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Research article

Effects of fasting on *FOXO3* expression as an anti-aging biomarker in the liver

Novi Silvia Hardiany [a,*], Muhammad Alifian Remifta Putra [b], Raya Makarim Penantian [b], Radiana Dhewayani Antarianto [c]

[a] *Department of Biochemistry & Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
[b] *Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
[c] *Department of Histology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

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ABSTRACT

Background: Aging is a multifactorial degenerative process that can be modulated by fasting through activation of the Fork-head transcription factor of the O class 3 (*FOXO3*), which plays an important role in increasing lifespans. However, the effects of different fasting durations on the expression of *FOXO3* in the liver has not yet been reported.
Objective: This study analyzed the effects of different fasting durations on the *FOXO3* expression and its pathway by measuring sirtuin1 (*SIRT1*), insulin-like growth factor-1 (*IGF-1*), and superoxide dismutase (SOD) activity in the liver.
Methods: New Zealand white rabbits were used to mimic the effects of fasting on humans. The rabbits were divided into the control, intermittent fasting (IF), and prolonged fasting (PF) groups. Both fasting groups were interspersed with the non-fasting phase for 8 h. This treatment was conducted for 6 days. On Day 7, all the rabbits were sacrificed, and their livers were taken to measure the *FOXO3* and *SIRT1* mRNA expressions, the *IGF-1* protein level, and the SOD activity level. ANOVA, multiple comparison, and Pearson's correlation were performed for statistical analysis.
Results: The *FOXO3* and *SIRT1* mRNA expressions were significantly higher in the IF group than in the control group. The *FOXO3* expression was also 2.5 times higher in the IF group than in the PF group. There was a positive correlation between the *FOXO3* and *SIRT1* mRNA expressions. The *IGF-1* protein level was significantly lower in the IF and PF groups than in the control group. The SOD-specific activity level was significantly higher in the IF group than in the control and PF groups.
Conclusions: Intermittent fasting significantly increased the *FOXO3* and *SIRT1* mRNA expressions and the SOD activity level in the livers of the rabbits and significantly decreased the circulating and hepatic *IGF-1*. Therefore, intermittent fasting may give a protective intervention effect towards aging.

1. Introduction

Aging is a complex process resulting from the accumulation of various factors, consisting of destruction in molecular, cellular, and

* Corresponding author.
E-mail address: novi.silvia@ui.ac.id (N.S. Hardiany).

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organ levels, which consequently leads to loss of function and increased vulnerability towards diseases, ultimately causing death [1]. The number of older people (people aged 60 or above) in the population continues to increase globally, and by 2050, is estimated to be more than 1.5 billion. Within the same period, the oldest group (people aged 80 or above) is predicted to triple in number. The increase in the aged population will definitely bring many challenges. For instance, an increase in lifespan is not always beneficial if not accompanied by an equal increase in health span, as the additional years of life will only be filled with diseases and disability. Moreover, there is a wide variability in the health status of older individuals; for instance, the mental and physical capacities of some 80-year-olds are similar to those of a 20-year-old, while those of other 80-year-olds decline at much earlier ages [2]. Unfortunately, the complex processes and reasons behind this variability are not yet fully understood.

Despite the complicated process of aging, it has been found that genetic and dietary changes can substantially increase the longevity of organisms [3]. First, with regard to genetics, many mutations that increase longevity affect stress-response or nutrient-sensor genes. One of these genes that have been extensively investigated and have shown a promising association with longevity is the fork-head transcription factor of the O class (*FOXO*), which is activated by upstream signals such as Sirtuin1 (*SIRT1*) when the organism faces low nutrient availability, such as during starvation [4,5]. *FOXOs* are crucial as they regulate several target genes that are involved in energy metabolism. In other words, they regulate metabolic balance. For instance, when we are starved, our resulting low insulin level activates the *FOXOs* in our liver to increase our glucose levels via gluconeogenesis and glycogenolysis [6].

Many studies have found that *FOXOs* also have important roles in various cellular processes. As transcription factors, they can either activate or inhibit downstream target genes, which eventually induce different cellular processes. One of the *FOXOs* subfamily, *FOXO3*, can respond to stimuli such as stress from metabolism, oxidation, reduction in insulin and growth factor number. These processes would further create response of anti-aging, by facilitating important processes such as stem cell homeostasis, differentiation, cell apoptosis regulation, and reactive oxygen species (ROS) destruction through activation of endogenous antioxidants such as superoxide dismutase (SOD) [6,7]. These processes collectively protect organisms from many diseases. Thus, it can be concluded that *FOXO3* gene expression prevents aging by regulating various cellular processes [7,8].

In terms of dietary changes, nutrient and stress sensors can mediate lifespan extensions by allowing the organism to adapt to different physiological and environmental signals. This leads to the selective investment of energy into various protective systems, which minimize damage that can reduce fitness. Caloric restriction and fasting have been proven to be the optimal signals that can extend lifespan and promote stress resistance effects that have been shown to be conserved throughout the course of the evolution of low-level organisms such as yeast all the way to higher organisms such as primates [3]. Fasting generally induces significant reduction of the level of circulating insulin-like growth factor-1 (*IGF-1*), whose signaling pathways, through the protein kinase B (PKB or Akt) pathway, negatively regulate *FOXO* transcription factors [3]. A better understanding of the relationship between *FOXO3* gene expression and dietary restriction in the form of fasting, both of which can boost an organism's longevity, would help determine a specific target for therapy. In addition, deeper insight into this area will allow us to further modulate *FOXO3* gene expression through dietary modification and to propose the optimal dietary restriction or form of fasting to slow down the decline in function and the onset of disease due to aging. Thus, this study expounded how different durations of fasting affect *FOXO3* expression and its pathway in the liver by implementing intermittent fasting (i.e., fasting for 16 h) and prolonged fasting (i.e., fasting for 40 h).

In this study, New Zealand white (NZW) rabbits were used to mimic the effects of fasting that are generally represented in humans and not in small laboratory rodents (i.e., mice and rats). Previous studies were more focused on low-level organisms [9], which limited such studies as these organisms are less capable of representing or simulating the effects of fasting on humans. The liver was chosen as the sample because it has shown a significant response to fasting to maintain homeostasis [10]. Moreover, aging decreases the liver function, and the elderly are more susceptible to liver disease [11]. In addition, the effects of different fasting durations on *FOXO3* expression in the liver have yet to be explored. The aim of this study was to analyze *FOXO3* expression and its pathway by measuring the *SIRT1* and *IGF-1* expressions as *FOXO3's* upstream signals, and the SOD activity as *FOXO3's* downstream target in the livers of rabbits whose diets have been restricted through intermittent fasting (for 16 h) and prolonged fasting (for 40 h). Therefore, this study is proposed to be the basis of *FOXO3* gene modulation and fasting in humans to achieve healthy aging.

2. Methods

This research was an experimental study conducted on 15 NZW male rabbits with weights between 1800 and 2500 g. They were divided into three groups of five. The most suitable rabbit age that can represent human age, the calculation was performed by setting the life expectancy of NZW rabbits as about 8–12 years in general. The disparity between the developmental stages of humans, and rabbits is also being acknowledge in which life phase of NZW rabbits are being considered to be able accurately correlates with the life phase in human. Age of the animals was 4 months which is about 12 years in human age. NZW rabbits are in the pubertal phase during this age, and one human year is equivalent to 13.04 days for NZW rabbits. This calculation was achieved by understanding that NZW rabbits attain their puberty around P150, thus it can be calculated as 11.5 years $\times$ 365 days equal to 4198 days. Further, 4198 days were divided by 150 which equals 27.98 human days equal to 1 rabbit day. Lastly, 365 days divided by 27.98 human days equal to 13.04 NZW rabbits day that equal to 1 human year. It is the shortest equivalent value for a rabbit's lifespan in its life phase [12].

The rabbits were maintained in a natural light and dark cycle at $25 \pm 2\ ^{\circ}C$. Each group was given a different treatment. The control group was normally fed ad libitum with a commercial pellet diet and given free access to water for six days. The IF group was not fed for 16 h (from 16:00 to 08:00 of the next day) for the same six days, interspersed by the non fasting phase for 8 h in diurnal time hours. Finally, the PF group was not fed for 40 h (starting on 16:00–16:00 for the first 24 h and continued until 08:00 in the second 24 h), with a similar 8 h non fasting phase interspersed in diurnal time hours. During the 8-h period when the rabbits could eat, the food that was given to them was 5% of their body weight, which was their daily normal intake [13]. The rabbits' body weights were measured every

day until Day 7, when they were anesthetized using 35 mg of ketamine per kg of body weight and 5 mg of intramuscular xylazine per kg of body weight to obtain their liver as the sample. At the end of the treatments, plasma glucose was detected in all the groups using the GLUC-PAP kit (Randox®). All the procedures were approved by the Ethics Committee of the Faculty of Medicine of Universitas Indonesia, with ethical approval number KET-249/UN2.F1/ETIK/PPM.00.02/2020.

2.1. RNA isolation for FOXO3 and SIRT1 mRNA expressions

From each of the liver samples of the three groups of rabbits, an RNA sample was isolated using an RNA isolation kit (Quick-RNA Miniprep Plus, Zymo®), according to the manufacturer's procedure. The isolated RNA of each liver sample was quantified using the Varioskan™ Flash Multimode Reader. Then, a Thermo Scientific μDrop Plate was wiped with lens tissue dampened with distilled water, followed by lens tissue dampened with 70% ethanol, to remove any contaminants, after which 2 μl of the purified RNA of each sample was pipetted onto the plate. Then, the concentrations (in ng/μl) and purities (A260/A280 and A260/A230) of RNA were measured, following which the quantified RNA samples were diluted in nuclease-free water to obtain the final concentration of 50 ng/μl.

2.2. FOXO3 and SIRT1 mRNA expressions via quantitative polymerase chain reaction

The mRNA sample (100 ng) was amplified using a quantitative polymerase chain reaction (qPCR) machine (Applied Biosystems 7500 Fast Instrument, USA) using a SensiFAST SYBR No-ROX One-Step Kit (Bioline®). First, the RNA was converted to complementary DNA (cDNA) by the reverse transcriptase enzyme for 10 min at 42 °C. Second, the reverse transcriptase was inactivated by setting the temperature at 95 °C for 5 min. Third, the cDNA was amplified through 40 cycles of PCR using the primers listed in Table 1. The primers were designed using BLAST primer tools, with 18S rRNA as the reference gene. The mRNA expressions of *FOXO3* and *SIRT1* were determined using the Livak ($2^{-\Delta\Delta Ct}$) method and written in the form of a ratio (a relative expression). The Livak formula was used to analyze the expression levels of *FOXO3* and *SIRT1* in the control, IF, and PF groups. The Livak method is one of the most common methods of analyzing changes in gene expression relative to the real-time qPCR result [14].

Note:
ΔΔCT = ΔCT(test) - ΔCT(calibrator)
ΔCT(test) = CT(target, test) - CT(reference, test)
ΔCT(calibrator) = CT(target, calibrator) - CT(reference, calibrator)
Targets = *FOXO3* and *SIRT1* genes.
Test = IF and PF groups.
Calibrator = control group.
Reference = *18S rRNA* gene (reference gene)

2.3. Analysis of the FOXO3 Protein Expression via Enzyme-Linked Immunosorbent Assay (ELISA)

The *FOXO3* protein was determined using the Rabbit Forkhead Box Protein O3 (*FOXO3*) ELISA Kit (MyBioSource®). This kit uses the Double Antibody Sandwich Technique, which depends on the tested antigen characteristics with more than two valences. Moreover, the kit can simultaneously identify both detection antibodies and coated antibodies. One hundred μL of rabbit *FOXO3* standards with different concentrations or samples were added into the corresponding wells and incubated for 90 min at 37 °C. One hundred microliters of biotinylated rabbit *FOXO3* antibody was added into each of the wells after the washing process and incubated at 37 °C for 60 min. Then, the plate was washed three times, after which 100 μL of the enzyme-conjugate liquid was put into each well and incubated for 30 min at 37 °C. This was followed by the addition of 100 μL of color reagent liquid after the plate was 5X washed. The occurrence of the chromogenic reaction was controlled within 30 min inside the dark incubator at 37 °C. The optical density was read at 450 nm within 10 min after 50 μL of the stop solution was added.

2.4. Analysis of the IGF-1 protein expression via ELISA

IGF-1 protein was detected in plasma in the forms of circulating *IGF-1* and *IGF-1* in the liver. It was determined using a rabbit *IGF-1* ELISA Kit (Cusabio, USA®). The rabbit *IGF-1* standard with different concentrations or samples was added into the corresponding wells

Table 1
List of primers.

No.	Gene	Sequence	Product Size
1.	*FOXO3*	F: TTGGAAGAACTCCATCCGACA	180 bp
	XM_008263339	R: CCACGGCTCTTGGTGTACTT	
2.	*SIRT1*	F: GAGCTGGGGTGTCTGTTTCA	153 bp
	XM_002718460	R: ACTTGAAGAATGGCCGAGGA	
3..	*18S rRNA*	F: AAACGGCTACCACATCCAAG	155 bp
	NR_033238.1	R: CCTCCAATGGATCCTCGTTA	

(with each well filled to 100 μL) and incubated for 2 h at 37 °C. One hundred microliters of biotinylated rabbit *IGF-1* antibody was added into each of the wells after the washing process and incubated at 37 °C for 60 min. Then, the plate was washed three times, and then 100 μL of the enzyme-conjugate liquid was put into each well and incubated for 60 min at 37 °C. The addition of 90 μL of color reagent liquid was performed to each well after the 5X washing process. The occurrence of the chromogenic reaction was controlled within 15 min inside the dark incubator at 37 °C. The optical density was read at 450 nm within 10 min after 50 μL of the stop solution was added.

2.5. Specific activity of the superoxide dismutase (SOD) enzyme

The SOD activity was determined using a Ransod kit (RANDOX,®, United Kingdom). This method uses xanthine along with xanthine oxidase to produce superoxide radicals that will further react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to produce red formazan dye. Thus, the SOD activity can be measured through the degree of inhibition of this reaction. A 50% inhibition of the rate of INT reduction in this assay was equal to one unit of active SOD. Thirty microliters of the samples were put in the cuvette, after which 1000 μL of mixed substrate was added, mixed, and followed by the addition of 150 μL of xanthine oxidase. The standards were prepared using the same protocol. The absorbance was read at 505 nm 2 times after the first 30 s and 3 min later. The specific activity of the SOD enzyme was determined by dividing the SOD activity by the protein level [15].

2.6. Statistical analysis

The results are presented in mean ± standard deviation (SD) values. Significance differences between the groups were analyzed using one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) post hoc multiple comparison. The parametric distribution assumption was tested first using the Shapiro–Wilk test for the test of homogeneity of variances. Correlation analysis was performed using the Pearson correlation test. The data were said to be significant when the p value was <0.05.

3. Results

3.1. Characteristics of rabbits

a. Body Weight Measurement

The changes in the body weights of the rabbits in the three experiment groups throughout the experiment period were measured. However, there was no significant difference in the body weights day to day within and among the groups, as shown in Table 2.

b. Plasma Glucose Levels

There was no significant difference between the plasma glucose levels of the three groups, as shown in Fig. 1 (control group, 116.3 ± 4.4 mg/dL; IF group, 143.3 ± 30.56 mg/dL; PF group, 106.1 ± 24.6 mg/dL).

c. Levels of *FOXO3* and *SIRT1* mRNA Expressions

Our research showed that a significant increase in the *FOXO3* mRNA expression in the IF group by approximately 2.17 times ($p < 0.01$) compared to the control group (Fig. 2a). The *FOXO3* mRNA expression in the PF group was lower, 0.86 times (Fig. 2a), in comparison with the control group. The *SIRT1* mRNA expression in the IF group increased by 1.55 times ($p > 0.05$) compared to the control group and significantly increased by 2.55 times ($p < 0.01$) compared to the PF group. On the other hand, the *SIRT1* mRNA expression of the PF group significantly decreased by 0.61 times compared to the control group (Fig. 2b). There was a significant positive correlation ($R = 0.641$, $p = 0.010$) between the *FOXO3* and *SIRT1* mRNA expressions.

d. *FOXO3* Protein Expressions

The *FOXO3* protein level in the control group was 7.45 ± 0.7 ng/mL, whereas in the IF group, it was 9.1 ± 1.2 ng/mL and in the PF

Table 2
Body weight of the rabbits.

Body Weight (g ± SD)								*p*-value
Experiment Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
Control ($n = 5$)	1912 ± 430.9	1984 ± 461.3	2058 ± 481.5	2024 ± 490.2	2124 ± 557.2	2170 ± 521.4	2160 ± 511.1	>0.05
IF ($n = 5$)	2210 ± 281.9	2228 ± 305.7	2138 ± 414.3	2192 ± 301.7	2316 ± 340.9	2304 ± 349.6	2288 ± 375.9	>0.05
PF ($n = 5$)	1910 ± 329	1968 ± 303.9	1858 ± 318.9	1914 ± 270.2	1878 ± 302.2	2018 ± 278.6	1966 ± 264.8	>0.05
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	

Note: From the one-way analysis of variance (ANOVA) test. IF = intermittent fasting; PF = prolonged fasting.

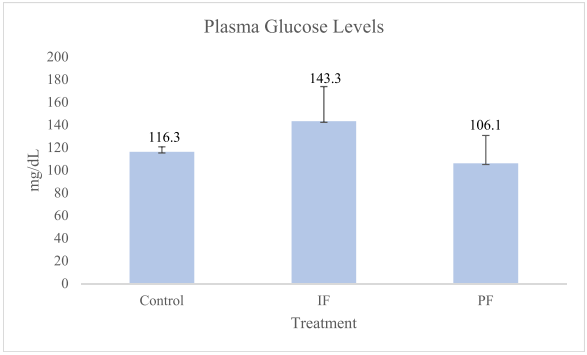

Fig. 1. The plasma glucose levels of the three groups. There was no significant difference ($p > 0.05$) between the groups [based on one-way analysis of variance (ANOVA)]. IF = intermittent fasting; PF = prolonged fasting.

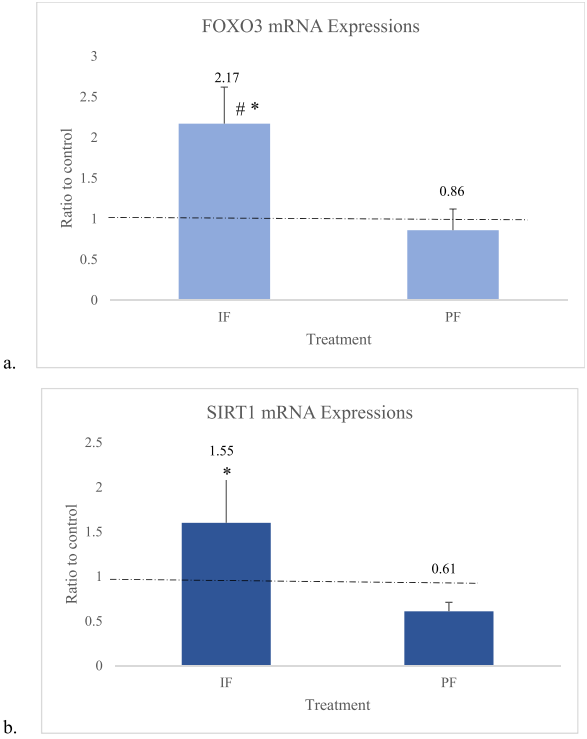

Fig. 2. a. *FOXO3* mRNA expressions. b. *SIRT1* mRNA expressions. #: significantly different between the IF and control groups, *: significantly different between the IF and PF groups. IF = intermittent fasting, PF = prolonged fasting. For the *FOXO3* mRNA expressions: analysis of variance (ANOVA) and Least Significant Difference (LSD post hoc multiple comparisons. For the *SIRT1* mRNA expressions: Kruskal–Wallis and LSD post hoc multiple comparisons. Each group consisted of five New Zealand white rabbits.

group, 8.3 ± 1 ng/mL, as shown in Fig. 3. The *FOXO3* protein expressions were determined to ensure that the increases in the mRNA levels corresponded with the increases in the protein levels. There was a significantly positive correlation ($R = 0.533$, $p = 0.041$) between the *FOXO3* mRNA and protein levels. Moreover, the *FOXO3* protein was significantly correlated with the *SIRT1* mRNA ($R = 0.530$, $p = 0.042$).

e. Plasma and Hepatic *IGF-1* Proteins

Both the plasma *IGF-1* protein, which represent the circulating *IGF-1*, and the hepatic *IGF-1* protein, were significantly lower in the IF and PF groups than in the control group, as shown in Fig. 4. The plasma and hepatic IGF-1 proteins in the control group were 11.15 and 10.73 ng/mL; in the IF were 7.98 and 8 ng/mL, and in the PF were 7.49 and 8.2 ng/mL, respectively.

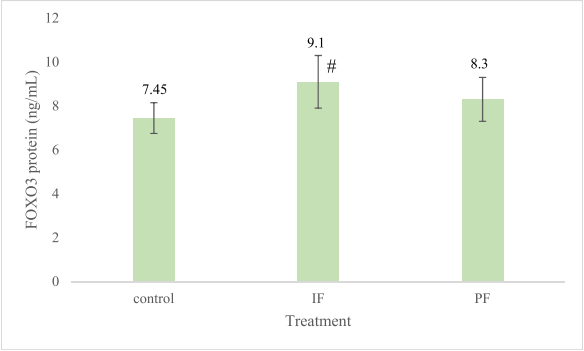

Fig. 3. *FOXO3* protein expressions in the three groups. The *FOXO3* protein expression in the IF group was significantly higher ($p = 0.025$) than that in the control group. #: significantly different between the IF and control groups. IF = intermittent fasting, PF = prolonged fasting. Based on analysis of variance (ANOVA) and Least Significant Difference (LSD) post hoc multiple comparisons. There were five New Zealand white rabbits in each group.

f. Specific Activity of the Superoxide Dismutase Enzyme

The specific activity of superoxide dismutase (SOD) in the IF group (10.67 ± 0.963 U/mg protein) was significantly higher than in the control group (6.97 ± 1.36 U/mg protein) and in the PF group (6.61 ± 0.913 U/mg protein), as shown in Fig. 5. There was no significant difference between the control and PF groups. This specific SOD activity was significantly correlated with the *FOXO3* mRNA ($R = 0.583$, $p = 0.023$).

4. Discussion

The body weights of the rabbits changed only slightly, fluctuating at around 50–60 g per day. Kawamura et al. [16] also found fluctuations of around 15–70 g per day in the body weights of male NZW rabbits aged 5–7 months. The daily fluctuations in the body weights of the rabbits is a common physiological phenomenon [17]. The short duration of the fasting (six days) in this experiment might explain the non-occurrence of weight loss in both the IF and PF groups. There was no significant decrease in the plasma glucose level in the fasting group compared to the control group. An insignificant decrease in the plasma glucose level was detected in the PF group. The plasma glucose level could have remained normal in all the groups due to the gluconeogenesis process, which is regulated by the *FOXO3* expression. Gluconeogenesis is the sole energy source of many tissues during fasting [8].

A vast body of evidence is surfacing that proves that IF lowers oxidative stress and inflammation. This trend was the result of the discovery that metabolic diseases such as diabetes start with oxidative stress and chronic inflammation. Various studies have attempted to discover the impact of IF, such as of Ramadan intermittent fasting (RIF), on the expressions of antioxidant genes and genes correlated with cellular metabolism, such as *SIRT1*. The research found that RIF could increase the genetic expressions of various genes, including of metabolic regulatory, anti-inflammatory, and antioxidant genes. Therefore, it was suggested that RIF might have a protective effect against adverse metabolism-related derangements and oxidative stress [18]. Another study supported this statement after finding that RIF lowered visceral adiposity and body weight, which reduced the risk factor of ROS accumulation [19]. This was

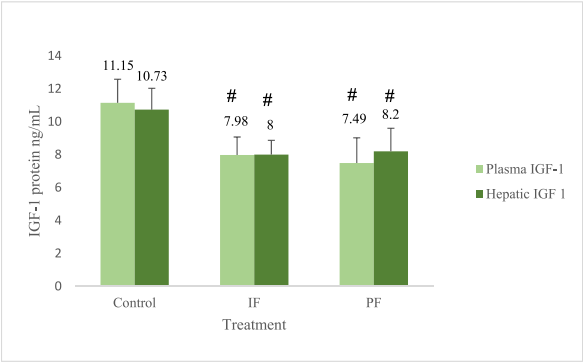

Fig. 4. IGF-1 protein levels. The plasma IGF-1 levels were significantly lower both in the IF group ($p = 0.010$) and in the PF group ($p = 0.004$) than in the control group. The hepatic IGF-1 levels were significantly lower both in the IF group ($p = 0.012$) and in the PF group ($p = 0.018$) than in the control group. #: significantly different between the IF or PF and control groups. Based on the analysis of variance (ANOVA) and Least Significant Difference (LSD) post hoc multiple comparisons. Five New Zealand white rabbits were in each group.

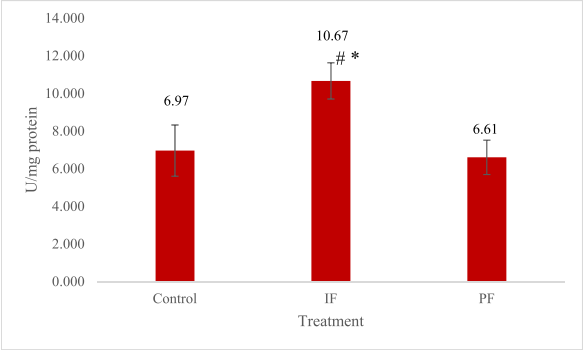

Fig. 5. Specific activities of the SOD enzymes in the liver tissues of the three groups. The SOD activity in the IF group was significantly higher ($p < 0.05$) than that in the control and PF groups. Note: significantly different between the IF and control groups. *: significantly different between the IF and PF groups. IF = intermittent fasting, PF = prolonged fasting. Based on analysis of variance (ANOVA) and Least Significant Difference (LSD) post hoc multiple comparisons. There were five New Zealand white rabbits in each group.

further confirmed by the finding from a systematic review and meta-analysis that diurnal IF during Ramadan had a protective effect against an increase in oxidative stress and inflammatory markers. Thus, it can suppress low-grade systemic oxidative stress and inflammation and prevent subsequent adverse health effects in humans [20].

Consistent with previous research, our experiment showed that the *FOXO3* expression both at the mRNA and protein levels increased more significantly in the IF group (i.e., with 16 h of fasting) than in the control group and the PF group. These data implied that IF in NZW rabbits had higher liver *FOXO3* expressions than in the two other groups. This finding is similar to that of Imae et al. [9], who examined the mRNA concentration in rat liver in response to internal and external triggers such as hormonal and nutritional factors. The rat group was subjected to fasting, after which significant increases were seen in the levels of its *FOXO1* mRNA (by 1.5 times), *FOXO4* (by 1.6 times), and *FOXO3* (by 1.4 times). After the rats were fed again for 3 h, the levels of their induced *FOXO1* and *FOXO3* mRNA expressions went back to the control levels, but not of their *FOXO4* mRNA expressions [9]. Furuyama et al. [21] also investigated the expression levels of mammalian daf-16 gene homologs, including of the mRNAs of the *FOXO* family genes examined in the rat skeletal muscle, which were found to have increased during fasting.

Higher *FOXO3* expression has a protective effect on the liver against the aging process. The main anti-aging mechanism of *FOXOs* is their being transcription factors, which are crucial for stem cell function in both adults and embryos [22]. In adults, *FOXOs* are needed for stem cell functionality in multiple tissues. *FOXO3* plays an important role in aging by maintaining stem cell homeostasis such as hematopoietic, neural, and muscle cell homeostasis. The correlation of stem cells with *FOXO* suggests the hypothesis that *FOXO* functions and regulations are pivotal in maintaining stem cell properties. Thus, it is proposed that the main contribution of *FOXO3* to longevity is its ability to modulate stem cells [22]. For instance, *FOXO3 plays a* role in satellite cells as the stem cells of skeletal muscle. In the event of cellular damage or stressful conditions, *FOXO3* proteins are expressed to induce Notch signaling activation to increase the rate of stem cell self-renewal. In normal conditions, they maintain the satellite cell pool so that it could actively replicate while remaining in its undifferentiated state [23]. However, in pathological conditions, overexpression of *FOXO3* is associated with the development and progression of several diseases, such as Alzheimer's disease [24] and cancer [25–27]. The pathogenesis of Alzheimer's disease involves activation of the *FOXO* signaling pathway, which induces neuronal cell death [24]. Moreover, activation of *FOXO3* stimulates chemotherapy drug resistance in glioblastoma multiforme [25] and hepatocellular carcinoma [26,27]. Therefore, the protective effect of FOXO3 mainly plays a role in normal cells.

In 1990, Straus et al. [28] bridged the knowledge between fasting (nutritional) and growth factors (hormonal) that may affect the aging process. Their research further investigated the effect of fasting on the level of *IGF-1* expression in animals based on its mRNA levels. Animal growth is fundamentally regulated by the intricate interplay between nutritional, hormonal, and even genetic variables. The hormones that are largely known to have important functions in regulating the somatic growth of cells are *IGF-I* and *-II.* Straus et al. measured the mRNA levels of *IGF-I* in six-week-old male control rats that were fed ad libitum and also fasted for 24, 48, or 72 h. The mRNA concentrations of several *IGF-I* species (8.0, 4.0, 1.7, and 1.0 kilobases) were reduced in the animals that fasted and further rebounded after 24 h of refeeding, even though they were not able to reach the initial control levels [28]. It was discovered that the insulin*/IGF-1* cascade was involved in inhibiting the activity of the *FOXO3* gene, the gene most widely known to be correlated to human longevity due to its role in the molecular signaling hub and chromatin conformation. Thus, decreasing the *IGF-I* level positively affects the organism by increasing its *FOXO3* expression [29]. Our study showed that the circulating and hepatic *IGF-1* levels more significantly decreased in the IF group than in the control group. Therefore, the increase in *FOXO3* in the IF group was probably due to the decrease in *IGF-1*.

Activation of the *FOXO3* expression was regulated by various types of post-translational modification (PTM). Modulation of *FOXO3* expression and subsequent controlled target genes greatly depends on the process of *FOXO3* translocation control between the cytoplasm and the nucleus, which can be done by utilizing the phosphorylation process with the help of various kinases. Nuclear *FOXO3,* which undergoes the serial process of PTMs, has the main function of modulating *FOXO3* transcriptional activity by altering the binding specificity of the promoter and changing the binding affinity of DNA [30]. In the nucleus itself, *FOXO3,* which remains, will

be further acetylated utilizing p300 and CREB-binding protein (CBP), or it can also go through a vice versa mechanism in which it is deacetylated by *SIRT1* [4]. Fasting results in low-calorie intake, which triggers the activation of sirtuins [31] and eventually activates *FOXOs*. Our results showed that *SIRT1* mRNA expression was higher in the IF group and was positively correlated with *FOXO3* expression. *SIRT1* deacetylates *FOXOs*, inducing the expression of genes correlated with mitochondrial biogenesis and stress resistance adaptation [32].

Increased activation of *SIRT1* gene expression in IF mediates the beneficial metabolic switch in various organs. During fasting, there is a period of starvation in which there is no dietary intake, meaning it induces low cellular energy status, which increases the NAD level$^+$ as well as the NAD$^+$/NADH ratio. Considering that NAD$^+$ is an absolute requirement for *SIRT1* enzymatic reaction, the increase in this cofactor increases the activation of *SIRT1*. However, Madkour et al. [18] found that relative mRNA expression of *SIRT1* insignificantly decreased by around 10.4% in 56 obese and overweight subjects (22 women and 34 men) who underwent Ramadan IF. The insignificant decline in the *SIRT1* expression in such research was related to the insignificant change in the total energy and the lipid dietary intake, accompanied by abundant simple carbohydrate intake during the non-fasting time at night during the Ramadan period. The low expression of *SIRT1* in that research was in line with insignificant changes in insulin and insulin resistance (determined via HOMA-IR) as markers of glucose homeostasis. Hence, the activity of *SIRT1* is said to reflect the metabolic state of cells [33]. When *SIRT1* increases, the deacetylation of Peroxisome proliferator-activated receptor-gamma coactivator (*PGC*)-1a increases, and it is activated. As *PGC-1a* regulates numerous genes involved in metabolism processes such as thermogenesis, biogenesis of mitochondria, and gluconeogenesis, its activation leads to an increase in mitochondrial activity and glucose metabolism [34]. Several studies with *SIRT1* knockout mice further revealed the importance of *SIRT1* in mediating response, as they lack the characteristic changes in *IGF-1* and longevity [35,36]. Therefore, previous studies have shown that deprivation of food during fasting can lead to protective and pro-longevity effects on different organisms, but the specific response to fasting differs from species to species [37].

Activation of *FOXO3* expression by IF plays an important role in maintaining redox balance and ROS detoxification [38]. *FOXO3* expression regulates SOD [38], an endogenous antioxidant that converts superoxide radicals to less reactive hydrogen peroxide (H_2O_2) [39]. Our results proved that the specific activity of the SOD enzyme in the IF group was significantly higher than in the control and PF groups. Preoperative fasting has been studied in mice to protect against renal ischemia–reperfusion injury, which is closely related to the production of ROS [40,41]. Ischemia-reperfusion cause oxidative stress, as can be seen in the increased levels of 4-Hydroxynonenal (4-HNE) and protein carbonyls in the kidney and the decreased activity of glutathione S-transferase, SOD, and glutathione peroxidase [42]. Mitchell et al. [41] proposed that the mRNA of specific antioxidant proteins involved in ischemia-reperfusion injury are increased during fasting, which suggests the protective effect of fasting against oxidative stress caused by reperfusion.

In contrast to the IF group, in the PF group, there was no significant change in the *FOXO3* expression, *SIRT1* mRNA expression, and SOD specific activity compared to the control, but the expressions were shown to have decreased compared to the IF group. It is possible that in PF, there is a reduction in the protein synthesis and overall proteolytic rates. Price et al. [43] demonstrated the effect of long-term calorie restriction on hepatic tissue in mice. Other studies postulated that chronic dietary restriction or PF itself has a negative effect on longevity, as already shown in several other studies on various types of organisms, such as rodents [44,45]. The significant decrease in the *IGF-1* levels in the PF group compared to the control group was unable to increase the *FOXO3* expression. Therefore, we can assume that other pathways besides *IGF-1* may be involved in regulating *FOXO3* expression during PF conditions, but that pathway is outside the scope of this study.

This study was limited by a relatively short period of treatment, which prevented full description of the effects of different fasting regimens with much longer durations. Similar experiments with longer durations could show if the effect could be sustained in the long term. Furthermore, in this experiment, the food intake and fecal production were not assessed, which made it unclear whether the metabolic changes in the NZW rabbits were due to the fasting regimen or the overall change or reduction in the total caloric and macronutrient intake. In addition, this study used a minimal sample for each group (five rabbits) and did not use older rabbits. This was because we wanted to analyze the effect of fasting on the expression of *FOXO3* as an anti-aging molecule at a young age to promote such intervention as stimulating anti-aging molecules from a young age, which is probably beneficial for achieving a healthy condition in old age. However, comparison of various age groups is needed to analyze the response of different ages in this research scheme. We also recommend that future researchers discover the other factors that may contribute to the expression rate of the *FOXO3* gene, especially in the fasting condition. In addition, further research should measure the protein levels of *FOXO3, SIRT1,* and IGF-1 using western blotting, immunochemistry, or immunoblot to validate the ELISA results in this study. Moreover, the exact and clear molecular pathway between fasting and *FOXO3* gene expression has not yet been fully discovered. This discovery would surely bring physicians one step closer to fully implementing fasting as a new alternative mode of therapy for humans with aging-related diseases. Exploration of *FOXO3* expression and other pathways that impact cellular aging, such as *PGC1a*, phosphoenolpyruvate carboxykinase (*PEPCK*), and glucose 6 phosphatase, in various tissues is also important in analyzing the effectiveness of fasting. Despite of several limitations, it was a first study which explore the effect of different fasting duration on the FOXO3 expression as anti-aging biomarker and its pathway in the liver.

5. Conclusion

IF significantly increased the *FOXO3* expression, *SIRT1* mRNA expression, and specific activity of the SOD enzyme in the liver, and significantly decreased the circulating and hepatic *IGF-1* levels. The increase in the *FOXO3* expression was mediated by the decrease in the *IGF-1* levels during fasting. Therefore, IF may prevent aging. On the contrary, PF did not significantly change the *FOXO3* expression nor its pathway compared to the control, and insignificantly increased the *FOXO3* expression compared to IF.

Author contribution statement

Novi Silvia Hardiany: Conceived and designed the experiment; Performed the experiment; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Muhammad Alifian Remifta Putra & Raya Makarim Penantian: Performed the experiment; Analyzed and interpreted the data; Wrote the paper.

Radiana Dhewayani Antarianto: Conceived and designed the experiment; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

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