Runx2 and sox9 expression on chondrocyte hyperthrophy formation in post-menopausal osteoarthritis mechanism

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RUNX2 and SOX9 Expression on Chondrocyte Hypertrophy Formation in Post-Menopausal Osteoarthritis Mechanism (An Experimentation on Rat Model)

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Abstract

In osteoarthritis post-menopause, decreasing estrogen influenced formation of chondrocyte hypertrophy. This study would explain role of RUNX2 and SOX9 to chondrocyte hypertrophy formation in decreasing estrogen condition. Fifty four rats were divided into six groups. Level of 17 ß estradiol and TGF-ß were examined by ELISA. SOX9 and RUNX2 used immunohistochemistry. Histopathology for chondrocyte hypertrophy. Level of 17 ß estradiol and SOX9 on treatment group decreased significantly, TGF-ß and RUNX2 increased significantly. Chondrocyte hypertrophy increased significantly in treatment group. The decreasing of 17 ß estradiol level caused increasing hypertrophy Chondrocyte through increasing level of TGF-ß and RUNX2.

Key words: Chondrocyte hypertrophy, Estrogen, RUNX2, TGF-ß.

Osteoarthritis is a degenerative disease that causes joint cartilage damages and joint movement inhibition. The formation of chondrocyte hypertrophy indicates "Point of No Return" leading to osteoarthritis. The impact of estrogen towards Type-2 collagen remains unclear. Chondroprogenitor cells found in the cartilage my become a factor that explains the effect of decreasing estrogen on the level of Type-2 collagen and answers the question why chondrocyte hypertrophy happens on osteoarthritis post menopause (Goldring *et al*., 2012).

The prevalence of osteoarthritis increases on post-menopausal women. Decreasing estrogen level causes changes on micro-environment, namely the increasing TGF-ß level that triggers differentiation on chondroprogenitor cells into chondrocyte hypertrophy by increasing of RUNX2 (Jayasuria *et al*., 2016), which initiates cellular changes towards osteoarthritis. The increasing number of chondrocyte hypertrophy enhances the level of MMP-13 so that the level of Type-2 collagen will decreases in cartilage (Roman Blast, *et al*., 2009). Role of RUNX2 and SOX9 on decreasing estrogen level and the number of Hypertrophic Chondrocyte, and the effects on TGF-ß level on chondroprogenitor cells and their hypertrophy is to be studied (Cheng and Genever., 2010).

Materials and Methods

The rats (Rattus norvegicus) from Veterinary Medical Faculty of Gajah Mada University used for the estimation. Monoclonal antibody for RUNX2 using Mouse anti-Rat RUNX2 Monoclonal Antibody (Santacruz Biotechnology, USA). Monoclonal antibody for SOX9 was estimated using Mouse anti-Rat SOX9 Monoclonal Antibody (LSBIO, USA), MMP-13 was estimated by Rabbit anti-Rat MMP-13 Polyclonal Antibody (BIOSS, USA). Collagen type 2 was estimated using Rabbit anti-Rat Collagen type 2 Polyclonal Antibody (BIOSS, USA). Levels of 17 ß estradiol were estimated using Rat 17 ß Estradiol Elisa Kit (Bioassay Technology Laboratory Shanghai, CHINA) and TGF-ß estimated using Rat Transforming Growth Factor ß Elisa Kit (Bioassay Technology Laboratory Shanghai, CHINA).

Blood samples of 2cc were collected with EDTA in the control group (K) and treatment

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group (P), and centrifuged at 3000 rpm/minute for 10 minutes to separate blood serum for estimation of 17 ß estradiol and TGF-ß level, at department of Science and Technology.

54 rats were divided into three groups (K1 to K3) and three treatment groups (P1 to P3) consisting of 9 rats each. Cartilage samples were taken from knee joint and calcified in 2% nitric acid for 3 weeks consecutively and they were examined at 4, 8 and 12th weeks.

Sandwich ELISA test was conducted to measure the levels of 17ß estradiol the preparation of all reagents, standard solution (50 µL contains 6 ng/L), and standard samples by considering the room temperature; 40 µL standard solution was mixed by 10 µL anti-estradiol antibody solution and added into 50 µL streptavidin HRP. The solution was incubated at 37°C for an hour. Plates were cleaned in 350 µL wash solution for five times. 50 µL solution A and 50 µL solution B were mixed and incubated at 37°C for 10 minutes until its color changed and then 50 µL stop solution was added. OD value was read using microplate reader set at λ 450 nm and measured. TGF examination was conducted based on the following procedures: preparing the preparations, samples, and standard solution (120 µL concentration 2400 ng/L). 50 µL aquadest was added into 50 µL standard solution until the concentration reached 75 ng/L. 50 µL streptavidin-HRP was added into 50 µL standard solution (composed of 40 µL sample and 10 µL TGF-ß antibody) and incubated at 37 °C for an hour. Plates were cleansed in 350 µL buffer solution. 50 µL substrate A and 50 µL substrate B were added and incubated at 37 °C in dark room condition. 50 µL stop solution was added until the color changed from blue to yellow. OD value was measured using microplate recorder set at λ 450 nm 30 minutes after stop solution added.

Immunohistochemistry examination comprised of dehydration phase, clearing phase, and embedding phase. Dehydration phase is reducing water molecules by using alcohol with concentrations of 70%, 80%, 90%, and finally 100% (absolute) for an hour consecutively. Clearing phase refers to cleaning the alcohol and chemical traces using xylol 98 % for an hour with three repetitions. Embedding phase is the process of creating tissue block by dipping cartilage tissue samples into paraffin to create paraffin block. Tissue samples were stored at 56-58°C temperature for 2 hours with three repetitions. The tissues in paraffin block, were cut using microtom with 4 µm thickness. The slice were put into water bath at 50°C temperature and stuck to the object glass. The preparations were heated to thaw the paraffin before coloration. Immunohistochemistry coloration technique applied in this experiment was biotin streptavidin technique based on the following procedures: first Betazoid Statrec Universal stainning kit (SCYTECH, CANADA) 1:100 in substrate for 3 minutes then the object glass was rinsed in aquadest, followed by Hematoxylin Meyers coloration for 30 second and the object glass was rinsed in water. Dehydration process was conducted by using ethanol 50 %, 70%, 80%, 90%, and 100% followed by xylol for 3 x 3 minutes. Then, mounting was conducted to close the tissue with glass decks and glued. Using the microscope, expression of RUNX2 and SOX9 were examined (Nikon H600L, Digital camera DS Fi2,300 megapixel)

Cartilage tissue block paraffins were made and then cut with a microtom with a thickness of 4 µm, then stained using hematoxylin and eosin stain. Preparations were read using a Nikon (Eclipse Ci microscope, 16.25megapixel Camera DS-Ri2) to see changes in the number of chondrocyte hypertrophy with 400x magnification.

The data was analyzed statistically by Anova test. The levels of SOX9 and RUNX2 were analyzed with Brown Forsythe using SPSS 23 for windows with significance level at ($p<0,05$).

Results and Discussion

This study showed the level of 17 ß estradiol significantly decreased on the treatment group compared with the control group, especially on P3 (12-week treatment) caused by ovarectomy in 4 weeks (Table I). 17 ß estradiol was mainly produced in the ovaries so ovarectomy treatment would reduce estradiol level and cause similar symptoms as menopause.

The level of TGF-ß significantly increased

Table I. Levels of 17 β estradiol, TGF- β, SOX9, RUNX2 on different groups

	17 β estradiol	TGF-β	SOX9	RUNX2
K1	9.888±1.6	143.3±56.21	5.28±3.5	8.25±1.74
K2	8.441±0.8	235.8±81.24	3.84±2	11.01±1.96
K3	8.125±1.39	339.5±87.61	4.55±2.2	11.79±1.77
P1	7.966±1.1	339.8±46.88	2.31±1.6	12.03±2.75
P2	7.201±1.16	382.8±90.64	1.9±1.1	14.19±2.31
P3	6.848±0.92	394.7±75.6	1.4±1.1	16.27±3.89

Table II. Chondrocyte Hypertrophy Number

Group	n	Mean ± SD
K1	9	17.78 ± 3.99^{ab}
K2	9	14.00 ± 5.15^{a}
K3	9	18.33 ± 6.86^{ab}
P1	9	27.22 ± 7.71^{c}
P2	8	25.75 ± 11.68^{bc}
P3	8	41.50 ± 10.89^{d}

*Different superscripts in the same column indicated a significant difference ($p<0.05$).

on the treatment groups (P) compared to control group (K) and seen in the 4th, 8th, and 12th week. Matsuda, *et. al*., (2001) Found that Estrogen level correlated with TGF-ß level on human cells. The study showed there was an increase of TGF-ß level caused by the decreasing level of estradiol. There was cross-reaction between estradiol and TGF-ß.

Ito, *et. al*., (2009) found that estrogen receptor α affected TGF-ß by inhibiting Smad proteins of cytoplasm. Cheng, *et. al*., (2016) found the effect of estrogen on cells through estrogen receptor ß that inhibited type-2 collagen and sclerostin expression, but he didn't explain how did estrogen affect the collagen.

Table I shows the concentration of estradiol was decreasing significantly (Kruskal-Wallis test) on treatment group compared control group ($p<0.05$). The level of TGF-ß in blood was increasing significantly on the treatment group (P1, P2, P3) ($p\leq0.05$). This result indicates that the decreasing estradiol level increase the level of blood TGF-ß. Level of SOX9 decreased significantly on the treatment group (P1, P2, P3) compared to the control group ($p<0.05$) and level of RUNX2 increased significantly in treatment group compared to control group ($p<0.05$). This result shows that decreasing level of estrogen was increasing TGF-ß and RUNX2.

Table II shows the number of chondrocyte hypertrophy cells significantly ($p<0.05$) increased in the treatment groups compared to the control groups.

The effect of increasing TGF-ß on chondroprogenitor cells is SOX9 and RUNX2 expression on the nuclei. RUNX2 and SOX9 are competing one another so that the differentiation depends on which factor is more dominant. If RUNX2 is dominant, the differentiation would be oriented to osteogenesis. But, if SOX9 is dominant, the differentiation would be orientated to chondrogenesis. The increasing of chondrocyte hypertrophy cells is commonly caused by RUNX2 expression. As an intracrine, RUNX2 stimulates differentiation towards chondrocyte hypertrophy. RUNX2 competes with SOX9 which functions as RUNX2 inhibiting factor (Cheng and Genever, *loc. cit*). In this research, the number of chondrocyte hypertrophy cells increased when RUNX2 level was increasing and SOX9 level was decreasing. It showed that the expression of RUNX2 influenced differentia-

tion of chondroprogenitor cell into hypertrophic chondrocyte.

The increasing TGF-ß level in blood may affect various extracellular matrix conditions and stimulate chondroprogenitor cells in cartilage. The effect of TGF-ß on chondroprogenitor cells would change micro-environment caused by decreasing estrogen level then stimulating differentiation towards chondrocyte hypertrophy (Dexheimer *et al*., 2016). Chondrocyte hypertrophy expresses several proteins, namely Alkali Phosphatase (ALP) Venous Endothelial Growth Factor (VEGF), Osteopontin, Matrix Metalloprotease 13 (MMP-13), and type-10 collagen. The role of MMP-13 as collagenase enzyme is clearly proven to degrading type-2 collagen. Degradation of type-2 collagen in cartilage caused by processes above will reduce the strength of cartilage because type 2 collagen is important to the extracellular matrix (ECM). Many opinions mentioned that mechanical, inflammation, pathological apoptosis and aging process might be the factors of hypertrophic chondrocytes formation. However, estrogen, as one of osteoarthritis factors, needs to be accounted considering the increased prevalence of osteoarthritis in post-menopausal period.

Summary

It can be concluded that decreasing level of 17 ß estradiol caused increasing amount of chondrocyte hypertrophy through increasing level of TGF-ß and RUNX2.

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