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The effect of D-galactose on inflammaging markers, appendicular muscle mass, fat-to-muscle ratio, and physical endurance to accelerate aging in mice

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ABSTRACT: D-galactose-induced aging is an effective accelerated aging method, but the doses used in various reports have been shown to vary. Therefore, this study aimed to determine the effect of varying doses of D-galactose on inflammaging markers (MDA, TNF-α, IL-6, and TGF-β), appendicular muscle mass, fat-to-muscle ratio (FMR), and physical endurance in mice. The sample population comprised 21 male mice aged 8 weeks (20–30 grams), which were divided into 3 groups (7 rats each). The control group (C) was monitored for 8 weeks, the T1 group received D-galactose at 100 mg/kg/day, and the T2 group was given D-galactose at 150 mg/kg/day for 8 weeks. Measurements of MDA, TNF-α, IL-6, TGF-β levels, appendicular muscle mass, FMR, and physical endurance were carried out at the end of week 8. The results showed that each treatment group showed a significant difference in MDA, TNF-α, IL-6, and TGF-β levels (p-value <0.05). In terms of appendicular muscle mass and FMR, C and T1 as well as T1 and T2 did not show a significant difference (p value> 0.05), while C and T2 had a significant variation (p-value <0.05). Based on the findings, there were significant differences in physical endurance between C and T1 as well as C and T2 (p value <0.05), while T1 and T2 did not show a significant variation (p value> 0.05). In addition, D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day could increase the inflammaging markers significantly. A dose of 150 mg/kgBW/day was reported to have the potential to decrease appendicular muscle mass and FMR significantly compared to 100 mg/kgBW/day. The results also revealed that doses of 100 and 150 mg/kgBW/day had equally significant reductions in physical endurance.

KEYWORDS: Aging; D-galactose; Frailty; Inflammaging.

1. INTRODUCTION

According to recent reports, 1 in 6 individuals worldwide is projected to reach the age of 60 years or older by 2030. This indicates that approximately 1.4 billion individuals are expected to reach this age, representing a significant increase from 1 billion recorded in 2020. In addition, the number is expected to experience a 2-fold increase to approximately 2.1 billion by 2050 [1]. Despite the demographic shift, the advancement in age for a significant proportion of the elderly has not been matched by successful aging outcomes. Various health problems have been reported to be associated with aging, leading to geriatric syndromes, with frailty being the most significant. Frailty significantly decreases quality of life, increases the incidence of hospitalization, and elevates health costs. At present, a comprehensive understanding of its pathogenesis remains unavailable due to various factors. Current hypotheses propose a complex interplay of organ, cellular, and molecular-level dysfunction. Although it is natural for physiological body reserves to decrease with age, frail individuals typically experience a hastened decline, leading to a breakdown in the body's compensatory mechanisms for maintaining homeostasis [2].

Frailty serves as a descriptor of biological aging in the elderly, characterized by a decrease in physiological reserves across multiple organs. This condition is typically driven by processes at the cellular and molecular levels. The chronic inflammatory process in the elderly, known as inflammaging, is one essential factor underlying aging at the molecular level, particularly in relation to several markers associated

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with oxidative stress, such as TNFα, IL-6, TGF-β, CRP, and MDA [3]. In the context of frailty, an increase in the proinflammatory cytokine TNF-α (Tumor Necrosis Factor-α) is often observed. In addition, IL-6 (interleukin 6) and CRP (C-Reactive Protein) are implicated in chronic disease pathogenesis and serve as biomarkers for predicting the risk of morbidity and mortality among the elderly. An increase in proinflammatory cytokines is a characteristic condition of aging, known as inflammaging [4]. In line with previous reports, sarcopenia (decreasing muscle mass) and frailty represent significant health concerns commonly affecting the elderly. These 2 conditions frequently overlap due to the frailty phenotype (decreased muscle mass and hand grip strength) that characterizes sarcopenia [4-6].

In response to these conditions, the chronic systemic infusion of D-galactose has been used in animal models to artificially induce brain aging, offering valuable insights for anti-aging therapeutic interventions [7]. This method has proven instrumental in comprehending the aging process and mitigating its effects on various organs. In addition, D-galactose, an aldohexose naturally produced by the body and present in the brain, plays an essential role in the mechanism of this method [8]. However, administration of exogenous D-galactose at levels surpassing normal concentrations can accelerate oxidative stress, apoptosis, and inflammation, leading to aging-related consequences across multiple organs [9–11]. In response, several studies have explored the use of varying dosages to determine the optimum concentration. When administered to laboratory animals, D-galactose causes accelerated brain aging, which is similar to the natural aging process [12]. This compound is often given intraperitoneally for 6 to 8 weeks to induce oxidative stress and decrease antioxidant expression, which in turn causes aging [13–16]. In some experiments, its induction did not cause significant aging effects. Therefore, this study aimed to examine the effect of D-galactose on appendicular muscle mass, fat-to-muscle mass ratio, physical endurance, and inflammaging markers (MDA, TNF-α, IL-6, and TGF-β) in mice.

2. RESULTS

2.1 Comparison of MDA levels based on D-galactose treatment groups

Based on Table 1 and Figure 1, 150 mg/kgBW/day D-galactose (11.98 ± 0.40) caused the highest increase in MDA levels compared to a dose of 100 mg/kgBW/day (9.79 ± 0.30). In addition, the levels obtained were higher compared to the controls (1.33 ± 0.18).

The results of the Kruskal-Wallis test showed a value of p = <0.001, indicating that the 3 treatment groups demonstrated significant differences. The post hoc Dunn test revealed that each treatment group had better outcomes compared to the controls, with the 100 and 150-dose groups showing a significant difference with a p-value <0.05. Based on these findings, administering D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day was effective in increasing MDA levels, with a dose of 150 mg/kgBW/day being the most effective.

Table 1. Differences in MDA Based on Treatment Groups Given D-galactose

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
MDA (nmol/ml)	1.33 ±0.18[a]	9.79 ±0.30[b]	11.98 ±0.40[c]	<0.001*

Note: Data do not meet normality assumptions; Kruskal Wallis test and post hoc Dunn test (a; b; c = different letters indicate significant differences at p<0.05); *significant at p<0.05.

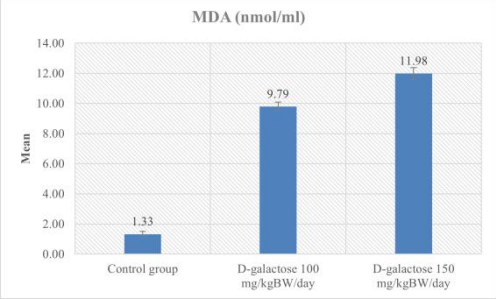

Figure 1. Bar chart comparison of MDA levels based on d-galactose treatment groups

2.2 Comparison of TNF-α levels based on D-galactose treatment groups

Table 2 and Figure 2 showed that D-galactose 150 mg/kgBW/day (18.80 ± 0.43) caused the highest increase in TNF-α levels compared to a dose of 100 mg/kgBW/day (13.33 ± 0.33). The results showed that the 2 groups had higher levels of TNF-α compared to the controls (6.57 ± 0.32).

The ANOVA test results obtained a p-value <0.001, indicating that the 3 treatment groups had significant differences. In addition, the LSD post hoc test showed that each treatment group had better outcomes than the controls, with the 100 and 150-dose groups showing significant differences with a p-value of <0.05. Based on these findings, administering D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day was effective in increasing TNF-α levels, with a dose of 150 mg/kgBW/day being the most effective.

Table 2. Differences in TNF-α Based on D-galactose Treatment Groups

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
TNF-α (pg/ml)	6.57 ±0.32[a]	13.33 ±0.33[b]	18.80 ±0.43[c]	<0.001*

Note: The data meets the assumptions of normality and homogeneity; ANOVA and Post hoc LSD tests (a; b; c = different letters indicate significant differences); *significant at p<0.05.

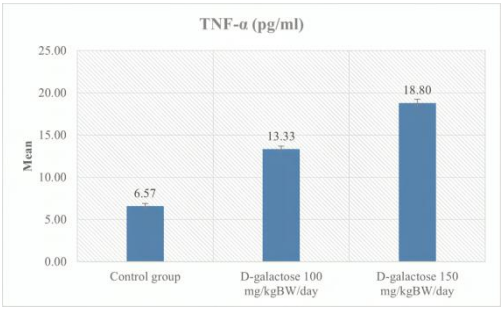

Figure 2. Bar chart comparison of TNF-α levels based on D-galactose treatment groups

2.3 Comparison of IL-6 levels based on D-galactose treatment groups

Based on Table 3 and Figure 3, D-galactose 150 mg/kgBW/day (133.07 ±2.99) gave the highest increase in IL-6 levels compared to a dose of 100 mg/kgBW/day (117.19 ±2.78). The results showed that both groups had higher IL-6 levels compared to the controls (68.48 ± 2.61).

The ANOVA test results obtained a value of p=<0.001, indicating that the 3 treatment groups had significant differences. Furthermore, the LSD post hoc test showed that each treatment group was better compared to the controls, with the 100 and 150-dose groups showing significant differences with a p-value of <0.05. Based on these findings, administering D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day was effective in increasing IL-6 levels, with a dose of 150 mg/kgBW/day being the most effective.

Table 3. Differences in IL-6 Based on D-galactose Treatment Groups

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
IL-6 (pg/ml)	68.48 ±2.61 [a]	117.19 ±2.78 [b]	133.07 ±2.99 [c]	<0.001*

Note: The data meets the assumptions of normality and homogeneity; ANOVA and Post hoc LSD tests (a; b; c = different letters indicate significant differences); *significant at p<0.05.

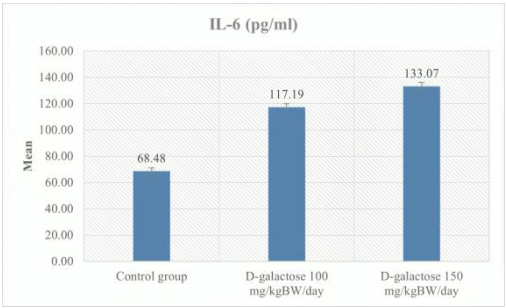

Figure 3. Bar chart comparison of IL-6 levels based on D-galactose treatment groups

2.4 Comparison of TGF-β levels based on D-galactose treatment groups

Table 4 and Figure 4 showed that D-galactose 150 mg/kgBW/day (18.96 ±1.26) caused the highest increase in TGF-β levels compared to a dose of 100 mg/kgBW/day (13.70 ±1.14). The findings showed that both groups had higher TGF-β levels compared to the controls (7.60 ± 0.94).

The ANOVA test results obtained a value of p=<0.001, indicating that the 3 treatment groups showed significant differences. In addition, the LSD post hoc test showed that each treatment group had better outcomes compared to the controls, with the 100 and 150-dose groups showing significant differences with a p-value <0.05. Based on these findings, administering D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day could increase TGF-β levels, with a dose of 150 mg/kgBW/day being the most effective.

Table 4. Differences in TGF-β Based on D-galactose Treatment Groups

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
TGF-β (pg/ml)	7.60 ±0.94 [a]	13.70 ±1.14 [b]	18.96 ±1.26 [c]	<0.001*

Note: The data meets the assumptions of normality and homogeneity; ANOVA and Post hoc LSD tests (a; b; c = different letters indicate significant differences); *significant at p<0.05.

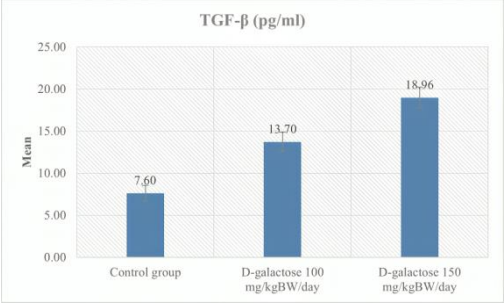

Figure 4. Bar chart comparison of TGF-β levels based on D-galactose treatment groups

2.5 Comparison of appendicular muscle mass based on D-galactose treatment groups

Based on Table 5 and Figure 5, D-galactose 150 mg/kgBW/day (0.45 ± 0.03) caused the highest decrease in appendicular muscle mass compared to a dose of 100 mg/kgBW/day (0.49 ± 0.09). The results showed that both groups had lower appendicular muscle mass compared to the controls (0.65 ± 0.15).

The Brown-Forsythe test results obtained a value of p = 0.009, showing that the 3 treatment groups showed significant differences. The Dunnet T3 post hoc test showed that the 3 groups did not show a significant difference with a p-value of > 0.05, while the control group with the 150 dose group showed a significant difference with a p value of <0.05. Therefore, the effective and significant treatment for reducing appendicular muscle mass was D-galactose 150 mg/kgBW/day.

Table 5. Differences in Appendicular Muscle Mass Based on D-galactose Treatment Groups

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
Appendicular muscle mass (gr)	0.65 ±0.15 [a]	0.49 ±0.09 [ab]	0.45 ±0.03 [b]	0.009

Note: The data meets the normality assumption but does not meet the homogeneity assumption; Brown Forsythe test and Post hoc Dunnet T3 (a; b; c = different letters indicate significant differences); *significant at p<0.05.

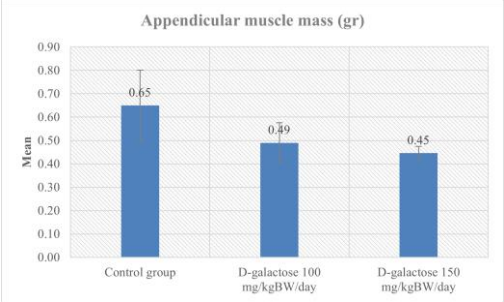

Figure 5. Bar chart comparison of appendicular muscle mass based on D-galactose treatment groups

2.6 Comparison of FMR based on D-galactose treatment groups

According to Table 6 and Figure 6, D-galactose 150 mg/kgBW/day (102.43 ±15.50) caused the highest decrease in FMR compared to a dose of 100 mg/kgBW/day (116.43 ±49.35). The 2 groups were reported to have lower FMR compared to the controls (168.57 ±51.90).

The Brown-Forsythe test results obtained a p-value = 0.028, indicating that the 3 treatment groups showed significant differences. The Dunnet T3 post hoc test showed that the control group and D-galactose 100 group as well as D-galactose 100 group with the D-galactose 150 group did not show a significant difference with a p-value of > 0.05. Meanwhile, the control group with the D-galactose 150 group showed a significant difference with a p-value of <0.05. Based on these findings, the effective and significant treatment in reducing FMR was D-galactose 150 mg/kg BW/day.

Table 6. Differences in FMR Based on Treatment Groups Given D-galactose

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
FMR	168.57 ±51.90 [a]	116.43 ±49.35 [ab]	102.43 ±15.50 [b]	0.028

Note: The data meets the normality assumption but does not meet the homogeneity assumption; Brown Forsythe test and Post hoc Dunnet T3 (a; b; c = different letters indicate significant differences); *significant at p<0.05.

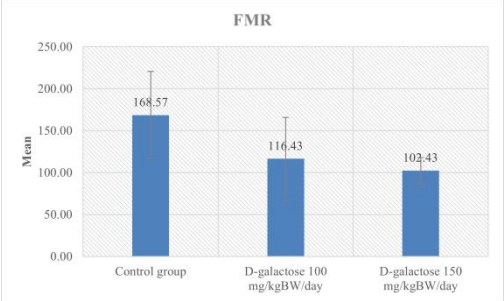

Figure 6. Bar chart comparison of FMR based on D-galactose treatment group

2.7 Comparison of physical endurance based on D-galactose treatment groups

Based on Table 7 and Figure 7, D-galactose 150 mg/kgBW/day (3.29 ± 0.76) caused the highest decrease in physical endurance compared to a dose of 100 mg/kgBW/day (4.43 ± 1.13). The results showed that both groups had lower physical endurance compared to the controls (16.14 ± 9.60).

The Brown-Forsythe test results obtained a value of p-value = 0.008, indicating that the 3 treatment groups showed significant differences. The Dunnet T3 post hoc test showed that the control group with the D-galactose 100 group and the control group with the D-galactose 150 group showed significant differences with a p-value <0.05. Meanwhile, the D-galactose 100 group and the D-galactose 150 group did not show a significant difference with a p-value of > 0.05. Based on these findings, the D-galactose 100 and 150 groups were equally effective or not significantly different in reducing physical endurance.

Table 7. Differences in Physical Endurance Based on Treatment Groups Giving D-galactose

Variable	Control group	D-galactose 100 mg/kgBW/day	D-galactose 150 mg/kgBW/day	p-value
Physical endurance (minutes)	16.14 ±9.60 [a]	4.43 ±1.13 [b]	3.29 ±0.76 [b]	0.008

Note: The data meets the normality assumption but does not meet the homogeneity assumption; Brown Forsythe test and Post hoc Dunnet T3 (a; b; c = different letters indicate significant differences); *significant at p<0.05.

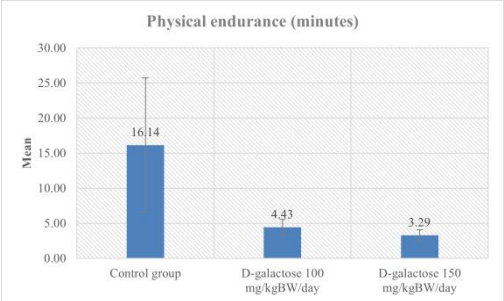

Figure 7. Bar chart comparison of physical endurance based on D-galactose treatment groups

3. DISCUSSION

Several studies had shown the occurrence of an inflammatory process throughout aging. Low-grade, persistent, systemic inflammation that occurred naturally with age was referred to as "inflammaging." This condition was primarily caused by tissue damage that was not fully repaired during inflammation, leading to the accumulation of macromolecular damage and cellular waste. In addition, a major factor in the initiation of the aging process was the release of proinflammatory cytokines from cells (senescence) that accumulated in different aging organs and were referred to as the "senescence-associated secretory phenotype" (SASP) [17].

An increase in MDA, TNF-, TGF-, and IL-6 levels was a sign of the inflammatory process in this study. During the aging process, there was an excessive accumulation of ROS due to the activated NADPH oxidase enzyme. The presence of these free radicals activated the TNF-α signaling pathway, causing inflammaging and the production of inflammatory cytokines, such as TNF-α, TGF-β, and IL-6. Among these cytokines, CRP, IL-6, and TNF-α were recognized as markers of the aging process [10].

Administration of D-galactose could induce oxidative stress and mitochondrial dysfunction in the brain. A previous study used D-galactose at a dose of 100-500 mg/kg/day for 6-8 weeks. The results in experimental animals showed an increase in inflammatory markers, such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), nitric oxide synthase 2 (NOS-2), IL-1β, IL-6, TNF-α, nuclear factor kappa B (NF-κB), p-NF-κBp65, pIκKα, pIκKβ, and thioredoxin-interacting protein (Txnip). The intake of D-galactose caused an increase in cardiac inflammatory markers, including tumor necrosis factor receptor (TNF-R), TNF-α, pNFκB, and COX-2. In addition, the expression of Ataxia Telangiectasia Mutated (ATM), Rad3-related protein (ATR), and Checkpoint kinase 1 (Chk1), an indicator of DNA damage, was also increased in cardiac tissue in D-galactose-treated animals. The dose used in this current study was 60-150 mg/kg/day intraperitoneally for 6-8 weeks [14].

Compared to mice without D-galactose, a significant increase in MDA, TNF-α, TGF-β, and IL-6 levels following D-galactose treatment at doses of 100 mg/kg/day and 150 mg/kg/day was observed. Increasing IL-6 levels could be achieved by administering D-galactose 100 mg/kgBW/day and 150 mg/kgBW/day, with a dose of 150 mg/kgBW/day being the most effective.

Previous studies had shown that administering D-galactose to cells also led to apoptosis, impaired autophagy, skeletal muscle atrophy (a big drop in muscle mass compared to body mass), and skeletal muscle fiber diameter. The dosage used varied between 50 and 400 mg/kg/day, with a treatment duration of 6–8 weeks [14].

Body fat tissue also underwent redistribution with increasing age, leading to an increase in body fat (specifically abdominal fat) and a decrease in appendicular fat (specifically subcutaneous fat). In addition, the accumulation of fat infiltration in organs, such as the liver, heart, bone marrow, and muscles also increased in the elderly, while subcutaneous fat tended to decrease. This phenomenon contributed to a condition known as age-related lipodystrophy [18, 19].

Previous studies had shown that the administration of D-galactose increased the expression of oxidative stress and decreased the expression of antioxidants. The presence of these free radicals activated the NF-κB signaling pathway, causing inflammation and the production of inflammatory cytokines, such as TNF-α, TGF-β, and IL-6. TNF-α, through activation of the caspase 9 pathway. Endothelial dysfunction and caspase 8 typically caused myocardial necrosis, impaired heart function, and contributed to frailty. TNF-α interacting with hormonal conditions caused a decrease in activity and change in muscle mass, which occurred simultaneously with the loss of muscle mass, then activated caspase-8 and TNFR1. This situation led to the activation of caspase-3, thereby causing apoptosis, which reduced muscle mass and contributed to frailty. TGF-β caused a decrease in heart muscle collagen, leading to myocardial fibrosis, impaired heart function, and frailty. IL-6 typically induced the activation of the MAPK and caspase-8 pathways, causing a decrease in muscle mass and muscle function, which led to frailty. D-galactose also led to apoptosis, impaired autophagy, skeletal muscle atrophy (a big drop in muscle mass compared to body mass), and skeletal muscle fiber diameter. The dosage of D-galactose administered varied between 50 and 400 mg/kg/day, with a study duration of 6–8 weeks [14].

In this study, administration of D-galactose at the dosage of 100 mg/kg BW/day did not result in a significant reduction in appendicular muscle mass or FMR. However, when given at 150 mg/kg BW/day, it showed a significant decrease in appendicular muscle mass and FMR. Administering 100 mg/kgBW/day and 150 mg/kgBW/day was both effective and significant in reducing physical endurance. This could indicate that muscle dysfunction preceded the emergence of macro abnormalities, as indicated by a decrease

in muscle mass. Administration of D-galactose 100 mg/kgBW/day could cause a decrease in muscle function, leading to a significant decrease in physical endurance.

This study still had several limitations, namely:

a) The analysis did not compare the aging process accelerated by induction with D-galactose with the natural aging process at the cellular level through tissue histology.
b) The only inflammaging marker measured in this study was TNF-α.
c) The effect of D-galactose administration on cognitive function that contributes to frailty was not measured.

Based on these findings, further studies were needed to assess organ histopathology and cognitive function with the D-galactose effective dose.

4. CONCLUSION

In conclusion, D-galactose at doses of 100 mg/kgBW/day and 150 mg/kgBW/day could significantly increase inflammaging markers. In addition, administration of D-galactose at 150 mg/kgBW/day reduced the appendicular muscle mass and FMR significantly. The results showed that the 100 and 150-dose groups exhibited equally significant reductions in physical endurance.

5. MATERIALS AND METHODS

5.1 Ethical approval

This experimental study was carried out under a protocol approved by the Health Study Ethics Committee of Dr. Moewardi General Hospital with number 625/V/HREC/2021.

5.2 Sample and Study Design

The study procedures were carried out using a post-test-only control group design. The sample population comprised 21 male mice of the sub-species Mus musculus Balb/C strain aged 8 weeks with a body weight ranging from 20–30 grams, which were equally divided into 3 groups of 7 mice each. These included 1) control group (C), which was observed for 8 weeks, 2) treatment group 1 (T1) given D-galactose intraperitoneally at a dose of 100 mg/kg/day for 8 weeks, and 3) treatment group 2 (T2) given D-galactose intraperitoneally at a dose of 150 mg/kg/day for 8 weeks. Examinations of MDA, TNF-α, IL-6, and TGF-β levels, appendicular muscle mass, fat-to-muscle mass ratio (FMR), and physical endurance were conducted at the end of the 8th week.

The mice were housed in 4 cages constructed from plastic tubs covered with wire lids. The environmental conditions during acclimatization and treatment were tightly controlled within a fixed specific range, namely a room that had 12 hours of light and 12 hours of darkness with a room temperature ranging from 23°C–26°C. This was carried out to ensure that the test animals could adapt according to the animals' biological time and the conditions to be occupied during the experiment. Temperature, water supply, the number of rats in the cage, and the change of husks were the same for all groups. The animals were kept in cages with a size of 28 x 30 x 12 cm to ensure free movement and prevent stress.

5.3 MDA, TNF-α, TGF-β, and IL-6 levels examination

Blood samples for MDA, TNF-α, IL-6, and TGF-β examinations were obtained from the mice through the orbital vein. The immunoassay method was used to analyze the levels of MDA, TNF-α, IL-6, and TGF-β.

5.4 Appendicular muscle mass

At the end of the 8th week, the mice were sacrificed, and the appendicular muscle mass was taken and weighed. Appendicular muscle mass was the muscle mass attached to the arms and legs of the animals, which was measured using a digital scale.

5.5 Fat to muscle mass ratio (FMR) examination

After the 8th week, the mice were sacrificed, and their fat and muscle mass were taken and weighed using a digital scale.

5.6 Physical Endurance Examination

At the end of the 8th week, the physical endurance of the mice was assessed using their swimming endurance. The instruments used for this evaluation included a stopwatch, a hair dryer, a towel, and a water tank that measured 50 cm long, 25 cm high, and 30 cm in breadth. The animals were swum until there were

signs of exhaustion, such as a bent body posture, a stretched tail, and a head submerged in the water for 4 to 7 seconds without any response from any of the 4 legs. Subsequently, each mice's tiredness duration was recorded, followed by hoisting and drying with a towel and hairdryer.

5.7 Statistic analysis

The data were assessed for normality using the Shapiro-Wilk test and for homogeneity using the Levene test. Statistical analysis comprised employing the ANOVA test, followed by post hoc LSD when the data met both normality and homogeneity assumptions. When the data was normally distributed but not homogeneous, the Brown Forsythe test was utilized, followed by the post hoc Dunnett T3. In cases where the data did not meet the assumption of normality, analysis was conducted using the Kruskal-Wallis test, followed by the post hoc Dunn test.

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Conflict of interest statement: There were no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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