CIRCULATING INSULIN-LIKE GROWTH FACTOR-I AND INDICATORS OF BONE AND CARTILAGE TURNOVER IN STEERS GIVEN TRENBOLONE ACETATE AND ESTRADIOL 17-BETA ALONE OR IN COMBINATION

by

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Abstract

Anabolic steroids are used extensively in beef cattle feeding management to take advantage of well-documented improvements in growth performance and efficiency of implanted cattle. In addition to muscle growth, steroids also impact changes in bone and cartilage formation. In general, these effects can be interpreted as hastening bone aging. The current study was designed to test the hypothesis that recently-identified peripheral indicators of bone and cartilage turnover could be detected in the peripheral circulation. Furthermore, it was hypothesized that these peripheral markers might reflect accelerated aging effects of the widely used steroidal implants trenbolone acetate (TBA) and estradiol-17β (E2). Circulating IGF-I was measured as a positive marker of steroid-induced enhancement of the somatotropic endocrine axis. Thirty-two crossbred yearling steers were blocked by BW and given one of four treatments: non-implanted controls; 25.7 mg estradiol-17β (E2); 120 mg trenbolone acetate (TBA); or a combination of 120 mg TBA and 24 mg E2 (T+E). Blood samples were collected on d 0, d 7, d 14 and d 28 and serum was analyzed by ELISA for IGF-I concentrations, as well as osteocalcin, C-terminal telopeptides of Type I collagen (CTX-I) and C-terminal telopeptides of Type II collagen (CTX-II), which serve as markers of bone formation, bone resorption and cartilage resorption, respectively. Circulating IGF-I was similar among treatments on d 0 and 28. At d 7 and 14, steers receiving E2 or T+E had greater circulating IGF-I than non-implanted control steers ($P < 0.05$). In contrast, steers receiving only TBA tended to have elevated IGF-I compared to controls on d 7 and 14 ($P = 0.10$). Although treatment did not affect serum osteocalcin, concentrations were increased on d 7, 14, and 28 compared to d 0 ($P < 0.005$ for all). Implant treatment did not affect circulating CTX-I, however CTX-II was affected by T+E treatment ($P<0.05$). The data suggest that, although selected markers of bone and cartilage turnover can be detected in circulation in cattle, implant-

induced changes in the concentrations of these markers are not directly evident in the peripheral circulation at least through 28 d following treatment.

Key Words: Bone, Cartilage, Cattle, Estradiol 17-β, Growth, Trenbolone acetate

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CHAPTER 1 - Review of Literature

Introduction

The primary focus of the following review will be on the administration of steroidal growth promotants to beef cattle and the resulting changes in concentrations of markers of bone and cartilage growth and resorption in the peripheral circulation. However, it is also important to consider the cellular components and local environment in which growth of long bones occurs, because it is within this environment that steroids are likely to exert their effects. This general topic will be reviewed first.

Growth of Bone and Cartilage

Bone is a living, dynamic tissue that functions not only for structure and protection, but also to maintain metabolism and provide support to other tissues. Most notably, bone provides attachment for skeletal muscle and flexibility for movement. Additionally, bone also maintains mineral homeostasis for calcium and phosphorus as well as produces hematopoietic cells in the marrow. Bones can be classified into long bones, such as the bones of appendages, or flat bones, such as those of the skull. Such flat bones develop through intramembranous growth, whereas long bones undergo endochondral growth (Farquharson, 2003). Both classifications employ separate mechanisms of growth and ossification, however the majority of this review will focus on the growth in length of long bones, as it is this system that is of particular interest in the support of accumulated skeletal muscle in livestock.

Long bone anatomy consists of the diaphysis or bone shaft, the epiphysis or the bolus end of the bone, the metaphysis that connects the diaphysis and the epiphysis, the periosteum and the

endosteum. The periosteum is a layer of connective tissue that surrounds the bone and when ruptured, activates a local population of osteoprogenitor cells to stimulate new bone growth in diameter. The endosteum lines the inside of the bone marrow cavity. Bone marrow is the site of hematopoietic cell development, as well as production of osteoprogenitor stem cells (Hossner, 2005).

Long bones also contain a unique canal system for vasculature. The Haversian system is a canal that runs the length of long bones that contains nerves and blood vessels. Lying perpendicular and connecting to the Haversian canal are Volkmann's canals that link to the periosteum (Junqueira and Carneiro, 2005). As bone matures, these canals lessen in diameter due to a network of Type I collagen fibers that are laid down in a circular ring manner at different orientations. These fibrous networks form lamellae, and their angular diversity provides greater strength at a lighter weight. Small gaps known as lacunae are left between the lamellae, and these gaps contain nerve endings and additional vasculature. Connecting lacunae and the Haversian canals are canaliculi, or thin ducts that allow intercommunication and nutrient transport (Junqueira and Carneiro, 2005).

The bone tissue itself is made up of living cells embedded in a mix of organic and inorganic material (Farquharson, 2003). The inorganic mineral creates ossification of the organic portion, or osteoid. Mineral provides strength and stability, while the collagen of the osteoid withstands tension and provides a flexible matrix (Farquharson, 2003). The tissue itself is 25% water, 45% ash and 30% organic bone matrix. The ash or inorganic portion contains minerals in the form of hydroxyapatite crystals made up of 37% calcium and 18-19% phosphorus. Of the osteoid, 90 to 95% is Type I collagen, with the remainder made up of proteoglycans and other proteins (Junqueira and Carneiro, 2005).

At the cellular level, mature bone consists of three major cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are mobile cells that are responsible for the synthesis and deposition of the Type I collagen-based osteoid. These cells are derived from mesenchymal cells embryonically, as well as from the periosteum or from osteoprogenitor stem cells in the marrow (Junqueira and Carneiro, 2005). In the remodeling of existing bone, osteoblasts also secrete collagenase to break down the Type I collagen portion of the organic bone matrix. Along with collagen and collagenase, osteoblasts also secrete other glycoproteins into the osteoid, such as the proteoglycans decorin and biglycan (Hossner, 2005). These molecules are thought to help organize the collagen in the osteoid through an interaction with transforming growth factor-β (TGF-β). As osteoblasts mature, they become encapsulated in calcified osteoid and are rendered immobile. The encapsulation forms lacunae, and these immobile cells are now termed osteocytes. Osteocytes are still capable of transcription and translation of growth factors involved in cellular activity and bone maintenance. These cells fill the canaliculi with cytoplasmic projections that physically connect one osteocyte to another for intercellular communication (Junqueira and Carneiro, 2005).

Osteoclasts are the final major bone cell type. These cells contain four to 29 nuclei and have phagocytic activity (Hossner, 2005). Circulating marrow monocytes fuse, forming the multinucleated osteoclast that is responsible for bone resorption. This resorption, or degradation of calcified osteoid, is essential to the remodeling, growth and repair of bone. Osteoclasts are uniquely designed for their phagocytic function. They have a large surface area due to multiple, deep folds in the plasma membrane. Through this membrane, osteoclasts degrade the crystalline structure by acidifying the mineralized osteoid. Carbonic anhydrase within the cell produces protons that are passed across the membrane via proton pumps. This lowers the pH of the zone

just outside the membrane. Additionally, the osteoclast secretes proteases such as acid phosphatase and collagenase to provide an additive degradation effect (Hossner, 2005). However, it is osteoblasts that express the receptors for signaling factors responsible for inducing bone resorption. Osteoblasts then act in a paracrine manner to stimulate or inhibit osteoclast activity. Once old bone matrix has been dissolved, osteoblasts can then replace it with new matrix.

Lying between the epiphysis and the metaphysis is a strip of cartilage called the epiphyseal plate, which is responsible for growth in length. This plate eventually ossifies as the animal reaches maturity and growth in length ceases. This process is commonly referred to as "closure" of the growth plate. The cartilage in the epiphyseal plate is comprised of chondrocytes, the cellular unit of cartilage, and their extracellular matrix, known as a cartilaginous matrix. This matrix is made up of proteoglycans and Type II collagen. The production of the cartilage model by chondrocytes is responsible for long bone growth, and the calcification of this model is known as endochondral ossification.

Unique characteristics of endochondral ossification are the five zones of chondrocyte differentiation and activity. These zones represent the maturation stages of the chondrocytes and their extracellular matrix, all which occur at specific locations within the plate. The first zone is known as the resting zone and is farthest away from a calcified matrix. The resting zone contains the stem cell progenitors of chondrocytes. As these cells mature, they form the proliferative zone, where they proliferate and stack into columns of chondrocytes. As they divide, the chondrocytes in the proliferative zone take on a flattened disc shape. These cellular columns then cease dividing, become more spherical in shape, and undergo hypertrophy, or an increase in cellular size, in the hypertrophic zone. It is the movement to the hypertrophic zone

that signifies terminal differentiation of these chondrocytes, and simultaneously, these cells decrease collagen Type II expression and increase expression of collagen type X (Farquharson, 2003). Phenotypically, these cells now have a greater amount of rough endoplasmic reticulum and Golgi apparatus, characteristic of their responsibility to secrete extracellular matrix. Next, the cartilage model calcifies and chondrocytes simultaneously begin to break down in the zone of degeneration. Finally, the ossification of the cartilaginous model is completed in the zone of vascular invasion (Farquharson, 2003). Osteoclasts resorb a portion of the cartilage matrix, and what remains is used as a scaffold for osteoblasts to thicken by deposition of osteoid. Chondrocytes are completely replaced by osteoblasts here, and capillary beds enter the matrix. Therefore, the five zones of chondrocyte differentiation mediate the creation of a cartilage model and the conversion of that model into bone matrix osteoid.

The chondrocyte moves from the proliferative zone into the hypertrophic zone at different rates and in different proportions depending on species and bone type (Farquharson, 2003). However, in the comparison of the rat and the pig, a strong, positive correlation was found for both (rat= 0.98, pig = 0.83) between the rate of long bone growth and the volume of chondrocytes found in the hypertrophic zone (Breur et al., 1991). It is possible that this difference in chondrocyte maturation and proportion is responsible for the differences in bone growth within and among species.

Conversely, flat bones utilize a second type of ossification. Intramembranous ossification does not use a cartilage model intermediate, and instead occurs through the aggregation of proliferating mesenchymal stem cells. These cells begin differentiation into osteoprogenitor cells that eventually express extracellular Type I collagen fibrils. Next, these

cell complete differentiation directly into osteoblasts, which secrete osteoid that once calcified, forms primary bone.

The mineralization of bone osteoid, whether from a cartilaginous model or from osteoblast secretion, occurs through a series of phases. First, the matrix vesicles combine calcium and phosphorus together into solid particles, and eventually form hydroxyapatite. Next, these crystalline structures translocate through the vesicle membrane into the extracellular fluid. The availability of calcium and phosphorus in the extracellular fluid is regulated by a homeostatic process involving the liver, kidney and existing bone.

Steroid Growth Promotant Implants

For nearly sixty years, estrogenic and androgenic steroid growth promotant implants have been used in the livestock industry to enhance muscle growth. It has been widely documented that cattle that receive androgenic and estrogenic implants have an increased carcass weight, a higher average daily gain and an increase in feed efficiency (Johnson et al., 1996a; Johnson and Chung, 2007; Pampusch et al., 2008). Several kinds and combinations of steroid implants have been approved for use in the United States in cattle, however there is only one approved for sheep (Beermann, 2003). Approval and usage are regulated by the Food and Drug Administration (FDA), and they are responsible for ensuring the safety of both synthetic and endogenous steroid hormones administered to animals. The hormones are administered as an implantable pellet given subcutaneously in the middle third of the animal's ear. Currently, the FDA has approved a total of 39 steroid implants for use in cattle and sheep in varying combinations of naturally occurring and synthetic steroids (FDA, 2009). The active components in these implants are zeranol for sheep, and zeranol, progesterone, estradiol benzoate, estradiol-

17β, testosterone propionate, and trenbolone acetate in cattle. These components are commercially available in a variety of dosage amounts and administration regimens, however estrogenic and androgenic implants usually provide an additive response when used in combination (Beermann, 2003).

Trenbolone acetate (TBA) is a commonly used synthetic androgen approved in the U.S. in 1987. It is 8 to 10 times stronger and has a longer half-life than testosterone propionate (Schiffer et al., 2001). Once released into circulation, TBA is metabolized into three forms, the first of which is hydrolysis into trenbolone-17β. In the heifer, further oxidation to trendione and then reduction to trenbolone-17α complete the metabolic path (Schiffer et al., 2001). The more active form is trenbolone-17β, which loses 95% of its potency in the metabolism to the alpha form (Pottier et al., 1981). TBA is widely accepted to have positive effects on muscle mass and feed efficiency in both steers and heifers. It was shown to shift energy requirements in beef cattle, increasing available energy for skeletal muscle growth by reducing maintenance needs 10 to 15% (Hunter and Vercoe, 1987). Commercially available forms of TBA not in combination with an estrogen include Finaplix-H® and Finaplix-S® (Intervet, Inc. Millsboro, DE) and Component T-H with Tylan® and Component T-S with Tylan® (Ivy Laboratories, Division of Ivy Animal Health, Inc. Overland Park, KS).

Estradiol-17β (E2) is an endogenous steroid mainly synthesized and secreted largely by the ovaries, but has also been basally found in the adrenal cortex and in the testes. In circulation, it is metabolized into estrone, which has much weaker potency. When exogenously administered to both steers and heifers, it also has positive effects on beef cattle growth performance. Interestingly, ruminants are the only animals that have a positive growth response when estrogens are administered (Hossner, 2005). Estrogenic compounds are somewhat more

effective in steers, and have been shown to increase average daily gain (ADG) by 10 to 15% and feed efficiency by 5 to 10% (Basson et al., 1985). Commercially available forms of E2 are available alone as Compudose® or Encore® (Ivy Laboratories, Division of Ivy Animal Health, Inc. Overland Park, KS). Other frequently used estrogenic implants include estradiol benzoate and zeranol, although E2 has a greater potency than both types.

Although both TBA and E2 are effective at inducing positive growth responses alone, they have an even greater synergistic effect. Steers administered a combination implant for 40 d have shown an 18% increase in ADG and a 13% increase in feed efficiency compared to nonimplanted animals fed the same number of days (Johnson et al., 1996a). Additionally, combination implants resulted in steers with an 82% increase in daily carcass protein accretion and larger longissimus muscle areas (Johnson et al., 1996a). In lambs, a combination TBA and E2 (T+E) implant resulted in a 50% increase in rate of live weight gain (Sulieman et al., 1988). Several studies report that E2 and T+E implants result in greater BW gain than TBA implants. A synergistic effect of T+E implantation was evidenced by an increase in BW gain compared to E2 and to TBA (Hayden et al., 1992). Skeletal muscle protein deposition also demonstrated the benefits of a T+E implant in the same study. The combination implant increased average muscle protein accretion by 25% over E2 implanted steers and 60% over TBA implanted steers (Hayden et al., 1992). These results provide further support that the benefits of an androgenic or estrogenic implant are increased when used in combination. Commercially available types of T+E implants include Revalor-G®, Revalor-S®, Revalor-IS®, Revalor-200®, Revalor-H®, Revalor-IH®, Revalor-XS® (Intervet, Inc. Millsboro, DE), Component TE-G®, Component TE-G with Tylan®, Component TE-ID®, Component TE-IS®, Component TE-IS with Tylan®, Component TE-S®, Component TE-S with Tylan®, Component TE-200®, Component TE-200

with Tylan®, Component TE-H®, Component TE-H with Tylan®, Component TE-IH® (Ivy Laboratories, Division of Ivy Animal Health, Inc. Overland Park, KS), Synovex T120®, Synovex T40®, and Synovex T80® (Ft. Dodge Animal Health, Ft. Dodge, IA).

The success of using these implants has been reported extensively, and although investigations into the mechanisms behind this process have been underway for nearly 50 yr, they remain to be fully understood. In 1960, Alexander Mauro discovered a population of mononucleated cells residing adjacent to myofibers, which he termed satellite cells (Mauro, 1961). Myofibers are the cellular unit of skeletal muscle and are unable to proliferate postnatally. Therefore, the only possible mechanism for growth is to increase in cell size. In order to sustain this hypertrophy, the myofiber must receive an external source of DNA for protein synthesis, and 60 to 90% of the DNA in the mature myofibers is acquired postnatally (Allen et al., 1979). To study this source of DNA, Moss and Leblond (1971) administered a [^{3}H]-thymidine injection to radiolabel proliferating rat muscle and satellite cell nuclei. They harvested the tibialis anterior at varying time intervals up to 72 hr and discovered that true muscle nuclei were not labeled, however nuclei of satellite cells were labeled, indicating that these cells were actively dividing. As time elapsed, labeled myofiber nuclei increased, and labeled satellite cells decreased, showing that the satellite cells were fusing with the myofiber and then losing their proliferative capacity. They determined that it is this fusion that is responsible for the increase in myofiber DNA, allowing muscle hypertrophy.

Insulin-Like Growth Factor I

Further investigation of this fusion process led to the discovery of the role of insulin-like growth factor I (IGF-I). Originally described by Salmon and Daughaday (1957) as somatomedin C, IGF-I is commonly stimulated by growth hormone to be secreted by a variety of tissues to

mediate many processes throughout the body. It is a 70 amino acid polypeptide with a highly conserved molecular structure similar to insulin (Hossner, 2005). IGF-I circulates at a very high concentration in the mature body, however it has the capability to induce cellular responses at low levels. Because of this discrepancy, nearly 99% of the IGFs are bound by one of six IGF binding proteins, most commonly by IGFBP-3 (Hossner, 2005). IGF-I was found to be a progression factor that not only keeps satellite cells in the cell cycle, but also simultaneously causes them to terminally differentiate as muscle cells and fuse with the myofiber (Florini et al., 1991). Barton-Davis et al. (1999) used gamma irradiation and IGF-I administration to further investigate the role of IGF-I in muscle hypertrophy. In mice treated with both, they found that the gamma irradiation only prevented half of the hypertrophy seen in the IGF-I only treatments. They determined that IGF-I, in addition to its action on satellite cells, also stimulates hypertrophy by acting to decrease protein degradation in the existing myofiber.

The knowledge that IGF-I can mediate satellite cell fusion and muscle hypertrophy, and that steroid implants result in increases in muscle protein accretion, brought the role of steroid implants and their effects on IGF-I under investigation. Blood serum from steers implanted with Revalor-S® (120 mg TBA and 24 mg E2) was analyzed for IGF-I and IGFBP-3, and it was determined that circulating IGF-I was increased 15 to 22% compared to non-implanted steers (Johnson et al., 1996b). Implantation also resulted in a higher concentration of IFGBP-3. Johnson et al. (1998) conducted a study to determine the effects of steroid growth implants on IGF-I levels and satellite cell activity. They administered Revalor-S to 7 steers and fed them *ad libitum* a high concentrate diet for 32 d. They found expected increases in ADG, feed efficiency and longissimus muscle area compared with nonimplanted steers fed the same number of days. By d 6, serum IGF-I levels had increased by 32% compared to d 0 in implanted animals, and

continued to stay elevated throughout the course of the study. In addition, they discovered an increase in satellite cell activity, both in proliferation and differentiation.

To further determine the mechanism by which implants stimulate an increase of IGF-I, Pampusch et al. (2003) conducted a similar study. They measured circulating IGF-I, but also IGF-I mRNA levels, and found that both were increased in implanted cattle by as early as 7 d after implantation. They hypothesized that the implant-induced increases in IGF-I came at a point in the growth curve where satellite cell number and activity were normally declining. As a result, it prolongs the period of rapid muscle growth resulting in the increased rate and efficiency of muscle deposition in implanted steers.

In 2008, the same group continued their investigation to further elucidate mechanisms by which implantation increased IGF-I. They implanted feedlot steers with TBA, E2, and a T+E implant that contained identical masses of steroid equal to each singular implant. They measured circulating IGF-I, as well as the mRNA levels of IGF-I receptor 25, estrogen receptors and androgen receptors of muscle tissue. Although they did not see an increase in receptor mRNA levels, they found that the TBA implant did not increase IGF-I, while the T+E and E2 implants did increase IGF-I mRNA expression. They concluded that it is the E2 component of the T+E implant that may be responsible for the increase in mRNA expression and circulating levels of IGF-I (Pampusch et al., 2008). Because of the variety of studies to date that have shown steroid implants to increase mRNA expression and circulating levels of IGF-I, it appears that IGF-I can be used as a marker of steroid growth promotant activity.

Steroid Implants and Markers of Bone Formation and Resorption

Although the effects and mechanisms of action of steroid implants on lean tissue development are well documented, less is known about the physiologic changes that affect other

tissues, such as bone. As an indicator of animal maturity, the analysis of bone weight, breaking load, size and ossification is a key factor in meat animal production. Because the chronological age of the animal is often unknown, physiological maturity is used in grading a carcass. In beef, the most obvious indicator is the cartilage located on the dorsal surface of the spinous processes of the thoracic vertebrae. Such areas are called buttons, and as an animal ages, these buttons undergo ossification, beginning at 30 mo of age (Hale, 1998). In sheep, the degree of ossification of the epiphyseal plate on the distal end of the metacarpal is used to classify a carcass as lamb, yearling mutton or mutton. If the cartilage has yet to ossify, it is considered a break joint, however if it has ossified, it is deemed a spool joint (Hutcheson et al., 1992).

The use of implants has been implicated as a factor in advancing bone maturity scores. In lambs, bone breaking load was increased by 10 to 11% in animals implanted with zeranol on d 0 and d 56 when compared to nonimplanted control animals (Hutcheson et al., 1992). This finding could be explained by the 4.4% increase in bone cortical area and the 2.6% increase in bone width in the same implanted animals. Spool joints were also more frequent in animals that received the zeranol implant (Hutcheson et al., 1992). When fed a restricted diet, lambs implanted with zeranol had a 55% increase in carcass maturity score compared to controls. When fed *ad libitum*, carcass maturity scores increased by 17% in implanted lambs (Hufstedler et al., 1996). The disparity between these percentage increases as a result of feed intake could indicate a shift in nutrient partitioning to biological processes other than bone growth and ossification. However, IGF-I levels in both *ad libitum* and restricted lambs were 53% higher on average in implanted sheep than in nonimplanted animals (Hufstedler et al., 1996). IGF-I secretion is greatly affected by nutritional status (Breier et al., 1988), however it appears that implantation can augment IGF-I concentrations in a restricted diet (Hufstedler et al., 1996).

Zeranol implantation in beef cattle has also been studied as an estrogenic activator of an increase in bone maturity. Steers implanted with a zeranol dose of 24 mg or greater had 12% smaller external anterioposterior diameters, and increasing levels of dosage reduced marrow cavity diameter linearly (Turner et al., 1995). When bulls received a zeranol implant, it was found that they had reduced femur lengths and circumference (Gray et al., 1986). Calcium concentration in the third metacarpal bones of steers responded quadratically to zeranol dose, decreasing as zeranol implants increased to 36 mg, and increasing when dosage reached 48mg or higher (Turner et al., 1995). Mirroring this relationship was the dosage effects on the load at flexure. Because of this relationship and the findings that 12 mg of zeranol resulted in an increase in breaking load in sheep (Hufstedler, 1991; Hutcheson et al., 1992), Turner et al. (1995) postulated that the currently approved dosage in sheep of 12 mg may not be optimal for carcass characteristics and that a dosage increase may result in a similar quadratic relationship.

In addition to zeranol, TBA and E2, both alone and in combination with each other, have been implicated in increase bone maturity scores. Skeletal maturity was advanced in all of 105 steers that received an estrogenic implant alone, a T+E implant, or a TBA implant (Foutz et al., 1997). This advancement however was not enough to push the animals into a higher maturity classification.

Estradiol benzoate was also found to hasten bone aging. Hardt et al. (1995) implanted 42 heifers and 38 steers as calves, at weaning, 84 and 169 d postweaning. As a group, they first received 10 mg of estradiol benzoate and 100 mg of progesterone in Synovex-C (Ft. Dodge Animal Health Ft. Dodge, IA). Heifers were reimplanted with Synovex-H containing 20 mg of estradiol benzoate and 200 mg of testosterone and steers were reimplanted with Synovex-S containing 20 mg of estradiol benzoate and 200 mg of progesterone. Upon slaughter, they found

the metacarpals had greater medio-lateral axis, cortical areas and cross-sectional areas in all implanted animals. Additionally, these metacarpals also had greater breaking loads than bones from nonimplanted controls. The steers had shorter metacarpals. Overall maturity, as a function of longissimus muscle area, skeletal, and lean maturity, was greater in implanted steers than nonimplanted steers. Conversely, skeletal or lean maturity was not affected by implantation in heifers (Hardt et al., 1995). It is possible to speculate from these findings that it is the estrogenic component of these implants that affect physiologic bone aging.

In a two-year experiment, 41 steers were implanted with zeranol on d 0 and re-implanted with T+E on d 56 or were implanted with T+E on both d 0 and d 56 (Reiling and Johnson, 2003). It was determined that the percentage of ash in cartilaginous buttons, as an indicator of ossification, increased from 2% in control animals to 3 to 4% in implanted animals. Similar to Foutz et al. (1997), these increases were enough to advance bone maturity scores but not enough to push the animals into a more advanced classification.

Several studies have indicated that implantation can hasten bone aging without advancing the carcass into a more advanced maturity grading classification (Foutz et al., 1997; Hardt et al., 1995; Reiling and Johnson, 2003). Although only slightly affecting the grades of these carcasses, there could have been a greater impact on those carcass grades if the steers were more physiologically mature on d 0. Because physiological maturity is most commonly used to determine the age of an animal, it is of specific importance to trade restrictions adopted after the international food safety issues surrounding animals with bovine spongiform encephalopathy (BSE). A neurological disease, BSE is more frequent in animals of older age. As a measure to protect against the possibility of importing infected carcasses, Japan trade stipulates that meat must be from animals less than 20 months of age to be imported from the United States. If

implantation does in fact cause a carcass to appear more mature that it truly is, it is possible that these trade restrictions could be falsely withholding meat that should be acceptable. As a result, evaluating the mechanisms and effects on bone aging now have additional economic implications.

Several authors concluded that the growth response associated with implants might repartition minerals such as calcium and phosphorus from bone deposition to meet the requirements of rapidly increasing lean tissue (Goodband et al., 1993; Turner et al., 1995). As those requirements increase, osteoclastic activity may increase to degrade bone, resolubilize the hydroxyapatite crystals and make calcium and phosphorus available in circulation. For this reason, measuring markers of bone formation and resorption could indicate the mechanisms by which this repartitioning occurs.

Osteocalcin

Osteocalcin, or bone Gla protein, is a peptide produced by active osteoblasts, and therefore serves as a marker of bone formation. Osteocalcin is vitamin-K and vitamin-D dependent and is the most common non-collagen protein in bone tissue (Lee et al., 2000). Its transcription is regulated by 1,25-dihydroxy-vitamin D_3, a vital part of the mineral homeostasis function of bone. In its mature form, osteocalcin has two anti-parallel helical domains stabilized by disulfide bonds and is very highly conserved across species (Lee et al., 2000). However, bovine osteocalcin is more stable and abundant than human, although both have a very short half-life.

Gene knockout of murine osteocalcin results in an increase in bone formation at six months of age (Ducy et al., 1996). This may indicate the regulatory role of osteocalcin in osteoblasts. After secretion by the osteoblasts, greater than 60% of osteocalcin is incorporated into the osteoid by hydroxyapatite absorption (Lee et al., 2000). Remaining osteocalcin can be sampled

and detected in serum or plasma, however serum samples are typically more accurate.

TGF-β has been found to inhibit the expression of osteocalcin by blocking promoter activity, however when the mitogen activated protein kinase pathway was inhibited, osteocalcin returned to control levels (Kwok et al., 2009). Additionally, osteoblast differentiation was also inhibited.

In vitro studies using osteoblastic cell culture have shown IGF-I to stimulate cell differentiation and proliferation in humans and rats (Li et al., 2009). Recombinant IGF-I and growth hormone were both added to human osteoblastic cell cultures and although growth hormone augmented cell growth, IGF-I was much more potent (Scheven et al., 1991). In addition to its ability to enhance cell growth, exogenous IGF-I promoted differentiation of osteoblastic progenitor cells, stimulating these cells to become post-mitotic and express markers of the osteogenic phenotype (Yeh et al., 1997). IGF-I effects on osteocalcin, as a common marker of osteoblastic activity, were further investigated. Li et al. (2009) added exogenous IGF-I to harvested osteoblastic cells cultured from neonatal calves. Osteoblast proliferation, osteocalcin concentration, and calcium nodule formation on d 1, 3, 5, and 7 were elevated. They found that osteoblast proliferation increased in response to IGF-I, with the greatest response increasing 110% on d 5 at 100 ng/mL, and then decreasing as dosage increased. Authors speculated that this could be the result of dose-limited receptor expression on osteoblasts. Osteocalcin concentrations increased from controls by 30% at 10 ng/mL of IGF-I, and by 70% at 100 ng/mL. This finding is consistent with other reports that IGF-I stimulates osteocalcin concentration and gene expression (Kudo et al., 1998).

An exercise protocol administered to Holstein bull calves resulted in changes to osteocalcin levels as well as bone characteristics (Hiney et al., 2004). In that study, exercised calves had

increases in thickness and a larger percentage of cortical bone in metacarpals. Bone mineral density was also increased, and had increased breaking points. Osteocalcin levels began at a lower average for exercised calves, however these levels increased daily and became consistently higher than control animals throughout the 42 d study. Multiple studies have shown that IGF-I levels increase with exercise (Berg, 2004; Desvigne et al., 2007) and it is possible that the exercise-induced increases in both IGF-I and in osteocalcin are correlated. When growth hormone injections were administered to aged rats in order to stimulate IGF-I concentrations, bone formation rate increased 11-fold, and when combined with exercise, an even further increase of 39% was reported (Oxlund et al., 1998). Because the growth hormone-IGF-I axis stimulates osteoblast precursor cells to proliferate and differentiate, it is probably that this is the mechanism by which exercise induces an increase in osteocalcin to mark the increase of bone formation.

C-terminal telopeptides of Type I collagen

As previously discussed, Type I collagen is the organic portion of mature bone osteoid. As osteoclasts act to remodel and degrade bone, products of that degradation are released into circulation. Osteoblasts, through their secretion of collagenase, also degrade Type I collagen to allow for deposition of new osteoid in bone remodeling. Collagen consists of three-peptide α-chains twisted into a triple helix stabilized by hydrogen and disulfide bonds (Becker et al., 2003). These helices line up in parallel rows and are held together by covalent linkages. When broken down by osteoclasts, C-terminal telopeptides (commonly represented by the abbreviation CTX-I) are released and can be detected to measure bone resorption. In circulation, these telopeptides are modified as β-isomerized and cross-linked di-peptides (Becker et al., 2003). A significant inverse correlation between bone mineral density of the spine and femur and CTX-I

concentration was found in women with osteoporosis (Trento et al., 2009). In men, it was determined that as age affects a decline in sex steroids, a correlated decline was also found in CTX-I concentration (Carnevale et al., 2005). Because bone remodeling initially requires degradation of existing bone prior to deposition of new bone, both osteoblasts and osteoclasts are involved. Initially osteoblast collagenase secretion results in a breakdown of Type I collagen. Most prominent however, osteoclasts mediate bone degradation, and the additive effects of this breakdown to remodel bone may normally lead to increases in CTX-I. However, when coupled to the increases in bone aging as a result of steroid implantation, it is possible that the bone remodeling process slows, resulting in greater physiologic maturity of those bones and a decrease in CTX-I concentrations.

C-terminal telopeptides of Type II collagen

In cartilage, the organic portion and the majority of the tissue is made up of Type II collagen, which varies from Type I by its amino acid sequence. In endochondral ossification, bone growth is mediated by chondrocytes and their secretion of a cartilaginous model that eventually ossifies. Osteoclasts enter the model and degrade all of the perpendicular layers and 60% of the parallel layers of Type II collagen that separate chondrocyte columns (Farquharson, 2003). The remainder is used as the scaffold for osteoblasts to deposit the Type I osteoid. As cartilage is eroded, the degradation products are released into circulation as described previously. This is of particular interest to human and animal subjects that are affected by arthritis, or the irreversible degradation of the cartilaginous tissue of joints. It has been documented that IGF-I stimulates chondrocyte hypertrophy, and as a result, a greater amount of Type II collagen can be found (Mushtaq et al., 2004). It is possible that given a larger endochondral model, osteocyte action in long bone growth to degrade the Type II collagen scaffold increases. This would likely

result in elevated CTX-II concentrations, however there are no studies to support this hypothesis.

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CHAPTER 2 - Circulating Insulin-Like Growth Factor-I and Indicators of Bone and Cartilage Turnover in Steers Given Trenbolone Acetate and Estradiol 17-beta Alone or in Combination

ABSTRACT: Anabolic steroids are used extensively in beef cattle feeding management to take advantage of well-documented improvements in growth performance and efficiency of implanted cattle. In addition to muscle growth, steroids also impact changes in bone and cartilage formation. In general, these effects can be interpreted as hastening bone aging. The current study was designed to test the hypothesis that recently-identified peripheral indicators of bone and cartilage turnover could be detected in the peripheral circulation. Furthermore, it was hypothesized that these peripheral markers might reflect accelerated aging effects of the widely used steroidal implants trenbolone acetate (TBA) and estradiol-17β (E2). Circulating IGF-I was measured as a positive marker of steroid-induced enhancement of the somatotropic endocrine axis. Thirty-two crossbred yearling steers were blocked by BW and given one of four treatments: non-implanted controls; 25.7 mg estradiol-17β (E2); 120 mg trenbolone acetate (TBA); or a combination of 120 mg TBA and 24 mg E2 (T+E). Blood samples were collected on d 0, d 7, d 14 and d 28 and serum was analyzed by ELISA for IGF-I concentrations, as well as osteocalcin, C-terminal telopeptides of Type I collagen (CTX-I) and C-terminal telopeptides of Type II collagen (CTX-II), which serve as markers of bone formation, bone resorption and cartilage resorption, respectively. Circulating IGF-I was similar among treatments on d 0 and 28. At d 7 and 14, steers receiving E2 or T+E had greater circulating IGF-I than non-implanted control steers ($P < 0.05$). In contrast, steers receiving only TBA tended to have elevated IGF-I compared to controls on d 7 and 14 ($P = 0.10$). Although treatment did not affect serum osteocalcin,

concentrations were increased on d 7, 14, and 28 compared to d 0 ($P < 0.005$ for all). Implant treatment did not affect circulating CTX-I, however CTX-II was affected by T+E treatment ($P < 0.05$). The data suggest that, although selected markers of bone and cartilage turnover can be detected in circulation in cattle, implant-induced changes in the concentrations of these markers are not directly evident in the peripheral circulation at least through 28 d following treatment.
Key Words: Bone, Cartilage, Cattle, Estradiol 17-β, Growth, Trenbolone acetate

Introduction

It is a well-established practice to administer androgenic and estrogenic steroid implants to increase lean tissue accretion and decrease adipose tissue deposition in feedlot cattle. Yearling steers implanted with a combined trenbolone acetate (TBA) and estradiol-17β (E2) implant show an increase in average daily gain (18 to 21%), feed efficiency (13%) and muscle size when compared to nonimplanted steers (Johnson et al., 1996a). In addition, steroid implants increase circulating concentrations of insulin-like growth factor I (IGF-I) and muscle IGF-I mRNA levels by d 7 after implantation (Pampusch et al., 2003). However, less is known about the impacts of these practices on bone growth and the metabolites that affect bone aging. The same implantation system increased the percentage ash content of the 9^{th} to 11^{th} thoracic cartilaginous vertebrae and consequently, advanced bone maturity scores (Reiling and Johnson, 2003). When implanted with E2 only, Kniffen et al. (1999) found a dosage-dependent advancement in epiphyseal plate closure of metacarpal bones III and IV in beef heifers. Together, these data may suggest that TBA, E2, or the combination, hastens bone aging and, in this way, indirectly impact carcass quality. Little is currently known about the mechanisms of action behind this effect and if peripheral indicators of bone and cartilage metabolism might be

reflective of implant-driven alterations in these tissues. The current study was undertaken with this particular question in mind. Specifically, we evaluated total IGF-I to document expected changes in the somatotropic axis in response to implantation and circulating osteocalcin and C-terminal telopeptides of Type I collagen (CTX-I) as peripheral markers of bone formation and resorption, respectively. C-terminal telopeptides of Type II collagen (CTX-II) were likewise evaluated as a systemic indicator of cartilage resorption.

Materials and Methods

All animal-related experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animals

Thirty-two crossbred yearling steers with an average initial BW of 379.0 ± 2.9 kg were divided by BW into four blocks and randomly assigned to one of four treatments within each block in a 2 x 2 factorial design: 1) non-implanted, control; 2) implanted with 25.7mg E2 (Compudose, Vetlife Inc., West Des Moines, IA; E2); 3) implanted with 120 mg of trenbolone acetate (Finaplix, Intervet Inc., Millsboro, DE; TBA); 4) implanted with 120 mg TBA and 24 mg E2 in combination (Revalor-S, Intervet Inc., Millsboro, DE; T+E). Steers began the experiment in two groups, 4 wk apart. Animals were acclimated to a 93% concentrate diet fed *ad libitum* in individual pens beginning 21 d before implantation. On d 0, steers received a steroidal implant subcutaneously in the middle third of the right ear whereas control steers received no implant. On d 0, d 7, d 14, and d 28, blood samples were collected by jugular venipuncture for serum analysis. Samples were obtained consistently from the right jugular vein (ipsilateral to the ear containing the implant). Samples clotted overnight at 4°C, then were warmed on the countertop

to approximately 20 to 25°C. Serum was then obtained by centrifugation (1,500 × g, for 20 min at 4°C), divided into aliquots and stored at −20°C until use. Steer management, allocation to treatments and implantation protocol were identical to a larger companion study reported previously (Pampusch et al., 2008).

Serum Analysis

Concentrations of IGF-I (OCTEIA IGF-I; AC-27F1), osteocalcin (Rat-MID Osteocalcin ELISA kit; AC-11F1), CTX-I (CrossLaps for Culture ELSIA kit; 6CRL4000), and CTX-II (Urine CartiLaps ELISA kit; AC-10F1) were measured by enzyme-linked immunosorbent assays (ELISA) according to the kit protocols or as modified according to suggestions from the manufacturer (Immunodiagnostic Systems Ltd., Fountain Hills, AZ). Internal data from the manufacturer were obtained to document immunoreactivity in bovine serum for osteocalcin, CTX-I, and CTX-II. The assays for CTX-I and CTX-II were modified slightly to accommodate measurement in serum. The modification to the protocol simply involved a five-fold dilution of the test serum following guidance from the manufacturer. No data, however, were available for the IGF-I ELISA in bovine serum, so the assay was validated in our laboratory. When IGF-I was added to bovine serum and tested in the ELISA, the concentration of IGF-I measured in the assay averaged 92.3 % of the expected concentration, demonstrating acceptable quantitative recovery of added mass. The IGF-I ELISA protocol called for 50 μL of serum. To evaluate the assay for the absence of interfering serum matrix effects, samples of bovine serum were diluted 1:2, 1:3.33, and 1:5 and evaluated in the ELISA. When corrected for dilution, the measured concentrations averaged 105.0 % of the expected concentration. The ELISA was sensitive to 14 ng/mL. All samples were assayed in two plates. The intra-assay CV averaged 6.4 % and the inter-assay CV was 6.9 %. The IGF-I ELISA protocol utilized a proprietary releasing agent to

allow release of IGF-I from binding proteins and, therefore, was assumed to estimate total IGF-I.

Statistical Analysis

All serum data were analyzed as a randomized block design with repeated measures using the Mixed model procedure (SAS Inst. Inc., Cary, NC). The model included effects of implant treatment, time after implant, replicate, block and all interactive terms. Effects of weight block, replicate, and their interactions were considered random effects in the model. Implant treatment, time and the treatment x time interaction were considered fixed effects. The heterogeneous compound symmetry option was used in the repeated measures command. Least squares means were compared by using the PDIFF option only when there was a significant F test ($P < 0.05$) for the main effects of treatment, time or the treatment x time interaction in the analysis of variance.

Results

Total IGF-I

There was an interaction of treatment and time ($P < 0.05$) for serum concentrations of IGF-I. Circulating IGF-I was similar among treatments on d 0 and 28. At d 7 and 14, steers receiving E2 or T+E had greater circulating IGF-I than non-implanted control steers ($P < 0.05$). In contrast, steers receiving only TBA tended to have elevated IGF-I compared to controls on d 7 and 14 ($P = 0.10$).

Osteocalcin

Osteocalcin serum concentrations were not affected by treatment, however there was a significant effect of time ($P < 0.01$). All treatment groups including the control, nonimplanted steers, had a significant increase in osteocalcin levels from d 0 to d 7, to d 14 and to d 28 ($P < 0.01$). However, concentrations did not differ between d 7 and d 14, nor between d 14 and d 28.

CTX-I

Estimates of bone turnover as reflected by Type I collagen degradation (CTX-I) were not affected by treatment or time. There was considerable variation among animals within day and treatment. For example, values ranged from 45.6 nM in a T+E implanted steer on d 0 to 345.0 nM in a different T+E implanted steer, also on d0.

CTX-II

Serum concentrations of CTX-II were not affected by time, however there was an effect of treatment in the analysis of variance ($P < 0.05$). When compared to the control group, the T+E implant had elevated CTX-II concentrations ($P < 0.05$). Concentration of CTX-II also tended to be increased in T+E steers compared to E2 ($P < 0.08$).

Discussion

It has been well documented that steroid growth promotant implants have positive effects on the efficiency and growth of livestock, and much is known about the cellular mechanisms behind the changes in muscle (Barton-Davis et al., 1999; Foutz et al., 1997; Johnson et al., 1998; Pampusch et al., 2008). However there are no published reports on peripheral markers of bone growth and bone resorption that are linked to the steroid-induced increases in bone maturity.

Identification of reliable markers of steroid-induced bone aging could have significant utility. Age is a contributor to carcass grading standards. Age also has implications in terms of international trade restrictions as the physiological indicators of maturity of an animal are used to estimate its age. Because visible ossification of cartilage is used to designate the degree of maturity, it is important to understand how implantation affects cartilage ossification, bone growth and resorption to understand the potential economic implications of steroid-induced bone aging.

The current study was not designed specifically to determine effects of implant treatment on growth performance of steers. However, we elected to measure circulating IGF-I as an indirect positive marker of responses of cattle to growth promoting steroid implants (Johnson et al., 1996b; Johnson et al., 1998). Additionally, implants containing E2 or T+E have a greater ability to stimulate circulating IGF-I than implants containing only TBA (Pampusch et al., 2008). Indeed, our results show a similar result, and may indicate a greater capability for E2 to affect serum IGF-I concentrations. However, IGF-I mRNA in bovine satellite cell cultures was equally stimulated by both E2 and TBA treatment (Kamanga-Sollo et al., 2008), which may suggest a different mechanism to stimulate locally produced IGF-I in these cells. It is possible that the disparity between local production and circulating IGF-I is due to receptor differences and/or differences in endocrine response pathways between E2 and TBA implants, and this could be consistent across tissues, including bone. The E2 implant used in this study is also a longer-acting implant, and it is possible that if measured for longer than 28 d, even greater changes in IGF-I may have been evident. When fed a high plane of nutrition similar to a feedlot finishing diet, plasma growth hormone, IGF-I, and the high-affinity hepatic somatotropic receptor were increased when increased by E2 treatment (Breier et al., 1988). This may be further evidence

that E2 and other estrogens act on the somatotropic axis to increase hepatic secretion of IGF-I, while TBA and other androgens may act through a yet unidentified mechanism. An increase in bone maturity scores when compared with nonimplanted controls has been associated with estrogens (Turner et al., 1995), androgens (Moran et al., 1991) and both in combination (Reiling and Johnson, 2003). Until now however, there has not yet been a comparative study that evaluated serum IGF-I concentrations resulting from E2, TBA and T+E implants in the same group with E2 and TBA singularly in amounts equal to those present in the combination implant. This potentially eliminates dosage differences and can help further distinguish treatment differences.

Similar to IGF-I, osteocalcin also increased over time. As a marker of osteoblast activity, osteocalcin levels can indicate ossification or formation of bone. Given the well-documented increases in bone maturity post-implantation, we hypothesized that osteocalcin might increase as well. Although there were obvious effects of implantation on IGF-I concentrations, as noted above, the general trend for all treatment groups, including the control animals, was an increase from d 0 to d 28. In fact, by d 28, serum IGF-I did not differ among treatments. This general rise in IGF-I could correlate with the rise in osteocalcin seen over time, regardless of implantation. It is possible that although osteocalcin, as a marker of bone formation, didn't respond to steroid treatment, it may have been responding to IGF-I effects on long bone growth and would be evident if measured in synovial fluid or another local medium. This effect of IGF-I has been shown to increase osteoblast proliferation, osteocalcin gene expression, as well as osteocalcin concentration in cultured osteoblasts in vitro (Kudo et al., 1998; Li et al., 2009). It is likely that the steroid-induced increases in bone growth and ossification of cartilaginous tissue is a result of stimulated osteoblast activity and osteocalcin secretion.

It has been established that the process of normal bone formation associated with somatic growth involves bone and cartilage turnover. For this reason, we felt it reasonable to hypothesize that both CTX-I and CTX-II might be at steady state in a normally growing steer. We further reasoned that, because bone growth and hastened maturity have been associated with steroidal implants, implantation might result in decreased CTX-I and elevated CTX-II, measures of bone and cartilage degradation, respectively. However, the results of our study did not confirm this hypothesis as there were no treatment by time effects for either CTX-I or CTX-II. We might speculate that changes in both bone and cartilage resorption triggered by steroid implantation and reflected in altered CTX-I and CTX-II might only be evident locally at the site of bone formation, but not evident in the peripheral circulation.

There was an unexpected effect of T+E on CTX-II with this treatment having increased circulating CTX-II compared to control and a tendency to be elevated compared to E2. The reason for this difference is not readily apparent as the mean CTX-II concentrations appeared to be elevated for other treatments even on d 0 before implantation had taken place. Additionally, if CTX-II was elevated as a result of implant-induced increases in IGF-I, it is highly unlikely that only the T+E implant and not the E2 implant would result in an increase, although it is possible that the TBA component of the combination implant is acting through an unknown mechanism to attenuate the effects of E2. It is also possible that a mechanical injury and repair of that injury, independent of treatment, may have accounted for that effect, although we feel that possibility is unlikely as there were no changes in CTX-I nor were there signs on injury in any of the cattle.

In summary, neither E2 nor TBA, alone or in combination, directly affected peripheral indicators of bone formation and resorption or cartilage degradation. We conclude that peripheral sampling of the indicators for bone and cartilage turnover in this study is not a reliable measure

to mark the cellular mechanisms of steroid-induced acceleration of bone aging. We feel it is unlikely that effects would have emerged with longer duration sampling as the effects of implantation on IGF-I had diminished by the final sampling day.

Figure 1. Serum insulin-like growth factor I (IGF-I) in steers implanted with estradiol-17β (E2), trenbolone acetate (TBA), a combination (T+E), and non-implanted controls. Serum was obtained by jugular venipuncture on d 0 prior to implantation and after 7, 14, and 28 d. Data points represent the least-square means (± SEM) of 8 steers per treatment. Asterisks indicate days on which IGF-I concentration was greater than controls for E2 and T+E (*; P < 0.05) and tended to be greater for TBA (; P = 0.10).**

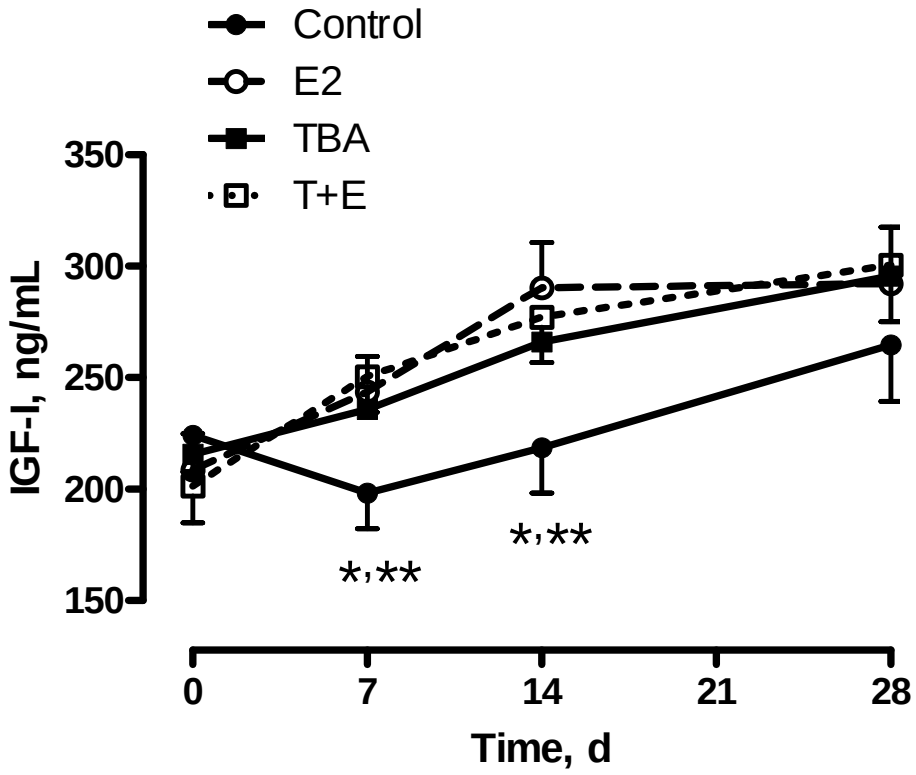


Figure 2. Serum osteocalcin in steers implanted with estradiol-17β (E2), trenbolone acetate (TBA), a combination (T+E), and non-implanted controls. Serum was obtained by jugular veinipuncture on d 0 prior to implantation, and after 7, 14, and 28 d. Data points represent the least-square means (± SEM) of 4 to 6 steers per treatment. Figure insert depicts time main effect means. Concentrations of osteocalcin were increased on d 7, 14, and 28 compared to d 0 (P < 0.005 for all comparisons).

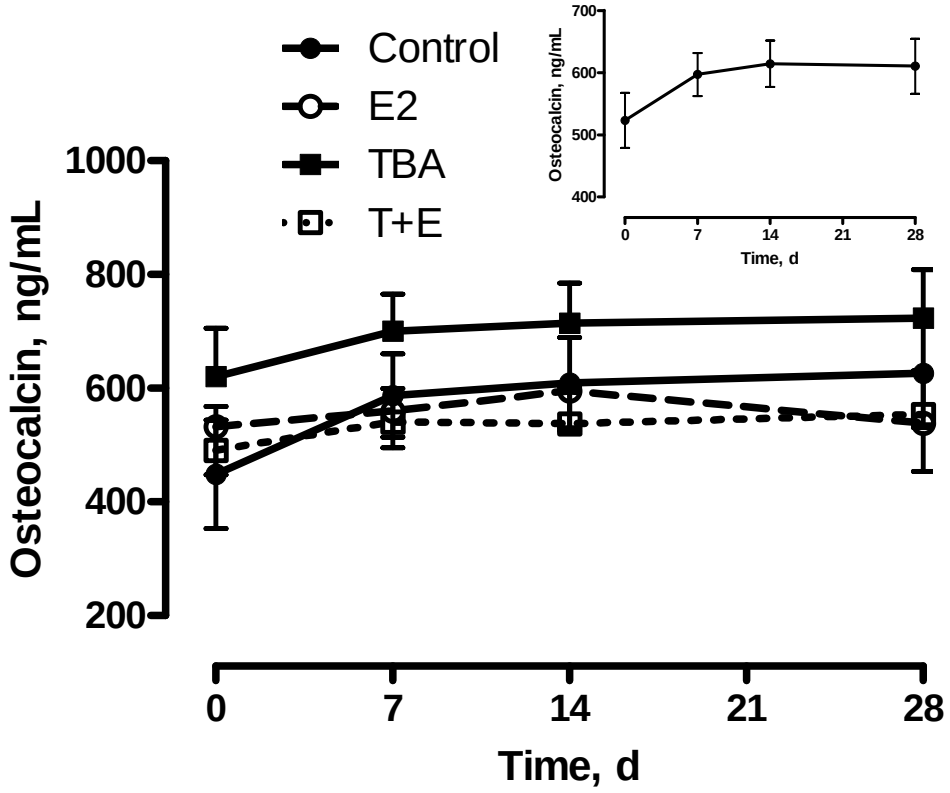


Figure 3. C-terminal telopeptides of Type I collagen, a measure of bone resorption, in steers implanted with estradiol-17β (E2), trenbolone acetate (TBA), a combination (T+E), and non-implanted controls. Serum was obtained by jugular venipuncture on d 0 prior to implantation, and after 7, 14, and 28 d. Data points represent the least-square means (± SEM) of 8 steers per treatment.

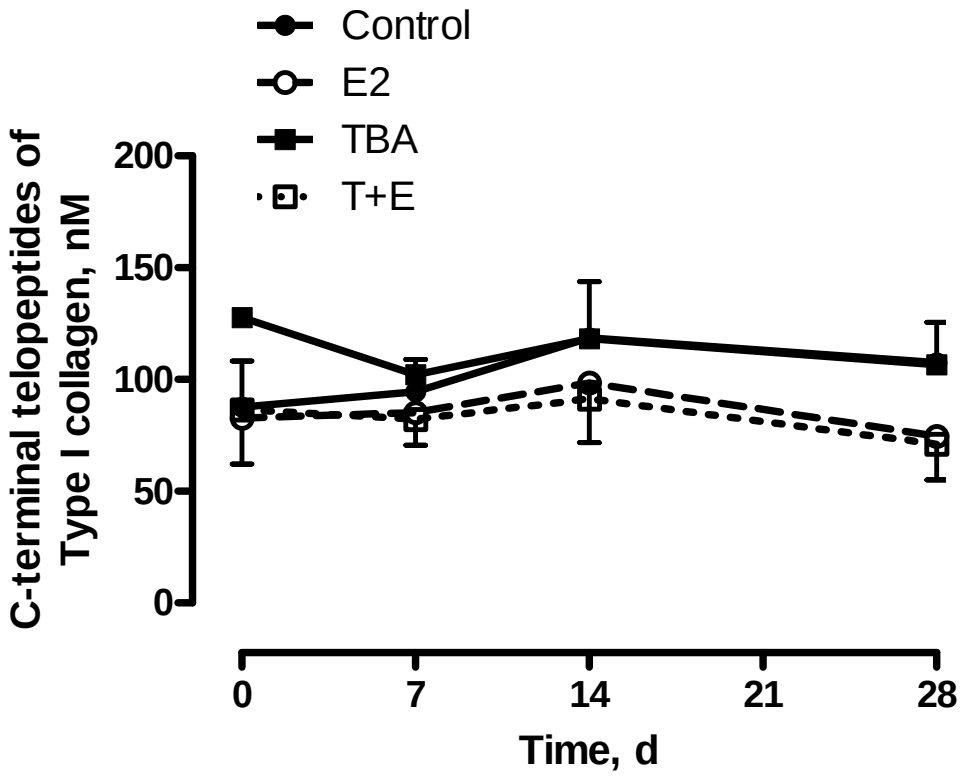
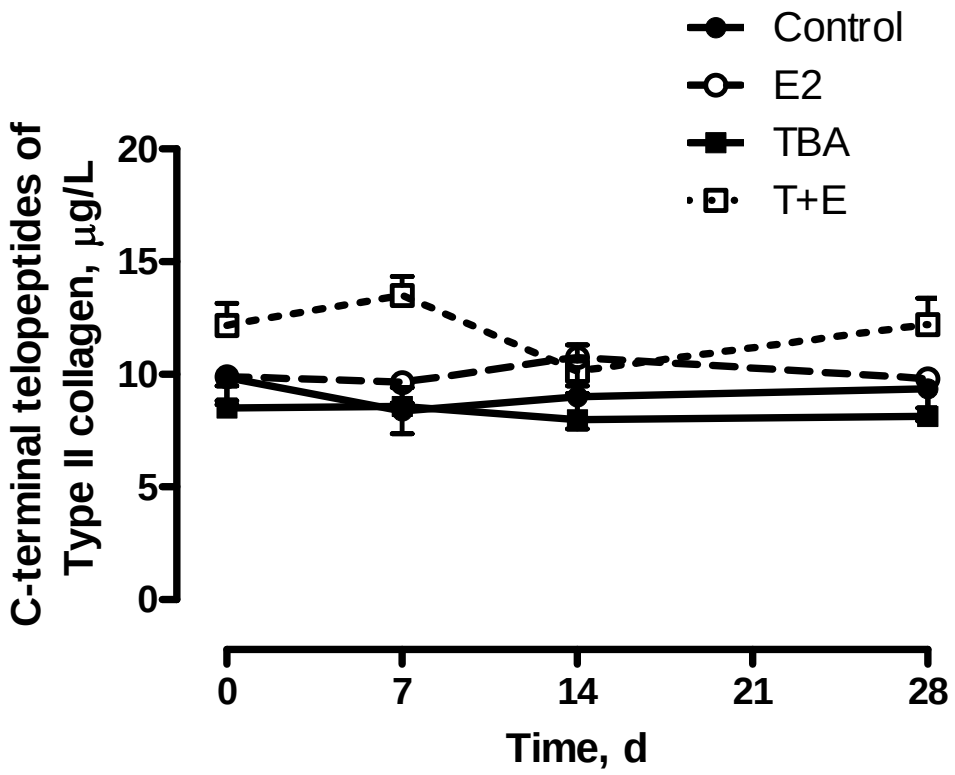


Figure 4. C-terminal telopeptides of Type II collagen, a measure of cartilage resorption, in steers implanted with estradiol-17β (E2), trenbolone acetate (TBA), a combination (T+E), and non-implanted controls. Serum was obtained by jugular venipuncture on d 0 prior to implantation, and after 7, 14, and 28 d. Data points represent the least-square means (± SEM) of 4 to 6 steers per treatment.



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