Effect of α-Tocopherol Deprivation on the Involution of Mammary Gland in Sheep

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ABSTRACT

The objective of this study was to investigate the effect of α-tocopherol deprivation on mammary gland involution and apoptosis in sheep. Two groups of four single lamb ewes were used. The control group received 100 mg/d of RRR-α-tocopherol supplementation and the experimental group received no vitamin E supplementation. After 3 mo of suckling, ewes were dried off, and blood samples from the jugular vein and tissue biopsies from the mammary gland were collected at d 1, 3, 5, and 8 after dry-off.

The experimental group had lower plasma concentrations of α-tocopherol (1.8 vs. 4.2 μmol/L), lower glutathione peroxidase activity in erythrocytes, and higher concentration of malondialdehyde in plasma than the control group.

Immunohistochemical analysis of tissue samples resulted in marked differences of bcl-2 and bax protein expressions during involution and between groups. The bax expression was decreased by α-tocopherol deprivation at 1, 3, and 5 d, but not at 8 d, while the bcl-2 score was higher only at 8 d (1.5 vs. 0.0 for experimental and control groups, respectively). As a result, the bcl-2 to bax ratios were increased for the experimental group at 1 and 8 d. During involution, apoptotic counts increased (from 0.12 to 4.06%), but no effects were detected in relation to bcl-2 to bax ratio and α-tocopherol. These results indicate that α-tocopherol can control bcl-2 expression, but not apoptosis in cells of the mammary gland during involution.

(**Key words:** sheep, mammary gland, α-tocopherol, apoptosis)

Abbreviation key: ED = vitamin E-deprived group, **GSHpx** = glutathione peroxidase, **MDA** = malondialdehyde, **ROS** = reactive oxygen species.

INTRODUCTION

In ruminants the role of apoptosis in tissue involution and remodelling is poorly defined even though papers have reported that histological and ultrastructural characteristics during bovine drying off are similar to those of rodents (1, 13, 33). The investigation of mammary gland apoptosis is intriguing because of possible links with reactive oxygen species (**ROS**) and different nutritional conditions. In practice, this could be related to the enhancement or reduction of lactation persistency in farm animals (33).

Nutrition can play an important role on oxidative stress, and recent studies have focused on dietary restriction as a modulator of oxidative stress, which can affect cell death and proliferation. These studies have demonstrated a protective effect of dietary restriction on aging and cancer (15). Both an imbalance of nutrients and excess of energy affect ROS generation and can alter antioxidant activity of cells and apoptosis (30). Many of the chemical and physical treatments capable of inducing apoptosis are known to evoke oxidative stress. Reactive oxygen species react with cellular macromolecules, such as polyunsaturated fatty acids and cholesterol in membranes, and can damage them directly, resulting in extensive damage to cellular structures. Cellular perturbations associated with programmed cell death may be the consequence of disrupted mitochondrial function as well as excessive production of ROS. Alternatively, oxidative stress can be induced by decreasing the ability of a cell to scavenge ROS or by a shortage of antioxidants. Regulation of mitochondrial and cytosolic ROS concentrations in mammalian cells can be mediated by bcl-2 gene products (11, 12, 16). The bcl-2 gene product inhibits apoptosis by interacting with mitochondrial superoxide dismutase (4). Another plausible role for bcl-2 would be as a free radical scavenger, serving as a nonreactive free radical trap, and as an inhibitor of ROS production (11, 12). Cells react to oxidative stress by increasing concentrations or activities of anitoxidants including the bcl-2 protein. The bcl-2 expression in endothelial cells does not affect ROS generation induced by hyperglycemia, but it does decrease lipid peroxidation and advanced glycation end-products formation (8).

Although nutritional factors may regulate apoptosis (17, 18, 23), research is required in ruminants to gain evidence about the relationship between nutrition and oxidative stress in vivo conditions. α-Tocopherol is one

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Table 1. Basal daily ration and diet composition (DM basis).[1]

	Days after lambing		
	0–30	30–50	50–80
Intake, kg/d			
Soybean meal	0.27	0.36	0.43
Sugar beet pulp, dehy.	0.36	0.46	0.50
Barley	0.35	0.44	0.55
Barley straw	1.35	1.35	1.35
Supplement[2]	0.08	0.08	0.08
Total intake	2.41	2.68	2.90
Diet composition			
CP, g/kg	118	130	125
Lipids, g/kg	19	18	16
Ash, g/kg	78	76	66
NDF, g/kg	575	547	473
Digestible energy, kcal/kg	2664	2761	2834
Calcium, g/kg	0.69	0.62	0.52
Phosphorus, g/kg	0.22	0.20	0.17
Vitamin A, IU/kg	2100	1900	1600
Vitamin D, IU/kg	750	700	600
Vitamin E, mg/kg	4.0	4.5	4.7

[1]The experiment started at dry-off (70 d after lambing).

[2]Supplement contained (per kg as fed): 64,000 IU of vitamin A, 6400 IU of vitamin D, 200 g of Ca, 60 g of P, 300 mg of Cu, 1000 mg of Fe, 1200 mg of Zn, and 15 mg of I.

of the most important lipid-soluble antioxidants and, as an integral component of all lipid membranes, protects lipid membranes from ROS (19). In peripartum cows, α-tocopherol supplementation reduces clinical mastitis and maintains adequate plasma concentration of α-tocopherol (31, 32).

This research investigates the effect of α-tocopherol deprivation on the generation of markers of oxidative stress and on the distribution and kinetics of apoptosis in mammary epithelial cells of sheep during the first 8 d after the end of lactation.

MATERIALS AND METHODS

Sheep and Diet

Eight multiparous nonpregnant sheep each with a single lamb were selected on the basis of general health and the absence of congenital or acquired abnormality of their mammary glands. Ewes were allotted in two paddocks of four animals each, and the vitamin E-deprived group (**ED**) was fed a daily ration consisting of barley, soybean meal, barley straw, and a α-tocopherol- and selenium-free mineral supplement, according to the requirements of NRC (20), from lambing to dry off. The control group received the same ration and was supplemented with 100 mg/d of RRR-α-tocopherol and 0.015 mg of selenium/d as sodium selenite during the last 60 d before dry-off. The amount of each diet component was adjusted during weaning to meet the decreasing nutrient demand of the ewes and the increasing nutrient requirements of lambs (Table 1). At the beginning and at the end of the experimental period, the live weight of animals was recorded.

The experimental protocol was approved and authorized by state and local laws and regulations. Mammary gland tissue and blood were obtained from the same sheep at 1, 3, 5, and 8 d after dry-off. Duplicate samples of tissue were collected by a biopsy needle (Magnum, Bardt, Covington, GA) after subcutaneous anaesthesia and skin incision of about 2 mm.

Blood samples were collected from the jugular vein in two tubes, containing heparin or K-EDTA, at 1 and 8 d. Plasma was immediately separated from the erythrocytes by centrifugation at $3500 \times g$ for 15 min. Heparinized plasma (500 μl) was transferred to vials that contained 150 μl of 0.2% butyl-hydroxy toluene in 95% ethanol and erythrocytes were transferred to another vial; both fractions were stored at –80°C until analysis.

α-Tocopherol, Malondialdehyde, and Glutathione Peroxidase Analysis

α-Tocopherol was measured in the K-EDTA plasma, with an HPLC pump (510, Waters Co., Milford, MA) and a fluorimetric detector (470, Waters Co.) set at 296 nm wavelength of excitation and 325 nm of emission. The system was equipped with an Hibar 250 × 4 mm Lichrosorb-CN, 5-μm particle size column (Merck, Darmstadt, Germany). Mobile phase was 96.05% *n*-hexane, 3.0% *t*-butyl methyl ether, and 0.05% 2-propanol, set at a flow rate of 2 ml/min (3). Peaks were converted to α-tocopherol (μmol/L) concentration with a calibration curve.

Malondialdehyde (**MDA**) in plasma was measured according to the thiobarbituric reactive compounds method outlined by Draper et al. (7). For the analysis, a Perkin Elmer HPLC (LC10, Perkin Elmer Co., Norwalk, CT) was set to a solvent flow rate of 1 ml/min and coupled to a filter fluorimetric detector (LS2, Perkin Elmer Co., Norwalk, CT). Fluorescence detection conditions were 510 nm excitation and 550 nm emission. A reverse phase C_{18} Nova-Pak HPLC column (4-μm particle size; Waters Co.) was used. The MDA standards were prepared by acid hydrolysis of malondialdehyde-bis (dimethyl acetal) and utilized at concentrations ranging from 0.607 to 0.150 n*M*.

Glutathione peroxidase activity (**GSHpx**) was measured on erythrocyte lysates with a commercial kit (RS 505, Randox Laboratories Ltd., U.K.). The method is based on the oxidation of glutathione by glutathione peroxidase in the presence of cumene hydroperoxide (22). Hemoglobin concentration of erythrocytes was measured by the method described by Drabkin and Austin (6), and the final value of GSHpx was expressed as units per gram of hemoglobin.

All chemicals employed in the analyses were obtained from Sigma-Aldrich (St. Louis, MO).

Immunohistochemistry

After rehydration, sections cut at 5 μm were subjected to inhibition of endogenous peroxidases and to enzymatic treatment with trypsin-$CaCl_2$ (0.5%) for 15 min at 37°C, and then incubated overnight at 4°C with a rabbit polyclonal antiserum which reacts with bax (Santa Cruz Biotechnology, Santa Cruz, CA) or bcl-2 proteins (Upstate Biotechonology, Lake Placid, NY). Slides were washed with Tris-buffered solution and then incubated with a peroxidase-conjugated goat anti-rabbit Ig (DAKO-immunoglobulins, Carpinteria, CA) diluted 1:100 in PBS. The stain was developed with 0.04% (w/v) diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in Tris-buffered saline containing 0.04% (vol/vol) hydrogen peroxide for 7 min at room temperature. For control, 10% of normal goat serum and 1% of BSA in PBS was substituted for the first antibody.

The bax and bcl-2 were scored on the basis of the strength of staining. Staining intensity was graded on a scale from 1 to 3, where 1 represented staining equal to that seen in negative control sections and 3 was equivalent to the intensity of human mammary gland positive control sections (9).

Apoptosis Detection

The tissue was fixed in 10% neutral formalin, embedded in paraffin wax by standard histological procedures. Sections were cut at 5 μm, stained with haematoxylin-eosin and methyl green pyronin and processed for in situ detection of cell death by terminal deoxynucleotide transferase-mediated deoxyuridine nick-end labeling (TUNEL) method according to the manufacturer's instructions (Frag-EL Kit, Calbiochem, San Diego, CA).

Only cells clearly positive by TUNEL detection were counted. A total of 10 microscopic fields for each samples were counted with a ×40 objective, and TUNEL positive cells were expressed as percentage of total cell counted.

Statistical Analysis

For statistical assessment of the differences in immunohistochemical expression of bax and bcl-2 proteins and apoptotic index between groups and sampling time, the Mann-Whitney test for two independent nonparametric samples was used and $P < 0.05$ were considered significant (27). Data of live weight of sheep, α-tocopherol, MDA, and GSHpx concentrations were processed using a repeated measure design (27):

$$y = \mu + T_{(1,2)} + A_{(1,4)}(T) + \varepsilon_2 + S_{(1,n)} + S{\times}T + \varepsilon_1$$

where:

μ = general mean
T = fixed effect for Treatment (1, ED; 2, control)
A = animal, nested within Treatments
ε_2 = A(T)-error
S = fixed effect for Sampling times (1 d and 8 d)
ε_1 = residual error

RESULTS AND DISCUSSION

Sheep and Diet

The estimated dietary concentration of α-tocopherol (Table 1) was far below the requirements (20) for ED group and greatly differed from that of control ewes. Dietary treatments were designed to maintain BW during lactation and ewes at the end of the experiment were similar to initial BW (beginning: 63.0 ± 4.0 vs. 62.3 ± 7.5 kg for ED and control; end: 62.0 ± 5.0 vs. 61.2 ± 5.6 kg for ED and control). This would indicate that nutrient availability, apart from the α-tocopherol, did not differ greatly.

The deprivation of α-tocopherol (ED group) resulted in an overall decrease ($P < 0.01$) in plasma concentration of α-tocopherol (Table 2), with higher differences be-

Table 2. Effect of supplemental vitamin E on mean glutathione peroxidase activity in erythrocytes and malondialdehyde (MDA) and α-tocopherol concentrations in plasma at 1 d and 8 d after dry-off.

	α-Tocopherol (μmol/ml)	GSHpx (U/g of Hb[1])	MDA (n*M*/L)
1 d after dry-off			
ED[2]	1.8	130	568
Control[3]	3.5	153	439
8 d after dry-off			
ED	1.9	138	704
Control	4.9	171	634
Mean			
ED	1.8	134	636
Control	4.2	162	537
Type II SS $P > F$			
Treatment	**	†	†
Day	**	NS	**
Treatment × day	**	NS	NS
Between subjects MS	1.16	794	15351
Model MS	0.16	791	2869

[1]Hb = Hemoglobin.

[2]ED = Vitamin E-deprived group (without α-tocopherol supplementation).

[3]With α-tocopherol supplementation.

† = $P < 0.1$.

* = $P < 0.05$.

** = $P < 0.01$.

tween groups at 8 than 1 d (interaction treatment × period, $P < 0.01$). The observed variation could be related to the end of α-tocopherol excretion in the milk. Weiss et al. (32) have observed a decrease of plasma concentration of α-tocopherol at the beginning of lactation in α-tocopherol supplemented cows. This variation was less evident when the concentration was expressed per unit of plasma cholesterol. The inverse situation, i.e., from lactation to dry-off, could result in increased concentrations of plasma α-tocopherol as reported in Table 2. The lack of variation in plasma α-tocopherol from 1 to 8 d for the ED group can be related to the very low plasma concentration. In a condition of α-tocopherol deprivation, the transfer from the plasma to the milk can be reduced (10, 21).

α-Tocopherol is a very active lipophilic radical scavenger, and in the present research the effect of its supplementation was evaluated measuring the end-product of lipid peroxidation (MDA) and GSHpx, an enzymatic scavenger that plays an important role in mammals. α-Tocopherol supplementation tended to increase ($P < 0.10$) erythrocyte GSHpx activity (Table 2). Saez et al. (26) did not find an effect of α-tocopherol administration on GSHpx in sheep. On the other hand, Yen et al. (34) reported a reduction of GSHpx in pigs, and Saez et al. (25) an increase in GSHpx activities in sheep after α-tocopherol supplementation. The GSHpx is a selenium-dependent enzyme and its increase could reflect both selenium status of the animal and α-tocopherol (14). Moreover, many compounds and enzymes are involved in the antioxidant defenses of cells, and they can act interactively or independently from each other. In fact, α-tocopherol can protect polyunsaturated fatty acids of membranes either by acting synergically with the glutathione enzyme systems or by trapping the oxygen-derived free radicals to halt a chain reaction (19). Whichever system was involved, a marked reduction of lipid peroxidation products would have been expected, and the analytical method of choice would be to determine MDA concentration. Higher plasma MDA concentration in the α-tocopherol deprived sheep were found (ED group), although the difference between groups was moderate ($P < 0.10$). Moreover, MDA increased from 1 to 8 d, in both groups ($P < 0.01$). Similarly to α-tocopherol, milk excretion could partially explain the increase of plasma MDA. Furthermore, other stressing events, such as lamb removal and dry-off and repeated animal manipulation, could affect ROS generation and lipid peroxidation. Finally, MDA can be further oxidatively metabolized by peroxidase enzymes (24), and this would prevent its accumulation in plasma.

Bax and Bcl-2 Expression and Apoptosis Detection

Family members of bcl-2 proteins are known to be involved in the protection of cells from oxidative stress (12, 16), but the mechanism is not well explained (2).

Immunohistochemistry was performed to evaluate the presence of bax and bcl-2 protein. Repeated biopsy samples from the same animal did not result in either morphological or structural damages to the mammary gland. At 8 d after dry-off, bax score was higher than d 1, 3, and 5 scores in the ED groups ($P < 0.05$) but remained unchanged over time in α-tocopherol supplemented sheep (Table 3). The control group had higher bax values than ED ewes at 1, 3, and 5 d. For both the groups, bcl-2 positivity was higher ($P < 0.05$) at 1 d,

Table 3. Effect of supplemental vitamin E on mean scores of bax and bcl-2 in mammary gland tissue from d 1 to 8 after dry-off.

	1 d			3 d			5 d			8 d		
	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Bax												
ED[1]	1.0[b]	±	0.0	1.5[b]	±	0.6	2.3[b]	±	0.5	3.0[a]	±	0.0
Control[2]	2.5	±	0.6	3.0	±	0.0	3.0	±	0.0	3.3	±	1.0
		*			*			*			NS	
Mean	1.8	±	0.9	2.3	±	0.9	2.6	±	0.5	3.1	±	0.6
Bcl-2												
ED	1.8[a]	±	0.5	0.5[ab]	±	1.0	0.0[b]	±	0.0	1.5[a]	±	1.0
Control	2.0[a]	±	0.8	0.5[ab]	±	1.0	0.0[b]	±	0.0	0.0[b]	±	0.0
		NS			NS			NS			*	
Mean	1.9	±	0.6	0.5	±	0.9	0.0	±	0.0	0.8	±	1.0

[a,b,c]Superscripts denote asymptotic difference ($P < 0.05$) according to the Mann-Whitney test within a row.

[1]ED = Vitamin E-deprived group (without α-tocopherol supplementation).

[2]With α-tocopherol supplementation.

*Denotes differences ($P < 0.05$) between treatments within time of sampling, according to Mann-Whitney test. NS: differences between treatments and within time of sampling are not significant, according to Mann-Whitney test.

Table 4. Effect of supplemental vitamin E on mean ratio of bcl-2 to bax scores and frequency of apoptotis (%) in mammary gland tissue from d 1 to 8 after dry-off.

	1 d			3 d			5 d			8 d		
	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Bcl-2 to bax												
ED[1]	1.75[a]	±	0.50	0.25[bc]	±	0.50	0.00[c]	±	0.00	0.50[b]	±	0.33
Control[2]	0.79[a]	±	0.25	0.17[ab]	±	0.33	0.00[b]	±	0.00	0.00[b]	±	0.00
		*			NS			NS			*	
Mean	1.27	±	0.63	0.21	±	0.40	0.00	±	0.00	0.25	±	0.35
Apoptotis												
ED	0.04	±	0.05	0.75	±	0.80	1.10	±	0.71	3.14	±	1.54
Control	0.20	±	0.19	0.40	±	0.33	0.49	±	0.32	4.98	±	3.61
		NS			NS			NS			NS	
Mean	0.12[c]	±	0.15	0.58[bc]	±	0.60	0.79[b]	±	0.61	4.06[a]	±	2.75

[a,b,c]Superscripts denote asymptotic difference ($P < 0.05$) according to the Mann-Whitney test within a row.

[1]ED = Vitamin E-deprived group (without α-tocopherol supplementation).

[2]With α-tocopherol supplementation.

*Denotes differences ($P < 0.05$) between treatments within time of sampling, according to Mann-Whitney test. NS: differences between treatments and within time of sampling are not significant, according to Mann-Whitney test.

decreased at 3 and 5 d in both groups, and then increased ($P < 0.05$) at 8 d only in the ED group.

Overexpression of bcl-2, as when ROS are present, leads to survival of the cells, but the effect is counterbalanced by bax protein, which triggers apoptosis (3). Therefore, the final fate of the cell will depend on the ratio of these two proteins. The bcl-2 to bax ratio was higher ($P < 0.05$) at 1 and 8 d for the ED treatment (Table 4). The highest ratio was observed at 1 d ($P < 0.05$) within each treatment. The ED group showed a decrease of the ratio until 5 d ($P < 0.05$) and then a slight increase at 8 d ($P < 0.05$), and the ratio for the control group decreased from 1 to 8 d ($P < 0.05$).

The involution of mammary gland in ruminants is an important factor in determining milk production for the successive lactation (17, 33). The remodelling process of the mammary gland is regulated by apoptosis (28) and biochemical inducers, including nutrient deprivations and ROS presence (15). During the involution of mammary gland in ewes, apoptosis occurs mostly between the 5th and 8th d after dry off (4, 24). The comparison of apoptotic index did not show significant variation (Table 4) between treatments within each sampling time. The apoptotic cells increased from 0.12%, at 1 d, to 4.06% at 8 d ($P < 0.05$). In particular, frequency of apoptosis greatly increased from 5 to 8 d in the glandular epithelium, and morphological evaluation revealed that apoptotic cells are concentrated in particular areas of the tissue observed (5). These results indicate that during involution of the sheep mammary gland, α-tocopherol deprivation gave rise to an increase of the bcl-2 to bax ratio but was not able to regulate apoptosis. In cultured murine lymphoma cells treated with α-tocopherol succinate, apoptosis was concomitant to high levels of bcl-2 mRNA (35), suggesting a ROS-independent regulation of apoptosis.

CONCLUSIONS

The results obtained in this experiment indicate that α-tocopherol deprivation can affect GSHpx and MDA in plasma and alter bcl-2 and bax (proteins that are naturally involved in involution and preventing lipid oxidation caused by ROS) expressions.

The control of apoptosis is under the coordination of diverse signals in large part mediated by external stimuli, such as the presence of ROS, that activate membrane-bound receptors on the cell surface. However, cellular alteration also is triggered by genetic programming, and other factors, so that involution can not be explained simply by the action of the antioxidants.

These results are intriguing because they document the in vivo involvement of oxidative stress on a physiological program of mammary involution. Further research would assess the effect of nutritional interventions on the genetic program of mammary glands during involution.

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