2006

Use of 25-hydroxyvitamin D3 and vitamin E and manipulation of dietary calcium to improve tenderness of beef

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Use of 25-hydroxyvitamin D₃ and vitamin E and manipulation of dietary calcium to improve tenderness of beef

by

Kristen Marie Carnagey

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Animal Nutrition

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Iowa State University
Ames, Iowa
2006

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GENERAL INTRODUCTION

Dissertation Organization

This dissertation is to partially fulfill the requirements of a Doctor of Philosophy degree. It consists of a general literature review followed by two manuscripts for submission to the Journal of Animal Science. The literature review introduces the topics included in the manuscripts. Each manuscript is complete on its own and has an abstract, introduction, materials and methods, and a combined results and discussion section followed by a bibliography. The manuscripts are followed by a general summary, general conclusion, and a future research section. The manuscripts are written in the style of the Journal of Animal Science. A reference section for references cited in the literature review, general summary, general conclusion, and future research sections concludes this dissertation.

The general hypothesis of this dissertation is that 25-hydroxyvitamin D3 and either vitamin E or manipulation of dietary calcium will improve the consistency of tenderness of beef from heifers or from cows. Two separate studies were performed to test this hypothesis. The goal of this research was to create a convenient, “on the farm” method to improve the consistency of beef tenderness. The first study in this dissertation was limited by the fact that the heifers that could be obtained for this research were specifically bred to produce tender beef. Although differences in tenderness were observed, beef from all of the cattle was very tender; so, the treatments did not make a large enough difference to benefit producers. In the second study, the number of cows in each treatment was only three; so, significant differences between treatments in many of the measured parameters were not observed.
Literature Review

Beef Tenderness

Meat tenderness is one of the most important factors that determine the palatability of meat (Huffman et al., 1996; Montgomery et al., 2000). Consumers are able to differentiate between degrees of tenderness and would be willing to pay a premium for beef that is tender (Boleman et al., 1997). In addition, consumers might be more likely to purchase beef more frequently if tenderness could be guaranteed.

Factors affecting beef tenderness. Tenderness of beef is affected by marbling, or presence of intramuscular lipid, by the concentration of collagen present in the muscle, and by the extent of postmortem myofibrillar degradation that occurs during aging. Marbling in beef is affected by breed of cattle and by feeding strategy. Generally, Bos taurus breeds produce beef that is more tender than that from Bos indicus breeds of cattle (Shackelford et al., 1994; Wheeler et al., 1994). Wheeler et al. concluded that beef from Bos taurus breeds of cattle had lower shear force values than did beef from Bos indicus breeds and that, in both Bos taurus and Bos indicus breeds, extent of marbling and Warner-Bratzler shear force values were related inversely. Higher marbling scores are associated with improved palatability regardless of cattle breed (Shackelford et al., 1994; Wheeler et al., 1994). In addition to being related to breed, extent of marbling also is increased when cattle are fed high concentrate feeds as opposed to grazing on pasture (Camfield et al., 1999). Also, the extent of collagen cross-linking increases in muscle as animals age, and increased cross-linking results in less tender beef (Morgan et al., 1991). Thus, beef from older cattle generally is less tender than beef from younger cattle. The last factor affecting tenderness of
beef that will be discussed is postmortem proteolysis. During postmortem proteolysis, enzymes present in the muscle cells remain active and hydrolyze myofibrils, thereby improving tenderness by disrupting muscle cell integrity (Koohmaraie, 1992b; Morgan et al., 1993; Wheeler and Koohmaraie, 1994).

At-home methods of beef tenderization. Consumers can purchase beef and then treat it at home by pounding to physically break apart collagen and myofibrils, which results in more tender beef but requires the consumers’ time. Another at-home solution to improve beef tenderness is the use of enzymatic meat tenderizers. These products are readily available, but, because of high sodium chloride content, they can alter the flavor of the meat. Additionally, if the tenderizers remain on the meat too long before cooking, an undesirable texture of meat results. Development of a method for tenderizing beef that is practical and could guarantee the tenderness of the product would benefit both producers and consumers. Consumer satisfaction with beef purchases likely would increase; hence, consumers may increase the instances in which they purchase beef. Producers could be benefited by an increase in demand that could lead to increased profits, especially if a niche market for “guaranteed tender” beef was developed.

Pre-consumer method of beef tenderization. Research has shown that injection of 0.3 M calcium chloride (10% wt/wt) into muscle following harvest results in improved Warner-Bratzler shear force values (Morgan et al., 1991). More recently, researchers have determined that injection of 0.2-0.25 M calcium chloride (5% wt/wt) up to 48 hours postmortem improves tenderness without altering other quality characteristics (Kerth et al.,
1995; Lansdell et al., 1995). Although this method successfully improves tenderness in treated beef, its effectiveness is limited to the muscles that are injected individually with the calcium chloride solution. Also, it is a cost-added method meaning that the producer will take deductions for the cattle they sell because the packing plant will need to purchase the calcium chloride and take the time to inject the meat. Developing a pre-harvest method for improving tenderness of beef may be more acceptable because the producers would not have to take discounts for their beef. Producers would, however, need to pay for the pre-harvest treatment; yet, if demand is increased, profits might be increased.

**Calpain System**

The mechanism by which injection of calcium chloride improves beef tenderness is presumed to relate to the calpain system. Calpains are calcium-dependent cysteine proteases found ubiquitously in mammalian systems (Belcastro et al., 1997). All calpain isozymes are heterodimers composed of an 80-kDa catalytic subunit and a 30-kDa regulatory subunit. Isozymes include m-, μ-, as well as several others. M- and μ-calpains are found in all tissues.

In m- and μ-calpain, the 30-kDa subunit is homologous and the 80-kDa subunit is similar but not identical (Saido et al., 1994). These two isozymes require different concentrations of calcium in the cytosol to become active; m-calpain requires near millimolar concentrations of calcium whereas μ-calpain requires only a micromolar concentration of calcium to induce activity. Five or six calcium ions must bind to m-calpain for the enzyme to become fully active; however, μ-calpain only needs to bind four calcium ions for full activity (Belcastro et al., 1997). Calpains are found primarily in the cytosol; however, some also are
associated with cell membrane components such as phospholipids, proteins, ion transfer systems, and receptors (Johnson, 1990).

**Calpain activity.** Calpain activity in muscle is affected by several factors (Belcastro et al., 1997). One factor that affects calpain activity is calcium concentration. Calpains are calcium-dependent; thus, calcium must be present in a concentration high enough to promote sufficient binding of calcium to calpain. The calcium requirement for calpain activity seems to decrease when calpain undergoes autolysis. Intact \( \mu \)-calpain requires \( 10^{-6} \) M calcium for activity; normal cell calcium concentration fluctuates between \( 10^{-8} \) and \( 10^{-6} \). Hence, it appears that the structure of calpain must be altered in some way to promote activity (Belcastro et al., 1997). Autolysis decreases the calcium requirement for \( \mu \)-calpain by more than 500%; the requirement for \( m \)-calpain decreases by more than 400% (Goll et al., 1992). Rate of autolysis is increased with decreasing pH, which means that more autolysis occurs postmortem because the pH decreases from neutral to approximately 5.7. The rate of autolysis of the 80-kDa subunit is slowed by decreasing temperature, but temperature does not affect rate of autolysis of the 30-kDa subunit (Koohmaraie, 1992a).

**Inhibition of calpain.** Interestingly, the enzymatic inhibitor specific to calpain, calpastatin, is also calcium dependent (Belcastro et al., 1997). Each molecule of calpastatin binds and inhibits up to four molecules of \( m \)- or \( \mu \)-calpain, depending upon the form of calpastatin. Calpastatin present in most mammalian tissues can inhibit up to four molecules of calpain; the calpastatin present in red blood cells inhibits only three molecules (Belcastro et al., 1997). Research has shown that calpastatin decreases the rate and extent of
myofibrillar hydrolysis by \( \mu \)-calpain (Geesink and Koohmaraie, 1999). Geesink and Koohmaraie (1999) discovered that, even in the presence of calpastatin, proteolysis proceeded for seven days in vitro. After seven days, little or no proteolysis occurred. The authors concluded that the action of calpastatin on \( \mu \)-calpain might be pH related; however, when this hypothesis was tested, \( \mu \)-calpain activity was inhibited slightly less when pH was 5.7 than when pH was 7.0. Therefore, the decrease in proteolysis that occurs after seven days is probably not related to pH; in fact, calpastatin was less inhibitory when pH was similar to that in aged meat, which is approximately 5.7. When the authors determined that pH was not responsible for the decline of \( \mu \)-calpain activity after seven days, the effect of ionic strength on \( \mu \)-calpain activity was investigated. Increasing ionic strength from 0 mM NaCl to 500 mM NaCl decreased stability of autolyzed \( \mu \)-calpain, resulting in diminished calpain activity (Geesink and Koohmaraie, 1999). These results are supported by a similar study that concluded pH does not affect calpastatin’s inhibitory effect on \( \mu \)-calpain; however, ionic strength does play a role in inhibition of \( \mu \)-calpain by calpastatin (Maddock et al., 2005).

In a separate study that evaluated the cumulative effects of pH, ionic strength, and oxidation on m- and \( \mu \)-calpain with or without the presence of calpastatin, researchers concluded that these factors, in combination, greatly affect calpain activity (Maddock Carlin et al., 2006). In agreement with Geesink and Koohmaraie (1999), Carlin et al. (2006) did not observe differences in the inhibition of \( \mu \)-calpain by calpastatin in response to differing pH alone. Some differences in inhibition of \( \mu \)-calpain were present when the interaction of differing pH with oxidation or the combination of calpastatin and oxidation was considered.
The results obtained by Carlin et al. (2006) are an example of the complexity surrounding the calpain system and how environmental factors play a role in myofibril degradation.

**Substrates of calpain.** All of the specific substrate proteins for calpain have not been identified; however, calpain seems to hydrolyze structural proteins. Five proteins that have been used as substrates to determine calpain activity are titin, nebulin, filamin, desmin, and troponin-T (Huff-Lonergan et al., 1996a). These structural proteins probably play a role in muscle integrity; so, hydrolysis by calpain results in disruption of muscle integrity and lower shear force values, which indicate improved tenderness.

**Vitamin E**

**Actions of vitamin E.** The actions of vitamin E as an antioxidant that prevents tissue damage by free radicals have been well documented (Burton et al., 1983; Combs, 1992; Shils et al., 1998). Vitamin E is a general term for a group of related compounds that all exhibit antioxidative properties. These compounds include α-, β-, γ-, and δ-tocopherols as well as α-, β- γ-, and δ-tocotrienols (Combs, 1992). The tocopherols and tocotrienols differ in that the tocopherols have a phytyl tail, whereas the tocotrienols have an unsaturated carbon tail. All of these compounds are derived from plants and are not synthesized by animals. Compounds that fall into the vitamin E category are hydrophobic and therefore fat-soluble, being present in the highest concentration in plant oils. Of these compounds, α-tocopherol is found in the highest concentration and is the most biologically active form in animals. For
this reason, α-tocopherol is often considered the most important member of the vitamin E family. The other tocopherols have different activities (Table 1).

Vitamin E acts as an antioxidant because its phenolic hydroxyl group reacts very quickly with organic peroxyl radicals to prevent oxidation of lipids. In the absence of vitamin E, autooxidation of lipids continues without interruption and can result in the disruption of cell membranes (Burton and Traber, 1990).

With vitamin E: ROO• + vitamin E-OH ➞ ROOH + vitamin E-O•

Without vitamin E: ROO• + RH ➞ ROOH + R•

   R• + O2 ➞ ROO•

_Vitamin E and beef._ In animal tissue, vitamin E is found primarily in adipose tissue but also in cell membranes of all tissues. Because animals do not synthesize vitamin E, all of the tocopherols and tocotrienols present are obtained through the diet. In the case of cattle, the requirement for vitamin E routinely is met by their consumption of plant substances. Even so, cattle may benefit from supplemental vitamin E in the diet. The antioxidant properties of vitamin E may be beneficial to prolonging the shelf-life and possibly to the extent of postmortem tenderization of beef.

_Vitamin E and beef quality._ Another important quality characteristic of beef is color. Acceptable beef color is bright, cherry red, and is caused by different forms of myoglobin present in meat. Deoxymyoglobin is the compound present upon harvest of beef
that soon reacts with oxygen in the environment and becomes oxymyoglobin (Livingston and Brown, 1981). Oxymyoglobin is the compound that gives the bright, cherry red color associated with fresh beef. After several days in an oxygen-rich environment, oxymyoglobin is oxidized further to met-myoglobin, which is brown in color is less acceptable to consumers (Liu et al., 1995). In beef, oxidation of lipids, resulting in rancid or warmed-over flavors and an undesirable change in texture, is related closely to the oxidation of myoglobin species (Liu et al., 1995). Additionally, oxidation of unsaturated fatty acids to saturated fatty acids increases the atherogenic index of beef (Ulbricht and Southgate, 1991). Beef naturally contains a higher concentration of saturated fatty acid than does pork or chicken; so, increasing the content of such fatty acids is highly undesirable.

**Color stability.** Many studies have been conducted to determine the effect of vitamin E on beef quality. In one study, 300 IU of vitamin E were fed daily to Holstein steers for nine months before harvest. Upon harvest, the strip loin was vacuum packaged and aged for seven days at 4°C before being sliced into 2.6-cm steaks and wrapped with oxygen-permeable wrap. The wrapped steaks were subjected to supermarket conditions for 11 days, and a trained panel assessed the discoloration daily. Beef from steers that were supplemented with vitamin E remained acceptable to panelists for 7.4 days, whereas steaks from control steers were acceptable, with regards to color, for only 4.9 days (Arnold et al., 1992). Results from subsequent studies have supported the findings by Arnold et al. (1992), as each supports the conclusion that vitamin E, when supplemented for an extended time before harvest, maintains the quality and improves the shelf-life of beef (Arnold et al., 1993; Liu et al., 1995; Rowe et al., 2004a).
Lipid oxidation. Supplementation with vitamin E before harvest also decreases the extent of lipid oxidation postmortem (Arnold et al., 1993; Liu et al., 1995). In a study by Arnold et al. (1993), three different daily dosages of vitamin E (0, 360, or 1290 IU) were supplemented to steers of different breeds for 252 consecutive days before harvest. The authors concluded that 360 IU of vitamin E are sufficient to prevent lipid oxidation as there was no improvement with supplementation with 1290 IU of vitamin E.

Beef tenderness. The effects of vitamin E on shelf stability of color and lipids have led researchers to consider other roles of vitamin E postmortem. Postmortem tenderization of beef occurs largely because of calpain activity as discussed previously. Because the calpain molecule has a region that is susceptible to oxidation, researchers have hypothesized that vitamin E could have a protective effect on calpain prolonging its active life and resulting in increased myofibrillar degradation and improved tenderness.

In one study designed to determine the effects of vitamin E and injection of calcium chloride on beef tenderness, researchers discovered that samples from calcium chloride-injected muscles from heifers that had been supplemented with 1,000 IU of α-tocopherol for 125 days before harvest exhibited signs of improved tenderness (increased troponin-T degradation, decreased Warner-Bratzler shear force) at 1 and 3 days of aging. Steaks from heifers that were supplemented only with α-tocopherol also exhibited increased proteolysis but at a slower rate (Harris et al., 2001). Harris et al. (2001) concluded that increased vitamin E concentration in calcium chloride-injected muscle of vitamin E-supplemented heifers might increase calpain activity and lead to improved beef tenderness.
Another study was designed to identify the effects of oxidative conditions postmortem on calpain activity and subsequent proteolysis and tenderization of beef (Rowe et al., 2004b). Vitamin E (0 or 1000 IU) was administered for 126 days prior to harvest. Strip loins were collected within 24 hours of harvest, and 2.54-cm steaks were vacuum packaged, irradiated at 0 or 6.4 kGy, and then aged at 4°C for 0, 1, 3, 7, or 14 days. Warner-Bratzler shear force values were higher at all aging periods for irradiated steaks than for non-irradiated steaks. Probably related to that finding is that irradiation decreased calpain activity, and therefore decreased myofibrillar proteolysis. The authors also noted that, at early aging times, oxidation of calpain resulted in decreased myofibrillar proteolysis and postmortem tenderization of steaks. Carlin et al. (2006), in a study that is discussed more thoroughly in the “Calpain” section of this literature review, found that the only single factor tested that influenced µ-calpain activity was oxidation. The fact that oxidation has such a remarkable effect on calpain is very important developing future research. Vitamin E could play a pivotal role in protecting calpains and promoting tenderness of beef.

**Vitamin D**

Vitamin D is an interesting fat-soluble vitamin in that it is actually a seco-steroid pro-hormone that is derived from 7-dehydrocholesterol and is synthesized in the skin of most mammals (Combs, 1992). Vitamin D₃ is produced when a precursor that is common to many steroid compounds and bile acids, 7-dehydrocholesterol, in the skin is exposed to ultraviolet radiation (Figure 1). Vitamin D₃ itself is not active in the body; rather, it is a storage compound. Vitamin D₃, obtained either via irradiation of 7-dehydrocholesterol in the skin or by ingestion, is transported to the liver where it undergoes hydroxylation to form 25-
hydroxyvitamin D₃ (25-OH D₃) (Figure 2). Although it has been hydroxylated, 25-OH D₃ also is not active and must be further hydroxylated in the kidneys, and to a lesser extent in other tissues, by the enzyme 1α-hydroxylase to form 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂ D₃]. 1,25-Dihydroxyvitamin D₃ is the biologically active form of vitamin D that acts as a steroid hormone. 1,25-Dihydroxyvitamin D₃ binds to the vitamin D receptor, which then binds retinoic acid X receptor (RXR) to form a heterodimer that interacts with certain vitamin D-responsive elements. This complex increases the transcription of mRNA for calcium binding protein in the intestine and other proteins, including osteocalcin, osteopontin, calbindin, and 24-hydroxylase, while decreasing mRNA transcription for IL-2 and IL12, decreasing cell proliferation, and decreasing stimulation of parathyroid hormone (PTH) synthesis (Bouillon et al., 1995; Darwish and DeLuca, 1993; Nagpal et al., 2005). The net results of these actions are an increase in intestinal calcium absorption, modulation of bone metabolism, modulation of intracellular calcium, and modulation of immune response.

Under normal conditions, when vitamin D is adequate and plasma calcium is maintained, 25-OH D₃ is hydroxylated to metabolically inactive, 24,25-dihydroxyvitamin D₃ (Combs, 1992). 25-Hydroxyvitamin D₃ 24-hydroxylase is up-regulated when PTH is present in low concentrations and when concentration of 1,25-(OH)₂ D₃ in plasma is sufficient.

**Vitamin D and immune response.** One of the most investigated actions of 1,25-(OH)₂ D₃, besides its role in plasma calcium homeostasis, is its immunologic effects. Many studies have been completed over the past 20 years to determine the effects of 1,25-(OH)₂ D₃ on certain immune cell types. Nearly all immune cells express 1,25-(OH)₂ D₃ receptors and
seem to respond in different ways. The general consensus of the usefulness of 1,25-(OH)$_2$ D$_3$ as a modulator of the immune system is that it suppresses the immune response and is used as treatment for autoimmune diseases and to prevent tissue rejection in the case of transplants (Lips, 2006; van Etten and Mathieu, 2005).

**Vitamin D and cardiovascular disease.** Although the mechanisms are not fully understood, research is emerging that indicates a relationship between vitamin D status and cardiovascular health. For example, an inverse relationship between circulating concentrations of 25-OH D$_3$ and blood pressure has been documented (Krause et al., 1998). One possible reason for this finding is that 1,25-(OH)$_2$ D$_3$ downregulates renin production in the kidney (Li et al., 2002). Renin is a hormone that stimulates an increase in blood pressure; so, down-regulating its synthesis results in decreased blood pressure.

**Hypo- and hypervitaminosis D.** Vitamin D$_3$ and its metabolites, 25-OH D$_3$ and 1,25-(OH)$_2$ D$_3$, play an integral role in the maintenance of plasma and extracellular calcium concentrations. When vitamin D is not sufficient either from sun exposure or from the diet for extended lengths of time, rickets can occur (Holick, 2005). More common effects of hypovitaminosis D are poor dietary calcium and phosphorus absorption, which results in weakened bones because of increased bone resorption to maintain plasma calcium concentration and poor bone mineralization leading to osteomalacia (Holick, 2005). Sufficient dietary vitamin D or exposure to sunlight or ultraviolet rays reverses the effects of vitamin D deficiency. The opposite of vitamin D deficiency also can occur. When very high amounts of vitamin D are consumed, hypervitaminosis D can occur, which can lead to
increased plasma calcium concentration and, if the duration is long enough, calcification of soft tissues and anorexia may result (Shils et al., 1998). However, sun exposure does not lead to hypervitaminosis D because of formation of inert metabolites.

**Vitamin D and beef.** The role of vitamin D in calcium homeostasis is of interest to beef researchers because of its action in increasing circulating calcium concentration. The general hypothesis surrounding this role of vitamin D is that the hypercalcemia that results after feeding supranatural dosages of vitamin D₃ increases calcium availability to calpains, thereby improving tenderness because of increased postmortem proteolysis.

Several studies have been conducted to determine the efficacy of feeding supranatural dosages of vitamin D₃ (0.5-7.5 × 10⁶ IU) to cattle before harvest on improving beef tenderness (Foote et al., 2004; Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Scanga et al., 2001; Swanek et al., 1999). See Table 2 for a summary of these studies. In general, vitamin D₃ improves tenderness of beef by way of increased postmortem proteolysis as a result of elevated calcium concentration, which results from increased calcium absorption in response to the active metabolite of vitamin D₃, 1,25-(OH)₂ D₃ (Combs, 1992). The calcium concentration in muscle from animals treated with vitamin D₃ in the studies listed in Table 2 vary widely among studies. Calcium ranges from about 14-500 µg/g of fresh tissue. According to the USDA National Nutrient Database for Standard Reference, Release 18, accessed on-line June 22, 2006 (http://www.nal.usda.gov/fnic/foodcomp/Data/SR18/nutrlist/sr18a301.pdf), calcium concentration in cooked beef is approximately 300 µg/g of beef. All of the studies show an increase in plasma calcium concentration with supplementation of vitamin D, but the wide
variation in calcium concentration is unexplained. As previously discussed, increased plasma calcium concentration can improve tenderness by increasing calcium availability to calpains (Delgado et al., 2001; Koohmaraie, 1992a, b).

The disadvantage of feeding a high dosage of vitamin D₃ close to time of harvest, however, is that excess vitamin D₃ results in a relatively high concentration of vitamin D₃ and its metabolite, 25-OH D₃, in muscle (Foote et al., 2004; Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Scanga et al., 2001; Swanek et al., 1999). Although it is unlikely that a person could consume enough beef to induce hypervitaminosis D, the concentrations in beef liver are so high (979 ng/g) that a person could consume enough liver (1.8 oz) to reach the upper limit (Montgomery et al., 2000). Hypervitaminosis D is undesirable because an increase in concentration of vitamin D can cause more calcium to be absorbed from the small intestine, leading to significantly increased calcium concentrations in blood and tissues, which is undesirable in humans because high calcium concentrations can lead to soft tissue calcification if the condition exists for an extended length of time (Shils et al., 1998). In effect, the same mechanism that works to improve tenderness of beef from cattle treated with vitamin D₃ can cause ill effects in humans if very high amounts of vitamin D are consumed regularly. Feeding large doses of vitamin D₃ to cattle a few days before harvest, however, is considered safe for cattle because they are not fed this high dosage of vitamin D₃ long enough to cause calcification of soft tissues.

25-Hydroxyvitamin D₃ and beef tenderness.
Feeding 25-OH D$_3$ before harvest of beef is an alternative to feeding vitamin D$_3$ in that it shows promise for eliciting responses similar to those of vitamin D$_3$ without high residues of vitamin D$_3$ or its metabolites in beef or in liver. However, the improvement in the tenderness of beef that results from feeding a supranatural dosage of 25-OH D$_3$ has shown some promise, but no effective dosage has been determined (Foote et al., 2004; Wertz et al., 2004). In both of these studies, 125 mg of 25-OH D$_3$ was sufficient to increase plasma, but not muscle, calcium concentration. In both cases, the concentration of 25-OH D$_3$ was increased in plasma and in muscle, and concentration of 1,25-(OH)$_2$ D$_3$ was increased only in plasma. However, this dosage of 25-OH D$_3$ did not increase postmortem proteolysis or improve tenderness in either study. The dosage of 25-OH D$_3$ administered probably was not high enough to increase muscle calcium concentration or result in increased postmortem proteolysis or improved tenderness.

**Dietary calcium and circulating calcium concentration**

Yet another method of altering plasma calcium concentration is the management practice of withdrawing and then replenishing dietary calcium in the diet. Researchers found that feeding a calcium-deficient diet to dairy cows for seven days prior to parturition up-regulates the calcium homeostatic mechanisms and, upon replenishing calcium in the diet, calcium absorption increases and milk fever (low plasma calcium concentration) can be prevented (Goings et al., 1974). Plasma calcium presumably is maintained during dietary calcium withdrawal by an increase in PTH and subsequent bone resorption. An increase in PTH also increases the synthesis of 1α-hydroxylase in the kidney (Combs, 1992; Shils et al., 1998). Because of increased 1α-hydroxylase, the concentration of 1,25-(OH)$_2$ D$_3$ increases,
resulting in increased transcription and translation of mRNA for calcium-binding protein in the small intestine. Because of increased calcium-binding protein in the small intestine, calcium absorption is more efficient when dietary calcium is replenished. The net result is an increase in plasma calcium concentration. Withdrawing and replenishing dietary calcium might be a very practical method for producers to use if it does, in fact, result in increased plasma and muscle calcium concentrations. The effectiveness of this feeding strategy in beef cattle has not been investigated.

Summary

Inconsistency of beef tenderness is one of the major quality concerns facing the beef industry, and there are many contributing factors. Researchers currently are developing “on the farm” methods of improving the beef tenderness. This dissertation focuses on the use of 25-OH D$_3$ in combination with vitamin E or dietary calcium concentrations as possible methods of improving tenderness of beef.
### Table 1. Biological and antioxidant activities of tocopherols

<table>
<thead>
<tr>
<th>Tocopherol</th>
<th>Biological activity(^a)</th>
<th>Peroxyl radical scavenging reactivity (mg/IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>β</td>
<td>60</td>
<td>0.75</td>
</tr>
<tr>
<td>γ</td>
<td>25</td>
<td>0.15</td>
</tr>
<tr>
<td>δ</td>
<td>27</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^a\) Biological activity expressed as percentage of activity of α-tocopherol. Adapted from Shils et al., 1998.
Figure 1. Conversion of 7-dehydrocholesterol to vitamin D₃
**Figure 2.** Metabolism of vitamin D₃

CT = Calcitonin
25-OH D₃ = 25-Hydroxyvitamin D₃
1,25-(OH)₂ D₃ = 1,25-Dihydroxyvitamin D₃
PTH = Parathyroid hormone
CBP = Calcium-binding protein
Table 2. Summary of studies using vitamin D₃ to improve beef tenderness (cont. on next page)

<table>
<thead>
<tr>
<th>Author (et al.)</th>
<th>Year</th>
<th>Dosage (x 10⁶ IU)</th>
<th>Duration (d)</th>
<th>Calcium conc. Plasma (mg/dl)</th>
<th>Vitamin D₃ conc. Plasma (ng/ml)</th>
<th>Muscle (µg/g)</th>
<th>25-OH D₃ conc. Plasma (ng/ml)</th>
<th>Muscle (µg/g)</th>
<th>1,25(OH)₂ D₃ conc. Plasma (pg/ml)</th>
<th>Muscle (pg/g)</th>
<th>Troponin-T degradation</th>
<th>Warner-Bratzler shear force (kg)</th>
</tr>
</thead>
</table>
| Swanek         | 1999 | 0                 | 7           | 9.23                        | na
|                |      | 5                 | 7            | 12.39                       | 19.9             | na              | na                          | na              | na                          | na              | na                     | 4.03                        |
|                |      | 1,25(OH)₂ D₃ conc. | 7.5         | 9                           | 12                           | na              | 529.7           | 91.1           | 610.4                        | 20.2            | 28.4                   | 20.1                        |
| Montgomery     | 2000 | 0                 | 9           | 3.1                         | 4.1                          | 48.1           | 1.4             | 20.8           | 43.9                          | 0.614           | 3.25                   | 3.87                        |
|                |      | 5                 | 9            | 11                           | na                           | 464.3          | 80.8            | 578.4          | 25.7                          | 27.2            | 26.2                   | 0.89                        |
|                |      | 7.5               | 9            | 12                           | na                           | 529.7          | 91.1            | 610.4          | 20.2                          | 28.4            | 20.1                   | 0.631                       |
| Karges         | 2001 | 0                 | 8           | 11.3                        | na                           | na              | na              | na              | na                          | na              | 4.64                   | 3.98                        |
|                |      | 1                 | 8            | 13.3                        | na                           | na              | na              | na              | na                          | na              | 4.2                    | 4.48                        |
|                |      | 2                 | 8            | 13.2                        | na                           | na              | na              | na              | na                          | na              | 4.53                   | 3.78                        |
|                |      | 3                 | 8            | 12                           | na                           | na              | na              | na              | na                          | na              | 4.68                   | 5.14                        |
|                |      | 4                 | 8            | 12.9                        | na                           | na              | na              | na              | na                          | na              | 5.14                   | 5.03                        |
| Scanga         | 2001 | 0                 | 8           | 11.3                        | na                           | na              | na              | na              | na                          | na              | 4.55                   | 4.48                        |
|                |      | 1                 | 8            | 13.3                        | na                           | na              | na              | na              | na                          | na              | 4.2                    | 4.48                        |
|                |      | 2                 | 8            | 13.2                        | na                           | na              | na              | na              | na                          | na              | 4.53                   | 3.78                        |
|                |      | 3                 | 8            | 12                           | na                           | na              | na              | na              | na                          | na              | 4.68                   | 5.14                        |
|                |      | 4                 | 8            | 12.9                        | na                           | na              | na              | na              | na                          | na              | 5.14                   | 5.03                        |
|                |      | 5                 | 8            | 12.7                        | na                           | na              | na              | na              | na                          | na              | 5.14                   | 5.03                        |
|                |      | 2 + 75 g CaCO₃    | 8            | 11.7                        | na                           | na              | na              | na              | na                          | na              | 5.03                   | 5.03                        |
|                |      | 4 + 75 g CaCO₃    | 8            | 12                           | na                           | na              | na              | na              | na                          | na              | 5.14                   | 5.03                        |
| Montgomery     | 2002 | 0                 | 9            | 8.7                         | 486                          | na              | 9.5             | 4.1             | 202.7                        | na              | 2.92                   | 2.92                        |
|                |      | 0.5               | 9            | 9.2                         | 572                          | na              | 18.3            | 5.6             | 229.2                        | na              | 2.79                   | 2.79                        |
|                |      | 1                 | 9            | 9.4                         | 779                          | na              | 23.7            | 6.6             | 130                          | na              | 2.81                   | 2.81                        |
|                |      | 2.5               | 9            | 9.8                         | 651                          | na              | 37.3            | 6.5             | 143                          | na              | 3.01                   | 3.01                        |
|                |      | 5                 | 9            | 10.2                        | 651                          | na              | 75.2            | 11.5            | 171.1                        | na              | 2.84                   | 2.84                        |
|                |      | 7.5               | 9            | 10.4                        | 766                          | na              | 65.9            | 12.4            | 172.3                        | na              | 2.97                   | 2.97                        |

a) Troponin-T degradation at 14 d aging.

b) Warner-Bratzler shear force of *longissimus dorsi* from the rib in all studies except Montgomery (2000) and Foote (2004). In those two studies, *longissimus dorsi* was obtained from the strip loin. All samples at 14 d aging.

c) Parameter was not measured.

* Value shown as µg/g of dry tissue
<table>
<thead>
<tr>
<th>Author (et al.)</th>
<th>Year</th>
<th>Dosage (x 10⁶ IU)</th>
<th>Duration (d)</th>
<th>Calcium conc. (mg/dl)</th>
<th>Muscle (µg/g)</th>
<th>Vitamin D₃ conc. (ng/ml)</th>
<th>Muscle (ng/g)</th>
<th>25-OH D₃ conc. (ng/ml)</th>
<th>Muscle (ng/g)</th>
<th>1,25-(OH)₂ D₃ conc. (pg/ml)</th>
<th>Muscle (pg/g)</th>
<th>Troponin-T degradation</th>
<th>Warner-Bratzler shear force (kg)</th>
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<tr>
<td>Foote</td>
<td>2004</td>
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<td>9</td>
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<td>3.3</td>
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<tr>
<td>Montgomery</td>
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<td>8</td>
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<td>4</td>
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<td>11.4</td>
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<td>217</td>
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<td>797.6</td>
<td>45.5</td>
<td>473</td>
<td>1.2</td>
<td>368.7</td>
<td>156.7</td>
<td>1.7</td>
<td>4.21</td>
</tr>
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</table>

- Troponin-T degradation at 14 d aging.
- Warner-Bratzler shear force of *longissimus dorsi* from the rib in all studies except Montgomery (2000) and Foote (2004). In those two studies, *longissimus dorsi* was obtained from the strip loin. All samples at 14 d aging.
- Parameter was not measured.
- Value shown as µg/g of dry tissue.
USE OF 25-HYDROXYVITAMIN D₃ AND VITAMIN E TO IMPROVE TENDERNESS OF BEEF FROM THE LONGISSIMUS DORSI OF HEIFERS¹

A manuscript to be submitted to the Journal of Animal Science

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¹Research funding granted by the Iowa Beef Industry Council and the National Cattlemen’s Beef Association.

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³The authors acknowledge Derrel Hoy and Duane Zimmerman in the Periparturient Diseases of Cattle Group of the National Animal Disease Center—USDA/ARS for their assistance with laboratory analyses.
ABSTRACT  The objective of this trial was to determine whether a single bolus of 25-hydroxyvitamin D$_3$ (25-OH D$_3$), vitamin E, or a combination of the two would improve the tenderness of steaks from the longissimus dorsi of beef heifers. Forty-eight Angus cross heifers were allotted randomly to 8 pens. Six heifers were in each pen, and there were two pens per treatment. The four treatments included Control, no 25-OH D$_3$ or vitamin E; 25-OH D$_3$, 500 mg of 25-OH D$_3$ administered as a one-time oral bolus 7 d before harvest; Vitamin E, 1000 IU of vitamin E administered daily as a top-dress for 104 d before harvest; or Combination, 500 mg of 25-OH D$_3$ administered as a one-time oral bolus 7 d before harvest and 1000 IU of vitamin E administered daily as a top-dress for 104 d before harvest. Blood samples were obtained on the day that heifers were allotted to treatments, on the day 25-OH D$_3$ was administered, and on the day before harvest. Plasma calcium concentration was increased in plasma when 25-OH D$_3$ was administered with or without vitamin E ($P < 0.007$). In longissimus dorsi muscle, calcium concentration tended to increase ($P = 0.10$) when 25-OH D$_3$ was administered alone but not when 25-OH D$_3$ was administered with vitamin E. Concentrations of 25-OH D$_3$ and 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$ D$_3$) in plasma were increased when 25-OH D$_3$ was administered with or without vitamin E ($P < 0.001$). Steaks from heifers treated with 25-OH D$_3$ or vitamin E, but not both, tended to be more tender than steaks in the control group after aging for 14 d ($P = 0.08$). Postmortem protein degradation as measured by western blot of the 30-kDa degradation product of troponin-T was increased with all treatments after 3 d of aging, but at 7 and 14 d there were no changes. Overall, use of 500 mg of 25-OH D$_3$ fed as an oral bolus 7 d before harvest or 1000 IU of vitamin E
administered daily for 104 d before harvest alone but not in combination may effectively improve tenderness of beef.

Key Words: Beef, 25-Hydroxyvitamin D₃, Tenderness, Vitamin E

INTRODUCTION

Tenderness is one of the most important quality characteristics affecting the beef industry in the United States. In fact, inconsistent tenderness costs the beef industry millions of dollars annually (Smith et al., 1995). Consumers do distinguish between tenderness categories (Boleman et al., 1997) and are willing to pay a premium for meat that is guaranteed to be tender (Shackelford et al., 2001). Recently, researchers have been searching for a practical, “on the farm” method of producing consistently tender beef that would result in increased profits.

Researchers have shown that feeding a supranatural dosage (0.5 to 7.5 million IU) of vitamin D₃ to beef cattle for 7 to 10 d before slaughter results in more tender beef (Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2000; Swanek et al., 1999). Feeding these dosages of vitamin D₃ results in elevated calcium concentrations in plasma and beef and improved tenderness presumably by enhanced action of calcium-dependent proteases, m- and μ-calpain, on myofibrillar protein degradation postmortem (Koohmaraie, 1992b).

25-Hydroxyvitamin D₃ (25-OH D₃) seems to elicit responses similar to those of vitamin D₃ without high residues of vitamin D₃ or its metabolites in beef. Feeding a supranatural amount of 25-OH D₃ has caused favorable changes in meat characteristics,
but improved tenderness has not resulted (Foote et al., 2004; Wertz et al., 2004). Most likely, the dosage of 25-OH D₃ was not sufficient to elevate muscle calcium concentration.

Recent data suggest that the antioxidant characteristics of vitamin E fed at 1000 IU/d for 125 d before harvest may protect the calpains from oxidation. By combining these two research foci of vitamins D and E, we hypothesized that feeding 25-OH D₃ and vitamin E in combination would result in elevated muscle calcium concentration and protection for the calpains so that maximal tenderness could be achieved with minimal accumulation of vitamin D₃ metabolites in the muscle. To test our hypothesis, we designed a study to examine the effect of 25-OH D₃, vitamin E, and a combination of the two on tenderness of beef from the longissimus dorsi muscle of market weight beef heifers.

**ANIMALS AND METHODS**

Forty-eight 13-mo-old Angus cross beef heifers, obtained from a breeding project at Iowa State University were housed at the Iowa State University Beef Nutrition Research Farm in Ames, IA. Approval for this project was obtained from the Iowa State University Animal Care and Use Committee, and all regulations were followed. Heifers were allotted randomly to one of four dietary treatments (Control, no 25-OH D₃ or vitamin E supplementation; 25-OH D₃, 500 mg 25-OH D₃ administered once as an oral bolus 7 d before harvest; Vitamin E, 1,000 IU of vitamin E administered daily for 104 d before harvest; and Combination, 500 mg 25-OH D₃ administered as an oral bolus 7 d
before harvest and 1,000 IU of vitamin E administered daily for 104 d before harvest). Heifers were housed in eight pens of six heifers, and all heifers in each pen were in the same dietary treatment. Two pens were allotted to each treatment. The vitamin E (Rovimix E-50 Adsorbate®) and 25-OH D₃ (Rovimix Hy-D 1.25®) were obtained from DSM Nutritional Products, Inc., Ames, IA. The vitamin E was mixed with soybean meal to provide 6000 IU of vitamin E daily per pen. The soybean meal containing vitamin E or soybean meal alone, for the control and 25-OH D₃ groups, was spread evenly over the feed each day for 104 d before harvest. Dosages of 25-OH D₃ and cornstarch, for the control and vitamin E treatments, were weighed and divided into five gelatin capsules. Five capsules were used to ensure that if a heifer unknowingly regurgitated one of the capsules, most of the dosage would remain in the rumen. No regurgitated capsules were found following administration of boluses. The capsules were administered 7 d prior to harvest. Blood samples were obtained by jugular venipuncture on the day that heifers were assigned to treatments, on the day 25-OH D₃ was administered, and on the day before heifers were sent to the abattoir. Blood was collected by using 3.81-cm, 20 gauge needles and sodium heparinized Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ). Blood samples were immediately stored on ice until centrifugation. Plasma was stored at -20°C until analyses. Heifers were transported 40 miles to be harvested at the Iowa Quality Beef meat packing plant in Tama, IA. Rib sections were obtained after 48 h and brought back to the Iowa State University Meat Laboratory, Ames, IA for further processing. The next day, rib sections were boned, sliced into six 2.54-cm steaks, and each steak was vacuum packaged individually. Two steaks from each heifer immediately were frozen at -20°C. Those steaks were considered aged for 3 d. The remaining four
steaks from each heifer were refrigerated at 4°C until 7 or 14 d of aging and then frozen at -20°C. Therefore, two steaks from each heifer were aged for 3, 7, and 14 d. One steak from each aging period was used for biochemical analyses, and the other was cooked and used for Warner-Bratzler shear force analysis.

**Calcium Concentration in Plasma and in Beef**

Plasma and meat calcium concentrations were determined by atomic absorption. Briefly, 5 mL of 0.1% lanthanum oxide was added to 100 µL of plasma from each heifer; the mixture was vortexed and analyzed by using atomic absorption spectroscopy (Perkin-Elmer Corp., Norwalk, CT). Meat samples were prepared for total calcium analysis by weighing 3 g of muscle into an acid-washed beaker and then using a modified HNO₃-H₂SO₄ wet combustion method (NMAM, 1994). The resulting solution was standardized to 25 mL with distilled-deionized water and then analyzed by using atomic absorption (Perkin-Elmer Corp., Norwalk, CT).

**25-Hydroxyvitamin D₃ and 1,25-Dihydroxyvitamin D₃ in Plasma and in Beef**

25-Hydroxyvitamin D₃ in plasma was extracted with acetonitrile and quantified by radioimmunoassay (RIA) using I¹²⁵ as the tracer (Hollis et al., 1993). Similarly, 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] was extracted by using acetonitrile, but that extract was treated with sodium periodate and purified by using solid phase extraction. 1,25-Dihydroxyvitamin D₃ was quantified from the purified sample by RIA using I¹²⁵ as the tracer (Hollis et al., 1996).
25-Hydroxyvitamin D₃ and 1,25-(OH)₂D₃ concentrations in meat were determined by using a series of extractions, HPLC, and RIA (Horst et al., 1981). Three grams of meat were homogenized in 12 mL of phosphate-buffered saline (PBS; 0.14 M NaCl and 0.01 M K₂HPO₄; pH 7.0). Two 2-mL aliquots of each sample were used for duplicate analysis. Approximately 1000 counts per minute of [³H]-25-OH D₃ and [³H]-1,25-(OH)₂ D₃ (Amersham Life Sciences, Arlington Heights, IL) were added to each aliquot for measuring recovery percentage. First, samples were extracted with chloroform:methanol (2:1); then, the extracts were dried by using a Savant SpeedVac concentrator (Thermo Electron Corp., Milford, MA). The resulting residues were dissolved in 1 mL of hexane and applied to silica columns (Varian, Harbor City, CA) to separate the 25-OH D₃ from the 1,25-(OH)₂ D₃.

The 25-OH D₃ fraction of each sample was dried and then suspended in 150 µL of the HPLC mobile phase (hexane:methylene chloride:isopropanol; 88:10:2; v:v:v). Samples were injected onto a Dupont Zorbax NH₂ 4.6-mm × 250-mm HPLC column (Mac-Mod Analytical, Chads Ford, CA). The flow rate of the mobile phase was 2 mL/min, and fractions containing 25-OH D₃ were collected according to the retention time of 25-OH D₃ in a standard. The collected fractions were stored at 4°C overnight. The following day, the fractions were dried in the Savant SpeedVac, and the residue was dissolved in ethanol and was divided for quantification by RIA (Hollis et al., 1993) and for determining recovery percentage by scintillation counting. Briefly, one third of each sample was used to determine recovery percentage, and two thirds were used for duplicate 25-OH D₃ quantification by RIA.
The 1,25-(OH)$_2$D$_3$ fraction of each sample was dried and then suspended in 150 µL of the HPLC mobile phase (hexane:isopropanol 99:8; v:v). Samples were injected onto a DuPont Zorbax NH$_2$ 4.6-mm × 250-mm HPLC column. The flow rate of the mobile phase was 2 mL/min, and fractions were collected according to the retention time of 1,25-(OH)$_2$D$_3$ in a standard. The collected fractions were stored at 4°C overnight. The following day, the fractions were dried in the Savant SpeedVac, and the residue was suspended in ethanol and was divided for quantification by RIA (Hollis et al., 1996) and for determining recovery percentage by scintillation counting. Briefly, one third of each sample was used to determine recovery percentage, and two thirds were used for duplicate 1,25-(OH)$_2$D$_3$ quantification by RIA.

**Vitamin E in Beef**

To show that the vitamin E that was administered to heifers in the vitamin E and combination treatment groups accumulated in the muscle of those animals, vitamin E concentration in meat was determined by analysis with HPLC. Three grams of meat were homogenized in 12 mL PBS. One mL of this solution was used for vitamin E analysis. Retinyl acetate (125 ng) was added to each aliquot as the internal standard. Samples were extracted two times with n-hexane. The extracts were added together and dried in the Savant SpeedVac®. The residue was suspended with methanol:chloroform:water (70:25:5; v:v:v), which was also the mobile phase for HPLC analysis. Samples and α-tocopherol standards to make a calibration curve were applied to a C-18 3µ 250 mm × 4.6 mm column (Alltech Associates, Inc. Deerfield, IL). The
flow rate of the mobile phase was 1.5 mL/min. The detection wavelength was set to 280 nm.

**Warner-Bratzler Shear Force**

Warner-Bratzler shear force values were analyzed to determine tenderness of beef. Steaks were thawed for 24 h at 4°C. An industrial broiler (model CNO2; General Electric, Chicago Heights, IL) was preheated to medium high heat, and the adjustable grill grate was set 11.2 cm below the heat source. Steaks were broiled to an internal temperature of 35°C, turned, and broiled until the internal temperature reached 71°C. Each tray of steaks was wrapped in plastic wrap, and all steaks were stored overnight at 4°C. The following morning, steaks were allowed to warm to the ambient temperature (approximately 22°C), and six 1.27-cm cores were cut from each steak parallel to the muscle fibers (AMSA, 1995). A texture analyzer (model TA-XTi; Texture Technologies Corp., Scarsdale, NY) fitted with a Warner-Bratzler cutting blade was used to cut the cores perpendicular to the muscle fibers at a penetration speed of 3.3 mm/s. The maximal force necessary to shear each core was recorded, and the values for the six cores for each steak were averaged for statistical analysis.

**SDS Polyacrylamide Gel Electrophoresis and Western Blotting**

Samples were prepared for electrophoresis by according to the procedure outlined by Huff-Lonergan et al. (1996b). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and western blotting were used to determine the extent of postmortem proteolysis in beef. Meat samples from each heifer aged 3, 7, and 14 d were prepared for
quantification of the 30-kDa degradation product of troponin-T that results from postmortem proteolysis (Huff-Lonergan et al., 1996a). Polyacrylamide gel electrophoresis was used to separate protein degradation products. Gels were 15% 100:1 acrylamide:bis-acrylamide (wt:wt). Every gel was loaded with 20 µg of each sample and internal standard for developing band density ratios, and a molecular weight marker to ensure that the protein moved through the gel appropriately. The internal standard used for this study was a 14-d sample from the control group. After the tracking dye reached the end of the gel, the proteins were transferred to a membrane for Western blotting (Huff-Lonergan et al., 1996b). The 30-kDa degradation product was detected by using antibody JLT-12 (Sigma Aldrich, St. Louis, MO) diluted 1:5,000 as the primary antibody and antibody A-2554 diluted 1:3,333 as the secondary antibody. The ECL-Plus chemiluminescent system (Amersham Biosciences, GE Healthcare, Piscataway, NJ) was used to detect troponin-T. Membranes were visualized by using a 16-bit megapixel charge-coupled device camera (Flour-Chem8800; Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Version 3; Alpha Innotech Corp.). The band density ratios of the 30-kD degradation product of troponin-T in each sample relative to the internal standard were used to determine the extent of troponin-T degradation and therefore the extent of postmortem proteolysis relative to samples from other treatments in this experiment.

2-Thiobarbituric Acid-Reactive Substances

2-Thiobarbituric acid-reactive substances (TBARS) indicate the extent of fatty acid oxidation of meat samples. 2-Thiobarbituric acid-reactive substances were
quantified by using a distillation method (Koniecko, 1985). Briefly, 10 ± 0.02 g of ground steak were weighed into round bottom flasks. The following were added to each flask: 97.5 mL of distilled water, 2.5 mL of 12 N hydrochloric acid:distilled water (1:2, v:v), Dow Antifoam C Emulsion (Sigma-Aldrich, St. Louis, MO):distilled water (1:1; v:v), and 5-7 boiling beads. The flasks were placed over burners, and a distillation apparatus was attached to each flask. The contents of the flasks were brought to a moderate boil, and 50 mL of distillate were collected. For analysis, 5 mL of distillate was pipetted into duplicate tubes, and 5 mL of 20 mM TBA reagent [0.1442 g 2-thiobarbituric acid (Sigma-Aldrich, St. Louis, MO) dissolved in 50 mL distilled water]. A standard curve consisting of 0, 2, 4, 6, 8, and $10 \times 10^{-8}$ M malondialdehyde was run with the samples for determining TBARS concentration and therefore oxidation in samples. Samples and standards were capped firmly and put in a water bath at 100°C for 35 min. Samples and standards were allowed to cool for 10 min before the absorbance was read at a wavelength of 532 nm. Absorbance was recorded, and mg of TBARS per 1000 g of beef was calculated as the TBARS score.

**Statistical Analysis**

All data were analyzed as a 2 × 2 factorial design with two dosages of 25-OH D$_3$ (0 or 500 mg) and two dosages of vitamin E (0 or 1000 IU), and the MIXED procedure of SAS (SAS Inst., Cary, NC) was used to determine ANOVA. Pen was the experimental unit for feedlot performance data, and animal was the experimental unit for all other measures. Warner-Bratzler shear force and troponin-T degradation were analyzed as a repeated measure with aging day as the repeated variable. Least squared means were
computed for all fixed effects and separated by using pair-wise $t$-tests (PDIFF) when a significant $F$-test ($P < 0.05$ unless otherwise noted) was detected.

**RESULTS AND DISCUSSION**

*Feedlot Performance*

No differences were observed in feedlot performance of heifers in the current study (Table 1). Initial weight, final weight, dry matter intake over the whole feeding period and during the last week of the study, average daily gain, and gain:feed did not change with dietary supplementation of 25-OH D$_3$, vitamin E, or both. Feed intake is of particular interest, because, in studies using vitamin D$_3$, cattle tend to go off feed as their calcium concentrations are increased (Karges et al., 2001; Karges et al., 1999). The current study indicates that the 500-mg dosage of 25-OH D$_3$ does not significantly affect feed intake, ADG, or market weight like vitamin D$_3$ has been shown to do. This fact is of significance because producers usually are not willing to use a procedure that will make cattle less efficient or decrease market weight, which both affect profitability.

*Calcium Concentration in Plasma and in Beef*

Calcium concentration in plasma of heifers was not different at baseline or on the day that 25-OH D$_3$ was administered (Figure 1). Six days after 500 mg of 25-OH D$_3$ or placebo was administered (1 d before harvest), calcium concentrations in plasma from heifers treated with 500 mg of 25-OH D$_3$ and a combination of 25-OH D$_3$ and vitamin E were increased by 20-25% ($P \leq 0.05$). The calcium concentrations in plasma at baseline
and 1 d before harvest in groups that were treated with a one-time bolus of 500 mg of 25-OH D$_3$ 7 d before harvest were similar to the plasma calcium concentration observed when $5 \times 10^6$ or $7.5 \times 10^6$ IU of vitamin D$_3$ were administered for nine consecutive days (Montgomery et al., 2000) and when $6 \times 10^6$ IU of vitamin D$_3$ were administered for 4 or 6 d (Karges et al., 2001). Other researchers have not successfully increased plasma calcium concentrations with supplemental 25-OH D$_3$ (Foote et al., 2004; Wertz et al., 2004), but the failure to induce hypercalcemia probably resulted because of the lower dosage of 25-OH D$_3$ (125 mg) that was administered.

Total calcium concentration in *longissimus dorsi* muscle tended to increase ($P = 0.10$) when 25-OH D$_3$ was administered alone, but the effect when 25-OH D$_3$ was administered with vitamin E was negligible (Figure 2). As with plasma calcium concentration, experiments by Foote et al. (2004) did not elicit an increase in muscle calcium. Again, this effect probably resulted because of the smaller dosage (125 mg) of 25-OH D$_3$ that was administered. Wertz et al. (2004) did not find an effect of treatment with 125 mg of 25-OH D$_3$ on water-soluble calcium concentration in *longissimus dorsi* muscle, but they did not analyze the samples for total calcium concentration. The possibility exists that the increase in muscle calcium that resulted in the present study is attributable to the water insoluble calcium concentration in the muscle. However, the likelihood of that possibility is small because Foote et al. (2004) used the same dosage of 25-OH D$_3$ as did Wertz et al. (2004) and did not find an effect of treatment on total muscle calcium concentrations. The calcium concentrations in the current study were similar to those obtained by Montgomery et al. (2002), and agreed with published values for expected calcium concentrations in beef according to the USDA National Nutrient

25-Hydroxyvitamin D₃ and 1,25-Dihydroxyvitamin D₃ in Plasma and in Beef

25-Hydroxyvitamin D₃ was increased in plasma and in longissimus dorsi muscle from heifers that were treated with 25-OH D₃ with or without vitamin E (P ≤ 0.05) (Figures 3 and 4, respectively). In plasma, 25-OH D₃ concentrations were 13 fold higher than concentrations of 25-OH D₃ in plasma of heifers not treated with 25-OH D₃, and these values were approximately two times the concentration observed by Wertz et al. (2004) when 62.5 or 125 mg of 25-OH D₃ were administered 21, 7, 4, or 0 d before harvest. The concentration of 25-OH D₃ in longissimus dorsi muscle was increased when 500 mg 25-OH D₃ was administered 7 d before harvest compared with concentrations in heifers in the control group. The concentration of 25-OH D₃ in the Vitamin E group was intermediate to those of the Control group and the groups that received 25-OH D₃. The concentrations of 25-OH D₃ observed in the present study were similar to the concentration observed by Wertz et al. (2004) even though the dosage for the present study was four times greater than the largest dosage used by Wertz et al. (2004). Although statistically significant (P ≤ 0.05), the concentration of 25-OH D₃ in steaks from heifers supplemented with 500 mg of 25-OH D₃ was only about three to four times the concentration in the control samples. This concentration of 25-OH D₃ in meat was still very low; thus, meat obtained from heifers treated with 25-OH D₃ would be safe to consume. The current upper limit of vitamin D intake is 50 µg of vitamin D₃/day (Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997). 25-
Hydroxyvitamin D₃ has about 1.4 times the activity of vitamin D₃; hence, given the concentrations of 25-OH D₃ found in this study, a person would have to consume more than 13.6 kg of meat per day to reach the upper limit. This consumption is not realistic or possible, and thus not a concern. In fact, consuming beef from cattle supplemented with 25-OH D₃ is safe and may be a reasonable approach to help ensure that consumers meet their daily recommended intake of vitamin D₃ activity, which is equivalent to 5-10 µg of vitamin D₃ per day (Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997).

1,25-Dihydroxyvitamin D₃ concentrations in plasma from heifers treated with 25-OH D₃ were 2-2.5 times higher than the concentrations observed in plasma from the control and vitamin E only groups (P < 0.001; Figure 5). The concentrations observed in the present study were similar to the concentrations observed by Montgomery et al. (2000) when 5 or 7.5 × 10⁶ IU of vitamin D₃ and to those observed by Wertz et al. (2004), but lower than the concentrations observed by Foote et al. (2004) when 125 mg of 25-OH D₃ were administered one time before harvest.

In agreement with previous studies (Foote et al., 2004; Montgomery et al., 2000; Wertz et al., 2004), the concentration of 1,25-(OH)_2 D₃ was not increased significantly in longissimus dorsi when 25-OH D₃ was supplemented (P ≥ 0.20) (Figure 6). However, in the current study, 1,25-(OH)_2 D₃ concentration was increased in longissimus dorsi muscle when vitamin E alone was supplemented. Although largely unexplained, this increase could be a result of the antioxidant property of vitamin E acting to protect 25-OH D₃ 1-α-hydroxylase activity in the kidney (Burton et al., 1983). The same result is not expected when 25-OH D₃ is supplemented, because transcription of the mRNA for
25-OH D₃ 1-α-hydroxylase would be decreased because of an already higher circulating concentration of 1, 25-(OH)₂ D₃ or, possibly because of the elevated concentration of 25-OH D₃ present (Combs, 1992; Shils et al., 1998). Another possible explanation of this increase in 1,25-(OH)₂ D₃ concentration in muscle from heifers treated with vitamin E is that activity of extra-renal 25-OH D₃ 1-α-hydroxylase might be preserved in muscle by accumulating vitamin E, and, therefore, results in increased concentration of 1,25-(OH)₂ D₃ in muscle. To date, researchers have determined that 25-OH D₃ 1-α-hydroxylase is present in smooth muscle (Somjen et al., 2005). Some researchers have considered the possibility of the presence of 25-OH D₃ 1α-hydroxylase in skeletal muscle, but results currently are not available (personal communication with M. Hewison, 2006).

Unexpectedly, concentrations of 1,25-(OH)₂ D₃ in muscle were intermediate in groups that were supplemented with 25-OH D₃ and were not significantly different from the concentration in muscle from heifers in the control group (P ≥ 0.20).

**Vitamin E in Beef**

Concentration of vitamin E was determined only in samples aged for 3 d. As expected, the vitamin E concentration was increased in steaks from the *longissimus dorsi* muscle when vitamin E was mixed with soybean meal and supplemented as a top-dress to heifers daily for 104 d before harvest (Figure 7). The increase in vitamin E concentration was not significant when vitamin E and 25-OH D₃ were both supplemented. This finding agrees with several studies (Harris et al., 2001; Roeber et al., 2001; Rowe et al., 2004a) in which α-tocopherol concentration in meat was increased after supplementation with 1000 IU of vitamin E for more than 100 d before harvest.
**Warner-Bratzler Shear Force**

Warner-Bratzler shear force was used to measure the tenderness of steaks from the *longissimus dorsi* muscle aged for 3, 7, or 14 d. Results are shown in Table 2. Supplementation with 500 mg 25-OH D$_3$ or vitamin E tended to improve tenderness when steaks were aged for 14 d ($P = 0.08$). After aging for 3 d, no difference in tenderness existed between treatments. By 7 d, however, steaks from the Control and Vitamin E treatment groups were more tender than steaks from the 25-OH D$_3$ or combination groups ($P \leq 0.05$). The reason for this is unclear, but all of the steak samples, regardless of treatment, already were very tender, even after aging for only 3 d. After 14 d of aging, steaks from heifers treated with 25-OH D$_3$ or with vitamin E tended to have lower shear force values than did steaks from the control or combination groups ($P = 0.10$). Wertz et al. (2004) did not observe a decrease in shear force necessary to penetrate steak samples after treatment with 125 mg of 25-OH D$_3$. Although the same statistical analysis was not performed, the data obtained by Foote et al. (2004) seem to agree with the current finding that 25-OH D$_3$ enhanced the effectiveness of aging as a method of meat tenderization. Results from the current study indicated that 25-OH D$_3$ improved tenderness to an extent that was similar to the improvement observed when vitamin D$_3$ is administered (Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2000; Swanek et al., 1999). The fact that the group treated with vitamin E for 104 d had lower shear force values agrees with a study in which steaks from cattle treated with vitamin E for 125 d were more tender than steaks that were not treated with vitamin E for 125 d (Harris et al., 2001).
After 3 d of aging, troponin-T degradation in steaks from heifers treated with 500 mg of 25-OH D$_3$ 7 d before harvest or 1000 IU of vitamin E for 104 d before harvest was nearly two times that in steaks from control animals (Table 3). Degradation in muscle from heifers treated with both 25-OH D$_3$ and vitamin E was intermediate to degradation in steaks from the control and single treatment groups. The amount of degradation product was decreased at 7 d postmortem in all treatment groups. At 14 d postmortem, the amount of degradation product was higher than that at 7 d postmortem but lower than that at 3 d postmortem. One explanation for this phenomenon could be that the 30-kDa degradation product, which is measured to determine postmortem proteolysis, is also a substrate for other proteases. As the proteases utilize the 30-kDa degradation product, the total amount of product measured decreases. Most other studies show an increase in troponin-T degradation with aging (Foote et al., 2004; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Wertz et al., 2004), but that increase was not evident in the current trial. Montgomery et al. (2004) showed that at 14 d postmortem, treatment with 0.5-5.0 × 10$^6$ IU of vitamin D$_3$ increased troponin-T degradation compared with controls. Treatment with 25-OH D$_3$ or vitamin E seemed to increase the rate of degradation because the greatest increase in product from postmortem proteolysis was present after just 3 d of aging. Many of the studies that have investigated postmortem proteolysis did not analyze samples aged for only 3 d. Rather, most have 7-, 14-, and 21-d aging periods. Therefore, the early dynamics observed in this study have not been fully investigated. Data obtained in the current study agree with proteolysis data.
from samples aged 7 and 14 d postmortem in other studies (Montgomery et al., 2004; Montgomery et al., 2000).

2-Thiobarbituric Acid-Reactive Substances

2-Thiobarbituric acid-reactive substances were quantified after steaks were aged for 3, 7, and 14 d. As expected, steaks from heifers treated with vitamin E, with or without 25-OH D$_3$, had lower concentrations of TBARS than did steaks from heifers not treated with vitamin E (Table 4). Concentrations of TBARS in steaks within each treatment were similar for all aging periods except in the Combination group, which exhibited less oxidation at 3 d than after 7 or 14 d of aging ($P = 0.05$). The concentration of TBARS likely did not increase in most treatments with aging because steaks were vacuum packaged before aging; so, limited oxygen was present. The results of TBARS and Warner-Bratzler shear force analyses in the present study are in agreement with the findings of Harris et al. (2001) who found that a decrease in TBARS resulted in less oxidation of calpains, and, therefore, increased postmortem proteolysis and, possibly, improved tenderness.

IMPLICATIONS

Results from the current experiment indicate that 500 mg 25-OH D$_3$ administered as a single bolus 7 d before harvest has the potential to improve tenderness of steaks from the longissimus dorsi muscle of beef heifers. Five hundred milligrams of 25-OH D$_3$ was as effective in the current study as $0.5-7.5 \times 10^6$ IU of vitamin D$_3$ is in previous research.
at increasing plasma calcium and improving the tenderness of beef without leaving a high concentration of vitamin D₃ or its metabolites in muscle. In fact, the concentrations of 25-OH D₃ found in steaks from the longissimus dorsi muscle may be beneficial as a dietary source of vitamin D for consumers. Additionally, results from the current study indicate that vitamin E also enhances the effect of aging on tenderness of beef. Surprisingly, the effects of vitamin E and 25-OH D₃ were not additive. This study indicates that supplementation with oral 25-OH D₃ or vitamin E can be used effectively to improve beef tenderness instead of vitamin D₃, which leaves very high concentrations of vitamin D₃ and its metabolites in meat. More research should be conducted to determine if these treatments are more effective under certain circumstances such as the breed of cattle or feeding regime (pasture or feedlot).

ACKNOWLEDGEMENTS

The authors would like to express thanks to DSM Nutritional Products, Inc., Ames, IA for their generosity in donating the Rovimix E-50 Adsorbate® that was used in this study. To the Iowa Quality Beef meat packing facility in Tama, IA, we send a special thank you for allowing us to come into their facility to obtain samples for transport to the Iowa State University Meat Laboratory. Thank you to Rod Berryman, manager of the ISU Beef Nutrition Research Farm for daily care of the cattle and for helping with blood samples collection and bolus administration. Laura Baseler, Katie Korn, and Audry Swinconos, thank you for your help in the laboratory.
LITERATURE CITED


Table 1. Effect of oral supplementation with 25-OH D$_3$, vitamin E, or both on feedlot performance of beef heifers$^a$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>25-OH D$_3$</th>
<th>Vitamin E</th>
<th>25-OH D$_3$ + Vitamin E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial wt, kg</td>
<td>392.59</td>
<td>390.51</td>
<td>391.83</td>
<td>389.32</td>
</tr>
<tr>
<td>Final wt, kg</td>
<td>536.44</td>
<td>516.21</td>
<td>546.21</td>
<td>519.92</td>
</tr>
<tr>
<td>ADG, kg/d</td>
<td>1.38</td>
<td>1.21</td>
<td>1.48</td>
<td>1.26</td>
</tr>
<tr>
<td>DMI, kg/d</td>
<td>8.45</td>
<td>8.54</td>
<td>9.03</td>
<td>8.43</td>
</tr>
<tr>
<td>Last week DMI, kg/d</td>
<td>7.33</td>
<td>6.35</td>
<td>7.05</td>
<td>5.91</td>
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<tr>
<td>G:F</td>
<td>0.16</td>
<td>0.14</td>
<td>0.16</td>
<td>0.15</td>
</tr>
</tbody>
</table>

$^a$ Means represent the average for each treatment (2 pens per treatment, and 6 heifers per pen).
Table 2. Effect of dietary supplementation of 25-OH D₃, vitamin E, or both on Warner-Bratzler shear force values of steaks from *longissimus dorsi* of heifers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 Day</th>
<th>7 Day</th>
<th>14 Day</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.79&lt;sup&gt;acx&lt;/sup&gt;</td>
<td>3.04&lt;sup&gt;bxy&lt;/sup&gt;</td>
<td>3.60&lt;sup&gt;cdx&lt;/sup&gt;</td>
<td>0.31</td>
</tr>
<tr>
<td>25-OH D₃</td>
<td>3.85&lt;sup&gt;x&lt;/sup&gt;</td>
<td>3.61&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>3.36&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>3.64&lt;sup&gt;x&lt;/sup&gt;</td>
<td>3.05&lt;sup&gt;by&lt;/sup&gt;</td>
<td>3.35&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>0.21</td>
</tr>
<tr>
<td>25-OH D₃ + Vitamin E</td>
<td>3.76</td>
<td>3.80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.95&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.32</td>
</tr>
<tr>
<td>SEM</td>
<td>0.29</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Shear force in kg.

<sup>b</sup> Denotes differences between treatments within a postmortem aging period (*P* ≤ 0.05).

<sup>c</sup> Denotes tendency to differ between treatments within a postmortem aging period (*P* = 0.08).

<sup>x,y</sup> Denotes tendency to differ within a treatment over aging periods (*P* ≤ 0.10).
Table 3. Effect of dietary supplementation of 25-OH D\textsubscript{3}, vitamin E, or both on troponin-T degradation in steaks from *longissimus dorsi* of heifers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Postmortem aging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Day</td>
</tr>
<tr>
<td>Control</td>
<td>0.92\textsuperscript{abx}</td>
</tr>
<tr>
<td>25-OH D\textsubscript{3}</td>
<td>1.75\textsuperscript{dx}</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.63\textsuperscript{cdx}</td>
</tr>
<tr>
<td>25-OH D\textsubscript{3} + Vitamin E</td>
<td>1.44\textsuperscript{cx}</td>
</tr>
<tr>
<td>SEM</td>
<td>0.28</td>
</tr>
</tbody>
</table>

\( a \) Values are ratios of densitometry of the 30-kDa degradation product as compared with a control sample aged for 14 d.

\( b,c,d \) Denotes differences between treatments within a postmortem aging period \( (0.03 \leq P \leq 0.07) \).

\( x,y,z \) Denotes a tendency to differ within a treatment over aging periods \( (0.05 < P \leq 0.10) \).
Table 4. Effect of dietary supplementation of 25-OH D<sub>3</sub>, vitamin E, or both on the concentration of 2-thiobarbituric acid-reactive substances in steaks from *longissimus dorsi* of heifers.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Postmortem aging</th>
<th></th>
<th></th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 Day</td>
<td>7 Day</td>
<td>14 Day</td>
</tr>
<tr>
<td>Control</td>
<td>1.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>25-OH D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
<tr>
<td>25-OH D&lt;sub&gt;3&lt;/sub&gt; + Vitamin E</td>
<td>0.26&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>0.51&lt;sup&gt;cy&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;cy&lt;/sup&gt;</td>
<td>0.08</td>
</tr>
<tr>
<td>SEM</td>
<td>0.24</td>
<td>0.11</td>
<td>0.13</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>TBARS (mg malondialdehyde/kg of meat).

<sup>b,c</sup> Denotes differences between treatments within a postmortem aging period (*P* ≤ 0.05).

<sup>x,y</sup> Denotes differences within a treatment over aging periods (*P* < 0.05).
Figure 1. Plasma calcium concentration for heifers in Control group (no 25-OH D$_3$ or vitamin E), 25-OH D$_3$ group (500 mg of 25-OH D$_3$ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D$_3$ + Vitamin E (25-OH D$_3$ and vitamin E). Letters a-c indicate that plasma calcium concentrations were different ($P \leq 0.007$) on the day before harvest. No differences exist at the start of the study (baseline) or on the day that 25-OH D$_3$ was administered (7 d before harvest). Error bars represent the standard error of means.
Figure 2. Calcium concentration in *longissimus dorsi* for heifers in Control group (no 25-OH D$_3$ or vitamin E), 25-OH D$_3$ group (500 mg of 25-OH D$_3$ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D$_3$ + Vitamin E (25-OH D$_3$ and vitamin E). Letters a and b indicate that muscle calcium concentrations tended to increase ($P = 0.10$) in heifers in the 25-OH D$_3$ group. No differences in calcium concentration exist ($P \geq 0.20$) in the Control, Vitamin E, or 25-OH D$_3$ + Vitamin E groups. Error bars represent the standard error of means.
Figure 3. Concentration of 25-hydroxyvitamin D₃ (25-OH D₃) in plasma for heifers in Control group (no 25-OH D₃ or vitamin E), 25-OH D₃ group (500 mg of 25-OH D₃ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D₃ + Vitamin E (25-OH D₃ and vitamin E). Letters a and b indicate that plasma calcium concentrations were different ($P < 0.001$) on the day before harvest. No differences exist at the start of the study (baseline) or on the day that 25-OH D₃ was administered (7 d before harvest). Error bars represent the standard error of means.
Figure 4. 25-Hydroxyvitamin D₃ (25-OH D₃) concentration in *longissimus dorsi* for heifers in Control group (no 25-OH D₃ or vitamin E), 25-OH D₃ group (500 mg of 25-OH D₃ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D₃ + Vitamin E (25-OH D₃ and vitamin E). Different letters (a and b) indicate that muscle 25-OH D₃ concentrations increased (*P* < 0.005) in heifers in groups that received 25-OH D₃. Error bars represent the standard error of means.
**Figure 5.** Concentration of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂ D₃) in plasma for heifers in Control group (no 25-OH D₃ or vitamin E), 25-OH D₃ group (500 mg of 25-OH D₃ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D₃ + Vitamin E (25-OH D₃ and vitamin E). Letters a and b indicate that plasma calcium concentrations were different \( (P < 0.001) \) on the day before harvest. No differences exist at the start of the study (baseline) or on the day that 25-OH D₃ was administered (7 d before harvest). Error bars represent the standard error of means.
Figure 6. 1,25-Dihydroxyvitamin D₃ concentration in *longissimus dorsi* for heifers in the Control group (no 25-OH D₃ or vitamin E), 25-OH D₃ group (500 mg of 25-OH D₃ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D₃ + Vitamin E (25-OH D₃ and vitamin E). Different letters (a and b) indicate that muscle 25-OH D₃ concentrations increased (*P* = 0.02) in heifers in the Vitamin E group. Error bars represent the standard error of means.
Figure 7. Vitamin E (α-tocopherol) concentration in *longissimus dorsi* for heifers in the Control group (no 25-OH D₃ or vitamin E), 25-OH D₃ group (500 mg of 25-OH D₃ as an oral bolus 7 d before harvest), Vitamin E group (1000 IU vitamin E for 104 d before harvest), or 25-OH D₃ + Vitamin E (25-OH D₃ and vitamin E). Different letters (a and b) indicate that muscle 25-OH D₃ concentrations increased (*P* = 0.02) in heifers in the Vitamin E group. Error bars represent the standard error of means.
Figure 8. Troponin-T degradation at 3, 7 and 14 d aging. Standard was a 14-d control sample.
USE OF 25-HYDROXYVITAMIN D$_3$ AND DIETARY CALCIUM TO IMPROVE TENDERNESS OF BEEF FROM THE ROUND OF BEEF COWS$^1$

A manuscript for submission to the Journal of Animal Science


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$^1$Research funding granted by the National Cattlemen’s Beef Association.

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$^3$The authors acknowledge Derrel Hoy and Duane Zimmerman in the Periparturient Diseases of Cattle Group of the National Animal Disease Center—USDA/ARS for their assistance with laboratory analyses.
ABSTRACT  The objective of this trial was to determine if 25-hydroxyvitamin D₃ (25-OH D₃), withdrawing and then replenishing supplemental dietary calcium, or both would improve the tenderness of beef from the round of cows. Twenty-seven Angus cows (3-7 years old) were allotted randomly to nine pens with three cows per pen. Treatments were arranged in a 3 × 3 factorial design with three dosages of 25-OH D₃ (0, 250, or 500 mg of 25-OH D₃ administered as a one-time oral bolus 7 d before harvest) and three percentages of supplemental limestone (0.5, 0.75, and 1.0%) replenished in the diet for 3 d before harvest and after a 2-wk limestone withdrawal. Plasma samples were obtained during the feeding period. Upon harvest, adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis muscles were obtained and aged for 1, 3, or 7 d. Calcium concentrations were increased in plasma when 250 or 500 mg of 25-OH D₃ were administered (P ≤ 0.05). Calcium concentrations in muscle increased (P ≤ 0.001) when 500 mg of 25-OH D₃ were administered. Concentrations of 25-OH D₃ in meat and in plasma and 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂ D₃] in plasma were increased when 25-OH D₃ was administered (P ≤ 0.05). The percentage of limestone replenished in the diet had no effect on 25-OH D₃ or 1,25-(OH)₂ D₃ in meat or in plasma. Calpastatin activity was significantly affected by treatments in the gracilus and vastus intermedius muscles (P ≤ 0.05). Among all muscles and aging periods, calpastatin activity and protein degradation, as indicated by troponin-T degradation, were related inversely (R² = -0.145, P = 0.02). Warner-Bratzler shear force values were variable, and significant improvement in tenderness was not observed. Results indicate that supplemental 25-OH D₃ influences muscle characteristics known to improve
tenderness, but its effectiveness on improving the tenderness of beef from the round of culled cows should be further investigated.

Key Words: Beef, Calcium, Cow, 25-Hydroxyvitamin D₃, Tenderness

INTRODUCTION

Beef from culled cows is generally less tender and, therefore, less desirable than that from younger cattle. Effectiveness of methods that are known to improve tenderness of beef from younger cattle has not been fully investigated in mature cows. Dietary vitamin D₃ has been shown to improve tenderness of beef from younger cattle (Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2000; Swanek et al., 1999). The proposed mechanism by which vitamin D₃ improves tenderness is via the calpain protease system, which is responsible for postmortem proteolysis and subsequent tenderization of beef (Koohmaraie et al., 1991). One possible concern of administering very large dosages of vitamin D₃ is that residues of vitamin D₃ activity in beef are high enough to cause hypervitaminosis D if large quantities of beef were consumed on a regular basis (Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2000; Swanek et al., 1999). 25-Hydroxyvitamin D₃ (25-OH D₃) has been investigated as a possible alternative to vitamin D₃ supplementation (Foote et al., 2004; Wertz et al., 2004). Results from the studies of Foote et al. (2004) and Wertz et al. (2004) indicate that dietary 25-OH D₃ has the potential to favorably alter conditions within the muscle to
support increased postmortem proteolysis and improve tenderness. Foote et al. (2004) and Wertz et al. (2004) did not observe improved beef tenderness with treatment with 125 mg of 25-OH D₃, but that was probably because of the rather small dosage administered.

Additionally, research has indicated that plasma calcium concentration can be increased by withdrawing and then replenishing dietary calcium (Goings et al., 1974). 25-Hydroxyvitamin D₃ has the potential to improve beef tenderness via the same mechanism as vitamin D₃ and dietary calcium manipulations can elevate plasma calcium concentration. Therefore, we hypothesized that 25-OH D₃ in conjunction with manipulations of dietary calcium would increase plasma calcium concentrations and improve tenderness of seven muscles from the round section of beef cows.

**MATERIALS AND METHODS**

**Animals**

Twenty-seven Angus cows (3-7 yr of age) were obtained from a breeding project at Iowa State University. The cows were housed at the Iowa State University Beef Nutrition Research Farm in Ames, IA. Approval for this project was obtained from the Iowa State University Animal Care and Use Committee, and all regulations were followed. Cows were allotted by weight to one of nine dietary treatments. Treatments were arranged as a 3 × 3 factorial design with three dosages of 25-OH D₃ (0, 250, or 500 mg of 25-OH D₃) and three manipulations of dietary calcium [calcium (limestone) withdrawn from all diets for two weeks and then replenished at 0.5%, 0.75%, or 1.0% of
diet dry matter] (Table 1). Cows were housed in nine pens of three cows, and all cows in each pen were in the same dietary treatment. The 25-OH D₃, Rovimix Hy-D 1.25®, was obtained from DSM Nutritional Products, Inc., Ames, IA. Dosages of 25-OH D₃ and cornstarch as the placebo (36.23 g; based on the weight of Rovomix Hy-D 1.25® to supply 500 mg of 25-OH D₃), were weighed and divided evenly into five gelatin capsules.

**Experimental design**

The experimental timeline is shown in Figure 1. On the day cows that had been on pasture since calving in the spring were brought to the feedlot, a blood sample was obtained, and then cattle were fed a high concentrate diet (Table 2) for 28 days. Seventeen days before harvest, supplemental calcium was withdrawn from the diet, and a blood sample was drawn. An additional blood sample was obtained 7 d after supplemental calcium was withdrawn (10 d before harvest). Seven days before harvest, another blood sample was obtained, and 25-OH D₃ (0, 250, or 500 mg 25-OH D₃) was administered by oral bolus. Additional blood samples were obtained 5, 3, and 1 d before harvest. Blood was collected by using 3.81-cm, 20 gauge needles and sodium-heparinized Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ). All blood samples immediately were stored on ice until centrifugation. Plasma was stored at -20°C until analyses. Supplemental calcium was replenished in the diet (limestone at 0.5%, 0.75%, or 1.0% of diet DM) 3 d before harvest. The diet composition for the diets is shown in Table 2. Calcium concentration was adequate (0.26% of diet DM) in the diet
containing 0.5% limestone and was increased to 0.35 and 0.44% in the 0.75% and 1.0%
DM diets, respectively.

Cows either were transported 40 miles to be harvested at the Amend Meat
Packing in Des Moines, IA or transported 5 miles to be harvested at the Iowa State
University Meat Laboratory, Ames, IA. Regardless of location of harvest, round sections
were obtained 20 h after harvest and processed at the Iowa State University Meat
Laboratory, Ames, IA. Seven muscles, adductor, gracilus, pectineus, sartorius,
semimembranosus, vastus intermedius, and vastus lateralis, were dissected by
experienced personnel. Each muscle was sliced into six 2.54-cm “steaks”, and each steak
was vacuum-packaged individually. Two steaks from each muscle from each cow
immediately were frozen at -20°C. Those steaks were considered aged for 1 d. The
remaining four steaks from each muscle from each cow were refrigerated at 4°C until 3
or 7 d of aging and then frozen at -20°C. Therefore, two steaks from each muscle from
each cow were aged for 1, 3, and 7 d. One steak from each aging period was used for
biochemical analyses, and the other was cooked and used for Warner-Bratzler shear force
analysis. A fresh piece from each muscle from each of the cows in the four most extreme
treatments (0 mg 25-OH D₃, 0.5% limestone; 500 mg 25-OH D₃, 0.5% limestone; 0 mg
25-OH D₃, 1.0% limestone; and 500 mg 25-OH D₃, 1.0% limestone; Table 1) was used
immediately for calpastatin analysis.

**Calpastatin Activity in Beef**

Following dissection, muscles from the 12 cows in the four most extreme
treatments mentioned previously were prepared for analysis of calpastatin activity
(Lonergan et al., 2001; Maddock et al., 2005). Briefly, 10 g of each muscle (adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis) from each of the 12 cows included in this analysis were homogenized in 30 mL of post rigor extraction buffer (100 mM Tris, 10 mM EDTA, pH 8.3) with 100 mg/L ovomucoid and 2 mM phenylmethylsulphonylfluoride (PMSF) added just before homogenization. Samples were homogenized for 30 s in a metal Waring® blender. Samples were allowed to “rest” for 30 s; then, the cycle of homogenizing and resting was repeated twice so that samples were homogenized three times. The homogenate of each sample was transferred equally to two centrifuge tubes, and the blender was rinsed with 10 mL of post rigor extraction buffer. The rinsate was added to the centrifuge tubes. Samples were centrifuged for 30 min at 20,000 × g. The supernatant was dialyzed overnight against 40 mM Tris and 1 mM EDTA. The dialyzed supernatant was transferred to a conical centrifuge tube, heated in a 95°C water bath for 20 min and then cooled in an ice bath for 15 min. While cooling, each tube was agitated to prevent protein coagulation. Each heated sample was divided into two 30-mL centrifuge tubes and centrifuged for 30 min at 20,000 × g. The supernatant from both tubes of each sample was filtered through cheesecloth into a 50-mL conical centrifuge tube. The volume of the supernatant was recorded, and the samples were stored at 4°C until analysis. Calpastatin activity was measured by using a known amount of calpain activity that had been extracted from muscle previously and by measuring the decrease in activity when a series of dilutions of the supernatant were added (Koohmaraie et al., 1995).

**Calcium Concentration in Plasma and in Beef**
Plasma and meat calcium concentrations were determined by atomic absorption spectroscopy. Briefly, 5 mL of 0.1% lanthanum oxide were added to 100 µL of plasma from each cow; the mixture was vortexed and analyzed by using atomic absorption spectroscopy (Perkin-Elmer Corp., Norwalk, CT). Meat samples were prepared for total calcium analysis by weighing 3 g of each muscle (aged 1 d) from each cow into an acid-washed beaker and then using a modified HNO₃-H₂SO₄ wet combustion method (NMAM, 1994). The resulting solution was standardized to 25 mL with distilled-deionized water and then analyzed by using atomic absorption (Perkin-Elmer Corp., Norwalk, CT).

**25-Hydroxyvitamin D₃ and 1,25-Dihydroxyvitamin D₃ in Plasma**

25-Hydroxyvitamin D₃ in plasma was extracted with acetonitrile and quantified by radioimmunoassay (RIA) using I¹²⁵ as the tracer (Hollis et al., 1993). Similarly, 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂ D₃] was extracted by using acetonitrile, but that extract was treated with sodium periodate and purified by using solid phase extraction. 1,25-Dihydroxyvitamin D₃ was quantified from the purified sample by RIA using I¹²⁵ as the tracer (Hollis et al., 1996).

**25-Hydroxyvitamin D₃ in Meat**

25-Hydroxyvitamin D₃ concentrations in meat were determined in the *semimembranosus* for all cows and in *adductor, gracilus, pectineus, sartorius, vastus intermedius*, and *vastus lateralis* for cows in the most extreme treatment (500 mg 25-OH D₃ and 1.0 % limestone) by using HPLC (Horst et al., 1981). Briefly, 3 g of meat were
homogenized in 12 mL of phosphate-buffered saline (0.14 M NaCl and 0.01 M K$_2$HPO$_4$; pH 7.0). Samples were extracted with chloroform:methanol (2:1, v:v); then, the extracts were dried by using a Savant SpeedVac concentrator (Thermo Electron Corp., Milford, MA). The resulting residues were dissolved in 1 mL hexane and applied to silica columns (Varian, Harbor City, CA) to separate the 25-OH D$_3$ from the 1,25-(OH)$_2$ D$_3$.

Known amounts of 25-hydroxyvitamin D$_2$ were added to each tube containing the 25-OH D$_3$ fraction from each sample, and the solvents were removed in the SpeedVac. Samples then were suspended in 150 µL of the HPLC mobile phase (hexane:isopropanol; 92:8; v:v). Samples were injected onto a Dupont Zorbax NH$_2$ 4.6 × 250-mm HPLC column (Mac-Mod Analytical, Chads Ford, CA). The flow rate of the mobile phase was 2 mL/min. A calibration curve of 25-OH D$_3$ was used to determine concentration of 25-OH D$_3$ in samples. 1,25-Dihydroxyvitamin D$_3$ was not determined in meat samples because other research has shown that its concentration in meat is not affected by treatment with 25-OH D$_3$ (Wertz et al., 2004), Carnagey et al., unpublished).

**SDS Polyacrylamide Gel Electrophoresis and Western Blotting**

SDS polyacrylamide gel electrophoresis and Western blotting were used to determine the extent of postmortem proteolysis in meat. Meat samples from each cow aged 1, 3, or 7 d were prepared for quantification of the 30-kDa degradation product of troponin-T that results from postmortem proteolysis (Huff-Lonergan et al., 1996a). Polyacrylamide gel electrophoresis was used to separate protein degradation products. Every gel was loaded with samples, an internal standard for developing band density ratios, and a molecular weight marker to ensure that the protein moved through the gel
appropriately. After the tracking dye reached the end of the gel, the proteins were transferred to a membrane for Western blotting (Huff-Lonergan et al., 1996b). The ECL-Plus chemiluminescent system (Amersham Biosciences, GE Healthcare, Piscataway, NJ) was used to detect troponin-T and its 30-kDa degradation product. Membranes were visualized by using a 16-bit megapixel charge-coupled device camera (Flour-Chem8800; Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Version 3; Alpha Innotech Corp.). The band density ratios of the 30-kD degradation product of troponin-T in each sample relative to the internal standard were used to determine the extent of troponin-T degradation and therefore the extent of postmortem proteolysis relative to samples from other treatments in this experiment.

**Warner-Bratzler Shear Force**

Warner-Bratzler shear force values were determined to evaluate tenderness of beef. Steaks were thawed for 24 h at 4°C. An industrial broiler (model CNO2; General Electric, Chicago Heights, IL) was preheated to medium high heat, and the adjustable grill grate was set 11.2 cm below the heat source. Steaks were broiled to an internal temperature of 35°C, turned, and broiled until the internal temperature reached 71°C. Each tray of broiled steaks was wrapped in plastic wrap, and all steaks were stored overnight at 4°C. The following morning, steaks were allowed to warm to the ambient temperature (approximately 22°C). A texture analyzer (model TA-XTi; Texture Technologies Corp., Scarsdale, NY) fitted with a Star probe blade was used to puncture steaks parallel to the muscle fibers at a penetration speed of 3.3 mm/s three times per steak. The maximal force necessary to penetrate the steak was recorded, and the values
for the three trials for each steak were averaged for statistical analysis. The coefficient of variance within each steak ranged from 2 to 12 g; so, three trials per steak were considered sufficient.

**Statistical Analysis**

All data were analyzed as a $3 \times 3$ factorial design with three dosages of 25-OH D$_3$ (0, 250, or 500 mg) and three dosages of dietary calcium replenished in the diet (limestone at 0.5%, 0.75%, or 1.0% of diet dry matter), and the MIXED procedure of SAS (SAS Inst., Cary, NC) was used to determine ANOVA. Cow was the experimental unit. Warner-Bratzler shear force and troponin-T degradation were analyzed as a repeated measure with aging day as the repeated variable. The age of the cows was used as a covariate to correct for the wide range of ages (3-7 yr). Least squared means were computed for all fixed effects and separated by using pair-wise $t$-tests (PDIFF) when a significant $F$-test ($P \leq 0.05$ unless otherwise noted) was detected.

**RESULTS AND DISCUSSION**

**Calpastatin Activity in Muscle**

Calpastatin is the inhibitor of calpain, which is responsible for postmortem proteolysis; so, its inhibitory activity is an indication of beef tenderness. When calpastatin activity is higher, calpain inhibition is increased and less proteolysis occurs; so, meat may be less tender (Geesink and Koohmaraie, 1999; Morgan et al., 1993). Calpastatin activities for each muscle are shown in Table 3. One unit of calpastatin
activity is defined as the amount of calpastatin necessary to inhibit one unit of calpain. In the present study, calpastatin activity was decreased in the *gracilus* muscle when 0 mg of 25-OH D$_3$ was administered and limestone was replenished at 1.0% of diet dry matter (40.89 U/g protein) and when 500 mg of 25-OH D$_3$ was administered in conjunction with limestone replenished at 0.5% of diet dry matter (44.91 U/g protein) as compared with activity in the control group ($P \leq 0.05$). Calpastatin activity was intermediate to the activities in the control group (83.28 U/g protein) and the groups previously listed when 500 mg of 25-OH D$_3$ was administered and limestone was replenished at 1.0% of diet dry matter (59.38 U/g protein). In the *vastus intermedius* muscle, calpastatin activity was decreased from 96.47 U/g protein in the control group to 44.85 U/g protein when 500 mg of 25-OH D$_3$ was administered and limestone was replenished at 0.5% of diet dry matter. Other treatments did not have an effect in the *vastus intermedius* muscle. Though not statistically significant, calpastatin activity in the *adductor* and *pectineus* muscles follows the expected pattern of activity with the highest activity in the control group and decreased activity in all other treatments. The relationship of calpastatin activity and other indicators of tenderness will be discussed in those sections of the Results and Discussion. In a recent study, expression of calpastatin mRNA was decreased greatly in *longissimus dorsi* muscle of culled Korean cows that were supplemented with 25-OH D$_3$ one time 6 d before harvest (Cho et al., 2006). In that study, even though calpastatin mRNA was decreased by 85%, there was no difference in the amount of calpastatin protein present or in the Warner-Bratzler shear force. Cho et al. did not measure calpastatin activity; so whether or not the calpastatin present was active remains unclear.
Calcium Concentrations in Plasma and in Meat

Calcium concentrations in plasma of cows were not different among treatment groups until 3 d before harvest (Table 4). When supplemental calcium was removed from the diets, concentration of calcium in the plasma decreased in all cases; cows, however, were able to maintain their plasma calcium concentration presumably by resorbing bone minerals. Another interesting point is that plasma magnesium concentration did not change when plasma calcium concentrations were maintained in the absence of supplemental dietary calcium but decreased when dietary calcium was sufficient (data not shown). That decrease in plasma magnesium concentration provides evidence that the increase in plasma calcium that is observed after supplementation with 25-OH D$_3$ results from increased absorption of dietary calcium and not mobilized from bone (Shils et al., 1998). On the day that calcium (limestone) was replenished in the diet (3 d after 25-OH D$_3$ was administered), blood samples were obtained before cattle were fed, and concentrations of calcium were increased, which indicates that cows were able to increase plasma calcium concentrations in response to dietary 25-OH D$_3$. After calcium was replenished in the diet by adding limestone at 0.5, 7.5, or 1.0% of diet dry matter, plasma calcium concentrations were increased to a greater extent in cattle that also were supplemented with 25-OH D$_3$ ($P \leq 0.05$). Whether cows were supplemented with 250 or 500 mg of 25-OH D$_3$ did not seem to make a difference as to the magnitude of increase in plasma calcium concentration ($P \geq 0.05$).

The calcium concentrations in plasma at the start of the study and 1 d before harvest in groups that were treated with a one-time bolus of 250 or 500 mg of 25-OH D$_3$ 7 d before harvest were similar to the plasma calcium concentrations observed when 5 ×
or 7.5 × 10^6 IU of vitamin D₃ are administered for 9 consecutive d (Montgomery et al., 2000) and when 6 × 10^6 IU of vitamin D₃ are administered for 4 or 6 d (Karges et al., 2001). The plasma calcium concentrations observed in this study are similar to those values obtained in an earlier study by the authors of this study (Carnagey et al., unpublished). Other researchers have not successfully increased plasma calcium concentrations with supplemental 25-OH D₃ (Foote et al., 2004; Wertz et al., 2004), but the failure to induce hypercalcemia in these previous studies probably resulted because of the lower dosage of 25-OH D₃ (125 mg) that was administered.

Each muscle was analyzed for total calcium concentration. Because no differences between muscles existed within treatments (P ≥ 0.20), data for all muscles were combined into one data set to determine treatment differences. Total calcium concentration in all muscles increased by approximately 12% when 500 mg of 25-OH D₃ was administered to cows as a single bolus 7 d before harvest (P ≤ 0.05) (Figure 2). Unexpectedly, when limestone was replenished at 0.75% of diet dry matter, muscle calcium concentrations were lower than when limestone was replenished at 0.5% or 1.0% diet dry matter (P ≤ 0.05), but were similar to calcium concentrations in plasma of control cows (P > 0.20). An interaction between the two treatments (25-OH D₃ and percentage of dietary limestone replenishment) existed (P ≤ 0.0001). The greatest increase in muscle calcium (15.8%) occurred when 500 mg of 25-OH D₃ was administered and limestone was replenished at 1.0% of diet dry matter. The muscle calcium concentrations observed in the current study agreed with those obtained by the same authors in an earlier study involving use of 25-OH D₃ and vitamin E to improve tenderness (Carnagey et al., unpublished). As with plasma calcium concentrations,
experiments by Foote et al. (2004) did not elicit an increase in muscle calcium. Again, this effect is probably attributable to the smaller dosage (125 mg) of 25-OH D$_3$ administered. Wertz et al. (2004) did not find an effect of treatment with 125 mg of 25-OH D$_3$ on water-soluble calcium concentration in *longissimus dorsi* muscle, but they did not analyze the samples for total calcium concentration. The possibility exists that the increase in muscle calcium that resulted in the present study is attributable to the water insoluble calcium concentration in the muscle, but the likelihood of that possibility is small because Foote et al. (2004) used the same dosage of 25-OH D$_3$ as did Wertz et al. (2004) and did not find an effect of treatment on total muscle calcium concentrations. In the present study, we found that increased plasma calcium concentrations were correlated with increased muscle calcium concentrations ($R^2 = 0.18$, $P < 0.001$), which supports the theory that the 125-mg dosage of 25-OH D$_3$ that was used by Foote et al. (2004), and by Wertz et al. (2004) was too low to elicit changes in muscle calcium concentrations.

**25-Hydroxyvitamin D$_3$ and 1,25-Dihydroxyvitamin D$_3$ in Plasma**

In plasma, 25-OH D$_3$ concentrations were increased from approximately 50 ng/mL to approximately 200 ng/mL when cows were supplemented with either 250 or 500 mg of 25-OH D$_3$ ($P \leq 0.05$) (Figure 3). The magnitude of increase in concentration of 25-OH D$_3$ in plasma is only about half of that observed in younger cattle (Wertz et al., 2004). For example, in a study conducted to determine the effects of feeding 25-OH D$_3$ and vitamin E to improve tenderness, concentrations of 25-OH D$_3$ in plasma were 13 fold higher in cows treated with 25-OH D$_3$ than those in control animals (Carnagey et al., unpublished). In the present study, the fold increase by the day before harvest was
approximately 2.5. Interestingly, the 25-OH D$_3$ concentration in control cows in this study was only slightly higher than that in a study conducted by Carnagey et al., (unpublished) in which younger heifers were used (80 vs. 60 ng/mL, respectively). The difference in baseline plasma 25-OH D$_3$ concentrations between these two studies probably can be attributed to the fact that the cows were housed outside on pasture before the start of this study (Hidiroglou et al., 1979). The concentrations of 25-OH D$_3$ observed in the current study are similar to those concentrations observed by Wertz et al. (2004) when 62.5 or 125 mg of 25-OH D$_3$ was administered 21, 7, 4 or 0 d before harvest; so, higher dosages of 25-OH D$_3$ do not seem to increase plasma concentrations more than smaller dosages do.

1,25-Dihydroxyvitamin D$_3$ concentrations in plasma from cows treated with 25-OH D$_3$ (either 250 or 500 mg) were 2.5-3 times higher than the concentrations observed in groups that were not supplemented with 25-OH D$_3$ ($P \leq 0.05$; Table 5). As expected, replenishing limestone in the diet did not affect the concentration of 1,25-(OH)$_2$ D$_3$ in plasma of cows ($P \geq 0.05$). Withdrawing calcium (limestone) from the diet of cows increased concentrations of 1,25-(OH)$_2$ D$_3$ in all cows on the day that 25-OH D$_3$ was administered. Blood samples were obtained before feeding; so, the increase in 1,25-(OH)$_2$ D$_3$ concentration observed on the day that 25-OH D$_3$ was administered is a result of internal homeostatic mechanisms that are responsible for maintaining plasma calcium concentrations. The most likely mechanism is that low plasma calcium stimulates the parathyroid gland to produce more parathyroid hormone (PTH), which increases 1α-hydroxylase activity in the kidney and thereby increases 1,25-(OH)$_2$ D$_3$ concentrations in the plasma (Shils et al., 1998). 1,25-Dihydroxyvitamin D$_3$ binds to nuclear receptors to
increase transcription of calcium-binding protein in the intestine, which, in turn, increases efficiency of absorption of calcium from the diet and maintains plasma calcium concentrations. In the present study, PTH concentration in plasma was increased in blood samples from the days during which calcium was withdrawn from the diet and before 25-OH D$_3$ was administered. Overall, the concentrations in plasma of 1,25-(OH)$_2$ D$_3$ observed in the present study are similar to those concentrations observed by Montgomery et al. (2000) when 5 or 7.5 × 10$^6$ IU of vitamin D$_3$ and those observed by Wertz et al. (2004), but lower than the concentrations observed by Foote et al. (2004) when 125 mg of 25-OH D$_3$ was administered one time before harvest. The slightly higher concentration of 1,25-(OH)$_2$ D$_3$ at baseline is likely explained by the fact that cows were exposed to sunlight daily until the start of the study (Hidiroglou et al., 1979).

25-Hydroxyvitamin D$_3$ Concentration in Meat

Samples from adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis of cows that were supplemented with 500 mg of 25-OH D$_3$ and that received 1.0% of their diet dry matter as limestone were analyzed for 25-OH D$_3$ concentration. No differences in 25-OH D$_3$ concentration were observed between muscles ($P \geq 0.10$) (data not shown); so, only the semimembranosus was used to determine differences between treatments. The concentration of 25-OH D$_3$ in semimembranosus muscle was increased from 25 to 37 ng/g of fresh tissue with supplementation of 500 mg of 25-OH D$_3$ 7 d before harvest compared with concentrations in cows treated with 0 or 250 mg of 25-OH D$_3$ ($P \leq 0.05$). The concentrations of 25-OH D$_3$ observed in the present study are much higher than those
concentrations observed by other researchers (Foote et al., 2004; Montgomery et al., 2000; Wertz et al., 2004) and the same authors in another study (Carnagey et al., unpublished). One possible explanation for this finding is that the present study required that cows were on pasture, in the sun from the time of calving until August when cows were brought to the feedlot. Because ultraviolet radiation from the sun converts 7-dehydrocholesterol in the skin to vitamin D$_3$, it is likely that the vitamin D status of these cattle is much higher than the status of the cattle that were housed in a sheltered feedlot during fall and winter months (Hidiroglou et al., 1979).

The mechanism for increasing 25-OH D$_3$ concentration in beef without increasing its concentration in plasma is not clear. One study reports that plasma 25-OH D$_3$ concentration is increased when exposure of skin to ultraviolet rays increases; however, the study did not report concentrations in the muscle and the plasma concentration was measured while cattle were being exposed to natural ultraviolet radiation (Hidiroglou et al., 1979). Although statistically significant ($P < 0.05$), the concentration of 25-OH D$_3$ in steaks from cows supplemented with 500 mg of 25-OH D$_3$ is less than two times the concentration in the control samples. This concentration of 25-OH D$_3$ is safe to consume even though its concentration is higher than that in other studies. The current upper limit of vitamin D consumption is 50 µg vitamin D$_3$/day (Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997). 25-Hydroxyvitamin D$_3$ has about 1.4 times the activity of vitamin D on a weight basis; so, at the concentrations of 25-OH D$_3$ found in this study, a person would have to consume more than 567.5 g of meat to reach the upper limit for daily intake of vitamin D$_3$ activity. Because most people do not consume that much beef in a day, or at least on consecutive days, this concentration is
still reasonable. In fact, consuming beef from cattle supplemented with 25-OH D₃ is safe and may be a reasonable method to help ensure that consumers meet their daily recommended intake of vitamin D₃ activity (equivalent to 5-10 μg of vitamin D₃/day depending upon age) (Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997). In fact, one 170-g serving of beef from treated cows would provide 7 μg equivalent of vitamin D activity and meet the daily recommended intake of vitamin D₃ for most people without any harmful effects.

**SDS Polyacrylamide Gel Electrophoresis and Western Blotting**

The extent of troponin-T degradation was determined by SDS polyacrylamide gel electrophoresis followed by Western blotting. Results are shown in Table 6. The internal standard used for determining extent of troponin-T degradation was a sample that had been prepared from the *longissimus dorsi* muscle from a feedlot heifer. Among all muscles and aging periods, calpastatin activity is correlated significantly with troponin-T degradation \((R^2 = -0.14, P = 0.02)\), which shows, as expected, that higher calpastatin values correspond to decreased troponin-T degradation. In sheep, calpastatin activity has been found to correlate with calpain activity (Delgado et al., 2001). The current study supports those findings because calpain activity and troponin-T degradation also are correlated positively.

The extent of myofibrillar degradation in the *adductor* at 7 d of aging was increased numerically with aging in all treatments and was increased significantly with treatment with 500 mg of 25-OH D₃. When 25-OH D₃ is used in combination with increased calcium, the extent of protein degradation was increased greatly \((P \leq 0.05)\). In
gracilus, in most cases the extent of myofibrillar degradation increased with aging time and was increased to a greater extent in the 0 mg of 25-OH D₃, 1.0% limestone and 500 mg of 25-OH D₃, 0.5% limestone groups. These groups were ones that had the lowest 24-h calpastatin activities in gracilus muscle. Degradation was increased numerically with aging in the sartorius muscle but was increased to a greater extent with treatment with 500 mg of 25-OH D₃ or 1% limestone. In the semimembranosus muscle, aging increased troponin-T degradation numerically in all treatments but increased degradation significantly when limestone was fed at 1% of diet dry matter. In the vastus intermedius muscle at 7 d aging, more myofibrillar hydrolysis had occurred with 0 mg of 25-OH D₃, 1.0% limestone than in other treatments. Very little troponin-T degradation was present in the vastus lateralis regardless of treatment or aging period. The greatest extent of protein degradation occurred when 500 mg of 25-OH D₃ was administered with 0.5% limestone. Previous studies have indicated that troponin-T degradation increases with aging (Foote et al., 2004; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Wertz et al., 2004), and that is the case for the current study too. Though total degradation was very low in this study, treatment with 500 mg of 25-OH D₃ and 0.50 % dietary limestone or 0 mg of 25-OH D₃ and 1.0% dietary limestone seemed to increase postmortem protein degradation in some muscles. In some cases, the effect of 25-OH D₃ seemed to be increased with increased dietary calcium, but, in others, increased calcium decreased the effect of 25-OH D₃.

Warner-Bratzler Shear Force
Warner-Bratzler shear force was used to measure the tenderness of steaks from the adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis muscles aged for 1, 3, or 7 d. Results for the main effects of 25-OH D₃ and dietary limestone for affected muscles are shown in Table 7. The muscles that showed the greatest response to treatments were the pectineus and the vastus intermedius muscles. In these muscles, most steaks aged for 7 d were more tender than those aged for 1 d. In the pectineus, replenishing calcium in the diet seemed to play a greater role in determining tenderness than did the dosage of 25-OH D₃. In the vastus intermedius, administering 250 mg of 25-OH D₃ or replenishing limestone at 1.0% of diet dry matter had a similar effect on improvement of tenderness as indicated by the value obtained when the Warner-Bratzler shear force at 7 d is subtracted from that at 1 d. The fact that none of these differences reached significance \( P \geq 0.10 \) is likely an effect of the small sample size in this study, as other studies (Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2000) have found that supplementation with vitamin D₃ improves tenderness because of increased postmortem proteolysis. And, the conditions supporting postmortem proteolysis are present in the current study.

**IMPLICATIONS**

Results from the current experiment indicate that 500 mg of 25-OH D₃ administered as a single bolus 7 d before harvest has the potential to improve tenderness of steaks from the round of beef cows based on increased calcium concentrations in plasma and in muscle. 25-Hydroxyvitamin D₃ is as effective as vitamin D₃ at increasing
plasma calcium and improving tenderness of beef without leaving high concentrations of vitamin D₃ or its metabolites in muscle. In fact, the concentrations of 25-OH D₃ found in beef from treated cows even could be beneficial to consumers. Withdrawing and replenishing limestone from the diet does not seem to increase calcium in the blood above the normal biological concentration of approximately 9 mg/dL; so, that treatment is not likely to increase calpain activity in muscle. Interestingly, troponin-T degradation was increased by 1.0% dietary limestone; therefore, the mechanism by which calpain is activated in this instance might not be related to muscle calcium. A study with a larger number of cows and fewer treatment combinations could determine more confidently the extent of the effects of these treatments on tenderness of beef from culled cows.

ACKNOWLEDGMENTS

The authors would like to express thanks to Amend Packing, Des Moines, IA and to the Iowa State University Meat Laboratory for their work in harvesting the cows in this study and for transporting the carcasses to and from Ames, IA. Thank you to Rod Berryman, manager of the ISU Beef Nutrition Research Farm, for daily care of the cattle and for helping obtain blood samples and administer boluses of 25-OH D₃. Laura Baseler, Katie Korn, and Audry Swinconos, thank you for your help in the laboratory.

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Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with an


Table 1. Experimental design

<table>
<thead>
<tr>
<th>Limestone treatments(^a) (% limestone, DM)</th>
<th>25-Hydroxyvitamin D(_3) (mg)</th>
<th>0</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>3 cows*</td>
<td>3 cows</td>
<td>3 cows*</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>3 cows</td>
<td>3 cows</td>
<td>3 cows</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>3 cows*</td>
<td>3 cows</td>
<td>3 cows*</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Limestone treatments refer to the percentage of limestone that was replenished in the diet 3 d before harvest.

* Indicates treatments used for determination of calpastatin activity.
Table 2. Diet composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of diet (DM basis)</th>
<th>Ca (% in DM of feed)</th>
<th>Ca (% in 0.50% limestone diet)</th>
<th>Ca (% in 0.75% limestone diet)</th>
<th>Ca (% in 1.0% limestone diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cracked corn</td>
<td>78.88</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Corn silage</td>
<td>15.00</td>
<td>0.08</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>2.64</td>
<td>0.28</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Steep liquor</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cane molasses</td>
<td>0.91</td>
<td>0.74</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Urea</td>
<td>0.64</td>
<td>0.09</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.50</td>
<td>37.1</td>
<td>0.19</td>
<td>0.28</td>
<td>0.37</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin A premix</td>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trace mineral premix</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rumensin®</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>-</td>
<td>0.26</td>
<td>0.35</td>
<td>0.44</td>
</tr>
</tbody>
</table>

a All cows were fed the diet containing 0.50% limestone until 17 d before harvest. When supplemental calcium was replenished 3 d before harvest, cows received either the 0.50, 0.75, or 1.0% limestone diet.
Table 3. Effect of treatment with 25-OH D$_3$ and dietary calcium on calpastatin activity in beef muscle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gracilus</th>
<th>Adductor</th>
<th>Pectinius</th>
<th>Semimembranosus</th>
<th>Sartorius</th>
<th>Vastus Lateralis</th>
<th>Vastus Intermedius</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 25-OH D$_3$; 0.5% limestone</td>
<td>83.28$^b$</td>
<td>44.71</td>
<td>53.89</td>
<td>46.23</td>
<td>40.07</td>
<td>61.76</td>
<td>96.47$^a$</td>
</tr>
<tr>
<td>0 25-OH D$_3$; 1.0% limestone</td>
<td>40.89$^c$</td>
<td>33.1</td>
<td>48.65</td>
<td>32.21</td>
<td>37.21</td>
<td>46.91</td>
<td>44.85$^a$</td>
</tr>
<tr>
<td>500 25-OH D$_3$; 0.5% limestone</td>
<td>44.91$^c$</td>
<td>36.86</td>
<td>43.19</td>
<td>44.04</td>
<td>41.3</td>
<td>55.58</td>
<td>83.44$^a$</td>
</tr>
<tr>
<td>500 25-OH D$_3$; 1.0% limestone</td>
<td>59.38$^b$</td>
<td>31.61</td>
<td>45.57</td>
<td>41.86</td>
<td>44.41</td>
<td>92.6</td>
<td>63.32$^{ab}$</td>
</tr>
<tr>
<td>SEM</td>
<td>6.98</td>
<td>4.68</td>
<td>4.75</td>
<td>7.98</td>
<td>4.04</td>
<td>16.16</td>
<td>11.91</td>
</tr>
</tbody>
</table>

$^a$ 1 unit of calpastatin activity is the amount of calpastatin required to inhibit one unit of calpain activity

$^b$ Values with different superscripts differ ($P \leq 0.05$).
Table 4. Calcium concentrations in plasma of cows treated with 25-OH D₃ and manipulations of dietary calcium

<table>
<thead>
<tr>
<th>25-OH D₃ (mg)</th>
<th>Limestone (diet DM)</th>
<th>Plasma calcium (mg/dL)</th>
<th>Time before harvest (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>0</td>
<td>0.50%</td>
<td>10.03</td>
<td>9.9</td>
</tr>
<tr>
<td>0</td>
<td>0.75%</td>
<td>9.25</td>
<td>9.33</td>
</tr>
<tr>
<td>0</td>
<td>1.00%</td>
<td>8</td>
<td>8.1</td>
</tr>
<tr>
<td>250</td>
<td>0.50%</td>
<td>8.48</td>
<td>8.95</td>
</tr>
<tr>
<td>250</td>
<td>0.75%</td>
<td>9.65</td>
<td>9.91</td>
</tr>
<tr>
<td>250</td>
<td>1.00%</td>
<td>9.11</td>
<td>9.24</td>
</tr>
<tr>
<td>500</td>
<td>0.50%</td>
<td>8.72</td>
<td>9.85</td>
</tr>
<tr>
<td>500</td>
<td>0.75%</td>
<td>8</td>
<td>9.23</td>
</tr>
<tr>
<td>500</td>
<td>1.00%</td>
<td>9.64</td>
<td>9.77</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.48</td>
<td>0.3</td>
</tr>
</tbody>
</table>

abc Values with differing subscripts are different ($P \leq 0.05$).
Table 5. 1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂ D₃] concentration in plasma of cows treated with 25-hydroxyvitamin D₃ (25-OH D₃) and differing amounts of dietary calcium

<table>
<thead>
<tr>
<th>25-OH D₃ (mg)</th>
<th>Limestone (diet DM)</th>
<th>Time before harvest (d)</th>
<th>1,25-(OH)₂ D₃ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45</td>
<td>17</td>
</tr>
<tr>
<td>0</td>
<td>0.50%</td>
<td>51.67</td>
<td>17.15</td>
</tr>
<tr>
<td>0</td>
<td>0.75%</td>
<td>43.28</td>
<td>15.55</td>
</tr>
<tr>
<td>0</td>
<td>1.00%</td>
<td>36.59</td>
<td>18.53</td>
</tr>
<tr>
<td>250</td>
<td>0.50%</td>
<td>63.41</td>
<td>35.31</td>
</tr>
<tr>
<td>250</td>
<td>0.75%</td>
<td>43.63</td>
<td>28.21</td>
</tr>
<tr>
<td>250</td>
<td>1.00%</td>
<td>51.48</td>
<td>29.93</td>
</tr>
<tr>
<td>500</td>
<td>0.50%</td>
<td>43.8</td>
<td>19.76</td>
</tr>
<tr>
<td>500</td>
<td>0.75%</td>
<td>48.65</td>
<td>38.48</td>
</tr>
<tr>
<td>500</td>
<td>1.00%</td>
<td>47.12</td>
<td>19.55</td>
</tr>
<tr>
<td>SEM</td>
<td>8.18</td>
<td>6.53</td>
<td>5.24</td>
</tr>
</tbody>
</table>

Letters (a-c) indicate different concentrations of 1,25-(OH)₂ D₃ in plasma (P ≤ 0.05).
Table 6. Effect of treatment with 25-OH D$_3$ and dietary calcium on troponin-T degradation in beef muscle

<table>
<thead>
<tr>
<th>Muscle/Aging</th>
<th>0 25-OH D$_3$; 0.5% limestone</th>
<th>500 25-OH D$_3$; 0.5% limestone</th>
<th>0 25-OH D$_3$; 1.0% limestone</th>
<th>500 25-OH D$_3$; 1.0% limestone</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adductor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.95*abh</td>
<td>0.23</td>
<td>0.22</td>
<td>0.58</td>
<td>0.14</td>
</tr>
<tr>
<td>3 d</td>
<td>0.35b</td>
<td>0.30b</td>
<td>0.19bc</td>
<td>0.02c</td>
<td>0.12</td>
</tr>
<tr>
<td>7 d</td>
<td>0.24</td>
<td>0.55</td>
<td>0.34</td>
<td>2.65*b</td>
<td>0.38</td>
</tr>
<tr>
<td>SEM</td>
<td>0.22</td>
<td>0.24</td>
<td>0.1</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td><strong>Gracilus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.01</td>
<td>0.02</td>
<td>0.16</td>
<td>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>3 d</td>
<td>0.02b</td>
<td>0.03b</td>
<td>0.64c</td>
<td>0.7bc</td>
<td>0.19</td>
</tr>
<tr>
<td>7 d</td>
<td>0.16*bc</td>
<td>0.23*bc</td>
<td>0.63c</td>
<td>0.09b</td>
<td>0.17</td>
</tr>
<tr>
<td>SEM</td>
<td>0.05</td>
<td>0.07</td>
<td>0.32</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td><strong>Pectineus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.15</td>
<td>0.07</td>
<td>0.16</td>
<td>0.32</td>
<td>0.19</td>
</tr>
<tr>
<td>3 d</td>
<td>1.03b</td>
<td>0.43</td>
<td>0.64</td>
<td>0.39</td>
<td>0.41</td>
</tr>
<tr>
<td>7 d</td>
<td>0.18b</td>
<td>1.03*c</td>
<td>0.66bc</td>
<td>0.17b</td>
<td>0.21</td>
</tr>
<tr>
<td>SEM</td>
<td>0.53</td>
<td>0.35</td>
<td>0.25</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td><strong>Sartorius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.15</td>
<td>0.21</td>
<td>0.57</td>
<td>0.11</td>
<td>0.37</td>
</tr>
<tr>
<td>3 d</td>
<td>0.57</td>
<td>0.18</td>
<td>0.08</td>
<td>0.06</td>
<td>0.17</td>
</tr>
<tr>
<td>7 d</td>
<td>0.11</td>
<td>0.39</td>
<td>0.42</td>
<td>0.46</td>
<td>0.27</td>
</tr>
<tr>
<td>SEM</td>
<td>0.28</td>
<td>0.15</td>
<td>0.35</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Semimembranosus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 d</td>
<td>0.01*bc</td>
<td>0.02b</td>
<td>0.17bc</td>
<td>0.56c</td>
<td>0.18</td>
</tr>
<tr>
<td>3 d</td>
<td>0.33bc</td>
<td>0.50*bc</td>
<td>0.05b</td>
<td>0.51c</td>
<td>0.19</td>
</tr>
<tr>
<td>7 d</td>
<td>0.67*bc</td>
<td>0.64b</td>
<td>1.38*bc</td>
<td>0.66bc</td>
<td>0.27</td>
</tr>
<tr>
<td>SEM</td>
<td>0.2</td>
<td>0.24</td>
<td>0.33</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td><strong>Vastus Intermedius</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>0.02</td>
<td>0.07</td>
<td>0.27</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>3 d</td>
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<td>0.08</td>
<td>0.08</td>
<td>0.94b</td>
<td>0.26</td>
</tr>
<tr>
<td>7 d</td>
<td>0.08c</td>
<td>0.10c</td>
<td>0.59c</td>
<td>0.14*bc</td>
<td>0.19</td>
</tr>
<tr>
<td>SEM</td>
<td>0.04</td>
<td>0.05</td>
<td>0.32</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td><strong>Vastus Lateralis</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
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<td>0.02</td>
<td>0.21</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>3 d</td>
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<td>0.52</td>
<td>0.12</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>7 d</td>
<td>0.08b</td>
<td>0.32c</td>
<td>0.17*ac</td>
<td>0.12*bc</td>
<td>0.1</td>
</tr>
<tr>
<td>SEM</td>
<td>0.07</td>
<td>0.22</td>
<td>0.12</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent band intensity as measured by densitometry as it relates to a standard beef sample chosen from another study.

*Denotes difference ($P \leq 0.08$) within a treatment during aging.

b,c Denotes difference ($P \leq 0.08$) between treatments at a given aging period.
Table 7. Effect of 25-OH D$_3$ and manipulations of dietary calcium on Warner-Bratzler shear force in selected muscles from the round section of beef cows

a. **Adductor**

<table>
<thead>
<tr>
<th>25-OH D$_3$ (mg)</th>
<th>Aging (d)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>5.48</td>
<td>6.02</td>
<td>5.62</td>
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</tr>
<tr>
<td>250</td>
<td>5.5</td>
<td>5.55</td>
<td>5.42</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>5.83</td>
<td>5.61</td>
<td>5.76</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.24</td>
<td>0.4</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Limestone (%DM)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>5.89</td>
<td>6.3</td>
<td>6.21</td>
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<tr>
<td>0.75</td>
<td>5.55</td>
<td>5.76</td>
<td>5.34</td>
<td></td>
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<tr>
<td>1.00</td>
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<td>5.23</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.24</td>
<td>0.4</td>
<td>0.52</td>
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</tbody>
</table>

b. **Gracilus**

<table>
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<tr>
<th>25-OH D$_3$ (mg)</th>
<th>Aging (d)</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>4.04</td>
<td>3.49</td>
<td>4.34</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>3.85</td>
<td>3.6</td>
<td>3.8</td>
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<td>4.17</td>
<td>4</td>
<td>4.59</td>
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</tr>
<tr>
<td>SEM</td>
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<td>0.3</td>
<td>0.45</td>
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<tr>
<td>Limestone (%DM)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
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<td>0.75</td>
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<tr>
<td>1.00</td>
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<td>4.86</td>
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</tr>
<tr>
<td>SEM</td>
<td>0.34</td>
<td>0.3</td>
<td>0.45</td>
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c. **Pectineus**

<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>4.47</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>5.02</td>
<td>5.08</td>
<td>4.65</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>4.72</td>
<td>4.89</td>
<td>4.46</td>
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</tr>
<tr>
<td>SEM</td>
<td>0.19</td>
<td>0.34</td>
<td>0.28</td>
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</tr>
<tr>
<td>Limestone (%DM)</td>
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<td>4.6</td>
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</tr>
<tr>
<td>1.00</td>
<td>4.7</td>
<td>4.99</td>
<td>4.61</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.19</td>
<td>0.34</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

d. **Vastus Intermedius**

<table>
<thead>
<tr>
<th>25-OH D$_3$ (mg)</th>
<th>Aging (d)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.19</td>
<td>5.03</td>
<td>5.28</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>5.32</td>
<td>5.57</td>
<td>4.66</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>5.51</td>
<td>5.47</td>
<td>5.12</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>0.21</td>
<td>0.32</td>
<td>0.38</td>
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</tr>
<tr>
<td>Limestone (%DM)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>5.42</td>
<td>5.21</td>
<td>5.3</td>
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<tr>
<td>0.75</td>
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<td>5.31</td>
<td>5.39</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
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</tr>
<tr>
<td>SEM</td>
<td>0.21</td>
<td>0.32</td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

Change in shear force from 1-7 d aging is greater in magnitude when 25-OH D$_3$ is administered in some cases.
Figure 1. Experimental timeline. Cows were brought in from pasture, a blood sample was drawn, and cows were fed for 28 days. On the day that limestone was withdrawn from the diet, a blood sample was obtained. An additional blood sample was obtained 7 d after limestone was withdrawn. Seven days before harvest, another blood sample was obtained, and 25-OH D$_3$ (0, 250, or 500 mg 25-OH D$_3$) was administered by oral bolus. Additional blood samples were obtained 5, 3, and 1 day before harvest. Limestone was replenished in the diet (limestone at 0.5%, 0.75%, or 1.0% of diet dry matter) 3 d before harvest.
Figure 2. Calcium concentration in muscle of cows treated with 25-OH D$_3$ and manipulations of dietary calcium. No muscle differences existed; so, data from all muscles were pooled. An asterisk (*) indicates that treatment with 500 mg of 25-OH D$_3$ increased calcium concentration in muscle compared with dosages of 0 or 250 mg of 25 OH D$_3$ and differing concentrations of replenished limestone ($P \leq 0.05$). Withdrawing and then replenishing limestone in the diet did not affect muscle calcium concentration ($P > 0.05$).
Figure 3. 25-Hydroxyvitamin D₃ concentration in plasma of cows treated with 25-OH D₃ and dietary manipulations of calcium. Non-overlapping error bars indicate that concentrations of 25-OH D₃ are higher at 5, 3, and 1 d before harvest for cows treated with 500 mg of 25-OH D₃ with either 0.5% or 1.0% of diet dry matter as limestone ($P \leq 0.05$). As expected, no differences in 25-OH D₃ concentration were observed from 45 to 7 d before harvest ($P > 0.10$).
Figure 4. 25-Hydroxyvitamin D₃ concentration in the *semimembranosus* muscle of cows treated with 25-OH D₃ and differing concentrations of dietary calcium. An asterisk (*) indicates that treatment with 500 mg of 25-OH D₃ increased calcium concentration in muscle compared with dosages of 0 or 250 mg of 25-OH D₃ (*P* ≤ 0.05).
**Figure 5.** 1,25-Dihydroxyvitamin D₃ concentration in plasma of cows treated with 25-OH D₃ and dietary manipulations of calcium. Non-overlapping error bars indicates that concentrations of 1,25-(OH)₂ D₃ in plasma are higher at 5, 3, and 1 d before harvest in cows treated with 500 mg of 25-OH D₃ with either 0.5% or 1.0% of diet dry matter as limestone ($P \leq 0.05$). As expected, no differences in 1,25-(OH)₂ D₃ concentration was observed from 47 to 7 d before harvest ($P > 0.10$).
Figure 6. Postmortem protein degradation is increased in samples from cows that were treated with 25-OH D$_3$ 7 d before harvest. This figure shows degradation in the sartorius muscle after 3 days of aging.
GENERAL SUMMARY

Collectively, the studies included in this dissertation investigate the use of 25-OH D$_3$ to improve beef tenderness. 25-Hydroxyvitmain D$_3$ (0, 250, or 500 mg) was administered to cattle once as an oral bolus 7 days before harvest, and plasma and muscle parameters were measured. 25-Hydroxyvitamin D$_3$ (250 and 500 mg) increased plasma calcium concentrations on the day before harvest in treated cattle. Muscle calcium concentrations were increased when 500 mg of 25-OH D$_3$ was administered. Concentrations of 25-OH D$_3$ in plasma were increased when 250 or 500 mg of 25-OH D$_3$ were administered, but concentrations in muscle were increased only if 500 mg of 25-OH D$_3$ were administered. Concentrations of 1,25-(OH)$_2$D$_3$ in plasma were increased by 250 or 500 mg of 25-OH D$_3$, but 500 mg of 25-OH D$_3$ did not increase concentrations of 1,25-(OH)$_2$D$_3$ in muscle. The concentrations of vitamin D activity in muscle is much lower when 500 mg of 25-OH D$_3$ is administered than when 0.5-7.5 $\times$ 10$^6$ IU vitamin D$_3$ are supplemented for 4-9 days (Foote et al., 2004; Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Scanga et al., 2001; Swanek et al., 1999).

The use of vitamin E alone, and in combination with 25-OH D$_3$, to improve tenderness also was investigated. Vitamin E (1000 IU) was administered daily for 104 days. Surprisingly, feeding vitamin E doubled the concentration of 25-OH D$_3$ and caused a 5-fold increase the concentration of 1,25-(OH)$_2$D$_3$ in muscle. No additive effect was observed when vitamin E and 25-OH D$_3$ were used in combination, and concentrations of both 25-OH D$_3$ and 1,25-OH D$_3$ in muscle were similar to those concentrations observed when only 25-OH D$_3$ was administered. Treatment with vitamin E, with or without supplemental 25-OH
D$_3$, increased vitamin E concentration in meat and decreased lipid oxidation at 3, 7, and 14 days of aging.

The manipulation of dietary calcium was used in combination with 25-OH D$_3$ to increase plasma and muscle calcium concentrations. Calcium (supplemental limestone) was withdrawn from the diet for 2 wk and then replenished for 3 days before harvest. When calcium was replenished in the diet, three different concentrations of limestone were used. Dosages of replenished limestone were 0.5%, 0.75%, or 1.0% of diet dry matter. All of these treatments were used in combination with 25-OH D$_3$ (0, 250, or 500 mg) with a goal of increasing calcium concentrations beyond the action of 25-OH D$_3$ alone. We hypothesized that the calcium homeostatic mechanisms would be increased by withdrawing supplemental calcium, and calcium-binding protein would be increased in the intestine and increased calcium absorption would result. No additive effect of manipulations of dietary calcium and 25-OH D$_3$ were observed on indicators of tenderness, and plasma calcium concentrations returned only to normal concentrations when calcium was replenished in the diet, regardless of the concentration of limestone.

In the study involving beef heifers, 25-OH D$_3$ and vitamin E both improved tenderness of beef after 14 days of aging. There was no additive effect as the beef from the heifers treated with both 25-OH D$_3$ and vitamin E was similar in tenderness to the beef from control heifers. In the study involving beef cows, treatment with 25-OH D$_3$ did not affect beef tenderness as indicated by Warner-Bratzler shear force.

Altogether, the results of these two studies indicate that 25-OH D$_3$ and vitamin E both have the potential for improving the consistency of beef tenderness. More research should be
conducted to define more clearly the effective dosages and the situations in which these treatment strategies will be most advantageous for improving the tenderness of beef.
GENERAL CONCLUSION

In general, the results of the two studies included in this dissertation indicate that 500 mg of 25-OH D$_3$ administered orally as a single bolus 7 days before harvest effectively does increase plasma and muscle calcium concentrations to the same extent as $0.5 \times 10^6$ IU of vitamin D$_3$ daily for 4-9 days before harvest (Foote et al., 2004; Karges et al., 2001; Montgomery et al., 2002; Montgomery et al., 2004; Montgomery et al., 2000; Scanga et al., 2001; Swanek et al., 1999). Because 25-OH D$_3$ does increase muscle calcium concentrations and leaves lower concentrations of vitamin D activity in muscle as compared with cattle that were treated with vitamin D$_3$, the possibility exists that 25-OH D$_3$ could be used for improving tenderness of beef. The relatively small impact on tenderness that was observed in this study can be explained at least partially by the fact that, in the heifer study, the beef from all of the cattle was already very tender, even after only 3 days of aging, and, in the cow study, that there was a relatively small number of cattle in each treatment combination.

Vitamin E (1000 IU/d for 104 days) may improve tenderness as effectively as 500 mg of 25-OH D$_3$. Vitamin E has other well documented benefits on beef quality—color stability and prevention of lipid oxidation (Arnold et al., 1992; Arnold et al., 1993; Liu et al., 1995); so, using vitamin E to improve tenderness may be accepted even more widely than will vitamin D or its metabolites.

Use 25-OH D$_3$ or vitamin E as a practical, on-the-farm method of improving beef tenderness may result in beef that is more consistently tender. Consumers may be willing to purchase beef more often or, possibly, even pay a premium, for beef that is “guaranteed tender”. Although such labeling may still be a few years in the future, the current research can point future research in a direction that can make beef consistently tender.
FUTURE RESEARCH

Effects of vitamin E on vitamin D status

The effects of vitamin E on vitamin D status should be investigated further because of the effect vitamin E has on 25-OH D$_3$ and on 1,25-(OH)$_2$ D$_3$ concentrations in beef. 1,25-Dihydroxyvitamin D$_3$ was increased in muscle by 5 fold with treatment with 1000 IU of vitamin E for 104 d before harvest. The results of the current study warrant an experiment in which 1000 IU of vitamin E is fed for 100 or more days and 25-OH D$_3$ and 1,25-(OH)$_2$ D$_3$ are quantified in plasma and in beef at the end of the study. Activity of 25-hydroxylase in the liver and 1-α-hydroxylase in the kidney should be quantified at the time of harvest. Additionally, an *in vitro* study should be designed to determine the activity of 25-hydroxylase and 1-α-hydroxylase in the presence of vitamin E. The current study indicates that activity of these enzymes may be extended in the presence of vitamin E.

Vitamin E could be at least as effective as vitamin D$_3$ and 25-OH D$_3$ at improving consistency of tenderness, and, when coupled with the positive impacts vitamin E has on beef quality (color stability and prevention of fatty acid oxidation), vitamin E could be the compound of choice when it comes to improving overall quality of beef.

Use of 25-OH D$_3$ to improve tenderness of pasture-fed cattle

Currently, a study is underway at Iowa State University to determine the effects of 25-OH D$_3$ on tenderness of beef from pasture-fed beef steers and feedlot beef steers. Some muscles, especially from the round section, are not consistently tender and, therefore, have lower marketability. Also, beef from conventionally fed cattle contains relatively high concentrations of saturated fatty acids, which are believed to be associated with human health
problems. Currently, research is being conducted by several research groups to improve beef
tenderness and healthfulness, but the research has not yet been combined to yield a product
that is both more tender and more healthful.

The working hypothesis for this experiment is that 500 mg of 25-OH D₃ will have a
similar effect on tenderness of beef from both feedlot and pasture-fed steers without negatively
affecting the perceived health benefits of pasture-fed beef such as increased conjugated linoleic
acid and an improved n-6 to n-3 ratio. Therefore, the overall objective of this study is to
determine the effects of dietary treatments intended to enhance tenderness of beef from selected
muscles from the round and rib section of feedlot and pasture-fed beef steers by increasing
calcium in muscle at time of harvest with oral administration of 25-OH D₃.

The rationale underlying this objective is that demand of pasture-fed beef is increasing; however,
beef from pasture-fed steers and heifers has been shown to be less tender than that from animals
fed high-grain diets in a feedlot. This project aims to enhance the tenderness of beef from
pasture-fed steers without negatively affecting the characteristics that beef from pasture-finished
cattle that are perceived to be more healthful than certain characteristics of conventional beef
(e.g., lower concentrations of saturated fatty acids). Consistently improved tenderness of beef
from pasture-fed cattle, which is often considered by U.S. consumers to be less tender and less
palatable than beef from grain-fed cattle, could lead to increased marketability and, therefore,
increased profits to producers.
Another study using fewer treatments to determine use of 25-OH D₃ for improving tenderness of beef from the round of cows

The study included in this dissertation that involves cows was confounded by many treatment combinations and a small number of cows. While data are encouraging, a study that involves more cows and fewer treatment combinations would likely increase the power of the experiment and lead to more conclusive evidence for the effect of 25-OH D₃ as it relates to improved beef tenderness. I would suggest a study in which a 2 × 2 factorial design was implemented. The treatments of 0 or 500 mg of 25-OH D₃ and 0.50% or 1.0% limestone should be used, and at least 10 cows should be included in each treatment. These four treatments are those referred to as “most extreme” in the study included in this dissertation, and results from the three cows in each treatment provide hints that some treatment effects exist. Because of the small number of cows used in the current experiment, no significant differences in improvement of tenderness were observed. The same treatments with a larger number of animals could help researchers determine the effectiveness of 25-OH D₃ on improving tenderness of beef from the round of cows.

Practical administration of 25-hydroxyvitamin D₃

To producers, bolusing cattle seven days before harvest could prove to be an inconvenience and possibly could result in decreased profits if cattle are excited and bruise their muscles shortly before harvest. Administration of 25-OH D₃ in the feed would be a more practical administration method than oral bolusing. A study should be designed to determine the effectiveness of 25-OH D₃ on beef tenderness when the 25-OH D₃ is administered as a component of a vitamin and mineral supplement. Probably, the 25-OH D₃
should be included for the last seven to 10 days before harvest at a concentration that would provide approximately 125 mg of 25-OH D₃ per animal each day. Because the producer would not be certain that each animal consumed the supplement everyday, the total dosage likely would have to be increased to be sure that each animal does consume the desired dosage of 500 mg of 25-OH D₃ before harvest. Beginning supplementation at 10 days before harvest and continuing through harvest would help ensure that 25-OH D₃ would be consumed early enough to increase plasma and muscle calcium concentrations and improve beef tenderness.
LITERATURE CITED


Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with an


human vascular smooth muscle cells and is upregulated by parathyroid hormone and estrogenic compounds. Circulation 111: 1666-1671.


