ORIGINAL PAPER

doi: 10.5455/medarh.2022.76.343-347
MED ARCH. 2022 OCT; 76(5): 343-347
RECEIVED: SEP 04, 2022
ACCEPTED: OCT 18, 2022

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Effect of Conjugated Linoleic Acid (CLA) Supplementation on Expression of B-Cell Lymphoma-2 (Bcl-2) in the Bladder Epithelium of Wistar (Rattus norvegicus) Rats Exposed to Cigarette Smoke

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ABSTRACT

Background: Bladder carcinoma is the 10th most common cancer in the world with an incidence about 3% of all cancers. The risk factor for smoking is found in 81% of all cases of bladder carcinoma. One of the protein groups associated with bladder urothelial carcinoma is B-Cell Lymphoma-2 (Bcl-2). Nicotine-derived nitrosamine ketone (NNK) contained in cigarette smoke would increase the proliferation of cancer cells through increased the expression of Bcl-2. The expression of Bcl-2 could be suppressed in the presence of Conjugated Linoleic Acid (CLA), a polyunsaturated fatty acid that has role in reducing the risk of cancer development which is reported in several studies, and then stimulate cell apoptosis. **Objective:** To determine the effect of CLA supplementation on Bcl-2 expression in the bladder of rats which is exposed to cigarette smoke. **Methods:** The study is an experimental study with true experimental posttest only control group design on Wistar rats. Sample was divided into 2 case groups: 0.5% of diet (125 mg) CLA supplementation in group A, 1% of diet (250 mg) CLA in group B; and 2 control groups: group without CLA supplementation (group C) as positive control and without cigarette smoke exposure (group D) as negative control. The study takes 60 days of exposure and then Bcl-2 expression on bladder epithelial was evaluated by immunohistochemistry staining. **Results:** The results descriptively showed that rats in group C has an average Bcl-2 expression of 25.8±7.33%, while rats in group D has an average Bcl-2 expression 14.1±7.73% which means cigarette smoke exposure has been shown to increase the expression of Bcl-2 by 45.35% (p=0.019) in the bladder mucosa of experimental animals. Group B obtained an average Bcl-2 expression was 14.2±9.6% and has a significant difference when compared to group C, it shows that the addition of 1% CLA would reduce the expression of Bcl-2 by 44.96% (p=0.032). However, for group A, group with 0.5% diet of CLA supplementation did not showed decrease of Bcl-2 expression when compared to the group C (p=0.37). **Conclusion:** Conjugated Linoleic Acid (CLA) supplementation 1% of diet can reduce Bcl-2 expression in bladder epithelium of wistar rats (Rattus norvegicus) exposed to cigarette smoke.

Keywords: Bcl-2, Cigarette smoke, CLA.

1. BACKGROUND

Bladder carcinoma is the 10th most common cancer in the world with an incidence about 3% of all cancers. The risk factor for smoking is found in 81% of all cases of bladder carcinoma (1). One of the protein groups associated with bladder urothelial carcinoma is B-Cell Lymphoma-2 (Bcl-2) which acts in the mitochondrial intermembrane space by activating cytochrome C, thereby inhibiting the apoptotic cascade (2).

Nicotine-derived nitrosamine ketone (NNK) contained in cigarette smoke can increase the proliferation of cancer cells by increasing the synthesis of thromboxane A2 (TXA2) and activating the TXA2 receptor which then activates the transcription factor cAMP Response Element-Binding Protein (CREB) through the ERK1/2 and ERK1/2 pathways. PI3K/AKT which will

increase the expression of Bcl-2 so that cell proliferation occurs. The expression of Bcl-2 can be suppressed in the presence of CLA (3).

Conjugated linoleic acid is a polyunsaturated fatty acid found naturally in diary products of ruminant animal. The mechanism of CLA in changing body composition involves metabolic changes that reduce lipogenesis and increase lipolysis accompanied by fatty acid oxidation in skeletal muscle and inhibition of adipose differentiation. Conjugated linoleic acid can inhibit cell proliferation through inhibition of DNA synthesis and stimulate cell apoptosis by decreasing Bcl-2 expression (4).

There are several previous studies that showed CLA plays a role in reducing the risk of cancer development. A study by Ou et al in 2007 suggested that there was a decrease in Bcl-2 levels in breast cancer cell in mice that had been given CLA supplementation (5).

There are studies showed that CLA reduced the risk of cancer, both colorectal and breast cancer in humans, however, there were no studies that explained the effect of CLA supplementation on Bcl-2 expression in the bladder (6).

2. OBJECTIVE

Therefore, this study was conducted to determine the effect of supplementation CLA on Bcl-2 expression in the bladder of rats which is exposed to cigarette smoke.

3. MATERIAL AND METHODS

Experimental Research

This research is a laboratory experimental study with a true experimental posttest only control group design which was carried out at the Pharmacology Laboratory and Pathology Anatomy Laboratory, Faculty of Medicine, Universitas Brawijaya from September to November 2021. The independent variables of this study were exposure to cigarettes and the administration of CLA supplementation with 2 doses, there were 0.5% and 1% of diet. Meanwhile, the dependent variable in this study was the expression of Bcl-2 in the bladder mucosa of Wistar rats. Observations of Bcl-2 levels were carried out under a microscope by previously stained with the immunohistochemical staining method.

Sample

A total of 20 Rattus norvegicus Wistar race rats aged 8-10 weeks weighing between 170-250 grams were obtained from *Pusat Veteriner Farma* (PUSVETMA) Surabaya and then adapted for 1 week at the Pharmacology Laboratory Faculty of Medicine Universitas Brawijaya before treatment. Furthermore, screening was carried out with the inclusion criteria of healthy adult rats, male, aged 8-10 weeks, and weighing between 170-250 grams. As for the exclusion criteria, the Wistar rats are not actively moving and have anatomical defects or tangled hair.

Experimental Animal Preparation

The acclimatized rats were divided into 4 groups each consisting of 5 rats. The rats were weighed before the study and on the 60th day. The rats's feed was formulated according to the AIN-93 standard feeding and each rat was given 25g a daily feed.

Experimental Animal Grouping

The rats that had been prepared were then divided into 4 groups. (A) Exposure to cigarette smoke + 0.5% diet of CLA supplementation. (B) Exposure to cigarette smoke + 1% diet of CLA supplementation. (C) Exposure to cigarette smoke only as positive control. (D) without CLA supplementation or cigarette smoke exposure as negative control (7).

Cigarette Smoke Exposure and CLA Supplementation

The filter cigarettes used in the study had 31mg of tar and 2.2mg of nicotine in each cigarette. Exposure was carried out using a smoking pump 8 times a day with an exposure duration of 15 minutes and a gap of 1.5 hours between exposures (8).

CLA supplementation was gavaged to the rats in two doses there were 125 mg for 0.5% and 250 mg for the 1% of diet in groupA and B respectively. The CLA used in this study was 95% CLA with a total content of 950 mg CLA consisting of 475mg of trans-10, cis-12 CLA and 475 mg cis-9, trans-11 isomers purchased from Allmax® in the form of oil (liquid).

Immunohistochemical Staining

Bcl-2 staining using an anti-Bcl-2 (C-2) primary antibody: sc-7382 purchased from Santa Cruz Biotechnology. First, the rat's bladder was isolated on the 61st day. The bladder mucosa sample that had been obtained was then fixed with a paraffin block. Next, the antibody solution was removed and washed. Furthermore, secondary antibody was added which was diluted with biotinylation of 100-400 ul and then 100-400 ul of Streptavidin-HRP reagent was added to each section and incubated for 30 minutes. Furthermore, washing three times in buffer and adding 100-400 ul of DAB media to each section. As soon as the sections were formed, immerse the slides in deionized water and then counterstain the sections in hematoxylin. Next wash the parts in deionized water and observe at 1000x magnification.

Bcl-2 Expression Assessment

Bcl-2 expression was observed and examined using a light microscope with 400x magnification from 10 high power fields of view at 500 cells.

Data Processing

Data on Bcl-2 expression were then collected and the mean ± standard deviation of Bcl-2 expression was obtained. Data analysis was performed with SPSS 23.0 statistical software using an independent T-test. The data was statistically significant if the p value <0.05 is obtained.

4. RESULTS

Difference in Body Weight of the Samples

There was a change of body weight in samples. The average body weight of samples in group A before the study was 212.8$\pm$12.87g and increased 26.2g to 239$\pm$14.58g after the study. Group B had similar mean weight before the study as group A, which was 212.8$\pm$19.71g and increased by 27.8g to 240.6$\pm$24.62g after the study. Group C had the smallest increase in average body weight,

Group	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	mean ± SD
A [CLA 0.5% (125mg)]	47	23	56	14	6	29.2 ± 21.46
B [CLA 1% (250mg)]	6	27	20	4	14	14.2 ± 9.6
C (Positive Control)	27	13	28	30	31	25.8 ± 7.33
D (Negative Control)	8	5,5	13	21	23	14.1 ± 7.73

Table 1. Mean of Bcl-2 Expression in Bladder Mucosa in each Group

Group	A [CLA 0,5% (125mg)]	B [CLA 1% (250mg)]	C (Positive Control)	D (Negative Control)
A [CLA 0.5% (125mg)]		0.09	0.37	0.08
B [CLA 1% (250mg)]	0.09		0.032*	0.49
C (Positive Control)	0.37	0.032*		0.019*
D (Negative Control)	0.08	0.49	0.019*	

Table 2. T-test Independent Analysis Results between Groups

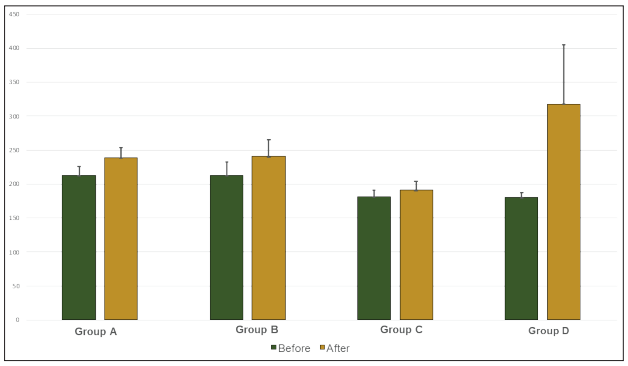

Figure1. Changes in the Average Body Weight of Experimental Animals for each Group.

which was only 8.6g from 182.2±9.47g before the study to 190.8±13.61g after the study, while group D had the largest increase in average body weight, which was 137g from 180.6±6.50g becomes 317.6±86.86g. Changes in the average body weight of experimental animals for each group can be seen in Figure 1.

Mean Expression of Bcl-2 in the Bladder Mucosa in Each Group

The Bcl-2 expression of each experimental animal was taken from an average of 10 high power fields of view with a magnification of 400x which was interpreted by experts from the Pathology Anatomy Laboratory.

Table 1 showed the average Bcl-2 expression in each sample from each group. The results descriptively showed that rats with exposure to cigarette smoke without CLA supplementation, in group C, obtained an average Bcl-2 expression of 25.8±7.33%, while rats in group D as negative controls, without exposure to cigarette smoke or CLA supplementation, showed a mean Bcl-2 expression of 14.1±7.73%. This result showed that exposure to cigarette smoke can increase the expression of Bcl-2 in the bladder mucosa of samples by 45.35%.

Decreased Bcl-2 expression was seen in group B rats, the group with exposure to cigarette smoke and 1% diet of CLA supplementation, which showed an average Bcl-2 expression of 14.2±9.6%. This result showed that the administration of 1% dietary CLA supplementation in experimental animals exposed to cigarette smoke can reduce the expression of Bcl-2 in the bladder mucosa by 44.96%.

Rats in group A, exposed to cigarette smoke with CLA supplementation of 0.5% diet, did not show any decrease in Bcl-2 expression however increase of Bcl-2 expression was found with mean 29.2±21.46%. The increase of expression was 11.64% when compared from the mean expression of Bcl-2 in group C as the positive control group. Figure 2 below showed the expression of Bcl-2 in representatives of each group using a 400x magnification microscope.

Differences in Bcl-2 Expression in Each Group

Based on the data obtained, further analysis was carried out to determine the effect of CLA supplementation on the expression of Bcl-2 in the bladder mucosa of wistar rats exposed to cigarette smoke. The results of the analysis of these differences can be seen in Table 2 below.

The data analysis between the positive control group (group C) and the negative control group (group D) showed a significant difference with a p-value 0.019. These data indicate that exposure of cigarette smoke can increased the expression of Bcl-2 in the bladder mucosa of samples significantly compared to without cigarette smoke exposure in the negative control group.

Furthermore, the administration of 1% dietary CLA supplementation was shown to significantly reduce the expression of Bcl-2 in the bladder mucosa of samples as seen from the analysis results which showed a significant difference between the group that was given 1% dietary CLA supplementation, group B, and the positive control (group C) with a p-value 0.032. An analysis was also carried out between group B and group D as a negative control with the result that there was no significant difference with a p-value of 0.49.

The data indicates that the Bcl-2 expression of the bladder mucosa between rats exposed to cigarette smoke with 1% dietary CLA supplementation almost resembled negative controls who were not exposed to cigarette smoke.

The T-test independent analysis between group A, the 0.5% dietary CLA supplementation group which experienced an increase in Bcl-2 expression, with group C as a positive control group showed a non-significant difference with a p-value of 0.37. Furthermore, analysis between group A and group B also found a non-significant difference with a p-value of 0.09, then an different

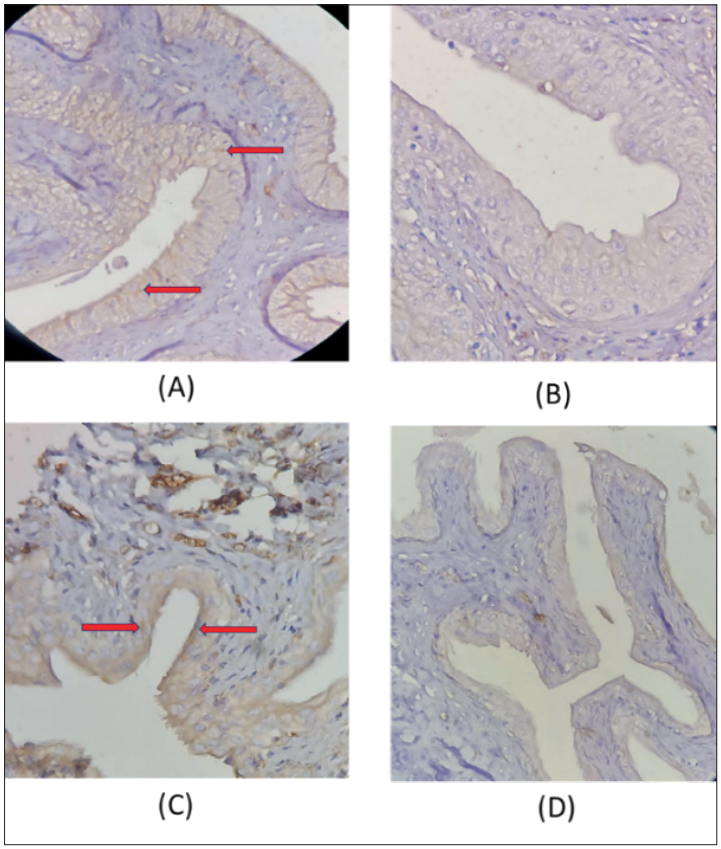

Figure 2. Histopathological results of Bcl-2 Immunohistochemistry in Bladder Mucosa of Samples with 400x Magnification: (A) Group A. Red arrows indicate Bcl-2 expression in the mucosa.; (B) Group B. The CLA 1% diet group showed lower Bcl-2 expression compared to groups C and A; (C) Group C as Positive Control Group, there was an increase in Bcl-2 expression in the bladder mucosa of samples exposed to cigarette smoke (red arrows).; (D) Group D. The Negative Control Group showed lower Bcl-2 expression than groups C and A, and there was no difference with group B, which means that the bladder mucosa of experimental animals exposed to cigarette smoke with 1% CLA supplementation diet was similar to the bladder mucosa of experimental animals without exposure to cigarette smoke or CLA supplementation.

test was carried out between group A and group D as a negative control and showed the same results, there was no significant difference with p-value 0.08.

5. DISCUSSION

The study showed that the most significant change in average body weight occurred in animal group without CLA supplementation diet and no cigarette smoke exposure with an average increase of 137 g. Groups which were given 0.5% diet and 1% diet of CLA supplementation experienced lower weight gain than group without exposure to cigarette smoke and CLA supplementation.

This result followed the theory from previous studies that CLA can reduce body weight and fat levels by increasing lipolysis activity and decreasing lipogenesis (5). Conjugated linoleic acid could modified body composition by decreasing body fat levels and has an essential role in lipid metabolism, primarily through the cellular oxidative system (9). Various studies had shown that the 10-trans and 12-cis isomers of CLA significantly increase lipolysis in adipose tissue and also have the ability to minimize fatty acid synthesis (10). Several other studies had proven that CLA can influence lipid metabolism and modify enzyme activity and hormonal profiles (11). The isomer of CLA significantly increased lipolysis in adipose, reduced fatty acid synthesis, and inhibited the expression of genes involved in the differentiation of pre-adipose to mature adipose to facilitate the process of lipogenesis (11). The effect of CLA on lipid and glucose metabolism is mediated by activation and inhibition of PPARs, particularly PPARγ (3, 5), a transcription factor in the nucleus that has a vital role in fatty acid catabolism and storage. PPARα and PPARβ are involved in the expression of proteins essential for fatty acid oxidation, and PPARγ was involved in adipose differentiation. The interaction of CLA with PPARγ could reduced body fat by altering the expression of genes that inhibit cell differentiation and modulate the activity of proteins involved in lipogenesis and lipolysis (11). In addition, CLA also significantly increased the oxidation of fatty acids to produced energy and some of the energy is used to form adenosine triphosphate (ATP). The rest is released in heat energy which can cause weight loss (5).

The results also showed that rats exposed to cigarette smoke without adding CLA as supplementation experienced an increase in Bcl-2 expression by 45.35% from the experimental group of animals that were not exposed to cigarette smoke or CLA supplementation. According to Nooshinfar E et al., exposure to cigarette smoke could increased the expression of Bcl-2 by increasing the synthesis of thromboxane A2 (TXA2) and activating the TXA2 receptor which then increased the expression of Bcl-2, thereby suppressed the process of apoptosis (3). Conjugated linoleic acid could inhibited cell proliferation by inhibiting DNA synthesis and stimulating cell apoptosis by decreasing Bcl-2 (12). In addition, CLA also inhibited the work of PPARβ in increasing cancer cell proliferation through PPARγ activation (9, 13) and can act as an anti-inflammatory by activating PPARα, which will then suppress NF-κB and STATs (14). Furthermore, Nicotine in cigarette smoke acts as a proliferator that inhibited apoptosis after various stresses, but the mechanism of intracellular signalling that mediates this function remains unclear. Bcl-2 is a cellular proto-oncogene that functions as a potent antiapoptotic molecule and tumour promoter. High levels of Bcl-2 were expressed in human lung cancer cells, whereas normal lung cells showed low levels. A report demonstrated a correlation between heavy smoking and an increase in Bcl-2. The expression of Bcl-2 in patients with lung, head, and neck cancer, indicated that Bcl-2 may be a significant target of carcinogens in tobacco smoke. Nicotine can stimulated endogenous Bcl-2 phosphorylation in cells and increased cell survival after chemotherapy drugs, including etoposide and cisplatin (15).

The result of the study reported that there was a significant difference of Bcl-2 expression on bladder mucosa

between the group that was supplemented with 1% diet of CLA and cigarette smoke exposure only group and there was no significant difference between the CLA 1% diet group and the negative control group. The study was indicated that the expression of Bcl-2 in the bladder mucosa of experimental animals exposed to cigarette smoke with 1% diet of CLA supplementation was almost similar to the negative control group. However, the increased of Bcl-2 expression in the CLA supplementation group at a dose of 0.5% diet may occur due to inadequate doses to reduced Bcl-2 expression. This result was similar with previous study conducted by Słowikowski BK et al., who conducted a study of administering CLA to non-small cell lung cancer cell cultures. Expression of Bcl-2 increased with minimal dose of CLA administration and over a short period of time although PPARγ was found to be significantly increased. After the CLA dose was increased and the study time was increased from 24 hours to 72 hours, the expression of Bcl-2 in these lung cancer cell cultures began decrease (16).

Overall, the results that have been obtained were accordance with several previous studies on CLA. Conjugated linoleic acid, a polyunsaturated fatty acid found in vegetable oils and dairy ruminant products, has been shown to suppressed proliferation and increased apoptosis in bladder cancer cell lines. According to Oh YS et al., CLA could inhibited cell proliferation through inhibition of DNA synthesis and stimulate cell apoptosis through inhibition of the IGF-IR signaling pathway characterized by increasing levels of Bcl-2 (12). Another study by Cesano A et al., showed that CLA supplementation in mice inoculated with prostate cancer cells had a smaller tumor size and reduced the incidence of lung metastases. These results support the view that dietary polyunsaturated fatty acids can affect the prognosis of prostate cancer patients (17). Until now there has been no study that specifically discusses CLA against bladder carcinoma so that the results of this study can be in line with the anti-carcinogenesis effect of CLA from other cancers, which can be used on the bladder mucosa.

6. CONCLUSION

Exposure to cigarette smoke could increased the expression of Bcl-2 in the bladder mucosa of wistar rats (Rattus norvegicus) as much as 45.35%. Conjugated inoleic acid supplementation with a dose of 1% diet can reduce Bcl-2 expression by 44.96% in the bladder mucosa of wistar rats (Rattus norvegicus) exposed to cigarette smoke.

- **Acknowledgement**: The study was supported by Urology Department Universitas Brawijaya and Dr. Saiful Anwar General Hospital, Malang. The study has already obtained ethical clearance from Health Research Ethics Commission (No: 400/167K.3/302/2021)
- **Authors contribution**: All authors were involved in the preparation of this article. Final proofreading was made by the first author.
- **Conflict of interest**: None declared.
- **Financial support and sponsorship**: Nil.

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