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The Effect of Trehalose Supplementation on Macrovascular Inflammation Biomarker in Old Rats by Assessing NFκB-p65 Expression

Pengaruh Suplementasi Trehalosa terhadap Biomarker Inflamasi Makrovaskular pada Tikus Tua dengan Menilai Ekspresi NFκB-p65

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Trehalose, Macrovascular Inflamation, NFκB-p65 Expression, Aging

ABSTRACT

Background: Vascular inflammation is one of contributing factors to the pathogenesis of arterial aging. Age-related activation of the inflammatory process can lead to various macro-and microvascular pathologies. The pro-inflammatory microenvironment generated in the vascular wall can lead to the pathogenesis of vascular diseases due to an increase in vascular dysfunction. Trehalose is a disaccharide that has several functions, protecting against stressors (one of them is reactive oxygen species/ROS) and preventing the inflammatory responses induced by endotoxic shock.
Objectives: To analyze the effect of trehalose supplementation on macrovascular inflammatory processes related to the aging process.
Methods: The experimental study used 28 male Wistar rats (*Rattus novergicus*) which were divided into 4 groups, young control group (Group A), old control group (Group B), 2% sucrose group (Group C), and 2% trehalose group (Group D); were then observed for 8 weeks.
Results: The results showed that there were no significant differences in aortic tissue NFκB-p65 expression between old and young subjects (p=0.247). The 2% trehalose group had 40% lower aortic tissue NFkB-p65 expression compared to the old control group (p=0.012); while the group given 2% sucrose solution had a 30% higher aortic tissue NFkB-p65 expression compared to the trehalose group (p=0.018).
Conclusion: Trehalose has a good effect on aging-associated vascular inflammatory processes that can be seen from the low aortic tissue NFκB-p65 expression in old rats.

ABSTRAK

Latar Belakang: *Inflamasi vaskular merupakan salah satu faktor penyebab terjadinya patogenesis penuaan vaskular. Aktivasi proses inflamasi terkait usia dapat menyebabkan berbagai patologi makro dan mikrovaskular. Lingkungan mikro pro-inflamasi yang dihasilkan di dinding pembuluh darah dapat menyebabkan terjadinya patogenesis penyakit vaskular karena terjadi peningkatan disfungsi vaskular. Trehalosa merupakan disakarida yang memiliki beberapa fungsi yaitu protektif terhadap stressor (salah satunya ROS) dan mencegah respon inflamasi yang diinduksi oleh syok endotoksik.*
Tujuan: *Menganalisis efek pemberian trehalosa terhadap proses inflamasi makrovaskular terkait proses penuaan.*
Metode: *Penelitian eksperimental menggunakan 28 tikus Wistar (Rattus novergicus) jantan yang dibagi menjadi 4 kelompok yaitu kelompok kontrol muda (Kelompok A), kelompok kontrol tua (Kelompok B), kelompok sukrosa 2% (Kelompok C), dan kelompok trehalosa 2% (Kelompok D); yang kemudian diamati selama 8 minggu.*
Hasil: *Hasil penelitian menujukkan tidak ada perbedaan yang signifikan antara ekspresi NFκB-p65 jaringan aorta pada kelompok kontrol tikus muda dan kontrol tikus tua (p=0,247). Kelompok trehalosa 2% memiliki ekspresi NFkB-p65 jaringan*

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aorta lebih rendah sebanyak 40% dibandingkan dengan kelompok kontrol tua (p=0,012); sedangkan pada kelompok yang diberikan larutan sukrosa 2% memiliki ekspresi NFkB-p65 jaringan aorta lebih tinggi sebanyak 30% dibandingkan dengan kelompok trehalosa (p=0,018).
Kesimpulan: *Trehalosa memiliki efek yang baik terhadap proses inflamasi vaskular terkait penuaan yang dapat dilihat dari rendahnya ekspresi NFκB-p65 jaringan aorta pada tikus tua.*

Kata Kunci: *Trehalosa, Inflamasi Makrovaskular, Ekspresi NFκB-p65, Penuaan*

INTRODUCTION

The most common cause of death worldwide is cardiovascular disease (CVD), which causes 17.9 million deaths each year. Heart attacks and strokes are the cause of four out of five deaths caused by CVD. In Central Asia, CVD causes fairly high mortality there.[1] In Indonesia, death, and morbidity in old individuals are commonly caused by CVD.[2] "Vascular aging" occurs with age, so it is associated with the risk of CVD. [3] Vascular aging will cause endothelial dysfunction due to increased vascular inflammation and reactive oxygen species (ROS). Thus, therapy that can inhibit inflammatory signals and oxidative in the elderly has benefits in improving arterial function, thereby reducing the risk of CVD.[4]

Trehalose has an α, α-1, 1-glycosidic bond that links 2 units of glucose and has several benefits, including protecting against stressors (one of them is ROS) and preventing inflammatory response induced by endotoxic shock.[5] Trehalose is found in small amounts in beans, mushrooms, seaweeds, honey, lobsters, shrimps, and yeasts.[6] As a sweetener, trehalose is made from corn starch using several bacterial enzymes such as alpha-amylase (which is obtained from *Bacillus licheniformis*) and isoamylase (from *Pseudomonas amyloderamosa*).[7]

The study by Echigo (2012) found that the production of inflammatory mediators, lipid peroxidation, and ROS can be inhibited by trehalose.[5] Another study by Svenkrtova (2017) and Seo (2018) also found the effect of trehalose in prolonging the life of yeast and worms (*Caenorhabditis elegans*).[8,9] In humans, trehalose is widely used as a “food ingredient” or “food additive” because trehalose has low sweetness and excellent physical properties, including an anti-aging effect on starch and protein stabilization.[6] In a study by Yoshizane (2020) found that daily consumption of 2,5 and 0,3% trehalose could reduce adipocyte hypertrophy and improve insulin resistance in mice given HFD.[6] Therefore, trehalose appears as a new therapy that can inhibit the inflammatory process so that it can repair vascular damages.[4]

From several theories and studies, it can be seen that trehalose has several protective effects that are much greater than other disaccharides (such as sucrose); so can be considered as a substitute for sugar that is often consumed daily. From several previous studies, no one has investigated the role of trehalose on macrovascular inflammation. Therefore, researchers are interested in research to find out the effect of trehalose supplementation on macrovascular inflammatory processes related to the aging process. This study can be a recommendation for choosing a healthy diet, especially sugar in elderly individuals to reduce the adverse effects of macrovascular inflammation that can occur with age.

METHODS

This study used male Wistar rats (*Rattus novergicus*) aged 3-6 months for young rats and those aged 12-18 months for old rats, then divided into 4 groups, namely: Group A (young control), Group B (old control), Group C (sucrose), and Group D (trehalose). This research was conducted for 8 weeks at the Entomology Laboratory, Faculty of Medicine, Hasanuddin University Makassar. This study has obtained research ethics from the Health Research Ethics Commission (KEPK) FKUH - RSPTN UH - Dr. RSUP. Wahidin Sudirohusodo Makassar with Number: 173/UN4.6.4.5.31/PP36/2021.

The number of rat samples in this study was determined using the Federer formula: (t-1)(n-1) $\geq$ 15; where t is the number of treatment groups and n is the number of samples per group. The ideal sample size according to the Federer formula calculation is 7 subjects/group. Thus, the total sample used was 28 rats which were divided into 4 groups.

Preparation of experimental animals

Acclimatization was carried out for 1 week. All subjects were given a standard diet and aqua dest for drinks, and we monitored their weight every day. The cages were cleaned every day to maintain a stable environment, all subjects were placed in a room with good air circulation and at a standard room temperature (28±2°C) with a humidity of 50%+10% and the room lights were set on a 12-hour dark and 12-hour light cycle.

Making a solution of 2% sucrose and 2% trehalose

From previous studies, the concentration used was 2%. To calculate the amount of sugar to be used, we used the formula M/V x 100%, where M is the mass of sugar (sucrose/trehalose) and V is the daily volume of water.[10,11] The daily water volume of rats >12 months old is 40ml/day.[12]

$$2\% = \frac{M}{V} \times 100\% \rightarrow 2\% = \frac{M}{40\ ml} \times 100\%$$

M = 0,8 gram

According to the calculation, the dose of sucrose/trehalose to be used is 0,8 grams. Thus, 0,8 grams of sugar (sucrose or trehalose) were dissolved in 40 ml of aqua dest and put into the rat’s drinking bottle.

Intervention

Group A consisted of young rats that were given 15-20 grams/day of standard diet and aqua dest as a drink; Group B consisted of old rats that were given 25 grams/day of standard diet and aqua dest as a drink; Group C consisted of old rats fed a standard diet and a 2%

sucrose solution as a drink, and Group D was old rats fed a standard diet and 2% trehalose solution as a drink. The standard diet given is the Van Der Vour diet which contains 20% Protein, 7% Fat, 15-20% Fiber, 1% Calcium, and 0,8% Phosphorus. The intervention was carried out for 8 weeks and was terminated at week 8 for aortic tissue sampling.

Aortic Tissue Sampling

Before taking the aortic tissue, the rats were first anesthetized using ether. The anesthetized rats were then euthanized using the cervical dislocation technique. Dead rats were necropsied for aortic organ sampling. The aorta was then stored in 10% buffered formalin for the histological preparations. After the procedure, the rats were buried in the ground.

Immunohistochemistry Examination (IHC)

Tissue samples were put into the paraffin and cut to a thickness of 4 µm, then deparaffinized and rehydrated in Phosphate Buffer Saline (PBS) solution. Primary rabbit polyclonal antibody NFκB-p65 (GeneTex, Inc, North America, GTX107678) was diluted 1:100 with Normal Antibody Diluent (Scytec) and incubated for 1 hour at room temperature. Then rinsed with PBS solution for 5 minutes. After that add Ultratek Anti-Polivalent Biotinylated Antibody (Scytec), and left for 10 minutes, and rinsed again with PBS solution. After that, add Ultratek HRP (Horse radish peroxidase) for 10 minutes. Use diaminobenzidine (DAB, brand Scytec) solution for visualization. Then, use haematoxylin for counterstaining. Then evaluations were made using a light microscope.

Immunohistochemical Evaluation

Interpretation of NF-κB p65 immunohistochemistry results was seen from the distribution and intensity of stained cells. Distribution is assessed based on the number of stained cells: 0 (not stained cell), 1 (less than 1/3 stained cells), 2 (1/3 to 2/3 of the stained cells), and 3 (spread over 2/3 of the stained cell area). The intensity was scored as: 0 (not stained cell), 1 (light), and 2 (solid). To assess the IHC interpretation, we summed the distribution scores and intensity scores of the results obtained and classified them as follows: 0 (negative), 2 (weakly positive), 3-5 (strongly positive).[13]

RESULTS

The characteristics of research subjects (n=28)

This research used aortic tissue from Wistar rats (*Rattus novergicus*) which were housed for 8 weeks (March to May 2021). The number of samples that met the research criteria was 28 samples; however, the number of samples that were subjected to immunohistochemical examination was only 16 samples (only 4 samples in each treatment group). The age of the research subjects used was divided into two groups, 3-6 months for young rats (n=7) and 12-18 months for old rats (n=21). The examination's characteristics for the average final bodyweight of the subjects were 264 grams (minimum weight 152 grams and maximum weight 368 grams). Characteristics of the immunohistochemical (IHC) results of NFκB-p65 expression were the average IHC results of subjects were 2.

Table 1. The characteristics of research subjects based on Age, Final Body Weight, and IHC (Immunohistochemical)

Variable	Minimum	Maximum	Median	Mean±SD
Age (months)	3	17	14	12±5.01
Final BW (gram)	152	368	287.5	264±69.43
IHC (NFκB-p65 expression)	2	5	4	2±0.91

BW = Body weight; IHC = Immunohistochemical

In this study, 28 Wistar rats (*Rattus novergicus*) were put into 4 treatment groups. Group A was a young control group that was given a standard diet (Van Der Vour) and was given aqua dest for drinks; Group B was an old control group that was given a standard diet (Van Der Vour) and drank aqua dest; Group C was old rats that given standard diet + 2% sucrose solution; and Group D was old rats that were given standard diet + 2% trehalose solution. This intervention was carried out for 8 weeks to know the differences in macrovascular inflammatory biomarkers by assessing the immunohistochemical expression of NFκB-p65 between the control group given distilled water and the group given sucrose or trehalose solution. Characteristics of research subjects based on treatment groups, include meaning final body weight, median age, and median IHC results.

Table 2 showed the characteristics of research subjects based on treatment groups, and it was found that there were significant differences between the study sample groups, both for the variables of age (p=0.017), final body weight (p<0.001), and IHC results (p=0.042).

Table 2. Descriptive table and F test on the variables of Age, Weight, and IHC

Variable	Group (Parametric: Mean ± SD; Non-Parametric: Median [Min-Max])				p-value
	A (young control) n=4	B (old control) n=4	C (sucrose) n=4	D (trehalose) n=4	
Age (months)	3 [3 -3]	14 [14 - 15]	14.5 [14 - 17]	14 [14 - 17]	0.017 [b]
Final body weight (grams)	162.8 + 9.18	290.0 + 15.10	305.3 + 24.25	292.5 + 63.22	<0.001 [a]
IHC (NFκB-p65 expression)	4.5 [4 - 5]	5 [4 - 5]	4.5 [4 - 5]	3 [2 - 4]	0.042 [b]

[a]: One Away ANOVA test; [b]: Kruskal-Wallis test

Comparison of IHC results between young and old controls

Using Mann-Whitney to compare the expression of NFκB-p65 in aortic tissue between young and old control groups; and obtained the following results:

Table 3. Comparison of IHC results between young and old controls

Group	n	Median (Min-Max)	p-value
Young control	4	4.5 (4 - 5)	0.247
Old control	4	5 (4 - 5)	

Based on the table above, it can be concluded that there is no difference between the IHC results in the young control group and the old control group significantly, with a p-value = 0,247 (p>0,05).

Comparison of IHC results between old controls and trehalose group

Using Mann-Whitney Test to compare the expression of NFκB-p65 in aortic tissue between trehalose group and old control group; and obtained the following results:

Table 4. Comparison of IHC results between trehalose and old controls

Group	n	Median (Min-Max)	p-value
Trehalose	4	3 (2 - 4)	0.012
Old control	4	5 (4 - 5)	

Based on the table above, it can be concluded that there is a difference between the IHC results in the trehalose group and the old control group significantly, with a p-value=0.012 (p <0.05).

Comparison of IHC results between trehalose and sucrose group

Using Mann-Whitney Test to compare the expression of NFκB-p65 in aortic tissue between trehalose group and sucrose group; and obtained the following results:

Table 5. Comparison of IHC results between trehalose and sucrose groups

Group	n	Median (Min-Max)	P-value
Trehalose	4	3 (2 - 4)	0.018
Sucrose	4	4.5 (4 - 5)	

Based on the table above, it can be concluded that there is a difference between the IHC results in the trehalose group and sucrose group, with p-value=0.018 (p <0.05).

Correlation between age and aortic tissue NFκB-p65 expression

To assess the correlation between age and aortic tissue NFκB-p65 expression, the Spearman's test was used, and it was found that there is no correlation between the two variables with p=0.319 (p>0.05) as shown in Table 6.

Table 6. Correlation between age and aortic tissue NFκB-p65 expression

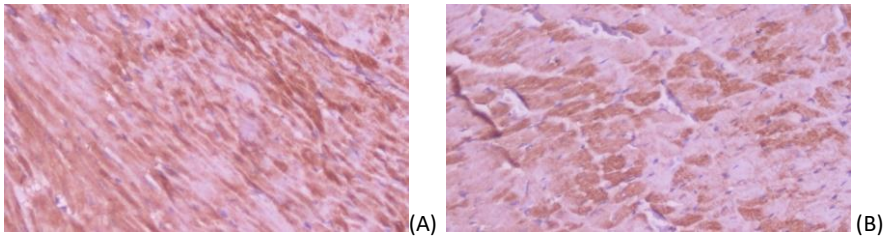
Variable	n	Median (Min-Max)	R-value	p-value
Age (months)	16	14 (3 - 17)	-0.266	0.319
IHC (NFκB-p65 expression)	16	4 (2 - 5)		

Correlation between aortic tissue NFκB-p65 expression and final body weight

To assess the correlation between aortic tissue NFκB-p65 expression and final body weight, the Spearman's test was used, and it was found that there is no correlation between the two variables with p=0.424 (p>0.05) as shown in Table 7.

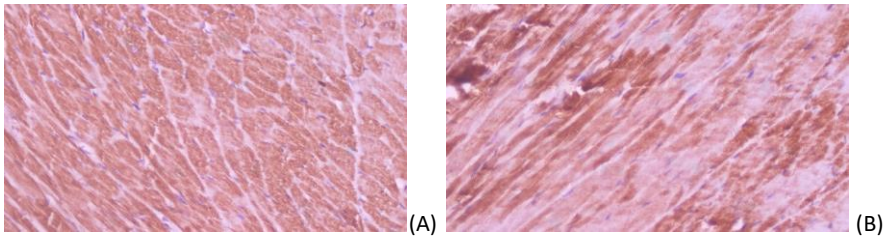
Table 7. Correlation between final body weight and aortic tissue NFκB-p65 expression

Variable	n	Median (Min-Max)	R value	p-value
Final body weight (grams)	16	279.5 (152 - 359)	-0.215	0.424
IHC (NFκB-p65 expression)	16	4 (2 - 5)		



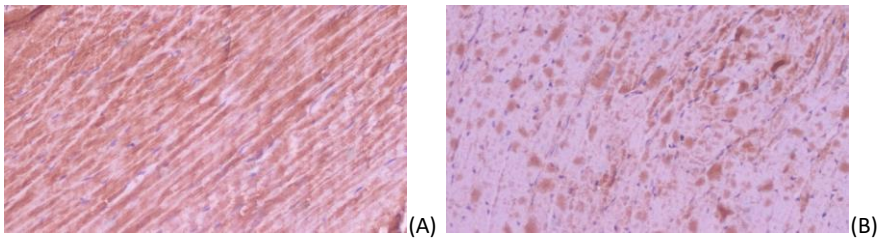
(A) (B)

Figure 1. Aortic tissue IHC results for a control group of young rats. **(A)** Diffuse positive cell distribution (value 3) and intensity of cells stained strongly (value 2) were seen, so the IHC interpretation was 5 which indicated a strong positive. **(B)** The distribution of multi-focal positive cells (value 2) and the intensity of cells stained strongly (value 2), so the IHC interpretation was valued at 4 which indicated a strong positive.



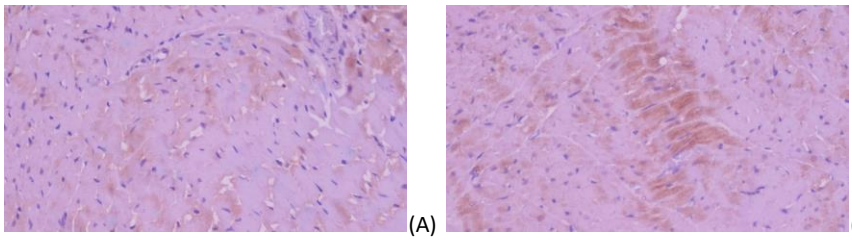
(A) (B)

Figure 2. Aortic tissue IHC results for control group of old rats. Both A and B showed a diffuse distribution of positive cell (value 3) and the intensity of cells stained strongly (value 2), so the IHC interpretation was 5 which indicated a strongly positive.



(A) (B)

Figure 3. Aortic tissue IHC results for sucrose group. **(A)** Diffuse positive cell distribution (value 3) and intensity of cells stained strongly (value 2) were seen, so the IHC interpretation was 5 which indicated a strongly positive. **(B)** The distribution of multi-focal positive cells (value 2) and the intensity of cells stained strongly (value 2), so the IHC interpretation was valued at 4 which indicated a strongly positive.



(A) (B)

Figure 4. Aortic tissue IHC results for the trehalose group. **(A)** The distribution of focal positive cells (value 1) and the intensity of stained cells is weak (value 1), so the IHC interpretation is 2 which indicates weakly positive. (B) The distribution of focal positive cells (value 1) and the intensity of cells stained strongly (value 2), so the IHC interpretation is 3 which indicated a strong positive.

DISCUSSION

Vascular inflammation is one of the factors causing the pathogenesis of vascular aging.[14] Age-related activation of the inflammatory process plays an important role in various macro-and microvascular pathologies, from atherogenesis and aneurysm formation to microvascular dysfunction.[15–17] Inflammation of the vascular wall will increase the occurrence of vascular dysfunction, impair cell metabolism, and increase apoptosis; thereby contributing to the pathogenesis of vascular diseases.[17]

In this research, the subjects were put into 4 groups, one group was a group of young rats (3-6 months) weighing 140-200 grams and the other groups were groups of old rats (12-18 months) weighing 200-400 grams. Young and old control groups did not receive any intervention, group C was a group that was given a 2% sucrose, and group D was a group that was given a 2% trehalose. The intervention with 2% sucrose and 2% trehalose solution refers to previous studies conducted by Sciarretta (2018) and Berry (2020) which used a similar concentration of trehalose (2%).[10,11]

The addition of 2% sucrose and 2% trehalose in this study as sweeteners were added to the drinking water of experimental rats and did not change the calories in the standard diet given. To make a solution of 2% sucrose and trehalose, as much as 0,8 grams of sugar (sucrose or trehalose) were dissolved in 40 ml of aqua dest and put into a rat's drinking bottle. If the given solution is not drunk, then the remaining solution is given by sonde to the experimental rat. This intervention was carried out for 8 weeks.

To assess the macrovascular inflammatory process that occurs in research subjects, the variable immunohistochemical examination (IHC) of aortic tissue NFB-p65 expression was used. The IHC method is often used for basic research which can detect antigens or proteins expressed in tissues accurately, and is a technique that is classified as sensitive and specific.[18] The results of IHC NFkB-p65 were obtained from the addition of the distribution score and the intensity of the stained cells. The interpretation of the IHC NFkB-p65 results was negative with a value of 0; weak positive with a value of 2; and strong positive with a value of 3-5.[13] The range of IHC results obtained in this study was 2-5 with a median of 4. So the higher IHC value indicates an increase in NFkB-p65 expression which will induce the inflammatory process and is related to atherosclerosis.[13,19]

The median value of IHC results for group A (young control rats) was 4,5 (min 4, max 5); for group B (old control rats) was 5 (min 4, max 5); for group C (sucrose group) was 4,5 (min 4, max 5); and for group D (trehalose group) it was 3 (min 2, max 4). Based on these results, among all study groups, group D, which is a trehalose group, had the lowest aortic tissue NFkB-p65 expression among the other three groups.

When compared between group A and group B, it showed no difference in IHC results between old and young groups (p=0.247). This indicates that age does not affect the expression of aortic tissue NFkB-p65 which is associated with vascular inflammatory processes. The study by Fernández-Friera (2019) found that arteries in young subjects showed an inflammatory process.[20] Although clinical signs and symptoms usually occur in late adulthood, postmortem studies conducted by Wissler (1993) suggest that from early childhood the atherogenic process has begun.[21]

Two important processes that occur in early atherosclerosis are increased adhesion of leukocytes to the vascular endothelium and migration through the vascular wall into the arterial intima. Leukocyte adhesion and migration is a multistep process involving various molecules. Leukocytes are attracted to the site of inflammation by chemokines after a chemotactic concentration gradient, MCP-1, and RANTES were involved in the process of leukocyte withdrawal to atherogenic areas. In addition to their chemotactic role, adhesion molecules on the leukocyte surface will be induced by MCP-1 and RANTES. On contact with activated endothelium, it expresses VCAM and ICAM, leukocytes adhere tightly and migrate through the vascular wall and into the arterial intima where they initiate smooth muscle cell proliferation and form lipid deposits and foam cells.[22]

The results for the group given 2% trehalose solution (Group D) had 40% lower NFκB-p65 expression than the old control group (Group B). Based statistical analysis showed that there was a difference (p=0,012). This indicates that the supplementation of trehalose sugar in old rats has a significant effect on decreasing the expression of NFkB-p65 in aortic tissue in this study. These results are consistent with a previous study by Sergin (2017) which showed that supplementation of trehalose sugar would activate autophagy in macrophages thereby reducing cytokine production and vascular inflammation. Trehalose appears to stimulate autophagy via activation of TFEB (EB transcription factor); increased aggregation of p62-protein cytotoxic

aggregates, reduced macrophage cell death and decreased atherosclerotic plaque formation.[23] Another study by Echigo (2012) also showed that trehalose will suppress the activation of the NF-κB so the inflammatory response can be inhibited, and protect cells from ROS induced by lipid peroxidation.[5] Prolonged use of trehalose was also found to have no metabolic side effects in human subjects, so it is safe to use as a supplement or sweetener in daily life.[10]

IHC results for the group given 2% sucrose solution (Group C) showed 30% higher NFκB-p65 expression compared to the trehalose group (Group D). Based on the analysis, it showed significant results with a p-value=0.018; which showed that there was a difference between the IHC results in the sucrose and trehalose groups. The supplementation of sucrose in old rats had a significant effect on increasing the expression of NFkB-p65 in aortic tissue which could increase the occurrence of vascular inflammatory processes in elderly subjects. Sucrose was used as a comparison in this study because sucrose (sugar) is a type of sugar or sweetener that is often used in daily life and is both a disaccharide group.

In aortic endothelial cells, hyperglycemia causes an increase in mitochondrial superoxide production. Endothelial function can be altered by ROS through various mechanisms, such as membrane lipid peroxidation, NF-κB activation, and decreased nitric oxide (NO) availability. VCAM-1 and ICAM-1 will be induced due to the activation of NF-κB, which will increase migration and adhesion to endothelial cells thereby inducing the inflammatory process. ROS and inflammatory factors will cause an increase in the formation of atherosclerotic lesions.[24]

This study also showed there was no correlation between age and aortic tissue NFκB-p65 expression with p-value = 0.319 (p>0.05). In addition, a correlation test was also conducted between final body weight and aortic tissue NFκB-p65 expression, and it was found that there was no correlation between the two variables (p=0.424). Various kinds of risk factors and lifestyles can cause inflammation, including obesity, smoking, and an unhealthy diet. All of that will trigger the inflammatory process and develop atherosclerosis.[25] Multiple comparison tests were also performed for the final bodyweight and showed no difference was seen between all groups of old rats (p>0.05). This indicates that the supplementation of 2% trehalose and 2% sucrose solution did not affect the bodyweight of the research subjects.

CONCLUSION

Trehalose has a good effect on aging-associated vascular inflammatory processes that can be seen from the low aortic tissue NFκB-p65 expression in old rats. Therefore, trehalose can be considered as a substitute for sugar consumed daily, so it can prevent or reduce vascular inflammatory processes and reduce the risk of cardiovascular disease.

Further research using human samples is needed to apply the findings of this study. It is also necessary to conduct further research using other non-invasive parameters to help diagnose vascular inflammation.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURE

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