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Original Article

Characterization of cytokines expression and relative signaling molecules after condylar fracture

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Abstract: Aim: To characterize the expression profiles of cytokines, including FGF1 (fibroblast growth factor 1), IGF-1 (insulin-like growth factor 1), IL-1 (interleukin 1), IL-6 (interleukin 6), TGF-beta (transforming growth factor 1 beta), BMP (Bone morphogenetic proteins) as well as PI3K (phosphoinositide 3- kinase) and AKT (protein kinase B) after condylar fractures. Methods: 15 healthy goats aged 6-8 months, weighed 10-12 kg were used for establishing the condylar facture model through hitting the temporomandibular joint region in one side. Condylar facture was validated by immediate CT scanning. Cartilage tissues in temporomandibular joint region from both fractured and un-hit control side were dissected for measuring the cytokine expression by immunohistochemical staining, western plot and RT-PCR. Results: The expression of BMP was 101.51 ± 2.156 in the experimental side and 101.23 ± 1.895 in the control side without significant difference (P = 2.64). Meanwhile, there was also no significant difference in the expression of FGF1 (P = 3.36) between the control side (91.03 ± 2.365) and the experimental side (91.19 ± 3.128). However, compared with the control side (97.26 ± 1.017), the expression of IGF1 in the experimental side (146.04 ± 1.235) was significantly higher (P = 0.001). In addition, the expression of IL-1beta (146.04 ± 1.235 vs 98.77 ± 1.826, P = 0.001) and IL-6 (130.08 ± 1.032 vs 83.05 ± 1.223, P = 0.001) was also significantly higher in experimental side than those in control side. Interestingly, no significant difference of TGF-beta expression was observed between experimental and control side (P = 4.43). However, the mRNA and protein expression levels of PI3K and AKT were significantly higher than those in healthy controls (P = 0.001). Conclusion: Enhanced expressions of IGF-1, IL1-beta, IL6, PI3K and AKT were observed in condylar fracture progression, suggesting targeting them might be a novel approach to promote the repair after condylar.

Keywords: Condylar fractures, cytokine, IL-1beta, IL-6, IGF-1, PI3K/AKT, IGF-1 signaling

Introduction

Condylar fracture is a common type of bone fracture in mandible, with incidence of one-third among total mandible fracture [1-4]. Thus it's of great importance to explore the biological events during recovering of condylar fracture, which might provide guidance on possible interventions to improve patients' outcome.

Bone and immune system are tightly related and inflammatory disorders are associated with bone loss [5-8]. Tissue damage and blood vessel rupture resulting from fracture can promote immune response [9, 10]. During fracture repair process, inflammatory responses are also initiated and contribute to the repair process with involvement of multiple cellular events within multiple cell types [11, 12]. Balanced inflammation at the fracture site can restrict tissue damage and initiate tissue repair through producing pro-angiogenic mediators and attracting mesenchymal progenitors cells, and is crucial for fracture healing [12, 13]. In contrast, fracture healing is disturbed when the inflammatory response is increased or prolonged [14]. Thus inflammatory responses are important for the recovery after bone fracture.

In the current study, we aimed to explore the possible involvement of immune response in condylar fracture goat model through measuring the expression profiles of cytokines, which play important roles in the regulation of immune response, including IL-1beta [15], IL-6 [16], IGF-1 [17], TGF-beta [18], and the related signaling molecules. Our study will provide clues of the involvement of cytokines in condylar fracture, which may provide theoretic basis for developing new treatment approach to promote

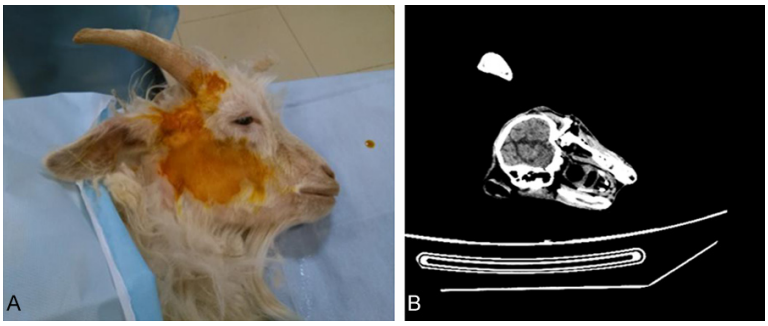

Figure 1. Model preparation. Condylar facture model in goat was established through hitting the temporomandibular joint region in one side followed by validation by CT scanning. (A) Photo of animal in operation desk. (B) CT image of a same animal as in (A).

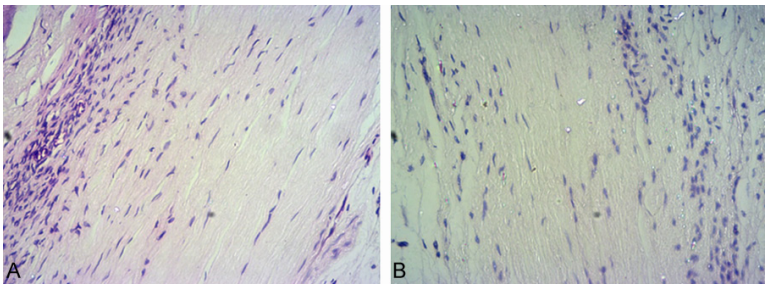

Figure 2. HE staining. 1 month after fracture, the joint cartilage and crystals were collected and fixed with 10% formalin overnight for paraffin embedded section followed by HE staining analysis. HE staining of control (A) and fractured sample (B).

recovery from condylar fracture through targeting immune response.

Methods

Sample preparation

15 healthy goats aged 6-8 months and weighed 10-12 kg were purchased and housed in the Animal Experiment Centre of the First Affiliated Hospital of Xinjiang Medical University. To produce condylar fractures, temporomandibular joint region of one side was hit to produce fracture which was further validated by immediate CT scanning (**Figure 1**). Cartilage tissues were resected from both the fractured and un-hit control under general anesthesia induced with Ketamine via intramuscular injection. The operation followed protocols of the Yang Chi's Experimental Approach from the Ninth Affiliated Hospital of Shanghai Jiaotong University in which the joint zone of the fracture was exposed by a side incision during which the cartilage of the condylar surface was exposed by separating. Same approach was applied for resecting for control preparation. All procedures were approved by the Ethic committee.

HE staining

1 month after fracture, the joint cartilage and crystals were collected and fixed with 10% formalin overnight for paraffin embedded section. Section was then deparaffined in Xylene for 2 X10 min. After infiltration in absolute alcohol for 5 min, 90% ethanol for 2 min and 70% ethanol for 2 min, sections were placed in distilled water for 2 min, stained with eosin for 2 min and then washed in tap water to remove remaining staining solution. Sections were then stained with Hematoxylin for 10 min and washed with tap water to remove remaining staining solution. After that, sections were further immersed in gradient ethanol of 70%, 90% and 100% for 2 min, 2 min and 5 min respectively.

Immunohistochemical staining

Deparaffined sections were burned with mid and high fire for 3 min respectively for antigen repair. After blocked in blocking solution for 2 hours at 37°C, sections were incubated with primary antibodies against BMP, FGF1 IGF1, IL-1beta, IL-6 or TGF-beta (Santa Cruz Biotechnology, Dallas, Texas, USA) overnight at 4°C. On the following day, sections were washed with PBS for 3X 5 min and then incubated with HRP-conjugated second antibody (1:1000) (Santa Cruz Biotechnology) for 2 hours at 37°C. DAB was applied for antigen visualization after incubation for 30 min-1 hour and remaining DAB solution (Thermo Fisher Scientific, Waltham, MA, USA) was washed out with tap water when signals were visible under a microscope. Sections were further re-stained with Hematoxylin, dehydrated for transparency and examined with neutral resin sheet.

Western blot test

Protein level of PI3K and AKT was detected by Western blot followed standard protocol. In

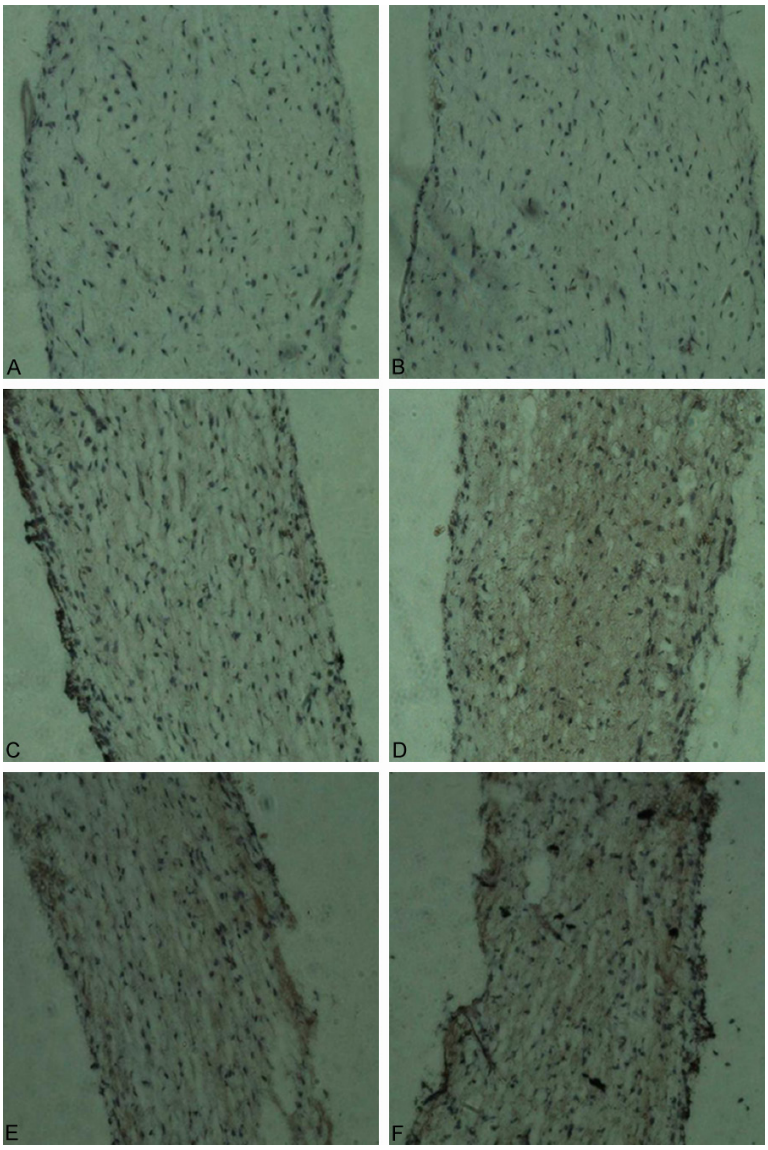

Figure 3. Expression of BMP (A & B), FGF1 (C & D) and TGF-beta (E & F) in control (left panels) and fractured samples (right panels) (x 40). Deparaffined sections were burned for antigen repair and blocked followed by incubation with primary antibodies and subsequent secondary antibody for immunohistochemical staining.

brief, total protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific) from tissues and quantified by BCA. 40 μg protein was separated by 10% SDS-PAGE and transferred to PVDF membrane (Gibco, Grand Island, NY, USA). After that, the membrane was blocked and incubated with primary antibodies (all from Santa Cruz Biotechnology) at 4°C overnight (PI3K, AKT and β-actin at 1:500, 1:500 and 1:800, respectively). Then the membrane was incubated with HRP-conjugated secondary antibody (1:5000) (Santa Cruz Biotechnology) for 60 min after washed by PBST for three times. At last, the protein expression was detected by ECL chemiluminiscence (Thermo Fisher Scientific).

RT-PCR experiment

The reverse transcription reagent kit and the RT-PCR reagent kit were purchased from TaKaRa and all procedures were performed according to the manufacture's manual. Primers for RT-PCR were as follows: AKT forward: 5'-GGCCCAGATGATCACCATCAC-3' & AKT reversed: 5'-CTATCGTCCAGCGCAGTCCA-3'; PI3K forward: 5'-AGCATTGGGACCTCACATTACACA-3' & PI3K reversed: 5'-ACTGGAAACACAGTCCATGCACATA-3'.

Statistical analysis

Statistical analysis was performed using SPSS software (version 20.0). All data was presented as mean ± standard deviation (SD) and analyzed by ANOVA. Independent t-test was used to compare the significance among the two groups. $P < 0.05$ indicates a statistical significance.

Result

Disrupted cellular arrangement after fracturing under HE staining

HE staining showed that cartilage and cartilage cell displayed light pink and blue, respectively. In control samples, cartilage cells lined orderly into upper, middle, columnar and cartilage layers (**Figure 2A**), whereas, in fractured samples, the arrangement of cell appeared to be disrupted (**Figure 2B**).

Expression of cytokines

The expression of BMP in experimental side was 101.51 ± 2.156, without significant differ-

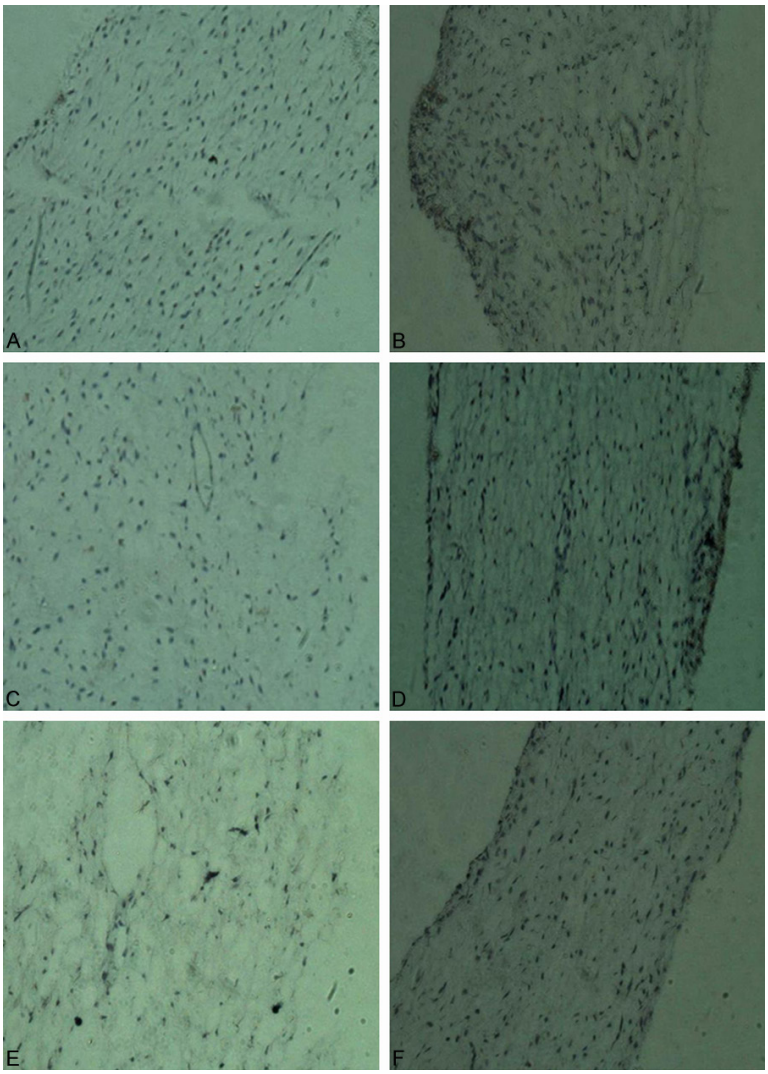

Figure 4. Expression of IGF1 (A & B), IL1-beta (C & D) and IL6 (E & F) in control (left panels) and fractured samples (right panels) (x 40). Deparaffined sections were burned for antigen repair and blocked followed by incubation with primary antibodies and subsequent secondary antibody for immunohistochemical staining.

ence to that in the control side (101.23 ± 1.895) (P > 0.05) (**Figure 3**). Meanwhile, no significant difference of the expression of FGF1 (91.03 ± 2.365 for control vs 91.19 ± 3.128 for experimental) was found between the two groups (P > 0.05) (**Figure 3**). However, a significantly higher expression of IGF1 (146.04 ± 1.235 vs 97.26 ± 1.017), IL-1beta (146.04 ± 1.235 vs 98.77 ± 1.826) and IL-6 (130.08 ± 1.032 vs 83.05 ± 1.223) was observed in experimental side than those in control side (P < 0.05) (**Figure 4**). However, no significant difference of TGF-beta expression was found between the experimental and control group (P > 0.05) (**Figure 3**).

The protein and mRNA expression of PI3K and AKT

To uncover the possible mechanisms underlying the elevated expression of cytokines, we measured the expression of PI3K and AKT to assess the status of PI3K/AKT signaling. We found that the mRNA (**Figure 5A** and **5C**) and protein (**Figure 5B** and **5D**) level of PI3K and AKT was increased significantly in fractured compared with control samples (P < 0.05), suggesting elevated cytokines expression may result from activated PI3K/AKT signaling.

Discussion

In this study, we successfully established a condylar fracture model in goat (**Figures 1** and **2**). To investigate the possible contribution of inflammatory response in disease progression, we compared the expression of classic cytokines in fractured samples with those in control samples. We found among BMP, FGF1, TGF-beta, IL-1beta, IL-6 and IGF-1, the expression of IL-beta, IL-6 and IGF-1 was increased significantly in fractured samples (**Figures 3** and **4**), indicating possible involvement of inflammatory responses in disease progression. Moreover, we found that the mRNA and protein level of PI3K and AKT was increased significantly in fractured samples, suggesting activated PI3K/AKT signaling may contribute to the increased cytokines' expression. Thus we revealed possible involvement of cytokines in condylar fracture in which elevated PI3K and AKT may play certain roles.

IL-1 plays multiple and diverse roles in inflammatory responses that can regulate the synthesis of IL-2, IL-6 and IL-8, which can increase IL-6 expression, thus forming a positive feedback to enhance the immune reaction and inflamma-

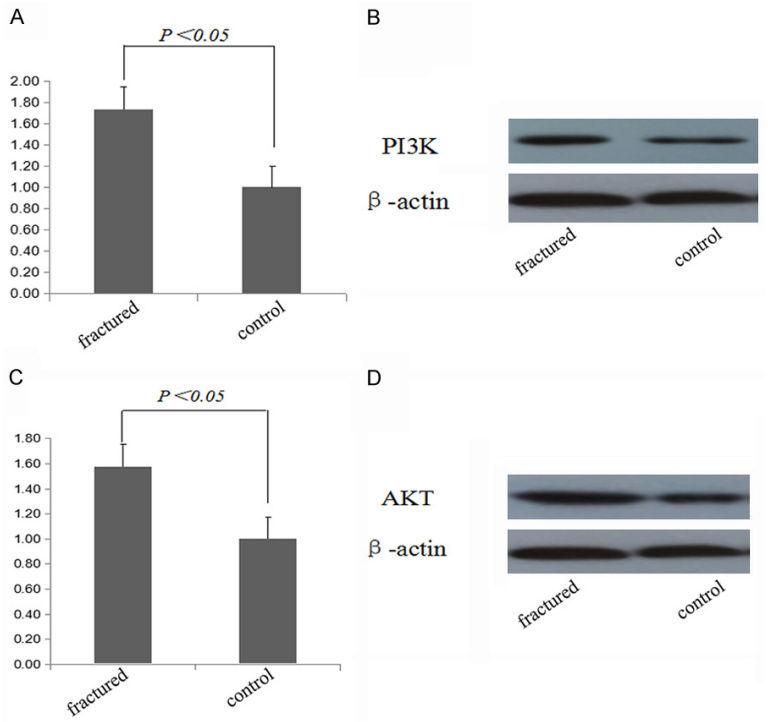

Figure 5. mRNA (A & C) and protein expression (B & D) level of PI3K (A & B) and AKT (C & D) in fractured and control samples. Total RNA or protein was isolated from tissues followed by analysis of the expression of PI3K or AKT by RT-PCR or western blot.

tion [19]. Hong-qiang Liu found that IL-1 could induce the degradation of matrix surrounding cartilage cells and apoptosis of cartilage cells in wet arthritis [20]. Consistent with this, our study showed increased IL-1beta level in fractured side, further confirming the role of IL-1beta in the progression of condylar fracture. IL-6 is an important pro-inflammatory cytokine with many biological activities. It can inhibit the differentiation of bone marrow mesenchymal stem cells into chondrocytes [21]. In addition, IL-6 plays an important role in the cartilage repair [22]. In our study, we found significantly increased IL-6 level in fractured side, revealing the contribution of IL-6 to the cartilage repair after fracturing. IGF-1 is also an important cytokine in promoting cell proliferation and inhibiting apoptosis in several malignant tumors [23, 24]. However, its role in post fracture recovery process remains poorly understood. In this study, we found IGF-1 level was increased after fracturing, indicating it might also participate in the progression of condylar fracture.

PI3K/AKT signaling is considered to be an important pathway in regulating the proliferation of malignant tumors, cell metabolism, cycle control as well as the formation of blood vessels [20, 25]. In the present study, we demonstrated that the protein and mRNA expression of PI3K and AKT were increased after fracturing, suggesting PI3K/AKT signaling might be involved in the development of condylar fracture. The increased PI3K/AKT expression might be due to the higher level of IGF-1 as IGF-1 has been reported to be capable to activate PI3K/AKT signaling [26-28]. In addition, increased PI3K/AKT expression might also be associated with the elevated expression of IL-1beta and IL-6, as a closely relationship has been observed among them [29-33].

In conclusion, enhanced expressions of IGF-1, IL1-beta, IL6, PI3K and AKT were observed in condylar fracture progression, suggesting targeting them might be a novel approach to promote the repair after condylar.

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Disclosure of conflict of interest

None.

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References

[1] Silvennoinen U, Iizuka T, Lindqvist C and Oikarinen K. Different patterns of condylar fractures: an analysis of 382 patients in a 3-year period. J Oral Maxillofac Surg 1992; 50: 1032-1037.

[2] Marker P, Nielsen A and Bastian HL. Fractures of the mandibular condyle. Part 1: patterns of distribution of types and causes of fractures in 348 patients. Br J Oral Maxillofac Surg 2000; 38: 417-421.
[3] Ellis E 3rd, Moos KF and el-Attar A. Ten years of mandibular fractures: an analysis of 2,137 cases. Oral Surg Oral Med Oral Pathol 1985; 59: 120-129.
[4] Fridrich KL, Pena-Velasco G and Olson RA. Changing trends with mandibular fractures: a review of 1,067 cases. J Oral Maxillofac Surg 1992; 50: 586-589.
[5] Nakashima T and Takayanagi H. Osteoimmunology: crosstalk between the immune and bone systems. J Clin Immunol 2009; 29: 555-567.
[6] Takayanagi H. New immune connections in osteoclast formation. Ann N Y Acad Sci 2010; 1192: 117-123.
[7] Takayanagi H. Osteoimmunology and the effects of the immune system on bone. Nat Rev Rheumatol 2009; 5: 667-676.
[8] Danks L and Takayanagi H. Immunology and bone. J Biochem 2013; 154: 29-39.
[9] Kolar P, Gaber T, Perka C, Duda GN and Buttgereit F. Human early fracture hematoma is characterized by inflammation and hypoxia. Clin Orthop Relat Res 2011; 469: 3118-3126.
[10] Schmidt-Bleek K, Schell H, Kolar P, Pfaff M, Perka C, Buttgereit F, Duda G and Lienau J. Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep. J Orthop Res 2009; 27: 1147-1151.
[11] Claes L, Recknagel S and Ignatius A. Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol 2012; 8: 133-143.
[12] Schmidt-Bleek K, Kwee BJ, Mooney DJ and Duda GN. Boon and bane of inflammation in bone tissue regeneration and its link with angiogenesis. Tissue Eng Part B Rev 2015; 21: 354-364.
[13] Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, Perka C, Buttgereit F and Duda GN. The early fracture hematoma and its potential role in fracture healing. Tissue Eng Part B Rev 2010; 16: 427-434.
[14] Bhandari M, Tornetta P, Sprague S, Najibi S, Petrisor T, Griffith L and Guyatt GH. Predictors of reoperation following operative management of fractures of the tibial shaft. J Orthop Trauma 2003; 17: 353-361.
[15] Santarlasci V, Cosmi L, Maggi L, Liotta F and Annunziato F. IL-1 and T helper immune responses. Front Immunol 2013; 4: 182.
[16] Scheller J, Chalaris A, Schmidt-Arras D and Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011; 1813: 878-888.
[17] Bilbao D, Luciani L, Johannesson B, Piszczek A and Rosenthal N. Insulin-like growth factor-1 stimulates regulatory T cells and suppresses autoimmune disease. EMBO Mol Med 2014; 6: 1423-1435.
[18] Worthington JJ, Fenton TM, Czajkowska BI, Klementowicz JE and Travis MA. Regulation of TGF beta in the immune system: an emerging role for integrins and dendritic cells. Immunobiology 2012; 217: 1259-1265.
[19] Abdel-Galil K and Loukota R. Fractures of the mandibular condyle: evidence base and current concepts of management. Br J Oral Maxillofac Surg 2010; 48: 520-526.
[20] Huang JG, Tong HJ, Liu HQ and Zhang XL. Effects of IL-1β and TNF-α on degradation of extracellular matrix of articular chondrocytes and related mechanism. Journal of Shanghai Jiaotong University (Medical Science) 2010; 30: 5.
[21] Quan JH, Chu JQ, Kwon J, Choi IW, Ismail HA, Zhou W, Cha GH, Zhou Y, Yuk JM, Jo EK and Lee YH. Intracellular networks of the PI3K/AKT and MAPK pathways for regulating toxoplasma gondii-induced IL-23 and IL-12 production in human THP-1 cells. PLoS One 2015; 10: e0141550.
[22] Liu YY, Mu R, Wang SY, Long L, Liu X, Li R, Sun JA, Guo JP, Zhang XP, Guo J, Yu P, Li CL, Liu XY, Huang ZY, Wang DP, Li H, Gu ZF, Liu B and Li ZG. Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 2010; 12: R210.
[23] Yang C, He DM, Chen MJ, Jiang B, Wang BL, Zhang XH, Qiu YT, Zhang SY and Cai XY. Study of the clinical character and classification of intracapsular condyle fracture. China Journal of Oral and Maxillofacial Surgery 2010; 8: 4.
[24] He D, Yang C, Chen M, Jiang B and Wang B. Intracapsular condylar fracture of the mandible: our classification and open treatment experience. J Oral Maxillofac Surg 2009; 67: 1672-1679.
[25] Oudart JB, Doue M, Vautrin A, Brassart B, Sellier C, Dupont-Deshorgue A, Monboisse JC, Maquart FX, Brassart-Pasco S and Ramont L. The anti-tumor NC1 domain of collagen XIX inhibits the FAK/PI3K/Akt/mTOR signaling pathway through αvβ3 integrin interaction. Oncotarget 2016; 7: 1516-1528.
[26] Wani ZA, Guru SK, Rao AV, Sharma S, Mahajan G, Behl A, Kumar A, Sharma PR, Kamal A, Bhushan S and Mondhe DM. A novel quinazolinone chalcone derivative induces mitochondrial dependent apoptosis and inhibits PI3K/Akt/mTOR signaling pathway in human colon cancer HCT-116 cells. Food Chem Toxicol 2016; 87: 1-11.

[27] Uckan S, Bayram B, Kecik D and Araz K. Effects of titanium plate fixation on mandibular growth in a rabbit model. J Oral Maxillofac Surg 2009; 67: 318-322.
[28] Li R, Pourpak A and Morris SW. Inhibition of the insulin-like growth factor-1 receptor (IGF1R) tyrosine kinase as a novel cancer therapy approach. J Med Chem 2009; 52: 4981-5004.
[29] Cahill CM and Rogers JT. Interleukin (IL) 1 beta induction of IL-6 is mediated by a novel phosphatidylinositol 3-kinase-dependent AKT/I kappa B kinase alpha pathway targeting activator protein-1. J Biol Chem 2008; 283: 25900-25912.
[30] Wegiel B, Bjartell A, Culig Z and Persson JL. Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. Int J Cancer 2008; 122: 1521-1529.
[31] Chou CH, Lai SL, Chen CN, Lee PH, Peng FC, Kuo ML and Lai HS. Il-6 regulates mcl-(1l) expression through the JAK/PI3K/Akt/CREB signaling pathway in hepatocytes: implication of an anti-apoptotic role during liver regeneration. PLoS One 2013; 8: e66268.
[32] Colakovic S, Lukic V, Mitrovic L, Jelic S, Susnjar S and Marinkovic J. Prognostic value of CA125 kinetics and half-life in advanced ovarian cancer. Int J Biol Markers 2000; 15: 147-152.
[33] Wang Q, Zhang B and Yu JL. Farrerol inhibits IL-6 and IL-8 production in LPS-stimulated human gingival fibroblasts by suppressing PI3K/AKT/NF-kappaB signaling pathway. Arch Oral Biol 2016; 62: 28-32.