Journal of Animal and Feed Sciences, 26, 2017, 226–235 *https://doi.org/10.22358/jafs/76595/2017*
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

Effects of dietary vitamin E (DL-α-tocopheryl acetate) and vitamin C combination on piglets oxidative status and immune response at weaning

A.I. Rey[1,3], C.J. López-Bote[1] and G. Litta[2]

[1] Complutense University of Madrid, Faculty of Veterinary Medicine, Department of Animal Production, 28040 Madrid, Spain
[2] DSM Nutritional Products, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland

KEY WORDS: immunoglobulins, vitamin E, vitamin C, stress conditions, piglets

Received: 2 February 2017
Revised: 3 May 2017
Accepted: 7 September 2017

[3] Corresponding author:
e-mail: anarey@vet.ucm.es

ABSTRACT. The aim of the study was to evaluate the effect of diet supplementation with vitamin E (40 or 250 mg α-tocopheryl acetate · kg^{-1}) and/or vitamin C (0, 200 or 500 mg ascorbic acid · kg^{-1}) for 40 days after weaning at 28 day of age on α-tocopherol concentrations in tissues, oxidative status and immune response in piglets (n = 144; 7.99 ± 0.12 kg). High level of dietary vitamin E addition into piglet diet resulted in increased concentration of α-tocopherol in serum and liver at days 42 and 68 after weaning when compared to the low-dose vitamin E-supplemented groups. Such effect was not found in piglets muscles and fat until day 68. At day 68 the antioxidant status in piglets fed high-dose vitamin E-supplemented diet increased and muscle oxidation (TBARS) decreased mainly at day 68 with no changes in the immune response in comparison to low-dose vitamin E-supplemented groups. Dietary vitamin C supplementation did not affect α-tocopherol levels in serum, liver and muscle, whereas α-tocopherol content in fat tended to be higher in piglets fed diets supplemented with both vitamins at day 68. Dietary vitamin C increased the serum antioxidant power (FRAP) and immunoglobulin (Ig) M tended to be higher at 42 day of age, while at day 68 IgA concentration increased. An interaction effect of both vitamins on the FRAP or muscle TBARS values was not observed; however, IgA increased only in groups fed diets supplemented with vitamin C and low dose of vitamin E. A long-term supplementation of the high-dose vitamin E-enriched diet is recommended for improving the oxidative status of piglets in the post-weaning period. An additional vitamin C-enrichment may have beneficial effects on oxidative status and the immune response, especially if the serum α-tocopherol concentration is close to values of 1 mg · ml^{-1}.

Introduction

Weaning is one of the most critical periods for piglets. After separation from sow, piglet suffers an increased stress which is not only accompanied by a low feed intake. It also leads to an unbalance between the oxygen reactive substances production and the organism antioxidant defence which results in a cellular damage (Debier, 2007). Consequently, the incidence of diseases is high due to lowered immune response (Bonnette et al., 1990) and results in high economical losses for the pig industry.

Many dietary additives have been studied in order to mitigate post-weaning stress. Antioxidant vitamins such as E and C could be considered for this. Vitamin C (ascorbic acid) acts as a catalyst in

oxide-reduction reactions and as a reducing agent that neutralizes free radicals in aqueous compartments (Hamilton et al., 2000). Supplementation of piglet diets with vitamin C is not essential since this vitamin is synthesised by these animals, nevertheless, the post-weaning stress may disturb this process (Lauridsen and Jensen, 2005). Hence, vitamin C supplementation in weaning piglets can increase immunoglobulin concentration (Zhao et al., 2002; Lauridsen and Jensen, 2005) and improve performance parameters (Bekenev et al., 2015). It was found that vitamin C is able to reduce stress effects in fish (Peng et al., 2013) and chicken (Chand et al., 2014). However, in weanling piglets there were only some or no beneficial effects (Mahan and Saif, 1983). Additionally, vitamin C may also regenerate vitamin E (α-tocopherol) which is considered to be the most effective lipid-soluble chain-breaking antioxidant that protects cellular membrane against oxidative damage (Buckley et al., 1995). Vitamin E is not only an efficient antioxidative agent in tissues which alleviates oxidative stress by capturing free radicals and other reactive substances (Buckley et al., 1995), but it may also increase the immune response (Fragou et al., 2004) by enhancing cell protection. So, as the serum vitamin E level declines after weaning (Amazan et al., 2012) due to lower feed intake and increased stress, the incorporation of both these vitamins may be promising.

However, the information on the combine effect of the dietary supplementation of vitamin E and C in piglets at weaning is lacking. Most of the studies in which the effects of both vitamins were examined aimed to improve meat quality parameters and lipid stability in growing pigs or during the fattening phase (Eichenberger et al., 2004; Gebert et al., 2006; Peeters et al., 2006). In a recent study conducted on piglets to prevent stress effects, it was found that the usage of both vitamins with *Eleutherococcus senticosus* extract may be useful to improve performance parameters (Bekenev et al., 2015). However, this study only tested one dose combination, hence additional investigations on these nutrient combination deserve more attention.

It was hypothesised that in piglets, dietary combination of vitamins E and C may preserve the antioxidant status decreasing post-weaning stress effects. The aim of the present research was to examine the effect of different dietary doses of vitamins E, C and its combination on serum and tissues tocopherol concentration, oxidative stress and immunoglobulin content at different times after weaning in piglets.

Material and methods

All experimental procedures were performed in accordance with the Spanish guidelines for the care and use of animals in research (BOE, 2013).

Animals and experimental diets

In total, 144 (72 male and 72 female) 7-day-old piglets (PIC Camborough × Pietrain; Pigchamp Pro Europa S.L., Segovia, Spain) weighting approximately 2–3 kg were used in the study. Piglets stayed with their mothers until weaning (28 days; 7.99 ± 0.12 kg body weight (BW)) and afterwards were randomly allocated to 24 experimental pens (6 piglets in each; 3 males and 3 females) and fed according to six different dietary treatments (Table 1) with a factorial arrangement: 3 doses of dietary vitamin C (0, 200 and 500 mg ascorbic acid · kg^{-1}) and 2 doses of dietary vitamin E (40 vs 250 mg α-tocopheryl acetate · kg^{-1}). Each treatment was replicated 4 times to obtain 24 piglets per treatment.

The experimental diets were formulated by Pigchamp Pro Europa S.L (Segovia, Spain) for two feeding periods: pre-starter – days 28–41 and starter – days 42–68. Diets, manufactured in a commercial feed mill (Nuri i Espadaler, S.L., Vic, Barcelona, Spain.) under the direct supervision of qualified personnel of Pigchamp Pro Europa S.L. DSM (Segovia, Spain), were calculated to be isonutritive, and to meet or exceed NRC (2012) nutrient requirements for piglets (Table 1).

Samples collection

Blood samples were collected from jugular vein on different days: immediately after weaning at day 28 (2 piglets per pen), at the final of the pre-starter period (day 42, 2 piglets per pen, 8 piglets per treatment) and at the end of the starter period (day 68, 2 piglets per pen, 8 piglets per treatment). After collection all blood samples were immediately placed on ice. The serum was then centrifuged at 600 *g* for 10 min at 4 °C and the supernatant was kept in a freezer at –80 °C until further analysis. Analyses were carried out within the next 2 months.

At the end of each experimental period (at 28, 42 and 68 day of age), 2 piglets per pen were euthanized in the farm. Samples of liver, *longissimus dorsi* muscle and subcutaneous fat were vacuum packed and stored at −20 °C until analysis.

Chemical analysis

The α-tocopherol concentration in serum was quantified as described by Rey et al. (2006) by direct extraction. Thus, serum samples were mixed

Table 1. Composition of experimental diets

Indices	days 28–41	days 42–68
Ingredients, %		
maize	40.19	40.00
barley	15.33	27.78
soyabean flour (44% CP)	10.00	17.76
Soycomil®	11.83	3.00
sweet serum	15.00	5.00
soyabean oil	4.14	2.50
bicalcium phosphate	1.64	1.83
L-lysine HCl 78	0.54	0.52
MHA Methionine 84	0.28	0.22
L-threonine	0.19	0.21
Valine	0.17	0.14
L-tryptophan 98	0.10	0.09
salt	0.20	0.31
calcium carbonate	0.09	0.34
premix[1]	0.30	0.30
Calculated composition[2], %		
dry matter	90.13	89.44
crude protein	18.47	17.78
crude fibre	3.00	3.31
crude fat	6.28	4.77
ash	6.39	5.76
net energy, kcal · kg^{-1}	2582	2457
Vitamin E, mg α-tocopherol · kg^{-1}		
α-Tocopheryl acetate (40 mg · kg^{-1})	48	36
α-Tocopheryl acetate (250 mg · kg^{-1})[3]	292	262
α-Tocopheryl acetate (40 mg · kg^{-1}) + + ascorbic acid (200 mg · kg^{-1})[4]	48	40
α-Tocopheryl acetate (250 mg · kg^{-1})[3] + ascorbic acid (200 mg · kg^{-1})[4]	280	246
α-Tocopheryl acetate (40 mg · kg^{-1}) + +ascorbic acid (500 mg · kg^{-1})[5]	51	39
α-Tocopheryl acetate (250 mg · kg^{-1})[3] + ascorbic acid (500 mg · kg^{-1})[5]	267	258

[1] premix 0.3% (3 kg · t^{-1}): IU: vit. A 15 000 000, vit. D_3 1 800 000; g: vit. E 40, vit. K_3 2, vit. B_1 2, vit. B_2 4, vit. B_6 3, nicotinic acid 20, pantothenic acid 10, folic acid 0.8, biotin 0.1, choline chloride 200, Mn (oxide) 40, I (calcium iodide) 0.5, Co (cobalte carbonate monohidrate) 0.25, Zn (oxide) 100; Cu (pentahidrate sulphate) 150, Fe 145, Se (sodium selenite) 0.1, Excipients (E562) 3; mg: vit. B_{12} 20, BHT and ethoxyquin E324 80; [2] according to de Blas et al. (2010); [3] to reach the final vitamin E concentration, 8.4 kg · t^{-1} of α-tocopheryl acetate was added to control diet; [4] to reach 200 mg vit. C · kg^{-1} diet, 0.60 kg · t^{-1} of pure vit. C (33 %) was added to control diet; [5] to reach 500 mg vit. C · kg^{-1} diet, 1.5 kg · t^{-1} of pure vit. C (33 %) was added to control diet

with 0.054 M dibasic sodium phosphate buffer adjusted to pH 7.0 with HCl and absolute ethanol. After mixing, tocopherol was extracted with hexane by centrifugation. The upper layer was evaporated to dryness and dissolved in ethanol prior to analysis. Tocopherols were analysed by reverse phase high-performance liquid chromatography (HPLC) (HP 1100, equipped with a diode array detector; Agilent Technologies, Waldbronn, Germany) as described by Rey et al. (2006). Identification and quantification were carried out by means of a standard curve set (R^2 = 0.999) using the pure compound (Sigma-Aldrich, St. Louis, MO, USA). All samples were analysed in duplicate.

The procedure described by Rey et al. (2006) was used to analyse α-tocopherols in subcutaneous fat and feeds. Samples were saponified in presence of pyrogalol (3% in ethanol), KCl (1.15%) and KOH (50%) and afterwards mixed with hexane. To extract α-tocopherol from *longissimus dorsi* muscle and liver, the direct extraction procedure and HPLC analysis was the same as described above for serum samples.

The ferric reducing antioxidant power (FRAP) was measured using the procedure described by Benzie and Strain (1999). The FRAP reagent was prepared fresh by mixing 10 vol of acetate buffer (300 mM) with 1 vol of 10 mmol TPZ solution (2,4,6-tripyridyl-s-triazine in 40 mM HCl) and 1 vol of 20 mM aqueous ferric chloride. A 100-µl aliquot of the sample extract was mixed with 3 ml of the working FRAP solution. The absorbance of the samples was recorded after 0 and 4 min at 593 nm. Results were expressed as µM.

Reduced and oxidized glutathione (GSH and GSSH, respectively) were quantified spectrophotometrically at 405 nm in deproteinized serum samples at weaning and after 5 and 20 days post-weaning using diagnostic colourimetric kit (Arbor Assays, Arbor, MI, USA). The concentration of GSSH was determined from 2-vinylpyridine-treated samples with 2-vinylpyridine-treated standard curve. Free glutathione (free GSH) concentrations were obtained by subtracting the GSSH levels obtained from the 2-vinylpyridine-treated standard from non-treated standards and samples (total GSH). The obtained concentrations were expressed as µM of glutathione.

The susceptibility of muscle and liver tissue homogenates to iron-induced lipid oxidation was determined by a modification of Kronbrust and Mavis (1980) method, in which 1 mM $FeSO_4$ was used as the catalyst of lipid oxidation (approximately 1 mg · ml^{-1} buffer). Tissue homogenates were incubated at 37 °C in 40 mM Tris-malate buffer (pH 7.4) with 1 mM $FeSO_4$ in a total volume of 10 ml. At fixed time intervals, 1 ml aliquots were removed for measurement of thiobarbituric acid-reactive substances (TBARS). Absorbance was measured at 532 nm and the values were expressed as nM malondialdehyde (MDA) · g^{-1}.

Immunoglobulins (Ig) A, G, and M were determined in piglet serum using a pig ELISA quantification kit (Bethyl Laboratories Inc., Montgomery, TX, USA). Briefly, for IgM determination, purified antibody (porcine IgM) was diluted in coating buffer (0.05 M carbonate-bicarbonate at pH 9.6) and incubated at room temperature for 60 min in a 96-well plate. The plate was then washed 5 times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) to remove the non-coated antibody. Blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was added to each well and the plate was incubated at room temperature for 30 min. The blocking solution was removed and the plate was washed again. Sample was then added and the plate incubated for 1 h, after which the diluted HRP-conjugated pig detection antibody was added. Finally, tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 min. After incubation with the substrate, the reaction was quenched and the absorbance measured at 450 nm. Similar protocols were used for the IgA and IgG quantifications. A standard curve was prepared for each ELISA batch, and the final values were expressed in mg · ml^{-1}.

Statistical analysis

The experimental unit for analysis of all data was the pen. Data were analysed following a completely randomized design using the general linear model (GLM) procedure included in SAS (version 8; SAS Inst. Inc., Cary, NC, USA). Dietary vitamin E and C supplementations were considered fixed effects according to the following model:

$$Y_{ec} = \mu + \alpha_e + \beta_c + (\alpha\beta)_{ec}$$

where: Y_{ec} – dietary treatment or age response dependent variable, μ – overall mean, α_e – dietary vitamin E supplementation effect, β_c – dietary vitamin C effect, $(\alpha\beta)_{ec}$ – corresponding interaction.

Data are presented as the mean of each group and root-mean-square error (RMSE) together with the significance levels (*P*-value). Differences between means were considered statistically significant at $P < 0.05$.

Results and discussion

Piglet performance. Dietary vitamin E or C did not affect average daily gain (ADG) or transformation index (TI) from day 28 to 68 (Table 2). The results of previous studies conducted on piglets and vitamin C did not reveal statistically significant differences in performance parameters (Mahan and Saif, 1983; Zhao et al., 2002). Also no effects of dietary vitamin E on performance were found by Bonnette et al. (1990) using four different doses of this vitamin (10, 110, 220 and 550 mg · kg^{-1}). The combination of both vitamins at different doses in the present study from day 28 to 68 did not modify the piglet growth. This result is in contrast to the results of the study of Bekenev et al. (2015) in which a combination of 80 mg of 25% tocopherol, 500 mg vitamin C and 3 ml *Eleutherococcus senticosus* extract in piglets (at 22–26 and 31–36 days of age until they reached 12–15 weeks) increased average daily gain and live weight at the end of the experiment.

Also, in piglets supplemented with high dose of vitamin E lower average daily feed intake (ADI) from day 42 to 68 of age ($P = 0.0272$) was estimated, whereas other performance parameters were not affected (Table 2). Peeters et al. (2006) also found that pigs supplemented with vitamin E (150 mg · kg^{-1}) ate less than control or vitamin C-supplemented pigs. However, these results cannot be conclusive since the experiment was replicated only four times.

Tocopherol concentration in tissues. The α-tocopherol concentration in serum (Table 3) in the group supplemented with dietary α-tocopheryl acetate was similar to the values reported by Moreira and Mahan (2002) who examined similar feed inclusion levels. The highest drop in α-tocopherol concentration was detected immediately after weaning (days 28–42) due to the increased stress and low feed intake – issues widely reported in the literature (e.g., Moreira and Mahan, 2002; Lauridsen and Jensen, 2005; Amazan et al., 2012). According to the present results, supplementation of 250 mg all-rac-α-tocopheryl acetate · kg^{-1} to piglet diet resulted in higher α-tocopherol serum concentration at 42 and 68 day of age ($P = 0.0001$) when compared to low-vitamin E- supplemented groups. Accumulation of α-tocopherol was higher in fat than in liver, followed by muscle (Buckley et al., 1995). Concentration of α-tocopherol in tissues was also affected by dietary α-tocopheryl acetate supplementation from days 42 to 68. However, α-tocopherol concentration in fat and muscle were not statistically affected by supplementation of high dose of dietary vitamin E to piglets at day 42 when compared to those receiving a low-dose supplementation. The highest amount of α-tocopherol was estimated in fat (Buckley et al., 1995). Also there was a lack of losses due to post-weaning stress which could be explained by the high previous concentration of α-tocopherol at day of weaning and the low mobilization of vitamin E from this tissue. Lauridsen and Jensen (2005)

Table 2. Performance parameters (body weight (BW, kg), average daily gain (ADG, kg), average daily intake (ADI, kg), feed conversion ratio (FCR) of piglets as affected by dietary vitamin E (as α-tocopheryl acetate; 40 and 250 mg · kg^{-1}) or vitamin C (ascorbic acid; 0, 200 and 500 mg · kg^{-1}) supplementation

Vitamin C	0		200		500		RMSE	*P*-value			*P* covariate
Vitamin E	40	250	40	250	40	250		vit. E	vit. C	vit. E x vit. C	
BW at day											
41	10.51	10.53	10.19	9.81	10.27	10.02	0.520	0.1980	0.7569	0.7021	0.0001
67	23.10	22.30	21.95	21.46	22.13	21.51	1.253	0.1519	0.4338	0.9872	0.0001
ADG, days											
28–41	0.19	0.20	0.18	0.14	0.17	0.14	0.040	0.1980	0.7570	0.7021	0.3571
42–68	0.48	0.45	0.45	0.45	0.46	0.44	0.043	0.3575	0.5223	0.8456	0.0118
28–68	0.39	0.37	0.36	0.34	0.36	0.34	0.032	0.1519	0.1503	0.9872	0.0119
28–41	0.26	0.24	0.24	0.22	0.27	0.23	0.038	0.0790	0.6142	0.5863	0.1345
42–68	0.83	0.80	0.84	0.77	0.78	0.77	0.061	0.1254	0.2777	0.3414	0.0010
28–68	0.61	0.56	0.59	0.55	0.57	0.54	0.048	0.0272	0.4060	0.7621	0.0018
FCR, days											
28–41	1.33	1.16	1.38	1.85	1.60	1.62	0.408	0.5331	0.1430	0.2827	0.7877
42–68	1.73	1.76	1.86	1.73	1.71	1.78	0.202	0.9215	0.8577	0.3189	0.8062
28–68	1.57	1.51	1.64	1.60	1.58	1.60	0.170	0.6563	0.6770	0.7728	0.6299

data presented as mean and root-mean-square error (RMSE)

Table 3. Concentration of α-tocopherol concentration in piglets tissues from 28 to 68 day of age as affected by dietary vitamin E (as α-tocopheryl acetate; 40 and 250 mg · kg^{-1}) or vitamin C (ascorbic acid; 0, 200 and 500 mg · kg^{-1}) supplementation

Vitamin C	0		200		500		RMSE	*P*-value		
Vitamin E	40	250	40	250	40	250		vit. E	vit. C	vit. E x vit. C
Serum α-tocopherol, μg · ml^{-1}										
28 days*	5.26	5.26	5.26	5.26	5.26	5.26				
42 days	1.35	2.69	1.50	2.83	1.46	3.06	0.35	0.0001	0.7041	0.8785
68 days	1.08	3.12	1.13	3.29	1.15	3.15	0.22	0.0001	0.8183	0.8957
Muscle α-tocopherol, μg · g^{-1}										
28 days*	5.43	5.43	5.43	5.43	5.43	5.43				
42 days	3.51	3.85	3.73	3.59	3.44	3.67	0.44	0.6916	0.9577	0.8499
68 days	1.51	3.34	1.20	3.45	1.63	3.40	0.21	0.0001	0.6722	0.4686
Liver α-tocopherol, μg · g^{-1}										
28 days*	8.25	8.25	8.25	8.25	8.25	8.25				
42 days	2.99	6.55	2.47	5.51	3.02	5.49	0.61	0.0001	0.4365	0.6776
68 days	2.35	11.76	3.42	11.44	3.03	8.62	1.26	0.0001	0.4157	0.3165
Fat α-tocopherol, μg · g^{-1}										
28 days*	11.96	11.96	11.96	11.96	11.96	11.96				
42 days	17.44	17.49	17.40	17.03	18.44	18.23	1.44	0.8829	0.7163	0.9893
68 days	12.88	24.89	11.93	27.59	14.58	23.37	1.40	0.0001	0.7911	0.0605

data presented as mean and root-mean-square error (RMSE); * – α-tocopherol concentration was determined at 28 day from two piglets per pen

studied the transfer of α-tocopherol from sows supplemented with different dietary doses of α-tocopheryl acetate to piglets and found a decrease in the tocopherol concentration in plasma, heart, liver and muscle from day 35 to 42, whereas an increase was observed in adipose tissue of piglets from sows supplemented with 250 mg α-tocopheryl acetate · kg^{-1}. In the present research, liver was the tissue in which α-tocopherol accumulation was more affected by supplementation of high dose of dietary vitamin E to piglets from day 28 to 42 (P = 0.0001), similarly as observed in serum. Hence, changes in liver tocopherol concentration were more marked due to dietary supplementation, and high dose of dietary vitamin E to piglets decrease the rate of post-weaning losses. This result indicate a different metabolic activity of tissues as observed by Lauridsen and Jensen (2005), with the liver as the most metabolically active tissue in which mobilization of α-tocopherol was faster than in the others to maintain serum α-tocopherol homeostasis.

Interestingly, similar concentration of α-tocopherol in serum, liver and muscle was found in post-weaned piglets (at different age) supplemented with dietary vitamin C to those supplemented with dietary vitamin E in the feed. However, α-tocopherol concentration in fat tended to be statistically affected ($P = 0.060$) at day 68 of age by the interaction effect of dietary vitamin E and vitamin C. Hence, α-tocopherol concentration in fat of piglets supplemented with a combination of high doses of vitamin E and vitamin C was numerically greater than those from piglets that were not supplemented with vitamin C or those that received basal levels of vitamin E. These results would indicate that dietary vitamin C probably enhances the tocopherol levels as observed by other authors *in vivo* (Lauridsen and Jensen, 2005) by its regeneration. However, according to the present study this effect was not observed in those tissues which suffer a higher mobilization such as liver or serum.

Oxidative status in piglet serum, muscle and liver. The total antioxidant capacity measured as FRAP was quantified in serum (Table 4) to evaluate oxidative stress in piglets supplemented post-weaning with dietary vitamin E or C. FRAP values were within the limits presented in the literature (Lauridsen and Jensen, 2005). Dietary vitamin C increased FRAP values at 42 day of age ($P = 0.05$), whereas it did not affect the serum antioxidant capacity at day 68. According to Mahan and Saif (1983), vitamin C supplementation may have a temporary effect upon weaning, since a trend to beneficial effects in two trials during the initial 2-week period was found. However, vitamin E supplementation to piglets only increased the antioxidant status at 68 day of age when compared to pigs fed basal levels of vitamin E ($P = 0.033$). Benzie and Strain (1999) reported that vitamin C contributes 15% to FRAP values, whereas vitamin E only 5%. In the present study, differences in serum α-tocopherol at 42 day of age were 50% in high-dose vitamin E-supplemented groups in comparison to groups supplemented with the basal dose of vitamin E in the diet; at 68 day of age were 65%. These results would indicate that due to the initial high concentration of serum vitamin E at weaning, FRAP values were not modified by the low contribution of vitamin E to its values. However, a long-term post-weaning vitamin E supplementation would maintain an optimum serum vitamin E concentration and antioxidant power. Hence, FRAP values increased from day 42 to 68 by the dietary vitamin E supplementation, being higher in those serum samples from piglets that received high vitamin E dose.

Some investigators have previously reported the effectiveness of dietary vitamin C (Benzie and Strain, 1999; Hamilton et al., 2000; Perai et al., 2014) or vitamin E (Benzie and Strain, 1999; Hamilton et al., 2000; Amazan et al., 2012) supplementation on controlling FRAP values. However, the studies on combination of both vitamins are lacking. In the present study, an interaction effect of both vitamins on the FRAP values was not observed, in accordance with the lack of interaction effect between vitamin E and vitamin C on serum α-tocopherol. These results are in disagreement with those reported by Hamilton et al. (2000) where total antioxidant properties of serum from adult humans were improved by ascorbic acid following α-tocopherol supplementation and *vice versa*. However, that study was not carried out in stressful conditions. In contrast, Zeferino et al. (2016) in the study on chickens found that diet supplementation with vitamins C and E as antioxidants did not mitigate any of the negative effects occurring under heat stress.

The GSH concentration was also quantified to evaluate the antioxidant properties of piglet serum (Table 4). Free GSH and GSSH concentrations in plasma have been considered to be an useful indicator of disease risk (Jones et al., 2000) since GSH is converted into GSSH during oxidative stress. In the present study, dietary vitamin C and/or E supplementation to piglets did not affect the GSH, GSSH and free GSH concentrations or the GSSH:GSH ratio at day 42 or 68 of age. Other authors (e.g., Rey et al., 2013) did not find any effect of dietary vitamin E supplementation on antioxidant enzymes such as GSH in stressful situations. However, Amazan et al. (2012) reported that vitamin E decreased glutathione in its reduced form. To our knowledge, there is not much information in the literature concerning the possible effects of dietary vitamin C or both vitamins on different forms of glutathione concentrations.

In order to evaluate the oxidation in other tissues, the MDA concentration was quantified in muscle at 42 and 68 day of age (Table 5). The MDA concentrations were numerically higher at day 42 when compared to day 68 which would indicate the most stressful situation close to the first post-weaning weeks. Dietary vitamin E supplementation improved the muscle oxidative status and lowered iron-induced oxidation in those muscles of high-dose vitamin E supplemented piglets ($P > 0.05$). The effect of dietary vitamin E was more effective at day 68, with differences in the vitamin E concentrations in muscle from low-supplemented and high-supplemented piglets. Some authors (e.g., Buckley

Table 4. Ferric reducing antioxidant power (FRAP) and reduced (GSH) and oxidized (GSSH) glutathione concentration in piglets serum at 28, 42 and 68 day of age as affected by dietary vitamin E (as α-tocopheryl acetate; 40 and 250 mg · kg^{-1}) or vitamin C (ascorbic acid; 0, 200 and 500 mg · kg^{-1}) supplementation

Vitamin C	0		200		500		RMSE	*P*-value		
Vitamin E	40	250	40	250	40	250		vit. E	vit. C	vit. E x vit. C
FRAP, μM										
28 days*	108.83	108.83	108.83	108.83	108.83	108.83				
42 days	59.66	64.33	71.02	74.61	71.95	76.00	5.207	0.340	0.0500	0.9946
68 days	81.90	102.31	76.92	91.82	83.99	103.47	10.151	0.033	0.6185	0.9586
GSH, μM										
28 days*	4.50	4.50	4.50	4.50	4.50	4.50				
42 days	4.78	4.83	4.63	4.94	4.30	5.01	0.3350	0.1968	0.8887	0.6181
68 days	4.93	5.33	4.68	4.81	4.48	4.87	0.3080	0.2308	0.2943	0.8799
GSSH, μM										
28 days*	0.94	0.94	0.94	0.94	0.94	0.94				
42 days	1.26	1.23	1.28	1.44	1.16	1.36	0.060	0.2595	0.5974	0.623
68 days	0.71	0.64	0.72	0.64	0.63	0.62	0.061	0.3026	0.6090	0.8184
GSH free, μM										
28 days *	3.56	3.56	3.56	3.56	3.56	3.56				
42 days	3.52	3.60	3.34	3.51	3.14	3.65	0.345	0.2230	0.9587	0.6581
68 days	4.22	4.68	3.96	4.16	3.84	4.25	0.312	0.2578	0.3243	0.8999
GSSH:GSH										
28 days *	26.27	26.27	26.27	26.27	26.27	26.27				
42 days	35.63	34.34	38.29	40.96	37.15	37.40	1.756	0.2156	0.6114	0.5713
68 days	16.84	13.76	18.33	15.42	16.43	14.68	1.409	0.2128	0.7167	0.9259

data presented as mean and root-mean-square error (RMSE); *α -tocopherol concentration was determined at 28 days from two piglets per pen

Table 5. Iron-induced lipid peroxidation at 37 °C (malondialdehyde (MDA) content) in piglets muscle at 42 and 68 day of age as affected by dietary vitamin E (as α-tocopheryl acetate; 40 and 250 mg · kg^{-1}) or vitamin C (ascorbic acid; 0, 200 and 500 mg · kg^{-1}) supplementation

Vitamin C	0		200		500		RMSE	*P*-value		
Vitamin E	40	250	40	250	40	250		vit. E	vit. C	vit. E x vit. C
Muscle MDA content, nmols · g^{-1}										
42 days of age										
0 min	0.042	0.038	0.027	0.027	0.022	0.033	0.019	0.7212	0.1862	0.5788
60 min	0.103	0.102	0.107	0.094	0.108	0.088	0.032	0.3088	0.9451	0.7770
120 min	0.193	0.167	0.172	0.159	0.188	0.148	0.032	0.0206	0.5068	0.5987
180 min	0.243	0.217	0.222	0.209	0.238	0.198	0.032	0.0190	0.5100	0.6200
68 days of age										
0 min	0.042	0.036	0.048	0.032	0.041	0.022	0.012	0.0013	0.1913	0.3618
60 min	0.085	0.068	0.081	0.073	0.087	0.076	0.011	0.0034	0.5020	0.6300
120 min	0.135	0.108	0.141	0.113	0.147	0.116	0.011	0.0001	0.1086	0.8952
180 min	0.185	0.158	0.191	0.163	0.197	0.166	0.011	0.0001	0.1310	0.8830

data presented as mean and root-mean-square error (RMSE)

et al., 1995) reported the efficacy of α-tocopherol in reducing the lipid oxidation of muscle by its inclusion in cell membrane, however, most of the studies on pigs were carried out during the fattening phase, close to slaughter age. Moreover, in the present study, neither dietary vitamin C nor the combination of vitamin C and vitamin E affected the lipid stability of muscle. Gebert et al. (2006) also found a lack of response of the dietary supplementation with vitamin C and E mixture on TBARS in growing pigs from 25–105 kg. In other study in which dietary vitamin E and/or C were combined (Eichenberger et al., 2004), dietary vitamin C resulted in higher TBARS whereas the combination of these vitamins reduced the TBARS production in *longissimus dorsi* muscle. In contrast, Lo Fiego et al. (2004) found that in rabbits 500 ppm of dietary vitamin C increased lipid stability of *longissimus dorsi* when the dietary vitamin E

Table 6. Immunoglobulins (IgA, IgM and IgG) concentration in piglets serum at 28, 42 and 68 day of age as affected by dietary vitamin E (as α-tocopheryl acetate; 40 and 250 mg · kg^{-1}) or vitamin C (ascorbic acid; 0, 200 and 500 mg · kg^{-1}) supplementation

Vitamin C	0		200		500		RMSE	*P*-value		
Vitamin E	40	250	40	250	40	250		vit. E	vit. C	vit. E x vit. C
IgA, mg · ml^{-1}										
28 days*	0.42	0.42	0.42	0.42	0.42	0.42				
42 days	0.41	0.53	0.43	0.39	0.59	0.43	0.061	0.593	0.2954	0.0859
68 days	0.58	0.71	0.80	0.66	0.79	0.78	0.050	0.843	0.0234	0.0367
IgM, mg · ml^{-1}										
28 days*	1.34	1.34	1.34	1.34	1.34	1.34				
42 days	2.02	2.18	2.44	2.03	2.90	2.60	0.284	0.431	0.0671	0.578
68 days	2.65	3.06	2.83	2.37	3.09	3.42	0.284	0.694	0.0775	0.2523
IgG, mg · ml^{-1}										
28 days*	10.32	10.32	10.32	10.32	10.32	10.32				
42 days	7.69	8.38	8.49	8.25	8.32	7.77	0.495	0.939	0.741	0.431
68 days	9.35	9.34	9.40	9.13	8.30	8.62	0.470	0.972	0.128	0.819

data presented as mean and root mean square error (RMSE); *α-tocopherol concentration was determined at day 28 from two piglets per pen

dose was 40 ppm. However, Castellini et al. (2001) reported that high levels of both vitamins could prevent oxidative stress during meat storage.

According to the results of the present study, dietary vitamin C supplementation during the first post-weaning stage may preserve the antioxidant capacity of tissues, whereas α-tocopherol concentration is sufficient in tissues at weaning. Its high-dose supplementation exert a long-term effect during the last post-weaning phase.

Piglet immune response. IgM, IgA and IgG levels in piglet serum at day 42 or 68 were not affected by high-dose vitamin E-supplemented diet when compared to low-dose supplemented diet (Table 6). Likewise, Bonnette et al. (1990) using four dietary levels of vitamin E (from 11 IU · kg^{-1} to 550 IU · kg^{-1} feed) found no effects of supplementation on the humoral and cell-mediated immunity in 4-week weaned piglets. Amazan et al. (2012) also reported that IgM, IgA and IgG levels in piglet serum were not affected by natural vitamin E supplementation in drinking water. In contrast, Fragou et al. (2004) found that dietary vitamin E provided to piglets improved immune status.

In the present study, dietary vitamin C increased the IgA concentration (P = 0.023) in piglets at day 68. Moreover, IgM tended to be higher at day 42 (P = 0.067) and 68 (P = 0.077) in piglets supplemented with dietary vitamin C when compared to unsupplemented animals, even though differences were not statistically significant. However, IgG was not modified by the vitamin C-enriched diets. Lauridsen and Jensen (2005) also found an increase in serum IgM in piglets supplemented with 500 mg vitamin C per kg diet. Similarly, Prinz et al. (1980) in guinea pigs reported the positive effect of dietary vitamin C on IgM levels in serum.

The interaction effect of dietary vitamin C and E on IgA levels at day 68 is also interesting. Hence, IgA was higher in groups supplemented with vitamin C and low doses of vitamin E to the diet, IgA was lower in groups supplemented with vitamin C and a high-dose vitamin E-supplemented diet (P = 0.036). A similar trend was observed in IgA levels at day 42 by the interaction effect (P = 0.085). As reported before, differences in the α-tocopherol serum concentrations between groups supplemented with low or high doses of dietary vitamin E were higher at day 68 than at day 42. These results would indicate that combination of dietary vitamin C to obtain beneficial effects on immunity depends on the presence of other antioxidants in serum such as vitamin E concentrations. According to Van Vleet (1980), the borderline deficiency of α-tocopherol concentration is considered to be lower than 1 mg · l^{-1} plasma. At day 68, serum α-tocopherol was close to the borderline level in low-dose vitamin E-supplemented groups. Consequently, dietary vitamin C supplementation improved the immune status of piglets in those groups fed diet supplemented with 40 mg vitamin E · kg^{-1}.

Conclusions

Long-term high-dose vitamin E supplementation of piglets decreases losses of α-tocopherol levels in serum and tissues, and improves the oxidative status of tissues during post-weaning phase. Dietary vitamin C supplementation has a potent antioxidant properties during the immediate post-weaning stress and it is also recommended to be used in combination

with vitamin E in diet to improve the oxidative status of piglet especially if serum α-tocopherol is below the needed values.

Acknowledgements

This research was supported by Project art. 83 270/2011 (ref. 4130750). We would like to thank DSM Nutritional Products (Basilea, Switzerland) for financial support and for providing the vitamin E and vitamin C, and PigChamp Pro Europa (Segovia, Spain) for the farm installations supply.

References

Amazan D., Rey A.I., Fernández E., López-Bote C.J., 2012. Natural vitamin E (D-α-tocopherol) supplementation in drinking water prevents oxidative stress in weaned piglets. Livest. Sci. 145, 55–62, https://doi.org/10.1016/j.livsci.2011.12.022

Bekenev V., García A., Hasnulin V., 2015. Adaptation of piglets using different methods of stress prevention. Animals 5, 349–360, https://doi.org/10.3390/ani5020349

Benzie I.F.F., Strain J.J., 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27, https://doi.org/10.1016/S0076-6879(99)99005-5

BOE, 2013. RD 53/2013, of 21st of October by which are stablish basic statements for animal protection used in experiments and other scientific purpose including teaching. Spain. B.O.E. 252, 34367–34391

Bonnette E.D., Kornegay E.T., Lindemann M.D., Hammerberg C., 1990. Humoral and cell-mediated immune response and performance of weaned pigs fed four supplemental vitamin E levels and housed at two nursery temperatures. J. Anim. Sci. 68, 1337–1345, https://doi.org/10.2527/1990.6851337x

Buckley D.J., Morrissey P.A., Gray J.I., 1995. Influence of dietary vitamin E on the oxidative stability and quality of pig meat. J. Anim. Sci. 73, 3122–3130, https://doi.org/10.2527/1995.73103122x

Castellini C., Dal Bosco A., Bernadini M., 2001. Improvement of lipid stability of rabbit meat by vitamin E and C administration. J. Sci. Food Agric. 81, 46–53, https://doi.org/10.1002/1097-0010(20010101)81:1<46::AID-JSFA777>3.0.CO;2-4

Chand N., Naz S., Khan A., Khan S., Khan R.U., 2014. Performance traits and immune response of broiler chicks treated with zinc and ascorbic acid supplementation during cyclic heat stress. Int. J. Biometeorol. 58, 2153–2157, https://doi.org/10.1007/s00484-014-0815-7

de Blas C., Mateos G.G., García-Rebollar P. (Editors), 2010. Spanish Foundation for the Development of Animal Nutrition (FEDNA) Tables of Composition and Nutritive Value of Food for Feed Manufactures. 3rd Revised Edition. Madrid (Spain)

Debier C., 2007. Vitamin E during pre- and postnatal periods. Vitam. Horm. 76, 357–373, https://doi.org/10.1016/S0083-6729(07)76013-2

Eichenberger B., Pfirter H.P., Wenk C., Gebert S., 2004. Influence of dietary vitamin E and C supplementation on vitamin E and C content and thiobarbituric acid reactive substances (TBARS) in different tissues of growing pigs. Arch. Anim. Nutr. 58, 195–208, https://doi.org/10.1080/00039420410001701413

Fragou S., Fegeros K., Xylouri E., Baldi A., Politis I., 2004. Effect of vitamin E supplementation on various functional properties of macrophages and neutrophils obtained from weaned piglets. J. Vet. Med. 51, 178–183, https://doi.org/10.1111/j.1439-0442.2004.00623.x

Gebert S., Eichenberger B., Pfirter H.P., Wenk C., 2006. Influence of different dietary vitamin C levels on vitamin E and C content and oxidative stability in various tissues and stored *m. longissimus dorsi* of growing pigs. Meat Sci. 73, 362–367, https://doi.org/10.1016/j.meatsci.2005.12.012

Hamilton I.M.J., Gilmore W.S., Benzie I.F.F., Mulholland C.W., Strain J.J., 2000. Interactions between vitamins C and E in human subjects. Br. J. Nutr. 84, 261–267, https://doi.org/10.1017/S0007114500001537

Jones D.P., Carlson J.L., Mody V.C. Jr., Cai J., Lynn M.J., Sternberg P. Jr., 2000. Redox state of glutathione in human plasma. Free Radic. Biol. Med. 28, 625–635, https://doi.org/10.1016/S0891-5849(99)00275-0

Kronbrust D.J., Mavis R.D., 1980. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content. Lipids 15, 315–322, https://doi.org/10.1007/BF02533546

Lauridsen C., Jensen S.K., 2005. Influence of supplementation of all-*rac*-α-tocopheryl acetate preweaning and vitamin C postweaning on α-tocopherol and immune responses of piglets. J. Anim. Sci. 83, 1274–1286, https://doi.org/10.2527/2005.8361274x

Lo Fiego D.P., Santoro P., Macchioni P., Mazzoni D., Piattoni F., Tassone F., De Leonibus E., 2004. The effect of dietary supplementation of vitamins C and E on the α-tocopherol content of muscles, liver and kidney, on the stability of lipids, and on certain meat quality parameters of the *longissimus dorsi* of rabbits. Meat Sci. 67, 319–327, https://doi.org/10.1016/j.meatsci.2003.11.004

Mahan D.C., Saif L.J., 1983. Efficacy of vitamin C supplementation for weanling swine. J. Anim. Sci. 56, 631–639, https://doi.org/10.2527/jas1983.563631x

Moreira I., Mahan D.C., 2002. Effect of dietary levels of vitamin E (all-rac-α-tocopheryl acetate) with and without added fat on weanling pig performance and tissue α-tocopherol concentration. J. Anim. Sci. 80, 663–669, https://doi.org/10.2527/2002.803663x

NRC, 2012. Nutrient Requirements of Swine. 11th Revised Edition. National Academies Press. Washington, DC (USA), https://doi.org/10.17226/13298

Peeters E., Driessen B., Geers R., 2006. Influence of supplemental magnesium, tryptophan, vitamin C, vitamin E and herbs on stress responses and pork quality. J. Anim. Sci. 84, 1827–1838, https://doi.org/10.2527/jas.2005-600

Peng S.M., Shi Z.H., Fei Y., Gao Q.X., Sun P., Wang J.G., 2013. Effect of high-dose vitamin C supplementation on growth, tissue ascorbic acid concentration and physiological response to transportation stress in juvenile silver pomfret, *Pampus argenteus*. J. Appl. Ichthyol. 29, 1337–1341, https://doi.org/10.1111/jai.12250

Perai A.H., Kermanshahi H., Nassiri Moghaddam H., Zarban A., 2014. Effects of supplemental vitamin C and chromium on metabolic and hormonal responses, antioxidant status and tonic immobility reactions of transported broiler chickens. Biol. Trace Elem. Res. 157, 224–233, https://doi.org/10.1007/s12011-013-9879-1

Prinz W., Bloch J., Gilich G., Mitchell G., 1980. A systematic study of the effect of vitamin C supplementation on the humoral immune response in ascorbate-dependent mammals. I. The antibody response to sheep red blood cells (a T-dependent antigen) in guinea pigs. Int. J. Vitam. Nutr. Res. 50, 294–300

Rey A.I., DazaA., López-Carrasco C., López-Bote C.J., 2006. Quantitative study of the α- and γ-tocopherols accumulation in muscle and backfat from Iberian pigs kept free-range as affected by time of free-range feeding or weight gain. Anim. Sci. 82, 901–908, https://doi.org/10.1017/ASC2006113

Rey A.I., Segura J., Arandilla E., López-Bote C.J., 2013. Short- and long-term effect of oral administration of micellized natural vitamin E (D-α-tocopherol) on oxidative status in race horses under intense training. J. Anim. Sci. 91, 1277–1284, https://doi.org/10.2527/jas.2012-5125

Van Vleet J.F., 1980. Current knowledge of selenium-vitamin E deficiency in domestic animals. J. Am. Vet. Med. Assoc. 176, 321–325

Zeferino C.P., Komiyama C.M., Pelícia V.C., Fascina V.B., Aoyagi M.M., Coutinho L.L., Sartori J.R., Moura A.S.A.M.T., 2016. Carcass and meat quality traits of chickens fed diets concurrently supplemented with vitamins C and E under constant heat stress. Animal 10, 163–171, https://doi.org/10.1017/S1751731115001998

Zhao J., Li D., Piao X., Yang W., Wang F., 2002. Effects of vitamin C supplementation on performance, iron status and immune function of weaned piglets. Arch. Tierernahr. 56, 33–40, https://doi.org/10.1080/00039420214176