

## Original Article

# SIRT1 Level Differences among Old Rats, Streptozotocin-Induced Diabetic Rats, and D-Galactose-Induced Aging Rats

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### Abstract

**Introduction:** The Sirtuin 1 (SIRT1) level is suspected to be lower in older ages. In a study of rats, aging can be induced by D-galactose. Various degenerative diseases can also develop from streptozotocin induction (rat models for diabetes mellitus). This study aimed to verify SIRT1 level differences among old rats, D-galactose-induced diabetic rats and streptozotocin-induced aging rats. **Material and Methods:** A total of 20 female Wistar rats aged four months was divided into five groups of 4 rats each. Group K1 was directly terminated, K2 and K3 were kept until 18 and 33 months old, respectively, and later terminated. Rats in the K4 group were induced with streptozotocin at a dose of 65 mg/kg BW and nicotinamide at 120 mg/kg BW and kept for one month. Rats in the K5 group were induced by 300 mg/kg BW of D-galactose for one month. Blood specimens were taken to examine SIRT1 levels using ELISA. The statistical test used in this study was the Kruskal-Wallis test (95% confidence level). **Results:** SIRT1 levels in rats aged 4, 18, and 33 months, diabetic rat models, and D-galactose-induced aging were  $0.43 \pm 0.12$ ,  $0.58 \pm 0.15$ ,  $0.30 \pm 0.01$ ,  $0.25 \pm 0.13$ , and  $0.21 \pm 0.05$ , respectively ( $p=0.028$ ). **Conclusions:** D-galactose-induced aging rat models had the lowest SIRT1 level compared to 33-month-old rats and diabetic rat models.

**Keywords:** Aging, D-galactose, rat model, SIRT1, streptozotocin.

### Introduction

The number of older people continues to increase in Indonesia as well as worldwide [1]. However, this condition reduces physiological functions and is commonly associated with the increasing prevalence of degenerative diseases [2]. To overcome various problems among the elderly and to improve their quality of life, different studies related to old age and its associated diseases are required.

Research involving humans requires an in-depth ethical review; consequently, studies involving experimental animals will be more frequently conducted. Animal models for old age are important and developed based on the pathophysiological process of aging [3]. The aging rat modeling is widely used because of its affordability and the easiness of D-galactose application. According to one of the theories of aging, the admin-

istration of D-galactose can increase reactive oxygen species (ROS), leading to the death of a large number of cells [4, 5]. However, the use of D-galactose in rats has yet to be effective [6]. Therefore, the parameters of the aging marker in D-galactose models should be proven.

Diabetic rats through streptozotocin induction can also be used as an aging animal model as it can lead to degenerative diseases, such as Alzheimer's disease [7], through the mechanism of telomere instability [8], which is in accordance with one of the aging mechanisms where telomere shortening occurs and ultimately causes cell apoptosis [9].

Aging animal models should be justified by the presence of aging markers. However, due to the complexity of the aging mechanism, no single valid marker is available [10], but one secure method to achieve is by assessing the markers in a peripheral blood specimen. For example, Rusanova et al. used plasma advanced



oxidation protein products (AOPP) and lipid oxidation (LPO) in their study [11]. Both markers indicate an increased level of ROS [12]. Aging has been recently associated with some metabolic disorders and leads to dyslipidemia and increased levels of triacylglycerol/TAG [13], triglycerides (TG), and low-density lipoprotein (LDL) as well as an increasing atherogenic index (AI) during the aging process [14]. However, the upstream of lipoprotein regulation contains Sirtuin 1 (SIRT1) [15].

SIRT1 is the most recent marker associated with the pathogenesis of aging [14, 16] and is a deacetylation enzyme [17]. The mechanism of SIRT1 deacetylation is associated with protection against the process of cell aging. SIRT1 also plays an essential role in cell cycle regulation, energy homeostasis, apoptosis, circadian rhythm, mitochondrial function, as well as lipid and glucose metabolism [18]. SIRT1 maintains the physiology of young age and extends lifespan [19]. SIRT1 also stimulates the sympathetic nervous system, maintains the morphology and mitochondrial function of skeletal muscles for physical activities, and also regulates body temperature and oxygen consumption during aging. Previous studies have identified the role of SIRT1 as an aging marker. SIRT1 decreases in Alzheimer's and mild cognitive impairment (MCI) conditions. Sirtuin levels are also lower in older people compared to young people [20]. Therefore, this study aims to determine the differences in SIRT1 blood levels among old-aged rats, diabetic rats (induced by streptozotocin and nicotinamide), and aging rats (induced by D-galactose).

## Material and Methods

### Study design and patients

This research was an experimental study with female Wistar rats, and the study was conducted from March 2018 to September 2019.

### Research Subjects

The rats were obtained from the Physiology Laboratory of the Faculty of Medicine, Universitas Islam Indonesia and taken care of at the Research Laboratory of the Faculty of Medicine, Universitas Islam Indonesia. All of the rats came from the same dam, and a total of 16 rats aged 4 months and 4 rats aged 18 months were involved in the study. The rats were randomized, except the 18-month-old rats, and divided into five groups,

each with four rats. The K1 and K2 groups consisted of 4-month-old rats, which were kept until termination at 4 months (K1) and 18 months (K2). The rats aged 18 months were kept and terminated at 33 months old (K3), while the K4 group with 4-month-old rats had diabetes mellitus (DM) induced with 65mg/kg BW streptozotocin and 120mg/kg BW nicotinamide through intraperitoneal injections, maintained for a month post-induction. The K5 group comprised a group of rats induced by 300mg/kg BW of D-galactose through daily intraperitoneal injection for one month and were then terminated.

### Determination of Sample Size

The number of experimental animals used in this study was determined using the formula [21]  $E = \frac{\text{total number of animals} - \text{total number of groups}}{10 - 20}$ . E is a constant within a range of 10-20. Based on the calculation, the total number of animals that should be used in this study was between 15-25 rats, with each group consisting of 3-5 rats. It was then decided for this study that the number of experimental animals used in each group should be four rats.

### Treatment

No specific treatment was given to the experimental animals, and they were kept up to a specified age. During the treatment, the rats were given ad libitum food and drink, treated in a group cage, and provided with a light-dark cycle. Two rats of group 4 were tested for the blood glucose levels before, after three days, and after one month of streptozotocin and nicotinamide induction. Meanwhile, the rats in group 5 were examined for malondialdehyde (MDA) levels before and after the induction with D-galactose.

### Measurement and Laboratory Analysis

On the predetermined termination day, the rats were anesthetized with ketamine injected intramuscularly. After they were unconscious, 2 ml of blood was taken through the heart. Each blood specimen was inserted into an Eppendorf tube to be centrifuged to obtain blood plasma.

Assessment of blood glucose levels was carried out using the rapid test method, whereas the MDA level was examined at the Food and Nutrition Laboratory of PAU UGM using the Thiobarbituric Acid Reactive Substance (TBARS) assay.

## ELISA

The blood specimens centrifuged at 3000 g for 15 minutes were stored at -80°C before the ELISA test was performed. A microplate was coated with the same amount of serum specimen in each well, and the standard protocol for SIRT1 testing using the FineTest ELISA kit was followed. The results were read on the ELISA reader at a wavelength of 450. Eight different concentrations of purified SIRT (0, 1.562, 3.125, 6.25, 12.5, 25, 50, and 100 ng/ml) were used to prepare the standard curve. The SIRT1 level was calculated using the formula  $y = 0.4492x^2 + 0.4795x + 0.0244$ ,  $R^2 = 0.983$ .

## Statistical analysis

The Kruskal-Wallis test was used to determine the significant differences in mean SIRT1 levels across the groups. The differences in every two groups were examined using the Independent t-test for normally distributed data and the Mann-Whitney U test for the remaining data.

## Ethical approval

The treatment of experimental animals used in this study was carried out by following animal welfare standards. This research protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Islam Indonesia (No. 85/Ka.Kom.Et/70/KE/IV/2018).

## Results

Three days after induction, the blood glucose level showed an increase (pre: 166.25 mg/dl, post: 216.25 mg/dl), indicating that the rats had DM since the blood glucose level exceeded 200mg/dl. The rats also experienced polydipsia, polyphagia, and polyuria. Prior to being terminated in the fourth week, the blood glucose level remained at > 200 mg/dl (310.25 mg/dl), while in group 5, the MDA level increased (pre: 1.08 nmol/ml and post: 9.35 nmol/ml).

The highest body weight at termination was found in the 18-month age group, and the lowest was in the 4-month age group. The rats' body weight decreased at old age (33 months) and after induction with streptozotocin and D-galactose (Table 1). The SIRT1 level was the highest at 18 months old and the lowest among the aging model rats induced by D-galactose (Figure 1).

Table 1: Body weight of the rats at termination.

Group (termination age)	Weight of the rat Mean $\pm$ SD
<b>K1 (4 month)</b>	118 $\pm$ 6.78
<b>K2 (18 months)</b>	158 $\pm$ 9.09
<b>K3 (33 months)</b>	140 $\pm$ 8.16
<b>K4 (5 months)</b>	121 $\pm$ 10.30
<b>K5 (5 months)</b>	120.75 $\pm$ 2.98

Note: Termination age of K1: 4 months, K2: 18 months, K3: 33 months, K4: 5 months, K5: 5 months.

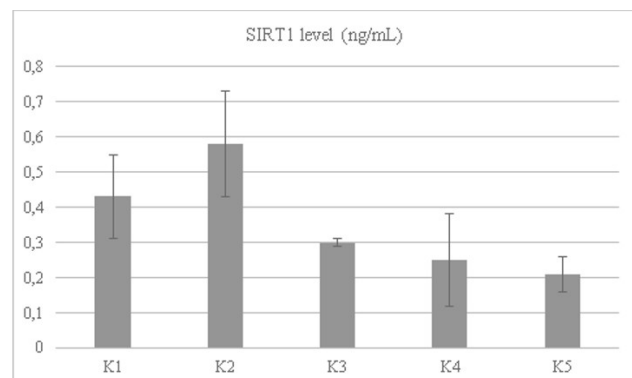


Figure 1: SIRT1 levels in the young age, old age, and aging models ( $p$  value=0.028). Note: Termination age of K1: 4 months, K2: 18 months, K3: 33 months, K4: Induced DM, K5: D-galactose-induced.

The post-hoc test showed that the level of SIRT1 in the D-galactose group was significantly lower compared to the age group of young rats) and even lower (significant) in old-aged rats (33 months). The DM model mice also had low SIRT1 levels and were not different from the D-galactose group or the old aged rat group (Table 2).

Table 2: The differences in SIRT1 levels between old aged, induced DM and D-galactose-induced rats.

Group	p value			
	K2	K3	K4	K5
<b>K1</b>	0.16	0.12	0.25	0.017
<b>K2</b>	-	0.032	0.08	0.004
<b>K3</b>	-	-	0.25	0.017
<b>K4</b>	-	-	-	0.386

Note: K: 4 months, K2: 18 months, K3: 33 months, K4: induced DM, K5: D-galactose-induced.

## Discussion

This study shows that the blood levels of SIRT1 change due to aging. There was a pattern of increasing SIRT1 levels among rats aged 18 months compared to those aged 4 months, whereas a decrease occurred at the age of 33 months. In addition, there were decreased SIRT1 levels in rats with DM (group 4) and at a high level of ROS in the body of rats from group 5. Of the five study groups, the highest SIRT1 levels were found in the 18-month age group, and the differences in SIRT1 levels among the five groups in this study were statistically significant. Significant differences were found among 18-month-old rats compared to the 33-month-old rats and among the high ROS-induced model group (group 5) compared to the 4-month, 18-month, and 33-month rat groups (Table 2). This shows that changes in SIRT1 levels are mainly influenced by the changes in age and ROS levels in the blood. In the DM group, there was a decrease in SIRT1 levels slightly above the D-galactose group, but no significant difference was found.

The decreased SIRT1 levels in this study are in line with the study of Lee and Yang [14], which suggests that decreasing SIRT1 levels can be found after the age of 40 years in women and after 50 years in men. There is no significant difference in SIRT1 levels between men and women. Aging also changes SIRT1 expression in body tissues as proven using immunohistochemistry, which shows that despite an increase in SIRT1 expression in the lungs and skin of 24-month-old rats, such expression decreases in the kidney and spleen [22]. This indicates that changes in the pattern of SIRT1 levels exist not only in the blood (systemic changes) but also in the tissues, and such changes are tissue-specific.

SIRT1 levels are evident in aging rat models. In aged animal models with genetic modification of senescence-accelerated mouse prone (a lifespan of 10-17 months) and senescence-accelerated mouse resistant 1 (a lifespan of 19-21 months), it was found that aging results in decreased SIRT1 levels in the white adipose tissue, brain, liver, and muscles. This is observable at both the transcription level and the translation level. Also, the lowest levels are found in the brain [23]. The results of this study indicate that the lowest decrease in SIRT1 serum levels is found in aging rat models (induction of increased ROS through D-galactose administration), and it is in line with the study of Gong et al. [23].

This study also shows that metabolic conditions also influence the decrease in SIRT1 levels due to a high blood glucose level. This is similar to the study by De Kreutzenberg et al., which found that insulin resist-

ance and metabolic disorders correlate with the low SIRT1 gene expression in peripheral blood mononuclear cells [24]. SIRT1 negatively correlates with the index for subclinical atherosclerosis, as low levels of SIRT1 are pro-atherogenic by nature. Decreased SIRT1 can also be found in pre-diabetic conditions, indicating that SIRT1 acts as a negative metabolic signal transducer. High glucose levels in the body result in low levels of nicotinamide adenine dinucleotide (NAD<sup>+</sup>). Because SIRT1 is an NAD<sup>+</sup>-dependent enzyme, a decline in this compound will result in low SIRT1 activities [24].

Aging will also cause mitochondrial dysfunction in muscle cells, adipocytes, and monocytes/macrophages as one of the pathogenesises of insulin resistance and type 2 DM in aging. Such conditions will result in the activation of monocytes in the circulation, as well as adipocytes and macrophage cells in fat tissues to induce an inflammatory process. Eventually, tumor necrosis factor (TNF)- $\alpha$ , Interleukin-6, and monocyte chemoattractant protein-1 (MCP-1) cytokine release will activate the inhibitor kappaB kinase (IKK) and c-Jun N-terminal kinases (JNK) inflammatory pathways. Existing hyperglycemia, inflammation, and hypercholesterolemia will also result in increased production of ROS by mitochondria [25]. The diabetic rat models seem to be able to model aging because of the low SIRT1 levels.

In the D-galactose group, SIRT1 levels reached the lowest values because of increased ROS levels. SIRT1 levels decrease extremely significantly when exposed to hydrogen peroxidase/H<sub>2</sub>O<sub>2</sub> [26]. In contrast, SIRT1 plays an essential role as a longevity factor and regulates autophagy and ROS production. High levels of ROS will inhibit SIRT1 activities by influencing its cysteine residues. High levels of ROS also increase cell inflammation [27]. On the other hand, overexpression of SIRT1 will result in milder inflammation [18] and inhibit nuclear factor-kappa B (NF $\kappa$ B), one of the pro-inflammatory cytokines [19]. The increase in such cytokines will raise the population of pro-inflammatory/M1 macrophages and oppositely reduce anti-inflammatory/M2 macrophages [25].

SIRT1 assists genetic stability in an oxidative stress condition made possible by SIRT1 deacetylation in the genes that play a role in DNA repair. SIRT1 will also reduce Bcl2 pro-apoptotic protein expression and prevent Caspase-3 breakdown [26]. Such a mechanism will eventually prevent early cell death. SIRT1 overexpression will improve the production of enzymes that play an essential role as antioxidants. One of the mechanisms of aging is increased apoptosis, while the SIRT1 mechanism of action occurs through stress resistance

and prevention of cell apoptosis due to SIRT1 deacetylation against p53, Foxo, and Ku79. The interaction of SIRT1 with Ku79 increases the sequestering of the Bax protein and also improves DNA repair activities [18].

This study has some limitations in that no further examination was conducted on other parameters (including levels of ROS, antioxidants, cholesterol, and others) that can support the mechanism of action of SIRT1 in the body. Therefore, the correlation between these parameters and changes in SIRT1 levels in the blood of aging animals or animals with certain metabolic disorders remains unknown. This study did not observe the pattern of changes in SIRT1 expression in body tissues either. Therefore, further research is expected in order to identify the mechanism of action of SIRT1 in an aging process and metabolic disorders.

## Conclusions

This research has proved that there was a change in the pattern of SIRT1 levels in the blood of aging animal models. The aging model using D-galactose indicated the existence of aging markers in the form of low SIRT1 levels and was proven to be more useful than waiting and caring for rats until old age. The streptozotocin model, although not as good as D-galactose, also showed signs of aging due to lower levels of SIRT1 compared to older rats and indicated no differences compared to the D-galactose rat models. Detection of SIRT1 blood levels can be an alternative to observe metabolic disorder risks, particularly those related to increased ROS levels.

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## Conflict of Interest

The authors declare no conflict of interest.

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