Transmission of relaxin and estrogens to suckling canine pups via milk and possible association with hip joint laxity

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Objective—To determine whether abnormal laxity of hip joints of canine pups with genetic predisposition to hip dysplasia (HD+) is related to ingestion of milk-borne hormones.

Animals—7 female Labrador Retrievers with HD+ and 8 with low predisposition to hip dysplasia (HD−) and their offspring.

Procedures—Immunoactive relaxin, estrogen, and estrogen precursor concentrations in milk of HD+ lactating bitches and in serum of their pups were compared with those of HD− bitches and pups. An aromatase inhibitor (CGS 16,949A) was injected into pups of HD+ bitches during lactation to inhibit estrogen synthesis from milk-borne precursors, and hip joint laxity was compared with that of control littersmates. Hip joint laxity of pups of HD− bitches, which received an injection with estradiol cypionate and canine relaxin, was compared with that of control littersmates to determine whether these hormones induced hip joint laxity.

Results—High concentrations of estrogens and relaxin were found in milk of HD+ and HD− bitches throughout lactation. Serum concentrations of milk-derived relaxin and total estrogens were similar in all pups, but estradiol-17β was detected only in pups of HD+ bitches. Hip joint laxity was reduced in pups that received CGS 16,949A. Hip joint laxity was increased in pups of HD− bitches that received estradiol cypionate and relaxin.

Conclusions and Clinical Relevance—Milk-borne maternal hormones and precursors were absorbed into the circulation of canine neonates and may play a role in hip joint laxity in HD+ pups. Phenotypic expression of hip dysplasia may therefore be preventable by antihormone treatment. (Am J Vet Res 2008;69:59–67)

Canine hip dysplasia is a polygenically inherited developmental abnormality that is associated with osteoarthritis in adulthood. Prevalence of hip dysplasia can be close to 50% in some breeds, according to statistics compiled by the Orthopedic Foundation for Animals. The hip joint is supported by muscles, ligaments, and a capsule, all of which must develop congruously to ensure joint stability, especially during the first 60 days after birth, prior to ossification, when the tissues are still pliable and readily deformed. Thus, it is likely that the factors that initiate the dysplastic process are expressed during this time period.

One of the early abnormalities detected in canine pups destined to develop hip dysplasia is a laxity of the hip joints that permits excessive movement of the femoral head in its acetabular socket, resulting in joint damage. Dysplasia frequently occurs in more than 1 joint, suggesting a possible involvement of blood-borne mediators, and a specific role of maternal hormones in abnormal hip development has long been suspected. Injection of large doses of estrogens into bitches in late stages of pregnancy or estrogens and a crude relaxin extract injected directly into newborn pups induce a dysplasia-like skeletal abnormality.

Relaxin is a 6-kd polypeptide hormone of mammalian pregnancy that, in concert with estrogens, induces profound changes in the composition and tensile properties of connective tissues of the pubic symphysis.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>HD+</td>
<td>Genetically predisposed to hip dysplasia</td>
</tr>
<tr>
<td>HD−</td>
<td>Not genetically predisposed to hip dysplasia</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>ECP</td>
<td>Estradiol cyclopentylpropionate</td>
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</table>
and sacroiliac joints. In several species, estrogens and relaxin induce formation of interpubic ligaments, increasing the diameter and flexibility of the birth canal; in others, estrogens and relaxin increase the extensibility of the pubic or sacroiliac joints. These hormonally induced changes in flexibility facilitate parturition and result from breakdown of connective tissues in association with increased activity of several proteases, including collagenase. The similarity of the relaxin-induced increase in flexibility of the pelvic ligaments to the abnormal laxity of prepubertal children observed in HD+ pups led us to postulate that maternal estrogens and relaxin might be the prime initiators of hip disease in these animals.

Relaxin receptors are members of the leucine-rich, repeat-containing, G-protein-coupled receptor group and are designated as LGR7 and LGR8. A previous study revealed that estrogens promote the synthesis of relaxin receptors and, conversely, that relaxin activates estrogen receptors. In addition, estrogens prime connective tissues for relaxin’s action by inducing synthesis and activation of key enzymes and mediators required for collagen and proteoglycan breakdown.

Crelin discovered that genetically normal neonates are protected against untoward effects of estrogen and relaxin. Thus, the pubic symphysis of newborn mice does not respond to exogenous estrogen and relaxin and remains refractory until the time of puberty. Moreover, pubic symphyses transplanted from nurslings to adults respond to estrogen and relaxin only if excised after the 12th day of birth. The refractoriness of newborn mice to estrogen and relaxin suggests that the pubic symphysis may lack specific receptors for one or both of these hormones.

In other mammalian species, specific estrogen binding sites have been found in adult articular cartilage, but not in epiphyseal plate or embryonic cartilage, suggesting that mature cartilage, but not transforming or developing cartilage, responds to estrogens through a receptor mechanism. In harmony with this finding, estradiol-17β enhanced, whereas an antiestrogen inhibited, cartilage destruction in experimental osteoarthritis in adult rabbits, purportedly acting through a receptor mechanism. Although radiolabeled relaxin specifically bound to connective tissues of the pubic symphyses of adult mice and rats, the relationship between age and first appearance of chemically identified relaxin receptors has not yet been investigated in rodents. However, LGR7 relaxin receptors were found in the cervices of neonatal pigs, which responded to injection of porcine relaxin as evidenced by induced tissue growth. This was proven a relaxin-specific effect because it was neither enhanced by a concomitant estrogen injection nor inhibited by an estrogen antagonist.

Crelin and Lavin’s mouse experiments suggest that the connective tissues of the neonatal pelvis remain refractory to estrogen and relaxin during the lactation interval, when these hormones could easily be absorbed from the milk across the immature gut to enter the circulation. If this is true for other species as well, then a genetic defect resulting in premature appearance of estrogen, relaxin receptors, or both might enable an inappropriate response to these hormones before puberty.

In support of this argument, reviewed found instability of the pubic symphysis in newborn children in association with congenital dislocation of the hip, suggesting sensitivity to a common blood-borne factor.

Immunoreactive relaxin persisted in the circulation of dysplasia-prone Labrador Retriever bitches for 5 to 6 weeks during the lactation period, whereas its concentration decreased below detectable limits after 1 to 2 weeks in nondysplastic Labrador Retriever bitches or in dysplasia-resistant Beagles. Thus, there may be a departure from normal in the secretion or metabolism of relaxin associated with the genetic predisposition for hip dysplasia in Labrador Retrievers.

By use of a heterologous porcine relaxin RIA, immunoactive relaxin was previously found in dog milk at concentrations 5 to 20 times those in dog serum. More recently, by use of a homologous canine relaxin RIA, immunoactive relaxin concentration in milk was found to be in the microgram per milliliter range; the pups thus ingested milligram quantities during suckling. The source of relaxin in dog milk is unknown. However, relaxin and its receptors have been immunolocalized and relaxin genes expressed in mammary glands of other species. Moreover, normal concentrations of relaxin were found in milk of 2 bitches following ovariohysterectomy at the time of cesarean section, a procedure that eliminated the ovaries and uteri as sources of relaxin. These observations suggest that the mammary glands, per se, may be the source of relaxin found in milk.

Estrogens and estrogen precursors have also been identified in milk of several species. Pups born to parents with hip dysplasia but hand-reared from birth, and therefore deprived of their dam’s colostrum and milk, had a lower prevalence and severity of hip disease than did their suckled counterparts. This finding supports the hypothesis that the transmission of milk-borne factors is related to the development of hip dysplasia in HD+ pups.

The objectives of the study reported here were to provide new data on milk-borne factors that might induce hip joint laxity in canine HD+ pups by use of RIAs for estrogens and estrogen precursors and a homologous canine relaxin RIA; to modulate hip laxity by treatment of HD+ pups with an estrogen synthesis inhibitor; and to attempt to induce laxity in hip joints of HD− pups by injections of the hormones postulated to be the causative milk-borne factors.

Materials and Methods

The study protocols were approved by the Institutional Animal Care and Use Committees of Cornell University and the NYU School of Medicine. Seven adult HD+ and 8 HD− Labrador Retriever bitches were mated with like males, as previously reported. Following whelping, colostrum and milk were collected from the bitches during the lactation interval. Serum was also collected from the suckling pups during the first 4 weeks after birth. Concentrations of relaxin; estrogens; and the estrogen precursors, testosterone and DHEA, in milk and serum were measured by use of specific RIAs. Daily milk consumption of the pups was estimated from a previous study in which similar Labrador Retriever litters were hand-reared on formula.
Effect of CGS 16,949A on hip joint laxity in HD+ pups—Two litters of HD+ pups were each allocated into 2 groups of littermates. The first group (5 pups; 2 male and 3 female) received an SC injection daily with aromatase inhibitor CGS 16,949A (4-(5,6,7,8-tetrahydroimidazo[1,5-alpha]pyridin-5-yl) benzotriazole monohydrochloride) at 2.5 mg/kg in saline (0.95% NaCl) solution; this dose inhibits estrogen synthesis in dogs. The second group (6 pups; 3 male and 3 female) received only the saline solution vehicle. The injections were started on day 3 after birth and continued for 6 weeks through the lactation interval. Hip joint laxity in treated and control pups was quantified at 4, 8, and 12 months by use of the method of Smith et al.23 Extended hip radiographs were evaluated for signs of hip dysplasia.23,31 Femoral head diameters in millimeters was also measured on the standard radiograph at each time interval.

Effect of estrogen and relaxin on hip joint laxity in HD– pups—A litter of 6 HD– pups was allocated into 2 groups of 3 pups each (2 females and 1 male in each group). Hip joints of both parents were free of signs of dysplasia. One group of pups received an SC injection with 300 µg of the long-acting estrogen ECP in 0.1 mL of sesame oil every other week (weeks 1, 3, and 5). In addition, these pups received a daily SC injection for 6 weeks with 10 µg of synthetic canine relaxin in 0.2 mL of Evans blue dye as a retardant vehicle.33 The 3 control pups received an SC injection with the vehicles (sesame oil and Evans blue dye) at the corresponding times. Joint laxity was evaluated by the method of Smith et al.31,34 Femoral head diameters in millimeters were also measured at 4, 8, and 12 months.

Measurement of canine relaxin and steroid hormones in milk and serum samples—Estradiol-17β and total estrogen content were determined in samples of milk of the HD+ and HD– bitches and serum from HD+ and HD– pups by use of commercially available, specific RIA kits, and canine relaxin was measured in unextracted milk and serum samples by use of a homologous RIA, as previously described.21 In addition, the samples were analyzed for differences in potential estrogen precursors, testosterone, and DHEA (free and sulfated) by use of specific commercially available RIAs (Table 1). With the exception of total estrogens, the steroid hormone assays were conducted on duplicate samples of serum or whole milk after validation by adding known amounts of the appropriate steroid hormone standards to representative serum or milk samples. Total estrogens were extracted from 0.6 mL of serum or milk with 10 volumes of ethylacetate-hexane (3:2) according to the manufacturer’s directions. The extracts were taken to dryness and reconstituted with 2.5 mL of assay buffer; the RIA was then carried out in duplicate on 0.5-mL volumes.

Extraction and bioassay of canine relaxin from milk—To determine whether the immunoactive relaxin-like substance in milk was biologically active, milk samples from HD+ and HD– bitches were pooled and extracted as follows. Thirty- to 50-mL milk samples were diluted to 100 mL with PBS solution (pH, 7.0) and defatted by centrifuging at 2,500 × g for 30 minutes at 4°C. To precipitate casein, 45 mL of distilled water and 6.6 mL of concentrated HCI were added to the defatted samples, and the mixture was stirred overnight in a 5°C cold box. The supernatant was transferred to a clean flask, 334 mL of cold acetone was added, and the mixture was again stirred overnight. The mixture was filtered, and 2 L of cold acetone was then added to the filtrate to precipitate the relaxin. After decanting the supernatant, the precipitate was dried and stored at −20°C until the time of bioassay and immunoassay.

Specific bioactivity of relaxin from milk extracts of HD+ and HD– bitches was determined against a synthetic canine relaxin standard by use of a mouse interpubic ligament assay.23 Briefly, immature female Charles River CD mice primed with 5 µg of ECP administered SC on day 0 received an SC injection with 0.5 or 2 µg of the canine

<table>
<thead>
<tr>
<th>RIA</th>
<th>Sample</th>
<th>Coefficients of variation (%)</th>
<th>Sensitivity</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>Serum</td>
<td>Interassay Intra-assay</td>
<td>10 pg/mL</td>
<td>E1 = 20, E2 = 100, and others &lt; 1</td>
</tr>
<tr>
<td>Total estrogens</td>
<td>Extract</td>
<td>11</td>
<td>2.5 pg/mL</td>
<td>E1, E2 = 100, and others &lt; 1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Serum or milk</td>
<td>11</td>
<td>0.2 ng/mL</td>
<td>T = 100, 5α-DHT = 8, and others &lt; 1</td>
</tr>
<tr>
<td>DHEA</td>
<td>Serum or milk</td>
<td>8</td>
<td>0.5 ng/mL</td>
<td>DHEA(S) = 100, DHEA = 59, Androst = 31, and others &lt; 1</td>
</tr>
<tr>
<td>Canine relaxin</td>
<td>Serum or milk</td>
<td>4</td>
<td>0.2 ng/mL</td>
<td>FSH, LH, PRL, hCG, and insulin = ND</td>
</tr>
</tbody>
</table>

E1 = Estrone, E2 = Estradiol-17β, T = Testosterone, 5α-DHT = 5α-dihydrotestosterone. DHEA(S) = Dehydroepiandrosterone (sulfate), Androst = Androsterone, FSH = Follicle-stimulating hormone, LH = Luteinizing hormone. PRL = Prolactin, hCG = Human chorionic gonadotropin. ND = Not detectable at 10 to 100 µg/mL.
relaxin standard or the milk extract on day 7 (doses of the extract were based on the RIA results). The next morning, the mice were killed by CO₂ asphyxiation, the pubic symphyses were exposed by dissection, and the interpubic ligament lengths were measured on a dissecting scope with transillumination and 10× magnification.33

Measurements of hip joint laxity via the distraction method and extended hip radiography—Hip joint laxity in HD+ and HD− pups at 4, 8, and 12 months of age was quantified by the method of Smith et al13 by use of a distractor.33,34 Each dog was positioned on its back during general anesthesia (pentothal sodium, IV, followed by inhalation of halothane), and the adjustable plastic distractor was placed between the thighs, which were positioned nearly perpendicular to the benchtop. Inward force was then applied to the stifle joints to displace the femoral heads laterally within the joint capsules. The displacement distance in millimeters divided by the radius of the femoral head in millimeters yields the distraction index. The distraction index is positively correlated with degree of joint laxity. A joint considered to be tight (ie, disease free) had a distraction index < 0.3.33,34

The dogs also were examined at 8 and 12 months by use of standard radiographic methods to determine which dogs developed early hip dysplasia or early osteoarthritis.33 In the standard method, the radiograph was obtained with the anesthetized dog positioned on its back and with the hind limbs extended beneath the dog, which dogs developed early hip dysplasia or early os
teoarthritis.

Statistical analysis—Mean values of hormone concentrations (relaxin and steroid hormones) and the various hip joint measurements (distraction lengths and indices and femoral head diameter) were compared by use of ANOVA and the Student or Welch t test after transforming the data to their natural logarithms. Significance of differences was set at P < 0.05. Relative potencies of serum and milk-borne hormones were calculated after logit-log transformation of the respective standard curves.

In the bioassays of relaxin-containing extracts of milk, ligament lengths of mice that received injections with the extracts were compared with those of mice that received injections with authentic canine relaxin standards. Differences between mean ligament lengths of control, extract-treated, and canine relaxin-standard–treated mice were tested for significance by use of the Student t test. Data are presented graphically or in tabular form as mean ± SD values.

Results

Hormone concentrations in colostrum and milk of HD+ and HD− bitches—Large quantities of immunoactive relaxin were found in colostrum and milk of HD+ and HD− bitches, with concentrations in colostrums in the range of 13 to 15 μg/mL (Table 2). The concentrations of relaxin in milk fluctuated widely over the course of lactation, but no significant differences between HD+ and HD− bitches were detected. Acid-acetone extracts of milk induced significant (P = 0.01) development of the interpubic ligaments of estrogen-primed mice at a dose of 2 μg/mouse (dose determined by use of RIA). However, the extracts were significantly less potent than a 2-μg dose of synthetic canine relaxin in this bioassay. Ligament lengths were as follows (n = 4 mice/group): control group, 0.32 ± 0.15 mm; 2 μg of synthetic canine relaxin, 1.72 ± 0.69 mm; and 2 μg of milk relaxin extract, 0.95 ± 0.3 mm.

Total immunoactive estrogen concentrations in milk were similar in HD+ and HD− bitches (Table 3). It was not possible to reliably measure estradiol-17β in milk, either directly or by following solvent extraction. However, the estrogen precursors, testosterone and progesterone, were measured. Levels of relaxin and estradiol-17β for the milk samples are presented in Table 3. The relaxin and estradiol-17β concentrations and their immunoactivity were measured in colostrums and milk from HD+ bitches. Milk and lactating milk were used in bioassays and immunoassay experiments. The relaxin standards were of pure origin. The estradiol-17β standard was 99% pure.

Table 2—Relaxin concentrations (mean ± SD [ng/mL]) in colostrum and milk of HD+ and HD− Labrador Retrievers.

<table>
<thead>
<tr>
<th>Group</th>
<th>Colostrum</th>
<th>Milk (wk of lactation)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>HD−</td>
<td>15,589 ± 5,611 (8)</td>
<td>4,346 ± 3,344 (5)</td>
</tr>
<tr>
<td>HD+</td>
<td>13,396 ± 5,387 (5)</td>
<td>3,019 ± 3,087 (5)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate No. of dogs.

Table 3—Concentrations (mean ± SD) of hormones in serum and milk of HD+ and HD− Labrador Retriever bitches.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dysphasia status</th>
<th>Estradiol-17β (pg/mL)</th>
<th>Total estrogens (pg/mL)</th>
<th>Testosterone (ng/mL)</th>
<th>DHEA(S) (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy serum</td>
<td>HD−</td>
<td>33.3 ± 1.5 (6)</td>
<td>83 ± 10 (6)</td>
<td>&lt; 0.2 (5)</td>
<td>5.75 ± 0.83 (4)</td>
</tr>
<tr>
<td>Pregnancy serum</td>
<td>HD+</td>
<td>30.2 ± 2.2 (9)</td>
<td>92 ± 11 (6)</td>
<td>&lt; 0.2 (5)</td>
<td>3.65 ± 0.58 (5)</td>
</tr>
<tr>
<td>Lactation serum</td>
<td>HD−</td>
<td>24.3 ± 5.2 (20)</td>
<td>51 ± 21 (3)</td>
<td>&lt; 0.2 (3)</td>
<td>6.35 ± 0.84 (4)</td>
</tr>
<tr>
<td>Lactation serum</td>
<td>HD+</td>
<td>19.0 ± 3.0 (26)</td>
<td>98 ± 21 (3)</td>
<td>&lt; 0.2 (5)</td>
<td>5.39 (1)</td>
</tr>
<tr>
<td>Milk</td>
<td>HD−</td>
<td>ND</td>
<td>46 ± 16 (6)</td>
<td>2.7 ± 1.0 (5)</td>
<td>1.4 ± 0.4 (5)</td>
</tr>
<tr>
<td>Milk</td>
<td>HD+</td>
<td>38 ± 24 (5)</td>
<td>2.3 ± 1.0 (5)</td>
<td>1.1 ± 0.4 (5)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD. ND = Not done (estradiol-17β could not reliably be determined in milk).

See Tables 1 and 2 for remainder of key.
DHEA, were readily detected by use of RIA in milk of HD+ and HD– bitches. There were no significant differences in total estrogens or estrogen precursors between milk samples of HD+ and HD– bitches.

The data were compiled to estimate the total daily intake of relaxin and estrogens by the pups Table 4). On the basis of daily mean milk consumption, the concentrations of immunoreactive estrogens and relaxin in colostrum and milk were such that the pups ingested approximately 1.6 mg of relaxin and 1,800 pg of estrogens daily during the first week of lactation. Estrogen consumption increased, whereas relaxin consumption decreased in the ensuing weeks. The pups also consumed about 225 ng of DHEA and 120 ng of testosterone daily during the first week of lactation.

Hormone concentrations in pup serum—Immunoreactive relaxin was readily measured in pup serum, but there were no significant differences in relaxin concentration between HD+ and HD– pups. Data from 1 HD+ litter are illustrated in relation to the concentration found in the milk of the dam (Figure 1). Absorption of < 5% of the milk-borne relaxin was sufficient to account for the immunoreactive relaxin concentrations observed in pup serum. Total immunoreactive serum estrogens were higher in pups than in their dams, but did not differ significantly between HD+ and HD– pups. Data from 1 litter of HD+ pups are illustrated in relation to the total estrogen concentration in the milk (Figure 1). Again, modest absorption of milk-borne estrogens could account for the serum estrogen concentrations in the pups. Immunoreactive estradiol-17β could not be detected by use of a specific RIA in serum of HD– pups, but was frequently detected in serum of HD+ pups. The concentration of

Table 4—Approximate daily intake of steroid hormones and relaxin in colostrum and milk by suckling Labrador Retriever pups from birth (day 1) to day 18.

<table>
<thead>
<tr>
<th>Variable</th>
<th>1</th>
<th>2–7</th>
<th>8–10</th>
<th>11–13</th>
<th>14–18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk ingested (mL/d)</td>
<td>90</td>
<td>90</td>
<td>125</td>
<td>165</td>
<td>240</td>
</tr>
<tr>
<td>Total estrogens (pg/d)</td>
<td>1,800</td>
<td>1,800</td>
<td>2,700</td>
<td>3,300</td>
<td>4,000</td>
</tr>
<tr>
<td>Total relaxin (µg/d)</td>
<td>1,620</td>
<td>654</td>
<td>22</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>Testosterone (ng/d)</td>
<td>220</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DHEA (ng/d)</td>
<td>120</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not done.

Figure 1—Concentrations of immunoreactive canine relaxin in serum of 5 suckling HD+ pups (circles [mean ± SD]) in relation to its concentration in maternal milk (bars; A) and immunoreactive total estrogen in serum of 6 suckling HD+ pups (circles [mean ± SD]) in relation to the estimated total daily estrogen consumption in maternal milk (bars; B).

Figure 2—Total estrogen concentrations (mean ± SD) in serum of 5 suckling HD+ pups (black circles) treated daily from day 2 until the end of lactation with the aromatase inhibitor CGS 16,949A (2.5 mg/kg) and in serum of 6 matching littermate control pups (white circles) and the estimated total daily estrogen consumption in maternal milk (bars).
estradiol-17β was 52 ± 30 pg/mL in serum of 6 HD+ pups at 1 to 2 weeks and 13.0 ± 12.2 pg/mL in serum of 7 HD+ pups on week 4 of lactation.

Effect of CGS 16,949A on hip joint laxity in HD+ pups—There were no differences in the serum total estrogen concentrations between treated and control pups during suckling (Figure 2), suggesting that the serum estrogens were derived primarily from ingested milk. Sufficient amounts of milk-borne estrogens were consumed during nursing to account for the serum concentrations of estrogen in the pups (the number of samples of milk available for analysis varied because not all bitches could always provide milk, which was attributable primarily to differences in number of suckling pups.) However, when hip joint laxity was quantified by distraction measurement at 8 months of age, significant decreases in the distraction lengths (P = 0.01) and distraction indices (P < 0.001) were observed in the CGS 16,949A-treated pups (Figure 3). No significant differences in femoral head diameters were recorded in these pups. However, radiographic diagnosis of hip dysplasia at 8 months revealed abnormal joints in 4 of 6 control pups, but in only 1 of 5 CGS 16,949A–treated pups (odds ratio, 8:1).

Effect of ECP and canine relaxin on hip joint laxity in HD− pups—To evaluate the possible hormonal induction of hip joint laxity, 500 µg of ECP was injected every other week and 10 µg of synthetic canine relaxin was injected SC daily into HD− pups starting on day 3 after birth, for a total of 6 weeks through the lactation interval. Hip joint laxity was quantified by use of the Smith-Biery distraction method at 4, 8, and 12 months of age (Figure 4). An increase in the distraction index was observed at 12 months (P = 0.015), and femoral head diameters were significantly reduced as early as 4 months in the pups treated with ECP and relaxin, compared with control pups. Radiographic diagnosis of hip dysplasia revealed 6 of 6 normal hip joints in the 3 control pups and 5 of 6 normal hip joints in the 3 treated pups; this difference was not significant.

Discussion

Hip joint laxity is an early measurable abnormality that presages later development of hip joint disease and osteoarthritis. In the present study, microgram quantities of immunoactive relaxin were detected in colos-
terpubic ligament direct measurement assay. However, no differences in milk relaxin concentration were detected between HD+ and HD– bitches. Similarly, nanogram quantities of immunoreactive estrogens were found in colostrum and milk, as were the estrogen precursors, testosterone and DHEA, but no differences were detected between milk samples of HD+ and HD– bitches. It was not possible to measure estradiol-17β in milk, so it is not known whether there were differences in milk content of this key steroid hormone between HD+ and HD– bitches.

Radioimmunoassays of the serum obtained from suckling pups revealed notable concentrations of relaxin and estrogens. However, the relaxin concentrations detected in pup serum (5 to 10 ng/mL) were only about 10% of those found in serum of pregnant bitches by use of the homologous canine RIA. Neither the minimum blood concentration required for hormonal activity nor the time of appearance of relaxin receptors in connective tissues of dogs is presently known.

In a previous study, blood drawn from pups immediately after natural or cesarean birth and before they suckled contained immunoreactive relaxin. When food was withheld from those pups for 4 to 5 hours, serum relaxin concentrations were undetectable. Those data suggest that relaxin enters the fetus before birth, in agreement with findings of a study in rhesus monkey infants that revealed transplacental passage of relaxin. The dog fetus is thus exposed to maternally produced relaxin before birth, as well as milk-borne relaxin during the suckling period.

The concentrations of relaxin in colostrum were remarkable, providing > 1 mg of relaxin/pup on the first day of life. Approximately 0.3% to 0.4% of ingested relaxin was absorbed into the circulation of the newborn pups, providing a dose of about 4 or 5 µg/kg/pup, which is sufficient to exert biological activity in standard relaxin bioassay systems.

In a prior study, orally administered porcine relaxin appeared in neonatal pup serum within 30 minutes, and the half-life in newborn pups was calculated to be about 40 minutes. It should be emphasized that the relaxin molecule consists of 2 polypeptide chains linked by disulfide bonds and that the tertiary structure is similar to that of insulin. The antiporcine relaxin antisem R6 (used in the prior study) does not recognize the molecule after it has been chemically reduced and after the 2 chains have dissociated. Therefore, it is probable that the substance detected in the serum of the pups had maintained its proper tertiary structure and thus its biological activity.

In a previous study, 1 HD– and 1 HD+ bitch were ovariohysterectomized at the time of cesarean section on day 63 of pregnancy. This procedure did not interfere with lactation, and the milk relaxin concentrations of these bitches were not different from those of the sexually intact bitches, indicating that the ovaries, uterus, and placentas were not required for relaxin secretion. These data support the view that the mammary gland may be the source of relaxin during lactation in dogs.

Substantial concentrations of immunoreactive estrogens were also found in pup serum. Although pup total estrogen concentrations did not differ significantly between HD+ and HD– pups, estradiol-17β was frequently detected in HD+ pups but not in HD– pups. Whether the immunoreactive estradiol-17β found in HD+ pup serum was a milk constituent or a metabolite of an ingested estrogen precursor is not known. It will thus be important to measure the appearance in pup serum of estradiol-17β following oral administration of estrogen precursors (testosterone and DHEA) in comparison with estradiol-17β itself.

The antiserum used for total estrogen measurements cannot discriminate between estrone and estradiol-17β; thus, the HD– pups were exposed primarily to estrone, which is only about 10% to 20% as potent biologically as estradiol-17β. The HD+ pups, conversely, were exposed to the same total estrogen concentration, but most of this was the more potent estradiol-17β. The milk-borne estrogens provided a serum concentration in pups similar to that observed in adult dogs that were not in estrus. Thus, aromatization of androgenic precursors may not be necessary to provide increased concentrations of estrogens in suckling pups, but may be the source of estradiol-17β detected in the HD+ pups.

To allow for the possibility that pup serum estrogens (especially estradiol-17β) were at least partially derived from estrogen precursors, the steroid aromatization inhibitor CGS 16,949A was administered to half of the pups from each of 2 litters born to HD+ bitches. The other pups from these litters served as controls. Significant decreases in hip joint laxity (distraction lengths and distraction indices) were observed in the CGS-treated pups, whereas there were no significant changes seen in their femoral head diameters. In addition, radiography revealed dysplastic changes in the hips of 4 of 6 control pups but only 1 of 5 CGS-treated pups at 8 months. We interpreted the data to suggest that more estradiol-17β was formed from milk-borne precursors in HD+ pups than in HD– pups and that this transformation was inhibited by the administered CGS-16,949A, leading in turn to the prevention of estradiol-17β–relaxin induction of hip joint laxity. We cannot, however, rule out the possibility that CGS 16,949A acted via some other mechanism. For example, CGS 16,949A suppresses aldosterone secretion and other mineralocorticoid antagonists (eg, spironolactone) inhibit fibrous tissue formation. Regardless of the mechanism of action, CGS 16,949A substantially inhibited laxity and, later, dysplastic changes in hips of HD+ pups and thus merits future consideration as a therapeutic agent.

In the experiment conducted to determine the effects of injected estradiol-17β and canine relaxin on hip joint laxity of HD– pups, the hip joints of the treated pups had increased distraction lengths and distraction indices at 12 months, compared with vehicle-treated littermate controls. However, hip joint laxity was associated with a decrease in the femoral head diameter as early as 4 months. Whether this ECP-induced reduction in femoral head diameter would eventually lead to hip dysplasia is not known, but the hip joints of the ECP- and relaxin-treated HD– pups did not differ radiographically from those of littermate controls at 12 months of age. Previously, Gustafsson and Beling used large doses of estradiol-17β to induce dysplasia-like
changes in the hip joints of Beagles, a hip dysplasia-resistant breed.

Taken together, these data suggest a mechanism whereby maternal hormones are transferred to the circulation of suckling canine pups, where they could induce laxity of the tissues supporting the hip joints of HD+ pups. Because no significant differences were detected in milk concentrations of total estrogens and relaxin between HD+ and HD− bitches or in serum concentrations of their pups, we postulate that hip joint laxity resulted from premature or inappropriate expression of estrogen receptors, relaxin receptors, or both in hip joint connective tissues of the HD+ pups. The data support the involvement of estrogens and relaxin because hip joint laxity was reduced by treatment of HD+ Labrador Retriever pups with glycosaminoglycan polysulfates, 41 a treatment proven to inhibit the actions of estradiol-17β and relaxin on the pubic symphysis of guinea pigs. 42 Therefore, it would be beneficial for future studies to evaluate the time of appearance of the specific receptors for relaxin (LGR7) and estrogens (ERα and ERβ) in hip joint connective tissue of HD+ pups, compared with HD− pups.

References


