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OSTEOARTHRITIS and CARTILAGE

Biochemical effects of estrogen on articular cartilage in ovariectomized sheep

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Summary

Cartilage is a sex-hormone-sensitive tissue but the role of estrogen in the pathogenesis of osteoarthritis (OA) remains controversial. In this study, intrinsic material properties and thickness of articular cartilage of the knee joint of ovariectomized (OVX) and estrogen-treated sheep were measured. Skeletally mature ewes ($N=36$, same breed, same housing, 4–5 years old) were divided into: sham treated (n = 9), OVX ($N=13$), OVX plus one estradiol implant (OVXE; $N=10$) and OVX plus two estradiol implants (OVX2E; $N=4$). Twelve months following sham procedure or OVX, sheep were euthanized and articular cartilage from a total of 216 points in the left femorotibial (knee) joints was tested for aggregate modulus, Poisson's ratio, permeability, thickness and shear modulus (six sites per sheep).

When all of the sites in each knee were grouped together, OVX had a significant effect on articular cartilage. The sham cartilage of all sites grouped together had a larger aggregate modulus ($P=0.001$) and a larger shear modulus ($P=0.054$) than the OVX tissue. No statistically significant differences were seen for permeability and thickness between OVX, sham, OVXE and OVX2E. Differences existed in biomechanical properties at the different sites that were tested Overall, no one location tended to be lowest or highest for all variables. This biomechanical study suggests that OVX may have a detrimental effect on the intrinsic material properties of the articular cartilage of the knee, even though the cartilage of the OVX animals appeared normal Treatment with estradiol implants ameliorated these deleterious effects and may have helped maintain the tissue's structural integrity. Our study supports epidemiological studies of OA in women after menopause The protective effect of estrogen and it's therapeutic effect remain to be further defined This model may allow the relationship of estrogen and estrogen antagonists to be studied in greater detail, and may be valuable for the study of the pathogenesis and therapies of OA of postmenopausal women, particularly in its early stages.

Key words: Estrogen, Articular cartilage, Sheep, Ovariectomy, Material properties, Biphasic theory

Introduction

In people over the age of 55 years, osteoarthritis (OA) is significantly more prevalent, and is more severe in women [1]. This suggests a relationship between OA and the alteration of sex hormones at menopause [2]. Although it is known that cartilage is a sex-hormone-sensitive tissue, the exact role of sex hormones in the pathogenesis of OA remains controversial [3]. Both *in vivo* and *in vitro* studies suggest that estrogen is detrimental to cartilage. The increased incidence of OA in obese post-menopausal women may be related to mechanical loading. However, although a gradual decline of estrogen and progesterone is usually associated with menopause, an increased frequency of OA in obese postmenopausal women may be associated with hyperestrogenism suggesting that estrogen may be chondrodestructive [2]. Furthermore, the existence of receptors for 17β-estradiol [E2] in rabbit chondrocyte and canine cartilage suggests that estrogen may be associated with OA [4]. Tsai and Liu [4] examined the effect of two different doses of intra-articular E2 in rabbits. Unlike the high dose, the low dose did not induce significant pathologic changes leading these authors to conclude that the direct interaction between E2 and rabbit articular cartilage was dose-dependent and duration-dependent. They concluded estradiol causes OA. E2 has been shown to inhibit proteoglycan synthesis in an experimental rabbit model of OA [5].

In 1952, Kellgren and Moore described a subset of primary OA that was generalized and associated

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with Heberden's nodes and occurred mainly in women. They found no correlation between this condition and menopause, and in a blind trial with estrogen therapy found no subjective evidence of benefit [6]. The relationship between estrogen use and the presence of knee OA in women who were members of the Framingham Heart Study cohort has been evaluated [7]. There was no positive association of estrogen use with radiographic knee OA after controlling for age, body-mass index, age at menopause, physical activity, history of knee injury, and smoking. Furthermore, a modest, but nonsignificant, protective effect for both radiographic OA and severe radiographic OA was seen in women who reported estrogen use [7].

Some experimental data have been reported that suggest estrogens may have a protective effect against OA, and in some OA-prone strains of rodents, the condition is more frequent in males and is suppressed by estrogens [1]. In a partial menisectomy model in the rabbit, estrogens appeared to exert no effect or an ameliorative effect on joint degeneration. It has been suggested that sex hormones have less effect on joint structures than on bony tissue and hormones may act by modulating genetic factors [1].

There are very few reports of the effects of ovariectomy or chronic sex steroid administration on properties of articular cartilage. A high prevalence of OA lesions (subchondral plate thickening, osteophytes, subchondral cysts, articular cartilage fibrillation and clefts, etc.) were observed in the knee joints of relatively young monkeys. However, this study failed to detect an effect of chronic sex steroid administration or ovariectomy on severity of OA [8]. It has not been determined whether estrogen deficiency initiates biomechanical changes in articular cartilage, and whether estrogen therapy alleviates or protects against these changes. E2 receptors have been found in articular cartilage [9]; however, we are unaware of any studies using a large animal model such as the sheep, to look at the effect of estrogen replacement therapy (ERT) on the intrinsic biomechanical properties of articular cartilage.

With the increased use of ERT by postmenopausal women for the prevention of osteoporosis, there has never been a greater need to investigate a possible link to OA. To address this issue, we studied the effects of ovariectomy and physiological doses of E2 on the intrinsic biomechanical properties of articular cartilage of the knee joint of sheep using a creep indentation test. Six sites were tested to represent a wide variety of joint locations where transmitted loading may differ. Following the linear biphasic theory for soft hydrated tissues [10], the following properties were determined: (1) the aggregate modulus (H_A in MPa), which is the tissue's compressive stiffness modulus; (2) the Poisson's ratio (v_s), which represents the tissue's apparent compressibility; (3) the permeability (k in m^4/Ns), which is indicative of the tissue's interstitial fluid flow inside the pores of the articular cartilage; and (4) the shear modulus (μ in MPa).

Materials and methods

This project was performed under the guidelines of the Colorado State University Animal Care and Use Committee. Skeletally mature ewes ($N=36$, same breed, same housing, 4–5 years old) were divided into four treatment groups; sham treated ($N=9$); ovariectomized (OVX) ($N=13$); OVX plus one estradiol implant (OVXE; $N=10$); and OVX plus two E2 implants (OVX2E; $N=4$).

OVARIECTOMY

Under general (halothane) anesthesia, sheep were subjected to OVX/sham procedure. In 27 sheep, ovaries were removed, while in nine sheep, ovaries were visualized and palpated but not removed (sham procedure). In 10 OVX sheep, one E2 implant was inserted subcutaneously while in four OVX animals, two E2 implants were inserted. Capsules were prepared from Silastic tubing, 0.335 cm i.d. × 0.465 cm o.d. (Dow Corning, Midland, MI, U.S.A.) and filled with pure crystalline E2. The length of the capsule was 5.0 cm. The capsule, when placed under the skin, has been shown to maintain a physiological serum E2 concentration [11, 12] and produce a serum E2 level of 5 pg/ml [13] with a rate of release of approximately 1.5 pg/day (T.M. Nett, personal communication).

SERUM LUTEINIZING HORMONE (LH) AND E2 MEASUREMENTS

Serum LH and E2 levels were measured using radioimmunoassay at 3, 6 and 12 months to evaluate the efficacy of ovariectomy and E2 implantation compared to the sham procedure. Ovariectomy was confirmed by measurements of serum LH levels > 4 ng/ml.

TISSUE SAMPLING

Twelve months following OVX or sham procedure, sheep were euthanized and the left knee joint of each animal was removed. Only the left knee was used for testing because the right knee

was used for an *ex-vivo* study of the effect of ovariectomy and estradiol on the bone mineral density (BMD) using a Hologic QDR 1000-W dual-energy X-ray absorptiometer (DXA; Hologic Inc., Waltham, MA, U.S.A.) [14]. Further, left–right differences in articular cartilage thickness have been observed in the knee joint of sheep [15]. All joints were disarticulated and stored at −80°C until evaluation. The joints were shipped in dry ice to the Orthopaedic Biomechanics Laboratory of the University of Texas Health Sciences Center at San Antonio for biomechanical testing.

PREPARATION OF TEST SAMPLES

For disarticulation and specimen preparation, each frozen specimen was thawed at room temperature for 1 h in normal saline solution (0.15 M NaCl) with protease inhibitors 10 mM, (N-ethyl malemide; 5 mM, benzamidine HCl; and 1 mM phenylmethylsulfonyl fluoride) (Sigma Chemical, St. Louis, MO, U.S.A.). Specimens from the knee joints were separated from the surrounding soft tissue by sharp dissection and sectioned horizontally, leaving the articular cartilage intact. All specimens were at least 25 mm × 25 mm.

Standard test sites were identified using measurement with calipers. The following sites were marked and tested: in the femur, the caudal centers of the medial condyle (FMP) and lateral condyle (FLP), and the central portion of the patellar groove (FCG). In the tibia, one site that was 'covered' by the medial meniscus (TMC) and one site not covered or 'uncovered' by the medial meniscus (TMU). The central portion of the patella (PAT) was also marked and tested. Test sites are shown in Fig. 1.

Each specimen was wrapped in gauze moistened with the solution and stored at −80°C. At the time of indentation testing, each specimen was thawed again for 1 h in normal saline solution with protease inhibitors. Thus all specimens underwent two freeze–thaw cycles, which do not affect the tissue's intrinsic material properties [16]. The osteochondral specimens were tested using an automated creep indentation apparatus (CIA) based on the methodology previously developed for indentation testing of articular cartilage [17, 18]. At the time of testing, the specimen was mounted onto an aluminium base plate with cyanoacrylate cement. A fiberoptic positioning system in-line was used for verifying that the loading shaft was perpendicular to the test point of the articular surface. A tare load of 0.1 N was then applied via a 1.0 mm diameter cylindrical rigid, porous, ultrasonically-cleaned tip, and when the slope of the tare creep became smaller than 1×10^{-6} mm/s, a test load of 0.049 N was automatically released onto the central portion of the test site. The small test load of 0.049 N was used because this load in conjunction with an indenter tip diameter of 1.0 mm, yielded applied strains that satisfied the small strain criterion of the linear biphasic theory. By using such a combination, the applied axial strain was consistently less than 0.10. On average, this strain was 6–7%. When the slope of the creep curve became smaller than 1×10^{-6} mm/s, as measured with a linear variable differential transformer, the load was automatically removed. The tissue was allowed to recover to equilibrium, and data acquisition ceased automatically. Thickness measurement was performed with a needle probe system. A total of 216 points were tested biomechanically using the CIA (six sites per sheep).

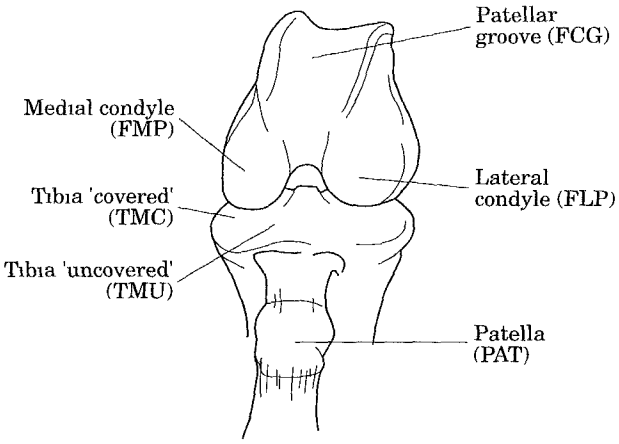

FIG. 1. Schematic diagram of the knee joint of a sheep, demonstrating the test sites for preparation of samples on the femur, tibia and patella. The sites in the femur are: the caudal centers of the medial condyle (FMP) and lateral condyle (FLP), and the central portion of the patellar groove (FCG). In the tibia one site was 'covered' by the medial meniscus (TMC) and the other was one not covered or 'uncovered' by the medial meniscus (TMU) The central portion of the patella (PAT) is also shown

To obtain the tissue's material properties, the aggregate modulus (H_A in MPa), the Poisson's ratio (v_s), the shear modulus (μ) obtained from H_A and v_s and permeability (k in m^4/Ns), a solution scheme [19, 20] based on biphasic finite element analysis [21] and non-linear optimization techniques [22] was used.

STATISTICAL ANALYSIS

The percentage of recovery and significance and magnitude of factors potentially influencing H_A, v_s, k, h, and μ were simultaneously assessed using analysis of variance. Factors tested included

Table I.
Least square means [standard errors (S E)] and p values of intrinsic material parameters of all treatment groups of the sheep knee joint pooled across all sites

	H_A	v_s	k	h	μ
Sham ($N=9$)	0.50 (0.03)	0.17 (0.01)	7.73 (0.99)	0 75 (0.05)	0.19 (0.02)
OVX ($N=9$)	0.39 (0.03)*	0.14 (0.01)	7 31 (0.83)	0.72 (0.04)	0.16 (0.01)*
OVXE ($N=10$)	0.53 (0 03)	0.15 (0.01)	7 42 (0.94)	0.70 (0.05)	0.21 (0.02)
OVX2E ($N=4$)	0.53 (0 05)	0.16 (0.02)	6.42 (1.49)	0 79 (0.07)	0.22 (0.02)
P	0.01	0.34	0.91	0.72	0 05

H_A = aggregate modulus (MPa), v_s = Poisson's ratio, k = permeability (m^4/N, s), h = thickness (mm), μ = shear modulus (MPa). *Significantly different from other groups

treatment, subject nested within treatment, location and the treatment by location interaction. Subject within treatment was considered a random effect and was used as the error term for testing treatment. Linear contrasts between individual treatment groups were tested using Fisher's protected least significant multiple comparisons test. Statistical significance was set if P rounded to 0.05 or less. Estradiol levels of different groups at 3, 6 and 12 months were compared using the non-parametric Kruskal–Wallis test.

Results

The average amount of recovery (the cartilage's ability to return to its original geometrical dimensions) for Sham, OVX, OVXE, OVX2E were 92.3 ± 9.0%, 90.4 ± 11.4%, 93.2 ± 11.4%, 89.0 ± 16.6%, respectively. These values were not significantly different ($P < 0.05$). The intrinsic material properties (H_A, v_s, k, μ) and *in situ* thickness (h) of articular cartilage of the knee joints are given in Tables I and II as least square means ±S.E.

The treatment by site interaction did not significantly influence any variable ($P < 0.30$). In other words, the effect of treatment was similar at all locations. Therefore, treatment effects were pooled across all locations and site effects were pooled across all treatments. Table I shows the treatment effects pooled together across all sites. Table II shows differences between sites, pooled across all treatment groups. Differences between treatment groups for v, k, and h were likely due to chance alone (Table I). For H_A, the OVX group was significantly lower than all other treatments. Sham, OVXE and OVX2E were not different from each other. For μ, OVX sheep had lower values than other treatment groups. The values for sham, OVXE and OVX2E were not different from each other. In summary, in the OVX group H_A and μ were lower than in other treatment groups. All other differences were likely due to chance alone.

Table II shows that the differences between least square means of the sites for all variables was highly significant. In other words, real differences exist in biomechanical properties at the different sites. Overall, no one site tended to be lowest or highest for all variables.

There were no significant differences between mean E2 levels of the sham and OVXE group at any time point. At 3 and 6 months, mean E2 levels of the OVX2E group were significantly higher than the levels of the other three groups ($P = 0.03$, 0 01).

Discussion

The biomechanical testing employed in this study has allowed the quantification of the intrinsic mechanical properties of the knee joint in

Table II
Least square means [standard errors (S E)] and p values of intrinsic material parameters of all test sites of the sheep knee joint pooled across all treatment groups

	H_A	v_s	k	h	μ
FCG	0 42 (0.03)	0.25 (0.02)	6.41 (1.10)	0 42 (0.05)	0.14 (0.01)
FLP	0.54 (0 03)	0.18 (0.02)	4.55 (1.10)	0.47 (0.05)	0 21 (0.01)
FMP	0.62 (0.03)	0.10 (0.02)	6.78 (1.10)	0.98 (0.05)	0.28 (0.01)
PAT	0.61 (0.03)	0.06 (0 02)	7.75 (1 10)	1.28 (0.05)	0.28 (0.01)
TMC	0.53 (0.03)	0.21 (0.02)	6.85 (1.10)	0.41 (0.05)	0.18 (0.01)
TMU	0.20 (0.03)	0.12 (0.02)	10.99 (1 10)	0.89 (0 05)	0.08 (0 01)
P	<0 001	<0.001	<0.003	<0.001	<0.001

H_A = aggregate modulus (MPa); v_s = Poisson's ratio, k = permeability (m^4/N, s), h = thickness (mm); μ = shear modulus (MPa) *Significantly different from the other groups

ovariectomized and E2 treated sheep. The results demonstrated that even though cartilage of OVX animals appeared normal, it had inferior structural integrity (smaller compressive modulus and small shear modulus) compared with the normal articular cartilage of the sham animals. Furthermore, the biomechanical properties of articular cartilage in OVX sheep treated with E2 implants seemed to maintain its structural integrity. In other words, cartilage material properties in OVXE and OVX2E animals were similar to those in the sham group.

This biomechanical study based on the biphasic creep indentation methodology, when results of all sites are pooled, suggests that OVX may have a detrimental effect on the intrinsic material properties of the articular cartilage of the knee. Significant differences were only present when the data was grouped, not when each site was examined. Treatment with E2 implants ameliorated these deleterious effects and may help maintain the tissue's structural integrity. Surprisingly, a change in cartilage permeability and thickness as a result of OVX were not observed. Our study supports epidemiological findings of OA in women after menopause, where either no effect or a mild protective effect of estrogen has been seen [7].

Indentation tests that have bone attached to the cartilage have some advantages, notably that the condition more closely resembles the physiological situation [23]. However, deficiency of estrogen may result in loss of bone mass [24] which may further reduce bone strength and stiffness [25] Further, postmenopausal bone loss can be prevented by treatment with low-dose estrogen with calcium [24]. Because the biphasic indentation theory assumes that the subchondral bone is a rigid body, a shortcoming of this project might be the decreases in magnitude of aggregate modulus and shear modulus of cartilage measured by indentation test may have been due to a decrease in subchondral bone stiffness. However, the stiffness of subchondral bone is much higher (three or four orders of magnitude larger) than the stiffness of articular cartilage, therefore a significant decrease in bone stiffness is not expected to affect the properties of the overlying cartilage. Further, our choice of applied strain (based on applied load, tip radius, and cartilage thickness) essentially discounted the effect of underlying bone stiffness. Comparison of the means of BMD of the opposite distal femur (and proximal tibia) of the different treatment groups were not statistically different at either of these locations [14]. In subsequent studies where the effects of ovariectomy and estrogen treatment will be examined for longer periods (e.g. 2 years), BMD measurement and mechanical evaluation of subchondral bone should be included in the protocol.

Estrogen deficiency is associated with accelerated bone loss in postmenopausal women but estrogens also play a major role in the regulation of cartilage growth, i.e. cartilage is a sex-hormone-sensitive tissue [26]. In growth plate chondrocytes, E2 inhibits cell proliferation and stimulates RNA synthesis, SO_4 incorporation, and collagen production depending upon E2 concentration and the model used [9]. These data suggest that estrogen deficiency in females might influence cartilage metabolism even though *in vitro* studies in small laboratory animals have implicated increased levels of estrogen and the development of OA [26]. We feel that the intra-articular E2 used in some *in vivo* animal studies may be unphysiological. Further, we hypothesized that physiological rather than pharmacological doses of E2 are indeed chondroprotective and are important in sustaining the mechanical properties of articular cartilage. The protective effect of physiological concentrations of estrogen and their therapeutic effect remain to be further defined. Using this model may allow the relationship of estrogen and estrogen antagonists (e.g. tamoxifen) to be studied in greater detail. Studies of longer duration, where histological and biochemical changes are characterized in this model, are recommended. Another important issue that needs to be investigated is the timing of ERT in the prevention of OA that may develop following menopause. Specifically, when ERT is begun and how much the preceding lack of estrogen has already influenced the tissue.

Our finding of a decrease in shear modulus and aggregate modulus in the OVX sheep are similar to those seen in an indentation study in Greyhound dogs where decreases of these parameters were observed at 6 and 12 weeks after transection of the anterior cruciate ligament [27]. These histological and biochemical changes of ligament-transection models have been well characterized but are they truly representative of the elderly postmenopausal estrogen-deficient woman with OA?

These results suggest that significant differences occur in the mechanical properties of ovine articular cartilage following ovariectomy and these changes are prevented by E2. The aged ovariectomized ewe may be a potentially valuable animal model for the study of the pathogenesis and therapies of OA of postmenopausal women, particularly in its early stages.

Problems associated with examining articular cartilage from postmenopausal women are the

different backgrounds and medical histories of the subjects, i.e. years since menopause, age, smoking history, medication (e.g. corticosteroids), diet, etc. This can be obviated by the use of a large animal model such as sheep of known age and breed. There are other reasons why sheep may be a good animal model for the study of the effects of estrogen deficiency on the mechanical effects of articular cartilage: although the sheep is a quadruped and its knee joint is smaller than that of humans, there are few anatomical differences between the knees of the two species. Further, another advantage of the sheep is that its knee joint is relatively extended as in man, whereas rabbits, dogs, rats and mice stand with their knees flexed [15]. This may explain why functionally, the knee joint of sheep and humans are relatively similar [28].

In conclusion, our biomechanical testing demonstrated that the cartilage of the knee joint from ovariectomized ewes when compared with that from sham or estrogen-treated animals, had inferior structural integrity. Real differences existed in the material properties at the different sites tested. Biomechanical and histological studies of the cartilage from ewes that have been estrogen-deficient for longer periods of time would provide more answers as to the role of sex hormones in the pathogenesis and treatment of OA.

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