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CONJUGATED LINOLEIC ACIDS (CLA) DECREASE THE BREAST CANCER RISK IN DMBA-TREATED RATS

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Abstract: The aim of this study was to investigate how supplementation of diet of female Sprague-Dawley rats with different doses of conjugated linoleic acids and for a varied period of time influences breast cancer risk, fatty acids profile and lipids peroxidation in chemically induced mammary tumors. Animals were divided into nine groups with different modifications of diet (vegetable oil, 1.0 or 2.0% of CLA) and period of supplementation, which lasted after (A), before (B) and before and after (BA) carcinogenic agent - 7,12-dimethylbenz[a]anthracene administration at 50th day of life. Mammary adenocarcinomas occurred in all groups, but CLA supplementation decreased the cancer morbidity. Two percent CLA seem to be excessive because of the coexisting cachexia. Two CLA isomers (cis-9, trans-11 and trans-10, cis-12) were detected in tumors but content of rumenic acid was higher. Dietary supplementation significantly influenced some unsaturated fatty acids content (C18:2 n-6 trans, C20:1, C20:5 n-3, C22:2), but the anti- or prooxidant properties of CLA were not confirmed. CLA can inhibit chemically induced mammary tumors development in female rats, but their cytotoxic action seems not to be connected with lipids peroxidation. CLA isomers differ with their incorporation into cancerous tissues and they influence the content of some other fatty acids.

Keywords: CLA, DMBA, mammary tumors, rats

Abbreviations: AOil = group of rats supplemented with vegetable oil after DMBA administration, A1% = group of rats supplemented with 1.0% of CLA after DMBA administration, A2% = group of rats supplemented with 2.0% of CLA after DMBA administration, BOil = group of rats supplemented with vegetable oil before DMBA administration, B1% = group of rats supplemented with 1.0% of CLA before DMBA administration, B2% = group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil = group of rats supplemented with vegetable oil before and after DMBA administration, BA1% = group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% = group of rats supplemented with 2.0% of CLA before and after DMBA administration, CLA = conjugated linoleic acids, DMBA = 7,12-dimethylbenz[a]anthracene, FA = fatty acids, FAME = fatty acids methyl esters, GC = gas chromatography, MDA = malonyldialdehyde, TBARS = thiobarbituric acid reactive substances, TEB = terminal end buds

Fatty acids profile of consumed fat determines both the composition and the functions of body lipids (1, 2). Moreover, the quality and the quantity of dietary fat is considered as one of the major factors influencing the risk of cancer. Conjugated linoleic acids (CLA), which are the positional and geometric isomers of linoleic acid (cis-9, cis-12 C18:2, n-6, LA) with two cis/trans unsaturated bonds separated by one single bond, are naturally present in milk and dairy products as well as in fat from ruminants. They are investigated since 1970' as a group of polyunsaturated fatty acids with numerous health-promoting properties (3, 4). CLA isomers can modify the risk of many diet-relating disorders, such as: obesity, atherosclerosis, cardiovascular disease, diabetes, osteoporosis and different types of cancer (5), but their action depends on the conformation of the isomer. Moreover, precise mechanisms of their action are still under investigation (6). Among many suggested ways of action, interactions with essential fatty acids in their metabolic pathways and anti- or prooxidant properties are very interesting.

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Table 1. Characteristics of experimental groups.

Group	AOil	A1%	A2%	BOil	B1%	B2%	BAOil	BA1%	BA2%
Diet I (before DMBA)	Lab. H	Lab. H	Lab. H	Lab. H + oil Lab. H	Lab. H + 1.0% CLA	Lab. H + 2.0% CLA	Lab. H + oil	Lab. H + 1.0% CLA	Lab. H + 2.0% CLA
Diet II after DMBA)	Lab. H + oil Lab. H	Lab. H + 1.0% CLA	Lab. H + 2.0% CLA	Lab. H	Lab. H	Lab. H	Lab. H + oil Lab. H	Lab. H + 1.0% CLA	Lab. H + 2.0% CLA
Number of individuals	8	9	9	13	15	10	14	17	9
Number of individuals with mammary tumors	7	6	3	13	11	10	13	15	1
Mammary tumor incidence	87.5%	67.0%	33.0%	100%	73.0%	100%	93.0%	88.0%	11.0%
Total number of tumors	13	8	3	28	24	18	63	28	3
Number of tumors per individual (range)	0 – 3	0 – 2	0 – 1	1 –	5	0 – 6	1 – 4	0 – 15	0 – 5 0 – 3
Mean number of tumors per individual	1.6	0.9	0.3	2.2	1.6	1.8	4.5	1.6	0.3
Weight of single tumor range) [g]	0.45 – 11.93	0.62 – 8.51	1.12 – 4.48	0.01 – 21.71	0.03 – 11.83	0.77 – 7.74	0.03 – 13.64	0.01 – 5.62	0.47 – 1.91
Total weight of tumors in group [g]	48.93	21.48	6.75	75.00	31.70	61.00	69.96	37.41	3.53
Age of first tumor appearance [day of life]	93	143	112	118	145	110	126	134	113

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration.

Many researchers emphasize that oxidative stress is connected with the etiology of many above-mentioned diseases. Lipids peroxidation, especially polyunsaturated fatty acids peroxidation, as a result of oxidative stress generates many biologically active compounds. They can increase the oxidative damage in cells as they can live relatively long and are highly reactive. Aldehydes, which are one of the groups of metabolites, interact with intracellular compounds, especially with proteins and nucleic acids, and can break the cell functions (7, 8). Estimation of the fatty acids metabolites generated during the lipids peroxidation facilitates the explanation of the mechanism of arising of pathological conditions, as well as the influence of fatty acids on them.

The aim of this study was to investigate how supplementation of diet of female Sprague-Dawley rats with different doses of conjugated linoleic acids and for a varied period of time influences the breast cancer risk. To find out the mechanism of CLA action we also assessed the fatty acids profile and concentration of lipids peroxidation products (thiobarbituric acid reactive substances – TBARS) in chemically induced mammary tumors.

MATERIALS AND METHODS

Animals

The guiding principles in the use and care of laboratory animals as well as the whole experiment were approved by The Local Ethical Committee on Animal Experiments. Female Sprague-Dawley rats (n = 104, age – 30 days) were purchased from Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Warszawa, Poland). They were kept in animal room at 21^{o}C, in a 12 h light : 12 h dark cycle and during the whole research they were fed *ad libitum* a standard laboratory diet Labofeed H (Fodder plant "Morawski", Żurawia 19, Kcynia, Poland) and water. Applied laboratory diet is composed of 22.0% protein, 4.0% fat, 30.0% starch, 5.0% fibre, 6.5% minerals. After the arrival and 1-week adaptation period, the animals were randomly divided into 9 groups with different dietary supplementation. The total characteristics of experimental groups is shown in Table 1. Moreover, at 50th day of life, all animals received intragastrically *via* gavage a single dose of carcinogenic agent - DMBA (7,12-dimethylbenz[a]anthracene, approx. 95%, Sigma-Aldrich) in the amount of 80 mg/kg body weight. Diet of three groups was supplemented with conjugated linoleic acid (Bio-C.L.A., Pharma Nord, Denmark), given *via* gavage in the amount of 0.15 mL/day (1% in diet) and diet of three other groups was supplemented with conjugated linoleic acid (Bio-C.L.A., Pharma Nord, Denmark), given *via* gavage in the amount of 0.30 mL/day (2% in diet). Bio-C.L.A. contained two main CLA isomers: cis-9, trans-11 (31.4 ± 0.0%) and trans-10, cis-12 (33.3 ± 0.1%) as main fatty acids, and also oleic acid (11.0 ± 0.0%), linoleic acid (10.2 ± 0.0%), palmitic acid (4.6 ± 0.0%) and stearic acid (2.2 ± 0.0%). Three control groups received intragastrically vegetable oil in the amount of 0.15 mL/day. Applied oil does not contain conjugated linoleic acids and was purchased from Pharma Nord, Denmark, where it is used as the substrate to Bio-C.L.A. synthesis. This allowed us to minimize the differences in fatty acids profile of applied diets. Table 2 shows the fatty acid composition of applied diets. Supplementation of diet was conducted after (AOil, A1%, A2%), before (BOil, B1%, B2%) and before and after (BAOil, BA1%, BA2%) DMBA administration. During the experiment, rats were weighed weekly and palpated to detect the appearance of tumors. The entire experiment lasted for the following 21 weeks. In the 21st week of the experiment, most of the animals were decapitated and exsanguinated, and the weight of internal organs were determined. Only animals of A2% and BA2% groups were decapitated in the 15th week of the experiment, because of their much lower body weight and cachexia.

Histopathological examination

DMBA treatment caused the mammary tumors induction, which effectiveness was determined as the percentage of animals with tumors in each group. Some of the tumors collected during necropsy (usually three in each group), selected randomly, were fixed in 10% formalin and were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland.

Preparation of experimental material

Mammary tumors were collected during necropsy and stored at -20^{o}C until being analyzed.

Fatty acids analysis

Fatty acid analysis was made by means of gas chromatography (GC) with capillary column and flame-ionization detection. Mammary tumors were thawed only once and three parallel samples of 0.2 g were taken for lipids extraction according to Folch et al. with minor modification (9). Purified organic extract was evaporated to dryness under a stream of

Table 2. Fatty acids composition of applied diets.

Fatty acid [%]	Fodder + oil	Fodder + 1.0% CLA	Fodder + 2.0% CLA
C6:0	0.11 ± 0.01	0.12 ± 0.00 –	
C8:0	–	0.02 ± 0.00	0.05 ± 0.03
C10:0	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
C11:0	0.01 ± 0.00	–	2.42 ± 0.01
C12:0	0.02 ± 0.00	0.06 ± 0.08	0.02 ± 0.00
C14:0	0.12 ± 0.01	0.12 ± 0.01	0.09 ± 0.05
C15:0	0.07 ± 0.01	–	0.07 ± 0.00
C16:0	10.49 ± 0.13	11.16 ± 0.23	10.12 ± 0.02
C16:1	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00
C17:0	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
C18:0	2.53 ± 0.01	2.47 ± 0.03	2.42 ± 0.00
C18:1 n-9	34.46 ± 0.10	15.87 ± 0.16	15.59 ± 0.04
C18:2 n-6 cis	35.11 ± 0.05	31.46 ± 0.31	27.17 ± 0.07
cis-9, trans-11 CLA	–	8.57 ± 0.34	12.41 ± 0.13
trans-10, cis-12 CLA	–	8.47 ± 0.38	12.37 ± 0.10
C18:3 n-3	14.15 ± 0.01	16.69 ± 0.19	13.70 ± 0.02
C20:0	0.24 ± 0.12	–	–
C20:1	0.36 ± 0.01	0.38 ± 0.00	0.49 ± 0.08
C20:3 n-6	0.04 ± 0.06	0.01 ± 0.00	–
C20:3 n-3	0.02 ± 0.00	0.04 ± 0.02	–
C20:5 n-3	0.06 ± 0.01	0.10 ± 0.02	0.08 ± 0.00
C21:0	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.01
C22:0	0.20 ± 0.10	0.16 ± 0.01	0.13 ± 0.01
C22:1 n-9	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
C22:2	0.12 ± 0.00	–	–
C23:0	–	0.12 ± 0.01	–
C24:0	0.15 ± 0.04	0.06 ± 0.05	0.09 ± 0.05
C24:1	0.10 ± 0.00	–	–

nitrogen and weighed to estimate the content of lipids in tumor tissue. The fat content was calculated as the percentage share of fat weight in tumor tissue [%]. The residue was taken for the preparation of fatty acids methyl esters (FAME) according to procedure of Bondia-Pons et al. with minor modifications (10), previously described for serum (11). Briefly, FAME were separated and quantified using Shimadzu GC-17A gas chromatograph with flame ionization detector. Injector was heated to 250°C and detector was heated to 270°C. Separation of FAME was performed on BPX70 capillary column (60 m × 0.25 mm i.d., film thickness: 0.20 µm, SGE) with helium as the carrier gas. The initial oven temperature was 140°C for 1 min, thereafter increased by 20°C/min to 200°C and hold for 20 min and then increased by 5°C/min to 220°C and hold for 25 min. Standards of CLA methyl esters: cis-9, trans-11 CLA and trans-10, cis-12 CLA were purchased from Nu-Chek-Prep. Inc., USA. Peaks of CLA isomers in examined samples were identified by comparison with retention time of standards and quantified by regression formula obtained with the standards. CLA content was expressed in relation to fat content and to tumor tissue weight.

TBARS analysis

From tumors thawed for fatty acids analysis, sample of 0.5 g was taken for TBARS analysis with spectrophotometric method (12). This sample was

mechanically homogenized with Teflon homogenizer in 2.5 mL of sodium chloride solution (0.9%). Afterwards, 2.5 mL of phosphate buffer (pH 7.0) and 1.5 mL of trichloroacetic acid (1.7 mol/L), were added to 0.5 mL of previously obtained homogenate. The whole sample was shaken vigorously and centrifuged for 15 min at 4000 rpm at 0°C, to separate the protein sediment. One milliliter of 2-thiobarbituric acid solution (69 mmol/L) was added to 3.0 mL of supernatant; the whole sample was shaken vigorously and heated for 15 min at boiling water bath. Three parallel samples were prepared for each tumor. The absorbance of samples was measured at λ = 530 nm after their cooling to room temperature. The reference sample was prepared analogously with 0.5 mL of sodium chloride solution (0.9%). TBARS content was quantified in relation to fat content and to tumor tissue weight.

Statistical analysis

All data are shown as the mean values ± standard deviation. For variables with skew distribution, data were transformed in logarithms and retransformed after calculations. Obtained results were evaluated with Statistica 9.0 (StatSoft, Poland) and GraphPad prism v.3.02 (GraphPad Software, USA). Due to the lack of normal distribution for some variables and to the relatively small number of examined tumors in some groups, the data were tested with Kruskal-Wallis test and verified with Dunn's multiple comparison test; p-value < 0.05 was considered significant.

RESULTS

DMBA given intragastrically in a single dose of 80 mg/kg body weight was effective in the induction of mammary tumors. They appeared during the experiment in all groups and were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. The percentage of tumor-bearing animals in each group is shown in Table 1.

The higher cancer morbidity was observed in groups BOil and B2%. In those groups all animals suffered from mammary tumors. Nevertheless, we

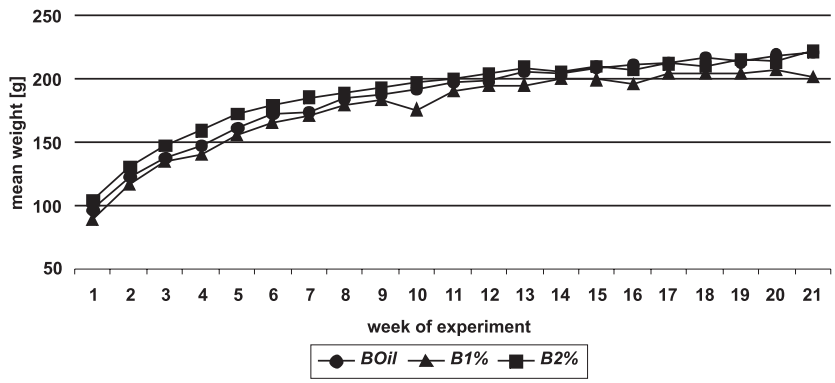

Figure 1. Changes of average body weight during the experiment of groups supplemented before DMBA administration

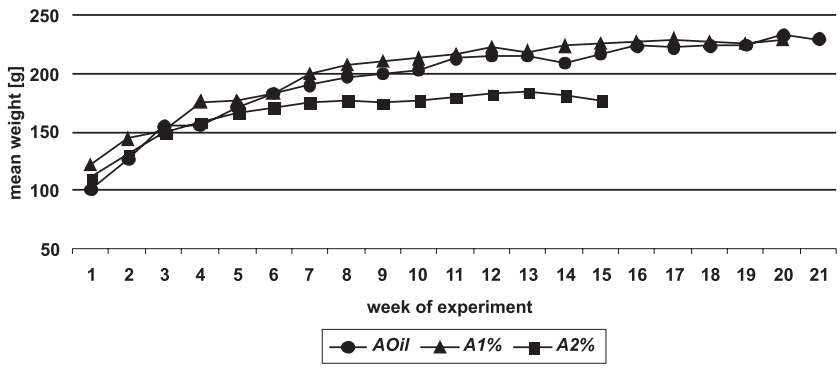

Figure 2. Changes of average body weight during the experiment of groups supplemented after DMBA administration

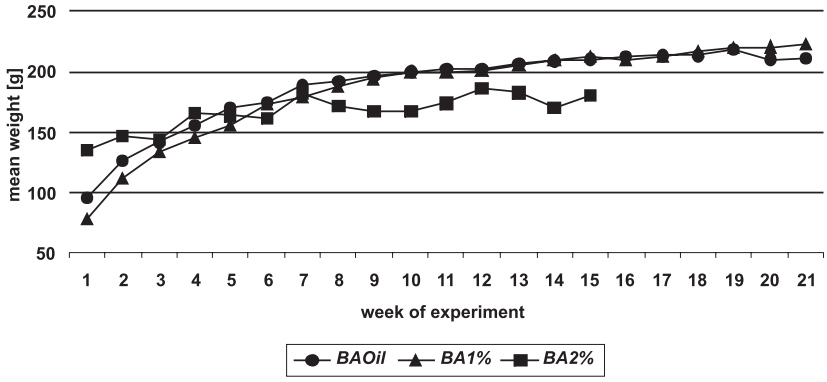

Figure 3. Changes of average body weight during the experiment of groups supplemented before and after DMBA administration

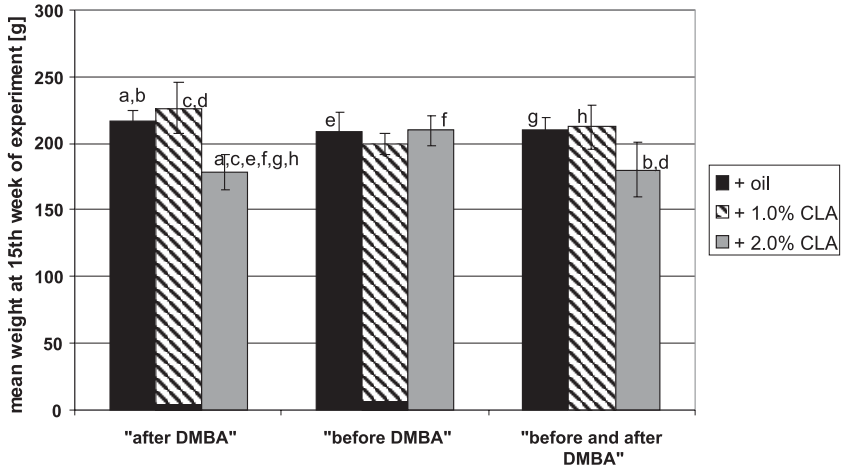

Figure 4. Mean body weight gained by each experimental group at 15th week of experiment. The same superscripts mean, that the values differ with $p < 0.05$

observed diminished induction of cancerous process in most of CLA supplemented groups. The preventive effect was the strongest in BA2% group, where breast cancer appeared only in one of nine animals. Also in A2% group the cancer morbidity was much smaller than in other groups. Furthermore, animals supplemented with the lower dose of CLA (1.0%) were less prone to chemically induced carcinogenesis than animals from similar groups receiving vegetable oil. The mammary tumors appeared and were palpated in 20-21 week of life, much later than in groups supplemented with oil. Taking into account other parameters characterizing the cancer morbidity, such as total weight of tumors and total number of tumors, we observed significant differences among all examined groups ($p = 0.0080$ and $p < 0.0010$, respectively). The preventive properties of CLA were also reflected by the diminished number and smaller size of tumors in A2% and BA2% groups.

During the whole experiment, the mean body weight of each group was checked weekly. There were no significant differences in body weight among those groups, which were fed the modified diets before the DMBA administration (Fig. 1). We observed the significant influence of diet supplementation with higher dose of CLA on body weight in A2% and BA2% groups (Figs. 2 and 3). From 7th week of the experiment, their mean body weight were much lower than body weight of other animals, which indicates that they did not utilize the forage as effectively as other groups. Moreover, the action of CLA was bound with the toxic effect of carcinogenic agent treatment and caused the exhaustion of the animals. The continuation of the experiment for A2% and BA2% group was impossible because of the cachexia of animals and for those groups the experiment was terminated at 15th week (Figs. 2 and 3).

We compared the mean weight gained at 15th week of experiment in each group (Fig. 4). There were significant differences among examined groups ($p < 0.0001$). The lowest weight gained two groups supplemented with the higher dose of CLA for a long period of time. Such body weight lowering effect was not observed with the supplementation with 1.0% dose of CLA. On the contrary, A1% group gained the highest weight of all examined groups at 15th week of experiment.

The earlier termination of the experiment for A2% and BA2% group also significantly influences the weight of internal organs, as their weights were the lowest in these two groups (Table 3). However, the applied modification of diet also had a significant impact on the organs' weight. The highest mean weight of liver was determined for BOil group, whereas A1% group gained the highest weight of kidneys and heart. As far as weight of spleen is considered, we observed great differences among individuals in all investigated groups but also significant differences among groups. The efficiency of DMBA action also modified the organs' weight, as the comparison of these parameters revealed their significantly higher values for individuals suffering from mammary tumors than in individuals without breast cancers (data not shown).

Fatty acids methyl esters (FAME) profiles in fat isolated from mammary tumors were determined using GC. In our experiment, we were able to identified 32 fatty acids in tumors. The following fatty acids were found to be the main fatty acids in the tumors of all investigated groups: palmitic C16:0, oleic C18:1 n-9 cis, linoleic C18:2 n-6 cis, arachidonic C20:4 n-6 and stearic C18:0 (Table 4).

There were significant differences in the concentration of some of the fatty acids among examined groups (Tables 4 and 5).

Two main saturated fatty acids were detected in the highest amounts in tumors from B2% group (C16:0) and BOil (C18:0), whereas their content in samples from BAOil (C16:0) and B1% (C18:0) was the lowest. In BAOil group we also detected the highest share of main monounsaturated acid - oleic acid, while the lowest concentration of this acid was characteristic for B2% group. Among polyunsaturated fatty acids, concentration of linoleic acid was the highest in fat fraction of mammary tumors but its content differed significantly among individuals in each group. We observed its highest content in tumors obtained from BAOil group. Moreover, cancerous tissues from this group were also characterized by the lowest content of arachidonic acid – the main metabolite of linoleic acid. Similar dependence was observed also for α-linoleic acid (C18:3 n-3), which content in BAOil was the highest among all investigated groups, and its metabolite - eicosapentaenoic acid (C20:5 n-3), which content in this group was the smallest. As far as docosahexaenoic acid (C22:6, n-3), the other ALA metabolite is concerned, its lowest amount was detected in adenocarcinomas from A2% and the highest – in BOil groups. The highest concentration of arachidonic acid was detected in a single analyzed tumor from BA2%.

Two main CLA isomers identified as cis-9, trans-11 CLA and trans-10, cis-12 CLA, were detected in mammary tumors from all investigated groups. Bio-C.L.A. used as the source of CLA consisted of several fatty acids, with prevailing share of two CLA isomers: trans-10, cis-12 CLA (33%) and cis-9, trans-11 CLA, rumenic acid (31%) (13). We observed that the applied dose of CLA supplement influenced their content in adenocarcinomas of mammary glands as the highest content of both CLA isomers were detected in groups supplemented with the higher dose of CLA and their content in groups supplemented with vegetable oil was minimal. Cis-9, trans-11 CLA was shown to be the predominant CLA isomer in fat from mammary tumors of all groups, while trans-10, cis-12 CLA was detected in lower amounts, although their concentration in applied diets was similar (Table 2). The highest content of rumenic acid was detected in A2% group ($1.16 \pm 0.98\%$) while trans-10, cis-12 constituted only $0.74 \pm 0.73\%$ of FA in mammary tumors from this group.

There were no significant differences in tumors fat content among examined groups, although some tendencies were observed – its content was elevated in mammary tumors obtained from groups supplemented with higher dose of CLA (Table 3). It was not possible to quantify the absolute content of CLA isomers in each group, as their amounts were below the limit of quantification. Only their percentage share in the total pool of fatty acids was evaluated. Especially in groups receiving vegetable oil or supplemented with CLA for a very short period of time, content of CLA isomers was below the quantification limit in some individuals. Moreover, in all investigated groups there were significant differences in both isomers content among individuals (Table 3). The highest concentration of rumenic acid (in fat fraction and in tissue) was determined in A2% group, whereas in BOil it was not possible to quantify its content. Adenocarcinomas of A2% were also characterized by the highest mean content of trans-10, cis-12 CLA. Furthermore, mean content of

Table 3. Comparison of mean weights of organs and fat, CLA and TBARS content in mammary tumors.

	Groups of animals									
	AOil	A1%	A2%	BOil	B1%	B2%	BAOil	BA1%	BA2%	p value
Liver [g]	6.48 ± 0.57[a]	6.20 ± 0.91	5.58 ± 0.47	6.73 ± 1.62	5.63 ± 0.41	5.75 ± 0.61	6.56 ± 1.34	6.13 ± 0.69	5.35 ± 0.63[a]	0.0013
Kidneys [g]	1.81 ± 0.07[a,b]	1.83 ± 0.18[c,d]	1.41 ± 0.08[a,c,e,f,g,h]	1.71 ± 0.18[e]	1.62 ± 0.13	1.74 ± 0.17[f]	1.70 ± 0.14[g]	1.63 ± 0.31[h]	1.50 ± 0.16[b,d]	< 0.0001
Spleen [g]	0.81 ± 0.25[a,b]	0.71 ± 0.23[c]	0.42 ± 0.04[a,c,d,e,f,g,h]	0.71 ± 0.28[d,e]	0.61 ± 0.30	0.67 ± 0.14[f]	0.76 ± 0.30[g]	0.71 ± 0.23[h]	0.49 ± 0.09[b]	< 0.0001
Heart [g]	0.84 ± 0.04	0.95 ± 0.15[a]	0.72 ± 0.04[a,b,c,d,e]	0.92 ± 0.09[b]	0.88 ± 0.09[c]	0.79 ± 0.05[f,g]	0.93 ± 0.10[d,f,h]	0.92 ± 0.12[e,g]	0.79 ± 0.08[h]	< 0.0001
Fat [%]	2.27 ± 0.84	2.13 ± 1.25	2.60 ± 0.76	2.32 ± 1.03	2.30 ± 1.30	1.68 (0.69 – 4.14)*	2.17 ± 1.35	2.52 ± 1.46	4.58	0.8079
cis-9, trans-11 CLA [mg/g of fat]	56.4 (14.0 – 227.6)*	2287.4 ± 1030.1	3690.7 (1355.9 – 10045.3)*	96.5 ± 0.00	78.8 ± 66.2	808.6 ± 749.5	269.6 (90.6 – 802.0)*[,a]	3068.9 ± 2507.9[a]	2903.8	0.0020
cis-9, trans-11 CLA [mg/g of tissue]	1.8 ± 1.4[a,b]	65.9 ± 43.4	112.2 (22.9 – 550.7)*[,a]	0.6 (0.0 – 13.4)*	2.6 ± 2.3	22.6 ± 21.5	5.8 ± 2.4[c]	63.8(16.8 – 242.4)*[,b,c]	72.1	0.0003
trans-10, cis-12 CLA [mg/g of fat]		1428.8 ± 885.3	4087.7 ± 3363.8	31.9 ± 0.0		632.4 (156.7 – 2552.3)*	265.7 (40.5 – 1742.2)*	1654.5 ± 1457.9	1462.1 0	.3018
trans-10, cis-12 CLA [mg/g of tissue]		46.0 ± 40.1	85.1 (14.0 – 516.2)*	2.7 ± 0.0		15.8 ± 3.7	5.8 ± 3.3	28.1 (4.5 – 173.5)*	35.4	0.1320
TBARS [nmol/g of tissue]	24.3 ± 17.6	20.6 ± 5.7	24.0 ± 15.0	32.1 ± 16.3	23.7 ± 16.4	17.8 ± 17.0	30.3 ± 17.9	36.2 ± 18.5	350.1	0.5042
TBARS [mmol/g of fat]	1.18 ± 1.11	1.17 ± 0.73	0.87 ± 0.30	1.58 ± 1.27	1.03 ± 0.8	1.20 ± 1.14	1.35 (0.29 – 6.19)*	1.67 ± 0.92	7.64	0.8284

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration. All data are shown as the mean values ± standard deviation. For variables with skewed distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as mean and confidence interval. p value < 0.05 - significant differences among groups in Kruskal–Wallis test; values with the same superscripts differ significantly.

this isomer in fat fraction of A2% tumors was higher than the content of cis-9, trans-11 CLA, although in other investigated groups the tendency was opposite – we detected higher amounts of rumenic acid than trans-10, cis-12 CLA. The highest concentrations of CLA isomers were revealed in mammary tumors obtained from groups supplemented with 2.0% of Bio-C.L.A., which indicates the influence of applied dose on the CLA isomers content in cancerous tissues.

TBARS content in mammary tumors was determined with spectrophotometric method and was express also in terms of fat content as it is considered as an indicator of lipids peroxidation processes. Results for TBARS are summarized in Table 3. Although we detected very high concentration of TBARS in one mammary tumor of BA2% group, we found this result as an outlier and this single result was not included into statistical analysis. The comparison of TBARS content in cancerous tissues from other investigated groups did not reveal any significant differences. Moreover, we observed large diversity in results among individuals in investigated groups.

DISCUSSION AND CONCLUSION

Some species of laboratory animals treated with carcinogenic agent become prone to cancer development. In our experiment, single DMBA treatment of Sprague-Dawley female rats at 50th day of life caused the mammary tumors appearance in all groups. They were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. Our previous experiment (14), as well as the experiments conducted by other researchers (15-18) revealed the usefulness of this model in breast cancer research. We observed the significant anticarcinogenic properties of CLA: diminished breast cancer morbidity and decreased weight and number of mammary tumors in CLA supplemented groups (Table 1). This observation is in accordance with our previous results (11, 19) and with the results of others. Corl et al. detected that CLA as well as its precursor - vaccenic acid administration decreased the mammary tumors risk in laboratory animals (20, 21). Also Ip et al. observed that 0.5%, 1.0% and 1.5% of CLA reduced the number of malign and benign breast tumors in female rats (17). Moreover, even smaller doses of CLA (0.05–0.5%) given to rats for 9 months effectively prevented the breast cancer development. They observed also that short (5 weeks) administration of CLA before DMBA treatment at 50th day of life diminished this cancer risk (17). Thompson et al., who compared 1-month supplementation with 1.0% CLA before DMBA treatment and 6-month supplementation, revealed the high effectiveness of short CLA administration followed by the carcinogenic agent administration (18). Our results – the lower cancer morbidity in B1% group, receiving 1.0% of CLA mixture only for 14 days (from 37th till 50th day of life) – confirm protective efficacy of CLA administered in early stage of life, before cancerous process induction. Ip et al. explained that CLA given early, when mammary glands maturate, cause a decrease in TEB cells' number and stimulate their differentiation (17). This brought about the decrease of the amount of potential places of cancerous process initiation, because TEB are the main places of adenocarcinomas induction in mammary glands of rodents (22). However, addition of CLA to diet after carcinogenic agent treatment also diminished the number of breast tumors and their growth (23). The activity of CLA depends on their dose in diet and its maximum is observed at 1.0%, regardless the carcinogenic agent and other dietary lipids presence (24). We observed the higher anticancer effectiveness of 2.0% CLA but this dose seems to be too high because of the cachexia stimulation. Our results ostensibly disagree with the statement of Pariza et al., who claimed that one of the CLA influence on cancerous process is through the cachexia inhibition (25). However, this effect is attributed to rumenic acid, whereas trans-10, cis-12 CLA does not demonstrate such activity. The fact, that applied preparation contained the mixture of two main CLA isomers, may cause such incompatibilities.

We observed much lower mean body weight of animals supplemented with higher dose of CLA since 7th week of experiments, what was the reason of their earlier decapitation. Many researchers emphasized the ability of CLA isomers to reduce the body mass. Especially trans-10, cis-12 CLA seems to be potent and its action has few potential mechanisms: reduction of lipoprotein lipase activity, diminished differentiation of preadipocytes into adipocytes through the genes inhibition and stimulation of lipids β-oxidation by activation of crucial enzymes expression (26, 27). Bio-C.L.A., which we used as a source of conjugated linoleic acids, consisted of few fatty acids with prevailing share of two CLA isomers: cis-9, trans-11 and trans-10, cis-12, which were present in equal amounts (Table 2). High intake of trans-10, cis-12 CLA seemed to be responsible for much lower weight gained by animals supplemented with 2.0% of CLA. Our results are in accordance with those obtained by He et al., who applied synthetic isomers of CLA to laboratory

Table 4. Fatty acids profile in mammary tumors of investigated groups.

Fatty acid [%]	Groups of animals									Kruskal-Wallis test p value
	AOil	A1%	A2%	BOil	B1%	B2%	BAOil	BA1%	BA2%	
C12:0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3	0.5 (0.13–2.04)*	0.2 ± 0.1	0.2 ± 0.1	0.2	0.1908
C13:0	0.3 ± 0.2	0.3 ± 0.2	0.1 (0.03-0.18)*	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	-	-	0.23	0.3632
C14:0	1.2 ± 0.3	1.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.2	1.7 ± 1.0	3.0 ± 5.6	1.1 ± 0.2	1.1 ± 0.2	1.2	0.1058
C15:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3	0.3584
C16:0	24.0 ± 2.0	23.3 ± 0.9	22.9 ± 1.7	22.1 ± 1.7	23.7 ± 1.0	24.1 ± 2.2	20.8 ± 1.6	22.9 ± 1.6	22.6	0.0093
C16:1	2.6 ± 1.4	1.7 ± 0.4	1.2 ± 0.0	1.6 ± 0.6	1.6 ± 0.2	2.1 ± 0.8	1.4 ± 0.3	1.6 ± 0.7	1.1	0.1308
C18:0	10.1 ± 2.0	9.2 ± 1.3	9.4 ± 2.2	10.5 ± 2.3	8.9 ± 1.6	9.4 ± 1.7	9.3 ± 2.1	10.3 ± 2.2	10.1	0.5336
C18:1 n-9 *cis*	18.7 ± 2.2	18.82 ± 2.46	20.0 ± 5.7	19.5 ± 2.8	19.6 ± 3.2	16.9 ± 4.3	23.3 ± 4.1	18.4 ± 2.7	19.4	0.0752
C18:2 n-6 *trans*	0.3 ± 0.1	0.1(0.03-0.18)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 (0.05–0.52)*	0.1 (0.03-0.15)*	0.1	0.0018
C18:2 n-6 *cis*	12.0 ± 3.4	14.7 ± 5.9	15.0 ± 1.2	13.6 ± 5.5	16.0 ± 5.1	9.9 ± 5.1	19.1 ± 5.2	15.1 ± 6.5	18.5	0.0731
cis-9, *trans*-11 CLA	0.03 ± 0.01	0.5 ± 0.15	1.16 ± 0.98	0.02 ± 0.00	0.05 ± 0.01	0.04 (0.02-0.08)*	0.05 ± 0.03	0.64 ± 0.34	0.84	< 0.0001
trans-10, *cis*-12 CLA	0.02 ± 0.00	0.22 ± 0.12	0.74 ± 0.73	0.01 ± 0.00	0.01 ± 0.00	0.02 (0.01-0.06)*	0.03 ± 0.02	0.32 ± 0.19	0.44	< 0.0001
C18:3 n-6	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 (0.02–0.12)*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0	0.5077
C18:3 n-3	1.8 ± 1.1	2.6 ± 1.7	2.5 ± 1.6	2.0 ± 1.4	2.8 ± 1.2	1.9 ± 1.2	3.2 ± 1.3	2.2 ± 1.1	2.4	0.3291
C20:1	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.5	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4	0.0003
C20:2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.3	0.3212
C20:3 n-3	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1	0.1315
C20:4 n-6	11.8 ± 3.0	12.0 ± 4.2	11.1 ± 7.9	12.0 ± 4.0	10.4 ± 5.7	12.7 ± 3.6	8.3 ± 3.8	11.3 ± 4.8	13.9	0.4732
C20:5 n-3	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3	0.0363
C21:0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3	0.1695
C22:2	0.1 ± 0.1	0.0 ± 0.0	0.0 (0.01-0.06)*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2	0.0001
C22:6 n-6	1.6 ± 0.0	1.5 ± 0.4	1.1 ± 0.7	1.6 ± 0.4	1.4 ± 0.4	1.5 ± 0.4	1.3 ± 0.4	1.5 ± 0.4	1.4	0.5657
C23:0	0.1 ± 0.1	0.1 ± 0.1	0.1 (0.02-0.41)*	0.2 ± 0.2	0.1 (0.04–0.24)*	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2	0.3951
C24:0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3	0.0314
C24:1	1.1 ± 0.3	0.9 ± 0.0	0.8 ± 0.2	1.3 ± 0.4	1.3 ± 0.8	1.0 ± 0.3	0.9 ± 0.3	1.0 ± 0.1	1.0	0.1528

A Oil – group of rats supplemented with vegetable oil after DMBA administration, A 1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A 2% – group of rats supplemented with 2.0% of CLA after DMBA administration, B Oil – group of rats supplemented with vegetable oil before DMBA administration, B 1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B 2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BA Oil – group of rats supplemented with vegetable oil before and after DMBA administration, BA 1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA 2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration. All data are shown as mean values ± standard deviation. For variables with skewed distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as mean and confidence interval. p value < 0.05 – significant differences among groups in Kruskal-Wallis test; percentage share of C10:0, C14:1, C15;1, C17:0, C17:1, C20:0 and C22:0 was = 0.1% and these fatty acids are not shown in the table.

Table 5. Results of Dunn's multiple comparison test for fatty acids content in mammary tumors.

	Compared groups	Dunn test p value
C10:0	A2% *vs*. BA1%	< 0.01
	B2% *vs*. BA1%	< 0.001
C16:0	AOil *vs*. BAOil	< 0.05
	B2% *vs*. BAOil	< 0.05
C18:1 n-9 *cis*	B2% *vs*. BAOil	< 0.05
C18:2 n-6 *cis*	B2% *vs*. BAOil	< 0.05
C18:2 n-6 *trans*	AOil *vs*. BOil	< 0.05
	AOil *vs*. B1%	< 0.05
	AOil *vs*. B2%	< 0.01
C20:1	AOil *vs*. A2%	< 0.01
	AOil *vs*. BA1%	< 0.01
C22:2	A2% *vs*. BA1%	< 0.05
	BOil *vs*. BA1%	< 0.01
	B1% *vs*. BA1%	< 0.05
	B2% *vs*. BA1%	< 0.01
cis-9, *trans*-11 CLA	AOil *vs*. BA1%	< 0.05
	A1% *vs*. BOil	< 0.01
	A2% *vs*. BOil	< 0.01
	BOil *vs*. BA 1%	< 0.001
	B2% *vs*. BA 1%	< 0.01
trans-10, *cis*-12 CLA	AOil *vs*. BA1%	< 0.05
	A2% *vs*. B1%	< 0.05
	B1% *vs*. BA1%	< 0.05
	B2% *vs*. BA1%	< 0.05
	BAOil *vs*. BA1%	< 0.05

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration.

animals, and observed much lower body weight in comparison with control group (28). However, Ip et al. did not detect any differences in total body weight gained by rats treated with DMBA and supplemented with different doses of CLA (0.5–1.0%) (16).

Applied modification of diet significantly influenced the weight of internal organs. These observations disagreed with those made by others researchers. After 8-week supplementation with two doses of cis-9, trans-11 CLA Turpeinen et al. did not revealed any differences in heart, liver, spleen and pancreas weight (29). Also in much longer experiment of Ip. et al., with usage of CLA mixture and carcinogenic agent, there were no differences in internal organs weight (16). However, when they gave single trans-10, cis-12 CLA to mice treated with chemical carcinogen, they observed significantly higher weight of livers, hearts and spleen. Moreover, after cis-9, trans-11 CLA application, there were no such effects (30). This may suggest that effects of two major CLA isomers are opposite. Furthermore, our results show that coexisting cancerous process significantly influences both the total body weight (especially by the mass of tumors) and internal organs weight.

As far as fatty acids profile in mammary tumors is considered, we detected significant influ-

ence of applied diet on the content of some of fatty acids. Significant differences referred only to 9 of 32 analyzed fatty acids. Many authors compared the fatty acids profile in adipose tissue of mammary glands and other tissues of animals receiving CLA. Banni et al. observed lack of influence of 1.0% CLA on linoleic acid amount in adipose tissue of mammary glands but their distinct influence on reduction of linoleic acids metabolites: C18:3, C20:3 and arachidonic acid (22). Eder et al. also detected that CLA supplementation decreased the concentration of arachidonic acid as well as all n-6 fatty acids in hepatic phospholipids (31). Experiment of Cao et al. showed that 1.0% CLA in maternal diet reduced the arachidonic acid concentration in hepatic phospholipids of progeny, whereas 2.0% CLA had the opposite effect. Moreover, the impact of above mentioned diet modification on α-linolenic concentration was opposed while the linoleic acid amount was raised in both groups (32). Influence of CLA on other n-3 and n-6 fatty acids concentration was also divers. CLA supplementation increased the content of docosahexaenoic acid and all n-3 fatty acids in hepatic phospholipids (31). However, information concerning the impact of CLA supplementation on fatty acids profile in cancerous tissues is limited. One of the causes is the shortage of sufficient amounts of experimental material. Because of very low content of many fatty acids in cancerous tissues compared with normal tissues, detection of some fatty acids is difficult. Especially in relation to n-3 fatty acids these reasons are important. Senkal et al. detected much lower content of docosahexaenoic acid and eicosapentaenoic acid in gastrointestinal tumors than in livers (33). Results obtained by Hoffman et al. also confirmed lower levels of docosahexaenoic acid in cancerous than in normal tissues (34). For CLA isomers incorporation into cancerous tissues, the tendency is opposite, as their concentration in such tissues is much higher than in normal ones (34). However, comparison of CLA content in malign and benign mammary tumors revealed the lack of significant differences among them (35) or higher CLA content in benign tumors (36). CLA are easier incorporated into triacylglycerols than in phospholipids, that is why their content in neutral lipids is higher than in phospholipids (37, 38). They are built into nucleus and cytosol and their content in cellular membranes and mitochondria is much lower. Their incorporation into cellular fractions is similar with that of monounsaturated fatty acids (34). We detected higher content of cis-9, trans-11 CLA than of trans-10, cis-12 CLA in mammary tumors. Tsuzuki et al. claim that these differences are the results of different metabolism of main CLA isomers, while only trans-10, cis-12 activates β-oxidation of fatty acids and facilitates its own metabolism (39).

Results of many studies emphasize the higher content of TBARS in serum of patients suffering from different types of cancer: breast, lungs, stomach, small intestine (7, 8). Czeczot et al. detected higher concentration of TBARS than in control tissues in all investigated types of tumors, except malign liver tumors. Obtained results indicate that malignancy and proliferation of cells are connected with low level of lipids peroxidation (40). Some authors claimed that CLA possess strong antioxidant properties. Ha et al. found CLA to be as potent antioxidant as α-tocopherol and almost as strong as butylhydroxytoluene (41). Ip et al. also confirmed that CLA can inhibit the lipids peroxidation, because the levels of malonyldialdehyde in tissues were much lower after CLA treatment (24). Furthermore, they observed the strongest anticancerogenic effect of 1.0% CLA, whereas 0.25% CLA was the most effective dose to inhibit the TBARS formation in tissues. CLA mixture given to animals caused the decrease of TBARS levels in their mammary glands (16). However, Chen et al. observed much higher concentration of TBARS in plasma of mice with gastrointestinal tumors treated with rumenic acid or trans-10, cis-12 CLA (42). Turpeinen et al. also detected much higher concentration of 8-isoPGF2α in urine of animals receiving both CLA mixture and single trans-10, cis-12 CLA, what suggests the pro-oxidative properties of CLA (29). Moreover, Stachowska et al. indicated that CLA increased formation of reactive oxygen species (ROS) in macrophages (43). According to many researchers, an increased peroxidation of lipids caused by CLA supplementation is connected with cytotoxic effect of conjugated fatty acids against cancer cells. An increased susceptibility to oxidation of n-3 polyunsaturated fatty acids is, according to many authors, the main cause of their chemopreventive properties. Products of their oxidation act toxic at cancerous cells (44). Osinsky et al., who detected higher concentration of malonyldialdehyde in tumors after administration of cobalt complexes, found this effect to be beneficial (45). We detected much higher content of TBARS in mammary tumor of BA2% group, but we did not observed any differences in concentration of TBARS among other investigated groups. Our results do not confirm neither prooxidant nor antioxidant properties of CLA. However, it seems to be advantageous to evaluate the TBARS and other biofactors content in serum or

other tissues. This will help to recognize the overall oxidation status of the organism and will explain the influence of CLA supplementation on lipids and other compounds oxidation.

Our results confirm that conjugated linoleic acids can inhibit chemically induced mammary tumors development in female rats, but their cytotoxic action seems not to be connected with lipids peroxidation. Cis-9, trans-11 CLA and trans-10, cis-12 CLA differ with their incorporation into cancerous tissues and they influence some fatty acids content in mammary tumors.

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