The Expression of HSP70 in Liver Cells of Mice Increases Due to Repeated Exposure of Foodstuff Containing Formalin

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***Abstract*—T**he use of formalin as a foodstuff preservative are still often found in Indonesia, although it has been banned by the government 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 stress in liver cells of mice. Stress cells can be measured through the expression of HSP70 protein. The purpose of this study was to determine the toxification of repeated exposure of fish flesh containing formalin on the expression of Heat Shock Protein 70 (HSP70) in liver cells of mice (Mus musculus).

The experimental design of this research using a design of 4 x 4 factorial experiment with treatment type and duration of exposure factors as independent variables, and the number of liver cells of mice that express HSP70 as the dependent variable. Factor of treatment type consists of: a negative control, positifve control of fish, positive control of formalin, and fish containing formalin treatment. Meanwhile, the factor of exposure duration consists of: repeated exposure for 0, 2, 14, and 62 days. The expression of HSP70 in the liver cells of mice were determined using immunohistochemical techniques (avidine-biotin assay). The data of HSP70 expression were analyzed using two-way ANOVA, followed by *post-hoc* test of Duncan's Multiple Range Test ($\alpha = 0.05$).

The results showed that there was a significant difference of the number of cells that express HSP70 between each group of treatment type, as well as each group of exposure duration factor. Based on the factor of treatment type, the higher number of cells that express HSP70 were the group of positive control of formalin and fish containing formalin, and the lower were the group of negative control as well as positive control of fish. Based on factor of exposure duration, the higher number of cells that express HSP70 were the group of repeated exposure for 2 days, and then 14 and 62 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 expression of HSP70. Based on the time factor, HSP70 expression was increased on 2 days of repeated exposure, and gradually decreased on 14 and 62 days. 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 cellular stress. Increased cellular stress has occurred since 2 days of repeated exposure, and slowly decreased at day 14 and 62, as a form of adaptation.

***Keywords*—**Formalin, HSP70, liver, repeated-exposure.

I. Introduction

FORMALIN, a cadaver preservative [1]-[3], as also used to preserve foodstuff now [4]-[6]. Formalin is still often found contained in foodstuffs, though this chemical have been banned from use as food additives [7]-[8]. Based on the Regulation of the Minister of Health of the Republic of Indonesia No. 722/MenKes/Per/IX/88 about Food Additive Materials, one of several preservatives that prohibited is formaldehyde, which is better known as formalin [9].

Formalin is used as a food preservative because its price is relatively cheap and relatively simple to use. From a survey conducted in Malang and surrounding areas in East of Java Provience, Indonesia, as in [10] found the information that to obtain one liter of formalin, the fishermen only had to spend 5000 rupiahs. The use of formalin in a relatively simple also motivates the fishermen for preferring formalin than ice block. The using of ice block requires a larger room and aggravates the weight of the fishing boats, as opposed to the use of formalin which requires only a small room.

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.

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 [10]-[12]. Our previous research has reported that

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repeated exposure of fish containing formalin may cause physiopathological effects on the digestive organs of mice [13], and behavioral disorders of mice [14]. 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 [15]-[16]. Further research about the influence of foodstuffs containing formalin needs to be done on the lower level of biological organization (cellular or molecular).

Every organism showing responses like homeostasis when faced with rapid changes in their environment. A concept associated with response to environmental changes, known as heat shock or stress response. When faced with increasing temperature or physiologically relevant (e.g., exposure of a variety of metals, amino acid analog, hypoxia, oxidative stress, nutritional deficiencies, ultraviolet radiation, viral infections, ischaemia reperfusion injury, and most of the agent or the treatment that can decreased the levels of ATP), cells from every living organism responses with increasing the synthesis of a protein, known as Heat Shock Protein (HSP) [17]- [21].

The group of HSP70 (Heat Shock Protein, 70 kD in weight) is a group of stress protein that most commonly found in mammals with its members that distributed throughout the cell. In the event of stress, usually when the cells know that they are under conditions that are not appropriate to double the protein, a member of the HSP70 is expressed at higher levels. Under normal conditions, HSP concentration ranging from 10% of the total protein content, and increased by approximately two or three times in stressful conditions [22]-[26].

Formalin that has oxidative characteristic, may activate the expression of HSP70 that act as stress marker. Based on the facts that the consumption of foodstuffs containing formalin is still dificult to avoid, and the detrimental effect that could be caused by formalin at the cellular level, and also because it is still a lack of research related to the toxification of repeated exposure of foodstuffs containing formaldehyde, so it is necessary to know the effect of repeated exposure of foodstuff containing formalin against the stress of an organ cells. Therefore, the purpose of this study was to determine the effect of repeated exposure of fish containing formalin on the expression of HSP70 in the liver of mice (*Mus musculus*).

II. Research Methods

A. Design of The Research

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

B. Animal 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 the institutional ethic regulations concerning the protection of animals [27]-[28].

The concentration of formaldehyde in fish flesh is 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), 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 add distilled water 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 concentration of each substances that induces to each mice was adjusted to the weight of the animals. Test substances were induced to mice using a gavage tube. At the time of the induces of test substances reach the specified time based on each time factor categories, the mice were euthanized by cervical dislocation and then dissected to taking the liver organ [29]-[30].

C. Tissue Fixation, Preparation, and Cutting of the Paraffin Block

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 ^{0}C) for 1 h, first paraffin (65-70 ^{0}C) for 1 h, and second paraffin (65-70 ^{0}C) 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 [31].

D. Immunohistochemistry Detection of HSP70

Immunohistochemical staining was performed on liver sections on slides that has deparaffinized. The endogenous peroxidase activity was blocked with 3% H_2O_2. For detection of HSP70, the slide were incubated overnight with monoclonal anti heat shock protein (HSP70) clone BRM 22 (Sigma-Aldrich, Germany). Biotinylated secondary antibody, an avidin-biotin complex and diaminobenzidine chromogene (Universal Dako LSAB®+ Kit Peroxidase, Dako, USA) were applied for visualization of the immunoreaction [32]-[33]. Histological expression of HSP70 were assessed on Meyer's

hematoxylin stained sections, with double-blind manner as in [34]-[35].

E. Data Analysis

The data of HSP70 expression 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's Multiple Range Test (DMRT) were applied to the data that shows difference significantly. All statistical tests (from normality to post-hoc test, with $\alpha = 0.05$) were done using SPSS software version 15 [36]-[38].

III. Results and Discussion

A. Immunohistochemical Analysis of the Expression HSP70

The expression of HSP70 as dependent variable refers to the number of liver cells of mice that express the protein HSP70. Determination of the expression of HSP70 performed using immunohistochemical techniques, based on the concept of specific antigen-antibody binding. In this case, cells expressing HSP70 as the antigen, will be recognized specifically by anti-HSP70 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 [39]-[41], so the specific color that describes the presence of antigen (HSP70 in this case) became stronger. The series of immunohistochemical and staining techniques will differentiates the cells that expressing HSP70 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-HSP70.

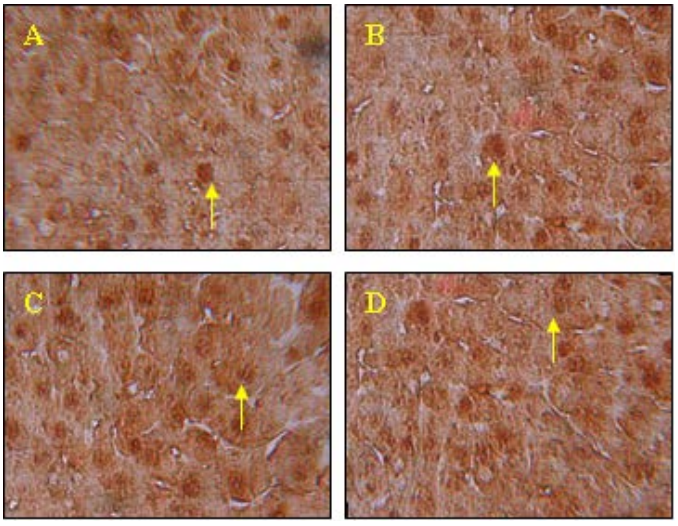

Fig. 1 Immunohistochemical staining using anti-HSP70 of the liver cells of mice (The yellow arrow indicates the hepatic cells of mice that express HSP70. Images created with the magnification of 1000 times. Picture with the notation: A) is a liver slide of mice exposed to distilled water (negative control); B) is a liver slide of mice exposed to meat fish (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 meat containing 100 ppm formalin (fish containing formalin treatment)).

The results of immunohistochemical staining of the liver cells of mice using anti-HSP70 and DAB visualization showing the existence of some brown large spots. Generally seen that this large spots is a brown ring-shaped spots with the inner also reflects the same color but in a higher intensity. The brown ring-shaped spots reflect the expression of HSP70 in the cytoplasm of liver cells of mice, while the brown color inside in a higher intensity reflect the expression of HSP70 in the nucleus of liver cells of mice. More or less the high-intensity-brown spots that was observed reflected the high or low the expression of HSP70.

B. The Expression of HSP70 in the Liver of Mice Based on Treatment Factor

The differences of HSP70 expression between the treatment of formalin (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 HSP70 expression which is a marker of cellular stress.

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

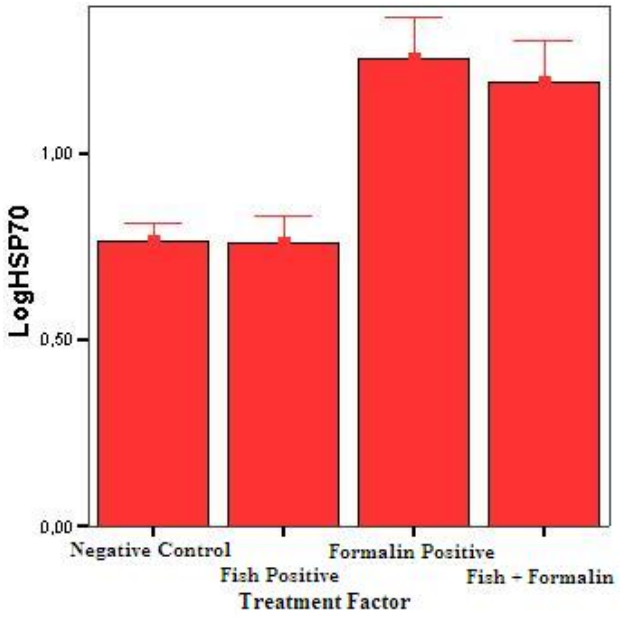

Fig. 2 Bar graph of logarithm of the mean of cell number that expressing HSP70 based on treatment factor (± standard error)

Formaldehyde can react with functional groups such as amine, thiol, hydroxyl, and amide forming various types of adducts, such as DPC (DNA-Protein Crosslink), one type of DNA damage [42]-[44]. In response to DNA damage, protein p53 (tumor suppresor protein) will be activated [45]-[46]. This protein, directly or indirectly, modulates the expression of some proteins that control mitochondrial membrane permeability, resulting the release of cytochrome *c* [47]-[48]. Mitochondrial cytochrome *c* together with the Apaf-1 (apoptotic protease activating factor 1) and dATP (nucleotide precursors) forming apoptosome that activates Caspase-9 (initiator capase). Furthermore, this initiator caspase begin the apoptosis process through the activation of executor caspase [49].

TABLE II
THE RESULTS OF DMRT TEST ON HSP70 EXPRESSION BASED ON TIME FACTOR

Categories of the Time Factor	Mean of HSP70 Expression
0 day	3,69[a]
2 days	16,22[c]
14 days	14,04[bc]
62 days	10,49[b]

The increase of HSP70 is often correlated with the emphasis on the process of apoptosis [50]-[52]. Apoptosis that can occur due to formaldehyde exposure as described above, could be suppressed by HSP70. Protein HSP70 suppress the apoptotic process by inhibiting the formation of apoptosome [53]-[54]. This protein can also suppress the apoptotic process through other pathway by blocking the activation of stress-induced kinases such as apoptosis signal-regulating kinase 1 (ASK1) [55], p38 [56]-[58], and c-Jun N-terminal kinases (JNK) [59].

C. The Expression of HSP70 in the Liver of Mice Based on Time Factor

The difference of HSP70 expression between groups of time exposure could be seen in Table II and Fig. 3. Observed difference between the control of time (0 day) with repeated exposure groups (2, 14, and 62 days) showed that repeated treatment of test substances significantly influence to enhance HSP70 expression. Next, the expression of HSP70 showed a slowly decline on day 14, and this decrease was also significantly observed on day 62 of repeated treatments.

Reference [60] (for comparison) shows that cigarette smoke can trigger the expression of HSP70 at high levels in lung fibroblast cells, which was observed from 4-16 h after exposure and peaked at eighth hour. Reference [61] also reported that HSP70 expression observed in vascular smooth muscle cells (which were isolated from thoracic aorta) from Sprague-Dawley rats, increases 5.91 times on 24 h after exposure to 200 μmol/L H_2O_2. From both studies, it is known that the expression of HSP70 has been observed even within hours after the execution of stress.

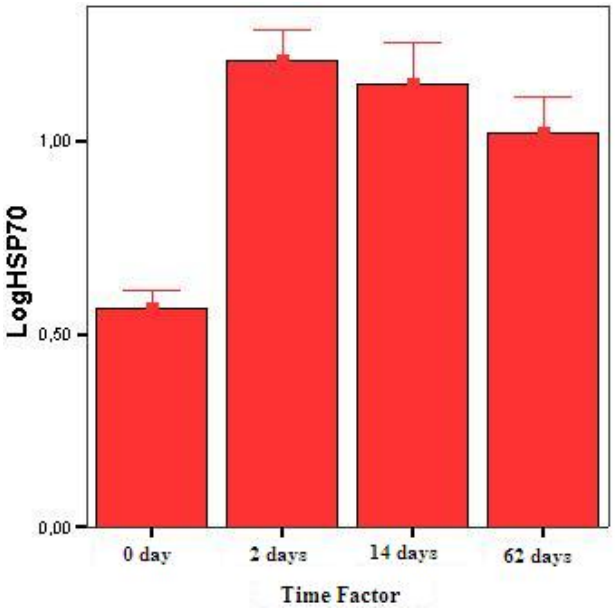

Fig. 3 Bar graph of logarithm of the mean of cell number that expressing HSP70 based on time factor (± standard error)

The decrease of stress response that slowly observed at day 14 and 62 after treatment showed that the repeated exposure of formaldehyde can gradually be adapted by the cell. The concept of adaptive response of cells against xenobiotics stressor or physiologically relevant has been discussed by many references [62]-[64]. Continuous decrease of the stress response as shown in the results of this study may be caused by a decrease in the ability of organism to cope with stress or unable to tolerate more stress due to the aging process [65]-[66].

Based on the fact that the repeated exposure of formaldehyde cause an increase in HSP70 expression in the

liver of mice and the consumption of foodstuffs containing formaldehyde in the midst of inhabitants are still difficult to avoid, then in the future it is necessary to prevent damaging effects from formaldehyde exposure on every biological levels of organism. The activation of HSP protein by substances like bioactive natural products can be exploited as new therapeutic strategies based on their ability to reduce damage trough protective function.

IV. Conclusion

The results showed that foodstuff containing formalin causes stress on the liver cell of mice characterized by increased expression of HSP70. The stress has been observed since 2 days of repeated exposure and then declined steadily until the repeated exposure reach the 62nd day. This case is due to the decreases in the ability of organism to cope with stress or unable to tolerate more stress due to the aging process. This work emphasizes previous researchs on the adverse effect of the consumption of foodstuffs containing formalin. Therefore, general public as consumers of foodstuffs should be more sensitive in addressing the use of formalin as a preservative.

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