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Pioglitazone Increase PPAR-γ Expression, Decrease MMP-9, MMP-13, VEGF, NO and TNF-α Secretion in IL-1β-induced Chondrocyte

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Abstract*:Osteoarthritis is the chronic musculoskeletal disorders. It is characterized by destruction of articular cartilage, proinflamatory mediator secretion and breakdown of cartilage matrix.MMP-9 and MMP-13 are biomarkers in matrix degradation in osteoarthritis. MMP-9 can activate pro MMP-13 and stimulates MMP-13 secretion, MMP-13 also can activate MMPs. NO (nitric oxide) is the essential production of catabolic factor in chondrocyte, it's caused by the cytokineproinflamatory responses. VEGF is the essential component for ossification and osteophyte formation.Recent studies suggest that activation of PPAR-γ with pioglitazone (agonist PPAR-γ) is an interesting target for the disease. PPAR-γ is a transcription factor that is also expressed in chondrocytes. The aim of this study to determine the effects of pioglitazone on MMP-9, MMP-13, NO, VEGF, TNF-αsecretion andPPAR-γ expressionby chondrocytes.The sample of this research is chondrocyte cell line induced by IL-1β then exposed with pioglitazone. MMP-9, MMP-13,VEGF and TNF-α were measured using ELISA, NOwas measured using colorimetry assay, PPAR-γ expression was measured by real time PCR. Pioglitazone exposure increased PPARγ expression significantly, and in contrast they are also decreaseMMP-9, MMP-13, NO, VEGF and TNF-αsecretion in all groups. This results show that pioglitazone have a role for treatmentin osteoarthritis by decreasing catabolic and inflamatory responses of chondrocyte.*

Keywords: *Pioglitazone, MMP-9, MMP-13, NO, VEGF, TNF-α, PPAR-γ*

I. Introduction

Osteoarthritis (OA) is the chronic musculoskeletal disorders. It is characterized by the destruction of articular cartilage, proinflamatory mediator secretion and breakdown of cartilage matrix. There are many change in OA, morphological change of chondrocyte OA are fibrillated, small crack and osteophyte formation. The biochemical change are decreasing of collagen tipe 1,2 and proteoglycan, Catabolic change are synthesis and secretion of many proteases and MMPs. The important MMPs in OA are MMP-9 and MMP-13 which are used as biomarkers in matrix degradation in osteoarthritis. MMP-9 can activate pro MMP-13 and stimulates MMP-13 secretion, MMP-13 also can activate MMPs. NO (nitric oxide) is the essential production of catabolic factor in chondrocyte, it's caused by the cytokineproinflamatory responses. VEGF is also the essential component for ossification in cartilage. It's can stimulate osteophyte formation[1]. In early osteoarthritis, chondrocytes are induced by cytokines such as IL-1 and TNF-α derived from synovial cells or macrophages. IL-1 is a potent pro-inflammatory cytokine which is able to stimulate chondrocytes to synthesize more IL-1 and other proinflammatory cytokines such as IL-6 and synthesize degradative enzymes MMP[2,3].

IL-1 is very important cytokine in early OA, increasing the destruction of the extracellular matrix, nitric oxide and induce apoptosis in chondrocyte. It can alsosuppress the synthesis of type II collagen and proteoglycans, and inhibits proliferation of chondrocytes stimulated by transforming growth factor-β (TGF-β)[4]. IL-1 have effect on increasing proliferation, activating inflamatory responses and inducing matrix degradation. IL-1 activates several transcription factors, such as NF-κB, AP-1, c-jun N-terminal kinase (JNK) and p38 MAPK. NFκB activation induces the transcription of several target genes involved in inflammation and the immune system, cell proliferation, cell cycle, and apoptosis. NFκB activation also induces several MMP genes, such as MMP-9 and MMP-13, stimulating VEGF production for angiogenesis, NO secretion caused by pro inflamatory mediator responses and also induce TNF-α[3,5,6].

Until now, there is no therapy to stop the progression of OA. According to other research suggest that peroxisome proliferator-activated receptor gamma(PPAR-γ) activationis an interesting target for the disease. PPAR-γ is a transcription factor that is expressed in chondrocytes.PPAR-γexpression in OA lower than normal chondrocytes. The induction of IL-1 down-regulates PPAR-γ expression[7]. Agonist PPAR-γ activated inhibition

of inflamatory processes and catabolic responses. The increasing of PPAR-γ expression in osteoarthritis cartilage reflects in the decreasing of inflammatory and catabolic factors such as MMP-9, MMP-13, NO, VEGF and TNF-α productions[2,8].

Pioglitazone is the PPAR-γagonist, these actions of pioglitazone were suppression of the activities of many transcriptional factors including NF-kB, activator protein 1 (AP-1), STATs and nuclear factors of activated T cells[8,9].PPAR-γ activators modulate the expression several genes influenceOA pathogenesis. PPAR-γ activation inhibits the IL-1 induced nitric oxide synthase, MMP-9, MMP-13, VEGF, TNF-α. The advantage effects of PPAR-γ activators has also reported in animal model of arthritis (in guinea pig model). The goal in this study is to identificate the effect of pioglitazone for increasing PPAR-γ expression, decreasing MMP-9, MMP-13, NO, VEGF, TNF-α secretions in IL-1βinduced chondrocyte.

II. Material And Methods

2.1 Chondrocyte cell culture

Chondrocytes cell line were obtained from NHAC-Kn, Lonza. Cell line were thawed in Chondrocyte basal media supplemented with R3-IGF1, bFGF, transferrin, insulin, FBS, and gentamicin/amphotericin-B. Cells were then culture in 25cm^{2}flask at a density of 10.000 cells/cm^2. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2, and the medium was changed once a week. The confluent cells were dispersed by trypsinization and were collected by centrifugation at 200g for 5 min. The cells were resuspended in alginate solution, aspirated into syringe, and released dropwise into 30ml polymerization solution. The cells will entrapped into alginate bead. Cells were fed every 2 to 3 days with differentiation media supplemented for 2 -3 weeks. At the end of incubation cells induced with IL-1β for 24 hours continued with exposure of pioglitazone dosage 0,1 μM, 1 μM and 10 μM.

2.2 Enzyme-linked immunosorbent assay (ELISA) and colorimetry assay

MMP-9, VEGF and TNF-α were assayed using Elisa kit from R&D System, Inc. This assay employs the quantitative sandwich enzyme immunoasssay technique. A monoclonal antibody spesific for MMP-9, VEGF TNF-α and have been pre-coated onto a microplate. Standards and samples are pipetted into the wells, The culture medium are used for the sample.MMP-9, VEGF and TNF-αare bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody spesific for MMP-9, VEGF and TNF-α is added to the wells. Followed by washing to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color development in proportion to the amount of MMP-9, VEGF and TNF-α bound in the initial step. The color development is stopped and color intensity is measured in 450 nmaccording to the manufacturer's protocol.MMP-13 were assayed in the conditioned culture media using ELISA kits from Abnova, cat no. KA0182 according to the manufacturer's protocol. NO were determined using colorimetry, the procedural of the assay were devided into two step, nitriteassay and nitratereductionassayaccording to the manufacturer's protocolfrom R&D System, Inc.

2.3 RNA isolation and cDNA synthesis

Total RNA from homogenized chondrocytes was extracted using TriReagent according to the manufacturer's procedures (Promega).IsolatedRNA was treatedin DEPC-water and quantified by nano-spectrophotometry at 260 and 280 nm. RNA samples werereverse-transcribed to cDNA using GoScript™ Reverse transcription System (Promega, Cat. A5000). Experimental RNA, primer and nuclease-free water were mixed and centrifuged into RNA tube. The tube preheated into 70ºC then immediately chilledon ice for 5 minutes. The reverse transcription mix was prepared by combining component: reaction buffer, $MgCl_2$, PCR nucleotide mix, reverse transcriptase, and nuclease-free water. The PCR conditions were as follows: annealing 25ºC for 5 minutes, 42ºC for 60 minutes, and 70ºC for 15 minutes. Then the tube immediately chilled on ice, and stored in -70ºC

2.4 Real Time PCR analyses

Relatif amounts of PPAR-γmRNA expressionwere analized by quantitative real-time RT-PCR(Light Cycler-Fast Start DNA Master SYBR Green I, Roche Applied Science) primers and LightCycler software (Roche Applied Science). The primers used were: PPAR-γ sense5–TGACCAGGGAGTTCCTCAAAA–3 and PPAR-γ antisense5 –AGCAAACTCAAACTTAGGCTCCAT–3; GAPDH sense 5 – CAG AAC ATC ATC CCT GCC TCT – 3 and GAPDH antisense 5 – GCT TGA CAA AGT GGT CGT TGA – 3. Preparation for master mix, added master mix and primer (according to the manufacturer's protocol). PCR conditions were: pre-incubation 95ºC 10 minutes 1 cycle; amplification 45 cycles of denaturation at 95ºC for 10 s, annealing at 56ºC for 20 s, and extension at 72ºC for 25 s; melting curve analysis 1 cycle of denaturation 95ºC 0 s, annealing 65ºC 15 s, melting 95ºC 0 s with slope 0,1ºC/s. Normalized gene expression was calculated as the ratio between PPAR-γ and GAPDH.

2.6 Statistical Analysis

Statistical analysis used Statistical Package for Social Sciences, v.16. The MMP-9, MMP-13, VEGF, NO, TNF-α and PPAR-γ level were measured and presented as mean ± SEM. The mean value was analysed by Kolmogorov Smirnov to determine the data distribution. Then analysed by one way anova to determine the effect of pioglitazone with dependent variable and to determine the differences between groups. The differences in each groups analyzed using post hoc tukey test. The statistical analysis correlation was performed by pearson correlation test. p(value) less than 0,05 was considered significant for the differences and correlation.

III. Result

Pioglitazone Increased PPAR-γmRNA expression

This study used a chondrocyte cell line - Normal Human Articular Chondrocyte (Lonza), which is divided into 5 groups: The first group normal control; the 2nd group induced by IL-1β 10ng/ml; the 3th group induced by IL-1β 10ng/ml and exposed to 0,1ng/ml pioglitazone; the 4th group induced by IL-1β 10ng/ml and exposed to pioglitazone 1 ng / ml; The5th groupinduced by IL-1β 10ng/ml and exposed to pioglitazone10ng/ml.All group were analysed the relatif amounts of mRNA PPAR-γexpression, MMP-9, MMP-13, VEGF, NO and TNF-α productions.

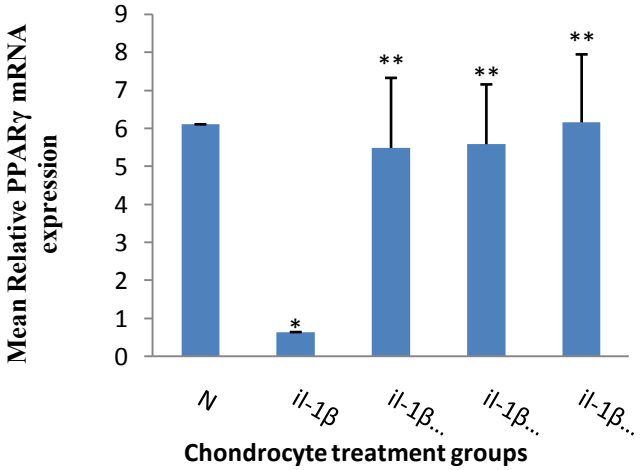

Figure 1 Effect of pioglitazone in relative PPAR-γ mRNA expression. This result is compared between mRNA PPAR-γ and GAPDH.IL-1β induction in chondrocyte increased PPAR-γ expression. Addition of pioglitazone slightly increase PPAR-γ expression in chondrocyte. *.Asterisks* (*) denote a significant effect of PPAR-γ expression compared to normal group, (**)($p< 0.05$)denote a significant effect of PPAR-γ expression compared to pioglitazone addition group. (N : no treatment; IL-1β : chondrocyte induced by IL-1β 10ng/ml; IL1B+P1 : chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 0,1μM; IL1B+P2 = chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 1μM; IL1B+P3 :chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 10μM. *Asterisks* (*) denote a significant effect compared to normal ($P < 0.05$), (**) denote a significant effect compared to IL-1β group ($p < 0,05$).

Figure (1) showed that IL-1β decreased PPAR-γ expression significantly. Mean and standart deviation of each groups were no treatment group (6,107±0,000) ; chondrocyte induced by IL-1β 10ng/ml (0,643±0,000);: chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 0,1μM (5,484±1,844); chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 1μM (5,584±1,572); chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 10μM (6,157±1,79).Pioglitazone increasedPPAR-γ expression significantly ($p < 0,05$). In the grouppioglitazone dosage 10 μM, pioglitazone show the high effect. Our investigation showed there is significant changes in PPAR-γ expression after pioglitazone addition ($p = 0,039$). Pioglitazone was one of the PPAR-γ ligands. A positive correlation was seen between pioglitazone and PPAR-γ expression ($R = 0,729$; $p < 0,040$).

Pioglitazone decreased MMP-9, MMP-13, VEGF, Nitric oxide and TNF-α secretions

This study after the chodrocyte in 80-90% confluence, then incubation cells induced with IL-1β for 24 hours continued with exposure of pioglitazone dosage 0,1 μM, 1 μM and 10 μM , we analyse the medium with ELISA and colorimetry to evaluate the decresion of MMP-9, MMP-13, VEGF, Nitric oxide and TNF-α secretions, MMP-9, VEGF and TNF-α were assayed using Elisa kit from R&D System, Inc. NO were determined using colorimetry, the procedural of the assay were devided into two step, nitritassay and

nitratereductionassay according to the manufacturer's protocol from R&D System, Inc. The result of the determination (see figure 2)

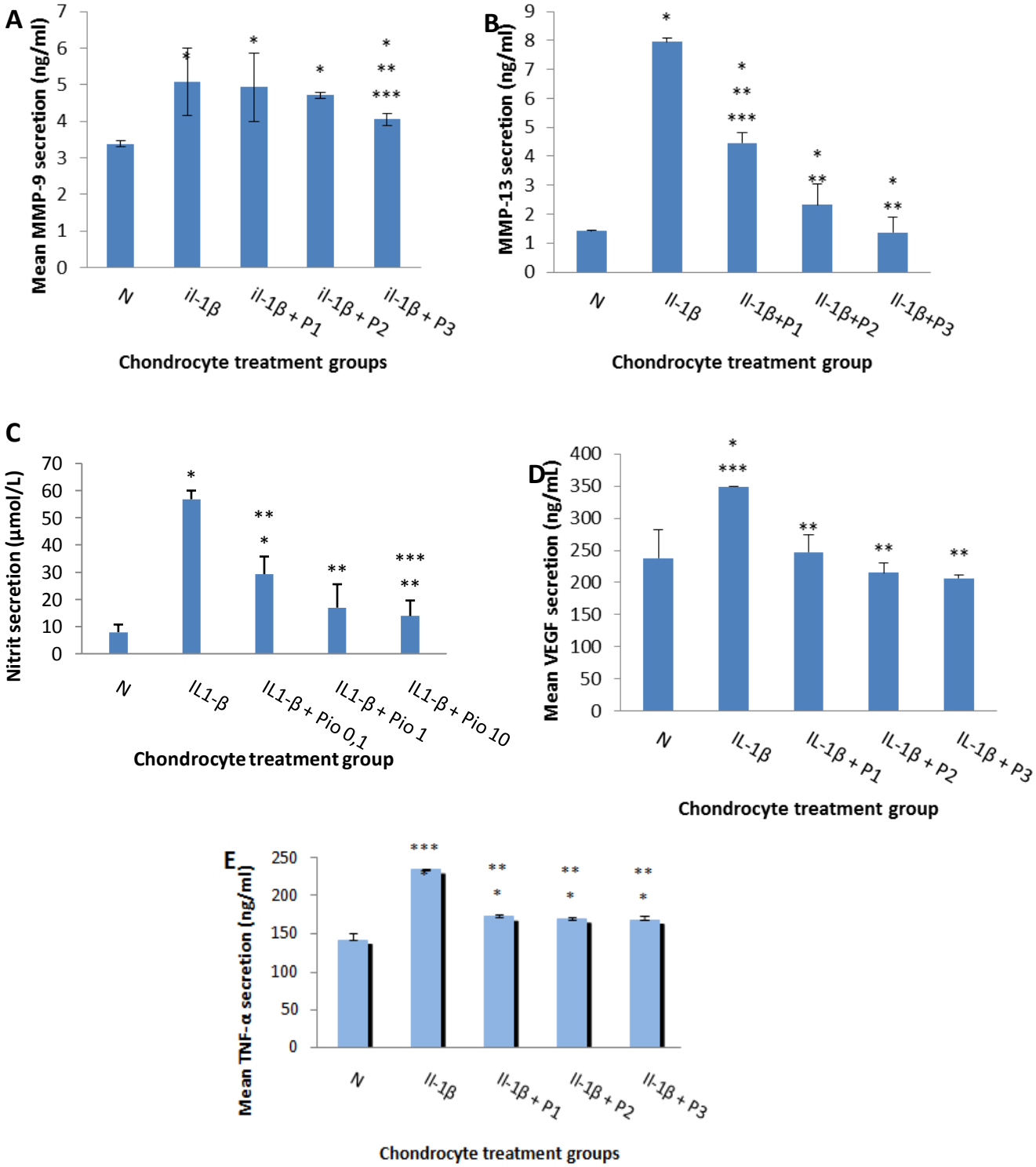

Figure 2. Effect of Pioglitazone in MMP-9, MMP-13, VEGF, NO and TNF-α secretion by chondrocyte in various treatmentgroups. (A) Pioglitazonedecreased IL-1β-induced MMP-9 secretion on chondrocytes in dose dependent manner, significant effect seen in higher dose. (B) Addition of pioglitazone slightly decrease MMP-13 secretion also in dose dependent manner. (C) Pioglitazone decreased nitric oxide secretions in IL-1β inducedin dose dependent manner. (D) Pioglitazone also decreased VEGF in IL-1β induced. Significant effect found at all given dose, the pioglitazone addition decreased IL-1β-induced MMP-9, MMP-13, NO, VEGF significantly.(N : no treatment; IL-1β : chondrocyte induced by IL-1β10ng/ml; IL1B+P1 : chondrocyte induced

by IL-1β 10ng/ml then treated with pioglitazone 0,1μM; IL1B+P2 = chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone 1μM; IL1B+P3 :chondrocyte induced by IL-1β 10ng/ml then treated with pioglitazone10 μM. *Asterisks* (*) denote a significant effect compared to normal ($P < 0.05$), (**) denote a significant effect compared to IL1 group ($p < 0,05$), (***) denote a significant effect compared to treatment with all dosage ofpioglitazone.

Figure (2A)showed that il-1β increased MMP-9 secretionsignificantly. Addition of pioglitazone decreased MMP-9 secretion significantly ($p = 0,000$). A significant change was also seen in all addition of pioglitazone (P1,P2,P3). Pioglitazone and MMP-9 also have positive correlation ($R = 0,855$; $p = 0,000$).Figure (2B)showed that IL-1β increased MMP-13 secretions significantly.Pioglitazonedecreased MMP-13 secretion, there was significant changes after IL-1 induction and added pioglitazone ($p =0,000$) addition. A significant change wasalso seen in alldose addition of pioglitazone (P1,P2,P3). A positive correlation was seen between pioglitazone and MMP-13 secretion ($R = 0,956$; $p < 0,05$).

Figure (2C) IL-1β induction increased nitric oxide significantly ($p < 0,05$).Addition of pioglitazone in all dosesdecreased the secretion of nitric axide significantly($p< 0,05$). Figure (2D) IL-1β induction increased VEGF significantly ($p < 0,05$).Addition of pioglitazonedecreased the secretion of VEGF significantly($p =0,000$). Pioglitazone dosage 0,1 μM was not significant with pioglitazone dosage 1 μM and 10 μM.Figure (2E) IL-1 induction increased TNF-α secretion significantly ($p < 0,05$)..Addition of pioglitazonedecreased the secretion of TNF-α significantly($p =0,000$). Pioglitazone dosage 0,1 μM was not significant with pioglitazone dosage 1 μM and 10 μM.

IV. Discussion

Osteoarthritis (OA) is the chronic musculoskeletal disorders. It is characterized by the destruction of articular cartilage, proinflamatory mediator secretion and breakdown of cartilage matrix. There are many change in OA, morphological change of chondrocyte OA are fibrillated and small crack and osteophyte formation. The biochemical change are decreasing of collagen tipe 1,2 and proteoglycan, Catabolic change are synthesis and secretion of many proteases and MMPs. It's means that in OA there is extracellular matrix degradation, The extracellular matrix degradation are done by many enzymes. Enzymes that degrade matrix, such as matrix metalloproteinase (MMPs) play an important role pathogenesis OA.MMPs have ability to damage type II collagen or aggrecans, proteoglycan and another component which are used for integrity of cartilage. Collagen tipe 2 and proteoglycan are the important components of ECM[10]. In pathogenesis of OA, chondrocyte metabolism are changedbythe presence of proinflammatory mediators. Thera many kinds of factors may initiate the degenerative cascade that generates a lot of changes in the characteristics of articular cartilage in osteoarthritis.The burden biomechanical, mechanical trauma,genetic and cytokineschanges, involved in the pathogenesis of OA. Pro-inflammatory cytokine that is a potent and play an important role on the pathogenesis of osteoarthritis is IL-1. IL-1 is able to induce chondrocytes to synthesize MMP[11]. IL-1 plays an important role in the inflamation process and connective tissue destruction. IL-1 have ability to activate expression of genes proteases (MMPs).

This research reported that induction of IL-1β decreased relative PPAR-γmRNA expression. According to the previous finding, IL-1 can inhibit PPAR-γ expression. Inhibitor MAPK, p38, C-Jun terminal kinase (JNK) andNFkBmediateddecreasing of PPAR-γ1 IL1-β induced.PPAR-γ is also expressed in chondrocytes and the expression in osteoarthritis decreased compared to normal chondrocytes. PPAR-γ activated by its agonist decreased the synthesis of catabolic response, inflammatory factors and reduce cartilage degradation in vivo and in vitro in osteoarthritis animal model[2,12]. In this research, the addition of pioglitazone (PPAR-γagonist) on IL-1 induced chondrocyte increased the relative mRNA PPAR-γexpression (figure 1). This suggests that pioglitazoneincreased relative mRNA PPAR-γexpression is influenced by the activation through many pathways. Pioglitazone is activator and ligand for PPAR-γ, activation of PPAR-γ suppressed the transcriptional activity of AP-1 and NFkB. PPARγregulatesgene expressionwith retinoid X reseptor (RXR)[13]. Heterodimer PPARγ/RXR boundspesificelemen responses of PPARγ in promotergenetarget andwork as transcription regulator.In patients with osteoarthritis found that the expression of PPAR-γ decreased in the cartilage. These findings suggest that decreased PPAR-γ expression in osteoarthritis cartilage will increase the gene expression of inflammatory and catabolic response, causing inflammation and degradation of articular cartilage[14]. The recent Studyproved that Egr-1 mediates the suppressiveeffect of IL-1 on PPAR-γexpression through a mechanisminvolving displacement of prebound Sp1[15,16]. In addition, research by Shan also explained that the PPAR-γ expression is regulated by IL-1β. In that study the induction of IL-1β may decrease the expression of PPAR-γ after 6 hours of induction[17].

PPAR-γ's pleiotropic physiological roles, its relativeabundance is predicted to have an importantmodulatinginfluence on a variety of homeostatic responses. A large number of soluble mediators are known toeffect PPAR-γ expression, stability and activity in a tissueandcell type-specific manner. Transcription

factors shown to regulate PPAR-γ expression include C/EBPs, EBF proteins, inhibitorof DNA binding (ID) 2 and NF-E2 related factor 2 (Nrf2). Several cytokines and chemokines regulate PPAR-γ expression in mesenchymal cells. The inflammatorycytokines TNF-α and IL-1 inhibit adipocyte differentiation by suppressing PPAR-γ expression[18,19,20].

MMP-9 and MMP-13 is the important MMPs in pathogenesis OA.MMP-9 and MMP-13 which are used as biomarkers in matrix degradation in osteoarthritis. MMP-9 can activate pro MMP-13 and stimulates MMP-13 secretion, MMP-13 also can activate MMPs.This research reported that induction of IL-1βincreased MMP-9 and MMP-13significantly, It's consistent with the previous finding, that induction of IL-1 activates several transcription factors, such as NF-κB, AP-1, c-jun N-terminal kinase (JNK) and p38 MAPK.. The activation ofNFκB induces several target genes transcription involved in the inflammation and immune system, cell proliferation, cell cycle, and apoptosis. Activation of NFκB also induces several MMP genes, such as MMP-3 and MMP-13[6.] In this research, the addition of pioglitazone (PPAR-γagonist) on IL-1 induced chondrocytedecreased MMP-9 and MMP-1significantly. According to the previous finding PPAR-γ interacts with other transcription factors and is not directly involved in DNA binding to regulate gene transcription. For example, PPAR-γ have interaction with AP-1 (activator protein-1), STAT (signal transducers and activators of transcription), and NF-κB, which all transcription factors also role in regulation of gene expression. Proinflammatory transcription factor NF-κB has a central role in immune responses and inflammation, which is NF-κBis the main target of PPAR-γ to suppress inflammation[21].

IL-1β induction also increased nitric oxide and TNF-α significantly. IL-1β activated transcriptional factor NF-kB, AP-1, c-jun n-terminal kinase (JNK) and p38 MAPK. Activation of NF-kB can induce transcriptional target gene in inflamatory process , imun system, cell proliferation, cell cycle and apoptosis. activation NF-kB induce target gene like MMPs. MMPs for example MMP-3, MMP-1, MMP-9 and MMP-13 stimulate matrix degradation and inflamation[22]. IL-1βInduction stimulate secretion proinflamatory cytokine especially TNF-α . TNF-αinduces chondrocyte to produce prostaglandin (PG), nitric oxide (NO),and the other protein which have effect in matrix synthase and matrix degradation[23]. NO is produced, can supressesaggrecane andincreased matrix degradation.In this study reported that TNF-α and NO increased significantly in IL-1β induced chondrocyte. It's consistent According to the previous finding. In this research, the addition of pioglitazone on IL-1β induced chondrocytedecreased TNF-α and NOsignificantly. The mechanism of this effect is same with MMP-9 and MMP-13, related with PPAR-γ. PPAR-γ interacts with other transcription factors and not directly involved DNA bindingto regulate gene transcription. For example, PPAR-γ have interaction with AP-1 (activator protein-1), STAT (signal transducers and activators of transcription), and NF-κB, which all transcription factors role in regulation of gene expression. Proinflammatory transcription factor NF-κB has a central role in immune responses and inflammation, which is NF-κBis the main target of PPAR-γ to suppress inflammation[24,25]. PPAR-γ have the opposite effect with IL-1β.

VEGF is also important in OA. VEGF roles in inflamatory respon and patologic angiogenesis OA. Increasing VEGF production also stimulate chondrocyte hypertrophy, matrix degradation and cell apoptosis. IL-1β induction also increased VEGF significantly. IL-1β activated transcriptional factor p38 MAPK, and c-jun terminal kinase (JNK) to stimulate VEGF gene transcription through SP-1[26]. In this research, the addition pioglitazone on IL-1β induced chondrocytedecreased VEGF significantly. Previous study suggest that IL-1β induces chondrocyte increased miR-146a and VEGF expression and decreased Smad4.In vivo conditions, increasing VEGF will increase synovial hyperplasia associated with increase number of blood vessels. Synovium hyperplasia and increase number of blood vessels coincided with progressive calcifications in calcified layer of articular cartilage. VEGF triggers ossification of cartilage calcification and subchondral bone remodeling. VEGF production by hyalin cartilage chondrocytes in the superficial and mid zones role in initiation and progression of OA[27].Addition various doses of pioglitazone in this study aims to suppress the inflammatory response played by chondrocytes VEGF, and proved administration of pioglitazone with various doses decrease VEGF production by IL-1β induced chondrocytes.

From the results it can be concluded that pioglitazone increasedrelative PPAR-γmRNA expression. This increase is followed by decreased of MMP-9, MMP-13, VEGF, NO and TNF-αsecretion.The activation of PPAR-γ by pioglitazoneisrelated to dosage, and mechanism in nuclear and transcription process[28]. The decreasing of MMP-9, MMP-13, VEGF, NO and TNF-αsecretionhas relation with the role of decrease PPAR-γcartilage degradation and inflamatory process.

V. Conclusion

Pioglitazone could decrease MMP-9, MMP-13, VEGF, NO and TNF-αsecretionand increase relative mRNA PPAR-γexpressionin IL-1β-induced chondrocyte. This results show that pioglitazone have a role in treatment of osteoarthritis by decreasing catabolic and inflamatoryresponse of chondrocyte. This study still needs further research using an animal model of osteoarthritis and preclinical study.

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