured using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). An aliquot of 20 μg of protein from each sample was subjected to 10% SDS-PAGE and immunoblotted as previously described [19].

Rat knee OA models
Eighteen ten-week-old male Sprague–Dawley rats were randomly divided into three groups (n = 6) comprising a sham-operated negative control group, an OA-induction group and an OA+LF (200 μg/mL) group. The OA model was created by anterior cruciate ligament transection with partial medial meniscectomy (ACLT+MMx) as previously described [20]. In the sham-operated negative controls, the right knee joint was exposed and incisions were closed following subluxation of the patella and washing of the joint surface with saline. From the 4th week after surgery, LF at 100 μg/rat was injected into the knee joints twice/week for 4 consecutive weeks, and rats were sacrificed 8 weeks after surgery. The knee joint of each rat was examined using hematoxylin and eosin staining as previously described [21]. This study was approved by the Scientific Investigation Board of Second Military Medical University (Shanghai, China).

Statistical analysis
SPSS 11.5 software was used for data analysis. Data are expressed as mean values ± SD from three separate experiments. Student’s t-test and one-way analysis of variance (ANOVA) were used to compare mean values from different samples. A value of P < 0.05 was considered significant.

Results
LF inhibits IL-1β-induced HAC apoptosis
We previously showed that LF is able to promote proliferation of HACs and reverse Dex-induced chondrocyte apoptosis [15]. Whether LF can prevent IL-1β-induced chondrocyte
apoptosis is not known. We first examined whether LF could increase the viability of human chondrocytes treated with IL-1β. As shown in Fig. 1, MTT assay revealed that IL-1β reduced the viability of HACs, while LF significantly ameliorated the deleterious effect of IL-1β in a dose-dependent manner. The viability of cells treated with 200 μg/mL of LF was increased to 93% in comparison to 71% in cells treated with IL-1β alone. Consequently, we selected a dose of 200 μg/mL LF for subsequent experiments, and evaluated the effect of LF (200 μg/mL) on IL-1β-stimulated HAC apoptosis. As expected, the IL-1β only group showed a
marked increase in the number of apoptotic HACs, whereas this effect was partially rescued by LF treatment (Fig. 2A). The influence of LF on apoptotic markers was further evaluated by western blotting analysis. As shown in Fig. 2B, LF treatment significantly reduced the levels of cleaved Caspase-3 and PARP, compared with those in the IL-1β group. These results demonstrated that LF inhibited IL-1β-induced apoptosis of HACs.

**LF triggers CREB phosphorylation in IL-1β-induced apoptosis of HACs**

Given that activation of CREB has been reported to counteract the pro-apoptotic effects of IL-1β [22], we investigated whether LF activated CREB in IL-1β-stimulated HACs. HACs were pretreated with IL-1β and then treated with LF at different time points. CREB protein was isolated and analyzed by western blotting. As shown in Fig. 3A, the level of p-CREB in IL-1β-stimulated HACs significantly increased 24 h after LF treatment, whereas total expression of CREB did not change. These results suggest that LF is capable of inducing the activation of CREB in IL-1β-stimulated chondrocytes.

To further confirm the involvement of CREB in LF-mediated inhibition of apoptosis, CREB1 siRNA was transfected into HACs. As shown in Fig. 3B, CREB1 siRNA, but not control siRNA, reduced the expression of CREB protein in HACs. Knockdown of CREB increased the percentage of apoptotic HACs and the amount of cleaved Caspase-3 and PARP in HACs treated with IL-1β and LF (Fig. 3C and D), suggesting that knockdown of CREB abolished the protective effects of LF on IL-1β-induced chondrocyte apoptosis. Therefore, these results indicated that CREB plays an important role in LF-mediated protective action.

**LF regulates CREB activity through AKT signaling**

Considering that AKT signaling is one crucial upstream signaling pathway affecting CREB expression [23, 24], we investigated whether LF required AKT signaling for the
activation of CREB. HACs were pretreated with the AKT inhibitor triciribine (30 μM) for 1 h or transfected with AKT1 siRNA for 48 h prior to treatment with IL-1β and LF. (A) The levels of p-AKT and p-CREB in the indicated groups were determined by western blotting. The histogram shows the relative expression levels of p-AKT and p-CREB analyzed by densitometry. (B) MTT assay in HACs transfected with AKT1 siRNA, CREB1 siRNA or negative control. (C) The percentage of apoptotic cells in the indicated groups was evaluated by flow cytometry. (D) The protein expression of cleaved caspase-3 and PARP in the indicated groups was analyzed by western blotting. The results represent the mean ± SD of three independent experiments. #P < 0.05 compared to the IL-1β and LF treatment group, *P < 0.05 compared to the control siRNA group.
triciribine or AKT1 siRNA treatment. The results showed that inhibition of AKT abolished the protective action of LF against IL-1β-induced cell apoptosis (Fig. 4C and D). Taken together, these data demonstrate that LF regulates CREB activity through the AKT signaling pathway in IL-1β-induced chondrocyte apoptosis.

**Discussion**

Chondrocyte apoptosis is considered a key pathological feature of OA, and the development of novel anti-apoptotic agents is important to prevent OA progression. Previous investigations have shown that LF has a powerful anti-apoptotic effect in many cells [12-14]. However, the effect of LF on chondrocyte apoptosis is still unclear. In this study, we report that LF exerts significant anti-apoptotic effects on IL-1β-stimulated HACs.
IL-1β is a proinflammatory cytokine which plays a critical role in the pathogenesis of OA [25]. In patients with OA, levels of IL-1β are elevated in the synovial fluid, synovial membrane, subchondral bone and cartilage [26]. In cartilage, IL-1β inhibits the synthesis of major extracellular matrix (ECM) components such as type II collagen and aggrecan by inhibiting the anabolic activities of chondrocytes [27, 28]. IL-1β also stimulates the production of a number of other inflammatory mediators implicated in OA pathology [26]. Recently, IL-1β has been widely used as a chondrocyte apoptosis-inducing agent. When exposed to IL-1β (10 ng/mL) for 2 h, chondrocyte apoptosis is significantly increased compared to the control group [9, 29]. Previously, we showed that LF inhibited Dex-induced OA apoptosis of chondrocytes at a concentration of 200 μg/mL. In the current study, we investigated whether LF can exert an anti-apoptotic effect in IL-1β-stimulated HACs, which are important in the occurrence and development of OA. Our results show that IL-1β-induced apoptosis of chondrocytes was also significantly ameliorated by treatment with 200 μg/mL LF.

Cyclic AMP-responsive element-binding protein (CREB), a leucine zipper-type transcription factor, is crucial for almost all known cellular processes such as growth, differentiation and apoptosis [30, 31]. It has been demonstrated that levels of Ser-133-phosphorylated-CREB are significantly elevated during cartilage formation [32]. CREB and its paralogs are phosphorylated and hence activated in a subpopulation of chondrocytes within the proliferative zone. Correspondingly, a CREB inhibitor reduced the height of the proliferative zone in developing limbs and lowered the proportion of BrdU-positive cells in the growth plate [33]. In addition, ATF-2 and CREB act as transcriptional co-activators and control Bcl-2 protein levels in growth plate chondrocytes. Bcl-2, a cell death inhibitor that regulates apoptosis, is expressed within the growth plate in proliferative and pre-hypertrophic chondrocytes [34]. In our study, western blotting was performed to investigate the possible involvement of CREB in chondrocyte apoptosis. Interestingly, the level of CREB phosphorylation was significantly increased in IL-1β and LF-treated HACs in a time-dependent manner. Furthermore, the anti-apoptotic actions of LF in IL-1β-stimulated HACs were prevented by knockdown of CREB1. These data indicate that LF inhibits chondrocyte apoptosis through activation of CREB.

AKT signaling performs a critical role in inhibiting chondrocyte apoptosis [35]. The ginsenoside Rg1 may protect chondrocytes from IL-1β-induced apoptosis via the PI3K/AKT signaling pathway [36]. Berberine ameliorates cartilage degeneration in IL-1β-stimulated rat chondrocytes and in a rat model of OA via AKT signaling [29]. It has been reported that AKT signaling is one crucial upstream signaling pathway for CREB expression [37, 38]. In this study, we investigated whether LF required the AKT signaling pathway for the activation of CREB. Our results show that triciribine or AKT1 siRNA treatment efficiently blocked the LF-induced increase in p-CREB and inhibited the LF-mediated protective effect on IL-1β-induced chondrocyte apoptosis. Importantly, LF promotes the repair of articular cartilage damage in a rat OA model, an effect accompanied by elevated levels of p-CREB and p-AKT expression. Further studies are needed to validate the role of AKT and CREB on apoptotic markers in vivo using knockdown experiments in the osteoarthritis knee model.

In conclusion, our study demonstrates that LF effectively suppresses chondrocyte apoptosis as well as articular cartilage damage at least partly through AKT-induced CREB activation. Therefore, LF may provide a new approach for OA therapy.

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

References

1. Felson DT, Neogi T: Osteoarthritis: is it a disease of cartilage or of bone? Arthritis Rheum 2004;50:341-344.
2. Rogers J, Shepstone L, Dieppe P: Is osteoarthritis a systemic disorder of bone? Arthritis Rheum 2004;50:452-457.
3. Urquhart DM, Soufan C, Teichtahl AJ, Wluka AE, Hanna F, Cicuttini FM: Factors that may mediate the relationship between physical activity and the risk for developing knee osteoarthritis. Arthritis Res Ther 2008;10:203.
4. Chen YJ, Tsai KS, Chan DC, Lan KC, Chen CE, Yang RS, Liu SH: Honokiol, a low molecular weight natural product, prevents inflammatory response and cartilage matrix degradation in human osteoarthritis chondrocytes. J Orthop Res 2014;32:573-580.
5. Heraud F, Héraud A, Harmand MF: Apoptosis in normal and osteoarthritic human articular cartilage. Ann Rheum Dis 2000;59:959-965.
6. Ryu JH, Shin Y, Huh YH, Yang S, Chun CH, Chun JS: Hypoxia-inducible factor-2alpha regulates fas-mediated chondrocyte apoptosis during osteoarthritic cartilage destruction. Cell Death Differ 2012;19:440-450.
7. Zhang X, Zhu Y, Chen X, Zhang Y, Zhang Y, Jia Y, Wang H, Liu Y, Xiao L: Baicalein ameliorates inflammatory-related apoptotic and catabolic phenotypes in human chondrocytes. Int Immunopharmacol 2014;21:301-308.
8. Thomas CM, Fuller CJ, Whittles CE, Sharif M: Chondrocyte death by apoptosis is associated with the initiation and severity of articular cartilage degradation. Int J Rheum Dis 2011;14:191-198.
9. Zheng X, Xia C, Chen Z, Huang J, Gao F, Li G, Zhang B: Requirement of the phosphatidylinositol 3-kinase/akt signaling pathway for the effect of nicotine on interleukin-1beta-induced chondrocyte apoptosis in a rat model of osteoarthritis. Biochem Biophys Res Commun 2012;423:606-612.
10. Sanchez C, Deberg MA, Piccardi N, Msika P, Regnier JF, Henrotin YE: Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin m pre-treated non-sclerotic osteoblasts. Osteoarthritis Cartilage 2005;13:979-987.
11. Caccavo D, Pellegrino NM, Altamura M, Rigon A, Amati L, Amoroso A, Jirillo E: Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. J Endotoxin Res 2002;8:403-417.
12. Francis N, Wong SH, Hampson P, Wang K, Young SP, Deigner HP, Salmon M, Scheel-Toellner D, Lord JM: Lactoferrin inhibits neutrophil apoptosis via blockade of proximal apoptotic signaling events. Biochim Biophys Acta 2011;1813:1822-1826.
13. Hou JM, Chen EY, Wei SC, Lin F, Lin QM, Lan XH, Xue Y, Wu M: Lactoferrin inhibits apoptosis through insulin-like growth factor i in primary rat osteoblasts. Acta Pharmacol Sin 2014;35:523-530.
14. Cornish J, Callon KE, Naot D, Palmano KP, Banovic T, Bava U, Watson M, Lin JM, Tong PC, Chen Q, Chan VA, Reid HE, Fazzari N, Baker HM, Baker EN, Haggarty NW, Grey AB, Reid IR: Lactoferrin is a potent regulator of bone cell activity and increases bone formation in vivo. Endocrinology 2004;145:4366-4374.
15. Tu Y, Xue H, Francis W, Davies AP, Pallister I, Kanamarlapudi V, Xia Z: Lactoferrin inhibits dexamethasone-induced chondrocyte impairment from osteoarthritic cartilage through up-regulation of extracellular signal-regulated kinase 1/2 and suppression of fas, fasl, and caspase 3. Biochem Biophys Res Commun 2013;441:249-255.
16. Yan D, Chen D, Shen J, Xiao G, van Wijnen AJ, Im HJ: Bovine lactoferricin is anti-inflammatory and anti-catabolic in human articular cartilage and synovium. J Cell Physiol 2013;228:447-456.
17. Yan D, Chen D, Hawse JR, van Wijnen AJ, Im HJ: Bovine lactoferricin induces timp-3 via the erk1/2-sp1 axis in human articular chondrocytes. Gene 2013;517:12-18.
18. Xia Z, Duan X, Murray D, Triffitt JT, Price AJ: A method of isolating viable chondrocytes with proliferative capacity from cryopreserved human articular cartilage. Cell Tissue Bank 2013;14:267-276.
19. Liang W, Ren K, Liu F, Cui W, Wang Q, Chen Z, Fan W: Periodic mechanical stress stimulates the FAK
mitogenic signal in rat chondrocytes through ERK1/2 activity. Cell Physiol Biochem. 2013;32:915-930.

20 Hayami T, FunakI H, Yaoeda K, Mitui K, Yamagita H, Tokunaga K, Hatano H, Kondo J, Hiraki Y, Yamamoto T, Duong LT, Endo N: Expression of the cartilage derived anti-angiogenic factor chondromodulin-i decreases in the early stage of experimental osteoarthritis. J Rheumatol 2003;30:2207-2217.

21 Hu N, Wang C, Liang X, Yin L, Luo X, Liu B, Zhang H, Shui W, Nan G, Wang N, Wu N, Chen X, He Y, Wen S, Deng F, Zhang H, Liao Z, Luu HH, Haydon RC, He TC, Huang W: Inhibition of histone deacetylases potentiates BMP9-induced osteogenic signaling in mouse mesenchymal stem cells. Cell Physiol Biochem. 2013;32:486-498.

22 Corbett GT, Roy A, Pahan K: Gemfibrozil, a lipid-lowering drug, upregulates il-1 receptor antagonist in mouse cortical neurons: implications for neuronal self-defense. J Immunol 2012;189:1002-1013.

23 Johannessen M, Delghandi MP, Moens U: What turns creb on? Cell Signal 2004;16:1211-1227.

24 Liang MH, Wendland JR, Chuang DM: Lithium inhibits smad3/4 transactivation via increased creb activity induced by enhanced pka and akt signaling. Mol Cell Neurosci 2008;37:440-453.

25 Goldring MB: Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep 2000;2:459-465.

26 Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H: Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat Rev Rheumatol 2011;7:33-42.

27 Diederichs S, Zachert K, Raiss P, Richter W: Regulating chondrogenesis of human mesenchymal stromal cells with a retinoic Acid receptor-Beta inhibitor: differential sensitivity of chondral versus osteochondral development. Cell Physiol Biochem. 2014;33:1607-1619.

28 Stowe J, Huch K, Gunther KP, Scharf HP: Interleukin-1beta induces different gene expression of stromelysin, aggrecan and tumor necrosis-factor-stimulated gene 6 in human osteoarthritic chondrocytes in vitro. Pathobiology 2000;68:144-149.

29 Zhao H, Zhang T, Xia C, Shi L, Wang S, Zheng X, Hu T, Zhang B: Berberine ameliorates cartilage degeneration in interleukin-1beta-stimulated rat chondrocytes and in a rat model of osteoarthritis via akt signalling. J Cell Mol Med 2014;18:283-292.

30 Giordano A, Avantaggiati ML: P300 and cbp: partners for life and death. J Cell Physiol 1999;181:218-230.

31 Ionescu AM, Schwarz EM, Vinson C, Puzas JE, Rosier R, Reynolds PR, O'Keefe RJ: Pthrp modulates chondrocyte differentiation through ap-1 and creb signaling. J Biol Chem 2001;276:11639-11647.

32 Zakany R, Szucs K, Bako E, Felszeghy S, Czifra G, Biro T, Modis L, Gergely P: Protein phosphatase 2a is involved in the regulation of protein kinase a signaling pathway during in vitro chondrogenesis. Exp Cell Res 2002;275:1-8.

33 Long F, Schipani E, Asahara H, Kronenberg H, Montminy M: The creb family of activators is required for endochondral bone development. Development 2001;128:541-550.

34 Ma Q, Li X, Vale-Cruz D, Brown ML, Beier F, LuValle P: Activating transcription factor 2 controls bcl-2 promoter activity in growth plate chondrocytes. J Cell Biochem 2007;101:477-487.

35 Wang L, Gai P, Xu R, Zheng Y, Lv S, Li Y, Liu S: Shikonin protects chondrocytes from interleukin-1beta-induced apoptosis by regulating pi3k/akt signaling pathway. Int J Clin Exp Pathol 2015;8:298-308.

36 Huang Y, Wu D, Fan W: Protection of ginsenoside rgl1 on chondrocyte from il-1beta-induced mitochondria-activated apoptosis through pi3k/akt signaling. Mol Cell Biochem 2014;392:249-257.

37 Socodato R, Brito R, Portugal CC, de Oliveira NA, Calaza KC, Paes-de-Carvalho R: The nitric oxide-cgkii system relays death and survival signals during embryonic retinal development via akt-induced creb1 activation. Cell Death Differ 2014;21:915-928.

38 Simao F, Matte A, Pagnussat AS, Netto CA, Salbego CG: Resveratrol prevents ca1 neurons against ischemic injury by parallel modulation of both gsk-3beta and creb through pi3-k/akt pathways. Eur J Neurosci 2012;36:2899-2905.