Original Article

Excess genistein suppresses the synthesis of extracellular matrix in female rat mandibular condylar cartilage

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Aim: To investigate the effect of excess genistein on the extracellular matrix in mandibular condylar cartilage of female rats in vivo.

Methods: Female SD rats were administered through oral gavage with genistein (50 mg/kg) or placebo daily for 6 weeks. The morphological changes of temporomandibular joints were studied with HE staining. The expression of cartilage matrix compounds (aggrecan and collagen type II), estrogen-related molecules (aromatase, estradiol, ERα and ERβ) and proliferating cell nuclear antigen (PCNA) in mandibular condylar cartilage was detected using immunohistochemistry, ELISA and real-time PCR.

Results: The genistein treatment significantly reduced the thickness of the posterior and middle regions of mandibular condylar cartilage, and decreased the expression of collagen type II, aggrecan and PCNA. Compared with the control group, the estradiol content and expression levels of the key estradiol-synthesizing enzyme aromatase in the genistein-treatment group were significantly decreased. The genistein treatment significantly increased the expression of ERβ, but decreased the expression of ERα.

Conclusion: Excess genistein suppresses extracellular matrix synthesis and chondrocytes proliferation, resulting in thinner mandibular condylar cartilage. These effects may be detrimental to the ability of mandibular condylar cartilage to adapt to mechanical loads.

Keywords: genistein; osteoarthritis; mandibular condylar cartilage; estrogen; estrogen receptors

Introduction

Osteoarthritis (OA) is the most common arthropathy in elderly people, and its socio-economic impact has increased as life expectancy has risen. OA is a degenerative disease characterized by the loss of collagens and proteoglycans as the main structural molecules of articular cartilage[1]. Epidemiological studies have shown that the prevalence and incidence of OA increase after menopause and that OA symptoms are more severe in postmenopausal women than in men[2–4], which suggests a link between OA and estrogen.

Although estrogen replacement therapy (ERT) has been proven to reduce the risk of OA[5–7], its application is hampered by an increased risk of breast cancer, myocardial infarction, and stroke[8, 9]. Therefore, using phytoestrogens, which lack the specific side-effects of estrogens, may provide an alternative therapy[10, 11]. Phytoestrogens are abundant in soybean products[12]; genistein, which structurally resembles 17β-estradiol, is particularly abundant. It has been shown that genistein can bind to estrogen receptors (ERs) with an affinity 100 to 1000-fold less than that of estradiol and that it competes with estradiol and displaces it from its binding sites[13]. A clinical study has suggested that consuming 88 mg phytoestrogen per day may benefit OA patients[14]. Hooshmand et al have also reported that genistein reduced the in vitro production of lipopolysaccharide-induced cyclooxygenase (COX)-2 in chondrocytes, indicating that genistein may be an attractive and viable alternative therapy for treating or preventing OA[15].

The temporomandibular joint (TMJ) plays an important role in craniofacial growth and function and shows a high incidence of OA[16]. In the literature, estrogen has been shown to play an important role in the development of TMJ and temporomandibular diseases[17–19]. These findings have been supported by the existence of estrogen receptors (ERs) in mandibular condylar cartilage[20, 21].

As an estrogen-targeted tissue[20, 21], cartilage is usually a solid connective tissue that covers subchondral bone tissue and plays an important role in the development of OA. To the best of our knowledge, however, few studies of the effect of genistein on cartilage have been reported, especially in vivo studies. The
aim of the present study was to investigate the effect of excess genistein on in vivo mandibular condylar cartilage.

Materials and methods
Genistein treatment in vivo
All the animal experimental procedures were approved by the Animal Research Committee of the Fourth Military Medical University. Thirty 7-week-old female SD rats, weighing 180–190 g, were provided by the animal center of the Fourth Military Medical University. All animals were housed at 22°C and 30%–60% relative humidity with a normal day-night rhythm (a 12:12 h light-dark cycle). The animals were randomly assigned to vehicle control and genistein-treatment groups (15 in each group). They had free access to tap water and a phytoestrogen-free diet in which corn oil was replaced with soybean oil. In the literature, the bone-protective effects of genistein (3–54 mg·kg⁻¹·d⁻¹) have been reported in OVX rats, mice and postmenopausal women[22]. There have been few studies focusing on the effect of genistein on normal or intact animals. Based on a series of genistein safety studies, the no observed adverse effect level (NOAEL) for genistein in rats has been estimated to be 50 or 100 mg·kg⁻¹·d⁻¹[13, 23]. Consistent with our previous study[22], 50 mg·kg⁻¹·d⁻¹ genistein was chosen as excess dose in the present study. The animals were treated once daily with genistein (50 mg/kg body weight, 99.5% pure, Winherb Medical Science Co Ltd, China) or placebo by oral gavage. The genistein was dissolved in a placebo solution (0.9% NaCl, 2% Tween 80, and 0.5% methyl cellulose in water). The application volume was 5 mL/kg body weight. Changes in body weight during the experimental period were considered when calculating the genistein dosage. All the animals were sacrificed 6 weeks after the initiation of treatment.

Tissue preparation
For the morphological and immunohistochemistry analyses 10 rats (5 from each group) received deep anesthesia from an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and were perfused with 200 mL of normal saline and 400 mL of paraformaldehyde (4% in phosphate buffer saline, pH 7.4) through their ascending aortas. The TMJs and the uterus were harvested without fatty tissue. The wet weight of uterus was measured by electron precision balance. In the literature, the bone-protective effects of genistein (3–54 mg·kg⁻¹·d⁻¹) have been reported in OVX rats, mice and postmenopausal women[22]. For the morphological and immunohistochemistry analyses.

The other six rats in each group were sacrificed for the real-time PCR analysis. To obtain enough total RNA, four sets of condylar cartilages from 2 randomly selected rats constituted one sample; hence, 12 sets of condylar cartilages from 6 rats in each group were randomly assigned to 3 samples.

Histological analysis
The hematoxylin and eosin (HE) stained sections were examined under a light microscope (Leica DM2500, Wetzlar, Germany). A true-color computer-assisted image analyzing system with a digital camera (Leica DFC420, Leica, Wetzlar, Germany) and software (Qwin Plus, Leica Microsystem Imaging Solutions Ltd, Cambridge, United Kingdom) were used for image capture and measurement. The thickness of the condylar cartilage was measured in each section. The condylar cartilage was divided approximately into anterior, middle and posterior regions. In every region, the thickness was measured at three quartering points. The means of the three measurements were used for the statistical analysis.

Immunohistochemical staining and analysis
Five commercially available primary antibodies were used for the immunohistochemical staining: anti-human collagen type II goat polyclonal IgG (SC7763, Santa Cruz Biol Inc, USA); anti-rat proliferating cell nuclear antigen (PCNA) mouse monoclonal IgG (MS-106, Labvision Ltd, USA); anti-human aromatase rabbit polyclonal IgG (AB69653, Abcam, UK); anti-human ERα rabbit polyclonal IgG (SC542, Santa Cruz Biol Inc, USA); and anti-human ERβ rabbit polyclonal IgG (SC8974, Santa Cruz Biol Inc, USA). The immunohistochemical staining was conducted using a three-step avidin-biotin complex method that has been previously described[24]. The sections were incubated overnight at 4°C with anti-human collagen type II goat polyclonal IgG (3 µg/mL), anti-rat PCNA mouse monoclonal IgG (3 µg/mL), anti-human aromatase rabbit polyclonal IgG (10 µg/mL), anti-human ERα rabbit polyclonal IgG (4 µg/mL), and anti-human ERβ rabbit polyclonal IgG (3 µg/mL).

The immunohistochemical staining for aromatase, ERα, ERβ, and PCNA was analyzed by the Qwin Plus software. Briefly, the stained sections were observed under a Leica DM2500 light microscope with a ×10 objective. The measurements were performed in middle and posterior regions of the condylar cartilage, where they regularly appeared among the animals. For the aromatase, ERα and ERβ analyses, the measurement area consisted of the complete mature and hypertrophic layers in which immunoreactive signals were obvious. The positively stained region and the complete selected region covering the mature and hypertrophic layers, respectively, were measured. The percentage of the entire region that stained positive was calculated. For the PCNA sections, 4 cubic regions (300 pixels x 300 pixels) were selected from approximately the middle and posterior regions of the condylar cartilage. The number of PCNA-positive chondrocytes was determined. Finally, the percent of the region that stained positive and the sum of the PCNA-positive cells in all of the cartilage were used for the statistical analysis.
RNA preparation, reverse transcription, and real-time PCR

The mandibular condylar cartilage samples were pulverized in liquid nitrogen. The total RNA was isolated from frozen tissues using a standard TRIzol® protocol (Invitrogen, Carlsbad, CA), followed by first-strand cDNA synthesis with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Inc, Maryland, USA). The real-time PCR was performed in an ABI 7500 Fast thermal cycler. The protocol consisted of 40 cycles of 94 °C for 5 s, 62 °C for 34 s, and 72 °C for 1 min each. The specific primers were designed with the primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) based on the cDNA sequences available at the Genebank database. Table 1 shows the sequences of primers used in this study. All the PCRs were run in triplicate for each sample, and the threshold cycles (CT) were measured. A ΔCT value was calculated for each sample by subtracting the CT value of the reference gene (18S) from the CT value of the gene of interest. 18S has been used as internal standard in several studies. All the samples were normalized to the ΔCT value of a control sample (ΔΔCT). The relative expression of the target gene was calculated using the expression 2^−ΔΔCT and is reported in arbitrary units[25].

Table 1. Primer sequences for aggrecan, collagen type II, aromatase, ERα, ERβ, and 18S.

| Gene     | Sequence                            | Fragment length (bp) | Accession number |
|----------|-------------------------------------|----------------------|------------------|
| Aggrecan | F: 5′-CCCTCCACCCAAGAATCAAGT-3′ R: 5′-TCATGGGACGCGAGGTGGC-3′ | 178 bp               | NM_022190        |
| Collagen II | F: 5′-AGCACTGGTGGAAGAGCAAGA-3′ R: 5′-ATCTGGACGTTAGCGGTG-3′ | 124 bp               | NM_012929        |
| Aromatase | F: 5′-TCATCAGCAATGCCTCAAGA-3′ R: 5′-GCATTCACGGCAGCAAT-3′ | 106 bp               | M33986           |
| ERα      | F: 5′-TGCGCAAGTACGAAAGTGC-3′ R: 5′-ATCAAGGCTCCGCCAGCTC-3′ | 108 bp               | NM_012689        |
| ERβ      | F: 5′-AAAAAATTCACGCTGATGCCT-3′ R: 5′-GCTGAATACGGAAGATGC-3′ | 124 bp               | NM_012754        |
| 18S      | F: 5′-CGGATACCCACATGAAAGA-3′ R: 5′-GCATGGGAGAACACT-3′ | 187 bp               | M11188           |

Enzyme-linked immunosorbent assay (ELISA) for determination of estradiol

For the ELISA analysis, the remaining 8 rats (4 from each group) were sacrificed under deep anesthesia. Two mandibular condylar cartilage sections from the same rat were used a single sample, giving 4 samples from each group. The cartilage samples were pulverized in liquid nitrogen and then placed RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) for 2 h at 4 °C for extraction. The insoluble material was removed by centrifugation at 10000 r/min for 10 min. The protein in the supernatant was estimated by the BCA method according to the manufacturer’s instructions (Pierce Biotechnology Inc, Rockford, IL, USA). Forty micromgrams of total protein from each group was assayed by enzyme-linked immunosorbent assay. The estradiol content was quantified by a goat anti-rat ELISA kit (QRCT-30130012133IE\UTL, Adlitteram Diagnostic Laboratories, Inc, San Diego, CA, USA) according to the manufacturer’s instructions. The minimum detectable estradiol concentration using this assay was less than 1.0 pg/mL.

Statistical analysis

The SPSS 13.0 software package (SPSS Inc, Chicago, IL, USA) was used to analyze and describe the data. Student’s t test was used to compare values between the groups. P-values were considered statistically significant when less than 0.05.

Results

Excess genistein increased the uterus weight index

The uterus weight index of the genistein-treatment group (2.2714±0.18875) increased significantly (P<0.01) compared to the control group (1.7884±0.11068).

Excess genistein suppressed the synthesis of extracellular matrix

In the mandibular condylar cartilage of both the control and genistein-treatment groups, the fibrous, proliferating, mature and hypertrophic layers were regularly arranged and had good continuity within the layers (Figure 1A and 1B). The thickness of the posterior and middle regions of the condylar cartilage in genistein-treated group decreased significantly (P<0.01) compared to the control group, however (Figure 1C). Consistent with the decreased cartilage thickness, the
expression of collagen type II and aggrecan was lower in the genistein-treatment group than in the control group \((P<0.01)\) (Figure 2).

![Figure 2](image)

**Figure 2.** The expression of extracellular matrix in mandibular condylar cartilage. Panels (A) and (B) show the collagen II expression in middle region of the condylar cartilage from the control and genistein treatment groups. Panel (C) shows the comparison of the condylar cartilage collagen II and aggrecan mRNA levels between the control and genistein treatment groups \((n=3)\). *c* \(P<0.01\) vs control.

Excess genistein suppressed the proliferation of chondrocytes
As shown in Figure 3, the density of PCNA-positive chondrocytes was lower in the genistein-treatment group \((90.4\pm12.7)\) than in the control group \((217.6\pm28.1)\) \((P<0.01)\).

![Figure 3](image)

**Figure 3.** The PCNA expression in mandibular condylar cartilage from (A) the control group and (B) the genistein treatment group.

Excess genistein decreased the level of estradiol in condylar cartilage
The ELISA results showed that the estradiol content was lower in the genistein-treatment group \((2.1310\pm0.20756 \text{ ng})\) than in the vehicle control group \((3.4293\pm0.23953 \text{ ng})\) \((P<0.01)\). Additionally, the expression of the key estradiol-synthesizing enzyme aromatase (as measured by both protein and mRNA levels) was lower in the genistein-treatment group \((P<0.01)\) (Figure 4) than in the control group.

![Figure 4](image)

**Figure 4.** The expression of aromatase (A and B), ER\(\alpha\) (C and D) and ER\(\beta\) (E and F) in mandibular condylar cartilage. (A, C, and E) are from the control group; (B, D, and F) are from the genistein-treatment group. (G) shows the comparison of the percent of positive area between the control and genistein treatment groups \((n=5)\). *c* \(P<0.01\). The scale bar is 100 \(\mu\text{m}\).

Excess genistein decreased the expression of ER\(\alpha\), but increased that of ER\(\beta\)
As for the ER expression, the ER\(\alpha\) mRNA levels and immunohistochemical signals were lower in the genistein-treatment group than in the control group \((P<0.01)\). However, both the mRNA levels and immunohistochemical signals for ER\(\beta\) were significantly higher in the genistein-treatment group than in the control group \((P<0.01)\) (Figure 4, 5).

Discussion
To the best of our knowledge, our study is the first to investigate the effects of genistein on mandibular condylar cartilage. Although the effect of phytoestrogens, especially genistein, on the extracellular cartilage matrix has been discussed in the literature, the data are not consistent. An in vitro study found that articular chondrocyte glycosaminoglycans (GAG) synthesis was significantly diminished following incubation with high doses of genistein \((10^{5}-10^{4} \text{ mol/L})\) but that the level of sulfate...
 increased at both the protein and mRNA levels. These results may indicate that genistein exerts its estrogenic activity in mandibular condylar cartilage mainly through ERβ, which is consistent with another recent finding that the effects of genistein on skeletal muscle major histocompatibility complex (MHC) expression was predominantly mediated through ERβ.[30]

In summary, the present study demonstrated that excess genistein can suppress extracellular matrix synthesis and chondrocyte proliferation in rat mandibular condylar cartilage, resulting in thinner mandibular condylar cartilage. This finding may imply decreased adaptivity to mechanical loads in mandibular condylar cartilage. Given the fluctuating differences in estrogen levels between individuals, the proper genistein dose should be studied further if genistein is to be widely used to treat OA or osteoporosis.

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Author contribution
Mei-qing WANG and Shi-bin YU designed the study and wrote the manuscript. Shi-bin YU and Xiang-hui XING performed the animal experiments, sample preparation and immunohistochemical staining. Guang-ying DONG performed the ELISA assay and PCR and revised the manuscript. Xi-li WENG performed the HE staining and data analysis and revised the manuscript.

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