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Effects of *pre-* and *post-partum* injections of Se, Zn and vitamin E on the concentration of cholesterol, CLA isomers and fatty acids in ovine milk*

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The study was conducted on 40 Polish Merino ewes divided into two groups: experimental (E) and control (C), each of 20 animals. Four weeks before, and then on day 1, week 3 and week 6 post lambing all ewes from group E were given intramuscularly 5 ml 0.1% Na_2SeO_4, 10 ml 10% $ZnSO_4$ and 250 mg vitamin E. In milk sampled 7 weeks after lambing the concentration of conjugated linoleic acid (0.66% of the fatty acid sum) was found higher ($P<0.05$) while cholesterol content (10.6 mg/100ml) lower ($P<0.01$) in group E than in group C. In group E, over the whole experiment, the level of total cholesterol of blood plasma dropped from 3.79 mmol/L to 3.02 mmol/L while HDL fraction increased from 0.85 mmol/L to 0.98 mmol/L. It is concluded that the *pre-* and *post-partum* intramuscular injections of Se, Zn and vitamin E improved the lipid profile of milk.

KEY WORDS: cholesterol / CLA isomers / milk / selenium / sheep / vitamin E / zinc

The quality of milk fat has been extensively studied in relation to the type of feed, primarily in cows and ewes. Known are the effects of forages, animal fats or marine oils on bovine milk fat secretion and composition. Special attention is given to fatty acids that could play a positive role for human health, such as butyric acid, oleic acid, C18 to C22 polyunsaturated acids and conjugated linoleic acid [Chilliard *et al.* 2001]. Isomers of conjugated linoleic acid (CLA) found in ruminants' milk and meat are products of incomplete biohydrogenation of unsaturated fatty acids by rumen bacteria

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Butyrivibrio fibrisolvens [Kepler *et al*. 1966]. CLA refers to a mixture of positional and geometric isomers of octadecadienoic (linoleic) acid with conjugated double bonds. The most extensively investigated CLA isomer is *cis-9,trans-11* octadecadienoic acid, which is thought to be biologically active [Chin *et al*. 1992, Bessa *et al*. 2000].

Selenium is implicated in antioxidant functions, and Se-Cysteine is essential in the active centres of Se-enzymes that carry out redox reactions, glutathione peroxidase (GPx), thyroid hormone deiodinase families and thioredoxinreductase [Tapiero *et al*. 2003]. Vitamin E is primarily active as an antioxidant protecting polyunsaturated fatty acids in *in vivo* and *post-mortem* animal tissues and muscle nutrients from free-radical attack [Morrissey *et al.* 1994]. Zinc is associated with enzymes, both as part of the molecule and as an activator. In its structural role, Zn usually stabilizes the structures of RNA, DNA, and ribosomes [McDowell 1992]. Zn superoxide dismutase (ZnSOD) is a dietary factor, which contributes to the antioxidant defence system [Morrissey *et al*. 1998].

Some studies suggest that the Zn content of diets for dairy cows can modify the level of blood lipids, as lower levels of Zn in the diet increase the lipid oxidation rate in the liver [Roussel *et al.* 1993]. In several studies using rats it was found that the content of polyunsaturated fatty acids (PUFA), especially in serum cholesterol esters and phospholipids were positively correlated with Se concentration in the diet [Crespo *et al.* 1995]. Czauderna *et al*. [2004] suggested that the interaction between Se and the CLA isomers mixture protected CLA from peroxidation damage in muscles and increased the level of CLA in the muscles of rats. Supplementing the cows with selenium significantly decreased the level of cholesterol in blood plasma, including HDL and LDL lipoproteins [Brzóska and Brzóska 2004]. Reklewska *et al.* [2002] indicated that supplementing the diet with minimum doses of linseed and mineral mixture (Mg, Fe, Cu, Co, Mn, Zn, Se, Cr, Ca) significantly increases CLA isomers and decreases cholesterol content of milk in cows.

The aim of the present experiment was to check the feasibility of decreasing the cholesterol level of blood and milk and increasing the CLA levels of milk in sheep by applying intramuscular *pre*- and *post-partum* injections of Se, Zn and vitamin E.

Material and methods

The experiment was conducted on 40 Polish Merino ewes divided into two groups: control (C) and experimental (E), each of 20 animals. Four weeks before lambing, and then on day one, week three and week six after lambing all 20 ewes from group E received intramuscular injection of 5 ml 0.1% Na_2SeO_4 (2.09 mg Se), 10 ml 10% $ZnSO_4$ (227 mg Zn) and 250 mg vitamin E (α- tocopherol). The ewes from both groups were kept in the same shed. Till the age of 8 weeks all the lambs were maintained with their dams. Feeding was based on local feeds: maize silage, meadow hay, cereal grains mix (oats – 20%, wheat bran – 60%, rapeseed oilmeal – 20%) and mineral premix (10 g per ewe daily). During the advanced pregnancy the daily intake of dry matter (DM) per ewe was 0.51 kg from meadow hay, 0.67 kg from maize silage and 0.86 kg from cereals while 0.68, 0.67 and 1.04 kg during lactation, respectively. The declared content of Se and Zn in the mineral premix was 12 ppm and 6000 ppm, respectively.

Milk from ewes was sampled on week 1 and week 7 after lambing, following the injection of one ml oxytocin to stimulate milk let-down. Proximate analysis of feeds was performed using standard methods. The level of metabolizable energy of feed was calculated on the basis of the results of proximate chemical analyses using the equation recommended by MAFF [1975].

The concentrations of SFA, MUFA, PUFA and CLA were investigated in milk. For determination of fatty acids samples were freeze-dried and extracted with chloroform-methanol-water mixture (4:2:1.v/v). Hydrolization and derivatization reaction was carried out according to Czauderna *at al.* [2001]. The derivatized samples were filtered through 0.2 μm membrane filter (WHATMAN) and the filtrates injected onto chromatographic column Spheri-5 RP-18, 5 μm, 220 × 4.6 mm (PERKIN ELMER, USA). The CLA isomer mixture standard (the *cis-9,trans-11* and *trans-10,cis-12*) of CLA and other fatty acid standards were provided by SIGMA (USA).

The blood was withdrawn six weeks after lambing. The total and HDL (high-density lipoprotein) cholesterol and triacylglycerol (TAG) in blood plasma were determined using enzyme-linked tests (Alpha Diagnostic S.A., Warsaw, Poland). For the determination of cholesterol in milk, samples were saponified and extracted with hexane according to Fletouris *et al.* [1998]. Total cholesterol was determined colorimetrically according to Searcy and Berquist [1960]. The Zn content in feeds was determined by atomic absorption spectrometry. Samples (0.5 g) were mineralized in a mixture of 5 ml HNO_3 and 1 ml H_2O_2 in hermetic high-pressure vessels by heating in microwave oven. Total Se content was determined by flame (air-acetylene) atomic absorption spectrometry using hydrogen generation system. Selenium hydride was generated with $NaBH_4$. Hallow cathode lamp (196.0 nm) with deuterium background correction was used.

Means and their standard errors were computed and differences between group means verified based on the t-test using Microsoft EXCEL and STATISTICA for Windows.

Results and discussion

The results of proximate analyses of feeds and their nutritive value indicators, as well as Zn and Se contents of DM are shown in Table 1. Daily Se intake per ewe (groups pooled) from feeds and mineral premix in the advanced pregnancy and lactation amounted to 0.39-0.44 mg while that of Zn to about 115-123 mg (figures not tabulated). Minimum dietary requirements for Se of particular animal species cannot be given with any accuracy. However, National Research Council (USA) suggested that Se requirements for sheep vary from 0.1 to 0.2 ppm of feed DM [McDowell 1992].

Table 1. Chemical composition and nutritive value of feeds (g/kg)

Component	Concentrate mixture	Meadow hay	Maize silage
Dry mater	862	850	336
In dry mater			
crude ash	52	67	48
crude protein	205	121	78
crude fibre	101	315	211
ether extract	39	18	34
N-free extractives	603	470	629
NDF	302	634	460
ADF	137	365	241
ADL	43	42	22
Metabolizable energy (MJ/kg)	12.6	10.2	11.8
Se (mg/kg DM)	0.16	0.10	0.12
Zn (mg/kg DM)	29.4	15.1	33.7

Some animal experiments have suggested that dietary Se may have some beneficial effects at levels above those generally accepted as adequate [McDowell 1992]. Current estimates put maximum tolerable level of Se at 2 mg/kg DM for the major livestock species, and no differentiation exists for tolerable levels between ruminants and nonruminants [McDowell 1992, Davis *et al*. 2006]. The maximum tolerable level of inorganic Se for sheep is much greater than 2 mg/kg DM as was suggested earlier. Feeding up to 12 mg of selenite/kg feed DM to ewes under the stress of production (*i.e*. gestation and lactation) for 72 weeks did not produce any clinical or pathologic signs of Se intoxication [Davis *et al*. 2006]. The NRC suggested that requirements of sheep for Zn vary from 20 to 33 mg/kg feed DM [McDowell 1992]. For ruminants, overt Zn toxicosis first appears when levels around 1000 ppm are incorporated into a natural-ingredient diet [McDowell 1992].

In our earlier study conducted in the years 1987-1988 intramuscular injection of 5 ml 0.1% Na_2SeO_4 and 250 mg of vitamin E four weeks before the mating season and four weeks before the lambing increased fertility when the Se plasma deficiency was deep. However, when the Se level of blood plasma was within the normal range, injection of both Se and vitamin E decreased fertility and lamb body live weight at birth [Gabryszuk 1994]. The Se, Zn and vitamin E intramuscular injections before and after lambing of ewes were effective in increasing the contents of these nutrients in milk [Gabryszuk *et al.* 2005].

In comparison to the control group, four intramuscular injections of Se (each of 2.09 mg), Zn (each of 227 mg) and vitamin E (250 mg) applied in group E led to significant decrease in the cholesterol content of milk and in total cholesterol of blood plasma with simultaneously higher level of HDL fraction (Tab. 2). Simultaneously the concentration of TAG of blood plasma in group E was lower than in control ewes

Table 2. The cholesterol content of milk, and total cholesterol, HDL fraction and TAG of blood plasma in ewes

Item	Group E		Group C	
	mean	SE	mean	SE
Milk week 7				
cholesterol (mg/100 g)	10.6^{A}	1.96	14.23^{B}	2.89
Blood week 6				
total cholesterol (mmol/l)	3.02^{A}	0.51	3.79^{B}	0.60
HDL (mmol/l)	0.98^{a}	0.13	0.85^{b}	0.14
TAG (mmol/l)	0.44^{A}	0.05	0.54^{B}	0.09

[aA...]Within rows means bearing different superscripts differ significantly at: small lettrs – P≤0.05; capitals – P≤0.01.

Table 3. Fatty acid composition (% of total fatty acids) of milk in ewes

Item	Group E		Group C	
	mean	CV	mean	CV
Milk week 1				
SFA (%)	70.4	17.4	71.3	10.2
MUFA (%)	22.4	21.1	22.2	16.6
PUFA (%)	7.2	28.8	6.4	19.3
CLA (%)	0.77^{a}	28.8	0.54^{b}	19.9
Milk week 7				
SFA (%)	72.2	22.8	74.4	14.3
MUFA (%)	21.5	22.8	20.2	16.6
PUFA (%)	6.3	7.3	5.4	19.9
CLA (%)	0.66^{a}	26.6	0.43^{b}	24.3

[ab]Within rows means bearing different superscripts differ significantly at P≤0.05.
SFA: C3:0, C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0 and C18:0.
MUFA: C10:1, C12:1, C14:1, C16:1, C18:1.
PUFA: C18:2, C18:3, C20:3, C20:4, C20:5.
CLA: a sum of CLA isomers: *cis*-9,*trans-11* CLA and *trans-10,cis-12* CLA.

(Tab. 2) while concentration of CLA in milk was higher in group E (Tab. 3).

Falkowska *et al.* [2000] showed that supplementation of a diet with Se and vitamin E significantly increased HDL fraction in blood of cows – from 0.440 to 0.552 mmol/l. Brzóska and Brzóska [2004] observed that when the level of dietary Se was increased from 0.04 to 0.48 mg/kg DM, the cholesterol content of blood plasma in cows dropped from 228.6 to 183.9 mg/dl, with simultaneous decrease in HDL and LDL. The level of Zn ranging from 39 to 59 mg/kg DM of the diet for dairy cows did not affect cholesterol or other metabolite levels in blood plasma or milk [Brzóska and Kowalczyk 2002]. The long-chain n-3 fatty acids are known to be a factor decreasing the LDL content of liver, which at their declining tendency in blood plasma, could promote the increased HDL blood plasma content [Brzóska and Kowalczyk 2002]. Reklewska *et al.* [2002] reported significant increase in CLA and decrease in cholesterol content of milk of cows fed diet with minimum doses of linseed and mineral bioplex mixture (Mg, Fe, Cu, Co, Mn, Zn, Se, Cr, Ca). Our earlier study [Gabryszuk *et al*. 2007] demonstrated that Se, Zn and vitamin E administered orally to growing ram-lambs induced a decrease in cholesterol content of blood and meat, and led to increased CLA isomers level in meat and liver. Emanuelson and Bertilsson [1995] reported a tendency to increased oxidation of milk fat when linseed was added to the cows' diet. Spontaneous oxidation of milk fat may occur at low concentration of milk antioxidants. Since increased dietary concentration of antioxidants may lead to improved oxidative stability of milk fat [Niki *et al.* 1989] it seemed worth trying to produce milk of increased content of antioxidants as well as of some functional fatty acids.

We suggest that the interactions between Se, Zn and vitamin E are factors which can modulate desaturase and chain elongase activity as well as inhibit fatty acid β-oxidation in mammary gland. The genetically determined ability of the subcellular membrane to assimilate and store peroxidation antagonists such as vitamin E, GSH-Px, catalase and superoxide dismutase. Crespo *et al*. [1995] reported that the concentration of PUFA was positively correlated with the level of Se in diets of rats. The *cis-9,trans-11* CLA found in ruminants milk and meat is an intermediate in the biohydrogenation of linoleic acid (*cis-9,cis-12* C18:2) to stearic acid (C18:0) – Bessa *et al*. [2000]. The major part of conjugated linoleic acids (*cis-9,trans-11* C18:2) is synthesized in the ruminant tissues and particularly in the mammary gland by the desaturation of trans-vaccenic acid (*trans-11* C18:1) resulting from the action of the stearoyl-CoA desaturaze (SCD). The introduction of *cis*-double bond is catalysed by the set of microsomal electron-transport proteins composed sequentially of NADH cytochrome β5 reductase, cytochrome β5, and the terminal SCD. Stearoyl-CoA desaturase is the rate-limiting component in this reaction. Its activity is regulated by different factors such as diet, hormones, temperature, metals, peroxisomal proliferators, vitamin A, and developmental processes [Ntambi 1999, Miyazaki and Ntambi 2003]. More than 80% of *cis-9*,*trans-11* CLA in milk is produced endogenously by Δ9-desaturase from *trans*-11 C18:1 in the mammary gland. Cows on the same diet have different milk fat *cis-9,trans-11* CLA concentrations that may be partially explained by differences in Δ9-desaturase activity between cows [Lock and Garnsworth 2002]. Increasing the activity of Δ9-desaturase in the mammary gland may offer greater potential for enhancing the *cis-9,trans-11* CLA content of milk fat [Lock and Garnsworth 2002].

We presume that supplementation of Se, Zn and vitamin E can regulate activity of SCD. We also suggest that dietary Se, Zn and vitamin E decreased β-oxidation of the CLA isomers in mammary gland. It can be hypothesized that the Se (as Na_2SeO_4), Zn and vitamin E as strong antioxidants, have a protective effect against peroxidation damage in CLA isomers metabolism.

This study demonstrated that Se, Zn and vitamin E administered intramuscularly to pregnant and lactating ewes induced a decrease in cholesterol content of blood and milk, and led to increased the CLA isomers level in milk. Based on the above observation, it is suggested that the intramuscular administration of Se, Zn and vitamin E improved the lipid profile of ovine milk as assessed from consumer's point of view.

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Wpływ iniekcji Se, Zn i witaminy E przed i po porodzie na zawartość cholesterolu, izomerów CLA i innych kwasów tłuszczowych w mleku owczym

S t r e s z c z e n i e

Badania przeprowadzono na 40 owcach merynosa polskiego, podzielonych na dwie grupy – doświadczalną (E) i kontrolną (C), po 20 zwierząt w grupie. Owcom z grupy E cztery tygodnie przed wykotem, a dalej po jednym dniu, po 3 tygodniach i po 6 tygodniach od wykotu podawano w iniekcji domięśniowej 5 ml 0,1% Na_2SeO_4, 10 ml 10% $ZnSO_4$ i 250 mg witaminy E (α-tokoferol). Oznaczono zawartość cholesterolu, SFA, MUFA, PUFA i izomerów CLA w mleku owiec, jak również cholesterolu całkowitego i frakcji HDL oraz trójglicerydów w osoczu krwi. W mleku owiec z grupy E stwierdzono istotnie więcej izomerów CLA (0,66%), a mniej cholesterolu (10,6 mg/100 ml) niż w mleku grupy kontrolnej. W badanym okresie stężenie cholesterolu całkowitego we krwi owiec z grupy E (3,02 mmol/l) obniżyło się istotnie, a frakcji HDL (0,98 mmol/l) istotnie wzrosło. Wnioskuje się, że czterokrotne domięśniowe podanie Se, Zn i witaminy E (za każdym razem odpowiednio po 2,09, 227 i 250 mg) poprawia profil lipidowy mleka owczego.