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The Effect of Fasting on Oxidative Stress in the Vital Organs of New Zealand White Rabbit

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Abstract

Background: Oxidative stress is defined as the condition in which balance between the synthesis and detoxification of reactive oxygen species in cells is disrupted. This research explored the effects of intermittent and prolonged fasting on malondialdehyde (MDA), carbonyl, reduced glutathione (GSH), and specific activity of catalase as biomarkers for oxidative stress in hearts, brains, and kidneys of New Zealand White (NZW) rabbits.

Methods: Fifteen NZW rabbits were divided into control, intermittent fasting (IF), and prolonged fasting (PF) groups. The controls were fed ad lib. IF and PF groups were fasted for 16 and 40 hours, respectively, followed by eight hours of non-fasting, for six days and were sacrificed on the 7th day. One hundred mg of heart, brain, and kidney tissues were homogenized in 1 ml of phosphate-buffered saline. MDA, carbonyl, GSH, and catalase were analyzed by spectrophotometry. Data were analyzed using One-way ANOVA and post hoc test.

Results: In heart, MDA was significantly greater in the control than in the IF and PF groups. In brain, GSH was greater in the IF than in the PF and control groups. Also, in brain, catalase specific activity was significantly greater in the control than in the IF and PF groups. In kidney, catalase specific activity was significantly less in the PF than in the control group.

Conclusions: The effect of fasting on oxidative stress in various organs showed various responses, however fasting reduced oxidative stress based on MDA and GSH levels in the heart and brain, respectively.

Keywords: Fasting, Oxidative stress, Vital organs, Rabbit.

Introduction

The term “oxidative stress” is used to describe a condition in which the balance between the synthesis and detoxification of reactive oxygen species (ROS) is disrupted in body cells and tissues. Although ROS are physiologically produced through the oxygen metabolism and play a role in cell signaling, excess amounts can damage cells and tissues (1). Free radicals are formed in cells either by accepting or losing a single electron, making them highly reactive due to the presence of unpaired electron(s). The production of ROS by aerobic cells contributes to the aging process and its accompanying diseases (2). Endogenous sources of ROS include myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, angiotensin II, and lipoxygenase. The mitochondria produce

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ROS in low levels from cellular respiration, specifically during electron transport. ROS can be in the form of free radicals such as superoxide (O2•−) and hydroxyl radical (OH•), or non-radicals such as hydrogen peroxide (H2O2) (3).

The radical superoxide is produced when the dioxygen molecule is reduced during cellular respiration facilitated by NADPH oxidase, leaving it with one unpaired electron. The enzyme superoxide dismutase facilitates the conversion of superoxide into hydrogen peroxide (H_2O_2), which is not a free radical itself, but can form hydroxyl radicals. These types of radicals are highly reactive, especially with the phospholipids in cell membranes. Hypochlorous acid is especially harmful to cellular proteins and is formed from hydrogen peroxide in the presence of MPO and chloride. The production of ROS may also be enhanced by exogenous factors such as radiation, pollutants, alcohol, cigarette smoke, drugs including gentamycin and cyclosporin, and heavy metals including iron, cadmium, arsenic, mercury, and lead (1, 4). Both exogenous and endogenous ROS are responsible for oxidative modification of the major cellular macromolecules including carbohydrates, proteins, lipids, and DNA. As such, they can be used as biomarkers for oxidative stress. The biomarkers that can be used are malondialdehyde (MDA), which is a result of lipid peroxidation, and carbonyl, which is a result of protein oxidation (5). To prevent oxidative stress, our cells are protected by endogenous antioxidants including catalase, reduced glutathione (GSH), glutathione peroxidase, and superoxide dismutase (SOD). Oxidative stress occurs when the ROS level exceeds antioxidant availability (5).

Degenerative diseases, including cardiovascular diseases, diabetes, neurodegeneration, and cancer, have been shown to be closely correlated with oxidative stress. In particular, ROS have negative effects on calcium regulation in the heart, which can lead to arrhythmias and cardiac remodeling (6). In the case of cardiovascular disease, many studies have demonstrated ROS involvement in atherosclerotic plaque development. The brain, which has rich lipid content and weak antioxidant capacity to combat ROS, may become an easy target for oxidative insult. Substances of ROS origin play a critical pathophysiological role when accumulated in the brain. They increase the blood-brain barrier permeability and alter brain morphology, leading to neuroinflammation and neuron death. Moreover, they are involved in various cell signaling mechanisms in the brain. This leads to ROS contribution in myriads of brain pathologies such as neurodegenerative diseases and cerebrovascular disorders (7-9). The kidney is vulnerable to oxidative stress because it handles wastes and toxins found in blood (10). Inflammatory conditions found in chronic kidney disease (CKD) stimulate the migration of leukocytes, which release O2•- and cause increased activity of ROS-producing enzymes (11). Combined with other factors such as mitochondrial dysfunction, oxidative stress is pivotal in the development and progression of CKD through endothelial dysfunction, glomerular damage, and renal ischemia (12). A vicious cycle then results from which these alterations cascade into further production of more cell-damaging ROS via apoptosis, further inflammation, and fibrogenesis—ultimately culminating in kidney failure if untreated (13).

A proven method of reducing oxidative stress effects is calorie restriction, including fasting. The definition of calorie restriction itself is an energy intake reduction well below the number of calories consumed *ad libitum* (14). The suggested mechanisms by which fasting decreases oxidative stress is by decreasing ROS, increasing antioxidant enzyme activities, or increasing the turnover rate of oxidized macromolecules, as well as reducing LDL cholesterol (15). The ability of fasting to improve physiological health markers has been broadly studied. Many fasting regimens are known, but one of the most heavily studied regimens is intermittent fasting (16). Intermittent fasting refers to no or very limited caloric intake in regular periods.

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It commonly lasts for 16 hr or two days each week on non-consecutive days, or a 24-hr fast on alternate days (17). Another fasting regimen, called prolonged fasting, can last up to 72 h (18). Even though the effects of fasting on oxidative stress has been well studied in rodents, other factors may affect the efficacy of fasting, such as species, age, sex, and restriction duration (19). Studies that observe the effects of various fasting durations have been inconclusive. Moreover, the studies have not analyzed the vital organs of higher-level animals such as rabbits. Therefore, this research aimed to examine the effects of intermittent and prolonged fasting versus controls using MDA, carbonyl, GSH, and catalase as oxidative stress biomarkers in the heart, brain, and kidney of New Zealand White (NZW) rabbits.

Materials and Methods

This was an *in vivo* study conducted on 15 NZW rabbits. All the rabbits were males, aged four months, with weights of 1800 – 2500 gm. The rabbits were divided equally into control, intermittent fasting (IF), and prolong fasting (PF) groups. The control group was fed ad lib. The IF group was fasted for 16 hours, followed by eight hours of non-fasting every 24 hr for six days. The PF group was fasted for 40 hours, followed by eight hours of non-fasting every 48 hr, for six days. All the groups were sacrificed on the seven days as shown in scheme below (Fig. 1).

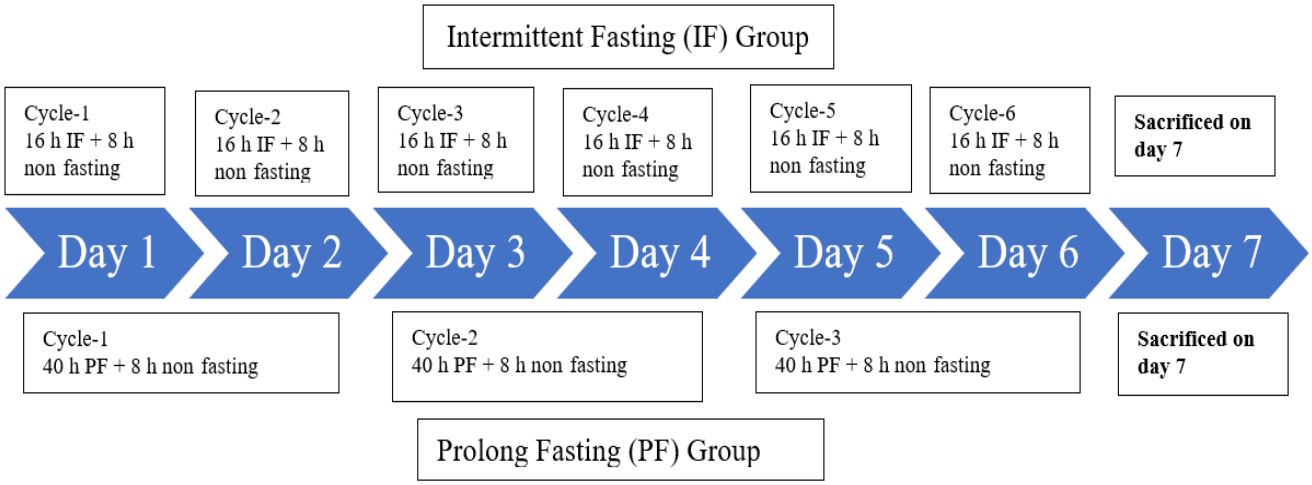

Fig. 1. Experiment plan.

The basis of the 16- and 40-hour fasts were previous studies that investigated the effects of fasting for 16-48 hours. Because fasting is suggested to reduce oxidative stress and its effects, the effects of the 16- and 40-hour fasts were compared (20-21). One hundred mg of heart, brain, and kidney tissues were analyzed for MDA, carbonyl, GSH, and catalase specific activity. The samples were homogenized using a homogenizer and a micro pestle in 1 ml of phosphate- buffered saline (PBS). The homogenate was then centrifuged at 1,500xG for 10 min, and the supernatant was transferred to a clean tube. All the procedures were approved by Ethic Committee from Faculty of Medicine Universitas Indonesia (Ethical number KET-249/UN2.F1/ETIK/PPM.00.02/2020).

MDA measurement

Two hundred µl of 20% trichloroacetic acid (TCA) were added into microtubes containing 400 µl of homogenate. The samples were centrifuged at 3,000 G for 10 minutes and the supernatants transferred to clean 2 ml microtubes. Four hundred µL of TBA 0.67% thiobarbituric acid (TBA) were then added to each tube and samples were incubated in a 100 °C water bath for 10 min. After cooling to room temperature, the absorbance was read at 530 nm. By this time, the reaction between MDA and TBA in acidic condition produced

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pink. The MDA concentration was calculated by plotting the absorbances of each sample into a standard linear curve of y= ax+b with y being the average absorbance and x being the MDA concentration in nmol/ml (22).

Carbonyl Measurement

Eight hundred µl of dinitrophenylhydrazine (DNPH) were added into microtubes containing 200 µl of homogenate, then incubated in the dark at room temperature for 45 min. To obtain the protein pellet, 800 µl of 20% TCA were added, the samples were centrifuged at 10,000xG for 5 minutes, and the supernatant was discarded. 10% TCA was added to the pellet and the samples were centrifuged at 10,000xG for 5 minutes. Ethanol-ethylacetate was used to wash the protein pellet three times. After washing, the pellet was dissolved in 800 µl of 9 M urea in 0.4 N NaOH, then incubated at 37 ºC for 10 min. Samples were then centrifuged at 10,000 x G for 5 min. Supernatants were read at absorbance 390 nm on a spectrophotometer. A blank was used for carbonyl calculation. For the blank, the steps were similar to above, however DNPH was replaced with 500 µl of 2.5 M HCl. The carbonyl level was calculated by subtracting the blank absorbance from the sample absorbance and then dividing by the extinction coefficient of 22 mM^{-1} cm^{-1} (23).

Reduced Glutathione (GSH)

Fifty µl of sample were added to the microtubes containing 200 ul of 5% TCA. The samples were centrifuged at 3,000xG for 10 min. Sample supernatants were collected and transferred to new tubes. Phosphate buffer pH 8.0 was added to the tubes. Last, 25 ul of DTNB was added to the sample tubes and samples were incubated in the dark for 1 hr. Absorbances were then read at 412 nm. GSH concentrations were calculated based on the glutathione standard linier curve (24).

Specific activity of catalase

The homogenate was diluted 20x in which a 5 µl of sample was diluted with 95 µl of 0.05 M PBS pH 7.0. Fifty µl of the diluted sample were mixed with 950 µl of 27.2 mM hydrogen peroxidase (H_2O_2) 1:4,000 and the absorbance was read at 210 nm after 30 (t0) and 150 (t1) sec. For the blank, 1,000 µl of 27.2 mM H_2O_2 1:4,000 was read at the same absorbance. Results of readings was calculated using the following formula:

$$\text{Catalase Activity (U/mL)} = \frac{(\Delta As - \Delta Ab)/Min}{H_2O_2 \text{ Molarity} \times \text{Sample Volume}} \times \text{Sample Dilution Rate}$$

ΔAs was obtained by subtracting the sample t0 absorbance from its t1 absorbance. ΔAb was obtained by subtracting the blank t0 from its t1 absorbance. As for the minute, since there was a 120-second difference between the two readings, two minutes was inserted into the formula. The sample dilution rate was 20x. The H_2O_2 concentration was 27.2 mM, and the sample volume was 50 µl. Catalase specific activity was calculated by dividing the catalase activity by the homogenate protein level (22).

Statistical Analysis

The results were analyzed using SPSS Statistics with cutoff at $p< 0.05$ to look for significant differences. The control, IF, and PF groups were compared by One-way ANOVA and post hoc test. Finally, the data was cleaned and checked for errors.

Results

The rabbits were weighed daily during the study. No significant weight differences were found between or within the groups (Table 1).

Table 1. Rabbit weights (gram ± SD).

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	p (within
Control (n= 5)	1912±430.95	1984±461.28	2058±481.48	2024±490.23	2124±557.16	2170±521.44	2160±511.12	> 0.05
IF (n=5)	2209±252.15	2228±273.45	2138±370.59	2192±269.84	2316±304.93	2304±312.7	2288±336.24	> 0.05
PF (n= 5)	2032±300.36	2078±277.45	1977±291.12	2012±246.67	1985±275.84	2108±254.32	2063±241.71	> 0.05
p (in group)	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	

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In heart tissue, MDA was significantly greater in the control than in the IF and PF groups; however, the IF and PF groups were not significantly different from each other. No significant differences were seen in heart tissue between any of the groups for carbonyl, GSH, or catalase (Table 2).

Table 2. Oxidative stress markers in the rabbit hearts.

	Control group	IF Group	PF Group	P
MDA (nmol/ml)	0.215±0.037	0.094±0.016#	0.090±0.018^	0.007$
Carbonyl (nMol/mg protein)	38.34±4.946	31.69±2.141	40.66±1.79	0.176
GSH (µg/ml)	0.928±0.122	0.898±0.124	0.693±0.036	0.250
Specific activity of Catalase (U/mg protein)	1.104±0.244	0.892±0.093	1.126±0.098	0.541

Note: # p= 0.006 (control vs IF group); ^p= 0.005 (control vs PF group); $= among group.

In brain tissue, no significant differences were seen between any of the groups for MDA or carbonyl. GSH was significantly greater in IF than in the PF and control groups, and catalase was significantly greater in IF and PF than in the control group (Table 3).

Table 3. Oxidative stress marker in the rabbit brains.

	Control	IF Group	PF Group	P
MDA (nMol/ml)	0.438±0.16	0.614±0.25	0.377±0.32	0.325
Carbonyl (nMol/mg protein)	547.7±38.15	365.4±24.2	409.1±44.7	0.448
GSH (U/ml)	0.015±0.004	0.028±0.006*,#	0.018±0.001	0.036$
Specific activity of Catalase (U/mg protein)	0.09±0.025	0.056±0.004#	0.049±0.009^	0.003$

Note: # p= 0.025 (control vs IF group for GSH), p= 0.004 (control vs IF group for specific activity of catalase); ^p= 0.001 (control vs PF group); *p= 0.009 (IF group vs PF group); $= among group.

In kidney, no significant differences were seen between any of the groups for MDA, carbonyl, or GSH. Catalase specific activity was significantly less in the PF than in the control group, but not significantly different between the IF and PF groups or between IF and control (Table 4).

Table 4. Oxidative stress marker in the rabbit kidneys.

	Control group	IF Group	PF Group	P
MDA (nMol/ml)	18.99±5.058	23.474±5.535	26.279±3.586	0.572
Carbonyl (nMol/mg protein)	0.255±0.051	0.222±0.054	0.275±0.047	0.292
GSH (µg/ml)	1.586±0.18	1.574±0.257	1.591±0.135	0.990
Specific activity of Catalase (U/mg protein)	0.105±0.012	0.089±0.014	0.081±0.011^	0.031$

Note: ^p= 0.011 (control vs PF group); $= among group.

Discussion

Several studies have shown that oxidative stress is closely related to degenerative disease including diabetes, cardiovascular problems, neurodegeneration, and cancer. In the context of cardiovascular problems, oxidative stress can lead to arrythmias, cardiac remodeling, and heart failure (6, 25, 26). Our research measured the extent of oxidative stress using MDA and carbonyl as biomarkers of lipid and

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protein oxidation, respectively, because ROS are responsible for oxidative modification of major cellular macromolecules. GSH and catalase were also measured to analyze endogenous antioxidant status. GSH acts as the substrate for glutathione peroxidase, which catalyze the reduction of H_2O_2 to H_2O (27). Moreover, catalase is the enzymatic antioxidant that also eliminates H_2O_2 (22). Previous studies have found that calorie restriction decreases the effects of oxidative stress by decreasing ROS production, increasing antioxidant enzyme activities, or increasing the turnover rate of oxidized macromolecules (19). However, calorie restriction is not a sustainable practice for most humans. The alternative method is fasting, which is a form of calorie restriction. The duration of fasting itself is less well studied, but the results show many subjects benefit from said fasting (28-30).

Our results showed a statistically significant decrease in heart MDA as the oxidative stress marker between the control and fasting groups. However, the MDA levels between intermittent and prolonged fasting were not significantly different. In brain and kidney, MDA did not differ between groups. It appears that the defensive response to oxidative stress differs between organs. A study conducted by Hernández-Moreno, et al. (31) to examine the effects of a single dose diazinon exposure on NZW rabbit kidneys, livers, and lungs revealed similar varying responses. After exposure to 25mg/kg mass of the pesticide, MDA concentrations were significantly increased only in kidney samples ($p< 0.01$), but not in the liver nor lungs, which required 125mg/kg of diazinon before MDA concentrations rose significantly. When given the higher dose, MDA concentrations rose 3-fold in kidney and lung samples, and 1.5-fold in liver.

Although some of our results were not statistically significant, one may note that MDA was greater in IF than in the control group in brain, and greater in both fasting groups than in control in kidney. Fasting and other forms of calorie restriction are stressful conditions that force the body to adapt to maintain homeostasis. Ristow, et al. (32) suggests how this stress caused by calorie restriction may serve as a stimulus for cellular adaptation, known as hormesis, which protects against ROS.

However, this stressor must be present in moderation, otherwise the cell fails to adapt, and oxidative stress rises as an indicator of this maladaptation to low energy availability (32). Stankovic, et al. (33) reported that MDA levels were significantly greater in livers of Wistar rats given less than 50% of their normal daily caloric intake than in those given 60% or more (33). Hence, we propose that the rabbits in our study experienced excessive calorie restriction that resulted in increased oxidative stress as seen in the trends. It is possible that MDA concentrations were just beginning to rise by the end of the 7-day fasting regimen, but at the experiment was ended before any significant changes had occurred.

No significant differences between groups were seen in carbonyl levels in heart, brain, or kidney, however carbonyl was lowest in the IF group in each tissue. Carbonylation has been linked to disease onset and progression, oxidative stress, and cell, tissue, and organ aging (34). Therefore, reducing the carbonyl level is important for organ health. A study that implemented IF on middle-aged rats for 12 weeks demonstrated that IF reduced oxidative damage. Middle-aged rats that underwent IF had a significant protein carbonyl reduction of 20-30% in the brain cortex, hippocampus, and hypothalamus compared to *ad libitum*-fed middle-aged rats. Motor coordination on those rats was improved and a correlation was found between the reduced protein carbonyl level and reduced oxidative stress. These findings suggest that oxidative stress reduction may be the result of early repair and maintenance of intermittent fasting (35).

For the antioxidant status, GSH in brain was significantly greater in the IF than in the PF and control groups. It has previously been demonstrated in several ways how IF could positively affect GSH levels. The first reason for increased GSH could be linked to a

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decrease in glucose when fasting for some time (36, 37). Because ROS are produced by glucose metabolism, the decrease in glucose levels is likewise associated with ROS production (37). Because the role of GSH as a reducing agent is less important in a situation of decreased ROS, especially in decreased H_2O_2, GSH levels in cells rise as a result of the decrease in ROS (38). In our study, however, no significant increase in brain GSH was seen in the PF group compared to control, contrast to what we found in the IF group. The PF finding showed same pattern as that seen in 24-hour IF mice (36); GSH levels increased, but not significantly. The non-significant PF finding agrees with previous studies in mice, which found that IF did not affect glutathione reductase, which restores GSH in mouse brain (36). Chausse et al. (36) concluded that IF does not boost antioxidant enzyme activity but does cause some tissue-specific reductions in oxidant buffering capability. In our study GSH levels in heart and kidney were not significantly different between groups. It is also possible that the statistically non-significant result found in our study was due to the relatively short fasting period of six days. A review of previous investigations exploring the protective effect of calorie restriction in the kidney revealed longer fasting periods ranging from 24 weeks (39) to 12 months (40). Hence, we assume that the fasting period was too short to produce significant effects. However, brain catalase specific activity was significantly less in the IF and PF groups than in the control group. This enzyme may inhibit oxidative stress; therefore, brain MDA and carbonyl did not increase, and to maintain the antioxidant level, GSH synthesis in the brain was increased.

In kidney, catalase specific activity in the PF group was significantly less than in controls. In this case, it is possible that the rabbit kidney had experienced excess calorie restriction, especially in the PF group. This may be worsened by the suggested relatively poor antioxidant defense system of the kidney. Another study found that PF decreased the activity of GSH and other antioxidant enzymes (41). A study using elephant seal pups showed that PF can be damaging because it increases the renin–angiotensin system (RAS), which induces oxidative damage and inflammation. The increase of RAS and pro-oxidant Nox-4 in muscle in PF elephant seal pups did not inhibit the increase of antioxidant expression and seemed to allow sufficient antioxidant supplies to suppress oxidative tissue damage. This is because PF is common in these seals, hence despite the increase in RAS, no associated inflammation or oxidative damage occurs (41). Another source of ROS is endoplasmic reticulum (ER) stress. Depletion of the GSH pool can cause redox imbalance. This can occur because GSH cooperates with ER oxidoreductin 1 (ERO1) and protein disulfide isomerases (PDI) to correct and prevent abnormal disulfide bonds. Large amounts of ROS are then produced, which depletes the GSH pool. Accumulation of misfolded and/or unfolded proteins is called ER stress, which causes a vicious cycle that leads to the disruption of ER function (42). Sorensen et al. (43) found that PF of 72 hours increased the oxidative stress level by increasing mitochondrial free radical production in rat liver.

This experimental research comes with its own set of limitations that must be addressed to analyze its findings. One limitation of this study is its relatively short duration. As has been discussed, studies proposing the beneficial effect of calorie restriction have used regimens lasting weeks to several months, giving ample time for cellular adaptations to occur and giving the said benefits of calorie restriction regarding oxidative stress. In this study, the fasting/non-fasting period lasted for only six days, which may not have been sufficient to cause significant changes for several oxidative stress markers in certain organs based on the discussed mechanisms. Nevertheless, the short duration of this study was sufficient to significantly decrease heart MDA in both the IF and PF groups and increase brain GSH in the IF group. Further research is needed to extend the fasting period and determine the

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best fasting duration for protective effects on the vital organs by including more groups based on a gamut of fasting durations.

Based on our results of MDA expression in heart, GSH in brain, and catalase in brain and kidney, we conclude that fasting reduces oxidative stress, and that IF appears to be more effective than PF in protecting these vital organs from oxidative damage.

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