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Original

The Effect of Estrogen on Type 2 Collagen Levels in the Joint Cartilage of Post-Menopausal Murine Subjects

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Abstract: Cartilage degradation frequently occurs during the postmenopausal period due to a decrease in type 2 collagen and constitutes a condition affected by the level of estrogen in the body. The purpose of this study is to prove that such postmenopausal reductions in estrogen levels influence the type 2 collagen expression in cartilage. Oophorectomies were performed on 54 *Rattus norvegicus* subjects and each of which was subsequently assigned to one of six different groups. 17β-estradiol present in the blood of the subjects was examined by means of ELISA technique, while the expression of MMP-13 and type 2 collagen in their knee joint cartilage was assessed immunohistochemically. A significant post-oophorectomy decrease of 17β-estradiol was detected in subjects ($p<0.05$), while the expression of MMP-13 increased appreciably ($p<0.05$) and a marked decrease in type 2 collagen expression occurred ($p<0.05$). The reduction in 17β-estradiol promoted an increase in the expression of MMP-13 and a decrease in type 2 collagen expression.

Key words: 17β-estradiol, MMP-13, Type 2 collagen

Introduction

Reduction in type 2 collagen is frequently encountered in postmenopausal women suffering from osteoarthritis. Type 2 collagen is distributed across all layers of the cartilage, especially in the superficial layer. Therefore, a reduction in the level of type 2 collagen can be used as an indicator of joint cartilage degradation. Collagen creates mesh formation as a support for cartilage, enabling it to withstand tensile force, shearing force and compression force[1)]. Studies have shown that reductions in blood estrogen levels reduce the cartilage collagen synthesis in joints[2)]. In postmenopausal women, the level of estrogen in the blood is reduced, thereby affecting the synthesis of chondrocyte collagen. Epidemiological studies conducted in Australia and the U.S.A. have indicated that osteoarthritis is more prevalent among postmenopausal women than in men[3)]. However, the mechanism of type 2 collagen reduction related to this condition in postmenopausal women remains unclear.

Reports from various countries contained similar data indicating that women suffer to a greater extent from osteoarthritis than do men. In 2015, the global prevalence of osteoarthritis among the people aged over 60-years-old stood at 9.6% for men, but the number is almost twice for women, at 18%. Australia reported that the total number of sufferers of the condition within its population amounted to 4 million. In 2016, the expenditure for the treatment of medical conditions related to osteoarthritis totaled US$ 5.5 billion. In 2013, 77 million Americans suffered from osteoarthritis, with a male to female ratio of 1:3, of which 10.5 million (13.6%) requiring surgical intervention, including joint replacement surgery, at a cost of US$ 6.35 billion per annum[4)].

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Materials and Methods

Location

This study was conducted at the Experimental Animal Sustenance Unit, Pathology Laboratory, Faculty of Veterinary Medicine and at the Faculty of Science and Technology, Airlangga University on February – May 2018. This research was conducted in accordance to the procedures that are already authorized by the Research Ethics Commission, Faculty of Veterinary Medicine, Airlangga University, with authorisation Number 246/UN3.1.6/SP/2018 and Ethical Clearance Number 2. KE.010.01.2018.

Study Subjects

This study was an experimental animal model study with Randomized Post-Test Only Control Group design. Samples of this study was female *Rattus norvegicus* (Wistar strain), obtained from Experimental Animal Care Unit at Universitas Gajah Mada, Indonesia. The inclusion criteria were female rats aged 3 months with approximate weight of 200 g and have never been pregnant. Exclusion criteria were sick rats during the samples recruitment. Drop-out criteria were infected rats after the Sham procedure or oophorectomy procedure.

There were 6 groups in this study. Sample size of each group was calculated using Lemeshow formula and corrected with Higgins formula

to anticipate the drop-out[5]. Sample size needed for each group was 9 rats, and the total samples for this study was 54 rats. At first, total samples were randomly divided into 2 groups, control group and intervention group. After that, Sham procedure was done on the control groups, while the bilateral oophorectomy procedure was done on the intervention groups. After the surgery, samples in control and intervention group randomly divided into 3 groups, namely C1, C2, and C3 group for the control group, and I1, I2, and I3 group for the intervention group. Subjects in C1 and I1 group were observed for 4 weeks and then sacrificed, subjects in C2 and I2 group were observed for 8 weeks and then sacrificed, subjects in C3 and I3 group were observed for 12 weeks and then sacrificed.

During sacrifice procedure, 2 cc of samples' intra-cardiac blood was aspirated, and then the blood was centrifuged for 3-5 minutes with 3,000x rotation per minute. The obtained serum was then used to evaluate 17β-estradiol level using ELISA method (Rat Estrogen ELISA Kit, Cat.No E0176 Ra, Bioassay Technology Laboratory, Shanghai, China). Cartilage on the knee joint was also obtained during the sacrifice procedure, and then preserved in the 10% formalin for immunohistochemistry evaluation of MMP-13 and type 2 collagen expression by using specific kit (Rabbit anti-Rat MMP-13 polyclonal and Rabbit anti-Rat Collagen Type 2 Polyclonal Antibody, BIOSS antibodies inc., Massachusetts, USA).

ELISA Examination Procedure

The ELISA examination involved the following procedures. All reagents, standard solutions and samples were prepared at room temperature. One hundred µl of standard solution was poured into a standard well and 100 µl of sample into a sample well, before adding 10 µl biotin conjugated anti E antibody. The diluted antibody was previously incubated for 15-18 hours in 4°C temperature. After that, 50 µl of streptavidin -HRP was added into the standard well prior to sealing it to prevent leakage and the well was incubated for 60 minutes at 37°C. The seal was opened and the plate was cleaned five times with a buffer before submerging it in 0.35 buffer solution for between 30-60 seconds. The plate was cleaned again five times with buffer solution and the area around the plate was cleaned with absorbent paper.

A and B substrate solution was added to each well and then the wells were incubated for ten minutes at 37°C until the color changed. Then the stop solution was added, and the result was read at 450 nm wavelength. The OD (Optical Density) value was measured after 10 minutes.

Immunohistochemical Staining

The histological specimen was deparaffinized and submerged in xylol solution four times for 3-5 minutes on each occasion in order to eradicate the endogenous peroxidase present. The specimen was then placed in absolute ethanol solution three times for 1-3 minutes, before being bathed twice in 70% ethanol for the same duration. It was subsequently washed three times using aqua bidestylata, while the slide border was cleaned with absorbent. Drops of hydrogen peroxide 3% were added before being incubated at room temperature for five minutes. The specimen was then washed three times with PBS 3 and the slide border cleaned. Drops of 0.025% trypsin were added and incubated at 37°C for six minutes before cleaning was carried out three times with aquabidestyilata. In addition, the slide borders were cleaned. Drops of Ultra V Block were added and incubated at room temperature for five minutes. Drops of diluted monoclonal (1:100) were then added, incubated at room temperature for 25-30 minutes, prior to washing three times with PBS and cleaning of the slide border with absorbent was completed.

Drops of Primary Antibody Enhancer appropriate to the examination concerned were added and incubated at room temperature for ten minutes. After washing three times with PBS, drops of Horseradish Peroxidase (HRP) polymer were added and incubated at room temperature for 15 minutes. After washing three times with PBS, the slide border was cleaned.

Drops of the chromogen (20 µ/1 ml substrate) in accordance with the proposed examination were added and incubated at room temperature for 5-15 minutes in a darkened room, prior to washing three times with aquabidestylata and cleaning. Staining with Mayer's hematoxylin solution, incubation at room temperature for 6-15 minutes and washing three times with running water was carried out, before bathing in water for ten minutes as the final cleaning. The results were dried and placed on a microscope object glass.

Statistical Analysis

All data acquired in this study was analyzed using SPSS Version 21. Shapiro-Wilk test was used to analyze the homogeneity of the data. Mann-Whitney test and Kruskall-Wallis test were used to analyze the difference in 17β-estradiol and type 2 collagen expression between groups. Brown-Forsythe test and Games-Howell test were used to analyze the difference in MMP-13 expression between groups.

Results

Table 1 shows the results of 17β-estradiol level measured by ELISA in the control and intervention groups. The estradiol level was decreased significantly in the intervention group compared to the control group ($p<0.05$). There was significant difference between C1 and I1 ($p=0.015$). There was significant difference between C2 and I2 ($p=0.030$) and between C3 and I3 ($p=0.048$), although there was no significant difference between the subgroups in the intervention group. The oophorectomy procedure resulted in a significant reduction in the level of estradiol.

Table 2 contains the results of MMP-13 expression using immunohistochemistry methods. It was found that MMP-13 expression increased significantly in the intervention group ($p<0.05$), but there was no difference between the intervention subgroups (I1, I2, I3). There wasn't significant difference between C1 and I1($p=0.107$), but there was significant difference between C2 and I2 ($p=0.012$). There was significant difference between C3 and I3 ($p=0.002$). It was concluded that oophorectomies cause increased MMP-13 levels in cartilage.

Table 3 shows the results of type 2 collagen expression using an immunohistochemical method. It was found that there was a significant decrease in type 2 collagen levels in the intervention group compared to the control group ($p<0.05$) due to the performing of oophorectomies. There was significant difference between C1 and I1 ($p=0.002$). There wasn't significant difference between C2 and I2 ($p=0.067$) but there was significant difference C3 and I3 ($p=0.004$).

Discussion

Estrogen is synthesized mainly in the ovaries and adrenal gland. About 98-99% circulate around the body in a form bonded to the carrier protein, β-globulin, also referred as sex-hormone-binding globulin (SHBG). The remaining 1-2% of free or unbounded hormones can enter the target cell and bind with a specific receptor, inducing biological effect[6].

Classic estrogen consists of estrone (E_1), estradiol (E_2) and estriol (E3). For women, 17β-estradiol is very important in maintaining the

Table 1. Level of 17 β estradiol

Groups	Level of 17 β Estradiol (E2)					P
	$\bar{x}$	SD	Median	Min	Max	
Control 4 weeks (C1)	9.888	1.596	9.833^{d}	7.238	12.783	0.001*
Control 8 weeks (C2)	8.441	0.797	8.214^{c}	7.264	10.05	
Control 12 weeks (C3)	8.125	1.393	7.881^{bc}	7.056	11.541	
Intervention 4 weeks (I1)	7.976	1.104	7.529^{abc}	6.354	9.802	
Intervention 8 weeks (I2)	7.201	1.156	6.996^{ab}	5.97	8.814	
Intervention 12 weeks (I3)	6.848	0.922	7.058^{a}	5.243	7.936	

*p < 0.05 was considered statistically significant (Kruskall-wallis test)
Values in a column with different superscripts were significantly different (P < 0.05) from each other (Mann-whitney test)

Table 2. Expression of MMP-13

Groups	Expression of MMP-13				P
	$\bar{x}$	SD	Min	Max	
Control 4 weeks (C1)	2.69^{ab}	0.86	1.4	3.8	0.000*
Control 8 weeks (C2)	1.67^{a}	1.06	0.4	3.6	
Control 12 weeks (C3)	1.36^{a}	1.02	0.2	3	
Intervention 4 weeks (I1)	5.11^{b}	2.3	2.8	8.6	
Intervention 8 weeks (I2)	5.55^{b}	2.24	3	10	
Intervention 12 weeks (I3)	3.63^{b}	0.81	2.6	5.2	

*p < 0.05 was considered statistically significant (Brown-Forsythe test)
Values in a column with different superscripts were significantly different (P < 0.05) from each other (Games-Howell test)

Table 3. Expression of type 2 collagen

Groups	Expression of Type 2 Collagen					P
	$\bar{x}$	SD	Median	Min	Max	
Control 4 weeks (C1)	7.33	2.84	6.60^{c}	3	11	0.000*
Control 8 weeks (C2)	5.04	3.29	4.80^{bc}	1.4	12	
Control 12 weeks (C3)	6.51	2.89	5.40^{b}	2	11	
Intervention 4 weeks (I1)	3.22	0.98	2.80^{ab}	2	5.2	
Intervention 8 weeks (I2)	2.55	1.25	2.50^{ab}	0.6	4	
Intervention 12 weeks (I3)	2.18	0.95	2.60^{a}	0.6	3	

*p < 0.05 was considered statistically significant (Kruskall-wallis test)
Values in a column with different superscripts were significantly different (P < 0.05) from each other (Mann-whitney test)

physiologic function of reproductive organs, especially in the growth of follicles, and play an important role in sexual development. Known peripheral effects of this hormone are maintaining secondary sexual characteristic; stimulation of hepatic protein synthesis such as renin substrate and globulin bound by sex hormone. The latest development showed that this hormone also maintains a good bone trabecular structure[7].

The hormone 17β-estradiol is more potent than estrone, so the level of 17β-estradiol becomes a marker for menopause. Estrone only has 1/50 of 17β-estradiol biological potential and estriol 1/80 of 17β-estradiol potential[8].

Estrogen and hypophyseal gonadotrophic hormone stimulated the proliferation of granulous cells, mainly for follicular growth and oocyte development. The main effects of estrogen on genital tissue include: (1) inducing endometrial proliferation in the uterus, (2) influencing the production of cervical secretion until it attains its maximum level at the mid-point of the menstrual cycle, and (3) maintaining the mucosal surface of the vagina in a healthy condition by a process of vaginal epithelium maturation[6].

Extragenital effects including secondary sexual characteristic development (estrogen constitutes the dominant stimulus in breast development at the onset of puberty), induction of protein synthesis (Sex hormone-binding globulin and renin substrate), maintenance of bone structure and prevention of osteoporosis. In menopausal women, the level of estradiol is frequently reduced leading to osteoporosis. This process is also thought to affect cartilage in osteoarthritic patients[7].

Menopause represents the cessation of the menstrual cycle in women due to the reduction in the level of estrogen. The postmenopausal period is one commencing at the cessation of menstruation until 12 months later.

Estrogen plays a major role in the functioning of the genital organs, including: sexual differentiation and sexual/reproductive organ development, in addition to exerting an extragenital effect on the endometrium, breast, non-joint bone and cartilage[6]. Estrogen also actively maintains the homeostasis of bone and cartilage, especially in postmenopausal os-

teoarthritic women[9]. In experimental subjects, oophorectomies cause an increase in cartilage turnover and erosion of cartilage surfaces. However, the mechanisms of this condition are still unclear[10].

This study showed that there was a significant post-oophorectomy reduction in 17β- estradiol in the experimental subjects compared to that following the sham procedure ($p<0.05$). It also indicated an increase in MMP-13 expression in oophorectomized subjects compared to those undergoing the sham procedure ($p<0.05$).

According to the study by Ito et al., estrogen could inhibit the TGF-β signaling pathway. In menopausal condition, in which estrogen level is decreased, the TGF-β signaling pathway would be disinhibited[9]. The effect of increasing TGF-β on chondroprogenitor cells is RUNX2 expression in the nuclei. An increase in hypertrophic chondrocyte cells is commonly caused by RUNX2 expression. As an intracrine, RUNX2 stimulates differentiation towards chondrocyte hypertrophy[6,11,12]. Hypertrophic chondrocyte expresses several proteins, namely: alkaline phosphatase (ALP), vascular endothelial growth factor (VEGF), osteopontin, matrix metalloprotease 13 (MMP-13). The effect of increased MMP-13 expression is the degradation of collagen, resulting in reduced expression of type 2 collagen in the joint cartilage of the subjects[13].

Several studies showed that administration of 17β-estradiol would decrease osteoclast activity by ERα activation, resulting in new bone formation and type-1 collagen synthesis. Moreover, estrogen or the receptor activation would upregulate osteoblast formation[14]. New bone formation would increase subchondral bone density which in turn will decrease the probability of OA. Another mechanism how estrogen may be protective to joint cartilage is by downregulation of inflammatory cytokines. Estrogen inhibits the formation of pro-inflammatory cytokines such as IL-1β and TNF-α and modulate the expression of anti-inflammatory cytokine like IL-10, independent from the HPA axis[15]. The production of MMP-13 is affected by said pro-inflammatory cytokines[16]. Thus, estrogen would decrease the expression of MMP-13, as demonstrated by Claassen et al[17].

Turner et al. demonstrated that oophorectomies conducted on sheep had a significant effect on the aggregate modulus and shear modulus of articular cartilage, without any change to cartilage thickness[6]. Christgau et al. found post-oophorectomy increased cartilage degradation and detected CTX II in mouse urine[18]. In postmenopausal women, there is an increase cartilage degradation and increased CTX II excretion in the urine which improved after the administering of levormeloxifene (SERM)[18].

Andersen et al. found that oophorectomized rats had increased joint cartilage erosion/degradation due to estrogen depletion and the administration of exogenous estrogen or SERM would inhibit the rate of degradation[10]. However, it didn't explain the exact mechanism of how estrogen depletion would lead to increased cartilage turnover as we aimed in our study. Our study found the similar result that oophorectomy would decrease the level of estrogen. The decrease of estrogen in turn would lead to increase the expression of MMP-13 which degrades the type 2 collagen in the joint cartilage.

Cartilage is formed by chondrocytes and its extracellular matrix (ECM). Cartilage degradation will cause turnover type 2 collagen, because forms a collagen mesh which determines the strength of the cartilage[16]. The type 2 collagen content of cartilage amounts to 90-95% with the result that its degradation will weaken the cartilage[13].

There are 28 types of MMP contained in body tissue, but it is mainly MMP-13 which induces the cartilage to degrade type 2 collagen[19]. MMP-13 is also produced by the synovial cell, macrophage and chondrocytes influenced by IL-1 and TNF- α[20]. Number study showed that inflammation influenced IL-1 and TNF- α production and caused increasing MMP- 13[21]. This study showed that the decrease of 17β-estradiol resulted in elevated MMP-13 expression in cartilage (Table 2). MMP mechanism will induce enzymes to destroy organ matrix, including oxidant, disulfide, alkylating agent, via a switching mechanism. In joint cartilage, this process is mainly due to oxidant activation which destroys collagen protein[19]. Therefore, 17β-estradiol affected type 2 collagen levels in cartilage leading to an increased risk of osteoarthritis on postmenopausal women[22].

The study by Asano et al. (2006) found that the production of MMP-13 in the synovium is affected by levels inflammatory cytokines, IL-1 and TNF-α. Thus, tissue inflammation would increase MMP-13 expression[16]. Our study showed that estrogen level depletion also increases MMP-13 expression trough hypertrophic chondrocyte formation. Further study is needed to assess whether this phenomenon is primarily caused by inhibition of TGF-β pathway or by the anti-inflammatory effect of estrogen.

Other studies found that there is a correlation between muscle mass and the incidence of OA. Estrogen in the body keep the muscle mass intact, and in post-menopause women, when the estrogen level is depleted, the muscle mass is reduced as well[23, 24]. However, in our study, we did not evaluate the effect of muscle mass. Further study needs to be done to determine the dominant factor of the OA, whether it is the muscle mass or the increase in MMP-13 and decrease in collagen-type 2.

In conclusion the decrease in 17β-estradiol will decrease type 2 collagen through the increase of MMP 13 on joint cartilage.

Competing interests

The authors declare that there is no conflict of interest with respect to research, sponsorship and/or publication of this article.

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