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The Effect of Estrogen on Ovine Anterior Cruciate Ligament Fibroblasts

Cell Proliferation and Collagen Synthesis

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Abstract: Estrogen has been implicated as a causal factor for anterior cruciate ligament injuries in women. Studies have demonstrated a decrease in anterior cruciate ligament fibroblast proliferation and collagen synthesis at supraphysiologic levels of estrogen in a rabbit model.

Hypothesis: The authors hypothesized that physiologic levels of estrogen would have no significant effect on anterior cruciate ligament fibroblast proliferation and collagen synthesis in an ovine model.

Methods: Anterior cruciate ligament fibroblasts were isolated from sheep knees using routine cell culture methods. The cells were exposed to 17β-estradiol at physiologic concentrations of 2.2, 5, 15, 25, 250, and 2500 pg/ml. Cell proliferation was determined by cell counts on days 4 and 6. Collagen synthesis was determined by ^{3}H-proline incorporation on day 4. Immunohistochemistry was performed to detect estrogen receptors.

Results: Immunohistochemistry demonstrated the presence of estrogen receptors in ovine anterior cruciate ligament fibroblasts. There was no significant difference in anterior cruciate ligament fibroblast proliferation or collagen synthesis regardless of 17β-estradiol concentration.

Conclusions: Based on results of this study, and given the low turnover of collagen in ligaments, it is unlikely that a 2- to 3-day per month increase in circulating estrogen would result in rapid, clinically significant alterations in material properties of the anterior cruciate ligament in vivo. The etiology of noncontact anterior cruciate ligament injuries is complex and multifactorial in nature, meriting further investigation.

Keywords: estrogen; collagen synthesis; anterior cruciate ligament (ACL); cell proliferation

It has been reported that female athletes have a 2 to 5 times greater incidence of ACL injuries compared to male athletes.[1,4,12,23] Thirty percent of these injuries are due to direct contact, whereas the remaining 70% result from noncontact mechanisms. The potential etiologic factors for noncontact ACL injuries can be broadly categorized into environmental factors, anatomic differences between males and females, biomechanical factors, and hormonal differences between males and females.[5]

These clinical observations have created interest in the role of hormonal influences in the apparent increased incidence of ACL injuries in women. Several laboratories have demonstrated the presence of estrogen receptors in fibroblasts from the human ACL.[9,15] In a rabbit model, a 2-week exposure to supraphysiologic levels of estrogen decreased fibroblast proliferation and collagen production in ACL fibroblasts in cell culture.[10] Slauterbeck et al demonstrated that administration of high-dose 17β-estradiol to ovariectomized rabbits significantly decreased the tensile strength of the ACL compared to ovariectomized rabbits that were not supplemented with estrogen.[16] Both of the aforementioned studies used supraphysiologic levels of estrogen using a rabbit model, an animal that has a markedly different estrus cycle than the human female menstrual cycle.

A study done in human ACL fibroblasts derived from 1 patient undergoing total knee replacement following traumatic arthritis showed a short-term increase in cell prolif-

eration and procollagen type I synthesis.[21] In a related study, with the addition of fibroblasts derived from a second patient following ACL rupture, these differences were unaffected by the addition of varying concentrations of progesterone.[22] The main conceptual difficulty with these studies is that they do not account for the known rates of collagen turnover because collagen performs the major load-bearing function of the ACL.[2,6,11,17] In general, collagen turnover in ligaments is low, on the order of 100 to 500 days.[3,13,14] Given the low turnover of collagen in ligaments, it is unlikely that a 2- to 3-day per month increase in circulating estrogen would result in rapid, clinically significant alterations in the material properties of the ACL in vivo. In addition, the first set of studies used a small-animal model with a unique estrus mechanism that is dissimilar to humans. Extrapolation of these results to the human situation is made more difficult due to these factors. Due to the difficulty in obtaining normal ACL tissue, the second set of studies used tissue derived from a small number of patients (1 and 2, respectively) undergoing treatment for prior soft tissue injuries. Although this tissue was viable, and cells were successfully isolated, these cells may behave abnormally. In addition, cultures may be contaminated with synovial fibroblasts, particularly in the patient with ACL rupture.

Ligament homeostasis is dependent on a balance between synthetic activity and degradative activity. Synthetic activity can be measured by the rate of cell proliferation and collagen synthesis, whereas degradative activity can be measured by protease activity such as matrix metalloproteinases (MMPs) that degrades collagen. This hypothesis-driven study was designed to evaluate the effects of physiologic levels of estrogen on ACL fibroblasts from a synthetic standpoint, using fibroblasts derived from sheep, which exhibit an estrus cycle that is comparable to the human menstrual cycle.

The hypothesis of this study is that physiologic estrogen levels have no significant direct effect on proliferation and collagen synthesis in ovine ACL fibroblasts.

MATERIALS AND METHODS

Animal Model

Careful consideration was given to the choice of animal model. Fibroblasts derived from the ovine (sheep) ACL were used for several reasons. An in vitro ovine cell culture model was chosen because sheep exhibit a well-defined estrus cycle with cyclic fluctuations in hormone levels comparable to the human menstrual cycle. The normal physiological estrogen levels in nonpregnant ewes range from 4 to 10 pg/mL.[8] In addition, there was easy access to tissue from skeletally mature 5- to 7-year-old parous ewes that were age matched and in the reproductive phase of their lives. These animals had not received exogenous steroids that could potentially affect the physiology of musculoskeletal tissues and were in anestrous at the time of sacrifice.

Power analysis was performed based on previous studies that involved quantification of collagen synthesis and cell proliferation. Based on our power analysis, we determined that 6 specimens were required for a power of 0.8 and α = .05. Thus, tissue from a minimum of 6 sheep was used for this study.

Harvesting Tissue and Cell Culture

Sheep knee specimens were obtained from the Colorado State University College of Veterinary Medicine and Biomedical Sciences at the conclusion of an Institutional Animal Care and Use Committees–approved study. The knees were harvested at the time of sacrifice of the animal. All extra-articular soft tissues were removed, taking care not to violate the joint capsule. The joints were placed on ice and shipped via overnight express mail from Colorado to New York. The ACL was harvested under sterile conditions and placed into sterile M199 tissue culture medium with 10% antibiotic/antimycotic (Ab/Am) solution (Life Technologies, Grand Island, NY). Four millimeters of ACL tissue at the femoral and tibial insertion sites were discarded to avoid chondrocyte or osteocyte contamination. Dissection of the outermost layers of the ACL was performed to prevent cell contamination from the surrounding synovial sheath. Fibroblasts were isolated from the inner core of the ligament. The tissue was minced with a surgical blade and placed into four 75-cm^2 tissue culture flasks (BD Falcon Labware, Bedford, Mass). The explants were washed twice for 20 minutes with M199 containing 10% Ab/Am. The final wash was aspirated, complete medium (M199, 10% Ab/Am, 10% fetal bovine serum [FBS]) was added, and the specimens were incubated at 37°C in an atmosphere of 5% CO_2 and 100% humidity. Once sufficient colonies of fibroblast were established, the remaining explant tissue was removed, and the medium was changed every 48 hours until a confluent monolayer was obtained. On reaching confluence, cells were treated with 0.25% trypsin in ethylenediamine-tetraacetic acid (Life Technologies) for 5 minutes and frozen in liquid nitrogen, according to standard cryogenic techniques, for future use.

Immunohistochemistry

Immunohistochemistry was performed to demonstrate the presence or absence of estrogen receptors on the ovine ACL. A central area of the ACL was embedded in optimal cutting temperature tissue-embedding medium (Tissue Tek, Miles Elkhart, Ind), frozen in liquid nitrogen, and stored at –70°C until sectioning. Eight-micrometer frozen sections were cut onto glass microscope slides, fixed in 100% acetone at –10°C for 20 minutes, and stored at –70°C until immunohistochemistry was performed.

In addition to the tissue samples, cytospin preparations were made from cultured cells, as described below.[19] Cytospin slides were prepared from a cell suspension of 1×10^5 cells. The cell suspension was placed in the cytospin apparatus (Cytospin, Shandon Inc, Pittsburgh, Pa) and centrifuged at 500 G. The resulting slides were air dried for 2 to 12 hours, fixed in acetone for 10 minutes, air dried, and frozen at –70°C until used for immunohistochemistry. Immunohistochemistry of cytospin specimens was performed to ensure

that the cultured cells continued to express estrogen receptor following harvest from the native ligament.

Immunohistochemistry was performed using standard immunoperoxidase techniques. The slides were thawed to room temperature and rehydrated in Tris Buffered Saline with 0.05% Tween-20, Trizma-Buffered Solution (TTBS) (Fisher Scientific, Pittsburgh Pa) over 10 minutes. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 10 minutes, and nonspecific binding sites were blocked with 3% normal goat serum (Vector Laboratories Inc, Burlingame, Calif). The tissue sections were incubated for 1 hour with a 1:50 dilution of primary monoclonal antibody (anti-estrogen receptor) (Dako Corp, Carpinteria, Calif) in a humid chamber at room temperature. Positive controls consisted of human breast cancer tissue known to have estrogen receptors (Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY). Negative control sections consisted of serial sections in which TTBS was substituted for the primary antibody. After incubation with the primary antibody, the sections were incubated for 25 minutes at room temperature with a biotinylated secondary antibody (Dako Corp). The slides were then incubated with streptavidin-peroxidase for 30 minutes at room temperature, and the reaction product was detected by addition of 0.05% 3,3'-diaminobenzidine containing 0.05% H_2O_2 for a total of 15 minutes. The slides were washed with TTBS between each step. The sections were counterstained with Mayer's Hematoxylin (Electron Microscopy Sciences, Fort Washington, Pa) and rinsed in water, and a cover slip was applied with Permount (Fisher Scientific). The slides were examined with light microscopy, and photomicrographs were made.

Administration of 17β-estradiol

The following protocol was used for exposure of ACL fibroblasts to estrogen. Cells cultured to confluence were released with trypsin, washed, and plated in 24-well Falcon cell culture plates at a density of 2.0×10^4 cells per well for all assays. Passage 2 cells were used for all experiments. Each group was plated onto 4 wells. Estrogen-conditioned medium was created with final concentrations of 2.2, 5, 15, 25, 250, and 2500 pg/ml and added on day 1 of cell culture. Cells were cultured in medium M199 containing 10% FBS. Because FBS contains a baseline level of 17β-estradiol, the batch selected for these experiments contained the lowest amount of 17β-estradiol available. The same batch of FBS was used for all experiments, resulting in a baseline level of 2.2 pg/ml of 17β-estradiol. Group 1 received the medium without additional 17β-estradiol and served as the control group. Group 2 was incubated in a total of 5 pg/mL of 17β-estradiol, which represented the low-normal level of serum estrogen in reproductive-age sheep. Group 3 was incubated in media containing 15 pg/mL 17β-estradiol and represented the high-normal sheep estrogen level. Group 4 received a total of 25 pg/mL 17β-estradiol and represented the low-normal serum estrogen level in the human. Group 5 received a total of 250 pg/mL 17β-estradiol, representing a supra-physiologic dose for the sheep and the high-normal serum estrogen level in the human. Group 6 received a total of 2500 pg/mL 17β-estradiol, which represented a supraphysiological dose for both sheep and humans.

Cell Proliferation Assay

Cell proliferation was determined by cell counts using a Coulter counter (Beckman Coutter Co, Miami, Fla). Cells were plated at an initial concentration of 2×10^4 cells per well. Following a 24-hour attachment period, cells were treated with known concentrations of 17β-estradiol. Cells were harvested from 4 wells per time point by incubation with 0.25% trypsin/ethylenediamine-tetraacetic acid for 10 minutes. Cell suspensions were then diluted in phosphate-buffered saline (PBS) with 0.1% sodium azide and counted using a Coulter counter. Each measurement was repeated 3 times. Cell counts were performed 4 and 6 days after plating. This technique has been used extensively in our laboratory and provides equivalent results to measurements of cell proliferation using ^{3}H-thymidine incorporation.

Quantification of Collagen Synthesis

Collagen synthesis was determined on day 4 by measuring the incorporation of ^{3}H-proline (Amersham, Arlington Heights, Ill). Cells were incubated with various concentrations of estrogen for 5 days with the addition of 25 μg/mL ascorbic acid and 5 μCi/mL of ^{3}H-proline (specific activity = 102 Ci/mmol). Cells were incubated for 24 hours, the labeled medium was aspirated, and the monolayers were washed 3 times with ice-cold PBS. Then, 300 μL of 10% trichloroacetic acid was added to each well to precipitate the proteins. The precipitate was washed twice with ice-cold PBS and solubilized in 300 μL of 3-N KOH. Radioactivity was determined using standard liquid scintillation techniques. ^{3}H-proline uptake was normalized to cell number and expressed as percentage of control. Statistics were performed via a 1-way analysis of variance (ANOVA).

Data Analysis

The effect of estrogen on cell proliferation was determined by comparing the mean cell counts between groups. The paired Student 2-sample *t* test was used for data comparison. The data for collagen synthesis were normalized by cell number and expressed as a percentage of the control group to allow direct comparison. The ANOVA analysis was performed using the SigmaStat program (Jandel Scientific Software, San Rafael, Calif).

RESULTS

Immunohistochemistry

Immunohistochemical staining of ACL tissue specimens and cytospin specimens from cultured fibroblasts demonstrated positive immunostaining for estrogen receptor in
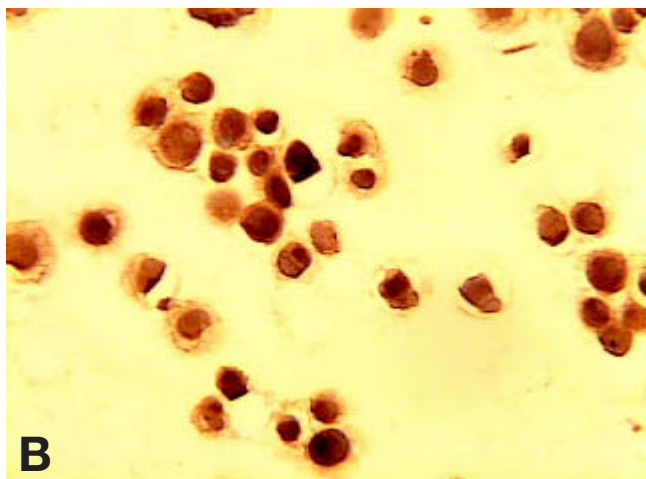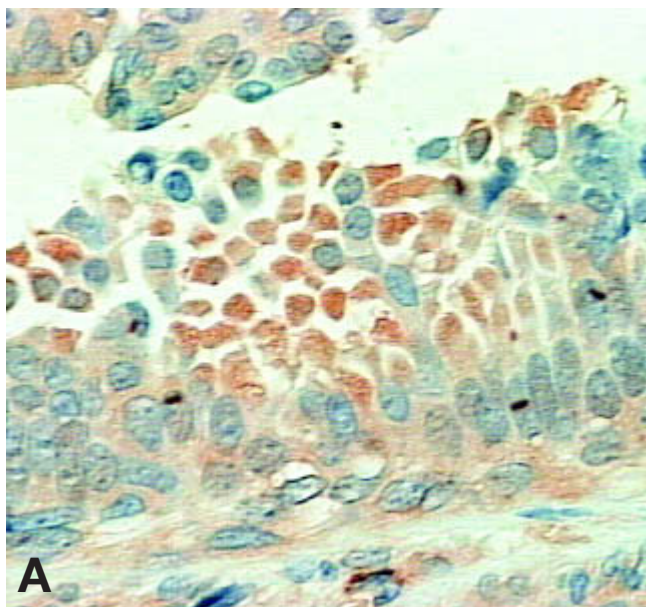
Figure 1. A, positive immunostaining for estrogen receptor is demonstrated in breast cancer tissue used as the positive control; B, positive nuclear immunostaining for estrogen receptors is demonstrated in this cytospin preparation of cultured ovine ACL fibroblasts.
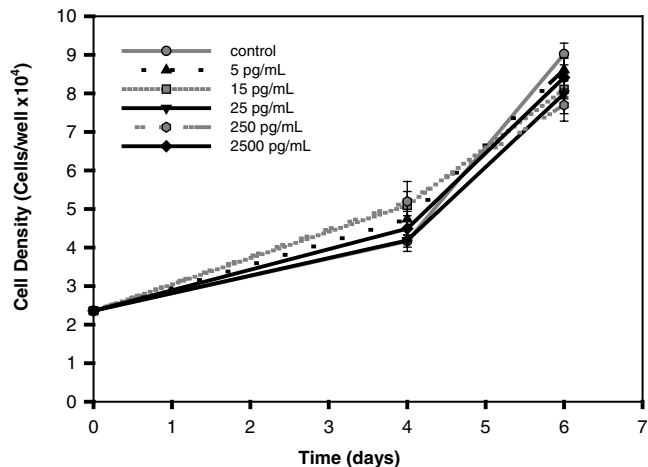

Figure 2. A representative graph demonstrating the insignificant effect of 17β-estradiol at varying concentrations on ovine ACL fibroblast proliferation.
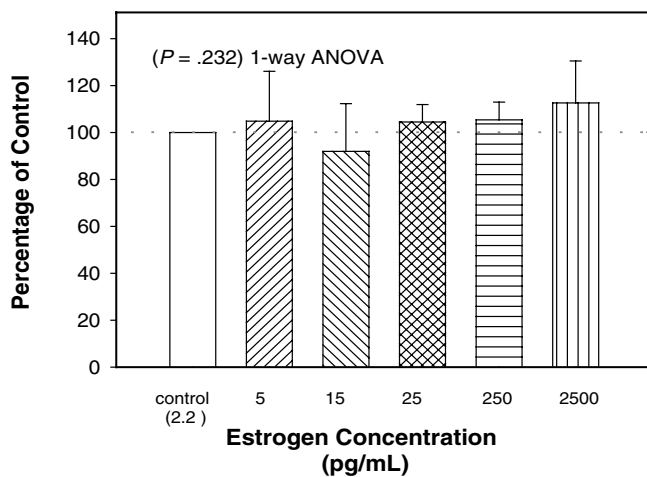

Figure 3. The effect of varying concentrations of 17β-estradiol on collagen synthesis measured by ^{3}H-proline uptake in ovine ACL fibroblasts, demonstrating no statistically significant effect.

all specimens tested (N = 6). Breast cancer tissue was used as a positive control. Brown nuclear staining indicated the presence of estrogen receptor (Figure 1A). Strong positive nuclear staining was also seen in cultured sheep fibroblasts (Figure 1B). Negative controls that were processed without incubation in the primary antibody showed no staining. These results demonstrate that ovine ACL fibroblasts express estrogen receptors.

Cell Proliferation

There were variations in cell proliferation rates seen in different experiments (using different cell batches); therefore,

a representative graph from 1 experiment is shown in Figure 2. There was no significant effect of estrogen on proliferation (N = 6). The remainder of the data are presented in Tables 1 and 2. There was no significant difference in cell proliferation of ACL fibroblasts at any 17β-estradiol concentration.

Collagen Synthesis

There was no significant difference in collagen synthesis by ACL fibroblasts at any of the 17β-estradiol concentrations tested ($P = .232$). The combined data for all specimens (N = 6) are demonstrated in Figure 3.

TABLE 1
Cell Proliferation Data Showing No Significant Difference in Cell Number
in Response to Estrogen Concentration After 4 Days in Culture

Estrogen Concentration (pg/mL)	Experiment Number				
	1	2	3	4	5
Control (2.2)	42 450 ± 1519	54 000 ± 3441	40 700 ± 4260	41 650 ± 1 561	30 890 ± 2718
5	39 945 ± 9627	69 800 ± 3398	45 750 ± 4281	47 400 ± 4147	28 355 ± 1958
15	46 175 ± 1390	64 800 ± 7855	37 900 ± 5615	50 950 ± 6167	30 235 ± 4128
25	35 335 ± 3519	67 300 ± 8146	35 200 ± 1376	51 950 ± 2579	28 720 ± 1374
250	34 385 ± 2524	62 150 ± 13 851	31 350 ± 3078	51 950 ± 2579	28 720 ± 1374
2500	34 805 ± 1928	51 350 ± 8899	27 300 ± 1510	44 900 ± 3333	28 330 ± 1861

TABLE 2
Cell Proliferation Data Showing No Significant Difference in Cell Number
in Response to Estrogen Concentration After 6 Days in Culture

Estrogen Concentration (pg/mL)	Experiment Number				
	1	2	3	4	5
Control (2.2)	86 655 ± 3482	149 600 ± 11 180	130 900 ± 10 426	90 200 ± 2814	81 180 ± 4713
5	84 090 ± 4054	151 300 ± 12 398	104 500 ± 3210	86 150 ± 2995	71 495 ± 4068
15	76 940 ± 4780	164 350 ± 9407	152 150 ± 14 929	81 050 ± 2473	65 970 ± 3498
25	81 895 ± 5413	167 550 ± 9379	134 050 ± 10 691	79 900 ± 5170	67 655 ± 6959
250	73 330 ± 3402	186 300 ± 4976	128 850 ± 7398	76 950 ± 4152	69 075 ± 2414
2500	75 290 ± 5064	163 500 ± 11 875	107 550 ± 12 663	84 100 ± 5053	63 160 ± 825

DISCUSSION

Noncontact ACL injuries have a higher incidence in women than in men. Many theories have been proposed to explain this difference. Hormonal influences have been cited as a possible cause based on in vitro studies in a small-animal (rabbit) model using supraphysiologic levels of 17β-estradiol.[10,16] The rabbit's estrus cycle occurs with sexual activity, and thus it is a very divergent model from the human menstrual cycle. Furthermore, the serum estrogen level in the rabbit remains ill defined, thus making it difficult to determine the physiologic range needed to perform these studies.

The results from this study failed to demonstrate differences in cell proliferation and in collagen synthesis of cultured ovine fibroblasts using a wide range of 17β-estradiol concentrations. These results are in contrast to a previously published study that documented a decrease in cell proliferation and collagen synthesis in cultured lapine ACL fibroblasts. It should be noted that in that study, these differences were demonstrated only at supraphysiologic estrogen concentrations. In addition, the authors measured collagen synthesis by the incorporation of ^{14}C hydroxyproline.[10] The use of this technique is open to criticism because proline hydroxylation is a posttranslational modification; thus, we chose to measure ^{3}H-proline incorporation to measure collagen synthesis and were unable to detect any differences.

In addition, biomechanical analysis of mechanical properties of sheep ACL performed in our laboratory demonstrated no difference in tensile strength in control versus specimens treated with estrogen.[18] Wentorf et al corroborated these findings with a similar study in a nonhuman primate model in which ACL and patellar tendons in ovariectomized and nonovariectomized Cynomolgus monkeys failed to show any difference in their material properties (presented at the 48th Annual Meeting of the Orthopaedic Research Society; Dallas, Tex; February 2002). In contrast, a study was performed that demonstrated a loss of the mechanical properties of ACL in response to exogenous estrogen in a rabbit model; however, levels of estrogen comparable to those seen in pregnancy were used in this study.[16]

The published and presented data on ACL injury incidence relative to phase of the menstrual cycle remain controversial. In a study conducted on 65 female athletes, it was noted that 2.5 times the expected number of ACL injuries occurred during midcycle in women who were not taking oral contraceptives than during either the follicular or luteal phase.[20] However, a recent study of 26 female high school athletes found no change in ACL laxity from the follicular to the luteal phases of the menstrual cycle, and the authors concluded that the menstrual cycle did not significantly affect ACL laxity in female athletes.[7] Boynton et al analyzed 81 female downhill skiers with ACL ruptures during the 1998-1999 and 1999-2000 ski seasons.

This study demonstrated that there was no statistically significant variation in ACL injury rate based on phase of menstrual cycle (presented at the 26th Annual Meeting of the American Orthopaedic Society for Sports Medicine; Sun Valley, Idaho; June 2000).

Noncontact ACL injuries in women remain a complex multifactorial problem. Based on the results of this study, and given the low turnover of collagen in ligaments, it is unlikely that short durations of cyclic change in estrogen levels would result in rapid, clinically significant alterations in the material properties of the ACL in vivo. However, as with any animal study, caution must be exercised when extrapolating these data to the human. Due to the difficulty in obtaining human ACL tissue from a sufficient number of donors, we chose a model that allowed us to investigate our hypothesis from a larger population. To our knowledge, this is the first study to use fibroblasts derived from sheep, an animal with a well-defined estrus cycle, to test the influence of estrogen on ACL fibroblast metabolism. Although no significant differences were found in ACL cell proliferation and collagen synthesis in this study, it remains possible that estrogen or relaxin may have an effect on the expression or activation of MMPs in ACL tissue. Experiments are ongoing in our laboratory to determine the effect of estrogen on MMP activation and expression by ligament fibroblasts. Further clinical research is also clearly necessary to definitively determine hormonal influence on ACL physiology. Until these studies are done, pharmacological intervention to decrease estrogen levels in female athletes at risk for ACL injuries is not warranted.

ACKNOWLEDGMENT

REFERENCES

1. Arendt E, Dick R. Knee injury patterns among men and women in collegiate basketball and soccer: NCAA data and review of literature. *Am J Sports Med.* 1995;23:694-701.
2. Burgeson RE, Nimni ME. Collagen types: molecular structure and tissue distribution. *Clin Orthop.* 1992;282:250-272.
3. Gerber G, Gerber G, Altman KI. Studies on the metabolism of tissue proteins. *J Biol Chem.* 1960;235:2653-2656.
4. Gray J, Taunton JE, McKenzie DC, et al. A survey of injuries to the anterior cruciate ligament of the knee in female basketball players. *Int J Sports Med.* 1985;6:314-316.
5. Griffin LY, Agel J, Albohm MJ, et al. Noncontact anterior cruciate ligament injuries: risk factors and prevention strategies. *J Am Acad Orthop Surg.* 2000;8:141-150.
6. Hama H, Yamamuro T, Takeda T. Experimental studies on connective tissue of the capsular ligament: influences of aging and sex hormones. *Acta Orthop Scand.* 1976;47:473-479.
7. Karageanes SJ, Blackburn K, Vangelos ZA. The association of the menstrual cycle with the laxity of the anterior cruciate ligament in adolescent female athletes. *Clin J Sport Med.* 2000;10:162-168.
8. Knobil E, Neill JD. *The Physiology of Reproduction.* New York: Raven Press; 1994.
9. Liu SH, al-Shaikh R, Panossian V, et al. Primary immunolocalization of estrogen and progesterone target cells in the human anterior cruciate ligament. *J Orthop Res.* 1996;14:526-533.
10. Liu SH, Al-Shaikh RA, Panossian V, et al. Estrogen affects the cellular metabolism of the anterior cruciate ligament: a potential explanation for female athletic injury. *Am J Sports Med.* 1997;25:704-709.
11. Liu SH, Yang RS, al-Shaikh R, et al. Collagen in tendon, ligament, and bone healing: a current review. *Clin Orthop.* 1995;318:265-278.
12. Nilsson S, Roaas A. Soccer injuries in adolescents. *Am J Sports Med.* 1978;6:358-361.
13. Nimni ME, De Guia E, Bavetta LA. Synthesis and turnover of collagen precursors in rabbit skin. *Biochem J.* 1967;102:143-147.
14. Nimni ME, Deshmukh K, Bavetta LA. Turnover and age distribution of a collagen fraction extractable from rat skin by mercaptoethylamine. *Arch Biochem Biophys.* 1967;122:292-298.
15. Sciore P, Smith S, Frank CB, et al. Detection of receptors for estrogen and progesterone in human ligaments and rabbit ligaments and tendons by RT-PCR. Paper presented at: 43rd Annual Meeting of the Orthopaedic Research Society; February 9-13 1997; San Francisco, California.
16. Slauterbeck J, Clevenger C, Lundberg W, et al. Estrogen level alters the failure load of the rabbit anterior cruciate ligament. *J Orthop Res.* 1999;17:405-408.
17. Smith BA, Livesay GA, Woo SL. Biology and biomechanics of the anterior cruciate ligament. *Clin Sports Med.* 1993;12:637-670.
18. Strickland SM, Belknap TW, Turner SA, et al. Lack of hormonal influences on mechanical properties of sheep knee ligaments. *Am J Sports Med.* 2003;31:210-215.
19. Watson P. A slide centrifuge: an apparatus for concentrating cells in suspension onto a microscope slide. *J Lab Clin Med.* 1966;68:494-501.
20. Wojtys EM, Huston LJ, Boynton MD, et al. The effect of the menstrual cycle on anterior cruciate ligament injuries in women as determined by hormone levels. *Am J Sports Med.* 2002;30:182-188.
21. Yu WD, Liu SH, Hatch JD, et al. Effect of estrogen on cellular metabolism of the human anterior cruciate ligament. *Clin Orthop.* 1999;366:229-238.
22. Yu WD, Panossian V, Hatch JD, et al. Combined effects of estrogen and progesterone on the anterior cruciate ligament. *Clin Orthop.* 2001;383:268-281.
23. Zelisko JA, Noble HB, Porter M. A comparison of men's and women's professional basketball injuries. *Am J Sports Med.* 1982;10:297-299.