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The effect of arrowroot diet on pancreatic and kidney histomorphology of mice induced by D-Galactose

Pengaruh diet umbi garut terhadap gambaran histomorfologi pankreas dan ginjal pada mencit yang diinduksi penuaan menggunakan D-Galaktosa

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Abstract

Arrowroot contains antioxidants that can potentially reduce d-galactose-induced aging of the pancreas and kidneys. This study aimed to determine the effect of arrowroot administration on the histomorphology of the pancreas and kidneys of d-galactose-induced diabetic mice. Experimental research that was conducted in 2023 using Musculus Balb/C mice was divided into four groups as follows: a healthy control group (HC), negative control (NC) with d-galactose induction and standard feed, P1 and P2 groups with d-galactose induction, and 45% and 60% arrowroot feed. Histological slides were stained with hematoxylin and eosin. Data analysis was performed under a microscope at a magnification of 40 × 10. Severe signs of aging in the pancreas (cell atrophy, cytoplasmic vacuolation, necrosis, congestion, and inflammation) were observed in the NC and P1 groups and minimal signs were observed in the P2 group compared to the HC group, The damage mean (%) of tubular, glomerular kidney and inflammation scores were 52,46 ± 1,46, 76,66 ± 9,87, 67,99 ± 2,12, and 60,93 ± 0,55 (p=0,005); 62,64 ± 11,13, 70,48 ± 13,32, 71,25 ± 8,04, and 65,53 ±14,26, (p=0,707); 8,75 ± 2,5, 27,25 ± 4,57, 8 ± 7,25, and 13,5 ± 6,19, (p=0,001), respectively. In conclusion, administration of arrowroot affects the histomorphological features of the pancreas and kidneys of mice induced by aging using d-galactose.

Keywords: Aging, arrowroot, D-galactose, histomorphology, pancreas, kidney

Abstrak

Umbi garut memiliki kandungan antioksidan yang berpotensi mengurangi terjadinya penuaan pada pankreas dan ginjal yang diinduksi d-galaktosa. Penelitian ini betujuan untuk mengetahui pengaruh pemberian umbi garut terhadap gambaran histomorfologi pankreas mencit yang diinduksi d-galaktosa. Penelitian eksperimen yang dilakukan pada 2023 menggunakan mencit Musculus Balb/C yang dibagi menjadi 4 kelompok yaitu: kontrol sehat (HC), kontrol negatif (NC) yang diinduksi d-galaktosa & diberi pakan standar, kelompok P1 dan P2 yang diinduksi d-galaktosa dan diberi pakan garut 45% dan 60%. Preparat histologi dibuat menggunakan pengecatan Hematoksilin-Eosin (HE). Analisis dengan pengamatan dibawah mikroskop perbesaran 40x10 **.** Tanda penuaan berat (atrofi sel, vakuolasi sitoplasma, nekrosis, kongesti, dan inflamasi) terlihat lebih banyak pada kelompok KN dan P1 dan minimal pada kelompok P2 dibandingkan kelompok HC. Rerata skor kerusakan (%) dan inflamasi tubulus dan glomerulus ginjal berturut-turut adalah 52,46 ± 1,46, 76,66 ± 9,87, 67,99 ± 2,12, dan 60,93 ± 0,55 (p=0,005); 62,64 ± 11,13, 70,48 ± 13,32, 71,25 ± 8,04, dan 65,53 ±14,26, (p=0,707); 8,75 ± 2,5, 27,25 ± 4,57, 8 ± 7,25, dan 13,5 ± 6,19, (p=0,001). Kesimpulan, pemberian umbi garut mempengaruhi gambaran histomorfologi pankreas dan ginjal mencit yang diinduksi penuaan menggunakan D-galaktosa.

Kata Kunci: D-galaktosa, ginjal, histomorfologi, pankreas, penuaan, umbi garut

Introduction

Aging is characterized by progressive loss of physiological integrity, leading to functional failure and an increased risk of death. During aging, biological processes result in reduced recovery capacity and anatomical changes in body components (Pyo et al., 2020). One of the main mechanisms that causes pathological aging is oxidative stress, which is characterized by the formation of cytotoxic reactive oxygen species (ROS). Oxidative stress can attack many cell types, including pancreatic and kidney cells (Maldonado et al., 2023; Tyagita et al., 2021).

Defects in pancreatic organs can cause damage to the pancreatic endocrine glands, such as diabetes mellitus. Enzyme insufficiency occurs when the exocrine pancreatic gland is damaged (hr et al., 2018). Between 2017 and 2045, the population with diabetes mellitus is estimated to increase from 122 million to 253 million, which is in line with the increase in old age from 653 million to 1,42 billion (Bellary et al., 2021). Aging of the kidneys can occur because of many factors such as free radicals and reactive oxygen species (ROS), which cause cell damage due to oxidative stress. Oxidative stress can cause damage and decrease kidney performance. The incidence of kidney damage that causes chronic kidney failure is 0,2% per 1000 people. This figure continued to increase until it reached 0,38% or 713,783 people experienced chronic kidney failure in 2018 (Lembaga Penerbit Badan Penelitian dan Pengembangan Kesehatan, 2019).

Organ damage from oxidative stress can be prevented by consuming foods high in antioxidants such as arrowroot tubers (*Maranta arundinacea L.*)(Ramadhani et al., 2017). Arrowroot tubers contain flavonoids, which act as antioxidants and anti-inflammatory agents. Apart from their content, which has the potential to delay the aging process, arrowroot tubers are affordable and easy to obtain in Indonesia, making them accessible to the public. Therefore, this study was conducted to examine the effect of arrowroot tubers on d-galactose-induced aging using histomorphological images of the pancreas and kidneys of mice (*Mus musculus*).

Methods

This experimental research was conducted meticulously using a true experimental research design and a post-test control group design. The research spanned a period of four months, during which mice treatment and histological observations were carried out at the Laboratory of the Faculty of Medicine and the Integrated Laboratory of the Faculty of Mathematics and Natural Sciences, UII. The research used experimental animals, namely *Mus musculus Balb/C*) with the following inclusion criteria: male mice, healthy (not disabled and moving actively) with a body weight of 25 ± 5 g and 12 weeks of age.

The number of samples was calculated using the formula E = 10 – 20 / E = total number of animals – total number of groups(Arifin & Zahiruddin, 2017). In this study, a minimum sample size of animals was obtained with E = $\frac{10}{4}$ + 1 = 4, and the maximum number of animal sample was E = $\frac{20}{4}$ + 1 = 6. This study used four mice per group plus one as a reserve, for a total of 20 mice. This study was designed and approved by the Ethics Committee of the UII Faculty of Medicine (number 15/Ka). Kom.Et/70KE/VII/2023, ensuring the highest standards of animal welfare were maintained.

After acclimation for one week, the mice were randomized and divided into four groups. The healthy control (HC) group received standard food, without any treatment. The negative control (NC) group was fed astandard feed with d-galactose induction treatment. The intervention group was fed 45% arrowroot (P1) with d-galactose induction and the intervention group was fed 60% arrowroot (P2) with d-galactose induction.

Feeding according to the groups was performed in the 2nd to 8th weeks(Fidianingsih et al., 2022). The feed administered was 10% of the mice's body weight daily (Bo-Htay et al., 2018), ensuring that the mice received the necessary nutrition for the study.

D-galactose was administered orally at 300 mg/kg BW/day via sondase once a day from the 3rd to the 8th week(Sulistyoningrum, 2017). At the end of the study, termination was performed to remove the pancreas, kidneys, and blood plasma samples to check oxidative stress levels using malondialdehyde (MDA) parameters. MDA levels were examined using a spectrophotometer at the UGM FKKMK Biochemistry laboratory.

The pancreas and kidney were soaked in 10% formalin in PBS for two days, and tissue processing and embedding were performed using paraffin. The block was cut using a

microtome with a thickness of 0,4 um in 1 slice. The preparations were stained with Hematoxylin Eosin. Histomorphological damage to the pancreas and kidneys of mice was assessed using an Olympus CX23 microscope and Optilab software at a magnification of 40 × 10.

Pancreatic preparations were assessed descriptively by observing the exocrine and endocrine glands throughout the field of view. Pancreatic damage is characterized by congestion, an increase in polymorphonuclear cells around the cells, cell degeneration, increased vacuolization, necrosis, or atrophy.

Kidney preparations were assessed by observing the tubules and glomeruli at five different locations in a rotating manner (Figure 1). From each location, four visual fields were obtained sequentially and side by side to obtain a total of 20 visual fields for each mouse. Tubules or glomeruli were considered to have lesions or damage if the cells were found to be experiencing degeneration, vacuolization, or necrosis, as indicated by the presence of pyknotic, karyorrhexis, or karyolysis by more than 50%(Wibowo, 2012). The number of damaged tubules or glomeruli divided by the total number of tubules or glomeruli both damaged and not damaged in one visual field was multiplied by 100 to determine the level of damage in one visual field.

Figure 1. The scheme of procedures for taking field of view

The level of inflammation in each area was calculated. The calculation was performed by considering a group of inflammation or one blocked blood vessel as one each, and then adding them to the other visual fields.

Result and Discussion

Characteristics of Research Subjects

At the start of the study, the mice were healthy, without any defects, and they moved actively. The mice adapted well, as indicated by an increase in body weight. At the end of the treatment period, d-galactose, especially in those that had been induced without the arrowroot diet, appeared less active than in the first week of the study. Plasma sampling was performed before termination followed by MDA analysis. The highest average MDA levels were found in the negative control group without the arrowroot diet (4,24 ± 1,50) and the lowest in the healthy control group (2,77 ± 0,37).

Description of Histomorphological Damage to the Pancreas

In the HC group, the histology of the islets of Langerhans showed minimal damage, namely, no inflammation, necrosis, atrophy, or fibrosis (Figure 2A). The negative control group showed severe damage owing to inflammation, necrosis, and atrophy (Figure 2B). The treatment group with 45% arrowroot feed appeared more damaged, and empty spaces were found due to necrosis, vacuolization, or degeneration, compared to the group with 60% arrowroot feed (Figures 2C and 2B).

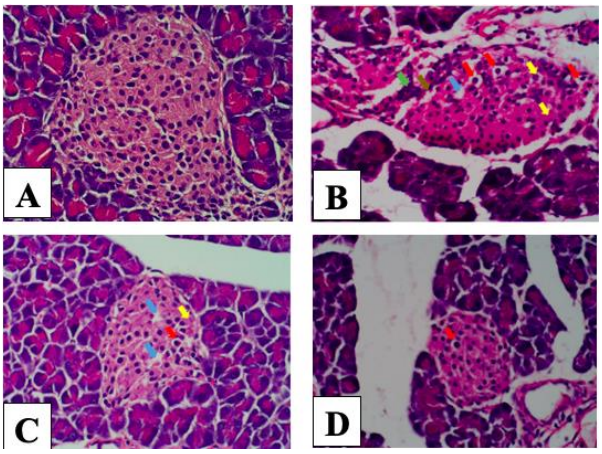

Figure 2. Differences in the picture of Langerhans Island between healthy controls, standard feed diets, and arrowroot diets

Note: Figure 2A shows the healthy control group (HC) with the average size of islets of Langerhans and minimal damage/degenerative signs. Figure 2B shows the negative control group (NC) with a severe degenerative process characterized by cytoplasmic vacuolization (red arrow), necrosis (yellow arrow), empty space due to necrosis (blue arrow), congestion (brown arrow), and inflammatory cell infiltration (green arrow). Figure 3C shows the 45% arrowroot feed group (P1) with cytoplasmic vacuolation

(red arrow), necrosis (yellow arrow), and empty space due to necrosis (blue arrow), the shape of the Langerhans islands still tends to be intact. Figure 2D shows the arrowroot 60% feed group (P2) with minimal degenerative processes, indicated by minimal cytoplasmic vacuolation (red arrow), and the shape of the islets of Langerhans, which were still intact.

The histology of the acini showed minimal damage in the healthy control group, as indicated by the absence of signs of inflammation such as inflammatory cell infiltration, vacuolation, congestion, necrosis, fat cell infiltration, and fibrosis (Figure 3A). The most severe damage was observed in the negative control group, with the discovery of atrophy of the acini, vast distances between acini, and acinar cell necrosis (Figure 3B). In the 45% arrowroot diet group, signs of inflammation were observed in the form of inflammatory cell infiltration, and the distance between the acini was wider and more damaged than in the 60% arrowroot diet group (Figures 3C and 3D).

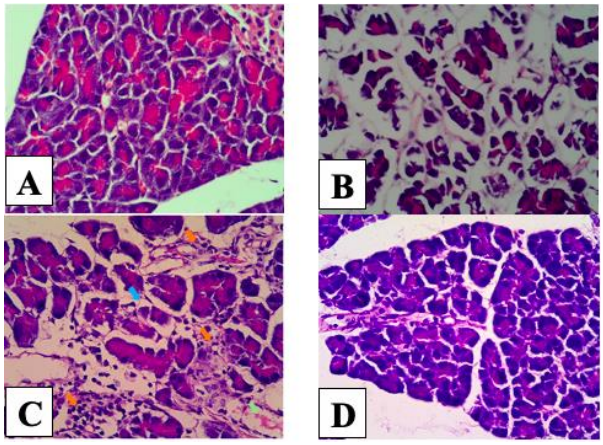

Figure 3. Differences in the appearance of the Pancreatic Exocrine Glands between healthy controls and those given a standard and arrowroot diet.

Note: Figure 3A shows that the healthy control group (HC) showed no signs of inflammation, necrosis, fat cell infiltration, or fibrosis with minimal degenerative signs. Figure 3B shows that in the negative control (NC) group, there was severe damage characterized by visible atrophy of the acini, distance between the acini widening, and disappearance of the acinus nucleus. Figure 3C shows that in the 45% arrowroot feed group (P1), there were signs of inflammation, characterized by the infiltration of inflammatory cells (orange arrow), congestion (light green arrow), and moderately widened acini (light blue arrow). Figure 3D shows that, in the arrowroot 60% feed group (P2), there were no visible signs of inflammation, atrophy, necrosis, congestion, or fibrosis in the acini. The distance between acini did not increase.

Description of Kidney Histomorphological Damage

In observing the kidney histology, it was found that the HC group showed the lightest tubular and glomerular damage (Figure 3A) compared to the other groups, followed by the 60% arrowroot treatment group (Figure 3C) and then the 45% arrowroot treatment group (Figure 3D). The NC group without arrowroot tuber intervention showed the most severe appearance of tubules and glomeruli compared to the other groups, namely necrosis, degeneration, and inflammation (Figure 3B).

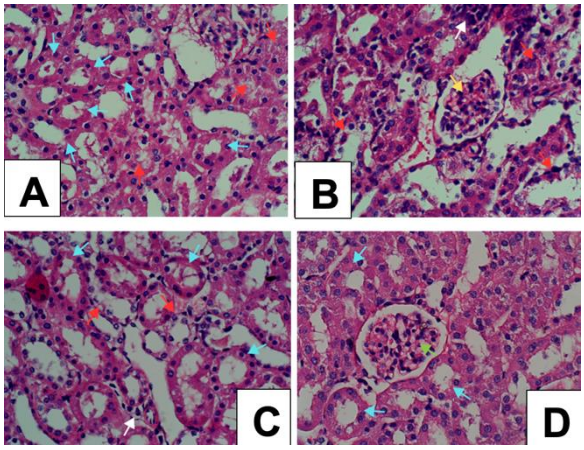

Figure 4. Histological image of kidneys of experimental animals

Note: A: HC (healthy control group); B:NC (negative control group); C: P1 (45% arrowroot intervention group); D: P2 (60% arrowroot intervention group). Light blue arrow:normal tubular cells; red arrow:abnormal tubular cells; white arrow: inflammation (congestion); yellow arrow: abnormal glomerulus; green arrow: normal glomerulus (40X Objective Magnification HE hematoxylin and eosin staining).

There were significant differences between the groups in the levels of tubular damage and inflammatory features ($p< 0,05$). In the glomerulus, the results showed no significant differences were observed between groups ($p> 0,05$) (Table 1). Post-hoc test results showed that administering arrowroot at 45% and 60% could improve inflammation caused by d-galactose induction; therefore, it showed the

same picture as healthy controls and was significantly different from negative controls (Table 2). The administration of arrowroot 60% improved tubular damage but was not as good as that of healthy controls (there was a significant difference between healthy and negative controls). Administration of 45% arrowroot did not improve tubular damage; there was no significant difference compared with the negative control and was significantly different from the healthy control (Table 3).

Table 1. Average total kidney damage score

Group	Number of Mice	Mean ± SD Tubule Abnormality	Mean ± SD Glomerular Abnormality	Mean + SD Inflammation
HC	4	52,46 ± 1,46	62,64 ± 11,13	8,75 ± 2,5
NC	4	76,66 ± 9,87	70,48 ± 13,32	27,25 ± 4,57
P1	4	67,99 ± 2,12	71,25 ± 8,04	8 ± 7,25
P2	4	60,93 ± 0,55	65,53 ± 14,26	13,5 ± 6,19
p-value		0,005*	0,707**	0,001**

Note: HC:healthy control group; NC: negative control group; P1: arrowroot root intervention group with 45% content; P2: arrowroot root intervention group with 60% content; * Kruskal-Wallis Test, ** Oneway ANOVA Test

Table 2. Post Hoc Test Differential picture test of renal cortex inflammation

Group	HC	NC	P1	P2
HC				
NC	0,003*			
P1	1	0,002*		
P2	1	0,023*	1	

*significant (p<0,05)

Table 3. Mann-Whitney test to assess tubular damage

Group	HC	NC	P1	P2
HC				
NC	0,021*			
P1	0,021*	0,248		
P2	0,021*	0,021*	0,021*	

*significant (p<0,05)

The results of this study showed that feeding arrowroot has the potential to inhibit damage to the pancreas and kidneys caused by induced aging. This can be seen from the scores and the most severe signs of aging in the NC group, followed by the P1, P2, and HC groups. These results are consistent with previous research which showed Kurniawan (2020), that administering an arrowroot flour diet containing antioxidants can repair kidney damage caused by 7,12 Dimeythylbenz-Alfa-Anthrahracene (DMBA). Similarly, a study showed Wu & Liao (2017) that the pure arrowroot tuber flour group with a consentration of 0.005 mg/ml had a higher antioxidant content (23,6%) than the control group or coupling arrowroot tuber flour.

Signs of aging in the mouse pancreas can be assessed by damage to the islets of Langerhans. In the NC group, severe degeneration was indicated by the appearance of loose or shrunken islets of Langerhans. Baset et al. (2020) and Omidi et al. (2020) showed severe degeneration of the islets of Langerhans in the form of sparse cells in mice induced by diabetogenic and aging agents using d-galactose. Another sign of aging is vacuolization in the Langerhans Island area. Vacuolization was obvious in the NC group, reduced in the P1 group, and minimal in the P2 and HC groups. This is consistent with the findings of Hassanien et al. (2020) and El-far et al. (2020), who showed vacuolization in rat islets of Langerhans induced by diabetogenic agents and aging using d-galactose. Cell necrosis was another sign of aging in the treatment group. The NC group showed a relatively higher number of cells experiencing necrosis than the P1 group, whereas the P2 group showed a relatively minimal number of necrotic cells. The following research by (López et al., 2018) and (El-Far et al., 2016) showed that one form of damage to the mouse pancreas is the occurrence of necrosis in the islets of Langerhans cells in mice, which is induced by d-galactose. The formation of empty

areas owing to necrosis was also observed in the NC and P1 groups. This follows the research conducted by Zubaidah et al. (2017), which showed the formation of several empty spaces on Langerhans Island owing to necrosis.

Pancreatic aging is also characterized by damage to exocrine glands and acini. In the CN group, severe damage was observed in the form of atrophy of the pancreatic acini in the form of a relatively reduced size, such that the distance between the acini widened. This follows research conducted by Müller et al. (2014) on pancreatic acini degeneration in the form of atrophy of the acini and widening of the distance between the acini in the rat pancreas induced by aging. Additionally, signs of aging were also shown by inflammatory cell infiltration in the acinar area in the P1 group, accompanied by vascular congestion. This research was conducted (Dong et al., 2017) in the form of d-galactose induction in mice and showed aging in pancreatic acini as a sign of inflammation, namely, inflammatory cell infiltration.

Continuous induction of d-galactose increases oxidative stress. Cells in the islets of Langerhans, especially beta cells, are damaged and their numbers are relatively reduced due to increased ROS production. In addition, oxidative stress damages the cellular organelle components. Simultaneously, cells cannot produce enzymes such as catalase and glutathione peroxidase to break down the toxic compounds formed. In this manner, the damage continues, causing cells to experience atrophy, leading to cell death/necrosis. In the NC group, the distance between the Langerhans Islands and the surrounding acini was wider than that in the other groups. This image shows atrophy of both acinus cells and cells in the islets of Langerhans and several areas experiencing necrosis(Aminah & Qomariyah, 2023; Dinić et al., 2022).

D-galactose induces damage by disrupting ion channel activity, which regulates the entry and exit of electrolytes and water into cells. Damage to the cell membrane structure due to oxidative stress causes an imbalance in the volume of intracellular and extracellular water. Excess water can enter the intracellular area, causing cytoplasmic vacuolization. Cytoplasmic vacuolization was observed in all groups, with the NC group showing more vacuolization than the other groups (Miller & Zachary, 2017).

Necrosis due to aging is accompanied by empty spaces in several areas of the Langerhans. Damage begins with the formation of ROS owing to oxidative stress, which disrupts cellular metabolism. Continuous production of toxic compounds causes cells to fail to adapt to compensate; therefore, damage occurs at the organelle level and protein synthesis in the cell nucleus. If this process continues, the cells undergo apoptosis and end in necrosis/cell death. In the necrosis stage, cell integrity was lost, causing an empty area to appear in the histological section. Thus, the area of the islets of Langerhans can decrease due to the loss of some cells that undergo necrosis (Dinić et al., 2022). Necrosis was observed in NC and P1 groups. However, necrosis was observed more frequently in the NC group than in other groups. In group P2, necrosis was minimal or non-existent. The necrosis process causes empty spaces to form because of damage to cell organelles, as seen in the NC and P1 groups.

The signs of inflammation found in the histological images of the pancreas and kidneys indicate the presence of inflammatory cell infiltration. This was due to the induction of d-galactose, which triggers oxidative stress. ROS produced during oxidative stress activates the inflammatory cascade and causes cell damage. ROS activates several inflammatory signaling molecules to increase the levels of pro-inflammatory cytokines. Proinflammatory cytokines are called inflammatory cells, resulting in the accumulation of inflammatory cells in areas experiencing inflammation(Meher & Rath, 2015). In addition, renal inflammation is influenced by tubular damage. Tubules that experience abnormalities such as injury, deformation, or death trigger endothelial activation and cell infiltration, and cell infiltration releases inflammatory mediators (Gyurászová et al., 2020).

In addition to the signs of inflammation, kidney damage can also be observed through tubular and glomerular cell nuclei that appear pyknotic, karyorrhexis, or karyolysis(Wibowo, 2012). This is because the induction of d-galactose increases the formation of ROS, which can cause DNA damage by breaking single strands of DNA and DNA-protein cross-links and modifying base residues, such as inserting the hydroxyl group on guanine into the C-8 position to form 8-hydroxy-deoxyguanosine (8-OH-dG).

DNA damage causes the cells to undergo apoptosis or necrosis (Park et al. 2014).

However, the sequence of glomerular damage differed from that of tubular damage. The P1 group showed a higher level of damage than the NC group. This is because tubules are the main target of damage due to oxidative stress in kidney cells. The tubules, particularly the proximal part, contain many mitochondria and play an essential role in the reabsorption of water and solutes. This role requires high-energy production by the mitochondria. Energy production by the mitochondria also produces ROS, which can cause oxidative stress (Gyurászová et al., 2020). As a result, glomerular damage in this study did not occur optimally; therefore, the results were not significant.

Feeding with modified arrowroot tuber flour had a positive effect on the histology of the mouse pancreas, as evidenced by the minimal signs of aging with 60% modified arrowroot tuber in the P2 group. This proves that flavonoid content can prevent and minimize damage to pancreatic cells by increasing the production of the enzymatic antioxidants, catalase, glutathione peroxidase, and superoxide dismutase. This component plays a role in converting the toxic compounds produced by oxidative stress into compounds that are safe for the body. In addition, flavonoids can inhibit the expression of pro-apoptotic genes, which causes apoptosis and necrosis in pancreatic cells (Ghorbani et al., 2019). One type of flavonoid is polyphenol, which can inhibit aging by scavenging ROS directly because polyphenol molecules contain phenolic hydroxyl groups. The number and position of the hydroxyl groups, substituent patterns, and glycosylation of phytochemical molecules influence the ROS-scavenging capacity of polyphenols. As a result, the amount of ROS formed due to d-galactose induction is reduced, and kidney damage is inversely proportional to the concentration of arrowroot tubers (Luo et al., 2021). Thus, cell damage in the pancreas and kidneys as a sign of aging can be minimized in the group given aging induction using d-galactose and modified arrowroot starch feed at 60% compared with 45%.

In addition, arrowroot has a high fiber content. It is a prebiotic and can increase the production of short-chain fatty acids (SCFA). SCFA are anti-inflammatory and improve the immune and digestive tract barriers. The improved gastrointestinal barrier prevents dysbiosis and inflammation of microbiota. This can improve the immune system and inhibit ageing (Warman et al. 2022).

The limitation of this study is that the observation of pancreatic histology was descriptive. The pancreas and kidney observations were not blinded; only one person observed them; therefore, they were subjective. This research took only one slice of preparation from the entire kidney and did not use stereological methods because of limited funds.

Conclusion

Based on the results of observations and statistical tests that have been carried out, it can be concluded that there is an effect of administration of arrowroot tuber flour on the histomorphological features of the pancreas and kidneys of *Mus musculus Balb/C* mice which were induced by aging using D-Galactose.

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IF, TA, SW, SH, and SS designed and implemented the study. IF analyzed the results and wrote the manuscript. TA conceived and supervised the original project.

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