Sex hormone regulation of collagen concentrations in cranial cruciate ligaments of sexually immature male rabbits

Victoria A. Light, BS; Ron D. Montgomery, DVM, MS; Benson T. Akingbemi, DVM, PhD

Objective—To investigate the effects of gonadectomy on collagen homeostasis in cranial cruciate ligaments of male rabbits.

Animals—30 sexually immature (16-week-old) male New Zealand White rabbits.

Procedures—Rabbits were randomly assigned to 5 groups of 6 rabbits each: sexually intact, placebo (control group); castrated, placebo; castrated, testosterone; castrated, dihydrotestosterone; and castrated, 17β-estradiol (E_2). Control rabbits underwent a sham operation, and all other rabbits underwent gonadectomy. At the time of gonadectomy, the placebo and sex hormones were administered via slow-release pellets implanted subcutaneously as assigned. After 21 days of hormone supplementation, measurements were obtained of serum testosterone and E_2 concentrations, ligament collagen characteristics, and androgen receptor, estrogen receoptor α, and matrix metalloproteinase expression.

Results—Following gonadectomy and hormone supplementation, the treatment groups differed in serum testosterone and E_2 concentrations to various degrees. Collagen concentrations were lower and fiber diameters higher in the absence of sex hormones, in association with the degrees of estrogen receptor α and androgen receptor expression. Although differences were detected among the groups in matrix metalloproteinase expression, these differences were not significant.

Conclusions and Clinical Relevance—Sex hormones appeared to play a role in cranial cruciate ligament homeostasis in male rabbits. Physiologic changes triggered by the lack of sex hormones following gonadectomy in sexually immature rabbits may potentially predispose those rabbits to orthopedic injuries. (*Am J Vet Res* 2012;73:1186–1193)

Rupture of the CCL is the leading cause of orthopedic disorders of the canine stifle joint,[1] and 20% of dogs admitted to university hospitals for lameness have this condition.[2–4] The economic impact associated with the treatment of CCL rupture in the United States in 2003 was estimated at > $1.3 billion.[4]

The pathogenesis of CCL rupture has been linked to degenerative changes within the ligament and may result from conformational abnormalities and traumatic events.[2,5–7] Studies[5,8,9] have demonstrated a positive correlation between CCL rupture, body weight, gonadectomy status, and age-related factors, such as development of synovitis and degenerative or adaptive changes in the ligament.

Abbreviations

AR	Androgen receptor
CCL	Cranial cruciate ligament
DHT	Dihydrotestosterone
E_2	17β-estradiol
ESR1	Estrogen receptor α
MMP	Matrix metalloproteinase
TEM	Transmission electron microscopy

The possibility exists that sex hormones impact CCL integrity. A review of data from > 1,000 dogs with CCL injuries at 23 veterinary clinics across North America revealed that gonadectomized dogs of both sexes had a higher prevalence of such injuries than did sexually intact dogs.[5] Another study[10] found that the prevalence of CCL injuries in sexually intact female dogs (5.15%) was higher than in sexually intact male dogs (2.09%). These findings were supported by those of observational studies[10,11] that found gonadectomy predicted an increased prevalence of CCL injuries in both sexes and in all sizes and breeds of dogs. Altogether, these observations suggest that the incidence of CCL rupture may be related, at least in part, to sex hormone action.

Received February 9, 2011.
Accepted July 21, 2011.
From the Departments of Anatomy, Physiology and Pharmacology (Light, Akingbemi) and Clinical Sciences (Montgomery), College of Veterinary Medicine, Auburn University, Auburn, AL 36849.
This manuscript represents a portion of a dissertation submitted by the first author to the Auburn University College of Veterinary Medicine as partial fulfillment of the requirements for a Doctor of Philosophy degree.
Supported by a Scott-Ritchey Research Center (SRRC) Interdepartmental Research Grant Program award to Dr. Akingbemi.
The authors thank Karen Wolfe for technical assistance.
Address correspondence to Dr. Light (lightva@tigermail.auburn.edu) or Dr. Akingbemi (akingbt@auburn.edu).

The mechanisms by which sex hormones affect CCL integrity are not clear, but studies[12–14] have shown that CCL remodeling is subject to sex hormone action. For example, high serum E_2 concentrations in women can decrease collagen production in anterior cruciate ligament cultures.[15] Other studies[16–19] found that expression of the MMP group of enzymes, which play a role in collagen metabolism, is regulated by hormones. Thus, it is possible that sex differences affecting ligament remodeling have an influence on the size, shape, and internal structure of the CCL and hence its ability to sustain loads.[20] It follows that altered ligament remodeling triggered by changes in blood sex hormone concentrations potentially predisposes CCLs to injury.[20]

Although castration has historically been performed as early as 4 weeks of age,[21] veterinarians in the United States now recommend that gonadectomy in dogs and cats be performed between 6 and 9 months of age.[22] Incidentally, gonadectomy prior to the attainment of sexual maturity has been suggested to result in sexual and developmental anomalies or to predispose dogs to certain diseases.[23,24] The interaction between E_2 and relaxin is believed to selectively contribute to degeneration of specific synovial joints in women, such as the knee joint, pubic symphysis, and temporomandibular joint.[25] Indeed, sex hormones appear to play a role in matrix turnover and collagen loss.[26] However, there is little or no information on the role of sex hormones in collagen homeostasis in males. The purpose of the study reported here was to investigate the effects of gonadectomy on collagen homeostasis in the CCLs of male rabbits.

Materials and Methods

Animals—Thirty 16-week-old sexually intact male New Zealand White rabbits[a] were obtained and allowed 3 days to acclimate to new housing conditions. This study protocol was approved by the Institutional Animal Care and Use Committee of Auburn University.

Procedures—Rabbits were randomly assigned to 5 groups of 6 rabbits each as follows: sexually intact–placebo (control group), castrated-placebo, castrated-testosterone, castrated-DHT, and castrated-E_2. On the fourth day after arrival, rabbits were anesthetized by IM administration of ketamine[b] (15 mg/kg) and dexmedotomidine[c] (0.5 mg/kg). To establish baseline values for serum sex hormone concentrations, a blood sample was obtained from a marginal ear vein and separated to obtain serum, which was stored at –20°C until analyzed.

Rabbits in the control group underwent a sham operation, whereas all remaining rabbits underwent gonadectomy. At the time of surgery, placebo and slow-release treatment pellets containing steroid hormones[d] were implanted subcutaneously in the lateral aspect of the neck and a suture was placed over the incision. Treatment pellets contained 50 mg of testosterone or DHT or 25 mg of E_2. Implants were left in place for a period of 3 weeks (21 days), at the end of which the rabbits were anesthetized for blood sample collection followed by euthanasia with a solution of pentobarbital sodium and phenytoin sodium[e] (150 mg/kg, IV).

The right CCL of each rabbit was aseptically harvested, snap frozen in liquid nitrogen, and stored at –80°C until immunoblot analysis of gene expression (n = 6 ligaments). The left CCL was collected and processed for electron microscopy to determine collagen fiber diameters (n = 3) or for collagen assays to measure collagen concentrations (n = 3).

Radioimmunoassays—Serum concentrations of testosterone and E_2 were determined with a tritium-labeled testosterone and E_2 radioimmunoassay[f] that involved use of antibodies against testosterone and E_2[g] (anti–testosterone-6 bovine serum albumin and anti–estradiol-6 bovine serum albumin, respectively). Interassay variation for the testosterone assay was 7.8%, with a lower limit of detection of 0.01 ng/mL, whereas the interassay variation for E_2 was 8.5%, with a lower limit of detection of 0.005 ng/mL.

Collagen assay—Concentrations of soluble collagen in CCLs were determined with a dye collagen assay kit[h] in accordance with manufacturer's instructions. This assay is based on the binding of Sirius red, which is an anionic dye, to the side chains of amino acids present in collagens fibers (I to IV). Briefly, ligament samples (50 mg) were placed in digestion solution containing a protease inhibitor mixture (10 µL/mL) and pepsin (1:10) in 0.5M acetic acid at a pH of 3.0 and incubated overnight on a rocker at room temperature (approx 22°C). The following morning, fresh solution was added and incubation was continued for 4 hours. At the end of incubation, 100 µL of the pepsin digest was mixed with 1 mL of dye and incubated for 30 minutes at room temperature. The collagen-dye complex was then precipitated by centrifugation at 12,879 × *g* for 10 minutes. The supernatants were drained and discarded, and the collagen-dye pellet was dissolved in 1 mL of alkali buffer provided by the manufacturer.

Aliquots of the dissolved collagen-dye complex (200 µL) for each sample were transferred to a 96-well plate and measured at a wavelength of 540 nm on a plate reader.[i] Ligament collagen concentrations were calculated by use of collagen standards provided by the manufacturer. The assays were performed in triplicate, and means were calculated for results in each treatment group.

Western blot procedures—Degrees of steroid hormone receptor and MMP expression were determined in CCL samples from all 6 rabbits in all 6 groups. Briefly, ligament samples (25 mg) were homogenized in lysis buffer[j] supplemented with 10 µL of protease inhibitor[k] in preparation for evaluation with the Bradford protein assay.[l] Ten- to 20-µg samples of the homogenized product were applied to 10% glycine-SDS-PAGE gels in a vertical electrophoresis system[l] and transferred by electrophoresis to nitrocellulose membranes[l] (0.45 µm). Subsequently, nitrocellulose membranes were preincubated for 60 minutes at room temperature in blocking buffer (5% whole milk in Tris-buffered saline [0.9% NaCl] solution containing 0.1% Tween 20). The nitrocellulose blots were incubated overnight at 4°C with primary antibodies against ESR1,[m] AR,[n] MMP-1,[n] MMP-2,[m] MMP-9,[m] and β-actin[n] at a dilution of 1:1,000 or 1:2,000 (β-actin). Subsequently, blots were

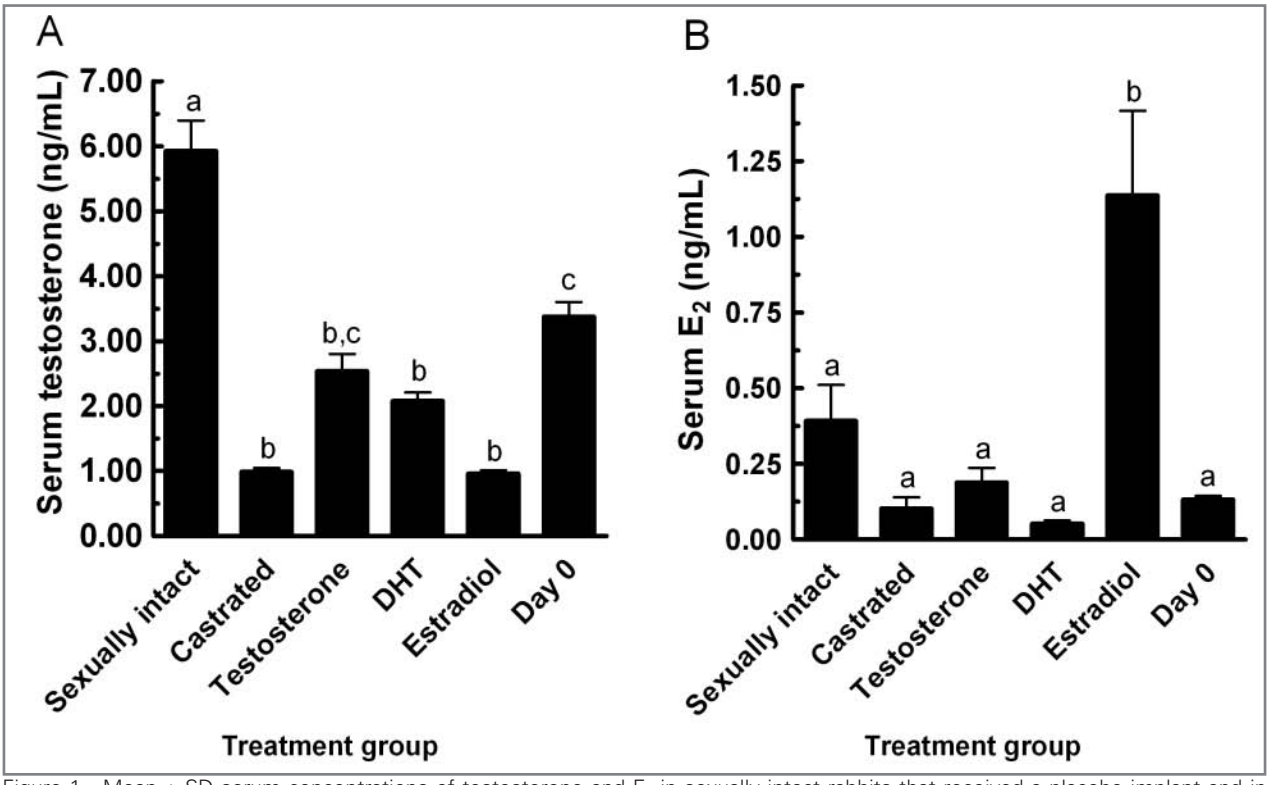

Figure 1—Mean ± SD serum concentrations of testosterone and E_2 in sexually intact rabbits that received a placebo implant and in castrated rabbits that were implanted with placebo, DHT, testosterone, or E_2 pellets (n = 6 rabbits/group), as measured with a radioimmunoassay. Data for day 0 represent precastration values. [a–c]Values with different letters are significantly ($P < 0.05$) different.

washed 3 times for 5 minutes each time and incubated in the appropriate horseradish peroxidase–conjugated secondary antibody[n] at a dilution of 1:2,000 in the blocking buffer for 90 minutes.

The resulting bands were made visible via chemiluminescence.[n] Relative protein amounts in identified immunoblots were estimated by measuring optical density of the bands displayed on exposed autoradiography films[o] with the aid of computer software[p] and were normalized to the degree of β-actin expression as an internal control. Six western blots were performed for each desired protein, with 1 sample from each of the 5 groups along with 2 positive control samples.

TEM—The diameter of collagen fibers was determined from ultraphotomicrographs after CCL samples were processed for TEM by use of standard procedures. Briefly, CCL samples were collected and immediately placed in a mixture of 4% paraformaldehyde–PBS solution and 0.5% gluteraldehyde solution. The samples were subsequently immersion-fixed in 2% O_sO_4–PBS solution overnight at 4°C. Following dehydration in a graded series of ethanol, samples were infiltrated and embedded with an epoxy resin.[q] Polymerization of capsules was performed at 60°C for 48 hours. Ultrathin sections (approx 85 to 90 nm) were collected on copper mesh grids, and photomicrographs were obtained via TEM[r] at a magnification of 45,000X. Three photomicrographs were obtained per sample, and 20 random fiber diameters were measured on each photomicrograph. Thus, 60 measurements/sample and 180 measurements/treatment group were obtained.

Statistical analysis—Data are reported as mean ± SD. Data were analyzed with 1-way ANOVA followed by the Tukey-Kramer test for multiple group comparisons.[s] Values of $P < 0.05$ were considered significant. Pearson interclass correlation coefficients (r) were determined for the relationship between collagen concentrations and fiber diameters.

Results

Following gonadectomy and implantation of hormone pellets (placebo, DHT, testosterone, and E_2) in the rabbits, several differences were evident in serum sex hormone concentrations among the various treatment groups (**Figure 1**; $P < 0.05$). For example, serum testosterone concentrations were low in the castrated-placebo, castrated-DHT, and castrated-E_2 groups and were high in the castrated-testosterone group, although concentrations were lower than in the sexually intact control group ($P < 0.05$). On the other hand, serum E_2 concentrations were higher in the castrated-E_2 group than in other groups ($P < 0.05$). Mean ± SD baseline serum testosterone and E_2 concentrations were 3.38 ± 1.73 ng/mL and 0.12 ± 0.09 ng/mL, respectively. Data on precastration serum testosterone and E_2 concentrations for all groups were pooled because there were no differences among groups ($P > 0.05$).

The collagen assay revealed that the lack of sex hormones had an effect on collagen concentrations in CCL samples (**Figure 2**; $P < 0.05$). For example, collagen concentrations in CCL samples from castrated rabbits were low, compared with concentrations in sexually in-

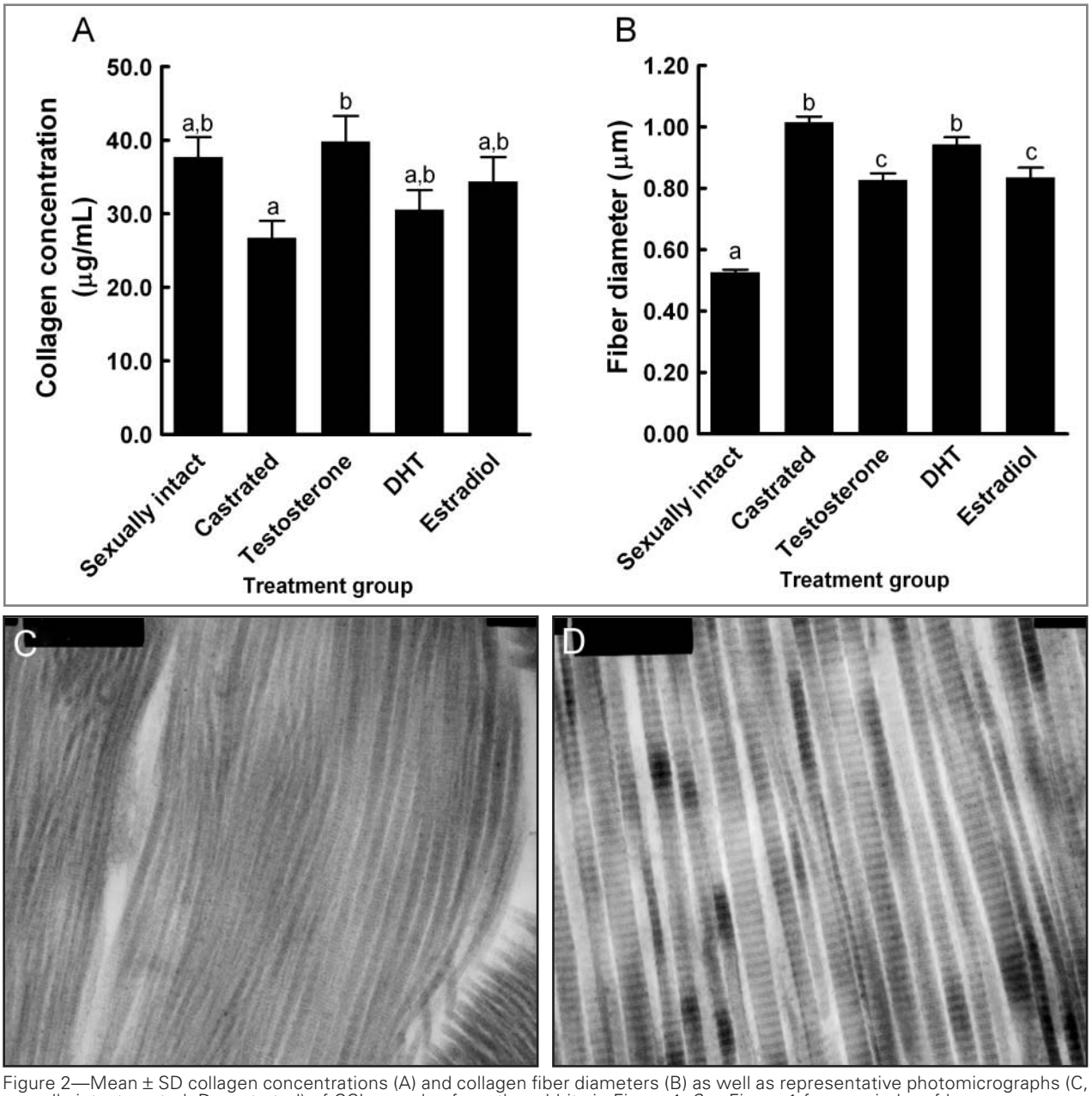

Figure 2—Mean ± SD collagen concentrations (A) and collagen fiber diameters (B) as well as representative photomicrographs (C, sexually intact control; D, castrated) of CCL samples from the rabbits in Figure 1. *See* Figure 1 for remainder of key.

tact rabbits in the control group. These concentrations were partially restored to baseline values in rabbits in the castrated-DHT and castrated-E_2 groups and were fully restored in rabbits in the castrated-testosterone group ($P < 0.05$). On the other hand, collagen fiber diameters were smallest in the sexually intact control group, were greatest in the castrated-placebo and castrated-DHT group, and were intermediate in castrated-testosterone and castrated-E_2 groups ($P < 0.05$).

The changes in collagen concentrations and fiber diameters were associated with differences in the degree of steroid hormone receptor (AR and ESR1) expression in CCL samples. For example, the degree of AR expression was greater in the castrated-placebo group than in castrated rabbits that received sex hormone implants (**Figure 3**; $P < 0.05$). Although ESR1 expression in the castrated-placebo group appeared greater than in the castrated-testosterone group, the difference was not significant. Similar to AR expression, the degree of ESR1 expression was greater in the castrated-placebo group than in the castrated-DHT and castrated-E_2 groups ($P < 0.01$). Furthermore, ESR1 expression in castrated-placebo rabbits was greater than in sexually intact rabbits ($P < 0.05$).

Differences in serum sex hormone concentrations appeared to affect MMP expression, although these findings were not significant ($P > 0.05$). However, the degree of MMP-1, -2, and -9 expression was similar in CCL samples from sexually intact control animals and in castrated rabbits that received testosterone and DHT implants but was higher in the castrated-placebo and castrated-E_2 groups (**Figure 4**). The degree of MMP ex-

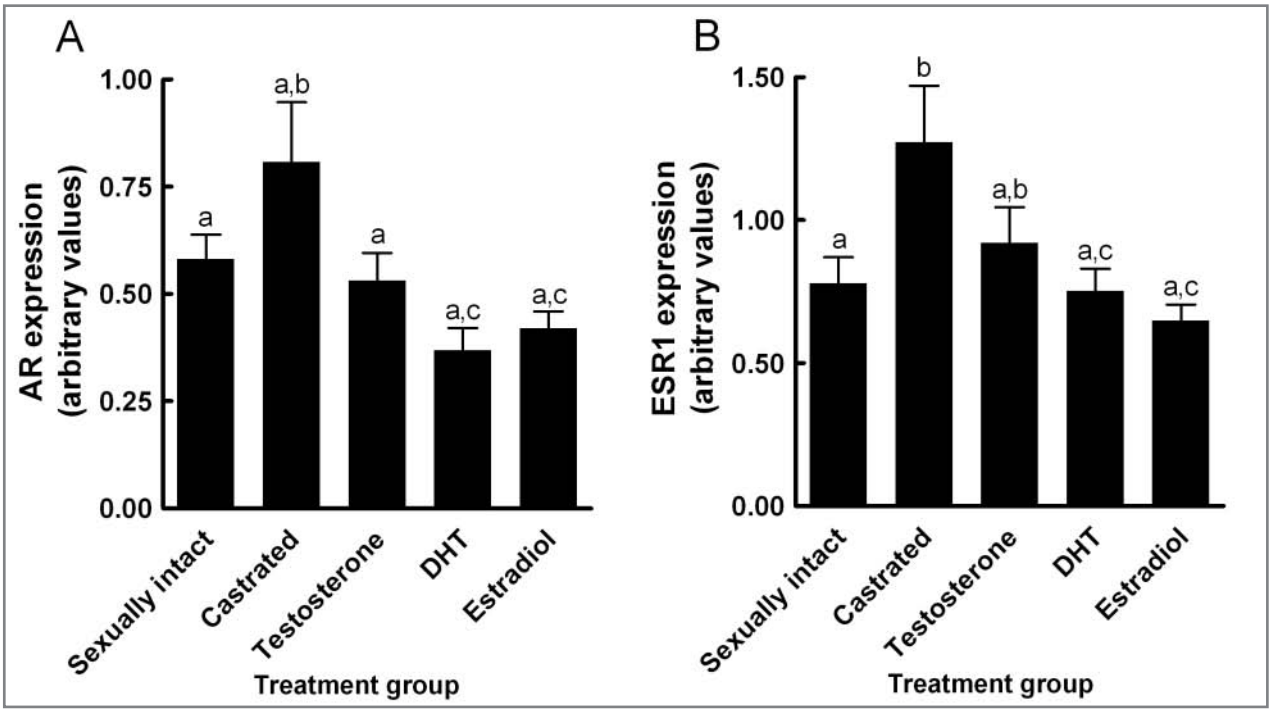

Figure 3—Mean ± SD degree of protein expression for AR (A) and ESR1 (B) in CCL samples (n = 6/group) from the rabbits in Figure 1. Values were calculated in relation to β-actin expression and are reported in arbitrary units. *See* Figure 1 for remainder of key.

pression was inversely related ($r = –0.73$; $P < 0.05$) to collagen concentrations in the castrated-placebo group (Figure 3).

Discussion

Understanding any effect that sex hormones have on CCL growth during sexual maturation could provide an informed basis for determining the appropriate age for gonadectomy in young animals. Through use of sexually immature male rabbits, the present study was designed to investigate the effects of sex hormones on collagen content in CCLs. Although castrated rabbits that received E_2 implants had higher serum concentrations of these hormones than did sexually intact control rabbits, the concentrations were within the physiologic range of < 20 pg/mL for male rabbits.[27]

The results of the present study suggest that gonadectomy decreases serum testosterone concentration and collagen concentrations but increases ESR1 expression and fiber diameters in the CCLs of rabbits. Sex hormone implantation resulted in partial or full alleviation of these effects. Nevertheless, the finding that collagen concentration was decreased after gonadectomy is important because collagen is known to be the main load-bearing component of ligaments. We also observed that following gonadectomy, rabbits that received testosterone or E_2 implants had collagen concentrations more similar to those of sexually intact control rabbits; this effect was not observed in DHT-supplemented rabbits. Consequently, aromatization of testosterone to E_2 could be an important regulating factor in CCL homeostasis. Although changes in MMP protein expression following gonadectomy were subtle, further elucidation of the interaction between sex hormones and MMP activity is warranted. Altogether, these results suggest that sex hormones are necessary for CCL homeostasis in male rabbits during sexual maturation.

In a study[28] of collagen fiber diameter in CCLs of 2 breeds of dogs with or without a predisposition to CCL rupture, investigators found that collagen fiber diameters of the predisposed dogs were significantly smaller than those of the nonpredisposed dogs. This finding led to the supposition that collagen fiber diameter might be an important factor in CCL insufficiency in dogs. In contrast, the present study showed that sexually intact rabbits, which are presumably less likely to be predisposed to CCL insufficiency, had smaller fiber dimensions than any castrated rabbit, including those that received hormone implants. An inverse relationship between collagen concentrations and fiber diameters was also evident. Although the mechanisms that bring about the increase in fiber diameter are not yet understood, the results of the present study do not suggest that fiber diameters predict CCL insufficiency.

The principal function of sex hormones in males is to support development of sexual characteristics and behavior and to maintain male fertility.[29] Although testosterone is the primary endocrine product of the testes, E_2, which is the predominant sex hormone in females, is present in small amounts in males (approx 20% of the concentration in nonpregnant females).[29] Conversion of testosterone to E_2 is due to action of the aromatase enzyme, which is expressed in several body tissues.[29] However, there is general consensus that, in addition to regulating reproductive activity, sex hormones affect development of the musculoskeletal system.[29,30]

Because blood testosterone concentrations gradually increase in parallel with other changes

that occur during sexual development in males, it is reasonable to expect that the surge in sex hormone concentrations that occurs during sexual maturation impacts physical development. Indeed, long-standing evidence suggests that testosterone influences collagen remodeling and structure.[31–33] There is also clear evidence that estrogens regulate the collagen content of CCLs[15] and affect the load needed for the ligament to rupture in women.[34] In this regard, several studies[35] have demonstrated that E_2 can modulate changes in collagen turnover and MMP expression. Therefore, an interesting observation in the present study was the increase in the degree of AR and ESR1 expression in CCLs from castrated rabbits. Although the basis for the increase in receptor expression after castration remains unclear, it is possible that increased degree of AR and ESR1 expression represents an adaptive response mechanism that serves to augment sex hormone signaling after gonadectomy.[36]

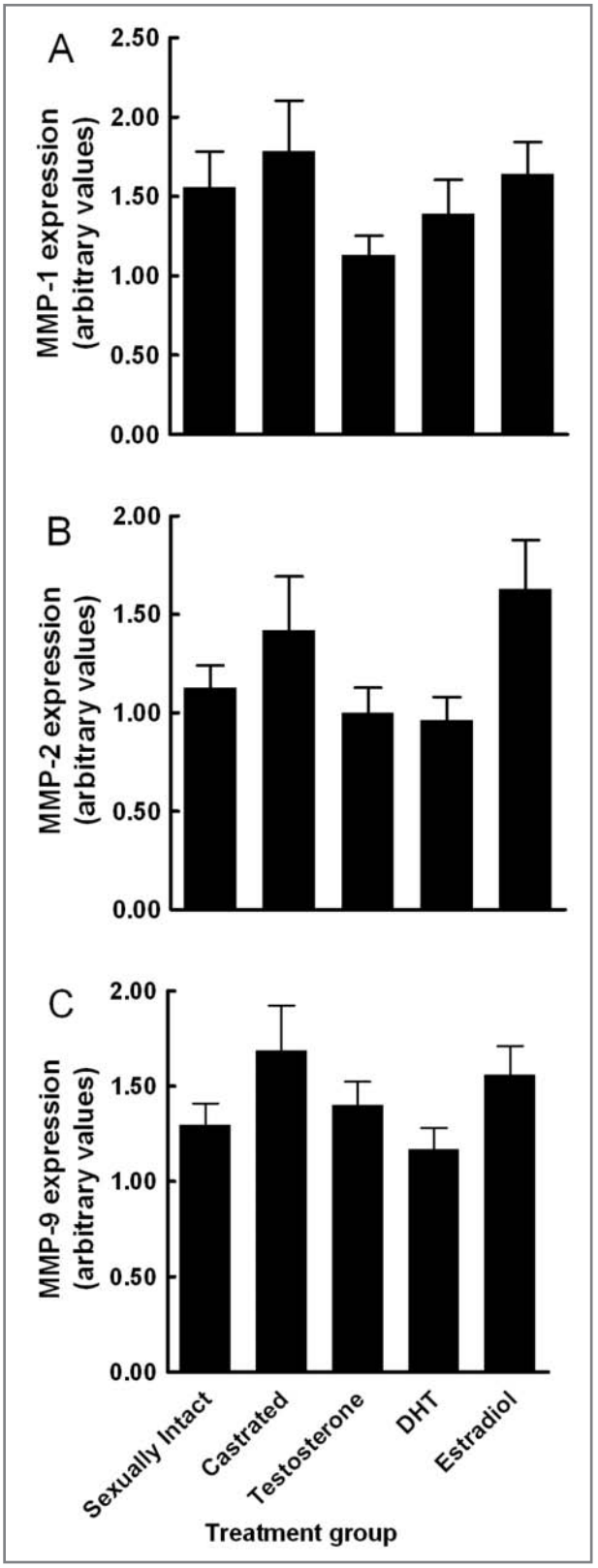

Figure 4— Mean ± SD degree of protein expression for MMP-1 (A), MMP-2 (B), and MMP-9 (C) in CCL samples from the rabbits in Figure 1. Differences between groups were not significant ($P > 0.05$). *See* Figures 1 and 3 for remainder of key.

The MMP family of proteolytic enzymes mediates the degradation and turnover of extracellular matrix macromolecules and collagen fibers.[19] Analysis of MMP expression in the presence and absence of sex hormones was important to the objectives of the present study because estrogens and estrogen receptor agonists are known to affect collagen content[37] as well as MMP-2 and MMP-9 activity.[38] However, a major limitation of the study was our inability to measure MMP enzyme activity or to analyze a wide spectrum of collagenases. Although increases in MMP-1, -2, and -9 expression were detected after gonadectomy, these changes were not significant. The lack of robust changes in MMP expression may have been due in part to the small sample size, which might have limited our ability to detect associations, or may have been the result of variations in the responses of individual rabbits to sex hormone implantation and action. Other uninvestigated MMPs might have had a greater role in mediating sex hormone action in CCLs.[31] This possibility remains to be investigated. Also considered was the idea that a longer period of sex hormone deprivation than used in the present study (3 weeks) might have yielded a different outcome.

In the United States, veterinarians commonly castrate nonbreeding dogs at < 6 months of age. However, results of several studies[21,39–41] in which the effects of early-age gonadectomy on skeletal development were investigated suggest that this standard practice may adversely affect dogs involved in sports (eg, racing or agility trials). For example, bone growth plate closure occurs later in dogs castrated at 7 weeks than in those castrated at 7 months of age.[21] In concert with growth factors, sex hormones promote closure of growth plates at sexual maturity,[40] suggesting that bones in dogs castrated before sexual maturity may continue to grow. Thus, dogs castrated at an early age can have longer limbs than dogs castrated later in life.[40] Abnormal growth in bone length commonly results in modifications in body proportions and distortions of bone-to-bone relationships. Excessive growth increases the lever arm (distance from the fulcrum or point of angular movement) in the lower portion of the hind limb, which exerts stress on the CCL. Together, these observations suggest that structural and physiologic changes triggered by the lack

of sex hormones following gonadectomy predispose these animals to orthopedic injuries.

Other evidence exists that castrated dogs have a higher incidence of CCL rupture,[10] which is potentially detrimental to performance in dogs used in sports. Additional studies are warranted to evaluate the effects of gonadectomy prior to sexual maturity on musculoskeletal function, particularly in canine athletes. Studies are also required to determine the relative roles and interactions of sex hormones and other physiologic factors in the regulation of CCL development prior to sexual maturity or cessation of physical development.

a. Myrtle's Rabbitry Inc, Thompsons Station, Tenn.
b. Fort Dodge Animal Health, Charles City, Iowa.
c. Orion Corp, Espoo, Finland.
d. Innovative Research of America, Sarasota, Fla.
e. Euthasol, Virbac AH Inc, Fort Worth, Tex.
f. T-Net 370, E_2-Net 317, Perkin Elmer Life and Analytical Sciences, Boston, Mass.
g. Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colo.
h. Sircol Collagen Assay Kit, Accurate Chemical and Scientific Corp, Westbury, NY.
i. SPECTRAmax Plus 384, Molecular Devices, Sunnyvale, Calif.
j. Lysis buffer, product No. 78510, Thermo Scientific, Rockford, Ill.
k. Protease inhibitor, product No. 78410, Thermo Scientific, Rockford, Ill.
l. Mini-PROTEAN, Bio-Rad Laboratories, Hercules, Calif.
m. Abcam, Cambridge, Mass.
n. Santa Cruz Biotechnology, Santa Cruz, Calif.
o. Denville Scientific, Metuchen, NJ.
p. Doc-It LS software, version 5.5.4, UVP Inc, Upland, Calif.
q. Ducopan Epoxy Resin, EMS, Hatfield, Pa.
r. Phillips 301, 60 kV, Eindhoven, The Netherlands.
s. Prism, version 4.0, GraphPad Inc, San Diego, Calif.

References

1. Marshall JL, Olsson SE. Instability of the knee. A long-term experimental study in dogs. *J Bone Joint Surg Am* 1971;53:1561–1570.
2. Moore KW, Read RA. Rupture of the cranial cruciate ligament in dogs—part 1. *Compend Contin Educ Pract Vet* 1996;18:223–234.
3. Palmer RH. Understanding tibial plateau leveling osteotomies in dogs. *Vet Med* 2005;100:426–430.
4. Wilke VL, Conzemius MG, Kinghorn BP, et al. Inheritance of rupture of the cranial cruciate ligament in Newfoundlands. *J Am Vet Med Assoc* 2006;228:61–64.
5. Whitehair JG, Vasseur PB, Willits NH. Epidemiology of cranial cruciate ligament rupture in dogs. *J Am Vet Med Assoc* 1993;203:1016–1019.
6. de Rooster H, de Bruin T, van Bree H. Invited review—morphologic and functional features of the canine cruciate ligaments. *Vet Surg* 2006;35:769–780.
7. Johnson JM, Johnson AL. Cranial cruciate ligament rupture. Pathogenesis, diagnosis, and postoperative rehabilitation. *Vet Clin North Am Small Anim Pract* 1993;23:717–733.
8. Duval JM, Budsberg SC, Flo GL, et al. Breed, sex, and body weight as risk factors for rupture of the cranial cruciate ligament in young dogs. *J Am Vet Med Assoc* 1999;215:811–814.
9. Bennett D, Tennant B, Lewis DG, et al. A reappraisal of anterior cruciate ligament disease in the dog. *J Small Anim Pract* 1988;29:275–297.
10. Slauterbeck JR, Pankratz K, Xu KT, et al. Canine ovariohysterectomy and orchiectomy increases the prevalence of ACL injury. *Clin Orthop Relat Res* 2004:301–305.
11. Amiel D, Ishizue KK, Harwood FL, et al. Injury of the anterior cruciate ligament: the role of collagenase in ligament degeneration. *J Orthop Res* 1989;7:486–493.
12. Dahlberg L, Friden T, Roos H, et al. A longitudinal study of cartilage matrix metabolism in patients with cruciate ligament rupture—synovial fluid concentrations of aggrecan fragments, stromelysin-1 and tissue inhibitor of metalloproteinase-1. *Br J Rheumatol* 1994;33:1107–1111.
13. Foos MJ, Hickox JR, Mansour PG, et al. Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament. *J Orthop Res* 2001;19:642–649.
14. Matrisian LM. Matrix metalloproteinase gene expression. *Ann N Y Acad Sci* 1994;732:42–50.
15. Yu WD, Liu SH, Hatch JD, et al. Effect of estrogen on cellular metabolism of the human anterior cruciate ligament. *Clin Orthop Relat Res* 1999;366:229–238.
16. Cho BS, Roelofs KJ, Ford JW, et al. Decreased collagen and increased matrix metalloproteinase-13 in experimental abdominal aortic aneurysms in males compared with females. *Surgery* 2010;147:258–267.
17. Woodrum DT, Ford JW, Ailawadi G, et al. Gender differences in rat aortic smooth muscle cell matrix metalloproteinase-9. *J Am Coll Surg* 2005;201:398–404.
18. Ehrlichman LK, Ford JW, Roelofs KJ, et al. Gender-dependent differential phosphorylation in the ERK signaling pathway is associated with increased MMP2 activity in rat aortic smooth muscle cells. *J Surg Res* 2010;160:18–24.
19. Kapila S. Does the relaxin, estrogen and matrix metalloproteinase axis contribute to degradation of TMJ fibrocartilage? *J Musculoskelet Neuronal Interact* 2003;3:401–407.
20. 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.
21. Salmeri KR, Bloomberg MS, Scruggs SL, et al. Gonadectomy in immature dogs: effects on skeletal, physical, and behavioral development. *J Am Vet Med Assoc* 1991;198:1193–1203.
22. Kustritz MV. Determining the optimal age for gonadectomy of dogs and cats. *J Am Vet Med Assoc* 2007;231:1665–1675.
23. Ware WA, Hopper DL. Cardiac tumors in dogs: 1982–1995. *J Vet Intern Med* 1999;13:95–103.
24. Panciera DL. Hypothyroidism in dogs: 66 cases (1987–1992). *J Am Vet Med Assoc* 1994;204:761–767.
25. Lekgabe ED, Royce SG, Hewitson TD, et al. The effects of relaxin and estrogen deficiency on collagen deposition and hypertrophy of nonreproductive organs. *Endocrinology* 2006;147:5575–5583.
26. Hashem G, Zhang Q, Hayami T, et al. Relaxin and beta-estradiol modulate targeted matrix degradation in specific synovial joint fibrocartilages: progesterone prevents matrix loss. *Arthritis Res Ther* 2006;8:R98.
27. Hanke H, Hanke S, Finking G, et al. Different effects of estrogen and progesterone on experimental atherosclerosis in female versus male rabbits. Quantification of cellular proliferation by bromodeoxyuridine. *Circulation* 1996;94:175–181.
28. Comerford EJ, Tarlton, JF, Wales A, et al. Ultrastructural differences in cranial cruciate ligaments from dogs of two breeds with a differing predisposition to ligament degeneration and rupture. *J Comp Pathol* 2006;134:8–16.
29. Guyton AC, Hall JE. *Textbook of medical physiology*. 10th ed. Philadelphia: WB Saunders Co, 2000;923–924.
30. Srinivasan N, Aruldhas MM, Govindarajulu P. Sex steroid-induced changes in collagen of the prostate and seminal vesicle of rats. *J Androl* 1986;7:55–58.
31. Lovering RM, Romani WA. Effect of testosterone on the female anterior cruciate ligament. *Am J Physiol Regul Integr Comp Physiol* 2005;289:R15–R22.
32. Savvas M, Bishop J, Laurent G, et al. Type III collagen content in the skin of postmenopausal women receiving oestradiol and testosterone implants. *Br J Obstet Gynaecol* 1993;100:154–156.
33. Varila E, Rantala I, Oikarinen A, et al. The effect of topical oestradiol on skin collagen of postmenopausal women. *Br J Obstet Gynaecol* 1995;102:985–989.
34. 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.
35. Dyer RF, Sodek J, Heersche JN. The effect of 17 beta-estradiol on

collagen and noncollagenous protein synthesis in the uterus and some periodontal tissues. *Endocrinology* 1980;107:1014–1021.
36. Antonio J, Wilson JD, George FW. Effects of castration and androgen treatment on androgen-receptor levels in rat skeletal muscles. *J Appl Physiol* 1999;87:2016–2019.
37. Oestergaard S, Sondergaard BC, Hoegh-Andersen P, et al. Effects of ovariectomy and estrogen therapy on type II collagen degradation and structural integrity of articular cartilage in rats: implications of the time of initiation. *Arthritis Rheum* 2006;54:2441–2451.
38. Nielsen RH, Christiansen C, Stolina M, et al. Oestrogen exhibits type II collagen protective effects and attenuates collagen-induced arthritis in rats. *Clin Exp Immunol* 2008;152:21–27.
39. Spain CV, Scarlett JM, Houpt KA. Long-term risks and benefits of early-age gonadectomy in dogs. *J Am Vet Med Assoc* 2004;224:380–387.
40. Grumbach MM. Estrogen, bone, growth and sex: a sea change in conventional wisdom. *J Pediatr Endocrinol Metab* 2000;13(suppl 6):1439–1455.
41. Howe LM, Slater MR, Boothe HW, et al. Long-term outcome of gonadectomy performed at an early age or traditional age in dogs. *J Am Vet Med Assoc* 2001;218:217–221.