

The impact of fasting toward oxidative stress marker in the liver and plasma of New Zealand White rabbit

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ABSTRACT

Background: Fasting may increase the activity of endogenous antioxidants and protect against oxidative stress. However, the effects of different fasting durations on the liver have not been reported.

Objective: The purpose of this study is to determine the effect of intermittent and prolonged fasting on oxidative stress markers in the liver tissue and plasma of New Zealand White rabbits.

Methods: New Zealand White rabbits were divided into three groups: control, intermittent fasting (IF), and prolonged fasting (PF), with each group consisting of five rabbits. The control group was provided with food and water *ad libitum*; the IF group fasted for 16 hours, while the PF group fasted for 40 hours, followed by an eight-hour non-fasting period for six days. In liver tissue and plasma, oxidative stress indicators (catalase, carbonyl, GSH) were evaluated.

Results: In the IF group, liver GSH was significantly higher than in the control group. However, neither liver carbonyl nor catalase levels changed significantly in the IF group. In the IF group, plasma carbonyl was significantly lower than in the PF group. In addition, there was no significant differences between groups in plasma catalase and GSH levels.

Conclusion: Intermittent fasting and prolonged fasting could significantly increase liver GSH levels of New Zealand White rabbits. In addition, intermittent fasting is more effective than prolonged fasting at preventing oxidative stress.

Keywords: fasting, oxidative stress, liver, plasma, New Zealand White rabbit

Introduction

In addition to producing energy, mitochondria also generate free radicals. However, when the number of these free radicals is within the normal range, antioxidants can neutralize them [1-4]. Accumulating free radicals can cause damage to cellular proteins, which are then carbonylated and rendered inert, which is called carbonyl [3,4]. This oxidative stress could promote several diseases, such as degenerative disorders, as well as exacerbate pre-existing diseases over time [1,2,5].

Typically, the effects of those diseases may manifest as senescence in the liver tissue alongside

a highly oxidative damage [3,4]. Oxidative stress may contribute to steatosis, chronic hepatitis, cirrhosis or fibrosis, and even hepatocellular cancer in liver tissue [6]. One of the body's defenses against oxidative stress is the endogenous antioxidant, which includes catalase and reduced-glutathione (GSH) [5,7,8]. The catalase protects the body against hydrogen peroxide (H₂O₂) by converting it into water and oxygen [7,8]. In a similar manner, GSH acts as a reducing agent that neutralizes hydrogen peroxide by turning it into water [9]. Additionally, GSH contributes to maintain mitochondrial activity, proliferation, and apoptosis [9].

Calorie restriction is another strategy for protecting against oxidative stress [10-12]. Calorie restriction has the potential to protect the liver against oxidative stress in humans [10-12]. However, malnutrition is a risk with calorie restriction, hence intermittent fasting is recommended as an alternative [13,14]. Multiple studies have shown that intermittent fasting can improve DNA repair mechanisms, increase mitochondrial function, reduce free radical generation, and minimize oxidative stress by increasing the activity and amount of endogenous antioxidants [7,8, 14-17]. However, the effect of the fasting duration on the liver organ has not been reported. Therefore, this study aimed to further investigate the effects of intermittent fasting (16 hours) and prolonged fasting (40 hours) on oxidative stress markers (carbonyl, GSH, and catalase) in the liver tissue and plasma of New Zealand White rabbits.

Methods

Ethical approval

This study has been approved by Ethic Committee from Faculty of Medicine Universitas Indonesia (Ethical number KET-249/UN2.F1/ETIK/PPM.00.02/2020).

Study design

New Zealand White rabbits were divided into three groups (each consists of 5 rabbits): control, intermittent fasting (IF), and prolonged fasting (PF), with each group consisting of five rabbits. The control group was provided with food and water *ad libitum*; the IF group fasted for 16 hours, while the PF group fasted for 40 hours, followed by an eight-hour non-fasting period for six days [19]. In liver tissue and plasma, oxidative stress indicators (catalase, carbonyl, GSH) were evaluated.

Carbonyl level measurement

Carbonyl level was analyzed by reacting liver tissue supernatant or plasma with 2,4-dinitrophenyl hydrazine (DNPH). A spectrophotometer with a wavelength of 390 nm was used to measure the

level of hydrazone protein which was formed from the reaction of DNPH with the carbonyl. The carbonyl content was determined as μmol per mg carbonyl [20].

GSH level measurement

The GSH level was determined by reacting a sample solution with TCA 5% and phosphate buffer pH 8.0, with 5,5'-dithiobis-(2-nitrobenzoic acid)/DTNB reagent or Ellman reagent to produce 2-nitro-5-thiobenzoate/TNB solution. The absorbance of the incubated TNB solution was measured using spectrophotometry with a wavelength of 412 nm. The levels of GSH are represented by the ratio between the absorbance of the sample and standard solution [21].

Specific activity of catalase

The catalase specific activity was determined by dividing the catalase activity with the protein levels. A total of 950 μL of H_2O_2 and 50 μL of the sample were mixed, and the catalase enzyme activity was measured by calculating the difference of H_2O_2 absorbance in a sample solution on 30 seconds and 210 seconds measured by spectrophotometry at 210 nm wavelength [22]. The protein levels were measured using spectrophotometry with 280 nm wavelength.

Statistical analysis

All data were then extracted with GraphPad Prism 9.0 (San Diego, USA). The Shapiro-Wilk test was used for the normality test, which continued with bivariate statistical analysis. The one-way ANOVA test or Kruskal Wallis test was used based on normality distribution data. $P < 0.05$ is considered significant.

Results

Oxidative stress marker in liver

Liver tissues were used because it has a high rate of GSH and catalase synthesis as well as a high level of carbonyl formation [3,4,9,14,15,18].

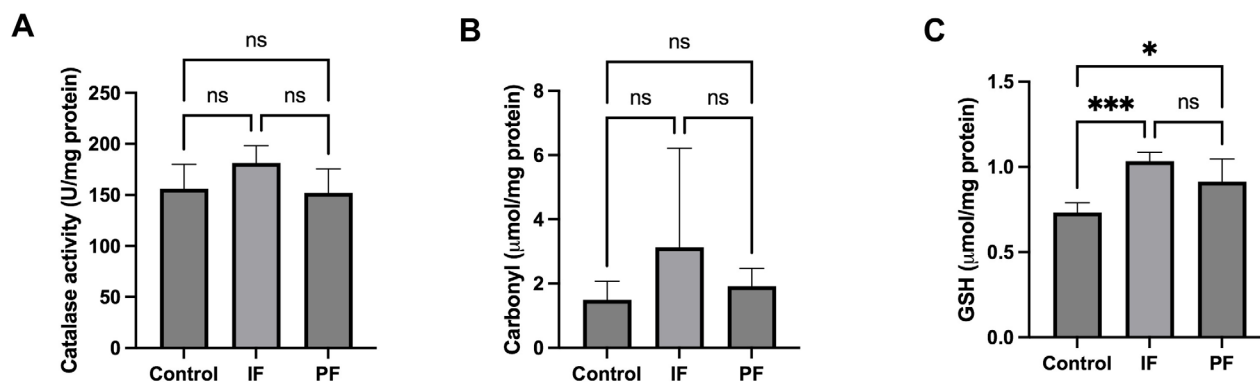


Figure 1. Oxidative stress marker in liver. A) Specific activity of catalase, analyzed using One-way Anova, B) carbonyl level, analyzed using Kruskal Wallis test, C) GSH level, analyzed using One-way Anova. IF: intermittent fasting, PF: prolonged fasting. *** $p < 0.005$, * $p < 0.05$, ns: not significant

Intermittent fasting did not significantly change specific catalase activity compared to the control group ($p = 0.1962$) and prolonged fasting group ($p = 0.9458$). Similarly, intermittent fasting did not significantly change carbonyl levels compared to the control group ($p = 0.4719$). Interestingly, the GSH level has significant changes between the control with both the intermittent fasting group ($p = 0.0004$) and the prolonged fasting group ($p = 0.0174$). The two duration conditions of fasting were not significantly different ($p = 0.1197$) (Figure 1).

Oxidative stress marker in plasma

Catalase specific activity did not differ significantly between the control with IF group ($p = 0.9094$), control with PF group ($p = 0.7716$), and IF with PF group ($p = 0.9587$). Interestingly, intermittent fasting decreased carbonyl levels relative to the control (0.6932), however prolonged fasting significantly increased carbonyl levels ($p = 0.0313$). There were no significant differences in GSH levels between the control, IF, and PF groups.

Discussion

In this study, we found that fasting increased GSH in the liver. Additionally, intermittent fasting decreased carbonyl levels, whereas prolonged fasting increased carbonyl levels. Previous research demonstrated that intermittent fasting raised GSH and catalase specific activity in the liver and plasma; however, only the liver GSH level rose significantly

[11,23]. Depletion of cellular energy increases antioxidant activity through activating the AMPK pathway, causing FOXO to activate catalase and superoxide dismutase 2. Moreover, intermittent fasting may have increased Nrf2 expression, hence increasing antioxidant activity [11].

Increasing GSH and catalase specific activity can prevent oxidative stress, as seen by the reduction in carbonyl level (Figure 2B). Furthermore, the decrease in ROS production may be attributable to the increased expression of the chaperon HSP-70 protein, which may prevent carbonyl accumulation [23, 24]. In addition to chaperon, the proteostasis system could also decrease ROS production via proteasome, autophagy, and chaperon-like mechanisms [24, 25]. As a result, the balance of ROS and antioxidant are maintained.

Increasing GSH levels may also correlate with decreased glucose levels and prevention against fasting-caused inflammation [14, 26]. It is believed that the decreasing glucose level is related to the decreasing ROS formation [26]. Decreasing ROS formation, especially H_2O_2 , would cause GSH level intracellularly increased, due to their function as reducing agent [9]. On the IF treated group itself, the level of GSH may increase as a result of an adaptive response to restore redox balance [14]. These increasing GSH synthesis is marked with a slightly increase of GSH regulating enzymes (GPx and GR), especially in the liver [14].

In contrast to plasma, the liver carbonyl level was tended to increase in the IF group compared

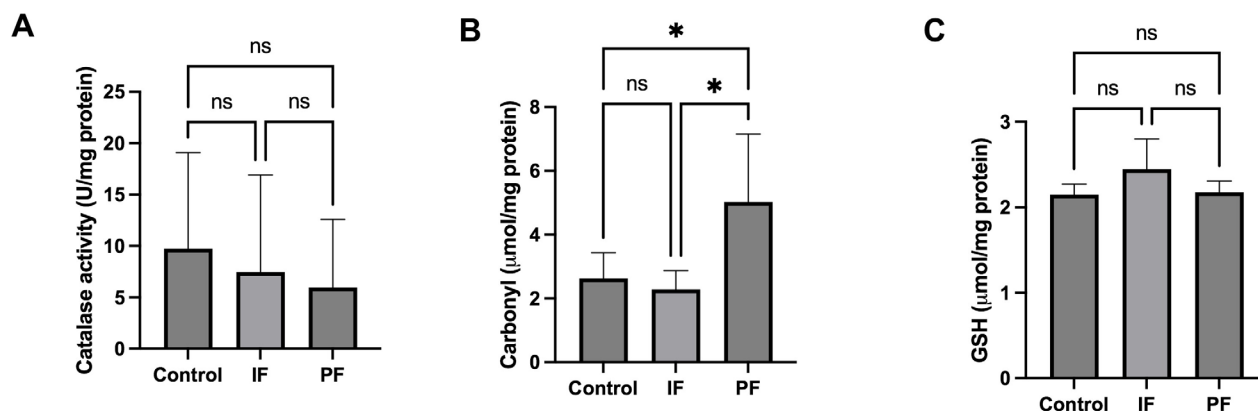


Figure 2. Oxidative stress marker in plasma. A) Specific activity of catalase, analyzed using One-way Anova, B) carbonyl level, analyzed using One-way Anova, C) GSH level, analyzed using Kruskal Wallis test. IF: intermittent fasting, PF: prolonged fasting. * $p < 0.05$, ns: not significant

to control and PF group, although this difference was not statistically significant. This result is similar to Chausse B *et al.* [14], who concluded that one month of intermittent fasting would have different effects on specific tissues. In addition, they demonstrated that one month of IF could increase mitochondrial respiration capacity, hence increasing carbonyl levels in the mice's liver tissue. Therefore, the highly increasing ROS production is unbalanced to the increasing antioxidant activation in the IF treated group's liver tissue.

Carbonyl levels in plasma are higher in the fasting group than in the control group. This may be due to a lack of glucose or triacylglycerol prompting liver cells to directly oxidize free fatty acids (FFA) and glycerol as an alternative source [23]. Therefore, lipolysis activity is increased to produce more FFA in the bloodstream [25]. However, the increased FFA concentration in the plasma would result in insulin resistance by preventing glucose transport, allowing lipolysis to occur [23].

On the other hand, the increasing autophagy activation could also generate FFA as a breakdown product, resulting in either systemic or local accumulation of FFA [24]. These high levels are lipotoxic and may cause tissue damage to the surrounding tissue [23, 25]. Incomplete FFA β -oxidation may cause specific damage of mitochondrial and endoplasmic reticulum by increasing ROS production and causing oxidative stress, including carbonyl formation.

GSH with protein disulfide isomerase (PDI) and ER oxidoreductin 1 (ERO1) work together as a chaperone-like mechanism in the endoplasmic reticulum [23]. Nevertheless, the mechanism would increase H_2O_2 production. Hence, the GSH level would decrease as compensation to prevent oxidative stress [23].

Suppose there is an imbalance between oxidants and antioxidants. In such a scenario, a vicious cycle of RE stress could disrupt the function of PDI and the RE itself, as well as increase carbonyl accumulations and protein malformation [23]. In other words, despite the fact that fasting could increase the activity of antioxidants to counteract the ROS level, the excessive amount of ROS generated in the PF-treated group exceeds antioxidant activity.

Prolonged fasting induces glucose and triacylglycerol shortage, resulting in increased lipolysis activity in the liver [23]. This excessive lipolysis results in an accumulation of FFA, which may promote ROS production. Thus, the GSH level in the liver of the PF-treated group was significantly higher than that of the control group. Due to prolonged fasting, the GSH level rises as a result of an increase in GSH biosynthesis enzyme activity, such as glutamate cysteine ligase (GCL) and GGT [27]. Nonetheless, the rising GSH level in the PF-treated group is only observed in liver tissue, not plasma, because GSH production occurs mainly in the liver [9]. However, when lipolysis occurs, GSH is required to maintain redox balance in the

liver tissue before being transported through the plasma [23].

Overall, the results demonstrate a significant rise in GSH level and a non-significant increase in catalase-specific activity, indicating that fasting may enhance the antioxidant capacity. In addition, fasting, particularly IF, may reduce carbonyl levels, one of the markers of oxidative stress. A rise in carbonyl levels were observed in the PF-treated group, may be due to ROS production from FFA accumulations caused by prolonged glucose shortage. The increasing level of carbonyl in the liver of the PF-treated group is significant, despite the fact that the GSH level has also increased marginally. This may occur as a result of the excess ROS production, which exceeds the antioxidant capacity [1,2]. However, the minor change of other oxidative stress indicators in the IF-treated group may be attributed to the short fasting duration.

This study was limited by the short duration of fasting and the limited number of stress oxidative markers that were only assessed in liver tissue and plasma. Therefore, future studies are recommended to extend the duration of the fasting and measure other stress oxidative parameters (such as glutathione peroxidase, glutathione reductase, superoxide dismutase) in various tissues.

Conclusion

Intermittent and prolonged fasting could significantly increase liver GSH level in New Zealand White Rabbit's. In addition, intermittent fasting decreased carbonyl levels, whereas prolonged fasting increased carbonyl levels, suggesting that intermittent fasting is more effective to prevent oxidative stress.

Author contributions

NSH, SG, DA collected the data and wrote the manuscript. EJG and RDA contributed to the completion of the manuscript. NSH obtained funding and reviewed and finalized the manuscript.

Declaration of conflict interest

None.

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