



### CARBON TETRACHLORIDE ADMINISTRATION INDUCES THE EXPRESSION OF HYPOXIA INDUCIBLE FACTOR-1 $\alpha$ (HIF-1 $\alpha$ ) IN RAT LIVER

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#### ABSTRACT

**Background:** There is now increasing evidence that HIF-1 is also responsive to a variety of non-hypoxic stimuli. However, the mechanisms by which these non-hypoxic stimuli induce HIF-1 $\alpha$  are not completely known, yet, although some evidence points to a role of ROS as messengers regulating HIF activity.

**Objective:** To determine the expression of HIF-1 $\alpha$  in liver rat tissue induced by carbon tetrachloride under normoxic conditions, with or without N-acetylcysteine protection.

**Methods:** Twenty five male Sprague-Dawley rats were divided into 5 group: normal control rats, normal rats orally administered with coconut oil (1 mL/200 g body weight) for 1 day, rats orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day, rats injected i.v. with NAC (0.15 mg/g body weight) for 8 days and then orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day, rats orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day and then injected i.v. with NAC (0.15 mg/g body weight) for 2 days. The expression of HIF-1 $\alpha$  mRNA was measured by real-time RT-PCR using the Livak method. The expression of HIF-1 $\alpha$  protein was measured by ELISA assay.

**Results:** The highest HIF-1 $\alpha$  mRNA and protein expression found in the group treated by CCl<sub>4</sub> and then was gradually lowered in the pre-NAC group, post-NAC group, control group, and last, in the oil group.

**Conclusion :** Our study shows the effect of CCl<sub>4</sub>-treated rats under normoxic conditions increased the mRNA and protein HIF-1 $\alpha$ . NAC post-treatment provide a better protective effect compared with NAC pre-treatment.

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**Keywords :** Hypoxia inducible factor-1 $\alpha$ , Carbon tetrachloride, Oxidative stress, N-acetylcysteine, Liver

## INTRODUCTION

Oxidative stress is a state in which an organism underwent an imbalance between prooxidants and antioxidants, which results in macromolecular damage and disruption of redox signaling and control.[1] In general, macromolecules such as nucleic acids, proteins, polysaccharide, and lipids are susceptible to free radical attack. Increased production of free radicals, which results in oxidative stress may occur by administration of various xenobiotic compounds, whose metabolism involves metastable, very active intermediates which are radicals. Carbon tetrachloride (CCl<sub>4</sub>) is one of such compound and well known as a hepatotoxic agent. This compound is Carbon tetrachloride is a lipid-soluble potent hepatotoxic agent that when bound to lipid and protein produces peroxidative degeneration of many tissues.[2]

Carbon tetrachloride metabolism begins with the formation of the trichloromethyl free radical (CCl<sub>3</sub>•), through the action of the mixed function cytochrome P450 oxygenase system of the endoplasmic reticulum. The CCl<sub>3</sub>• radical reacts with various biologically important substances such as amino acids, nucleotides, and fatty acids, as well as proteins, nucleic acids, and lipids. In the presence of oxygen, radical CCl<sub>3</sub>• radical is converted to the trichloromethyl peroxy radical (CCl<sub>3</sub>O<sub>2</sub>•) via oxidation activity in the liver. This radical is more reactive and is capable of abstracting hydrogen from polyunsaturated fatty acids (PUFA) to initiate the process of lipid peroxidation.[3,4]

HIF (hypoxia-inducible factor) is a heterodimer transcription factor that consists of one of the regulatable HIF-1 $\alpha$  subunits and the constitutively expressed HIF-1 $\beta$  (also known as aryl hydrocarbon

receptor nuclear translocator or ARNT). Subunit - $\alpha$  is a component regulator of HIF-1 complex that can be regulated through the mechanism of O<sub>2</sub>-dependent and O<sub>2</sub>-independent. In the presence of O<sub>2</sub>, the overall levels of - $\alpha$  subunits are low due to the rapid degradation by a complex mechanism. While hypoxia stabilized the - $\alpha$  subunit and then translocates to the nucleus, dimerizes with subunit - $\beta$  recruits the coactivators p300/CBP, and induces expression of its transcriptional targets via binding to hypoxia-responsive elements (HREs).[5]

According to Morel and Barouki[6], HIF-1 $\alpha$  is constitutively expressed but is rapidly degraded under normoxic and oxidative conditions. Conversely, HIF-1 $\alpha$  is stabilized by hypoxia and by antioxidants through an as yet unknown mechanism. The effect of antioxidants to the stability of HIF-1 $\alpha$  was also raised by Kaluz et al.[5], it was reported that attenuation of oxidative stress by antioxidants can also stabilize HIF-1 $\alpha$ . On the other hand, Giaccia et al.[7] and Giordano[8] stated that during hypoxia, reactive oxygen species (ROS) formation process will increase and this can cause oxidative stress.

Although HIF-1 has initially been described as a transcription factor regulated by hypoxia, there is now increasing evidence that HIF-1 is also responsive to a variety of non-hypoxic stimuli among them hormones such as insulin, growth factors such as platelet-derived growth factor (PDGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), and insulin-like growth factors-1 (IGF-1), coagulation factors such as thrombin, vasoactive peptides such as angiotensin II, cytokines, or carbachol which activates muscarinic acetylcholine receptors. Moreover, HIF-1 has also been shown to

be activated by metal ions such as cobalt, chromium, and arsenite as well as by mechanical stress. However, the mechanisms by which these non-hypoxic stimuli induce HIF-1 $\alpha$  are not completely known, yet, although some evidence points to a role of ROS as messengers regulating HIF activity.[9] Nitric oxide (NO) has been reported to stabilize HIF-1 $\alpha$  under normoxia. Adverse reports have been published showing that NO treatment interferes with HIF-1 activity. This contradiction may be due to the cell culture model diversity and the physiological situation.[10]

To determine the expression of mRNA and protein HIF-1 $\alpha$  under normoxic and oxidative conditions, we conducted the study using liver rat induced by CCl<sub>4</sub> as a model toxicant and N-acetylcysteine (NAC) as an antioxidant.

## **MATERIAL AND METHODS**

Twenty-five male Sprague Dawley rats, 6-8 weeks old, weighing 150-200 grams kept in the animal house of Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. The rats were obtained from Balitvet, Ministry of Agriculture. Rats were provided with food and water ad libitum. Each experimental group consisted of five animals. Group 1. Normal control rats ; Group 2. Normal rats orally administered with coconut oil (1 mL/200 g body weight) for 1 day ; Group 3. Rats orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day ; Group 4. Rats injected i.v. with NAC (0.15 mg/g body weight) for 8 days and then orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day ; and Group 5. Rats orally administered with CCl<sub>4</sub> (0.55 mg/g body weight) for 1 day and then injected

i.v. with NAC (0.15 mg/g body weight) for 2 days.

All procedures were approved by Panitia Tetap Penilai Etik Penelitian, Faculty of Medicine, Universitas Indonesia with ethics approval number (Ethical Clearance) 438/PT02.FK/ETIK/2009. Animals were sacrificed by ether anesthesia. Liver tissues were taken out, weighed, divided into aliquots, and immediately frozen -84° C.

### **Isolation RNA**

Total RNA was extracted from liver tissues by using TriPure Isolation Reagent (Roche). RNA concentration was determined using UV spectrophotometer.

### **Real-time RT-PCR**

Eighty nanograms of RNA were reverse transcribed and amplified to cDNA using real-time RT-PCR with iScript One-Step RT-PCR Kit with SYBR Green (BioRad).  $\beta$ -actin gene was used as internal control. Primers (1st BASE, Ltd.) used for HIF-1 $\alpha$ : forward [5'-ACA GTG GTA CTC ACA GTC GG-3'] and reverse [5' - CCC TGC AGT AGG TTT CTG CT - 3'];  $\beta$ -actin primers: forward [5'- CAC TGG CAT TGT GAT GGA CT - 3'] and reverse [5'- CTC TCA GCT GTG GTG GTG AA - 3']. Real-time RT-PCR product for each primer pair was 466 bp for HIF-1 $\alpha$  and 174 bp for b-actin. Real-time RT-PCR reaction mix consists of 12,5  $\mu$ L 2x SYBR Green RT-PCR reaction Mix; 0,75  $\mu$ L of each primer, 9,5  $\mu$ L nuclease-free water; 1  $\mu$ L RNA template and 0,5  $\mu$ L iScript Reverse Transcriptase. Real-time RT-PCR conditions were: synthesis cDNA at 50°C for 10 minutes; inactivation of iScript Reverse Transcriptase at 95°C for 5 minutes; 40 cycles at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A melting curve was performed to verify the presence of a single amplicon. Nontemplate control (NTC) was used as a

negative control. Real-time RT-PCR data were calculated according to the livak method.

### Preparation of tissue homogenates

One hundred milligrams of liver tissue was homogenized using 250  $\mu$ L lysis buffer (R&D System, Inc.) in microtube with micropestle, followed by centrifugation 5000 rpm for 10 minutes at 4oC. Each supernatant was collected and stored at -20oC.

### Determination of protein concentration

The liver protein concentration was measured by spectrophotometry at  $\lambda$  280 nm using a series of standard bovine serum albumin (Santa Cruz Biotechnology) from 0,1 – 10 mg/mL.

### Measuring protein HIF-1 $\alpha$ using ELISA

Samples that have measured levels of the protein was diluted with carbonate-bicarbonate buffer pH 9.6 with concentration 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, then incubate 20 hours at 4oC. Wash the microplate 3 times with 300  $\mu$ L PBS-Tween20 0.1% 0.1 M pH 7.2. Blocking conducted with 200  $\mu$ L solution of 5% BSA (Santa Cruz Biotechnology), then incubate 1.5 hours at room temperature. Wash the microplate 3 times with 300  $\mu$ L PBS-Tween20 0.1% 0.1 M pH 7.2. Add 100  $\mu$ L mouse monoclonal antibody anti-HIF-1 $\alpha$  (Santa Cruz Biotechnology) diluted 1:500 in PBS-Tween20 0.1% 0.1 M pH 7.2, then incubated 1.5 hours at room temperature. Wash the microplate 3 times with 300  $\mu$ L PBS-Tween20 0.1% 0.1 M pH 7.2. Add 100  $\mu$ L goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) diluted 1:1000 in PBS-Tween20 0.1% 0.1 M pH 7.2, then incubate 1 hour at room temperature.

Wash the microplate 4 times with 300  $\mu$ L PBS-Tween20 0.1% 0.1 M pH 7.2. Add 100  $\mu$ L ABTS chromogenic substrates (KPL) ratio 1:1 between solution A and solution B and incubate for 30 minutes at room temperature. Read the microplate at  $\lambda$ 405 nm.

### Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by multiple comparisons least significant difference (LSD). p-values < 0.05 were considered as significant.

## RESULTS

The purity index of RNA isolation from majority samples is >1.75 (mean 1.94  $\pm$  SD 0.27). Analysis of melting curve, using HIF-1 $\alpha$  and  $\beta$ -actin primers showed 1 peak for each primer pairs (Figure 1), 81.08 $^{\circ}$ C for HIF-1 $\alpha$  and 84.68oC for  $\beta$ -actin.

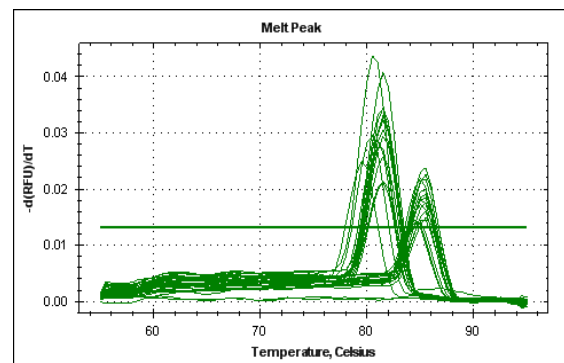


Figure 1. Melting curve HIF-1 $\alpha$  and  $\beta$ -actin.

Electrophoresis on 2.5% agarose showed only one band each for  $\beta$ -actin and HIF-1 $\alpha$  equal to 174 bp and 466 bp respectively (Figure 2). It is proved that there was no primer dimer present.

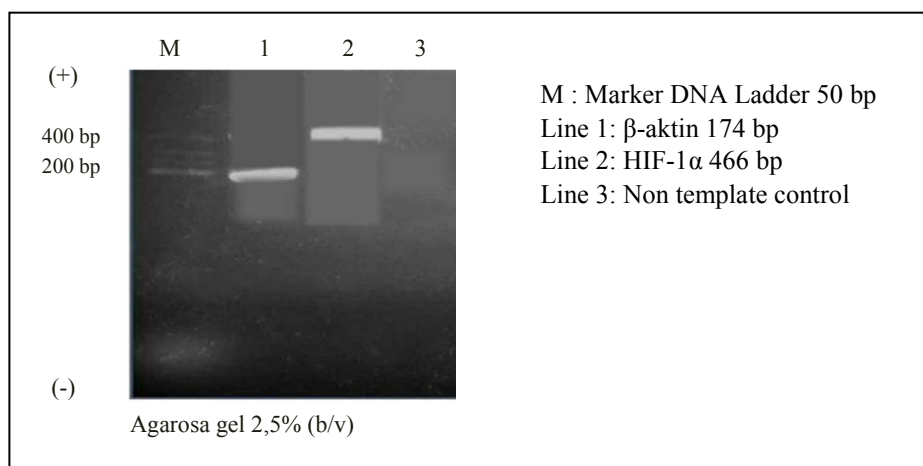


Figure 2. Visualization of RT-PCR product β-aktin and HIF-1α.

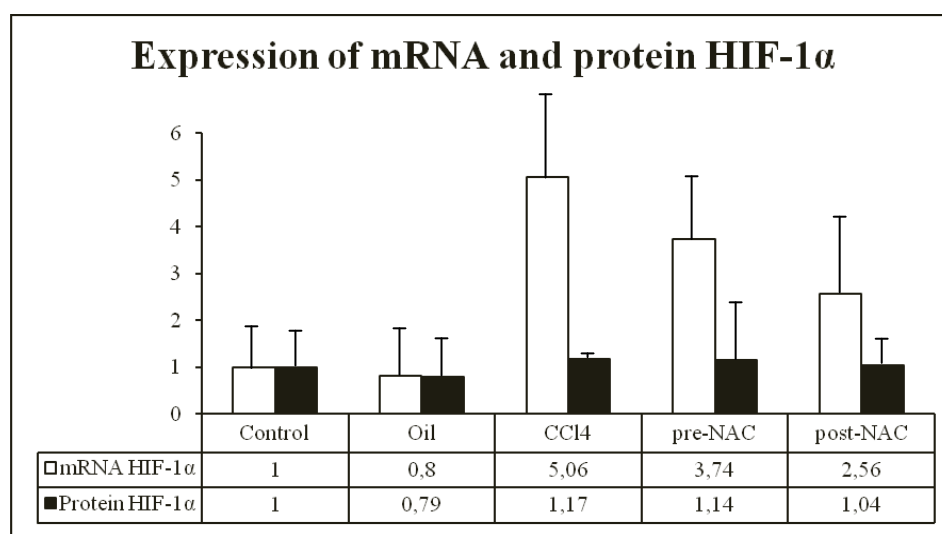


Figure 3. Expression of mRNA and protein HIF-1α in rat liver tissue

Table 1. Real time RT-PCR and ELISA analysis of HIF-1α expression in the liver of rats (n = 5)

Group	mRNA expression	Protein expression
Normal	6.10 ± 0.95	5.8 ± 0.84
Normal + coconut oil	5.77 ± 1.01	4.6 ± 0.89
CCl <sub>4</sub>	8.44 ± 2.20	6.8 ± 0.45
Pre-NAC + CCl <sub>4</sub>	8.01 ± 1.22	6.6 ± 1.14
CCl <sub>4</sub> + post-NAC	7.46 ± 1.91	6.0 ± 0.71

Expression of HIF-1α mRNA and protein. The effect of oral administration of CCl<sub>4</sub> on mRNA and protein HIF-1α expression of NAC-treated rats and normal rats is presented in Table 1.

Figure 3 shows the expression of HIF-1α mRNA and protein. HIF-1α mRNA relative expression level was elevated 2.56 times in the post-NAC group, then the pre-NAC group as much as 3.74 times, and last, mRNA expression of HIF-1α is the highest found in group

treated by CCl<sub>4</sub> (5.06 times) compared to the control group. In the oil group, the expression of HIF-1 $\alpha$  was decreased 0.8 times compared with the control group.

The HIF-1 $\alpha$  protein expression was elevated 1.04 times in the post-NAC group, then the pre-NAC group as much as 1.14 times, and last, protein expression of HIF-1 $\alpha$  is the highest found in group treated by CCl<sub>4</sub> (1.17 times) compared to the control group. In the oil group, the expression of HIF-1 $\alpha$  was decreased 0.79 times compared with the control group.

The result of statistical test (ANOVA, LSD, < 0.05) showed there was a significant differences in the mRNA HIF-1 $\alpha$  expression between CCl<sub>4</sub>-treated group and control (p=0.026), also between CCl<sub>4</sub>-treated group and oil control group (p=0.013). While, the pre-NAC group was a significant differences between oil control group (p=0.033). No significant differences in the mRNA HIF-1 $\alpha$  expression were observed between the post-NAC group with the entire treatment group.

There was a significant differences in the protein HIF-1 $\alpha$  expression between oil control group with the entire treatment group [control group (p=0.035), pre-NAC group (p=0.001), post-NAC group (p=0.016), CCl<sub>4</sub>-treated group (p=0.000)].

## **DISCUSSION**

CCl<sub>4</sub> has long been known as a model toxicant and has been the focus of many in vitro and in vivo toxicology studies. The primary site of toxicity and carcinogenesis is the liver. CCl<sub>4</sub> consistently causes liver toxicity, resulting in fatty degeneration, cellular necrosis, fibrosis, and cirrhosis. This occurs in multiple species and through multiple

routes of exposure.[4] The hepatotoxic effects of CCl<sub>4</sub> have been widely studied in animals. Indeed, carbon tetrachloride is used as a model chemical in many laboratory investigations of the basic mechanism of action of hepatotoxic chemicals. All other toxic effects of CCl<sub>4</sub> are related to its biotransformation catalyzed by cytochrome P-450 dependent monooxygenase, specifically CYP2E1. The liver and kidney (especially in humans) are especially vulnerable because of the abundance of CYP2E1, which is also present in the respiratory and nervous systems, and various isoforms of CYP3A. Considerable data are available for hepatic toxicity, but similar cellular damage would be expected in other tissues with a high abundance of CYP2E1. There is considerable evidence that hepatic injury produced by carbon tetrachloride is mediated by two major processes resulting from bioactivation in the endoplasmic reticulum and mitochondria of centrilobular hepatocytes, which have the highest concentration of CYP2E1: haloalkylation of cellular macromolecules by reactive metabolites such as trichloromethyl free radical or trichloromethyl peroxy free radical and lipid peroxidation, which impairs cellular functions dependent on membrane integrity. Both haloalkylation and lipid peroxidation contribute to loss of cellular functions and subsequent cell death.[3]

In response to parenchymal cell damage, perisinusoidal cells may be stimulated to release extracellular matrix proteins (type-I collagen) that contribute to hepatic fibrogenesis, which is largely mediated by hepatic macrophages (Kupffer cells). Kupffer cells activated by carbon tetrachloride release tumor necrosis factor-alpha (TNF- $\alpha$ ), nitric oxide, transforming growth factor-beta (TGF- $\beta$ ), and interleukins (IL)-1, -6, and -10.[3]

Kupffer cells (the major component of the hepatic sinusoid) sensitized to CCl<sub>4</sub> intoxication release tumor necrosis factor (TNF), which may aggravate the toxicity of CCl<sub>4</sub> to the hepatocytes. Also, products of peroxidation (malondialdehyde and 4-hydroxynonenal) are known to inhibit protein synthesis and the activities of certain enzymes. Enhanced free radical concentration resulting from oxidative stress conditions can cause loss of enzymatic activity. A significantly large amount of free radicals were detected in the liver after a single intraperitoneal dose of CCl<sub>4</sub>. These findings suggest that in the liver oxidative stress caused by CCl<sub>4</sub> intoxication would lead to damage of antioxidant enzymes such as SOD and GPX, or reactive intermediates formed in the course of bioactivation of CCl<sub>4</sub> may bind to those enzymes that are responsible for their inactivation.[2]

Kupffer cells, the resident macrophages of the liver, normally protect the hepatocyte phagocytosing incoming particles. However, activated Kupffer cells release toxic secretory molecules that have the potential to damage hepatocytes. It is possible that modest damage to parenchymal cells (due to the metabolism of CCl<sub>4</sub> in those cells) participates in the activation of Kupffer cells, thereby causing them to release harmful cytokines that may contribute to further damage to hepatocytes.[11]

The results obtained by Jiang et al.[12] using immunohistochemical detection demonstrated the increased expression of TGF- $\beta$  and collagen type I proteins in the liver 129/SvpcJ mice after 1 mL/kg intraperitoneal dose of CCl<sub>4</sub> for 4 weeks.

Many growth factors and cytokines are known to stabilize HIF-1 $\alpha$  under normoxic conditions, including insulin,

insulin-like growth factors, transforming growth factor, platelet-derived growth factor, epidermal growth factor, interleukin-1 $\beta$ . In addition, nitric oxide (NO) has been reported to stabilize HIF-1 $\alpha$  under normoxic conditions.[10]

Treatment with 0.55 mg/g CCl<sub>4</sub> increased the expression of mRNA and protein HIF-1 $\alpha$  even though the protein expression was not as high as the mRNA expression. The highest expression was in the group treated by CCl<sub>4</sub> and then was gradually decreased in pre-NAC group, post-NAC group, control group, and coconut oil group.

Oral exposure to carbon tetrachloride has been observed to result in a wide spectrum of adverse effects on the liver, the most prominent of which is a destruction of the smooth and rough endoplasmic reticulum and its associated enzyme activities, inhibition of protein synthesis.[3]

The products of CCl<sub>4</sub> metabolism by CYP2E1 include trichloromethyl and trichloromethyl peroxy radicals. Studies with radical scavengers, such as N-acetylcysteine, and spin-trapping agents, such as N-tert-butyl- $\alpha$ -(4-nitrophenyl)nitron (PBN), have shown that these agents confer a protective effect against carbon tetrachloride-induced toxicity, indicating that carbon tetrachloride toxicity is produced by the free radicals released via metabolism of carbon tetrachloride. Treatment with cysteine, which is a precursor of glutathione, also protected against carbon tetrachloride hepatotoxicity when given orally 30 minutes before or 1 hour after intraperitoneal injection of CCl<sub>4</sub>. [4]

The data obtained from this study shows that NAC post-treatment provide a better protective effect compared with

NAC pre-treatment. NAC administration seems to protect the hepatic parenchyma against the action of trichloromethyl and trichloromethyl peroxy radicals, due to its antioxidant potential. NAC acts as an antioxidant which effectively reduces oxygen reactive species. It presents potent ability to interact directly with oxidant agents, acting as a scavenger of free radicals, and it exerts an indirect effect on the antioxidant mechanism since it contributes to restoring glutathione. This drug is defined as a precursor for the synthesis of this antioxidant enzyme.[13]

This finding was also verified by Sari[14] using the same sample. Sari reported that the decrease in catalase specific activity is accompanied by elevated levels of dicarbonyl compounds. CCl<sub>4</sub> administration increased the level of dicarbonyl compounds and decreased the activity of catalase. In addition, NAC administration after CCl<sub>4</sub> induction has better protection against cell damage when compared with the pre-treatment effect.[14] Decreased in catalase enzyme activities indicates that this enzyme in many cells use to cope with free radicals from CCl<sub>4</sub> metabolism, while elevated levels of dicarbonyl compounds describe the severity of cell damage to cells.

In this study, the rats of experimental groups were given carbon tetrachloride 0.55 mg/g in coconut oil for 1 day. The liver from rats fed with coconut oil showed the lowest expression of mRNA and protein HIF-1 $\alpha$ . Sari also finding the increase in catalase specific activity is accompanied by decreased levels of dicarbonyl compounds in the coconut oil group.[14] Thus, indicates the lowest stress oxidative level in the rat fed coconut oil compared to controls and other groups.

Investigators reported previously that the cardiac mitochondria from Sprague-Dawley rats fed with coconut oil for 16 weeks showed the lowest concentration of oxidized proteins and peroxidized lipids. While, the fish oil diet leads to the highest oxidative stress in cardiac mitochondria, an effect that could be partly prevented by the antioxidant probucol. A diet enriched in saturated fatty acids offers strong advantages for the protection against oxidative stress in heart mitochondria.[15] A study using New Zealand white rabbit fed with coconut oil for 6 months offers better antioxidant capacity than sunflower oil as indicated by the lower lipid peroxidation rate among coconut oil-fed rabbits.[16]

The beneficial effect of coconut oil may come from the saturated fatty acids, the vitamin E content, or from other antioxidants present in the oil.[15] Polyunsaturated fatty acids, due to the presence of double bonds, are more prone to be attacked by free radicals and become oxidized readily. Monounsaturated and saturated fatty acids are more resistant to free-radical attack than polyunsaturated fats.[16]

## **CONCLUSION**

The overall result of our study shows the effect of CCl<sub>4</sub>-treated rats increased the mRNA and protein HIF-1 $\alpha$  under normoxic condition. NAC post-treatment provide a better protective effect compared with NAC pre-treatment.

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