

Aging Parameters of the Accelerated Aging Procedure through D-Galactose Induction

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ABSTRACT

Background and Objectives. Intraperitoneal injection (i.p.) of D-galactose (D-gal) accelerates aging and develops aging models. A low dose of long-term use and a high dose of short-term use of D-gal can induce natural aging in mice, like brain, cardiac, liver, renal, and skin aging, and erectile dysfunction. Our research aims to determine whether a high dose of short-term use of D-gal. i.p. in rats can induce natural aging and affect the following parameters: body weight (BW), Superoxide Dismutase (SOD), Vascular endothelial growth factor (VEGF), C-reactive protein (CRP), and myostatin.

Methods. A daily D-gal i.p. dose of 300 mg/ml/kg for seven days was carried out to induce aging parameters in the rats. After seven days, the body and gastrocnemius circumference of the rats were weighed, and biochemical analysis for SOD, VEGF, CRP, and myostatin in the blood plasma was done.

Results. The data obtained were analyzed using nonparametric statistics Friedman test and Mann-Whitney test. After the seven day-intervention, both the control (NaCl 0.9% i.p.) and the high dose of short-term use of D-gal i.p. groups showed no significant difference in the body weight and gastrocnemius circumference. However, D-gal administration could increase the blood plasma level of SOD, VEGF, CRP, and myostatin.

Conclusion. We conclude that a high dose of short-term intraperitoneal D-galactose can be administrated to induce aging in rat models. The SOD, VEGF, CRP and myostatin can be used as aging parameters.

Keywords: aging, CRP, D-Galactose, myostatin, SOD, VEGF

INTRODUCTION

D-galactose (D-gal) is a monosaccharide in dairy products or fruits and vegetables. As reducing sugar, at low or average concentrations, D-gal is metabolized into glucose but, at high concentrations, can be metabolized into aldoses and hydroperoxides through galactose oxidase. This process results in the formation of superoxide anion and oxygen derivatives as free radicals.¹ Induction of D-gal can be used to lead to natural aging. Therefore, it helps induce accelerated aging in a rat model, either as chronic or acute induction. Both chronic induction (a low dose of long-term use of D-gal)² and acute induction (a high dose of short-term use of D-gal)³ can induce aging in mice, like brain, cardiac, erectile dysfunction, liver, renal, and skin aging.⁴ The pathomechanism of D-gal inducing the aging process is still unclear, particularly when we use high doses of D-gal in a short time. D-gal usage is usually within 4-6 weeks at a 60-150 mg/gram BW dose. Excess intake of D-gal may contribute to the formation of reactive oxygen species (ROS) through the oxidative metabolism of D-gal and glycation end products. Interestingly, the aging rats

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model was possible when chronically injected with D-gal.⁵ However, using small doses of D-gal for a long time often makes it difficult for researchers because it requires a long research time.⁶

Since aging people and age-related illnesses are increasing, research is needed to attenuate age-related diseases. Aging studies need a technically challenging and expensive aging model. In this case, D-gal can accelerate the aging process in various tissues in mouse models and enhance the oxidative changes that occur in the natural aging process in D-gal aging models of skeletal muscle. Evaluating gastrocnemius muscle aging includes measuring protein carbonyl groups, advanced oxidation protein products, lipid hydroperoxides, thiols total, and Cu, Zn-superoxide dismutase activity. A significant increase in oxidative stress markers exists in the D-gal-induced aging of the gastrocnemius muscle model. In the aging process, there is also an increase in the number of dead cells in the cell cycle, called cellular senescence.⁷ Senescent cells are often called the Senescence-Associated Secretory Phenotype, which will induce the production of proinflammatory cytokines (TNF- α , IL-6, and NF- κ B over activation).⁸ The telomere/telomerase system triggers cellular senescence. The trigger of cellular senescence by very short telomeres indicates DNA damage. In addition to increased inflammation, there is an increase in myostatin protein as a marker of aging muscle in the aging process.⁹

The main signs of sarcopenia are muscle mass and strength reduction, often observable in aging. Besides muscle mass, four serum marker parameters are essential in evaluating muscle aging: SOD, VEGF, CRP, and myostatin. Particular biomarkers are involved in the presence of low muscle mass and function in sarcopenia. The involvement of biomarkers is essential in pathogenesis and early detection. CRP level is higher in sarcopenic patients.¹⁰ SOD is an influential antioxidant enzyme for superoxide removal. SOD reduction stimulates superoxide overproduction, delaying skeletal muscle regeneration.¹¹ VEGF insufficiency indicates progressive deterioration of organ or tissue function that is observable in aging. VEGF signaling insufficiency is related to organ aging.¹² Myostatin levels in the blood are related to muscle regeneration. Myostatin plays an active role in the regulation of myogenesis during aging.¹³ The antagonism of myostatin is needed in the alleviation of sarcopenia.¹⁴

This study investigated whether the pathways of muscle aging in male rats can result from administering a high dose of D-galactose in a short-time. The emergence of muscle aging was assessed through the changes in rat body weight and four biochemical markers, namely SOD, VEGF, CRP, and myostatin levels in the blood.

MATERIALS AND METHODS

Animal and Condition

Rattus norvegicus strain Sprague Dawley, male, 16-20 weeks old, 200-300 grams weight, was used in this study. The

room temperature was at 24°C with air conditioning. The rats were obtained from the Drug and Food Supervisory Body (Badan Pengawas Obat dan Makanan, BPOM), Jakarta. The rats were in healthy status. No information is available about genetic modification, genotype. During acclimation, the rat obtained citrafeed (contains 15% protein, 16% crude fiber, 5% fat, 1.35% calcium and 0.7% phosphor) and clean water. After seven-day adaptations, the rat was injected with D-Galactose 300 mg/gram body weight /day.³

D-Galactose solution as much as 300 mg/kg body weight is dissolved (BW) in 1 mL of aquabidest liquid (according to the optimal dose of D-Galactose intraperitoneally in rat 200 gram BW).

1 mL NaCl 0.9% was also injected intraperitoneally (control) which was the same volume with the intervention group. The blood samples were taken seven days after injection. They were obtained from the retroorbital sinus with a capillary tube and collected in sterile tubes. The experiment was conducted in the Integrated Laboratory, Faculty of Medicine and Health Science, Atma Jaya Catholic University Jakarta. Finally, the rats were decapitated by cervical dislocation.

Research Design

The sample size in each group) was calculated using the *Resource Equation Approach* formula, minimum size = $(10/k)+1 = (10/2)+1 = 6$ and maximum size = $(20/k)+1 = (20/2)+1 = 11$. Twenty two Sprague Dawley rats were randomly and equally divided into two groups. The first group, eleven rats, was induced with intraperitoneal (G-ip) D-galactose 300 mg/kg/day for seven days. The second group, the remaining 11 rats, was induced with NaCl 0.9% i.p. (N-ip).³ Each group was measured for body weight (BW) and gastrocnemius muscle circumference at the beginning (day 0) and after seven days (day 7). The first author and the technical assistant controlled the possibility of confounders so that there were no disturbing events during the experiment.

Body Weight and Gastrocnemius Circumference Measurement

Muscle loss progress was observed by measuring the diameter of the thigh. The systemic effects of D-gal administration were seen from the changes in body weight and gastrocnemius circumference, which were calculated on day 0 and day 7.

Measurement of SOD, VEGF, CRP, and Myostatin with ELISA

The blood samples were taken seven days later after injection of high dose short-term intraperitoneal D-Galactose. They were obtained from the retroorbital sinus with a capillary tube and collected separately in sterile tubes. SOD, VEGF, CRP, and myostatin in the blood plasma were analyzed from the beginning (day 0) and after seven days (day 7). The blood samples were allowed to clot, and

centrifuged at 1,200 g for 10 min at 4°C. The obtained serum was analyzed. Serum profiles were measured using ELISA kits according to the manufacturer's protocol. SOD was measured by Rat Super Oxidase Dimutase ELISA, BIOENZY, BZ-08188610-EB. VEGF was measured by Rat Vascular Endothelial Cell Growth Factor ELISA KIT, BIOENZY, BZ-08189560-EB. CRP was measured by Rat C-Reactive Protein ELISA, BIOENZY, BZ-18183500-EB. Myostatin was measured by Mouse Myostatin MSTN ELISA Kit, BZ-08142901-EA, BIOENZY.

Data Analysis

Nonparametric statistics, Friedman test and Mann-Whitney test, were applied to compare the treatments. Body weight, SOD, VEGF, CRP, and myostatin, were analyzed separately. $P < 0.05$ was considered significant. SPSS 2.0 and GraphPad Prism8 were used for the statistical analysis. The statistical distribution of the data was normal, therefore we examined the mean and SD (Standard Deviation).

Ethical Clearance

Ethical approval for this experiment was obtained from Atma Jaya Catholic University, Jakarta, August 21, 2022, No:11/08/KEP-FKIKUAJ/ 2022.

RESULTS

The Effect of D-gal Induction on the Rat Body Weight and Gastrocnemius Circumference

D-gal induction did not affect the body weight and gastrocnemius circumference of the rat (Figure 1). Both the control (NaCl) and D-gal did not show significant differences on the body weight of the rats after Friedman test (Table 1). Further analysis with Mann-Whitney test between the groups at the beginning of the experiment (day 0) and seven days after treatment (day 7) was also insignificant (Table 2). Data on gastrocnemius and foot circumference showed similar patterns of body weight.

Changes in SOD, VEGF, CRP, and Myostatin Levels after Induction of D-galactose

The Friedmann tests showed that both control and intervention groups had an increase in SOD and VEGF (Table 1). The Mann-Whitney tests confirmed the increase of SOD and VEGF due to the D-gal intervention.

The Friedmann tests for the overall data CRP and myostatin showed no significant difference among groups. The groups that received the D-gal intervention on day 0 and day 7 differed significantly, as revealed by the Mann-Whitney tests (Table 2).

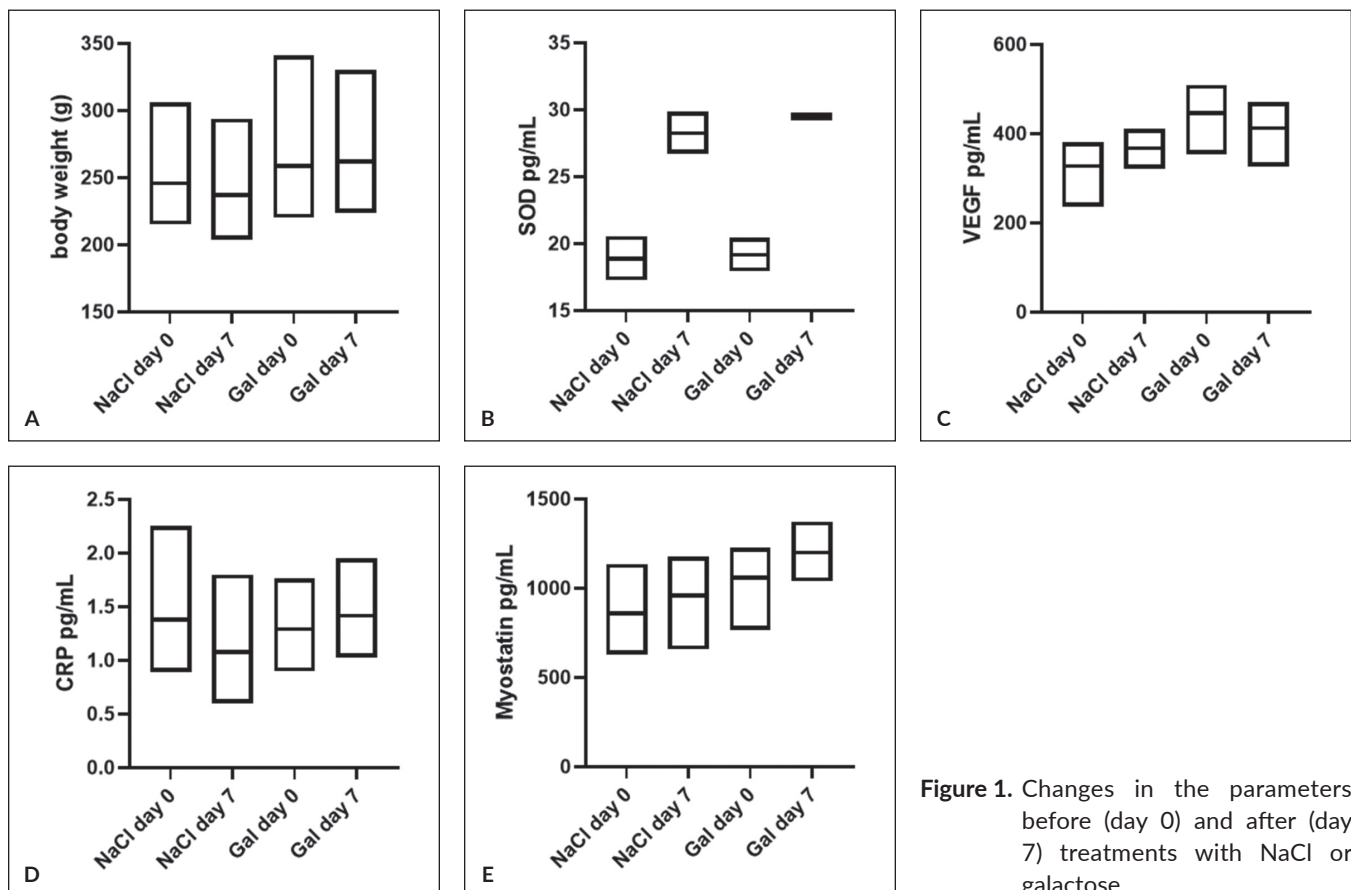


Figure 1. Changes in the parameters before (day 0) and after (day 7) treatments with NaCl or galactose.

Table 1. Results of Friedman Test

	Parameter				
	Body weight	SOD	VEGF	CRP	Myostatin
<i>P value</i>	0.3069	0.0014	0.0055	0.6522	0.0666
<i>Exact or approximate P value</i>	Approximate	Exact	Exact	Exact	Exact
<i>Are means significantly different? (P<0.05)</i>	No	Yes	Yes	No	No
<i>Friedman statistics</i>	3.609	12.120	10.680	2.040	7.080
<i>Number of treatments</i>	4	4	4	4	4
<i>Number of subjects</i>	7	5	5	5	5

Table 2. Results of Mann-Whitney Test

Parameter	Treatment	<i>P value</i>	Significantly different (<i>P</i> <0.05)
<i>Body weight</i>	NaCl day 0 VS day 7	0.4347	No
	Gal day 0 VS day 7	0.8275	No
<i>SOD</i>	NaCl day 0 Vs day 7	0.0079	Yes
	Gal day 0 VS day 7	0.0079	Yes
<i>VEGF</i>	NaCl day 0 VS day 7	0.3095	No
	Gal day 0 VS day 7	0.0079	Yes
<i>CRP</i>	NaCl day 0 VS day 7	0.5476	No
	Gal day 0 VS day 7	0.0079	Yes
<i>Myostatin</i>	NaCl day 0 VS day 7	0.4206	No
	Gal day 0 VS day 7	0.0079	Yes

DISCUSSION

In this study, D-gal induction did not change the body weight significantly. This finding was in accordance with the study of Haider et al. that reported body weight was not affected after administering a high dose D-gal.³ However, Fatemi et al. reported that D-gal-induced mice have a weight loss effect.¹⁴ After administration of large doses of D-gal, weight loss happens due to increased hydration (increased drinking due to increased thirst), leading to weight loss due to decreased food intake and loss of fat through increased lipolysis.^{14,15} According to Thornton et al., high doses of D-gal can cause oxidative damage to various tissues and organs because systemic D-gal exposure accelerates the biochemical and morphological processes of aging, including the central nervous system. Damage to the central nervous system will affect the central or peripheral renin-angiotensin. This situation will increase drinking response and weight loss. This situation is related to chronic hypohydration (extracellular dehydration) and increased levels of the hormone angiotensin II.¹⁶

In this study, D-gal intervention may increase ROS which will trigger the increase of SOD activity. This study showed that the control group that received NaCl 0.9% i.p. showed a significant increase in SOD (13.41+4.49 pg/mL) compared to the group that received D-gal (10.22+1.61 pg/mL). Subacute administration of D-gal significantly altered the antioxidant enzyme activities. This result is in accordance with the report of Hadier et al. that after D-gal

administration, the SOD was increased significantly.³ D-gal can cause mutations in mitochondrial DNA, decline enzymes for DNA repair, and result in mitochondrial disturbances that trigger aging. Exposure to D-gal also causes a decrease in antioxidant enzymes such as glutathione, catalase, SOD which can increase oxidative stress. Galactose oxidase oxidizes D-gal to hydrogen peroxide (H₂O₂), which causes a decrease in superoxide dismutase (SOD), while H₂O₂ reacts with reduced iron and forms hydroxide ions (OH⁻)/free radicals. Oxidative stress will reduce ATP synthesis, which can damage mitochondrial membranes, damage mitochondrial structures, and ultimately induce apoptosis.^{17,18} This study shows that NaCl 0.9% i.p. can function as an antioxidant to reduce the effects of free radicals caused by exposure to D-galactose i.p. The NaCl substance also functions as an antioxidant as a superoxide anion scavenging. Another effect of NaCl is the catalysis of fat oxidation (lipid oxidation).^{19,20}

D-gal-induced rat group showed a decreased VEGF level after seven days of intervention (Mann-Whitney test). This result followed the report of Sun et al. that the expression of VEGF in the D-gal model group was significantly decreased.⁴ The decrease in VEGF level is associated with the decrease of angiogenesis during sarcopenia induced by D-gal. VEGF insufficiency causes deterioration of tissue function due to the increase of decoy receptors.¹² Therefore, using the D-gal short-term rat model may help further research on VEGF signaling insufficiency, preventing age-associated capillary loss, improving organ perfusion and function, and extending life span. Healthier aging is evidenced by good metabolism and body composition, and amelioration of aging-associated pathologies, including hepatic steatosis, sarcopenia, osteoporosis, “inflammaging” (age-related multiorgan chronic inflammation), and increased tumor burden.

CRP is an inflammatory biomarker. This study showed that D-gal induction could increase CRP levels in the blood (Mann-Whitney test). This CRP increase shows the increase of inflammation after D-gal induction. This result follows Hadzi-Petrushev et al., that rats given a D-gal solution of 100 mg/kg orally for 42 days increased the 8-iso-PGF(2 α), IL-6, TNF- α , which is a sign of increased inflammation.¹⁹ Inflammatory markers with higher circulating enzymes, such as IL-6, TNF- α , and CRP, are significantly positively correlated with skeletal muscle strength and decreased muscle

mass in sarcopenia.²¹ In line with Azman and Zakaris's research²², administration of 100 mg/kg/day D-gal and 150 mg/kg/day D-gal both increased the IL-6 levels, with 150 mg/kg/day D-gal administration having even higher IL-6 levels.²³

This study showed that the group that received D-gal had a significant increase in myostatin (88.8 ± 18.7 pg/mL) (Mann-Whitney test) compared to the group that received NaCl 0.9% (-121.3 ± 34.6 pg/mL). Myostatin is a member of transforming growth factor- β (TGF- β) that functions as an autocrine inhibitor of skeletal muscle growth and inducer of muscle fiber atrophy by activating Smad2 and Smad3, and suppressing protein synthesis by inhibiting protein kinase B. Protein kinase B is one of the key proteins in the Akt signaling pathway or PI3K-Akt signaling pathway as a signal transduction pathway that promotes survival and growth in response to extracellular signals.²⁴ Increased myostatin expression is central to integrating and balancing anabolic and catabolic responses. Myostatin can negatively regulate the Akt signaling pathway, which promotes protein synthesis, and increases the activity of the ubiquitin-proteasome system in inducing atrophy. In addition, myostatin can modulate major catabolic pathways, including the ubiquitin-proteasome and autophagy-lysosome systems. Thus, the myostatin pathway inhibits muscle growth through cross-regulation between myostatin, growth-promoting pathways, and proteolytic systems; muscle hypertrophy; and regulation of translation.^{25,26} The increase in myostatin protein due to D-gal administration will cause a risk of muscle atrophy. Even though in this study, there has not been a change in the diameter of the gastrocnemius muscle.²⁷

CONCLUSION

Giving D-galactose at high doses in a short time on rats can induce natural aging. The presence of these parameters – SOD, VEGF, CRP, and myostatin, confirms this aging. This short-term high-dose D-gal-induced aging rat model with its parameters is the novelty of this study. Regular and quantitative injection of D-gal solution into rats can produce symptoms of natural aging models that are used to screen antiaging drugs and their pharmacological activities, like sarcopenia. The methods of building and evaluating the aging models are provided. The outcome of this study can facilitate subsequent researches and experiments on the mechanism of aging.

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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