Effects of dietary vitamin E and vitamin C supplementation on the level of α-tocopherol and L-ascorbic acid in muscle and on the antioxidative status and meat quality of pigs

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ABSTRACT: In total thirty pigs (Slovak Meaty) defined by DNA based test as not susceptible to malignant hyperthermia (non-mutant on *RYR1*) were used in the experiment. Treatment consisted in supplementation of vitamin E (500 mg α-tocopherol/kg diet as α-tocopherol acetate) (group E) and the same doses of vitamin E plus vitamin C (200 mg L-ascorbic acid/kg diet) (group E + C) to finishing pigs for the last 30 days before slaughter. The higher dietary vitamin E level resulted in higher levels of α-tocopherol in fresh (24 h), chill-stored (5 days, 4°C), chill-stored and cooked (80°C) and frozen meat (3 months, –25°C), ($P < 0.05$). Higher dietary vitamin C resulted in higher levels L-ascorbic acid in fresh and chill-stored meat ($P < 0.05$) but no significant differences vs. control pigs were observed in cooked and frozen meats. Supplementation with vitamins E and C (group E + C) had positive effects on pH (45 min) ($P = 0.06$) and on drip loss ($P < 0.05$) values as compared to control group. The rate of oxidation (malondialdehyde-MDA production) by stimulation with Fe^{2+}/ascorbate (incubation of muscle LD for 0 and 30 min) was higher in control group as compared to both experimental groups ($P < 0.05$). Positive effects of vitamin E on oxidative stability measured as thiobarbituric acid reactive substances (TBARS, MDA) were observed mainly in chill-stored meat ($P < 0.05$). Using TBARS method, no additional effect of vitamin C on oxidative stability of fresh, chill-stored, cooked and frozen meat was found. In conclusion, supplementation of the combination of vitamin E (500 mg α-tocopherol/kg diet) and vitamin C (200 mg L-ascorbic acid/kg diet) for 30 days before slaughter improved meat quality values (drip loss, pH), however, it seems to depend on the genetic background of animals (occurrence of mutation on *RYR1*). Oxidative stability of meat lipids measured as TBARS value can be improved by vitamin E supplementation to feed.

Keywords: pigs; feed; vitamin E supplementation; vitamin C supplementation; α-tocopherol in meat; L-ascorbic acid in meat; lipid peroxidation; meat quality

Phospholipids of biological membranes are particularly susceptible to peroxidation because of their high content of polyunsaturated fatty acids (PUFA) and the association with enzymatic and nonenzymatic systems capable of generating prooxidative free-radical molecules (Halliwell, 1997). Peroxidative changes in meat are initiated at the membrane level and it has been suggested that the membrane integrity and fluidity are affected (Monahan *et al.*, 1994; Storrey, 1996; Hayam *et al.*, 1997; Isabel *et al.*, 1999). Vitamin E is considered as the principal antioxidant defence agent against lipid oxidation in cell membranes in mammals. The advantages of vitamin E supplementation (commonly used as α-tocopheryl acetate) at supranutritional levels in the diet of pigs in terms of increasing the oxidative stability of phospholipids in the subcellular muscle membranes have been shown (Asghar *et al.*, 1991; Monahan *et al.*, 1994; Benzie, 1996; Jensen *et al.*, 1997). Dietary supplementation of vitamin E increases the concentration of α-tocopherol in muscle and reduces the susceptibility of

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the muscle to lipid oxidation (Buckley *et al*., 1995). The recommended level of dietary α-tocopheryl acetate supplementation in growing pigs is 15 to 40 mg/kg of feed (Albers *et al*., 1984), however, some meat quality parameters and oxidative stability are improved when dietary α-tocopheryl acetate levels are higher (200 to 500 mg/kg diet) as was shown by several researchers (Asghar *et al*., 1991; Monahan *et al*., 1992; Buckley *et al*., 1995; Cheah *et al*., 1995; Lauridsen *et al*., 1999; Turek *et al*., 1999; Isabel *et al*., 1999; Lahučký *et al*., 2000, 2001). Such studies showed the beneficial effects of vitamin E on lipid peroxidation in meat, usually evaluated by TBARS (thiobarbituric reactive substances) concentrations though there are some limitations of the factors discussed (Benzie, 1996). Skeletal muscle is particularly susceptible to oxidative reactions since it contains high concentrations of prooxidants (transition metals, haem-containing proteins, i.e. myoglobin, haemoglobin). Similarly susceptible is the lipid membrane which contains a higher proportion of polyunsaturated fatty acids (Kanner, 1994). Lipid oxidation is the primary cause of rancidity during storage of meat and meat products and pork-based products are much more susceptible to rancidity development than the other types of meat products (Buckley *et al*., 1989).

Vitamins E and C are primary antioxidants in biological systems and break the chain of lipid peroxidation. Many studies suggest that vitamin C and vitamin E act synergistically (Gey, 1998). Previous studies evaluating the efficacy of relatively high levels of vitamin C (>300 mg/kg) were inconsistent in saying that the growth or feed efficiency response may be due to the instability of vitamin C to a large extent. L-ascorbic acid is unstable when exposed to either oxygen or certain minerals, and this can result in rapid oxidation to the dehydroascorbic acid derivative. Later (Mahan *et al*., 1994) a stable source of vitamin C (magnesium-L-ascorbyl-2-phosphate) was used in pig feeding experiments. Another stable source of vitamin C (L-ascorbyl-2-polyphosphate, Rovimix® Stay-C® 25, Roche) was introduced (de Rodas *et al*., 1998). The supplementation of 75 mg/kg dietary vitamin C during the high-stress post-weaning period in pigs provides at least 25% ascorbic activity and improves performance and serum iron status. The pig synthesizes vitamin C and does not require it in the diet, but it is possible that endogenous synthesis is inadequate to maximize vitamin C's contribution to oxidative stability in some situations and studies of the effect of new stabilized forms on oxidative stability of pork would thus be useful (Pettigrew and Esnaola, 2000). Data from a pig experiment reported by Kremer *et al*. (1999) suggest that vitamin C supplementation before slaughter can improve parameters of meat quality.

Therefore the objective of this study was to evaluate the effects of vitamin E and C supplementation on the level of α-tocopherol and ascorbic acid and on the lipid peroxidation status in fresh, cooked and frozen pork and on some meat quality parameters.

MATERIAL AND METHODS

Animals and feeding

Thirty Slovak White Meaty pigs were used in this experiment. Control group (n = 10) and two experimental groups were homozygotes negative on malignant hyperthermia with equal number of gilts and castrates. The genotype (malignant hyperthermia, mutation on ryanodine receptor gene *RYR1*) was determined by a DNA based test (Bauerova *et al*., 1999). The pigs were penned in double boxes in Research Institute of Animal Production (RIAP, Nitra, Slovak Republic) facilities to minimise the influence of stress. Control group was fed a diet supplemented with basal level of α-tocopherol (Table 1). Experimental groups received a supplemental level of α-tocopherol (500 mg/kg) for 30 days before slaughter (group E, n = 10) and a supplemental level of α-tocopherol (500 mg/kg) and ascorbic acid (200 mg/kg) (group EC, n = 10) for 30 days before slaughter. Vitamin E (Rovimix® E-50 SD, stable source of vitamin E in feed) and vitamin C (Rovimix® Stay-C® 35, produced for use as a stabilized source of vitamin C in feed) were provided by a commercial company (Roche, Germany). Levels of α –tocopherol and ascorbic acid in diet are in Table 1 (Protocol 185–186/2004).

Animals were stunned, slaughtered and exsanguinated in the slaughterhouses of the RIAP (transportation about 200 m) at the average live weight 110 kg. After slaughter, the carcasses were chilled at 4°C for 24 h, and the *m. longissimus dorsi* (part *lumborum*, LD) was then removed from each carcass. A portion of the sample was used immediately (24 h) and the remaining samples were wrapped in aluminium film and stored in a refrigerator at 4°C for 5 days and in a freezer at –25°C for three months until analysed.

Table 1. Composition and nutritive value of diet

Item	% (weight)	Item	Control	Vitamin E + C
Wheat	24.0	Organic matter (%)	82.15	82.15
Barley	40.0	Crude protein (%)	17.42	17.42
Oat	10.0	Crude fat (%)	2.79	2.79
Soybean meal	12.0	Crude fibre (%)	4.51	4.51
Wheat meal	4.0	N-free extract (%)	57.43	57.43
Lucerne meal	3.0	Ash (%)	5.63	5.63
Meat and bone meal	2.0	Metabolisable energy (MJ)	12.38	0.91
Fish meal	1.0	Lysine (%)	0.91	12.38
Mineral supplement	3.0	α-tocopherol – added (mg/kg)	–	500.00
Fodder salt	0.4	– analysed (mg/kg)	33.6	515.0
Biofactor supplement	0.6	Vitamin C – added (mg/kg)	–	200.00
		– analysed (mg/kg)	90.3	189.20

Chemical analysis

The concentration of vitamin E (α -tocopherol) in (fresh, cooked, frozen) samples was determined by HPLC (Veterinary and Food Institute, Bratislava, Slovak Republic) using a modified method of Berlin *et al*. (1994). A mixture of 1.5 ml sample homogenate and 2 ml absolute ethanol and 0.5 ml 1% ascorbic acid was heated at 70°C for 5 min. After adding 1 ml 10 N KOH, the mixture was heated for 30 min. After cooling, 5 ml n-hexane was added for extraction. The solvent was removed by evaporation under nitrogen, and the residue was immediately resolved in absolute ethanol and HPLC analysis was performed employing methanol/water 97 : 3 v/v as mobile phase at a flow rate of 1 ml/min and a Hypersil WP 300 C4 column with a precolumn (12.5 × 0.4 cm i.d., 5μm particle size). Detection was performed by fluorescence at 292 nm excitation and 366 nm emission. Peaks were quantified upon calibration with authentic samples of α -tocopherol (Sigma).

For vitamin C (ascorbic acid) the methodology of Davidek *et al.* (1981) and Omaye *et al.* (1979) with 2,4-dinitrophenylhydrazine as a colour reagent was used.

To evaluate the stability of skeletal muscle lipids (fresh samples) against stimulated lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were determined using the modified method previously described by Buege and Aust (1978). To stimulate lipid peroxidation, 3 ml of muscle homogenate were incubated in 0.1 mM ascorbate and 5 μM $FeSO_4$ for different time intervals, at 37°C. Volumes of 0.5 ml were withdrawn and pipetted into 0.25 ml of 20% trichloroacetic acid (TCA) in 100 mM KCl at 0 and 30 min incubation time. These samples were centrifuged at 10.000 × g for 10 min and 0.5 ml of the supernatant was mixed with 0.5 ml thiobarbituric acid (0.67%) and boiled for 15 min in a water bath. The absorbance was determined at 535 nm immediately after cooling.

Lipid oxidation in (fresh, 5 days chill-stored, cooked, frozen for 3 months) samples was assessed by the 2-thiobarbituric acid method of Salih *et al.* (1987). Thiobarbituric acid reactive substances (TBARS) were expressed in terms of malondialdehyde (MDA, mg/kg tissue).

Meat quality measurements

The pH value of the carcass (*m. longissimus* – between the 13th and 14th rib) was determined in 45 min *post mortem* using the combined pH electrode (ingold). Electrical conductivity (Biotech instrument) was measured 3 h *post mortem*. Colour changes after refrigerated storage were measured on the freshly cut surface of a sample by using

Miniscan and L, a and b values were evaluated. Total water, protein and intramuscular fat were measured by the Infratec-Analyser (ash values were calculated). Drip loss analyses were made according to Honikel (1998). Shear force was determined in chill-stored and cooked samples (internal temperature 80°C, used also for further analyses) with a Warner-Bratzler (W-B) apparatus. The experiments were in accordance with the RIAP guidelines for animal care (1999).

Statistical analyses were calculated as mean values (of three repeated measurements) and standard deviations and differences were evaluated by *t*-test.

RESULTS AND DISCUSSION

The supplementation of vitamin E (α-tocopheryl acetate) to pigs increased the α-tocopherol levels (Figure 1) of fresh, chill-stored and frozen meat about twice compared to those observed in pigs fed the basal level of α-tocopheryl acetate (33.6 mg per kg of feed, Table 1). The levels of α-tocopherol in the *m. longissimus* in the present study when pigs received α-tocopheryl acetate 500 mg/kg feed (analysed 515 mg/kg, Table 1) are higher or comparable with previously reported results (Buckley *et al.*, 1995; Jensen *et al.*, 1997; Isabel *et al.*, 1999; Lahučký *et al.*, 2000, 2001; Harms *et al.*, 2003). The levels of α-tocopherol in muscle changed with the time of storing, however, the differences between fresh, chill-stored, cooked and frozen samples were not significant ($P > 0.05$). Dietary supplementation of vitamin C increased vitamin C concentrations in fresh meat (Figure 2) and to some extent in stored meat ($P < 0.05$). However, the level of vitamin C was lower ($P > 0.05$) in cooked meat. Sahin *et al.* (2002) also reported a higher level of vitamin C in serum and liver after dietary vitamin C supplementation (200 mg/kg) to Japanese quails using 2,4-dinitrophenylhydrazine as a colour reagent to measure the vitamin C content in tissue.

Table 2 shows contents of water, crude protein and intramuscular fat of meat (LD) 24 h after slaughter. The values were not influenced by dietary treatments as was shown earlier with supplementation 200 mg α-tocopherol/kg feed (Lahučký *et al.*, 2001). The level of total protein is comparable or a little lower than it was reported recently in heavier pigs by Corino *et al.* (2002) using Kjeldahl method for crude protein analysis.

It is known (Den Hertog-Meischke *et al.*, 1997; Krška *et al.*, 2001) that the effect of vitamin E supplementation on meat quality values is different for different muscles. As follows from Table 3, pH

Table 2. Chemical composition of the *m. longissimus dorsi*

Item	Control		Vitamin E supplemented		Vitamin E + C supplemented		Significance
	mean	S.D.	mean	S.D.	mean	S.D.	
Total water (%)	74.23	0.70	73.82	0.84	73.93	0.75	–
Total proteins (%)	22.41	0.41	22.53	0.50	22.46	0.89	–
Intramuscular fat (%)	2.76	0.76	2.81	0.94	2.81	0.81	–

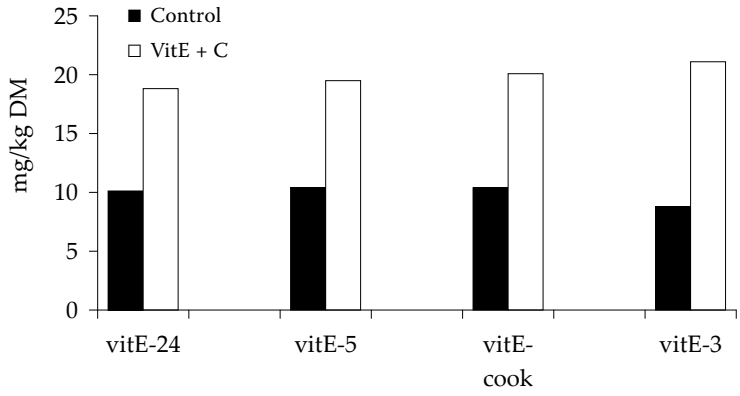

Figure 1. Content of α-tocopherol in muscle

vitE-24 = fresh meat 24 h
vitE-5 = chill-stored meat 5 days
vitE-cook = cooked meat
vitE-3 = frozen meat 3 months

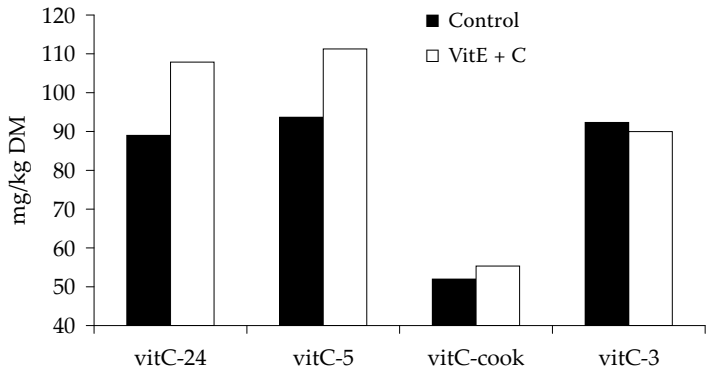

Figure 2. Content of ascorbic acid in muscle

vitC-24, 5, cook and 3 as vitE in Figure 1

value and colour (L value) in the *m. longissimus dorsi* were not significantly ($P > 0.05$) influenced by supplementation of vitamin E (group E). The results are in agreement with previous reports (Honikel *et al.*, 1998; Turek *et al.*, 1999; Lahučký *et al.*, 2000). The improvement in pH value ($P = 0.06$) after vitamin C and vitamin E supplementation (group E + C, Table 3) can support experimental data (Kremer *et al.*, 1999). They suggested that the addition of sodium oxalate or vitamin C to final meal given to pigs before slaughter resulted in higher early *post mortem* pH, but further studies on glycolysis and glycogen metabolism would be useful as vitamin C is known as a precursor of oxalic acid and sodium oxalate inhibits a key glycolytic enzyme, pyruvate kinase.

More papers (Asghar *et al.*, 1991; Monahan *et al.*, 1994; Cheah et al., 1995; Lahučký *et al.*, 2000) reported most evident reductions in drip loss when high levels of vitamin E were added to the diet, but there are also contradictory results about positive effects of vitamin E supplementation on drip loss (Buckley *et al.*, 1995; Jensen *et al.*, 1997; Honikel *et al.*, 1998; Waylan *et al.*, 2002) or the level of fluid in muscles (Lauridsen *et al.*, 1999). Using a higher level of vitamin E supplementation (500 mg/kg diet) administered for 46 days could reduce a drip loss in unfrozen *m. longissimus thoracis* in heterozygotes and in normal on malignant hyperthermia pigs as was shown by Cheah *et al.* (1995). We received a tendency of improving drip loss (lower value) in *m. longissimus dorsi* in 24 h *post mortem* of normal on malignant hyperthermia pigs supplemented with vitamin E (Table 3) and significantly lower value ($P < 0.05$) in pigs supplemented with vitamin E and vitamin C (group E + C). It could be a synergistic effect of vitamin C and vitamin E. Results reported by Sahin *et al.* (2002) also suggested that feed supplementation with a combination of dietary vitamin C (200 mg) and vitamin E (250–500 mg) might be a good management practice for reducing a heat stress-related decrease in the performance

Table 3. Pork quality (*m. longissimus dorsi*)

Trait	Time	Control		Vitamin E supplemented		Vitamin E + C supplemented		Significance
		mean	S.D.	mean	S.D	mean	S.D.	
pH	45 min	6.27	0.22	6.38	0.19	6.45	0.26	–
El. conductivity (μS)	3 h	4.06	1.01	3.67	1.22	3.93	1.12	–
Colour (L)	24 h	48.67	3.64	48.58	2.36	48.60	2.14	–
Free water (%)	24 h	37.84	2.95	36.73	3.33	36.41	3.25	–
Drip loss (%)	24 h	4.86	1.03	4.12	1.05	4.05	0.88	*
Colour (L)	5 days	51.63	2.82	51.69	3.75	50.84	2.66	–
Free water (%)	5 days	35.87	2.90	34.26	2.94	33.75	2.86	–
Shear force (kg)	5 days	4.09	1.15	4.82	0.71	4.66	0.66	–

*$P < 0.05$

of Japanese quails. It seems that supplementation of a high level of vitamin E and/or vitamin C level to the diet will reduce drip loss in some situations, but perhaps not in all situations. The genetic background (occurrence of mutation on ryanodine receptor gene, malignant hyperthermia status) of experimental pigs could influence the results as was discussed earlier (Lahučký *et al.*, 2000). Van Laack and Spencer (1999) also suggested that the fatty acid composition of the membrane phospholipids might influence the oxidative state of the muscle as well as of water-binding capacity. There were reported contradictory results on the shear force (tenderness) value in pigs supplemented with vitamin E (Mitsumoto *et al.*, 1995; Cannon *et al.*, 1996; Maiorano *et al.*, 1999; Waylan *et al.*, 2002). As results from the data of Table 3, the tendency of a higher level of the shear force value in pigs supplemented with vitamin E (Lahučký *et al.*, 2001) or with vitamin E and vitamin C could influence the aging time of meat. For further experiments the results reported by Vargas *et al.* (1999) and Montgomery *et al.* (2000) that dietary vitamin D_3 given 9 days before slaughter improved the tenderness (lower W-B shear force values) of 14 days post mortem aged beef could be interesting (interaction vitamin E and D_3).

It has been proposed that a high level of vitamin E in the diet might reduce the damaging oxidation of meat, and this area has been reviewed extensively (Morrissey *et al.*, 1994; Buckley *et al.*, 1995). The development of lipid oxidation measured as TBARS (MDA, mg/kg tissue) values in fresh, chill-stored, cooked and frozen meat is presented in Figure 3. Dietary levels of vitamin E (group E) and vitamin E and vitamin C (group E + C) did not substantially affect the development of lipid oxidation in the fresh meat (24 h), but significant differences ($P < 0.05$) were mainly received in chill-stored meat between control vs. supplemented pigs (group E and group E + C) and in tendency ($P = 0.06$) in frozen meat. In cooked meat (internal temperature 80°C) there were higher values of MDA in both control and supplemented groups but differences between control vs. supplemented groups were not significant ($P = 0.1$). No significant ($P > 0.05$) differences were received between group E vs. group E + C in fresh, chill-stored, cooked and frozen meats. It seems that using the TBARS method for the evaluation of oxidative stability the additional effect of vitamin C could not be sufficient as was also discussed by Pettigrew and Esnaola (2000) and by Yancey *et al.* (2002). In contrary, Kucuk *et al.* (2003) observed an additional effect of vitamin C supplementation on the MDA level in serum of laying hens. For further studies, using the TEAC (Trolox equivalent antioxidant capacity) method for the evaluation of watersoluble antioxidants would be useful (Furll and Rohl, 2003). Our results are comparable with observations of Asghar *et al.*, 1991; Buckley *et al.*, 1995; Jensen *et al.*, 1998; Corino *et al.*, 1999 and Isabel *et al.*, 1999, 2003), who used a higher level of α-tocopheryl acetate/kg feed for growing pigs, and a significantly higher level of α-tocopherol in muscle and reduced lipid oxidation of pork chops during chill storage regardless of packaging was reported. Thus, the level of α-tocopherol in muscle, which seems to depend on its level in feed and time of feeding, may be an important factor in determining the shelf-life of pork chops.

For lipid oxidation in fully cooked meat (in our experiment internal temperature 80°C), the pigment oxidation issue would be irrelevant, because meat pigments, whether deoxymyoglobin, oxymyoglobin or metmyoglobin, are all denatured when meat is cooked and could be also further factors for susceptibility to lipid oxidation after cooking (Rhee *et al.*, 2003).

Lipid peroxidation can be induced and enhanced by employing systems containing prooxidants like

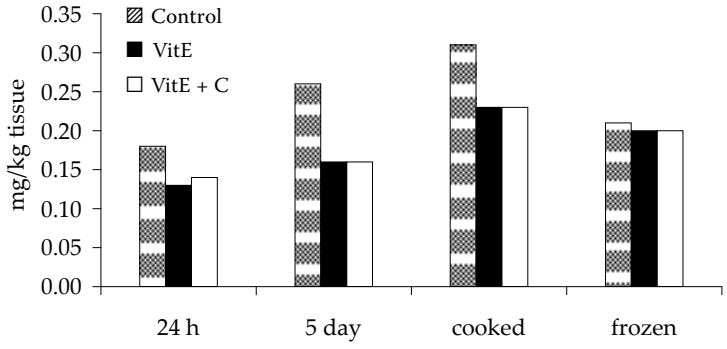

Figure 3. Level of thiobarbituric acid reactive substances (TBARS, MDA) in muscle

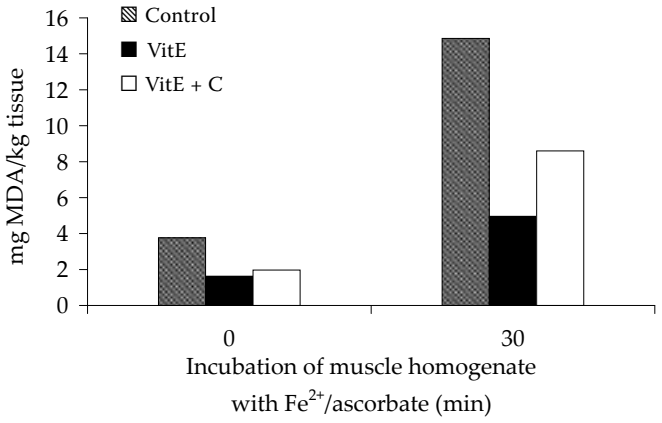

Figure 4. Antioxidative stability of muscle (incubation of muscle homogenate with Fe^{2+}/ascorbate)

Fe^{2+}/ascorbate. The possibility of a sample to slow the formation of peroxidative degradation products (MDA) in such systems is an indication of its antioxidative capacity (Nuernberg *et al.*, 2002). Figure 3 shows the accumulation of TBARS (MDA) after the incubation of fresh (24 h) muscle homogenates. Whereas the MDA of the control were increasing during 30 min of incubation, the increase was significantly ($P < 0.05$) lower in supplemented groups. Significant differences ($P < 0.05$) were also found between vitamin E group and vitamin E + C group. The illustrated differences (Figure 3) substantiate the occurrence of the protective action of vitamin E incorporated into the muscle tissue against peroxidation. Such a beneficial effect of dietary vitamin E on the oxidative stability of pork chops and ground meat after storage was also shown by others (Asghar *et al.*, 1991; Lauridsen *et al.*, 1999; Lahučký *et al.*, 2001; Nuernberg *et al.*, 2002). The higher level of MDA after 30 min incubation in iron-induced lipid oxidation of LD homogenate (group E + C) as compared to vitamin E group can be explained by findings that additional vitamin C can exhibit either antioxidative or prooxidative (at a higher level of Fe) effects (McDowell, 1989). As was shown (O'Sullivan *et al.*, 2003), vitamin E in conjunction with vitamin C promoted non-supplemental iron absorption in the vitamin E-treated group for *m. longissimus dorsi* and this effect may prove useful nutritionally for both animals and humans as a means of improving the iron stores of individuals with a reduced iron status.

It is clear from the data that a high level of vitamin E in the pig diet improves the oxidative stability of pork. However, the appropriate duration of feeding a high level of vitamin E can be discussed. In agreement with Isabel *et al.* (2003), dietary strategies for improving meat quality and antioxidative stability characteristics are not commonly used due to extra feeding costs, which are difficult to be recovered in the commercial setting. However, the relatively higher added value of processed meats compared to fresh meat could provide an economic advantage to the use of dietary strategies for reducing deterioration during storage, or for improving quality characteristics. This provides an interesting area of research to define dietary strategies to optimise quality, and their possible interactions with technological processes.

In conclusion, dietary supplementation of vitamin E (500 mg α-tocopheryl acetate/kg feed) and vitamin C (200 mg/kg feed) to growing-finishing pigs increases the concentrations of α-tocopherol and ascorbic acid in meat (*m. longissimus dorsi*). Supplementation of vitamin E and vitamin C improves meat quality parameters (drip loss, pH), but the results can be influenced by the genetic background of animals (occurrence of mutation on ryanodine receptor gene, malignant hyperthermia status). Lipid oxidation measured as TBARS (MDA) and antioxidative capacity (Fe^{2+}/ascorbate induced) of meat can be positively influenced by supplementation of vitamin E to growing-finishing pigs. Further research is continuing to determine if the *post mortem* application (by injection) of ascorbic acid (calcium ascorbate) at different level of α-tocopherol (supplementation by feeding before slaughter) in meat has beneficial effects on quality and antioxidative parameter development during chill-storage.

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