RESEARCH ARTICLE

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Effects of Conjugated Linoleic Acid (CLA) Supplementation and Exposure of Cigarette Smoke on *Bcl-2, P53* Protein, and *TNF-α* Expression in the Epitels of Wistar Rats (Rattus Norvegicus) Bladder

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Abstract

Objective: To determine the effect of Conjugated Linoleic Acid (CLA) supplementation on Bcl-2, P53 protein and *TNF- α* expression in the bladder of rats which is exposed to cigarette smoke. **Methods:** The study is a true experimental in Wistar rats. Sample was divided into 4 groups: group A had a 0.5% CLA supplementation (125 mg) diet, group B had a 1% of CLA diet (250mg). 2 other was control groups group without CLA supplementation (group C) as positive control and without cigarette smoke exposure and group D as negative control. The study takes 60 days of exposure and then Bcl-2, P53 protein and *TNF- α* expression on bladder epithelial was evaluated by immunohistochemistry staining. Data analysis was then performed using the One-way ANOVA test. **Result:** It showed that rats in group C has an average Bcl-2 expression of 25.8±7.33%, while Group B obtained an average Bcl-2 expression was 14.2±9.6% and had a significant difference when compared to group C (p=0.032). TNF-α expression in the four groups showed that *TNF- α* expression in group C was the highest with an average of 87.80±8.20. For *TNF- α* group B of 28.80±13.88 expression has a significant difference when compared to group C (p=0.000). Increase expression of wild-type p53 in group B compared to group A and decrease expression of wild type p53 in groups C and D compared to group B. Meanwhile, mutant type p53 expression showed no expression in all study groups. **Conclusion:** Exposure of cigarette smoke can increase the expression of Bcl-2, P53 protein and *TNF- α* in the bladder mucosa and CLA supplementation can reduce Bcl-2, wild-type P53 protein and *TNF- α* expression.

Keywords: Bcl-2- Cigarette smoke- CLA- p53- TNF-α.

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Introduction

Bladder cancer is the seventh most frequently diagnosed cancer in male population worldwide, with a mortality prevalence of 416,000 cases annually [1]. The incidence of bladder cancer increases with age, diagnosed at 72 years of age on average, and rarely diagnosed at <40 years of age [2]. Bladder carcinoma was the 12th most common cancer incidence, and the 8th most cancer incidence in men in 2020 [3]. RSUD Dr. Saiful Anwar Malang (2012-2016) recorded a total of 216 bladder cancer patients, with 81% of the cases were Transitional Cell Carcinoma (TCC) [4].

Smoking is the most important risk factor for bladder cancer. One of the substances of cigarettes isnicotine which can causeoxidative stress and disrupts the balance of oxidants-antioxidants levels in blood cells and tissues. Nicotine increases the production of hydrogen peroxide and anion superoxide, which causes disturbances in the mitochondrial metabolism [5, 6]. Nicotine-derived nitrosamine ketone (NNK) present in cigarette smoke increase the proliferation of cancer cells by activating alveolar macrophages and triggering the release of Tumor Necrosis Factor alpha (*TNF-α*). Further pathways includes the Mitogen-Activated Protein Kinase (MAPK) and the synthesis of thromboxane A2 (TXA2), which activates the TXA2 receptorand the transcription factor cAMP Response Element-Binding Protein (CREB) through the ERK1/2 and ERK1/2 pathways [7, 8]. On the other hand, PI3K/AKT protein will also increase the expression of *Bcl-2* and *P53* expression, so that cell proliferation occurs [8]. The expression of *Bcl-2*, *P53*, and *TNF- α* can be

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suppressed in the presence of Conjugated Linoleic Acid (CLA) [8-10].

CLA is a polyunsaturated fatty acid found naturally in dairy products of ruminant animals. CLA affects metabolic changes by inhibiting adipose differentiation, reducing lipogenesis, and increasing lipolysis accompanied by fatty acid oxidation in skeletal muscle [11]. CLA can inhibit cell proliferation through inhibition of DNA synthesis and stimulate cell apoptosis [10]. CLA has proven to influence the inflammatory cascade in breast cancer, colon cancer, skin cancer, and prostate cancer [12]. However, no study has been performed to examine the effect of CLA supplementation on the expression of *Bcl-2*, *P53* protein, and *TNF- α* levels in bladder cancer models. Thus, this study was conducted to determine the effect of CLA supplementation on *Bcl-2*, *P53* protein, and *TNF- α* expression in the bladder of rats exposed to cigarette smoke.

Materials and Methods

This research is an experimental study with rat models carried out at the Pharmacology Laboratory and Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya, since September to November 2021.

Sample

A total of 20 Rattus norvegicus Wistar race rats aged 8-10 weeks weighing between 170-250 grams were obtained from PUSVETMA Surabaya and then adapted for 1 week at the Pharmacology Laboratory of the Faculty of Medicine before treatment. Furthermore, screening was 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 mice are not actively moving and have anatomical defects or tangled hair.

Experimental Animal Preparation

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

Experimental Animal Grouping

The rats that had been prepared were then divided into 4 groups. (A) Exposure to cigarette smoke and 0.5% CLA supplementation, (B) Exposure to cigarette smoke and 1% CLA supplementation, (C) Exposure to cigarette smoke, (D) Negative control.

Cigarette Smoke Exposure and CLA Supplement Supplementation

The filter kretek cigarettes used have a tar content of 31 mg and nicotine 2.2 mg/stem. 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 for 60 days [14].

CLA supplementation was carried out using a probe. Prior to supplementation, mice were weighed. The two doses of CLA used in this study were 125 mg for the 0.5% supplementation group and 250 mg for the 1% supplementation group [15]. 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 Stain

Bcl-2 staining using an anti-*Bcl-2* (C-2) primary antibody: sc-7382. Wild-type *p53* using anti-*p53* antibody (AA 345-390), while the other slide is to analyze mutant-type *p53* using *p53* monoclonal antibody (PAb241). *TNF- α* staining using the monoclonal antibody *TNF-α* (52B83): sc-52746 100 g and all staining purchased from Santa Cruz Boitechnology. First, the rat bladder was isolated on the 61st day. The bladder mucosa sample that had been obtained was then fixed with 4% paraformaldehyde.

For the *Bcl-2* staining, the antibody solution was removed and washed. Then, antibody that has been diluted with biotinylation of 100-400 ul and then 100-400 ul of Streptavidin-HRP reagent added to each section and incubated for 30 minutes. Furthermore, washing it for 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. The *p53* protein each group was fixed with a paraffin block, then stained with hematoxylin and eosin (HE), and assessed quantitatively with an objective lens magnification of 400x using a light microscope from 10 high power field [13]. Then dewaxing *TNF-α* staning with dimethylbenzene and rehydration with ethanol. Staining with monoclonal antibody *TNF-α* diffusion 1:100 and incubated at 40°C after administration of H2O2 and washing with PBS. Antibody then administered with peroxidase label for 15 minutes at 37°C. The peroxidase activity was then observed using Diaminobenzidine (DAB) which would appear as a brownish yellow color [14].

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.

Wild-type and mutant-type p53 expression

The expression of wild-type and mutant-type *p53* was indicated by the presence of brown color in the nucleus. The amount of wild-type and mutant-type *p53* expression was assessed quantitatively with an objective lens magnification of 400x using a light microscope from 10 high power field [13].

TNF-α Expression Assessment

TNF-α expression was observed and quantified using an Olympus BX-51 microscope with 400x magnification per 10 large visual fields by counting the number of brown-stained epithelial or stromal cells [14].

Data Processing

Bcl-2, *P53* protein and *TNF- α* expression data were then collected and obtained a mean ± standard deviation. Data analysis was performed using SPSS 24.0 statistical software. The test uses One-Way ANOVA analysis of variation and Tukey's Post-Hoc analysis. The data is said to be statistically significant if the p value <0.05 is obtained.

Results

Characteristic of Research Subjects

A total of 20 experimental animals were involved in this study, 15 of it were exposed to cigarette smoke. There were 10 experimental animals that were given CLA supplementation. At the end of the study, there was an increase in body weight for all treatment groups, including the control group. The average increase in body weight before and after treatment is presented in Table 1.

Expression of Bcl-2

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 (Figure 1). Table 2 showed the average *Bcl-2* expression in each sample from each group. The results showed that rats in group C as positive controls, obtained an average *Bcl-2* expression of 25.8+7.33%, while rats in group D as negative controls, 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%.

Furthermore, the administration of 1% dietary CLA supplementation in group B was shown to significantly reduce the expression of *Bcl-2* in the bladder mucosa of

Table 1. Average Body Weight of Rats during the Study

Group	Body Weight Average Before Treatment (gr)	Body Weight Average After Treatment (gr)
A. Cigarette Smoke + CLA 0.5%	212.80 ± 12.87	239.00 ± 14.58
B. Cigarette Smoke + CLA 1%	212.80 ± 17.79	240.60 ± 24.62
C. Cigarette Smoke	182.20 ± 9.47	190.80 ± 13.60
D. Control	180.60 ± 6.04	317.60 ± 86.86

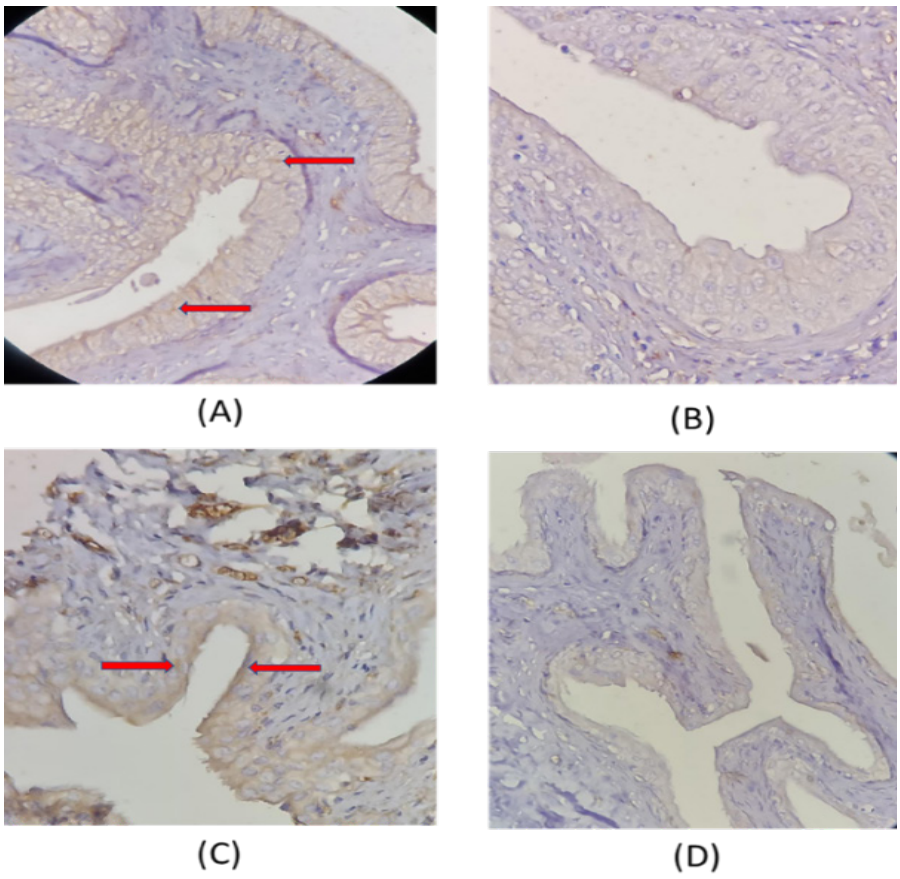

Figure 1. Histopathological Results of *Bcl-2* Immunohistochemistry in Bladder Mucosa of Samples with 400x Magnification: (A) Group A. Red arrows indicate *Bcl-2* expression in the mucosa.; (B) Group B. The CLA 1% diet group showed lower *Bcl-2* expression compared to groups C and A; (C) Group C as Positive 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

Table 2. Analysis Results of *Bcl-2* Expression

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*	

Note: (*) indicates a significance ($p < 0.05$).

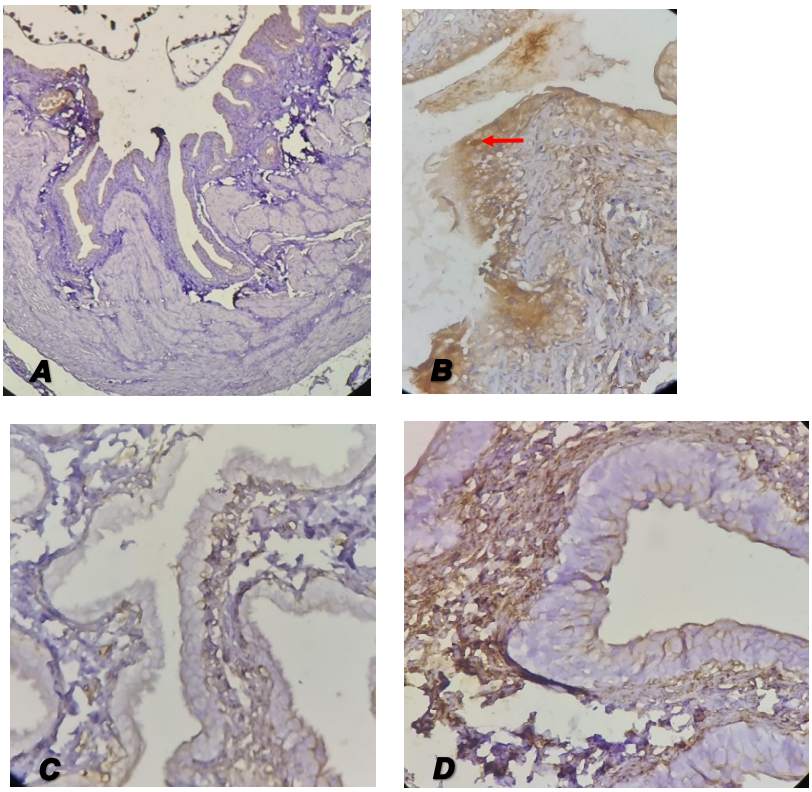

Figure 2. Wild-type *p53* Protein Expression in Rat Bladder Tissue on Immunohistochemistry Staining at 400x Magnification. The number of cells that expressed p53 wild-type was indicated by the presence of brown color in the nucleus (Red Arrow). Group A showed no expression of wild-type p53, Group B showed expression of wild-type P53, Group C showed no expression of wild-type p53, Group D showed no expression of wild-type p53.

samples as seen from the analysis results which showed a significant difference from group B and C with a p-value 0.032.

Expression of P53 Protein

In this study, it was found that the highest mean wild-type *p53* expression, which was 3.08/10 HPF, was found in group B (Figure 2). Meanwhile, group A was 0/10 HPF. This indicates that there is positive effect of cigarette smokes on the increase of wild-type *p53* expression in rat bladder mucosa. This study showed that the mean of wild-type *p53* expression in the group that exposed to cigarette smoke and given oral CLA supplementation 0.5% and the group that exposed to cigarette smoke and given oral CLA supplementation 1% was 0/10 HPF (Table 3). It is indicating that there is a positive effect of CLA administration on decreasing the wild-type *p53* expression in the bladder mucosa of rats exposed to cigarette smoke.

The conditions for the sample group to be normally distributed and homogeneous were not met, so the next test used the Kruskal-Wallis test. Based on the Kruskal-Wallis test for wild-type *p53* expression, a significance value ($p = 0.000$) <0.05 was obtained, so it can be concluded that there was a significant difference in the average of

Table 3. Analysis Results of Wild-Type *p53* Expression.

Group	A [CLA 0,5% (125mg)]	B [CLA 1% (250mg)]	C (Positive Control)	D (Negative Control)
A [CLA 0.5% (125mg)]		1,000	0.002*	1,000
B [CLA 1% (250mg)]	1,000		0.002*	1,000
C (Positive Control)	0.002*	0.002*		0.002*
D (Negative Control)	1,000	1,000	0.002*	

Note: (*) indicates a significance ($p < 0.05$).

Table 4. Analysis Results of *TNF-α* Expression

Group	A [CLA 0,5% (125mg)]	B [CLA 1% (250mg)]	C (Positive Control)	D (Negative Control)
A [CLA 0.5% (125mg)]		0.008*	0.006*	0.000*
B [CLA 1% (250mg)]	0.008*		0.000*	0.257638889
C (Positive Control)	0.006*	0.000*		0.000*
D (Negative Control)	0.000*	0.257638889	0.000*	

Note: (*) indicates a significance ($p < 0.05$).

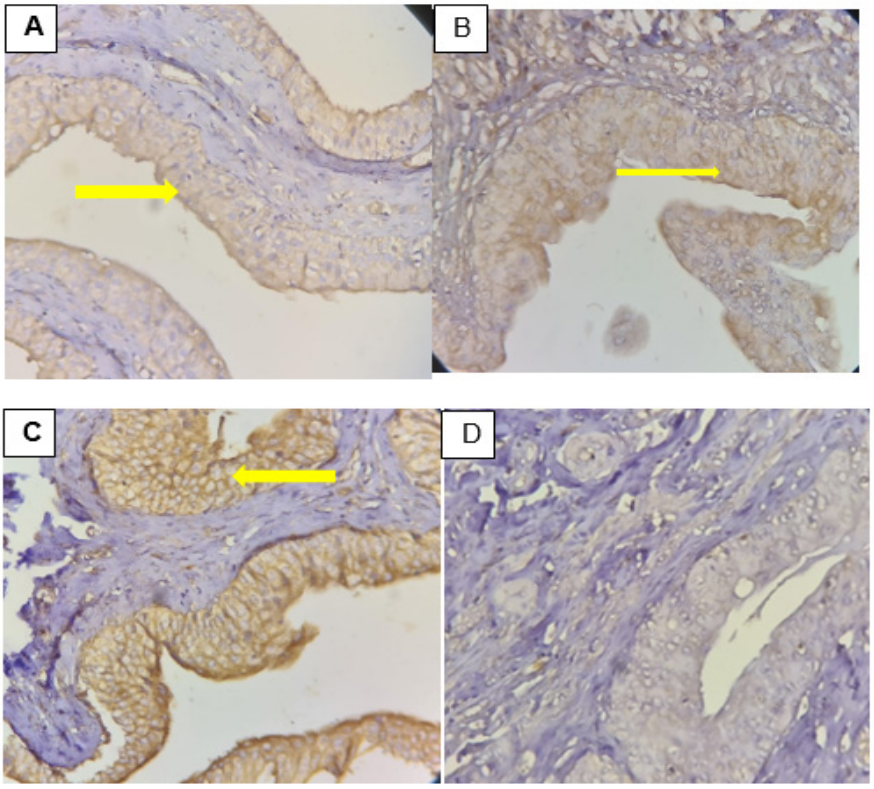

Figure 3. Immunohistochemistry *TNF-α* Staining Results. 400x magnification. (A) Exposure to cigarette smoke plus 0.5% CLA; (B) Exposure to cigarette smoke plus 1% CLA; (C) Exposure to cigarette smoke; and (D) Control without treatment. *TNF- α* expression in brown colored cells (yellow arrow).

the 4 study groups. Then, the test was continued with the Mann Whitney test with the administration of 1% dietary CLA significantly reduce the expression of *P53* Protein between group B and C with p value 0.002. In this study, no mutant-type *p53* expression was found in all study groups. Thus, it shows that there is no effect of cigarette smokes exposure with a frequency of 8 times a day, with each exposure session lasting 15 minutes and given for 60 days on the mutant-type *p53* expression in the bladder mucosa of rats.

Expression of TNF-α

The *TNF- α* expression was observed then calculated the average of 10 visual fields with 400x magnification which had been interpreted. *TNF-α* will be colored as dark brown. There appears to be a microscopic morphological difference as shown in Figure 3.

TNF-α expression in the four groups showed that TNF- expression in group C was the highest with an average of 87.80±8.20. For TNF- expression in groups A and B were 57.60±17.16 and 28.80±13.88, respectively (Table 4). Additionally, the introduction of a 1% dietary CLA supplement notably decreased the *TNF-α* expression in the bladder mucosa, as evidenced by the analysis findings. There was a substantial variance observed between the group receiving the 1% dietary CLA supplement and the positive control, with a significant p-value of 0.000.

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 is aligned with the previous studies, which found that CLA can reduce body weight and fat levels by increasing lipolysis activity and decreasing lipogenesis [15]. Conjugated linoleic acid could modify body

composition by decreasing body fat levels. It also has an essential role in lipid metabolism, primarily through the cellular oxidative system [15]. 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 [16]. Several other studies had proven that CLA can influence lipid metabolism and modify enzyme activity and hormonal profiles. 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 [17]. In addition, CLA also significantly increased the oxidation of fatty acids to produce energy. Some of the energy is then used while the other is released as heat energy which can cause weight loss [15].

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% compared to the experimental group that were not exposed to cigarette smoke nor CLA supplementation. According to Nooshinfar E et al. [8], exposure to cigarette smoke could increase the expression of *Bcl-2* by increasing the synthesis of thromboxane A2 (TXA2) and activating the TXA2 receptor, thereby suppressed the process of apoptosis [8]. Conjugated linoleic acid could inhibited cell proliferation by inhibiting DNA synthesis and stimulating cell apoptosis by decreasing *Bcl-2* [12]. There was a significant difference of *Bcl-2* expression on mucosal bladder in the group which supplemented with 1% diet of CLA and cigarette smoke exposure among other groups. However, the increase 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 is similar with a previous study conducted by Słowikowski BK et al. [18], about administering CLA to non-small cell lung cancer cell cultures. Expression of *Bcl-2* increased with a minimal dose of CLA administration and over a short period of time.. After the CLA dose was increased and the study time was also increased from 24 hours to 72 hours, the expression of *Bcl-2* in these lung cancer cell cultures began to decrease [18].

For the *p53* protein, the mean expression in groups C and D was lower than group B. This effect was due to CLA affecting arachidonic acid metabolism and decreasing LOX and COX, leading to a decrease in prostaglandin E2 (PGE2) and inhibition of NF-kB activation, so it has an anti-inflammatory effect. In addition, CLA has antioxidant properties with a mechanism as a free radical scavenger [19, 20]. It is possible that these two properties can prevent DNA damage due to exposure to cigarette smoke, so that it did not trigger the activation of wild-type *p53*.

However, a study by Kemp et al., showed that CLA can increase the expression of wild-type *p53* and p21WAF1/CIP1 mRNA (three- to five-fold and twofold, respectively) in breast cancer cell lines (MCF- 7) and colon (HCT-116) [21]. This difference in results probably due to the insufficient duration of cigarette smokes exposure to cause *p53* gene mutations.CLA may play a role in preventing oxidative stress and inflammation caused by cigarette smoke, so it did not stimulate the activation of wild-type *p53*.

This study showed that in all study groups there was no expression of mutant-type *p53*. It is probably due to the insufficient cigarette smoke exposure to cause *p53* gene mutations in the rats bladder. This result is supported by a study showed that *p53* mutations occur after the tumor genesis process. A previous study also showed that tumor genesis in humans is estimated to occur after approximately 20 years of mutagens exposure, such as cigarette smoke in the human body [22, 23]. In this study, cigarette smoke exposure was carried out for a duration of 60 days with a frequency of 8 times a day and each exposure for 15 minutes, and if the ratio of the age of rats to humans is 1 day in rats compared to 34.8 days in humans so in this study cigarette smoke exposure which acts as a mutagen when converted is 5.8 years of human age [24, 25]. Therefore, it is possible that mutant-type *p53* has not yet emerged due to the lack of duration of exposure. Further research is needed with a longer duration of cigarette smoke exposure to determine the role of CLA on *p53* mutant type.

Exposure to cigarette smoke for 30 days will significantly affect the level of catalase and *TNF-a* in the kidneys compared to the control group [26]. This increase in *TNF-a* expression is associated with increased activator protein (AP) [27]. Increased expression of *TNF-a* was also reported by Churg et al., stating that inflammation associated with *TNF-a* causes long-term damage to connective tissues in the lungs and can lead to emphysema [28].

In a study by Mohammadi et al., administration of CLA in the C2C12 cell line, administration of CLA at concentrations of 50, 100, and 150 μM increased the cell survival rate compared to the control group. The pre-treatment of CLA to theinduced *TNF-a* cells result in the decrease of cell death due to the inhibition properties of CLA [29]. Macrophages released under inflammatory conditions are believed to be the primary source of *TNF-a* in an in vivo trial. In addition, lipopolysaccharide (LPS) is one of the stimuli for macrophages in *TNF-a* production, given that CLA can inhibit LPS and suppress the production of *TNF-α* [30].

This study found that administration of CLA significantly reduced *TNF-a* expression in bladder cells exposed to cigarette smoke. The CLA 1% treatment groups showed significant results. An increase in dose was negatively correlated with an increase in dose. When administered with a dose of 1% CLA, *TNF-a* expression was significantly lower compared to the0.5% CLA dose. However, *TNF-a* expression between the A group and the control group was similar. The relationship between dose and *TNF-* expression requires further research.

In conclusion, exposure of cigarette smoke can increase the expression of *Bcl-2*, *P53* protein and *TNF- α* in the mucosal bladder. CLA supplementation can also reduce *Bcl-2*, *P53* protein and *TNF- α* expression.

Author Contribution Statement

BD: Conceptualization, writing-original draft, supervision, validation, visualization. EW:

Conceptualization, methodology, supervision, validation. NGP: Conceptualization, methodology, writing-original draft, investigation, data curation, visualization, project administration, resources. AD: Conceptualization, methodology, writing-original draft, investigation, data curation, visualization, project administration. TNB: Methodology, formal analysis, project administration. PN: Writing - Review & Editing, AK: Writing - Review & Editing. KPS: Validation, visualization. HSY: Validation, visualization.

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General

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Conflict of Interest

The authors declare no conflict of interest

Ethical Declaration

The author has ethical clearance (No.400/152/K.3/302/2021) from Health Research Ethics Committee, Saiful Anwar General Hospital, Malang, Indonesia.

Availability of data and material

All data underlying the results are available as part of the article and no additional source of data are required.

Scientific body

This research is part of thesis that approved by Universitas Brawijaya

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