Phytoestrogen (Daidzein) Promotes Chondrogenic Phenotype of Human Chondrocytes in 2D and 3D Culture Systems

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Abstract Clinical investigations have shown a significant relationship between osteoarthritis (OA) and estrogens levels in menopausal women. Therefore, treatment with exogenous estrogens has been shown to decrease the risk of OA. However, the effect estrogen has not been clearly demonstrated in the chondrocytes using phytoestrogens, which lack the specific side-effects of estrogens, may provide an alternative therapy. This study was designed to examine the possible effects of phytoestrogen (daidzein) on human chondrocyte phenotype and extracellular matrix formation. Phytoestrogens which lack the specific side-effects of estrogens may provide beneficial effect without causing hormone based side effect. Human chondrocytes cells were cultured in 2D (flask) and 3D (PCL-CA scaffold) systems. Daidzein cytotoxic effect was determined by MTT assay. Chondrocyte cellular content of glycosaminoglycans (GAGs), total collagen and chondrogenic gene expression were determined in both culture systems after treatment with daidzein. Daidzein showed time-dependent and dose-independent effects on chondrocyte bioactivity. The compound at low doses showed significant ($p < 0.05$) increase in total collagen and GAGs production at similar levels in 2D and 3D culture environment. The mRNA levels of Collagen II and Sox9 were increased significantly ($p < 0.01$) after the treatment while the upregulation in COMP expression was statistically insignificant ($p > 0.05$). The expression levels of Fibronectin, Laminin and Integrin $\beta 1$ were significantly increased especially in 3D culture system. This study was illustrated the potential positive effects of daidzein on maintenance of human chondrocyte phenotype and extracellular matrix formation suggesting an attractive and viable alternative therapy for OA.

Keywords Phytoestrogens · Human chondrocyte · Extracellular matrix · PCL-CA scaffold · Osteoarthritis

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Abbreviations

CTX-II	C-terminal crosslinked telopeptide type II collagen
CA	Citric acid
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
DMEM	Dulbecco's Modification of Eagle's Medium
DMMB	Dimethylmethylene blue

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DMSO	Dimethyl sulfoxide
ERT	Estrogen replacement therapy
ECM	Extracellular matrix
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAGs	Glycosaminoglycans
h	Hour
IL-1 β	Interleukin-1 β
NO	Nitric oxide
MMP	Matrix metalloproteinase
MNTD	Maximal non-toxic dose
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PCL	Polycaprolactone triol
PCL-CA	Polycaprolactone triol-citrate
SEM	Scanning electron microscopy
17 β -estradiol	17Beta-estradiol

1 Introduction

The characteristic pathological changes in osteoarthritis (OA) involve articular cartilage degeneration, subchondral bone thickening, osteophyte development and synovial inflammation, which are accompanied with capsule laxitude and decreased muscle strength [1]. Over a prolonged period of time, chondrocytes senescence and death lead to low cartilage cellularity in aging joints and thus promoting pathogenesis of OA [2]. In addition, as chondrocytes is the sole source of ECM synthesis, chondrocytes apoptosis, which also indicates the absence of the renewal of chondrocyte population, causes a failure in turnover of cartilage ECM [3].

In both genders, the incidence of OA elevates with age but postmenopausal women possess a higher prevalence of OA [1, 4, 5]. As shown in a nationwide survey, radiographic OA is three times more prevalent among women within the age of 45 to 64 years old [6]. Other than the multiple physiopathological mechanisms implicated in OA, sex hormone particularly estrogen has been attracting much attention. A significant increase in OA prevalence among postmenopausal women is accompanied with the existence of estrogen receptors in joint tissues, implies a relationship between OA and postmenopausal state [6]. A large epidemiological study conducted in Italy has supported the statement that estrogen deficiency increases the risk of OA [7]. Animal models exhibit that the depletion of estrogen promotes the incidence of OA [8]. In a rat model, ovariectomy which causes estrogen deficiency has accelerated cartilage degradation and erosion [9].

Several *in vitro* studies have demonstrated that the cartilage metabolism is regulated by estrogens. In the cultures of rabbit joint chondrocytes, the 17 β -estradiol (E₂), the naturally occurring estrogen, improves glycosaminoglycan (GAG)

synthesis by upregulating the uridine diphosphate glucose dehydrogenase gene [10]. Furthermore, E₂ impedes the cyclooxygenase-2 mRNA expression in bovine articular chondrocytes and defends the chondrocytes from reactive oxygen species-induced damage [11]. Nevertheless, high concentrations of E₂ suppress DNA synthesis in human chondrocytes [12] and inhibit proteoglycan synthesis [13]. *In vivo* studies on the effects of estrogen on cartilage in ovariectomized Sprague-Dawley rats [14] and ovariectomized cynomolgus macaques [15] showed an increased in cartilage turnover and surface erosion. Concisely, both low and high concentrations of estrogen have a deleterious effect on the normal balance of joints and estradiol concentration in cartilage microenvironment dictates the final effect [6].

In the context of therapy for postmenopausal OA, estrogen replacement therapy (ERT) is employed. Estrogen enhances chondrocyte proliferation and maturation, inhibits pro-inflammatory cytokines and matrix metalloproteinases formation and protect the chondrocytes against reactive oxygen series [8]. A cross-sectional study showed that ERT significantly decrease the risk of radiographic hip OA, especially in long-term user [1]. As a result of cartilage degradation, fragments of C-terminal crosslinked telopeptide type II collagen (CTX-II) were released into circulation and eventually secreted into urine. Study has discovered that the CTX-II concentration in postmenopausal women was greater than that of the premenopausal women, significantly lower level of CTX-II was observed in women underwent hormone replacement therapy [6]. Although ERT could decrease the severity and incidence of postmenopausal OA, ERT is impeded by an increased risk of breast cancer, myocardial infarction and stroke [1, 4, 5].

Phytoestrogens are plant-derived compounds that can be isolated from various plants such as Leguminosae plant, *Psoralea corylifolia* [8]. Interestingly, the phytoestrogen daidzein showed an ability to bind to the estrogen receptors and serve as natural selective estrogen receptors [4, 8]. Thus, this study was designed to evaluate the potential effects of daidzein, which lack of the side effects of estrogen, as an alternative to treat postmenopausal OA through activation of chondrocytes functions and enhance its ability in extracellular matrix formation.

2 Materials and methods

2.1 Human chondrocytes expansion

Human chondrocytes (Purchased from Lonza, USA) were cultured in growth medium consisting of Dulbecco's modified Eagle's Medium (DMEM; Gibco-Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen, USA) or 2% FBS in maintenance

medium and incubated at standard conditions (humidified atmosphere, 5% CO₂, 37 °C) for enough expansion.

2.2 Cell proliferation assay

Daidzein (Sigma-Aldrich UK, Cat# D7802) was dissolved in DMSO to prepare 10 mg/ml, then a stock concentration of 1 mg/ml was prepared. The maximal non-toxic dose (MNTD value) was determined by MTT assay method after adjusting the final concentration of DMSO to be less than 1%. Human chondrocytes were seeded at 1×10^4 cells per well in triplicate at optimal conditions (5% CO₂, 37 °C in humidified incubator) in 96 well plates incubated for 24 h. Following 24, 48 and 72 h of incubation with increasing concentrations of the compound (0–400 µg/ml), a 10 µl solution of freshly prepared 5 mg/ml MTT in PBS was added to each well and allowed to incubate for an additional 2 to 4 hours (h). Next, the media was aspirated and DMSO was added at 100 µl/well. Then, the plates were swirled gently to facilitate formazan crystal solubilization. By using a microplate reader (Tecan Infinite M200 Pro, Switzerland), the absorbance was measured at 570 nm. The percent of cell viability was calculated by using the formula below.

Percent of cell viability

$$= 100 - \left(\frac{\text{Absorbance of phytoestrogen of treated cells}}{\text{Absorbance of untreated cells}} \right) \times 100$$

2.3 Polymer scaffold preparation

The polymer was produced by mixing equimolar amounts of both the citric acid (CA) and polycaprolactone triol (PCL), PCL; Mw = 300 (Sigma-Aldrich) together and the mixture was heated up to 160–165 °C until CA crystals melted. The reaction mixture was further mixed at 140–145 °C for 1 h under a constant stream of nitrogen. Thus formed polycaprolactone triol-citrate (PCL-CA) pre-polymer was processed into scaffolds by solvent-casting/particulate leaching method as we described previously [16, 17].

2.4 Seeding of human chondrocyte into PCLT-CA scaffolds

The scaffold was placed in 24 well plates, washed 3 times with PBS and left to dry at room temperature and placed under UV for overnight. The sterilised scaffold was soaked with FBS-free DMEM for 3 h, the media were removed and the scaffold was left for another 3 h in the incubator. Then, the desired amount of the cells were dropped into the scaffold and left for another 1 h in the incubator (37 °C and 5% CO₂) before top up with the media.

2.5 Cellular content of total collagen and GAGs

Cells were seeded into T25 flask (4×10^5 /T75 flask) or into scaffolds (4×10^5 /scaffold) and grown in maintenance medium 2% FBS as control or supplemented with daidzein. To study the effect of increasing concentrations of daidzein on total collagen and GAGs production, chondrocytes were incubated with 10, 20 and 30 µg/ml of daidzein for 72 h. While the cells were incubated with 20 µg/ml of daidzein for 24, 48 and 72 h to evaluate the effects of the time of exposure to phytoestrogens. After the cell-conditioned medium was removed, cells were detached by trypsinisation and cell number was evaluated by Hemocytometer method and equal number of cells were used for the assays.

Cellular content of GAGs was determined using the dimethylmethylene blue (DMMB) assay as described previously [18]. GAGs concentrations were estimated from a standard curve of chondroitin sulphate (Sigma-Aldrich UK). The DMMB solution was added to the sample and standards prior to reading absorbance values at 525 nm. To measure total cellular soluble collagen, total collagen kit was used (QuickZyme BioSciences, Leiden-Netherlands) as described previously [16]. The cells were incubate overnight at 4 °C on a rotating platform with 0.5 M Acetic acid. The supernatant of cellular extract was tested in the assay in 1-fold to 10-fold dilutions made in dilution buffer and read at a wavelength of 540 nm. All the measurements were performed in triplicates using Tecan Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland).

2.6 Western blot

Western blot analysis was carried out to determine the expression levels of chondrogenic protein markers. Briefly, cells lysates for immunoblotting were prepared, using ice-cold lyses buffer. Equal amount of protein was loaded (20 µg) in Western blot gels using 2-D Quant Kit (GE Healthcare BioSciences, USA) according to the manufacturer's instructions. The separated proteins were shifted on to nitrocellulose membrane and subsequently, sealed with blocking buffer. The membrane and specific antibody were incubated overnight to detect the expression levels of COMP (abcam, USA, cat. #ab42225), Sox9 (abcam, USA, cat. #ab26414) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (abcam, USA, cat. #ab8245) as a loading control protein. The membrane can then be further processed with washing again with Tris Buffered Saline since described previously, and finally build up using Western Blue®stabilized substrate (Promega, USA).

2.7 Indirect immunostaining

Cells were grown in T75 flask (5×10^5) and treated with 20 µg/ml of daidzein and incubated for 24, 48 and 72 h to study

the effect of the treatment on collagen II expression. Then, cells were trypsinised and reseeded on cover slides fixed in 6-well plates and for 24 h. Next, the cells were washed three times with PBS and fixed with ice-cold methanol for 15 min at -20°C . After the washing steps, the cells were incubated with a coating buffer for 1 h at room temperature. A mouse antibody specific to collagen II (abcam, USA, cat. # ab3092) was added and the cells were incubated for overnight at 4°C . The cells were washed three times with PBS and incubated for 30 min with an anti-mouse IgG labeled with FITC fluorescent dye (Invitrogen, USA, cat. # 62-6511).

2.8 Real time PCR

Real time was employed to study the expression levels of chondrogenic (collagen II, COMP and Sox9) and extracellular gene markers (Collagen I, III, Fibronectin, Laminin and Integrin Beta1) after the treatment with daidzein in reference to GAPDH expression levels. Briefly, human chondrocytes were seeded in flask or scaffold and incubated with maintenance DMEM medium supplemented with 20 $\mu\text{g/ml}$ of daidzein (Treatment group) or without (Control group). Cells were harvested after 72 h and total RNA was extracted using Trizol method (Invitrogen, USA), purified by RNA purification kit (Promega, USA) and quantified by nanospectrophotometer. The amount of 1 μg of pure RNA was used for gene expression analysis. Reverse-transcription was performed using cDNA synthesis kit (Invitrogen, USA) to prepare first strand cDNA. Real time-PCR was performed using SYBR Green PCR kit (Applied Biosystems). The PCR program consisted of an initial step of 10 sec at 95°C , followed by 40 cycles of denaturing at 95°C , annealing at 50°C for 5 second and extension at 60°C for 31 sec. The relative level of genes expression was quantified using GAPDH gene as an endogenous control.

2.9 Statistical analysis

All the assays were done in triplicates and the statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P values of <0.05 were considered significant. Error bars are expressed as $\pm$ SD.

3 Results

3.1 Cell viability

The results of cell viability assay showed that the MNTD value of daidzein was 30 $\mu\text{g/ml}$ for 24, 48 and 72 h of the treatment with daidzein. This dose showed approximately 90% of cell viability. Therefore, to eliminate the cytotoxic effects of daidzein on human chondrocyte, the doses equal

or less than 30 $\mu\text{g/ml}$ were used in the following experiments (Fig. 1).

3.2 Daidzein showed dose-independent effects on the production levels of GAGs and total collagen in human chondrocytes

In both 2D and 3D culture under the same condition, daidzein showed a dose-independent effect on the GAGs and total collagen production by human chondrocytes. In the context of daidzein concentrations 10, 20 and 30 $\mu\text{g/ml}$ of daidzein increased the production of GAGs and total collagen significantly in which the 20 $\mu\text{g/ml}$ of daidzein treatments exhibited the most significant positive anabolic effects in both 2D and 3D culture. In the aspect of culture systems, daidzein treated human chondrocytes cultured in 3D scaffold showed produced higher level of GAGs and total collagen production than that of 2D culture, suggested that the 3D culture condition is more significant in inducing GAGs and total collagen formation by human chondrocytes. Human chondrocytes cultured in 3D scaffold treated with 20 $\mu\text{g/ml}$ of daidzein displayed the most significant increase in GAGs and total collagen production, followed by 2D chondrocytes culture supplemented with 20 $\mu\text{g/ml}$ of daidzein (Fig. 2).

3.3 Daidzein showed time-dependent effects on the GAGs and total collagen production

Human chondrocytes were cultured in 2D and 3D culture environment to determine the effect of daidzein on ECM formation, including the GAGs and total collagen production. In both 2D and 3D culture systems, daidzein has a time-dependent effect on the GAGs and total collagen production by human chondrocytes. Daidzein treatments increased both

Fig. 1 Determination of cell viability after the treatment with daidzein. An MTT assay was used to determine the *in vitro* cells viability. Human chondrocytes were incubated for 24, 48 and 72 h of incubation with increasing concentrations of the compound (0–400 $\mu\text{g/ml}$). The MNTD value of daidzein was 30 $\mu\text{g/ml}$ for all of time points. This dose showed approximately 90% of cell viability

Fig. 2 The effect of daidzein concentration on the production levels of GAG and total collagen in human chondrocytes. Cells were seeded into T25 flask (4×10^5 /T75 flask) or into scaffolds (4×10^5 /scaffold) and grown in maintenance medium 2% FBS as control or supplemented with 10, 20 and 30 $\mu\text{g/ml}$ of daidzein for 72 h. Cellular content of GAGs was determined using the dimethylmethylene blue

(DMMB) assay. Total collagen kit was used to measure total cellular soluble collagen. The concentration of 20 $\mu\text{g/ml}$ of daidzein showed the highest levels of GAG production in the 2D and 3D culture systems ($p < 0.05$, Two way ANOVA). Similarly the same concentration induced collagen production in human chondrocytes compared to low and high doses ($p < 0.05$, Two way ANOVA)

GAGs and total collagen production significantly with increasing incubation time. Compared to incubation time of 24 h, 72 h of treatment showed the most significant increase in GAGs and total collagen production, followed by 48 h of treatment. In 2D and 3D condition, daidzein treated chondrocytes produced similar level of GAGs and total collagen. Moreover, the production level of GAGs was significantly higher than that of total collagen (Fig. 3)

3.4 Effect of daidzein on chondrogenic phenotype in 2D and 3D culture systems

Prominent morphological differences were observed between chondrocytes cultured in 2D and 3D systems. After 72 h of daidzein treatment, chondrocytes in 2D culture whether in sparse (Fig. 4A) or confluent status

(Fig. 4B) displayed a fibroblast-like morphology. The SEM images illustrated the pore size, pore distribution and the surface morphology of PCL-CA scaffold. The pores are homogeneously dispersed in the scaffold with an average diameter 200 to 300 μm (Fig. 4C, D). Human chondrocytes showed rounded shape of chondrocytes in PCL-CA scaffold which are similar to the chondrocytes in native cartilage (Fig. 4E). Western blotting and gene expression analysis were employed to confirm these speculations. Results of western blotting showed that Sox9, an earliest marker in chondrogenesis, was upregulated in both 2D and 3D culture systems compared to control. On the contrary, COMP, a cartilage specific marker regulated by Sox9, showed insignificant changes in both treated groups in 2D and 3D culture conditions compared to control (Fig. 4F). Indirect immunostaining of collagen II, the marker of

Fig. 3 The effect of time of incubation with daidzein on the production levels of GAG and total collagen in human chondrocytes. Cells were seeded into T25 flask (4×10^5 /T75 flask) or into scaffolds (4×10^5 /scaffold) and grown in maintenance medium 2% FBS as control or supplemented with 20 $\mu\text{g/ml}$ of daidzein for 24, 48 and 72 h to evaluate the effects of the time of exposure to phytoestrogens.

Cellular content of GAGs was determined using the dimethylmethylene blue (DMMB) assay. Total collagen kit was used to measure total cellular soluble collagen. GAG production increased two-fold at 72 h compared with 24 h ($p < 0.01$, Two-way ANOVA). The total collagen production showed significant increase ($p < 0.05$, Two-way ANOVA) at 72 h compared to 24 h

Fig. 4 Morphological differences of chondrocytes cultured in 2D and 3D systems and the expression levels of chondrogenic protein markers after treatment with daidzein. After 72 h of daidzein treatment, chondrocytes in 2D culture whether in sparse or confluent status displayed a fibroblast-like morphology **A** Sparse form of the cells; **B** The confluent form of the cells while in the scaffold; **C** The external appearance of PCL-CA scaffold and the internal appearance under SEM (1 mm); **D** The internal appearance PCL-CA scaffold under SEM (0.4 mm) as 3D culture system the cells showed rounded shape; **E** PCL-CA scaffold seeded with chondrocytes as pointed by white arrows. Expression of chondrogenic markers **F** Western blot analysis of chondrogenic markers showed upregulation of SOX9 in 2D and 3D culture systems, while insignificant changed were observed in the expression level of COMP protein in both culture systems. Protein expression of chondrogenic markers was evaluated by western blotting and normalized to the expression levels of GAPDH. (C control, T treatment)

chondrogenesis, with FITC fluorescent dye showed considerable upregulation due to the treatment with daidzein for 24, 48 and 72 h (Fig. 5).

3.5 Gene expression analysis

Total RNA isolated from both 2D and 3D culture was purified and quantified by using RT-PCR. Gene expression of chondrogenic markers were normalized to the GAPDH expression level. Gene expression analysis was used to determine the maintenance of cells as chondrocytes and expression of ECM genes in both culture systems. The gene markers are categorized into 3 groups namely transcription factor (COMP and Sox9), collagen network (collagen type I, II and III) and receptor and its ligand (integrin β 1 and the fibronectin and laminin as ligands). In both 2D and 3D culture systems, as compared with the controls, daidzein treatment increased the expression level of Sox9, collagen type I, II and III, fibronectin, laminin and integrin β 1, in which Sox9 and collagen type II showed the most significant upregulation (Fig. 6).

In 2D culture system, phytoestrogen treatment upregulated Sox9 gene expression level significantly, which was higher than that of COMP. Collagen type II gene expression was the most significant compared with collagen type I and III. The upregulation in the gene expression of fibronectin, laminin and integrin β 1 were equally significant. Similarly, in 3D culture system, phytoestrogen treatment upregulated the Sox9 and collagen type II expression significantly. Compared to daidzein treatment in 2D culture, 3D culture system showed a more significant upregulation in the expression level of fibronectin and integrin β 1. In both 2D and 3D culture systems, collagen type I and III gene expression level did not show comparable differences (Fig. 6).

4 Discussions

Our findings defined the important of daidzein in inducing the expression level of Sox9, which is essential for chondrogenic differentiation and matrix production. Several

Fig. 5 Indirect immunostaining of collagen II with FITC fluorescent dye. Human chondrocytes were grown in T75 flask (5×10^5), treated with 20 $\mu\text{g}/\text{ml}$ of daidzein and incubated for 24, 48 and 72 h to study the effect of the treatment on the expression levels of collagen II. The cells were trypsinised and reseeded on cover slides fixed in 6-well plates and for 24 h, washed with PBS and fixed with ice-cold

methanol. The fixed cells were incubated with mouse antibody specific to collagen II and then incubated with anti-mouse IgG labeled with FITC fluorescent dye. The results showed considerable up regulation of collagen II due to the treatment with daidzein for all time points

Fig. 6 Gene expression analysis of chondrogenic and extracellular gene markers after the treatment with daidzein 2D and 2D culture systems. Daidzein induced the expression levels of chondrogenic gene markers SOX9 and Collagen II ($p < 0.01$), while insignificant difference in COMP expression in both culture systems. The expression levels of extracellular matrix genes were upregulated after the treatment with daidzein. Fibronectin and integrin beta1 were significantly upregulated in 2D ($p < 0.05$) and 3D ($p < 0.01$) systems. Similar upregulation of laminin ($p < 0.05$) was observed in both systems. Insignificant difference in the expression levels of collagen I and III were observed in both culture systems after the treatment with daidzein

studies have showed that the *in vitro* chondrocytes expansion in 2D culture system resulted in an inevitable chondrocytes dedifferentiation which is characterized by the fibroblastic-like morphology of

chondrocytes, decreased ECM production, upregulation of collagen type I gene expression and downregulation of collagen type II gene expression as well as a changed in chondrocyte cell surface antigen [19–21]. In this study,

despite of the altered chondrocyte morphology, the significant increase in GAGs and total collagen production, prominent upregulation in collagen type I, II and III gene expression suggested that chondrocytes in 2D culture retained its chondrogenic properties and did not undergo dedifferentiation. In other words, it may be the positive effects of daidzein in maintaining the properties of monolayer cultured chondrocytes in the aspect of ECM production and chondrogenic gene markers. Other studies reported that the chondrocytes dedifferentiation process can be decelerated by supplementing growth factors to the culture medium [22–24]. Thus, this study had implied the potentiality of daidzein in retaining the differentiated state of chondrocytes and in promoting ECM deposition. The maintenance of the rounded chondrocyte phenotype depicted in 3D culture is in agreement with the results of several studies [25–28], which had proved that the 3D culture system favors the maintenance of chondrocyte phenotype. In addition, if the scaffold-cell composite is used for in vivo engineering after implantation, the implanted tissue constructs are a subject to repetitive shear forces induced by joint movements and compression under the body weight. Since these forces are vital for chondrocyte cells and maintenance of healthy cartilage metabolism, the mechanical integrity of the scaffolding material should be conducive to physical strain in multiple directions. In particular, CA-based elastomers have demonstrated flexibility, hydrophilic surface and substantial swelling in water, with mechanical properties that are proven to be beneficial to chondrocyte cells [29].

COMP plays its structural role in assembly and stabilization of ECM by interacting with collagen fibrils (collagens I, II, and IX) and matrix components such as fibronectin via carboxyterminal globular domain [30]. From the western blotting results, the insignificant changes of COMP in both 2D and 3D culture system implied that daidzein treatment did not increase the expression of COMP. Studies have reported that the human articular chondrocytes express estrogen receptors alpha and beta (ER α and ER β) which implied that cartilage is an estrogen-targeted tissue [8, 31]. The results of this study showed that chondrocytes in both 2D and 3D culture systems responded to the phytoestrogen and the phytoestrogen (daidzein) treatment exerted positive effects on human chondrocytes by upregulating the GAGs and total collagen production as well as chondrogenic gene expression significantly.

Despite physiological doses of phytoestrogen are not known [32], we have demonstrated that a 20 μ g/ml of daidzein optimal in enhancing the GAGs and total collagen production in both culture systems, indicated the anabolic effect on articular cartilage. In both 2D and 3D culture systems, 10 μ g/ml of daidzein increased the GAGs and total collagen production minimally whereas 30 μ g/ml of

daidzein repressed the GAGs and total collagen production which seemed to be destructive for articular cartilage. Under the same concentration of daidzein, higher production of GAGs and total collagen were observed in 3D culture, which suggested that daidzein further enhance the chondrogenic environment imposed by 3D culture. The 3D culture system resembles the natural microenvironment of chondrocytes in cartilage where the cell-cell and cell-matrix interactions are promoted thereby the signals enhance ECM production. Supplementation of daidzein further stimulated the chondrocytes to produce higher level of GAGs and total collagen.

Daidzein treatment in both 2D and 3D culture system increased the COMP gene expression but Sox9 gene expression level was higher than COMP. This can be explained by the role of Sox9 as a regulator for the genes encoding COMP. Sox9 is an early marker which mediates the activation of COMP by binding itself to the positive regulatory regions with the association of transcription factor Sox5 and Sox6. In this study, treatment of daidzein has upregulated the expression of Sox9 but not COMP. It may be due to the time point selected for the analysis of chondrogenic gene expression could be the initial stage of COMP gene expression. This speculation can be supported by a finding stating that, in chondrogenic medium, COMP gene expression increased gradually with Sox9 within the first week of incubation [33].

In both 2D and 3D culture systems, daidzein treatment on chondrocytes upregulated upregulated collagens type I, II and III gene expression. Collagen type III has been described as a modulator in collagen type I fibril formation [34], thus the increased collagen type III gene expression in this study showed the association between collagen type I and II in mRNA level and resulted in an increased total collagen production in protein level.

Fibronectin promotes cell-cell interaction thereby defines the development of a chondrogenic differentiation promoting microenvironment [35]. The expression pattern of integrin subunits varies during chondrogenesis where the expression of integrin β 1 is prominent in mature chondrocytes [36]. Integrin β 1, the heterodimeric transmembrane glycoprotein, modulates the interactions between ECM proteins and chondrocytes, in other words, regulating the cell-matrix adhesion and cell-cell contacts [37]. Daidzein upregulated the integrin β 1 gene expression and this greater expression of integrin β 1 in protein level was essential for chondrocytes adherence to ECM. In addition, the attachment of chondrocytes to ECM including fibronectin and laminin is primarily stimulated by daidzein.

In addition to positive effects of 20 μ g/ml of daidzein on GAG and total collagen production shown in this study, 8 demonstrated that high concentration (10 – 20 μ M) of purified phytoestrogen, bavachin reduced IL-1 β -induced

production of chemokines, thereby hindered inflammatory cell migration indirectly [8]. Furthermore, *in vitro* study showed that genistein, a phytoestrogen which structurally resembles estrogen, decreased the formation of lipopolysaccharide-induced cyclooxygenase (COX)-2 in chondrocytes [38]. Clinical study showed that daily consumption of 88 mg of phytoestrogen is beneficial for OA patients [39]. Taken these together, we have showed the positive effects of phytoestrogens suggests that the phytoestrogens would be an appealing and feasible therapeutics compound for maintenance of chondrocyte phenotypes, therefore may provide a beneficial effect for treating OA.

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Compliance with ethical standards

Conflict of interest We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Ethical statement There are no human or animal specimens carried out for this study. The cells were purchased from Lonza, USA.

References

- Roman-Blas JA, Castaneda S, Largo R, Herrero-Beaumont G. Osteoarthritis associated with estrogen deficiency. *Arthritis Res Ther*. 2009;11:241.
- Mobasheri A, Kalamegam G, Musumeci G, Batt ME. Chondrocyte and mesenchymal stem cell-based therapies for cartilage repair in osteoarthritis and related orthopaedic conditions. *Maturitas*. 2014;78:188–98.
- Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res*. 2001;3:107–13.
- Claassen H, Briese V, Manapov F, Nebe B, Schunke M, Kurz B. The phytoestrogens daidzein and genistein enhance the insulin-stimulated sulfate uptake in articular chondrocytes. *Cell Tissue Res*. 2008;333:71–9.
- Ham KD, Oegema TR, Loeser RF, Carlson CS. Effects of long-term estrogen replacement therapy on articular cartilage IGFBP-2, IGFBP-3, collagen and proteoglycan levels in ovariectomized cynomolgus monkeys. *Osteoarthritis Cartil*. 2004;12:160–8.
- Martín-Millán M, Castañeda S. Estrogens, osteoarthritis and inflammation. *Joint Bone Spine*. 2013;80:368–73.
- Parazzini F. Menopausal status, hormone replacement therapy use and risk of self-reported physician-diagnosed osteoarthritis in women attending menopause clinics in Italy. *Maturitas*. 2003;46:207–12.
- Cheng C-C, Chen Y-H, Chang W-L, Yang S-P, Chang D-M, Lai J-H, et al. Phytoestrogen bavachin mediates anti-inflammation targeting I κ B kinase-I κ B α -NF- κ B signaling pathway in chondrocytes in vitro. *Eur J Pharmacol*. 2010;636:181–8.
- Mahajan A, Tandon V, Verma S, Sharma S. Osteoarthritis and menopause. *J Indian Rheumatol Assoc*. 2005;13:21–5.
- Maneix L, Beauchef G, Servent A, Wegrowski Y, Maquart FX, Boujrad N, et al. 17 β -oestradiol up-regulates the expression of a functional UDP-glucose dehydrogenase in articular chondrocytes: comparison with effects of cytokines and growth factors. *Rheumatology (Oxford)*. 2008;47:281–8.
- Claassen H, Schunke M, Kurz B. Estradiol protects cultured articular chondrocytes from oxygen-radical-induced damage. *Cell Tissue Res*. 2005;319:439–45.
- Scranton PE Jr, McMaster JH, Diamond PE. Hormone suppression of DNA synthesis in cultured chondrocyte and osteosarcoma cell line. *Clin Orthop Relat Res*. 1975;112:340–8.
- Talwar RM, Wong BS, Svoboda K, Harper RP. Effects of estrogen on chondrocyte proliferation and collagen synthesis in skeletally mature articular cartilage. *J Oral Maxillofac Surg*. 2006;64:600–9.
- Høegh-Andersen P, Tankó LB, Andersen TL, Lundberg CV, Mo JA, Heegaard A-M, et al. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. *Arthritis Res Ther*. 2004;6:R169–80.
- Ham KD, Loeser RF, Lindgren BR, Carlson CS. Effects of long-term estrogen replacement therapy on osteoarthritis severity in cynomolgus monkeys. *Arthritis Rheum*. 2002;46:1956–64.
- Rothan HA, Djordjevic I, Bahrani H, Paydar M, Ibrahim F, Abd Rahman N, Yusof R. Three-dimensional culture environment increases the efficacy of platelet rich plasma releasate in prompting skin fibroblast differentiation and extracellular matrix formation. *Int J Med Sci*. 2014;11:1029–38.
- Djordjevic ICN, Dutta NK, Kumar S. Synthesis and characterization of novel citric acid-based polyester elastomers. *Polymer*. 2009;50:1682–91.
- Ratcliffe ABP, Saed-Nejad F, Shurety W, Catterson B. Synovial fluid analyses detect and differentiate proteoglycan metabolism in canine experimental models of osteoarthritis and disuse atrophy. *Agents Actions Suppl*. 1993;39:63–7.
- Xu F, Xu L, Wang Q, Ye Z, Zhou Y, Tan WS. 3D dynamic culture of rabbit articular chondrocytes encapsulated in alginate gel beads using spinner flasks for cartilage tissue regeneration. *Biomed Res Int*. 2014;2014:539789.
- Caron MMJ, Emans PJ, Coolen MME, Voss L, Surtel DAM, Cremers A, et al. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. *Osteoarthritis Cartil*. 2012;20:1170–8.
- Lin L, Shen Q, Xue T, Duan X, Fu X, Yu C. Sonic hedgehog improves redifferentiation of dedifferentiated chondrocytes for articular cartilage repair. *PLoS ONE*. 2014;9:e88550.
- Chua KH, Aminuddin BS, Fuzina NH, Ruszymah BH. Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. *Eur Cell Mater*. 2005;9:58–67 (**discussion 67**).
- Mandl EW, Jahr H, Koevoet JL, van Leeuwen JP, Weinans H, Verhaar JA, et al. Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture. *Matrix Biol*. 2004;23:231–41.
- Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. *J Bone Miner Res*. 2000;15:166–74.
- Gigout A, Jolicœur M, Buschmann MD. Low calcium levels in serum-free media maintain chondrocyte phenotype in monolayer culture and reduce chondrocyte aggregation in suspension culture. *Osteoarthritis Cartil*. 2005;13:1012–24.
- Bittencourt RAdC, Pereira HR, Felisbino SL, Ferreira RR, Guilherme GRB, Moroz A, et al. Cultura de condrocitos em arcabouço tridimensional: hidrogel de alginato. *Acta Ortop Bras*. 2009;17:242–6.
- Yates KE, Allemann F, Glowacki J. Phenotypic analysis of bovine chondrocytes cultured in 3D collagen sponges: effect of serum substitutes. *Cell Tissue Bank*. 2005;6:45–54.

28. Bernardini G, Chellini F, Frediani B, Spreafico A, Santucci A. Human platelet releasates combined with polyglycolic acid scaffold promote chondrocyte differentiation and phenotypic maintenance. *J Biosci.* 2015;40:1–9.
29. Kang Y, Yang J, Khan S, Anissian L, Ameer GA. A new biodegradable polyester elastomer for cartilage tissue engineering. *J Biomed Mater Res A.* 2006;77:331–9.
30. Tseng S, Reddi AH, Di Cesare PE. Cartilage oligomeric matrix protein (COMP): a biomarker of arthritis. *Biomark Insights.* 2009;4:33–44.
31. Yu SB, Xing XH, Dong GY, Weng XL, Wang MQ. Excess genistein suppresses the synthesis of extracellular matrix in female rat mandibular condylar cartilage. *Acta Pharmacol Sin.* 2012;33:918–23.
32. Takahashi T, Ogasawara T, Asawa Y, Mori Y, Uchinuma E, Takato T, et al. Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. *Tissue Eng.* 2007;13:1583–92.
33. Hamid AA, Idrus RBH, Saim AB, Sathappan S, Chua K-H. Characterization of human adipose-derived stem cells and expression of chondrogenic genes during induction of cartilage differentiation. *Clinics.* 2012;67:99–106.
34. Halasz K, Kassner A, Morgelin M, Heinegard D. COMP acts as a catalyst in collagen fibrillogenesis. *J Biol Chem.* 2007;282:31166–73.
35. Tavella S, Bellese G, Castagnola P, Martin I, Piccini D, Doliana R, et al. Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. *J Cell Sci.* 1997;110:2261–70.
36. Fukumoto T, Sanyal A, Fitzsimmons JS, O'Driscoll SW. Expression of beta1 integrins during periosteal chondrogenesis. *Osteoarthritis Cartil.* 2002;10:135–44.
37. Knudson W, Loeser RF. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol Life Sci.* 2002;59:36–44.
38. Hooshmand S, Soung do Y, Lucas EA, Madhally SV, Levenson CW, Arjmandi BH. Genistein reduces the production of proinflammatory molecules in human chondrocytes. *J Nutr Biochem.* 2007;18:609–14.
39. Arjmandi BH, Khalil DA, Lucas EA, Smith BJ, Sinichi N, Hodges SB, et al. Soy protein may alleviate osteoarthritis symptoms. *Phytomedicine.* 2004;11:567–75.