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ARTICLE

Vitamins E and C May Increase Collagen Turnover by Intramuscular Fibroblasts. Potential for Improved Meat Quality

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Vitamins influence collagen metabolism in animals grown for meat. This study investigated whether vitamins E and C regulate collagen turnover in muscle by the balance of effects on the synthesis of collagen and its degradation by secretion of matrix metalloproteinases (MMPs) by bovine intramuscular fibroblasts. Fibroblasts isolated from longissimus dorsi (LD) and semitendinosus (ST) muscle were treated with different concentrations of vitamins. Pro-MMP-2, MMP-2, and total soluble collagen (TSC) synthesis were determined. Vitamins E and C each preferentially increased ($P < 0.05$) MMP-2 in cells derived from LD relative to those derived from ST. Higher TSC values ($P < 0.05$) were found for ST cells than for LD cells. Both vitamins may increase collagen turnover exerted by intramuscular connective tissue fibroblasts. These results may have implications in vivo on animal production, as a high rate of collagen turnover may lead to increased collagen solubility in muscles, which can affect meat tenderness.

KEYWORDS: Vitamins C and E; collagen turnover; MMPs; fibroblast; meat tenderness

INTRODUCTION

Vitamin E is a generic term that includes a group of tocopherols and tocotrienols that function as the principal lipid-soluble chain-breaking antioxidants in biological membranes (*1*). Animals are unable to synthesize tocopherol and depend exclusively on dietary sources. The naturally occurring form of vitamin E in feedstuffs is *RRR*-α-tocopherol (also known as *d*-α-tocopherol), whereas the acetate ester of *RRR*-α-tocopherol (*RRR*-α-tocopheryl acetate or *d*-α-tocopheryl acetate) is the most common supplemental form of vitamin E in ruminant diets (*2*). Because dietary tocopherol accumulates in most tissues including skeletal muscle, vitamin E is an important factor that could affect meat quality.

Fresh-growing forages have adequate amounts of vitamin E to maintain high levels of vitamins in foraging ruminants; however, current research indicates that grains such as corn, as well as stored feeds, such as dry hay, may not contain adequate amounts of vitamin E to meet animal requirements (*3*). For this reason, producers are recommended to supplement their feed with about 400−1250 IU/day for finishing steers (*4*). The supplementation of cattle feed with vitamin E during the growing and finishing periods may decrease lipid oxidation and drip loss while improving meat color (*5*). Additionally, Carnagey et al. (*6*) reported that the use of 1000 IU of vitamin E administered daily for 104 days before slaughter effectively decreased toughness (Warner−Bratzler shear force) in longissimus steaks from heifers. This lipid-soluble vitamin may also influence intramuscular collagen characteristics, improving collagen solubility (*7*), which could affect meat tenderness.

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Collagen is the major connective tissue protein and accounts for about 30% of the total protein in the mammalian body. The collagen content of most skeletal muscles is relatively low (about 2−6% dry matter); however, this protein plays an important role in determining the textural properties of meat and is believed to contribute to the background toughness, one factor responsible for the variability in meat tenderness (*8*, *9*). Matrix metalloproteinases (MMPs) are good markers for collagen turnover as they represent a large family of 28 structurally related zinc-dependent endopeptidases responsible for the degradation of connective tissue components during tissue remodeling, turnover, and growth. These enzymes are synthesized and secreted mainly as pro-enzymes by fibroblasts and, upon activation, the collagenases (MMP-1 and MMP-13) denature fibrillar collagen, which can then be degraded to small peptides by gelatinases (MMP-2 and MMP-9) (*10*).

In addition to vitamin E, pasture diet has been reported to increase the level of other antioxidants, including ascorbic acid, in muscle tissue of cattle in comparison to grain-fed animals (*11*). Vitamin C is a free radical scavenger that reduces the α-tocopheryl radical, thereby regenerating vitamin E, and is an indispensable cofactor for collagen synthesis, maturation, and secretion (*12*). Recently, it has been reported that administration of L-ascorbic acid to Japanese cattle improved the fat marbling, firmness, and texture of the meat (*13*).

The aim of this study was to investigate the effect that vitamins E and C may have on the balance of activity of MMPs and collagen synthesis by fibroblast cells derived from two bovine muscles, longissimus dorsi (LD) and semitendinosus (ST), that differ in both function and fiber type distribution. This study demonstrates the impact that vitamins C and E may have on meat

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quality by increasing collagen turnover at the cellular level, suggesting that the animals' diets may be an important factor regulating collagen turnover in vivo. This research further highlights the complex nature of collagen metabolism in the living animal and re-emphasizes that the same cell types isolated from different muscles may show phenotypic differences in their response to vitamins C and E.

MATERIALS AND METHODS

Intramuscular Fibroblast Isolation and Culture. Primary cultures of intramuscular connective tissue fibroblasts were isolated directly from LD and ST muscles from a 9-month-old bovine animal at the University of Guelph's abattoir within 1 h post-mortem. Muscle samples were washed thoroughly and placed in Hank's balanced salt solution (HBSS) with 1% antibiotic/antimycotic solution (Sigma Chemical Co.). Samples were finely minced and incubated in 0.1% trypsin (Sigma Chemical Co.) in HBSS at 37 °C for 50 min with strong shaking. Samples were then centrifuged at 400*g* for 10 min and the supernatant discarded. The pellets were washed three times with phosphate buffered saline (PBS) and centrifuged. After the last washing step, sediments were resuspended in Dulbecco's Modified Eagle Medium (DMEM, 4500 mg of glucose/L, L-glutamine, sodium bicarbonate, and pyridoxine HCl; Sigma Chemical Co.) and supplemented with 1% antibiotic/antimycotic solution (10000 U penicillin, 10 mg of streptomycin, 25 μg of amphotericin B/mL, 0.1 mg of kanamycin/mL, 20 mM Hepes, and 10% fetal bovine serum; Sigma Chemical Co.). Fibroblasts were then allowed to grow until about 95% confluence in 100 × 20 mm tissue culture dishes (Sarstedt, Newton, NC) in a humidified atmosphere at 37 °C with 5% CO_2, before passaging by splitting cultures in a 1:10 ratio into new tissue culture dishes with fresh media. At this ratio a confluent monolayer was obtained in early passage cultures within 7−10 days after seeding. All cultures were serially subcultured until the cells reached passage 4. For the study, cells were used within the first four passages to avoid changes in their original phenotype during subculture (*14*).

Treatments. Before experimental treatments, cells were trypsinized (to detach adherent viable fibroblasts) and counted with a hemocytometer, replated in 60 × 15 mm tissue culture plates (Sarstedt) in triplicate at a density of 2×10^5 cells, and allowed to adhere for 24 h. When close to 80% confluence, cells were induced to quiescence by serum starvation with a gradual replacement of normal DMEM with 5, 2.5, 1, or 0.1% serum until reaching 24 h of complete acclimatization with serum-free DMEM. During this 24 h of serum-free period, cell cultures received one of the following four treatments: (1) (control) fibroblasts were not treated with vitamins; (2) 50 μM vitamin E (+ α-tocopherol acetate, Sigma Chemical Co.); (3) 100 μM vitamin E; (4) 50 μM vitamin C (ascorbic acid, Sigma Chemical, Co.); (5) 50 μM vitamin E + 50 μM vitamin C; (6) 100 μM vitamin E + 50 μM vitamin C.

Cell culture media from cells under each treatment were immediately collected and centrifuged for 5 min at 1000 rpm to remove any dead cells and debris, and then aliquots of equal volume were distributed into 1.5 mL conical tubes and stored at −80 °C until analysis of gelatinases. After medium collection, fibroblasts were washed twice with PBS and lysed in a buffer containing 20 mM Tris-HCLl, pH 7.5, 1% Triton X-100, 100 mM NaCl, 0.5% Nonidet P-40, and 1 mg/mL protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation at 13000 rpm for 10 min and stored at −80 °C until analysis of total soluble collagen synthesis.

Sulforhodamine B Cell Survival Assay. Any potential antiproliferative (cytotoxic) effects of vitamins E and C were evaluated on the two groups of cells by the sulforhodamine B (SRB) assay of cell survival. SRB is a widely accepted method based on the ability of the protein dye SRB to bind electrostatically basic amino acid residues of trichloroacetic acid fixed cells (*15*). Briefly, cells were plated in 96-well microtiter plates at a cell density of 5000 cells per well and were left to resume exponential growth for 24 h in an incubator. Thereafter, an equal amount of serum-free medium (the negative controls) or a serial dilution of tested vitamins was applied. The cell numbers were then estimated 24 h later by means of the SRB assay. Briefly, cell monolayers were fixed with 50% (w/v) trichloroacetic acid for 1 h at 4 °C and stained for 30 min with 0.4% (w/v) SRB (Sigma) in 1% acetic acid solution, after which the excess dye was removed by washing repeatedly with 1% (v/v) acetic acid before being air-dried. The protein-bound dye was dissolved with 10 mM unbuffered Tris-base solution (Sigma), and plates were left on a plate shaker for at least 15 min until complete dissolution. Absorbance was read in a multidetection microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT) at 570 nm. Eight replicate wells were prepared for each adhesive and vitamin tested. The results were expressed as a survival fraction, and the dilution that resulted in 50% reduction in cell numbers (DL_{50}) after 24 h of exposure was calculated from dose−effect curves derived using Graph Pad Prism 4 software (GraphPad Software Inc., La Jolla, CA).

Gelatin Zymography. Zymographies were performed following the protocol used by Siwik et al. (*16*). Volumes normalized by the number of cells were loaded under nonreducing conditions in 4% stacking/10% separating SDS polyacrylamide gel with 1 mg/mL gelatin (type A from porcine skin, Sigma-Aldrich) as the substrate. Samples were mixed with double volumes of nonreducing sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 0.3 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.03% bromophenol blue. During electrophoresis, both the latent pro-MMP-2 ($M_r \approx 71$ kDa) and active MMP-2 ($M_r \approx 62$ kDa) migrate throughout the gel according to their different molecular weights. The gels were then washed twice for 30 min each at room temperature in 2.5% Triton X-100 (Fisher Scientific) to remove SDS and renature the enzymes. Although activation in vivo of pro-MMPs involves both the disruption of the $Cys^{73}-Zn^{2+}$ bond and proteolytic clipping of the cysteine-containing terminal region, unfolding due to SDS disrupts the $Cys^{73}-Zn^{2+}$ bond and so in renaturing conditions the pro-MMP-2 is fully activated (*17*). This allows a useful quantification of both the natural level of MMP-2 activity and its total expression (sum of active + pro forms). The gels were then incubated for 18 h at 37 °C with gentle shaking in an incubation buffer consisting of 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, and 0.05% Brij-35. The gels were stained for 1 h in 0.05% Coomassie Blue, 10% acetic acid, and 40% methanol and then destained in a solution of 10% acetic acid and 40% methanol. Clear, digested regions representing MMP activity were quantified using an imaging densitometer. Molecular weights were estimated using prestained molecular weight markers. The identity of the enzymes as Zn-dependent MMPs was confirmed by the incubation of a duplicate gel in an incubation buffer containing the metal chelator EDTA (10 mM) (data not shown) (*16*). Pro-MMP-2 and MMP-2 levels were expressed as percentage change in every treatment versus the levels found in a control group of cells.

Total Soluble Collagen Synthesis. Total soluble collagen concentration in the cell supernatants was measured using the Sircol collagen assay kit (Biocolor, Carrickfergus, U.K.). Briefly, a 100 μL aliquot of cell supernatant was mixed with 1 mL of Sircol dye and incubated at room temperature for 30 min and then centrifuged at 13000*g* for 10 min to pack the collagen−dye complex. The pellet obtained was dissolved in 0.5 M NaOH to release the collagen−dye complex, the absorbance of which was measured at 540 nm by spectrophotometry (Genesys 10 Bio, Thermo Electron Corp.). The concentration of collagen was determined from a collagen standard curve prepared with the collagen standard provided with the assay. Total soluble collagen was expressed as a concentration (μg of collagen/100 μg of protein) relative to control fibroblasts that did not receive any treatment. Protein determination of cell lysates was performed with a Bio-Rad dye-binding assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Immunocytochemistry. To confirm that the cells isolated were fibroblasts, immunocytochemical localization of vimentin and collagen type I was performed on the two groups of cells. At the same time, both cell groups were pretreated for 24 h with 50 μM vitamin E + 50 μM vitamin C to reveal any difference in fluorescence with regard to a control group of cells that had not received any vitamin treatment. The immunocytochemical localization of the antibodies was performed as explained before (*14*). Briefly, bovine intramuscular fibroblasts were fixed in 2.0% (w/v) paraformaldehyde (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), permeabilized with 0.2% Tween 20, and blocked for 30 min with 10% v/v goat serum (Sigma-Aldrich Canada Ltd.). Culture slides were incubated overnight at 4 °C with either rabbit-polyclonal antibody to vimentin (1:50; Cell Signaling, Danvers, MA) or mouse monoclonal [COL-1] to collagen type I (1:150; Abcam, Inc., Cambridge, MA), and subsequently incubated for 2 h in the dark with an FITC-labeled goat polyclonal antibody either to rabbit IgG (1:1000; Abcam, Inc.) or to mouse IgG (1:500; Abcam, Inc.).

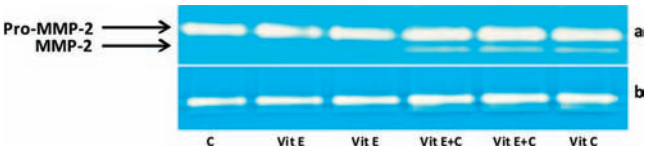

Figure 1. Representative zymogram depicting the effects of different concentrations of vitamins E (50 and 100 μM) and C (50 μM) and their mixture (50 μM vitamin E + 50 μM vitamin C; 100 μM vitamin E + 50 μM vitamin C) on latent (pro-MMP-2) and active matrix metalloproteinase 2 (MMP-2) in media from cultured cells derived from bovine (**a**) longissimus dorsi and (**b**) semitendinosus muscles. C, control group.

Statistical Analysis. Results were statistically analyzed by analysis of variance (ANOVA) using the Predictive Analytics Software (PASW) Statistics 17 program (SPSS Inc., Chicago, IL). When the F value from this indicated that the null hypothesis (no treatment differences) should be rejected, Fisher's protected least significant difference (LSD) procedure was applied post hoc on pairwise comparisons of each treatment to find which of the treatment means differed from each other at a significance level of 5%. Correlations between levels of latent and active MMP-2 and total soluble collagen synthesis were examined using Pearson's correlation coefficient. Statistical significance was defined as $P < 0.05$. The results are presented as the mean of each variable for the individual experiments ± standard error.

RESULTS

The immune-staining technique applied to LD and ST cells tested positive for vimentin and collagen type I (data not shown), which together with the typical fibroblast-like shape of the cells confirms that the isolated cells were actually fibroblasts. Cytotoxic concentrations (LD_{50}) of vitamin E were 1166.5 ± 120.28 and 1060.8 ± 100.64 μM for intramuscular fibroblasts derived, respectively, from LD and ST muscles ($P > 0.05$). LD_{50} for vitamin C differed significantly between both groups of cells ($P < 0.0001$) with values of 239.5 ± 16.83 and 357.2 ± 10.8 μM for LD and ST, respectively. The concentrations of each vitamin used in the present study were well below their LD_{50} on IMCT fibroblasts, which ensures no toxicity in the study.

Effects of Vitamins on Latent MMP-2. For illustration purposes, representative zymograms of the culture media from the two groups of cells on the five treatments studied and the control are shown in **Figure 1**. It is important to note that, of the two principal gelatinases, only MMP-2 was detected in the zymograms. No other gelatinase activity bands were observed on the gels, suggesting that the other major gelatinase, MMP-9, was not present in detectable amounts in these experiments. The unique detection of MMP-2 in culture of IMCT fibroblasts may indicate the important role of this collagen-degrading enzyme in the remodeling of ECM in IMCT in vivo. In the zymogram two bands can be seen; an upper ≈71 kDa band corresponding to the nonactivated (or latent) pro-MMP-2 and a second band (fully activated MMP-2, of approximately 62 kDa) clearly seen just after treatment with a mixture of vitamins E and C. MMP-2 is expressed in an inactive or latent form (pro-MMP-2), which contains an extra peptide sequence folded over the active site of the enzyme and which is cleaved by other enzymes to activate the MMP extracellularly. In the zymography process the higher molecular weight of pro-MMP-2 is retained but the active site is exposed, so permitting its quantification separate from the lower molecular weight active form.

The expression of latent (nonactivated) MMP-2 by the cells derived from LD and ST muscles is shown in **Figure 2** expressed as a percentage change in comparison to levels found in the control cells. The addition of either 50 or 100 μM of vitamin E affected the expression of pro-MMP-2 to different extents ($P < 0.01$) in the

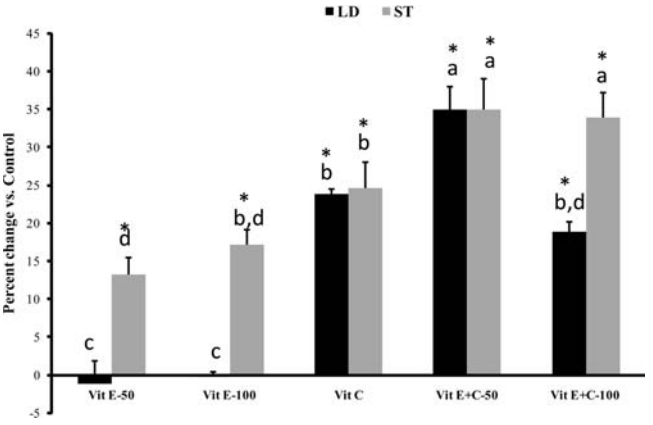

Figure 2. Effects of the presence of different concentrations of vitamins E (50 and 100 μM) and C (50 μM) and their mixture (50 μM vitamin E + 50 μM vitamin C; 100 μM vitamin E + 50 μM vitamin C) on latent matrix metalloproteinase 2 (pro-MMP-2) in media from cultured cells derived from bovine longissimus dorsi and semitendinosus muscle. Values are expressed as the percentage change versus control. Results are the mean ± SE of at least five experiments. ∗ indicates $P < 0.05$ comparing control versus addition of vitamins. Bars with different letters differ significantly ($P < 0.05$).

two groups of cells; pro-MMP-2 from ST cells responded differently from the control to vitamin E in a dose-dependent manner (50 μM, $P = 0.014$; 100 μM, $P = 0.004$), whereas pro-MMP-2 from LD cells did not differ ($P > 0.05$) from the control group of cells. Both cell groups expressed more pro-MMP-2 ($P < 0.001$) than the control when vitamin C was present. A synergistic enhancement of pro-MMP-2 expression was observed for LD and ST cells when vitamin C was combined with vitamin E; however, the vitamin E effect was no longer dose-dependent for ST.

Effects of Vitamins on Active MMP-2. When vitamin E was present, active MMP-2 secreted (**Figure 3**) by LD cells was higher ($P < 0.05$) than the values shown by the control. Although not statistically significant ($P > 0.05$), a trend of dose dependence was observed for this treatment. In contrast, MMP-2 secreted by ST-derived cells was similar ($P > 0.05$) to the control when either concentration of vitamin E was present in the cultures. Vitamin C induced an enhancement ($P < 0.0001$) of the levels of active MMP-2 for both groups of cells with regard to the control. The synergism between these two vitamins was not statistically evident with regard to MMP-2 activity; nevertheless, there was a trend showing higher values of active MMP-2 when LD ($P = 0.096$) and ST ($P = 0.082$) cells were exposed to the mixture of vitamin C and 50 μM vitamin E.

Effects of Vitamins on Total Soluble Collagen (TSC). The addition of vitamin E to fibroblast cells increased ($P < 0.05$) total soluble collagen synthesis to a greater extent in ST versus LD fibroblasts (**Figure 4**). However, in both cell groups and at either concentration of vitamin E (50 μM, $P < 0.0001$ for both cells; 100 μM, LD, $P = 0.002$, and ST, $P < 0.0001$) there was a significant increase in the production of TSC in comparison to the control. The collagen concentration for neither ST nor LD cells responded to vitamin E in a dose-dependent manner. Vitamin C induced a higher ($P < 0.05$) production of TSC in ST cells versus LD cells; however, in both cases, TSC values were higher (by 35% in ST ($P < 0.0001$) and by 25% in LD ($P < 0.01$)) in comparison to the control. When the responses of the cells to the mixture of vitamins E plus vitamin C were compared, it was noted that there were no synergistic effects ($P > 0.05$). Cytoplasmic staining for pro-collagen type I tended to increase for both cell types

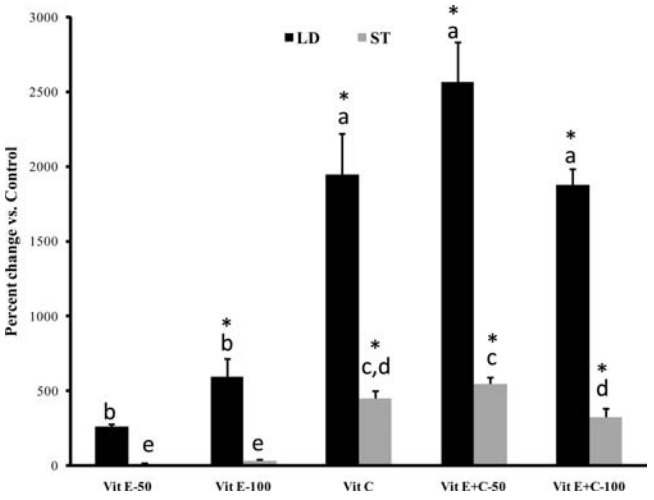

Figure 3. Effects of the presence of different concentrations of vitamins E (50 and 100 μM) and C (50 μM) and their mixture (50 μM vitamin E + 50 μM vitamin C; 100 μM vitamin E + 50 μM vitamin C) on matrix metalloproteinase 2 (MMP-2) activity in media from cultured cells derived from bovine longissimus dorsi and semitendinosus muscle. Values are expressed as the percentage change versus control. Results are the mean ± SE of at least five experiments. * indicates $P < 0.05$ comparing control versus addition of vitamins. Bars with different letters differ significantly ($P < 0.05$).

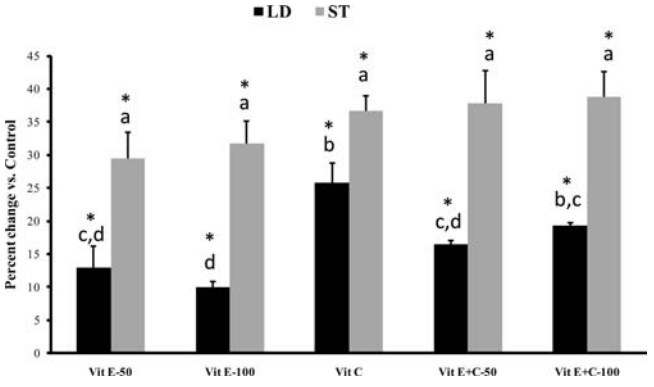

Figure 4. Effects of the presence of different concentrations of vitamins E (50 and 100 μM) and C (50 μM) and their mixture (50 μM vitamin E + 50 μM vitamin C; 100 μM vitamin E + 50 μM vitamin C) on total soluble collagen synthesis in lysates from cultured cells derived from bovine longissimus dorsi and semitendinosus muscle. Values are expressed as the percentage change versus control. Results are the mean ± SE of at least three experiments. * indicates $P < 0.05$ comparing control versus addition of vitamins. Bars with different letters differ significantly ($P < 0.05$).

when cells were exposed to a mixture of vitamins C and E (50 μM + 50 μM, respectively) in comparison to control cells (data not shown). A strong positive correlation was present for collagen production and levels of MMP-2 (LD, $r = 0.758$, $P = 0.0001$; ST, $r = 0.556$, $P = 0.007$) for both muscles (**Figure 5**).

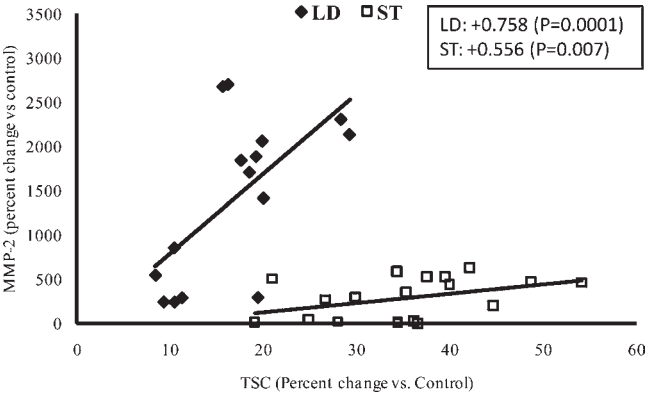

Figure 5. Correlations between levels of MMP-2 activity with total soluble collagen synthesis (TSC) from cultured cells derived from bovine longissimus dorsi (LD) and semitendinosus (ST) muscles. Statistical significance was defined as $P < 0.05$.

DISCUSSION

Improved meat tenderness has been reported when animals are killed at the moment of maximum collagen turnover (*18*). Turnover of collagen is rapid in young animals and, unlike other proteins, falls to a very low level as animals age and/or their growth rates decline (*19*). To increase collagen turnover and hence affect positively meat toughness, factors that increase the expression and activation of MMPs can be sought to trigger the degradation of older collagen, which may stimulate the production of more soluble immature collagen. In this study, a cell biology model was used to examine the effects that vitamins C and E may have on the activity of MMPs and collagen synthesis by fibroblast cells derived from two commercially important bovine muscles, LD and ST. Vitamin E is routinely supplemented in cattle feedlot diets, whereas the potential effects of vitamin C on animal performance have not been extensively studied. Furthermore, a synergistic effect between these two vitamins has been reported before in different models (*1*). This study demonstrated the impact that these vitamins alone and together may have in controlling the collagen metabolism in fibroblasts from the skeletal muscle of beef animals. To clearly demonstrate effects, the levels of both vitamins C and E used in this study are above blood serum levels normally found in cattle, but are very substantially below the levels reported above that cause cytotoxicity for the fibroblasts studied here.

Studies regarding the effect of vitamins on MMPs in diverse human cells have shown either no effect of α-tocopherol in the expression of MMP-2 (*20*) or, in some cases, results contrasting to those in the present study, in which vitamin E reduced MMP-9 expressions (*21*). However, Asbun et al. (*22*) obtained results similar to the present investigation's in that pretreatment of primary adult rat cardiac fibroblasts with vitamin E (45 μM) stimulated MMP activity. Asbun et al. (*22*) attributed this stimulatory effect to the ability of vitamin E to modify second-messenger systems, such as protein kinase C, that may affect the production of MMPs and/or tissue inhibitors of metalloproteinases (TIMPs).

In this study, treatment with vitamins increased TSC synthesis in fibroblasts isolated from both muscles; however, these results are contrary to those reported in the literature (*22*, *23*). Ahuja et al. (*23*) reported that the administration of 2.5 mM α-tocopherol decreased collagen production up to 50% in human cavernosal myofibroblasts. In contrast, Asbun et al. found that 45 μM vitamin E was not able to affect collagen synthesis in adult rat cardiac fibroblasts.

The in vitro data presented in this paper may have in vivo implications on animal production. Cattle cannot synthesize vitamin E and normally obtain it by dietary means. Because fresh-growing forages have adequate amounts of vitamin E, requirements are easily met in pasture-fed cattle. In contrast, stored feeds and grains are relatively low in vitamin E, and extended feeding of grain-based diets may deplete tissue α-tocopherol levels in cattle (*24*) such that supplementation is required. Animals finished on a concentrate diet may reach values

of muscle α-tocopherol similar to those of pasture-fed animals if receiving a supplementation of at least 1000 IU/day of vitamin E for a minimum period of 100 days (*25*). This may ensure the lowest concentration of α-tocopherol needed to maximize its antioxidant capacity in skeletal muscle. Therefore, cattle grazed on good pasture or on diets adequately supplemented with α-tocopherol can achieve sufficient concentrations of vitamin E in their muscles, which may help in maintaining a high rate of collagen turnover during animal production with positive implications for meat tenderness.

Most studies examining the effects of vitamin E supplementation on meat quality have focused mainly on color and lipid stability (*5*, *11*). Only a few researchers have investigated the effect of vitamin E on meat tenderness, with mixed results. Arnold et al. (*26*) studied the effect of vitamin E supplementation on longissimus lumborum steaks from steers fed a diet supplemented with *dl*-α-tocopheryl acetate to provide at least 300 IU/day of vitamin E for 9 months and found no tenderness differences between the vitamin E treatment and the control as determined by taste panel evaluation or WBSF measurements. In contrast, other studies have found positive effects with the use of this vitamin on beef tenderness (*6*, *7*). Carnagey et al. (*6*) found that 1000 IU/day of vitamin E administered for 104 days effectively improved meat tenderness of longissimus muscle steaks from beef heifers. Maiorano et al. (*7*) focused on the effect of vitamin E on collagen and demonstrated that intramuscular injections of vitamin E (total dose of 1200 IU/animal) given to growing lambs may improve intramuscular collagen properties by reducing the IMCT maturity. The authors attributed the lower maturity of IMCT to an increased rate of collagen turnover induced by the fastest preslaughter growth rate observed in lambs on the low-energy diet supplemented with vitamin E.

Similarly, findings in the present study regarding the effect of vitamin C in MMPs contrast with past studies wherein addition of ascorbic acid suppressed MMP activity in various cell types (*27*, *28*). On the contrary, different studies have found an increase in mRNA levels and secretion for collagens type I and type III in tissue cultures of different kinds of cells after treatment with vitamin C, which supports our findings (*29*). Ascorbic acid, apart from being a critical cofactor for collagen metabolism (*12*), is a potent cellular antioxidant, and, therefore, great care must be taken to ensure its bioavailability in vivo. Vitamin C deficiency will first impair collagen synthesis and then impair quenching of reactive free radicals, which may cause oxidative stress at the cellular level.

There is limited research examining the effect of vitamin C on beef quality. Our findings provide insight to the positive effect that this vitamin may have on an animal's collagen metabolism, which may influence the background toughness of beef. Synthesis of vitamin C by the ruminant liver apparently satisfies the metabolic requirement for ascorbic acid (*30*). Nevertheless, conditions such as housing stress on calves and the fattening process have been reported to dramatically decrease vitamin C levels in cattle (*31*, *32*). In certain situations, the endogenous vitamin C production in cattle may be insufficient to fight free radical production, which may influence meat quality. Furthermore, the supply of vitamin C for ruminants depends mostly on the synthesis of ascorbic acid in the liver because dietary vitamin C is easily degraded in the rumen; for this reason, when levels of ascorbic acid are impaired, cattle are more prone to vitamin C deficiency than other domestic animals.

Descalzo et al. (*11*) reported that levels of ascorbic acid in psoas major muscle from animals fed pasture presented significantly higher values of this vitamin in comparison to the same muscle from grain-fed animals. thereby increasing the overall antioxidant status in beef muscles from pasture-fed animals. This may help in the scavenging of free radicals, avoiding oxidative stress in cattle, which may further have implications in meat tenderness (*33*). Although vitamin C is not commonly used as a supplement to cattle feed, researchers have proposed administering vitamin C in a coated form to prevent degradation in the rumen but to be available for absorption from the intestinal tract (*34*). Use of a coated from of vitamin C has improved marbling and the firmness and texture of meat from Japanese cattle (*13*). Apart from the coated form of ascorbic acid, some researchers have also found that the administration of a more stable form of ascorbic acid such as ascorbyl-2-phosphate may increase the levels of this vitamin in muscle (*35*).

Vitamins C and E interact synergistically on an intracellular basis to provide antioxidant protection. In cell membranes, vitamin E is oxidized as it quenches peroxyl free radicals or reactive oxygen species (ROS). The intracellular vitamin C reduces the oxidized vitamin E to regenerate its activity so that vitamin E does not need to be replaced in the membrane (*1*). In addition, the positive relationships between collagen production and MMP-2 activities further suggest that vitamins may increase net collagen turnover by fibroblasts in bovine muscles. If animals are killed when maximum collagen turnover is attained, background toughness may be reduced considerably, which may be attributed to increased collagen solubility and the activation of MMP-2, with the latter being involved also in post-mortem degradation of collagen (*36*). Sylvestre et al. (*36*) found high values of active MMP-2 in muscles of fast-growing lambs and were also able to detect this enzyme even after 21 days of post-mortem aging. These authors suggested that due to additional MMP activity, collagen may become more soluble during post-mortem maturation of meat with some collagen being completely degraded to free hydroxyproline. Furthermore, these authors found a negative significant correlation between the levels of active MMP-2 and collagen insolubility in meat from lambs.

The signaling mechanism by which vitamins may increase MMP activity in bovine intramuscular fibroblasts is not clear. At the level of transcription, it has been reported that activation of mitogen-activated protein kinase (MAPK) plays an important role in the regulation of MMP expression (*37*). Additionally, MT1-MMP has also been implicated in the collagen-induced MMP-2 activation, suggesting that the presence of a considerable amount of active MT1-MMP protein on the fibroblast surface may be essential for MMP-2 activation (*38*). However, additional research is necessary to clarify the signaling mechanisms by which vitamins may increase MMP activity specifically in bovine intramuscular fibroblasts.

Vitamins affected collagen and MMP-2 expression to different extents in both groups of fibroblasts studied. These results correspond to our previous observations that fibroblast-like cells isolated from different beef muscles in the same animal presented different proliferative capacities and different expression of MMP-2 in vitro (*14*). These results may be of extreme importance because phenotypic differences in growth pattern and MMP-2 expression among fibroblasts from the same tissue but different locations may give rise in vivo to different extracellular matrix characteristics, including degree of collagen turnover. The different responses of the two muscle cells observed in the present study when vitamins were present may reflect in vivo the muscle-specific responses to other stimuli that some researchers have found within the same animal (*18*, *39*).

On the basis of the data obtained from the present work, it is probable that the MMP-2 activation induced by vitamins in intramuscular fibroblasts would favor synthesis of the extracellular matrix (ECM), which may support the reorganization of the

newly deposited collagen and other constituents of the ECM in a muscle-specific manner. This raises the possibility that, in vivo, the interaction between the collagen network and bovine intramuscular fibroblasts may induce the activation of MMP-2, which may, in turn, be involved in intramuscular extracellular matrix turnover, which could affect meat collagen solubility positively.

These findings have been obtained by deliberate comparison of fibroblasts isolated from different muscles within the same animal; unknown interanimal variations may therefore exist, but the current results do point to a need to continue investigations to better understand the effects that micronutrients may have on extracellular matrix remodeling and their possible impact on an animal model in vivo.

ABBREVIATIONS USED

DL_{50}, lethal dose 50; DMEM, Dulbecco's Modified Eagle Medium; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IMCT, intramuscular connective tissue; IU, international units; LD, longissimus dorsi; MAPK, mitogen-activated protein kinase; MMP(s), matrix metalloproinase(s); mRNA, messenger ribonucleic acid; PBS, phosphate buffer saline; pro-MMP, proenzyme matrix metalloproteinase; SDS, sodium dodecyl sulfate; SRB, sulforhodamine B; ST, semitendinosus; TSC, total collagen; TIMP(s), tissue inhibitor matrix metalloproteinase(s); X, xanthine; XO, xanthine oxidase.

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