The Activation of Caspase-9 in Liver Cells of Mice Increases Due to Repeated Exposure of Formalin-containing Fish

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***Abstract*—T**he case of formalin abuse as a foodstuff preservative are still frequently found in the midst of society, in Indonesia, although it has been banned since the twenties of years ago. Our previous studies have shown that foods containing formalin causes an increase in functional disorder of liver in tissue level measured by elevation of the SGOT, SGPT, and the ratio of these two enzymes. Through this research we intend to examine the toxification of repeated exposure of foodstuffs containing formalin at the cellular level by measuring the activation of Caspase-9. Caspase-9 is an initiator of programmed cell death, which is more known as apoptosis. The purpose of this study was to determine the effect of repeated exposure of fish containing formalin against Caspase-9 activation in the liver cell of mice (Mus musculus).

Caspase-9 was determined using immunohistochemical techniques (avidin-biotin sandwich assay). The data of Caspase-9 (the number of liver cells of mice that activates Casdpase-9 as dependent variable) were analyzed using two-way ANOVA with treatment factor and time factor as independent variable. Treatment factor consists of four categories, that was: negative control, positive control of fish, positive control of formalin, and treatment of fish containing formalin. Time factor consists of four categories, that was: exposures that repeated daily during 0, 2, 14, and 62 days. When the results of ANOVA analysis showed a signifcant difference, the analysis followed by *post hoc* test using Duncan's Multiple Range Test (α = 0.05).

The results showed that there was a significant difference of the number of liver cells that activates Caspase-9 between each group of treatment, as well as time factors. Based on the treatment factor, the highest number of liver cells that activates Caspase-9 was the group of positive control of formalin, subsequently occupied by the group of fish containing formalin treatment, the group of positive control of fish, and the lowest was the group of negative control. Based on time factor, the higher number of cells that activates Caspase-9 were the group of repeated exposure for 62, 14 and then 2 days, while the lowest was the group of repeated exposure for 0 day. Results of data analysis showed that the formalin treatment either in the form of single compounds or mixtures with fish flesh can increase the activation of Caspase-9. Based on the time factor, Caspase-9 activation was increased on 2 days of repeated exposure and stagnation until 62 days of repeated exposure. Thus, it can be concluded that repeated exposure to foods containing formalin can cause toxification at the cellular level of the liver that measured through increased apoptosis initiator. Increased apoptosis initiator has occurred since 2 days of repeated exposure and stagnation until 62 days.

***Keywords*—**Caspase-9, formalin, liver, repeated exposure.

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I. Introduction

Cases of formalin abuse as a preservative of food are still frequently found in the midst of society, although it has been banned since 23 years ago through the Regulation of the Minister of Health of the Republic of Indonesia No. 722/MenKes/Per/IX/88. For example, based on observations at several fishing activities centers in Malang area (East Java, Indonesia) and its surroundings [1], the fishermen adds formalin deliberately in the fish that will be sold in the market or the fish auction. Meanwhile, there was no formalin addition for the fish that will be sold at the fish processing industry and that will be consumed by their families.

Justification of the use of food additives, especially preservatives, depending on the technical needs of the use of the additive materials, the benefits obtained by consumers, and the experiment on the safety of food. The use of formalin as preservative, does not meet these criteria, especially related to the safety. Formalin when consumed together with foodstuff can react with molecules in the cell and ultimately change the function, thus causing damage to the cellular, tissue, organ, until the organism level [1]-[7].

There are many publications that reports the effect of formalin on animal experiments. This effect occurs first at the molecular, then cellular, tissue, organ, and until the organism level [1]-[3]. Our previous research has reported that repeated exposure of fish containing formalin may cause physiological as well as pathological effects on the digestive organs of mice [5]-[6], and behavioral disorders of mice [7]. Our studies also reports that foods containing formalin causes an increase in functional disorder of liver in tissue level measured by elevation of the SGOT, SGPT, and the ratio of these two enzymes [8]-[9].

The damage will increase with the repeated consumption of food. At the molecular level, formalin can cause DNA-protein crosslink, which is one type of DNA damage [10]-[11]. This crosslink can systematically activate a variety of proteins (via the mitochondrial pathway) that modulate the cascade of

Caspase [12]. Caspase plays a role in preparing programmed cell death (apoptosis) sistematically through the direct dismantling of cellular structures and other various destructive mechanisms [13].

Caspase is a group of enzyme that acts as a trigger of death program of cells, as a regulatory element in the program's death, and finally as part of the effector elements of the program itself [14]. Caspase groups initially expressed as single chain zymogen. When activated, these enzymes can break down the group of cellular proteins proteolitically that lead to apoptosis. Caspase grouped into two types, namely the initiator (Caspase-8, -9, and -10) and the executor (Caspase-3, -6, and -7). The activation of this enzyme is a key of the cells to undergoing the apoptosis [12].

So far, no studies have reported on the influence of the foodstuff that contains formalin against structural and functional disorder at the molecular level in the cell, especially about the activation of Caspase. Whereas, this information is necessary to construct the general public perception, both of which act as producers as well as consumers of foods, regarding the negative effects of formalin on health. Therefore, this study aims to determine the effect of repeated exposure of fish containing formalin against the activation of Caspase-9 that observed in liver cells of mice.

II. Research Methods

A. Design of The Research

This research was experimental research that uses randomized block design, 4 x 4 factorial, with treatment and time factors as independent variables. Four categories of treatment factor consist of: negative control; positive control of fish flesh; positive control of formaldehyde; and fish flesh containing formaldehyde. While four categories of time factor consist of: repeated exposure over 0; 2; 14; 62 days.

B. Animals Cultivation, Preparation, and Induction of Test Substances

Fourty-eight male Balb/c mice 2.5 months old with 15-25 g in weight were used. They were maintained in Animal Physiology Laboratory of Biology Department, Mathematics and Natural Science Faculty, University of Brawijaya, Malang. Mice were housed in polyethylene plastic container, managed in 27±2 ^{0}C with 12 hour photoperiod and provided with fodder and water *ad libitum*. All animal procedures conformed to institutional regulations concerning the protection of animals [15]-[16].

The concentration of formaldehyde in fish flesh was 100 ppm (mg/kg). The fish that used as food models in this study was *Oreochromis niloticus*. The solution of fish containing formaldehyde (100 ppm) was made by mixing 10 mL of 1% formaldehyde solution (diluted from 37% formaldehyde PA grade, Merck, Germany) and 1 kg of refined meat of *O. niloticus* in 1 L volumetric flask, and then the distilled water was added until the gauge line. Principally, the preparation of other test substances is equal with this formaldehyde solution preparation, just adjust to the type of substances. Furthermore, the concentrations of each substances that induces to each mice (using gavage tube) were adjusted to the weight of the animals. The mice were euthanized by cervical dislocation and then dissected to taking the liver organ, when the time of the induces of test substances reach the specified time based on each time factor categories [17]-[18].

C. Tissue Fixation, Preparation, and Cutting of The Parafin Block

Each of liver specimen were washed with PBS (phosphate buffered saline), inserted into fixative solution (10 % formaldehyde) for a day, and then dehydrated in 85% alcohol for 1-2 h (hours), 96% alcohol for 1-2 h, and absolute alcohol for 2-3 h. The specimens were cleared with xylol:absolute alcohol = 1:3 for 1 h, 2:2 for 1 h, 3:1 for 1 h, first pure xylol for 1 h, and second pure xylol for 1 h. Infiltration was done in the oven with xylol:paraffin = 1:1 (45-50 0C) for 1 h, first paraffin (65-70 0C) for 1 h, and second paraffin (65-70 0C) for 1 h. The specimens were inserted in paper box, given a liquid paraffin, and then labeled. Finally, the paraffin block was cut with a rotary microtome (4 μm thickness), and placed on the slide [19].

D. Immunohistochemistry Detection of Caspase-9

Immunohistochemical staining was performed on liver sections on slides that has deparaffinized. The endogenous peroxidase activity was blocked with 3% H2O2. For detection of Caspase-9, the slide were incubated overnight with rabbit anti Caspase-9 mouse specific antibody (Cell Signaling Technology, Inc., USA). Biotinylated secondary antibody, an avidin-biotin complex, and diaminobenzidine chromogene (Universal Dako LSAB®+ Kit Peroxidase, Dako North America, Inc., USA) were applied for visualization of the immunoreaction [20]-[21]. Histological expression of Caspase-9 were assessed on Meyer's hematoxylin stained sections, with double-blind manner as in [22]-[23].

E. Data Analysis

The data of Caspase-9 activation in the liver of mice (dependent variable, in three replicates) were analyzed using two way analysis of variance, after fulfilling normality (Kolmogorov-Smirnov test) and homogeneity (Levene test) requirements. Duncan Multiple Range Test (DMRT) were applied to the data that shows difference significantly. All statistical tests (from normality to post-hoc test, with $\alpha = 5\%$) were done using SPSS version 15 [24]-[26].

III. Results and Discussion

A. Immunohistochemical Analysis of The Activation of Caspase-9

The activation of Caspase-9 as dependent variable refers to the number of liver cells of mice that activates the initiator of apoptosis, Caspase-9. Determination of the activation of Caspase-9 performed using immunohistochemical techniques, based on the concept of specific reaction of antigen-antibody.

In this case, cells that activates Caspase-9 as the antigen, will be recognized specifically by anti-Caspase-9 antibody. This bond is further stained by a sandwich assay using avidin-biotin complex compounds. Sandwich assay plays a role in amplification of specific antigen-antibody reaction [27]-[29], so the specific color that describes the presence of antigen (Caspase-9 in this case) became stronger. The series of immunohistochemical and staining techniques will differentiates the cells that activating Caspase-9 with another normal cells, so the counting of the number of cells are more specifics and easy. Here are presented Figure 1 which contains the immunohistochemical stained liver cells of mice using anti-Caspase-9.

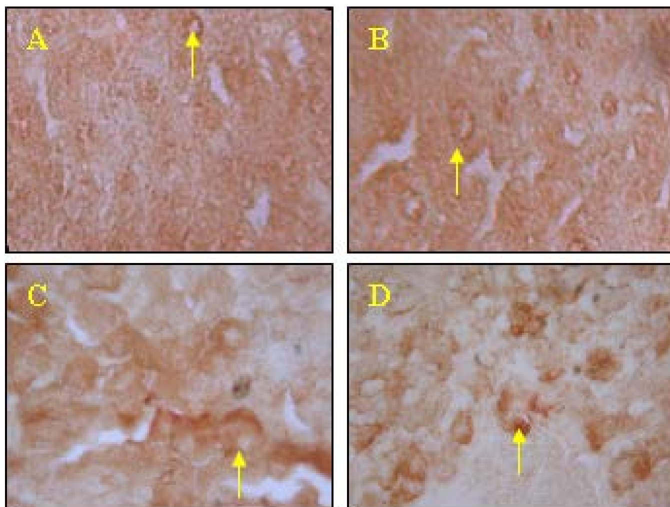

Fig. 1 Immunohistochemical staining using anti-Caspase-9 of the liver cells of mice (The yellow arrow indicates the hepatic cells of mice that activate Caspase-9. Images created with the magnification of 1000 times. Picture with the notation: A) is a liver slide of mice exposed to distilled water (negative control); B) is a liver slide of mice exposed to fish flesh (positive control of fish); C) is a liver slide of mice exposed to a 100 ppm formalin stock solution (positive control of formalin); and D) is a liver slide of mice exposed to the fish flesh containing 100 ppm formalin (fish containing formalin treatment)).

The results of immunohistochemical staining of the liver cells of mice using anti-Caspase-9 and DAB visualization showing the existence of some brown large spots. Generally seen that this large spots is a ring-shaped with high intensity of brown color. The brown ring-shaped spots reflect the activation of Caspase-9 in the cytoplasm of liver cells of mice. More or less the high-intensity-brown spots was observed, it reflected the high or low the activation of Caspase-9.

B. The Activation of Caspase-9 in the Liver of Mice Based on Treatment Factor

The differences of Caspase-9 activation between the treatment of formaldehyde (either in the form of single compounds as positive control of formalin or in mixtures with fish flesh) and without formalin (negative control and positive control of fish) could be seen in Table I and Fig. 2. This difference indicates that the treatment of fish flesh containing formalin was significantly influential in increasing Caspase-9 activation.

TABLE I
THE RESULTS OF DMRT TEST ON HSP70 EXPRESSION BASED ON TREATMENT FACTOR

Categories of the Treatment Factor	Mean of HSP70 Expression
negative control	5,78^{a}
positive control of fish	5,74^{a}
positive control of formaldehyde	17,86^{b}
fish flesh containing formaldehyde	15,38^{b}

Note: The numbers that are followed by the same letters indicate no significant differences, whereas the numbers that are followed by different letters indicate significant differences between groups of treatment factor. The data of HSP70 expression are the number of cells that expressing HSP70 per unit area of view field in microscope slide with the same magnification. This note also applies to Table 2.

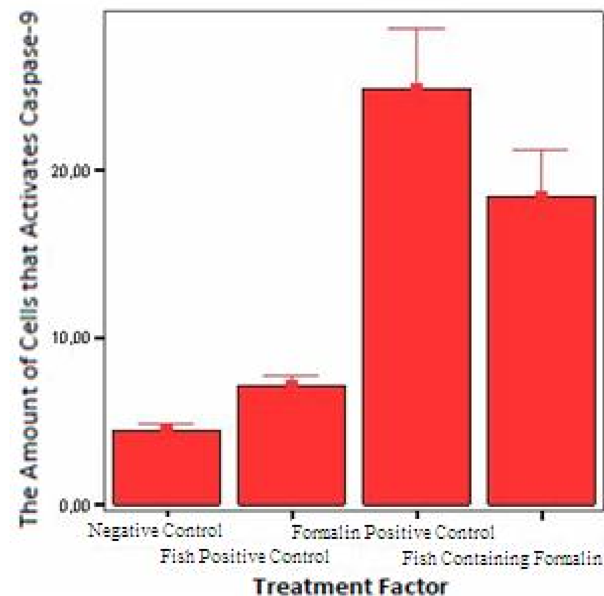

Fig. 2 Bar graph of the mean of the amount cell that activates Caspase-9 based on treatment factor (± standard error)

C. The Activation of Caspase-9 in the Liver of Mice Based on Time Factor

The difference of Caspase-9 activation between groups of time exposure could be seen in Table II and Fig. 3. The difference between the control of time (0 day) with repeated exposure groups (2, 14, and 62 days) implied that repeated treatment of test substances significantly influence to enhance

TABLE II
THE RESULTS OF DMRT TEST ON CASPASE-9 ACTIVATION BASED ON TIME FACTOR

Categories of the Time Factor	Mean of Caspase-9 Activation
0 day	4,833 ± 0,289^{a}
2 days	15,750 ± 1,455^{b}
14 days	16,167 ± 1,715^{b}
62 days	18,091 ± 2,085^{b}

the activation of Caspase-9. The activation of Caspase-9 showed a stagnant pattern starts on 2 to 62 days of repeated treatments.

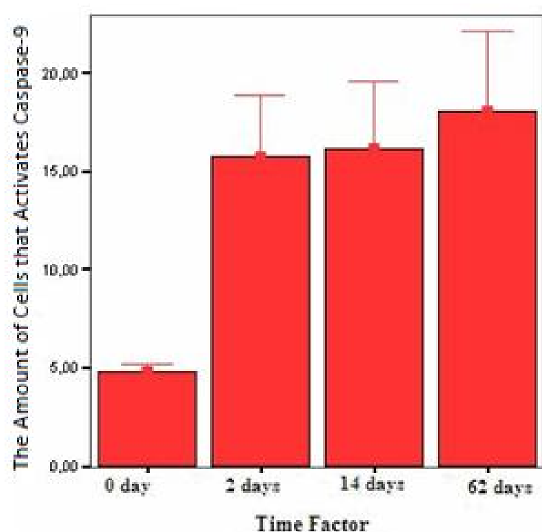

Fig. 3 Bar graph of the mean of the amount cell that activates Caspase-9 based on time factor (± standard error)

IV. Discussion

Preservatives may be added on foodstuffs as long as not being used to disguise poor quality of the food ingredients. Justification of the use of preservatives depending on the technical needs of the use of additional materials, the benefits obtained by consumers, and testing of a safety [30]. The use of formalin did not meet these criteria, especially related to food safety.

Based on in vitro experiments, formalin can trigger the formation of DNA-Protein Crosslink (DPC) [2] [10] [31]-[34], DNA single-stranded breakage, chromosomal aberrations, sister chromatid exchange, and gene mutations in human cells and mice [3] [33] [35]. Furthermore, based on in vivo experiments in mice [36], formalin that exposes through the respiratory system can trigger the abnormalities of chromosomal of lung cells. Reference [33] also reported that the exposure of formaldehyde through the digestive system of rats can cause the occurrence of micronuclei in the gastrointestinal mucosa.

Formaldehyde can react with amine, thiol, hydroxyl, and amide group forming various types of adducts, such as DPC, which is a specific form of DNA damage [34]. In response to DNA damage, the p53 protein will be activated [12], which directly or indirectly modulate the expression of proteins that control mitochondrial membrane permeability, resulting in release of mitochondrial proteins such as cytochrome c. This protein will join together with Apaf-1 (apoptotic protease activating factor 1) and dATP (nucleotide precursors) to form apoptosom which activates Caspase-9 from its proenzyme. Caspase-9 is a cysteine aspartate protease enzyme that acts as an initiator of programmed cell death, or apoptosis. Caspase-9 can initiate apoptosis by activates the executor Caspase, such as Caspase-3. Furthermore, the executor Caspase will prepare to dismantling the cellular structures, disrupt the cellular metabolism, deactivate the proteins that inhibit the cell death, and activate additional destructive enzymes [13] [37].

The results obtained in line with the concept of cellular signaling through the mitochondrial pathway (intrinsic) as described above. Exposure to formaldehyde, either in the form of a single compound (positive control of formalin) or in the mixed form with fish (treatment of fish containing formalin) is thought to cause DNA damage (DPC) specifically, affecting the increased activation of Caspase-9 in liver cells of mice.

In accordance with the factors of exposure time, this study showed an increase in the number of cells undergoing apoptosis even since 2 days of repeated exposure. Reference [38] explains that the occurrence of DNA damage depends on the exposure time of mutagens/carcinogens. Exposure to a single carcinogen will cause DNA damage increased over time, reaching a maximum point, and then declined gradually over time as a form of damage repair. In comparison to the case of formalin exposure, reference [10] found that the half time required by the DPC that formed by exposure to formalin (once exposure) is ranged from 18.3 to 26.3 hours as measured on several human cell lines. This fact implies that in a period of less than the half time, formaldehyde has caused damage at the DNA level. Application of the model of repeated exposure leads to DNA damage (observed by the high activation of Caspase-9 in hepatic cells) remains observed on the second days of exposure. Associated with the reference [38] above, the repair process was not effectively normalize the DNA damage caused by repeated exposure of formaldehyde.

The accumulation of repeated exposure of formalin, not only cause damage to the molecular/cellular (that observed through increased activation of Caspase-9), but also cause damage to the higher levels like tissues, organs and even organisms. Based on our previous studies, we has reported that the exposure of fish containing formalin leads to increased the values of SGOT (serum glutamic oxaloacetic transaminase) and SGPT (serum glutamic pyruvic transaminase) [8] as well as the ratio of these two enzymes [9] in the blood of mice, which are the marker of liver functional disorders. At the organ level, exposure of fish containing formalin causes physiological disorder which observed through the intestines and stomach weight, and pathological disorders which observed through the formation of tumors in liver organ [5]-[6]. Furthermore, at the organism level, exposure of fish containing formalin causes behavioral disorder [7], and even cause death [6].

V. Conclusion

The results of this study and our previous studies emphasize the facts about the dangers of consuming formalin-contaminated foodstuffs. The use of formalin for preserving foods can not be tolerated anymore. It has been proven that the consumption of foodstuffs containing formalin (albeit in

low concentrations) may cause interference or damage even to the molecular level. Therefore, to reduce contact between people (as consumers) with formalin-containing foodstuffs, socialization of the negative effects caused by food (foodborne diseases) needs to be done. In addition, it is necessary to find the natural product-based antidote to bind formaldehyde which are already entering the body. Both of these prevention are going to be our concern for future studies.

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