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Full Length Research Paper

Evaluation of mutagenic/antimutagenic activity of conjugated linoleic acid in mice by micronucleus test

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Conjugated linoleic acids (CLAs) are positional and geometrical isomers of linoleic acid and some researchers have shown biological activities including modulation of lipid metabolism, atherogenesis, diabetes, and immune functions. In this study, the animals were supplemented with 2% of the average feed consumption with CLA (G1 = positive control) and safflower oil (G2 = negative control) and the test groups were supplemented with CLA at the concentration of 2 and 4% of the average feed consumption. To assess the CLA potential protective effect, two groups were used; G3 = CLA 2% + cyclophosphamide (CP) and G5 = CLA 4% + CP. To assess the mutagenic effects of CLA, two groups were used; G4 = CLA 2% + NaCl 0.9% and G6 = CLA 4% + NaCl 0.9%. In order to investigate the mutagenic/antimutagenic effects of CLA, micronucleus test was used. The results showed variation of feed consumption in the groups that received 4% of CLA, when compared to the control group (G1 and G2) and CLA groups (G3 and G4) ($p<0.05$), during the period studied. It was observed that CLA did not show mutagenic effect at the concentrations tested (2 and 4%). Also, CLA showed antimutagenic effect at the same concentrations. However, the animals that received 4% of CLA, presented clinical signs of malnutrition.

Key words: Conjugated linoleic acid, antimutagenicity, cyclophosphamide.

INTRODUCTION

Conjugated linoleic acid (CLA) is a dietary adjuvant for its anticarcinogenic properties and other biological activities such as modulation of lipid metabolism, atherogenesis, diabetes, hypertension, asthma and immune functions (Degner et al., 2006; DeClercq et al., 2011; Ing and Belury, 2011; MacRedmond and Dorscheid, 2011).

Conjugated linoleic acid (CLA) is a general term referring to a mixture of geometrical and position isomers of the octadecadienoic acid and is found in food derived from ruminants such as beef and lamb, as well as the dairy products from these sources (Lee et al., 1994). Most of the published studies have used a mixture of CLA isomers with two major forms; *cis*-9, *trans*-11-CLA (*c*9, *t*11-CLA) and *trans*-10, *cis*-12-CLA, (*t*10, *c*12-CLA), and a number of minor isomers (*t*7, *t*9-CLA; *c*9, *c*11-CLA; *t*9, *t*11-CLA;*c*10, *c*12-CLA; *t*10, *t*12-CLA; *t*11, *t*13-CLA; and *c*11, *c*13-CLA) (Kelley, 2007; DeClercq et al., 2011; Guler and Aktumsek, 2011).

The first investigation of the possible health promoting properties of CLA was published by Pariza et al. (1979), who found unidentified anticarcinogenic factors in fried ground beef. Ground beef extract contained anti-mutagenic compounds that selectively inhibited rat liver S-9-mediated mutagenesis (Pariza et al., 1979; Pariza, 1983). In a subsequent study, a factor that inhibited the initiation of mouse epidermal carcinogenesis induced by 7, 12-dimethylbenz[a]anthracene (DMBA) was found in grilled ground beef (Pariza and Hargraves, 1985). The factor was isolated and identified as an isomeric mixture of conjugated dienoic derivatives of linoleic acid (Ha et al., 1987).

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Among the several tests of assessment of carcinogenic compounds, the rodent bone marrow micronucleus test is largely investigated and could be referred to as a cytogenetic method based on counts of micronucleus in the cytoplasm of newly formed cell (Hayashi et al., 1994). Evaluation of micronucleus frequency *in vivo* is the primary test in a battery of genotoxicity tests and is recommended by the regulatory agencies worldwide the globe to be conducted as part of product safety assessment (Krishna and Hayashi, 2000). Micronucleus test has many advantages: reliable identification of cells that have completed only one nuclear division, sensitivity and precision, quickness and simplicity, the ability to screen large numbers of cells, and good reproducibility.

Micronucleus which appear in the cytoplasm of the divided cells as small additional nuclei result from chromosome fragments or whole chromosomes that are left behind during mitotic division.

Thus, the presence of micronucleus is an indication of exposure to clastogenic and/or aneugenic agents (Ramírez, 1999).

Thus, after determining the fatty acid profile of the CLA and placebo (safflower oil), the objective of this study was to evaluate the effects of mutagenic and antimutagenic effects of conjugated linoleic acid on bone-marrow cell of mice through micronucleus test.

MATERIALS AND METHODS

Supplements

Supplements used were commercial conjugated linoleic acid Tonalin® TG 80 and safflower oil (oil arising from the seed of *Carthamus tinctorius* L.), both from Cognis Brazil Limited.

Determination of the fatty acid profile of the CLA and placebo (safflower oil)

The methylation of fatty acids was performed using the method described by Christie (1982). For determination of the fatty acid profile, a gas chromatograph equipped with fused silica capillary columns, CP SIL 88 (0.25 mm x 0.2 µm x 100 m) and flame ionization detector (FID) was used. A temperature gradient was used, in which the first temperature was 70°C for 4 min, and then, an increase of 13°C per min until 175°C (maintained for 27 min) was performed, followed by an increase of 4°C per min until 215°C for 11 min, and followed again by an increase of 4°C until 240°C during 4 min; total of 70 min for the run. The temperatures of injector and detector were 250 and 300°C respectively. The injection mode was split, with 50:1 ratio. Carrier gas was hydrogen with flow rate of 1.8 ml per min, and pressure of 36.3 psi on top of the column.

Animals and diets

For biological assays, three weeks old male Swiss mice (25±5 g) were obtained from Central Animal Facility of the Federal University of Alfenas. The animals were housed in wire topped opaque polycarbonate cages and maintained under constant environmental conditions on a 12 h light/dark schedule. The environmental temperature was 20 ± 2°C and 50% humidity. Commercial feed and water were provided *ad libitum*. All experiments were conducted according to Brazilian regulations for animal experimentation (COBEA), after approval by the Ethics Committee on Animal Experimentation at the Federal University of Alfenas (protocol 201/2008).

Experimental design

The 36 Swiss albino male mice were distributed in six groups with six animals each, according to the following distribution: G1 (positive control) = safflower 2% + cyclophosphamide (CP); G2 (negative control) = safflower 2% + NaCl 0.9%; G3 (experimental group) = CLA 2% + CP; G4 (experimental group) = CLA 2% + NaCl 0.9%; G5 (experimental group) = CLA 4% + CP; G6 (experimental group) = CLA 4% + NaCl 0.9%. To assess the CLA potential protective effect, two groups were used; G3 and G5. To assess the mutagenic effects of CLA, two groups were used; G4 and G6. Throughout the experimental period, animals were supplemented by orogastric gavage with disposable syringe of 1ml (Figure 1). Cyclophosphamide (50 mg/kg body weight) was injected in three groups (G1, G3 and G5) 24 h before the euthanasia and the other three groups received NaCl 0.9% (G2, G4 and G6). Both femur bones were excised and their bone marrow flushed into test tubes using a syringe containing bovine fetal serum. All animals were sacrificed 24 h after treatment by cervical dislocations under ether anesthesia (Figure 1).

Supplementation

Animals were supplemented by orogastric gavage with 1 ml disposable syringes and gavage needles. The amount of supplement was calculated every other day, based on the average feed consumption of each group, so that supplementation followed normal feed ingestion. Supplements were aspirated with the syringe and kept away from light until the moment of administration. Mice were removed group by group from the experiment room, placed in plastic boxes, and taken to the supplementation room. This procedure was done daily during daytime and always at the same time, since rodents have nocturnal habits.

Sampling preparation for micronucleus test

For the conventional assessment of micronucleus frequencies, two slides for each animal were prepared according to the method described by MacGregor et al. (1987). Briefly, femurs were dissected, cleaned of any adhering muscle and bone marrow cells were flushed with bovine fetal serum into a centrifuge tube. The cells were stained with Leishmann and centrifuged at 2000 rpm for 5 min and supernatant was removed. The slides were coded, and the cells blindly scored by light microscope at 1000X magnification. The frequency of micronucleated polychromatic erythrocytes (MNPCE) in each mouse was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group. Polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio was also determined as a total of 1000 erythrocytes counted.

Statistical evaluation

Statistical analysis was performed using the Sisvar package® (Ferreira, 2004). The frequencies of MNPCE between treatment groups and their respective controls were compared by the chi-square test (χ^2). The results were considered statistically significant

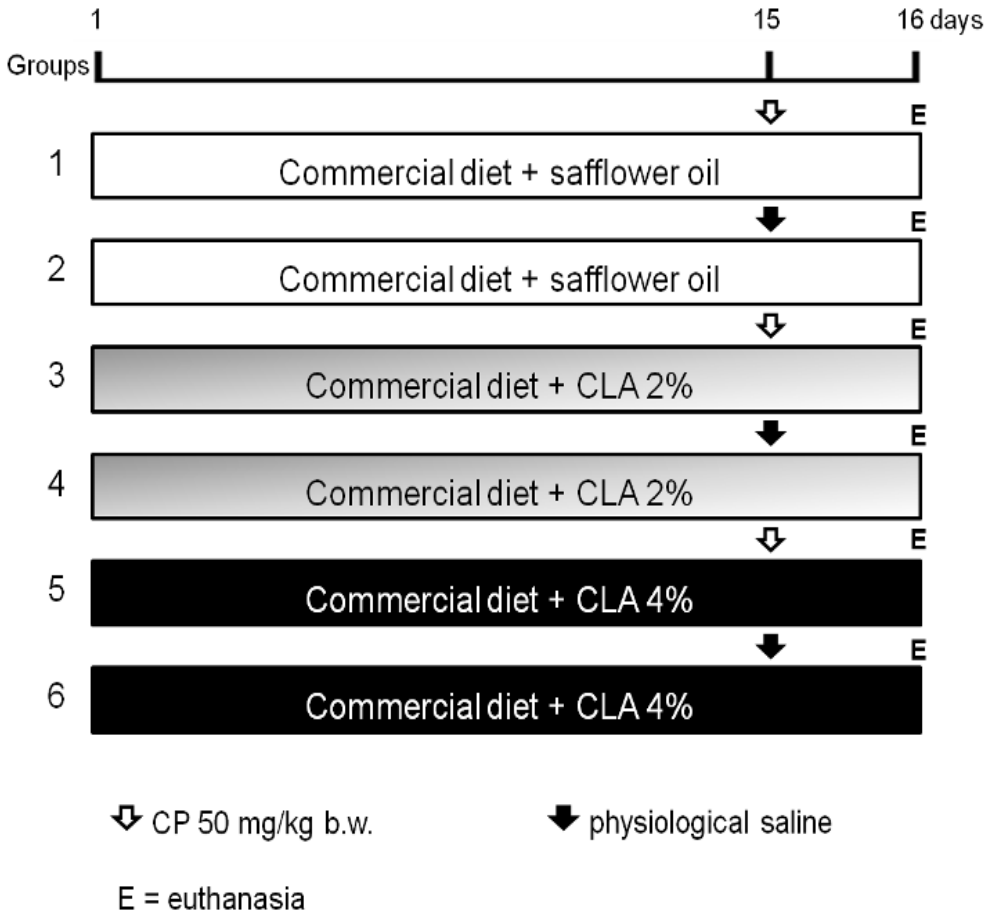

Figure 1. Experimental design for the micronucleus test. CLA, conjugated linoleic acid; CP: cyclophosphamide.

if the p values obtained were less than 0.05. Evaluations of mean body weight and diet consumption, were performed using one-way analysis of variance (ANOVA/Tukey, $p<0.05$).

The percentage of reduction in the frequency of cyclophosphamide-induced DNA damage was calculated as follows:

(Mean frequency of damage in A) - (mean frequency of damage in B) % reduction = x 100

(Mean frequency of damage in A) - (mean frequency of damage in C)

Where, A = positive control group treated with CP, B = group treated with the CLA + CP and C = negative control group.

RESULTS AND DISCUSSION

Determination of the fatty acid profile of the CLA and placebo (safflower oil)

Table 1 provides the composition of fatty acid from safflower oil supplement and mixture of CLA isomers. The amount of linoleic acid and CLA isomers was 77.75 and 80.01% in the capsule of safflower oil and CLA, respectively.

Animals and diets

During the experiment, the animals were weighed and the consumption of ration was controlled. These results are shown in Table 2, which shows the variation of weight gain and feed consumption in the groups that received 2 and 4% of CLA when compared to the control group ($p<0.05$), during the period studied.

The frequency of micronucleus (MN) in MNPCE of mice bone marrow, was lower ($p<0.05$) after administration of the substances tested (2 and 4% of CLA), as presented in Table 3.

According to profile of fatty acid indicated on Table 1, the safflower oil supplement used for orogastric gavage by control group contained 77.75 g of linoleic acids per 100 g of total fatty acids. The supplement of conjugated linoleic acid used for orogastric gavage by the test groups

Table 1. Profile of the fatty acid of safflower oil and CLA.

Fatty acid	Safflower oil (g/100 g)	Conjugated linoleic acid Tonalin® (g/100 g)
C4:0	-	-
C6:0	0.004	-
C8:0	0.025	-
C10:0	0.021	0.029
C10:1	-	-
C11:0	-	-
C12:0	0.037	0.032
C12:1	-	-
C13:0	-	0.227
C13:0 isso	-	-
C13:0 anteiso	-	-
C14:0	0.146	-
C14:0 isso	-	-
C14:1 *cis*-9	-	-
C15:0	-	-
C15:0 isso	0.005	-
C15:0 anteiso	-	-
C15:1	-	-
C16:0	6.347	2.733
C16:0 isso	-	-
C16:1 *cis*-9	0.086	-
C17:0	0.029	0.035
C17:0 isso	-	-
C17:1	0.024	-
C18:0	2.191	2.767
C18:1 *trans*-6	-	-
C18:1 *trans*-10	-	-
C18:1 *trans*-16	0.068	0.24
C18:1 *cis*-9	12.147	10.148
C18:1 *cis*-11	0.528	1.511
C18:1 *cis*-12	0.239	0.787
C18:1 *cis*-13	0.1	0.474
C18:1 *cis*-15	-	-
C18:2 *trans*-11. *cis*-15	-	-
C18:2 *cis*-9. *cis*-12	77.757	-
C18:2 *cis*-9. *trans*-11 CLA	-	38.426
C18:2 *trans*-10. *cis*-12 CLA	-	41.592
C:18:3	0.059	-
C20:1	-	-
C20:3	-	-
C20:4	-	-
C20:5	-	-
C22:0	-	-
C22:1	-	-
C22:5	-	-
C22:6	-	-
C24:0	-	-
C24:1	-	-
Total	99.948	99.142

Table 2. Body weight and food consumption in the different experimental groups of animals used in the micronucleus test.

Group/treatment*	Body weight (g)‡		Total feed consumption (g)
	Initial	Final	
G1	28.21±3.76^{Aa}	32.35±3.90^{Aa}	98.42±4.33^{A}
G2	24.81±2.99^{Aa}	27.35±4.65^{ABa}	92.44±3.11^{A}
G3	26.96±0.53^{Aa}	21.05±4.39^{Ba}	83.50±4.72^{B}
G4	26.46±2.46^{Aa}	22.39±3.37^{Ba}	81.66±3.93^{B}
G5	26.42±2.35^{Aa}	21.55±2.07^{Bb}	51.77±5.95^{C}
G6	27.46±1.30^{Aa}	21.17±1.17^{Bb}	60.97±6.42^{C}

*G1 (positive control) = safflower 2% + CP; G2 (negative control) = safflower 2% + NaCl; G3 = CLA 2% + CP; G4 = CLA 2% + NaCl; G5 = CLA 4% + CP; G6 = CLA 4% + NaCl. ‡ Values sharing similar capital letter in the same column and minuscule letter in the same line are not different ($p > 0.05$) by Tukey test.

Table 3. Frequency of MNPCEs of bone marrow of mice Swiss of the experimental groups treated with conjugated linoleic acid and safflower oil.

Group/Treatment	MNPCEs		
	Nº	%	% of reduction
G1	122	2.03	
G2	20	0.33	
G3	17	0.43	94.60
G4	18	0.45	
G5	40	1.00	60.78
G6	21	0.53	

*G1 (positive control) = safflower 2% + CP; G2 (negative control) = safflower 2% + NaCl; G3 = CLA 2% + CP; G4 = CLA 2% + NaCl; G5 = CLA 4% + CP; G6 = CLA 4% + NaCl. Analyzed cells = 6000.

contained a mixture of CLA isomers. The major CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) were present at the ratio of 1:1, approximately.

Among the other CLA isomers, *cis*-9, *trans*-11 and *trans*-12, and cis-12 are regarded as biological active. Isomers *cis*-9 and *trans*-11 showed anti-carcinogenic effects (Ip et al., 1999), and isomers *trans*-10, and *cis*-12 are particularly involved in the change of body composition (Park et al., 1999).

It is very difficult and expensive to isolate each of these isomers; this is the reason why most of the studies with CLA have used commercial mixture made from vegetable oil with predominantly CLA isomers *cis*-9, *trans*-11and *trans*-10, and *cis*-12 at the ratio of 1:1 (Funck et al., 2007). Therefore, it is not possible to award results for each isomer in isolated form.

The results of weight gain and food consumption suggest that the consumption of CLA at different concentrations interferes with animal development, growth and food intake. These effects appear to correlate with a control of the satiety-related factors such as leptin, ghrelin, adiponectin and resistin. Leptin is a hormone synthesized and secreted by adipocytes in the plasma, while ghrelin is secreted from the gastrointestinal tract. Adiponectin and resistin are adipokines secreted by preadipocytes and mature adipocytes, respectively (Hermsdorff and Monteiro, 2004; Romero and Zanesco, 2006). Supplementation with 2% of CLA on food intake was able to reduce leptin levels in rats (Botelho et al., 2008). In addition, CLA supplementation was able to inhibit the activity of the activated peroxisome proliferator-activated receptors γ (γ PPAAR) and this in turn reduced the expression of genes for leptin in rodents and adipocytes (Zhang et al., 1996; Ing and Belury, 2011). The t10,c12-CLA isomer attenuates development of obesity-related hypertension, at least in part, by stimulating adiponectin production, which subsequently activates vascular endothelial nitric oxide synthase (DeClercq et al., 2011).

In studies with Wistar rats, Botelho et al. (2005) evaluated the effect of dietary CLA at concentrations of 1, 2 and 3% of its daily diet. When compared with the control (linoleic acid 2%), animals that received 1% of CLA showed less weight loss. In the animals that received 2 and 4% of CLA, there was reduction on fat mass content. In this study, consumption of CLA influenced the feed intake and consequently the weight loss of the animals that received 4% CLA, when

compared to the control groups and 2% CLA.

With the aim to investigate the potential mutagenic/ antimutagenic effects, this research adopted the 2 and 4% of CLA, due to the results of fat loss found by Botelho et al. (2005) at the same concentrations. However, in this study, in animals that received a concentration of 4% CLA on food intake during the experiment, an aggressive behavior and at the end of the experiment, clinical signs of malnutrition as a change of coat and apathy were observed.

When Sprague-Dawley mice were fed with a high-hydrogenated soybean oil food with conjugated linoleic acid at 1.5, 3.0 and 5.0%, they did not show different diet intake, weight gain and feed efficiency, when compared with the control group after six weeks of experimentation (Choi et al., 2004; Akahoshi et al., 2004; Bissonauth et al., 2006; Purushotham et al., 2007).

Wistar adult mice fed with food added with 1.5% CLA during three weeks did not show different diet intake, weight gain and feed efficiency when compared with the control group (Purushotham et al., 2007). Bissonauth et al. (2006) did not find any difference in diet intake and weight gain, neither in mice that received separately *trans*-10 and *cis*-12 CLA isomers and *cis*-9 and *trans*-11CLA isomers nor in the control group that received linoleic acid.

Santos-Zago et al. (2011) evaluated the effects of the consumption of two commercial conjugated linoleic acid (CLA) mixtures on lipid content and liver histology of healthy rats. The concentration of CLA was 2% of feed consumption, and the animals were daily supplemented for 42 days. The results of total liver lipid contents did not show significant differences between the groups. Regarding the hepatic histology, it was observed that although fat globules were visibly present in higher numbers and bigger size in the CLA groups, the organ histology was considered normal since both cytoplasm and organelles showed integrity.

Among the methods of cytotoxicity investigation in genetic level and *in vivo* experiment, micronucleus test is performed on bone marrow and has been widely used and accepted by regulatory agencies and scientific community. This test detects genomic changes and/or influences in mitosis; in which micronucleus indicates irreversible loss in DNA. Although genetic toxicity is not a measure of carcinogenicity, its frequency is associated with the development of cancer, therefore this is a good correlation between the high frequency of micronucleus and the appearance of tumors in mice and humans (Jena et al., 2002).

In our study, the frequency of micronucleus found in positive control group was higher than that in negative control group, proving the efficiency of micronucleus test in this research. In the same way, damage-inducing agent, CP, was shown to be effective in inducing the chromosomal damage from immature erythrocytes (PCEs).

CLA did not show mutagenic effect at any of the concentrations studied (2 and 4%). The frequency of MNPCE from the groups that received CLA was not different from that of the negative control group (safflower oil). Antimutagenic activity was observed at the two concentrations (2 and 4%), when compared to the control group that received CP intraperitoneal administration.

CLA played a role in tumor growth and tumor development in different levels (initiation, promotion, progression and metastasis) by the decrease and retention of cellular multiplication. CLA has also acted in increasing programmed cellular death (apoptosis) and necrosis. Complementing this, CLA shows activity on immune system, as well as on the reduction of body fat accumulation, in which it indirectly influences the tumor appearance and improves cachexia (Field and Schley, 2004; Pariza et al., 1999).

In addition to its antimutagenic activity, CLA shows a protective activity, because the groups fed with CLA had a decreased MNPCEs number compared to the positive control group. The group that received intraperitoneal physiological solution of 0.9% showed decreased MNPCEs number.

Similar results regarding anticarcinogenic/protective effect of CLA were obtained by Ip et al. (1991). Female mice fed with 10 mg of 7.12 dimethylbenzanthracene (carcinogenesis-inducing substance) had inhibition of 32, 56 and 60% on mammary tumor rate when fed with 0.5, 1.0 and 1.5% of CLA, respectively. This CLA supplement was started two weeks before administration of carcinogenic agent and kept receiving until the end of the experimentation. This research observed that *c*-9, and *t*-11 CLA could be the most active isomer against carcinogenesis inhibition, as it was the major compound present in the mixture.

After this, Ip et al. (1994) fed female mice with increased amounts of CLA (0.05, 0.1, 0.25, or 0.5%) during two weeks before administration of 5 mg of 7,12 dimethylbenzanthracene. After 36 weeks of supplementation, a dose-dependent reduction in the incidence and mammary tumor size were observed, proving a good biological activity of CLA on carcinogenesis treatment. Although "*in vitro*" results and animal experiments had showed beneficial effects using CLA in this kind of cancer, studies with women have not proved this association (Voorrips et al., 2002; Larsson et al., 2009). Research with gastrointestinal and colon tumor was done specially by '*in vitro'* assays and animal experiments (Palombo et al., 2002; Cohen et al., 2003; Ochoa et al., 2004; Bhattacharya et al., 2006; Song et al., 2006). In one of the rare researches with humans, Larsson et al. (2005) suggested that the intake of high-CLA milk products decreases rectum cancer in 13% and colon cancer in 34%. Kuniyasu et al. (2006) found peritoneal metastasis inhibition of gastrointestinal cancer in humans.

Therefore, although more evidences are needed, CLA

has shown some effects on carcinogenesis in different tissues; however, the mechanisms involved could vary case by case: lipid peroxidation, fatty acid composition of tissue, eicosanoid metabolism, genetic expression, cell cycle regulation cell proliferation and apoptosis could play a role (Kelley, 2007).

Conclusion

In this study, the supplementation of CLA at 2 and 4% on the diet of Swiss albino male mice showed antimutagenic effects, as assessed by micronucleous assay. No mutagenic effects to chemically induced DNA damage were observed. It is noteworthy that the mice that received 4% of CLA on feed intake showed clinical signs of malnutrition. Considering these results, further studies should be made to elucidate the mechanisms of how CLA has such effects, mainly regarding the clinical signs of malnutrition observed, as well as studies in humans.

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