Clinical investigations have shown a significant relationship between osteoarthritis and estrogens levels in menopausal women. Therefore, treatment with exogenous estrogens has been shown to decrease the risk of osteoarthritis. However, serious side-effects are considered as a main concern of its application such as increasing the risk of breast cancer, myocardial infarction, stroke - to name but a few. Instead, 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 differentiation and extracellular matrix formation. 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) and total collagen were determined in both culture systems after treatment with daidzein. Western blotting, Immunostaining and Real time PCR was applied to evaluate the expression levels of chondrogenic and extracellular gene markers after the 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). There were insignificant effects of the treatment with daidzein on the expression levels of collagen I and III in both culture systems. Whereas, the expression levels of Fibronectin, Laminin and Integrin β1 were significantly increased especially in
3D culture system. This study was illustrated the potential effects of daidzein on human chondrocyte differentiation and extracellular matrix formation suggesting an attractive and viable alternative therapy for osteoarthritis.

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Phytoestrogen (daidzein) promotes the chondrogenic phenotype of human chondrocytes in 2D and 3D culture systems

Abstract

Clinical investigations have shown a significant relationship between osteoarthritis and estrogens levels in menopausal women. Therefore, treatment with exogenous estrogens has been shown to decrease the risk of osteoarthritis. However, serious side-effects are considered as a main concern of its application such as increasing the risk of breast cancer, myocardial infarction, stroke - to name but a few. Instead, 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 maintenance and extracellular matrix formation. 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) and total collagen were determined in both culture systems after treatment with daidzein. Western blotting, Immunostaining and Real time PCR was applied to evaluate the expression levels of chondrogenic and extracellular gene markers after the 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). There were insignificant effects of the treatment with daidzein on the expression levels of collagen I and III in both culture systems. Whereas, the expression levels of fibronectin, laminin and integrin β1 were significantly increased especially in 3D culture system. This study
was illustrated the potential effects of daidzein on human chondrocyte differentiation and extracellular matrix formation suggesting an attractive and viable alternative therapy for osteoarthritis.

**Key words:** chondrogenesis, glycosaminoglycans, extracellular matrix, PCL-CA scaffold, cartilage repair, osteoarthritis.

**Abbreviation**

C-terminal crosslinked telopeptide type II collagen: CTX-II; Citric acid: CA; cyclooxygenase: COX; Dimethylmethylene blue: DMMB; Estrogen replacement therapy: ERT; Extracellular matrix: ECM; Glyceraldehyde-3-phosphate dehydrogenase: GAPDH; Glycosaminoglycans: GAGs; Interleukin-1β: IL-1β; Nitric oxide: NO; Maximal non-toxic dose: MNTD; Osteoarthritis: OA; Polycaprolactone triol: PCL; polycaprolactone triol-citrate: PCL-CA; Thin-layer chromatography: TLC; Tumour necrosis factor-α: TNF-α

**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]. In normal human cartilage, flattened and rounded chondrocytes are founded in superficial and upper-middle zones respectively while in the fibrillated human cartilage, more than 20 rounded chondrocytes cluster in a large lacuna. The increased number and size of chondrocyte clusters are localized adjacent to the cartilage fissures. This condition allows the nutrients and cell mediators from synovial fluid to gain a faster access to chondrocytes, leading to proliferation of
chondrocytes in osteoarthritic cartilage. The number of proliferating chondrocytes elevated gradually with OA progression [2].

Inflamed synovium produces catabolic and pro-inflammatory mediators including cytokines, nitric oxide (NO), prostaglandin E₂ and neuropeptides and modify the balance of cartilage extracellular matrix (ECM) degradation and repair, thus causing excessive formation of proteolytic enzymes that mediates for cartilage breakdown [3-5]. Interleukin-1β (IL-1β) increases enzyme activity and inhibits synthesis of enzyme inhibitors, thus contributing cartilage destruction. IL-1 and tumour necrosis factor-α (TNF-α) hinder the production aggrecan and collagen by stimulating chondrocytes to produce NO, MMPs and aggrecanases [4].

In the aspect of cytokine and growth factor expression pattern, as compared to chondrocytes from the normal cartilage, chondrocytes from fibrillated OA cartilage show upregulated level of intracellular IL-1α, IL-1β and plasma membrane-bound IL-1RI whereas the receptor IL-1RII is downregulated. This demonstrated the higher concentration of IL-1 in OA joints and greater sensitivity of OA chondrocytes towards IL-1 which eventually leads to IL-1 induced cartilage damage. Compared to chondrocytes from normal cartilage, chondrocytes adjacent to OA lesions display a higher binding of TNF-α and IL-1β [4].

A vascular condition of articular cartilage, limited chondrocytes in mature and aged cartilage and inability of chondrocytes to migrate to the damaged sites suppress the healing of cartilage. Cartilage repair is feeble and the fibrocartilage would replace the damaged cartilage [6, 7]. The number of chondrocytes reduces progressively with age. Despite the fact that chondrocytes apoptosis is essential during normal skeletal growth, the chondrocyte-derived apoptotic bodies deliver degradative properties that promote ECM degradation and calcification [3]. Over a
prolonged period of time, chondrocytes senescence and death lead to cartilage cellularity in aging joints and thus promoting pathogenesis of OA [3]. 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 [8].

In both genders, the incidence of OA elevates with age but postmenopausal women possess a higher prevalence of osteoarthritis [1, 9, 10]. As shown in a nationwide survey, radiographic OA is three times more prevalent among women within the age of 45 to 64 years old [11]. 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 loss of ovarian function [11]. A large epidemiological study conducted in Italy has supported the statement that estrogen deficiency increases the risk of OA [12]. Animal models exhibit that the depletion of estrogen promotes the incidence of OA [13]. In a rat model, ovariectomy which causes estrogen deficiency has accelerated cartilage degradation and erosion [14].

Several *in vitro* studies have demonstrated that the cartilage metabolism is regulated by estrogens. In the cultures of rabbit joint chondrocytes, the 17β-estradiol (E2), the naturally occurring estrogen, improves glycosaminoglycan (GAG) synthesis by upregulating the uridine diphosphate glucose dehydrogenase gene [15]. Furthermore, E2 impedes the cyclooxygenase-2 mRNA expression in bovine articular chondrocytes and defends the chondrocytes from reactive oxygen species-induced damage [16]. Nevertheless, high concentrations of E2 suppress DNA synthesis in human chondrocytes [17] and inhibit proteoglycan synthesis [18]. *In vivo* studies on the effects of estrogen on cartilage in ovariectomized Sprague-Dawley rats [19] and
ovariectomized cynomolgus macaques [20] 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 [11].

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 [13]. 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 [11]. 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, 9, 10].

Phytoestrogens are plant-derived compounds that can be isolated from various plants such as Leguminosae plant, *Psoralea corylifolia* [13]. Interestingly, the phytoestrogen daidzein showed an ability to bind to the estrogen receptors and serve as natural selective estrogen receptors [9, 13]. Thus, this study was designed to evaluate the potential effects of daidzein, which lack of the side effects of estrogen, as an alternatives to treat postmenopausal AO through activation of chondrocytes functions and enhance its ability in extracellular matrix formation.

**MATERIALS & METHODS:**
**Human chondrocyte expansion**

Human chondrocytes (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% CO$_2$, 37°C) for enough expansion.

**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% CO$_2$, 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. 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), the absorbance was measured at 570 nm. The percent of cell viability was calculated by using the formula below.

\[
\text{Percent of cell viability} = 100 - \left(\frac{\text{Absorbance of phytoestrogen of treated cells}}{\text{Absorbance of untreated cells}}\right) \times 100
\]

**Polymer scaffold preparation**
The polymer was produced by mixing equimolar amounts of both the citric acid (CA) and polycaprolactone triol (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 [21, 22].

**Cellular content of total collagen and glycosaminoglycans (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 Glycosaminoglycans (GAGs) was determined using the dimethylmethylene blue (DMMB) assay as described previously [23]. 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 [21]. 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).

**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 Bio-Sciences, 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 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).

**Indirect Immunostaining**

Cells were grown in T75 flask (5x10^5) 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 -20ºC. 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 4ºC. 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).

**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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. Briefly, human chondrocytes were seeded in flask or scaffold and incubated with maintenance DMEM medium supplemented with 20 µg/ml of daidzein (Treatment group) or without (Control group). Cells were harvested after 72 hours 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.

**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 ± SD.

**RESULTS:**
Cell Viability

The results of cell viability assay showed that the maximal non-toxic dose (MNTD value) of daidzein was 30 \( \mu \)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 \( \mu \)g/ml were used in the following experiments (Fig. 1).

**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 GAGs and total collagen production significantly with increasing incubation time. Compared to incubation time of 24 hours, 72 hours of treatment showed the most significant increase in GAGs and total collagen production, followed by 48 hours 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. 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 \( \mu \)g/ml of daidzein increased the production of GAGs and total collagen significantly in which the 20 \( \mu \)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 μg/ml of daidzein displayed the most significant increase in GAGs and total collagen production, followed by 2D chondrocytes culture supplemented with 20 μg/ml of daidzein (Fig. 3).

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

Prominent morphological differences were observed between chondrocytes culture in 2D and 3D systems. After 72 hours of daidzein treatment, chondrocytes in 2D culture (Fig. 4A and 4B) displayed a fibroblast-like morphology. The SEM images illustrated the pore size, pore distribution and the surface morphology of PCL-CA scaffold (Fig. 4C and 4D). This observation is in contrast with the rounded shape of chondrocytes in PCL-CA scaffold (Fig. 4E), which are similar to the chondrocytes in native cartilage. 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 2D and 3D culture (Fig. 4F). Indirect immunostaining of collagen II, the marker of chondrogenesis, with FITC fluorescent dye showed considerable upregulation due to the treatment with daidzein for 24, 48 and 72 h (Fig. 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 β1 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 COMP, Sox9, collagen type I, II and III, fibronectin, laminin and integrin β1, 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 β1 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 β1. In both 2D and 3D culture systems, collagen type I and III gene expression level did not show comparable differences (Fig. 6).

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 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 [24-26]. 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 [27-29]. 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 [30-33], which had proved that the 3D culture system favors the maintenance of chondrocyte phenotype.

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 [34]. From the western blotting results, the insignificant changes of COMP in both 2D and 3D culture system implied that daidzein treatment maintained the level of COMP which is essential for the preservation of ECM environment generated by chondrocytes.

Studies have reported that the human articular chondrocytes express estrogen receptors alpha and beta (ERα and ERβ) which implied that cartilage is an estrogen-targeted tissue [13, 35]. 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 [36], we have demonstrated that a 20 μg/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 μg/ml of daidzein increased the GAGs and total collagen production minimally whereas 30 μg/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, daidzein upregulated the of Sox9 gene expression which in turn increases the COMP gene expression. The time point selected for the analysis of chondrogenic gene expression may 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 [37].

In both 2D and 3D culture systems, daidzein treatments on chondrocytes upregulated the gene expression of COMP which in turn upregulated collagens type I, II and III gene expression. COMP, as the catalyst in collagen fibrillogenesis driven the upregulation of collagens type I, II and III gene expression. COMP catalyzes fibrillogenesis by interacting primarily with free collagen type I and II molecules thereby enhancing the collagen molecules assembly. Collagen type III has been described as a modulator in collagen type I fibril formation [38], 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 [39]. The expression pattern of integrin subunits varies during chondrogenesis where the expression of integrin β1 is prominent in mature chondrocytes [40]. Integrin β1, the heterodimeric transmembrane glycoprotein, modulates the interactions between ECM proteins and chondrocytes, in other words, regulating the cell-matrix adhesion and cell-cell contacts [41]. Daidzein upregulated the integrin β1 gene expression and this greater expression of integrin β1 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 μg/ml of daidzein on GAG and total collagen production shown in this study, Cheng et al., 2010 demonstrated that high concentration (10 – 20 μM) of
purified phytoestrogen, bavachin reduced IL-1β-induced production of chemokines, thereby hindered inflammatory cell migration indirectly [13]. Furthermore, \textit{in vitro} study showed that genistein, a phytoestrogen which structurally resembles estrogen, decreased the formation of lipopolysaccharide-induced cyclooxygenase (COX)-2 in chondrocytes [42]. Clinical study showed that daily consumption of 88 mg of phytoestrogen is beneficial for OA patients [43]. Concisely, the positive effects of phytoestrogens suggest that the phytoestrogens would be an appealing and feasible alternative therapy for OA.

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\textbf{Conflict of interest:} The authors declare that they have no competing interests.

\textbf{Ethics statement:} There is no ethics approval needed for this study.

\textbf{References}


**Figures legends**
Figure 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 hours of incubation with increasing concentrations of the compound (0-400 µg/ml). The maximal non-toxic dose (MNTD value) of daidzein was 30 µg/ml for all of time points. This dose showed approximately 90% of cell viability.

Figure 2: The effect of time of incubation with daidzein on the production levels of GAG and total collagen in human chondrocytes. 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.

Figure 3: The effect of daidzein concentration on the production levels of GAG and Total collagen in human condrocytes. The concentration of 20 µg/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).

Figure 4: The expression levels of chonrogenic protein markers in human chondrocytes after treatment with daidzein. Human chondrocytes were cultured in flask as a 2D culture system (A and B) and in scaffolds as 3D culture system (C and D, PCL-CA non-seeded scaffolds; E, PCL-CA seeded-scaffolds). Expression of chondrogenic markers (F) showed upregulation of SOX9 in 2D and 3D culture systems, while insignificant changed were observed in the expression level of COPM 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)

Figure 5: Indirect immunostaining of collagen II with FITC fluorescent dye. Human chondrocytes were grown in T75 flask (5×10⁵), treated with 20 µg/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 results showed considerable upregulation of collagen II due to the treatment with daidzein for all time points.

Figure 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 lamanin (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.
Gene expression after the treatment with daidzein in 2D culture

Gene expression after the treatment with daidzein in 3D culture
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Manuscript Title: Phytoestrogen (daidzein) promotes chondrogenic differentiation and extracellular matrix formation in 2D and 3D culture systems

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Phytoestrogen (daidzein) promotes chondrogenic phenotype of human chondrocytes in 2D and 3D culture systems

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