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Effects of in utero pestivirus infection on bovine fetal bone geometry, biomechanical properties and composition ☆

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ABSTRACT

Transplacental viral infection of the fetus can result in abnormal trabecular and cortical bone modeling in long bones through impaired bone resorption and formation. Although such infections are frequently associated with neonatal fractures in humans and animals, their effect on the biomechanical properties of the developing skeleton remain poorly understood. The goal of this study was to determine the effects of transplacental bovine viral diarrhea virus (BVDV) infection on the biomechanical properties of fetal femora. Pregnant heifers were inoculated intranasally with non-cytopathic BVDV or media alone on day 75 of gestation to produce persistently infected (PI) and control fetuses, respectively, which were then removed on days 192 and 245 of gestation.

Histomorphometry, compositional analysis and 'four-point bending until failure' were performed on fetal femora. Altered cortical geometry largely accounted for differences in calculated elastic modulus (PI vs. control, and day 192 vs. day 245) and ultimate stress (day 192 vs. day 245). Fetal infection with BVDV did not significantly impair inherent biomechanical properties of bone but rather resulted in decreased periosteal apposition rates, manifested as smaller femoral mid-diaphyseal diameters. There were no differences between PI and control fetuses in cortical thickness ratio, ash density or calcium/phosphorous content; however, cortical thickness ratio decreased with fetal age. Thus even when cortical thickness ratios are similar, differences in mid-diaphyseal diameter affect the error associated with the calculation of stress and strain by classical beam theory equations.

Introduction

Fetal bone development involves a complex series of processes working in concert to produce substantial longitudinal and appositional growth in a relatively short period of time. Viral infections represent unique developmental insults that can result in growth restriction and abnormal bone development (Williams and Carey, 1966; Graham et al., 1970; Smirnova et al., 2008). The effects of such infections on the skeleton remain largely unknown for many common viruses with demonstrated ability to cross the placenta and infect the developing fetus. Transplacental infection with human cytomegalovirus and rubella virus can result in abnormal fetal bone development, characterized by irregular radiodense zones within the metaphyses, and are frequently associated with pathologic fractures in the neonate (Williams and Carey, 1966; Graham et al., 1970; Kopelman et al., 1972; Sacks and Habermann, 1977; Smith and Specht, 1979).

Bovine viral diarrhea virus (BVDV), an important cause of disease in cattle, is capable of transplacental infection. If maternal infection occurs prior to the development of fetal adaptive immunity, the fetus can become persistently infected (PI) leading to lifelong viral shedding and impaired fetal bone development characterized by regularly spaced transverse radiodense bands within long-bone metaphyses (Nuss et al., 2005; Webb et al., 2012). The radiodense bands represent regions of increased bone and calcified cartilage volumes termed 'growth retardation lattices', formed as a result of impaired modeling of primary spongiosa secondary to reduced numbers of osteoclasts (Webb et al., 2012). These lesions are similar in morphology to those caused by congenital rubella virus and cytomegalovirus infections in humans. Persistent infection with BVDV also results in reduced periosteal apposition rates which lead to reduced femoral mid-diaphyseal diameter in affected fetuses (Webb et al., 2012).

Bone fragility has previously been suspected as the underlying cause of long-bone fractures in neonatal calves associated with transplacental infection with BVDV (Constable et al., 1993; Nuss

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et al., 2005; O'Toole, 2006). Based on these findings it was hypothesized that bones from PI fetuses would have impaired biomechanical properties. The objectives of this study were to characterize the biomechanical effects of intra-uterine BVDV infection on bovine fetal femora and to determine the architectural and/or compositional factors responsible for any potential differences in mechanical properties.

Materials and methods

Fetal infection

All animal experiments were approved by the Colorado State University Animal Care and Use Committee (Approval No. 08-168A-01, 6/20/2008). Pregnant BVDV-naïve Hereford heifers ($n = 19$), ranging in weight from 295 to 430 kg, were inoculated intranasally on day 75 of gestation with either 2 mL of MEM media containing 4.4 $\log_{10}TCID_{50}$ non-cytopathic BVDV type 2 strain 96b2222 (van Campen et al., 2000) to produce PI fetuses, or MEM media alone to produce controls as described previously (Webb et al., 2012). Six of the 19 pregnant heifers (controls, $n = 3$; PIs, $n = 3$) received IV oxytetracycline (9 mg/kg) on days 175 and 185 of gestation in order to fluorochrome-label bone formation. Fetuses were collected via caesarean section on days 192 (oxytetracycline labeled, $n = 3$/group; non-labeled, $n = 4$ control; $n = 2$ PI) and 245 (non-labeled, $n = 4$ control; $n = 3$ PI) of gestation.

Following euthanasia left femora were dissected free of soft tissue, wrapped in saline-soaked gauze and frozen for later mechanical testing. Right femora were dissected free of soft tissue and fixed in 10% neutral buffered formalin. The overall length of the femora, width of the distal epiphysis at its widest point in the mediolateral plane and average mid-diaphyseal diameter were calculated by measuring the mid-diaphyseal diameter in the mediolateral and craniocaudal axes with 'dial' calipers and dividing the sum by 2. Average mid-diaphyseal diameter divided by overall length and average mid-diaphyseal diameter divided by width at the distal epiphysis was used as an index of bone geometry. Total cross-sectional area and cortical thickness ratio, an index of cortical geometry, were calculated, the latter using the formula developed by van Lenthe et al. (2008):

$$2 * \text{average cortical thickness} / \text{average diaphyseal diameter}.$$

Biomechanical testing

Testing was performed on a servohydraulic mechanical testing system (858 Mini Bionix II, MTS) using a custom-built four-point bending fixture. Since there was no significant difference in length between control and PI femora at either day of collection, standardized fixture settings were used for each collection day. The distances between the outer and inner contacts were as follows: day 192 fetuses – 41 and 22 mm; day 245 fetuses – 73 and 48 mm, respectively.

Whole femora were then loaded in four-point bending to a load of 150 N for two pre-conditioning cycles, then loaded in four-point bending at a cross-head displacement rate of 0.15 mm/s until failure in the cranial-to-caudal axis. Force and displacement were recorded at 100 Hz with custom software. Strain and stress were calculated from standard beam-bending equations for four-point bending of a cylindrical tube:

$$\text{Stress} = \sigma = \frac{Fac}{2I}$$

$$\text{Strain} = \varepsilon = \frac{6cd}{\alpha(3l - 4\alpha)}$$

where a is the distance between the outer and inner contacts, c is equal to one-half of the femoral diameter, I is the area of the moment of inertia ($I = \frac{\pi}{4}(r^4_{\text{Outer}} - r^4_{\text{inner}})$, r is the radius), d the displacement and l the distance between the outer contacts (Turner and Burr, 1993). Stiffness and elastic modulus were calculated by determining the slope of the linear-regression-fit of the force displacement and stress/strain curves, respectively, at three segments of the curve corresponding to 100–500, 500–900, and 900–1300 N of force.

Histomorphometry

Mid-diaphyseal sections of right femora were sectioned and 'hand-ground' to 100 ± 20 μm thickness for light microscopic evaluation. Fluorochrome-labeled sections were used to determine the portion of the outer cortex containing plexiform bone capable of chelating fluorochrome (newly mineralized bone) within the woven bone cores. This proportion was determined at four standardized sites, on the same axes utilized for determining the diaphyseal diameters, by measuring from the endocortical aspect of the first label to the periosteal surface and dividing by cortical thickness (Fig. 1). Average plexiform plate separation was determined by averaging the distance from the osteoid surface to the equivalent surface of the adjacent plate for the four outermost plexiform plates at four standardized sites. Cortical porosity was determined using a modified Mertz grid and simple point-counting method under 200× magnification at four anatomically standardized areas (<1.1 mm^2) of interest, which extended from the periosteum to the endocortical surface. A minimum of 300 points were counted at each site.

Ash and mineral analysis

Ash and mineral analysis was performed on cortical bone from the left femora (mechanically tested femur) utilizing previously described methods (Norrdin et al., 1977). A transverse section of mid-diaphysis measuring approximately 5 mm in width was ashed and calcium (Ca) and phosphorus (P) content determined. Tissue volume (cortical cross-sectional area * slice thickness), ash density (g ash/cm^3 tissue volume * [1 − porosity]), apparent density (g ash/cm^3 tissue volume), percent calcium (g Ca/g ash weight), percentage phosphorus (g P/g ash weight), and Ca:P molar ratio were calculated for each sample.

Statistical analysis

Data analysis was performed using SAS software (SAS Institute) using the general linear models procedure. Comparisons were performed with the least square means procedure followed by Student's t test. Only probabilities associated with a priori comparisons were used to ensure overall protection. For mechanical data, linear regression analysis was used to determine the effect of independent variables. All data are presented as means ± standard deviation and assumptions for use of applicable statistical tests were satisfactorily met. All differences described were significant at $P < 0.05$.

Results

All PI fetuses on both days of collection were positive and all controls negative for BVDV RNA in blood as determined by qRT-PCR (Bielefeldt-Ohmann et al., 2012). Fetal sex by day of collection was recorded: day 192 controls (three males, four females); day 192 PIs (one male, four females); day 245 controls (two males, two females); day 245 PIs (three males). The long bones of 3/5 PI fetuses on day 192 and 2/3 PI fetuses on day 245 contained radiographic focal densities and transverse radiodense bands within metaphyses as previously described (Webb et al., 2012). Femoral dimensions are listed in Table 1.

PI fetuses had smaller mid-diaphyseal diameters when compared with controls, but overall femoral length and width at the distal epiphyses did not differ. The two indices of bone geometry

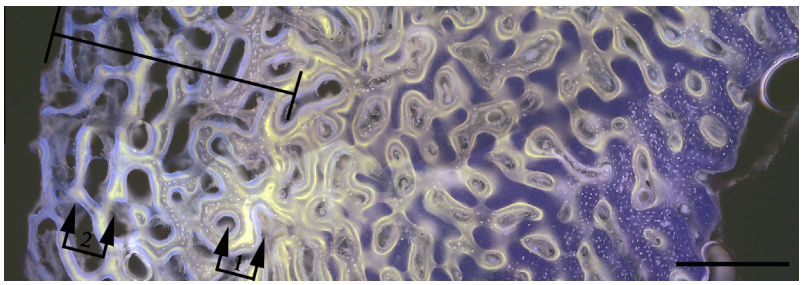

Fig. 1. Cross-section through femoral cortex from oxytetracycline-labeled day 192 fetus depicting greenish-gold oxytetracycline label within woven bone cores of outer plexiform bone: (1) first label, day 175 of gestation; (2) second label, day 185 of gestation. Site of measurement for determining portion of newly mineralized cortex (longer line) extends from periosteal surface to the endocortical aspect of first label. Unstained, 100 μm-thick section under ultra-violet light. Scale bar, 0.5 mm.