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The Protective Effects of Trehalose on Gene Expression Linked to Oxidative Stress and Inflammation in Liver Tissue of Adult and Aged Wistar Rats

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Abstract

Background: Aging is associated with chronic diseases and increased oxidative stress, particularly affecting liver function. Aging is linked to chronic diseases and heightened oxidative stress, particularly impacting liver function. Recent research has demonstrated that oral administration of trehalose offers multiple benefits for various tissues and organs.

Methods: This study investigated the effects of oral trehalose (2% in water) on the gene expression of key markers linked to liver oxidative stress and inflammation in 4-month-old adult and 24-month-old Wistar rats. Thirty-two male Wistar rats (n=8) were randomly assigned into four groups: adult control, aged control, adult trehalose (2% in water), and aged trehalose, over a treatment period of one month. Following treatment, liver tissues were analyzed using real-time PCR for genes related to oxidative stress (*PGC-1α*, *NRF2*, and *SOD*) and inflammation (NF-κB, IL-1β, TNF-α, and TGF-β).

Results: Our findings revealed a significant up-regulation of *PGC-1α*, *NRF2*, and *SOD* in aged trehalose group compared to the aged control ($P<0.001$); however, SOD expression increased by trehalose administration in aged rats compared to other 3 groups. Inflammatory markers (*NF-κB, IL-1β, TNF-α*) were significantly reduced by trehalose, and *TGF-β* expression, involved in fibrosis, was attenuated exclusively in aged rats compared with controls ($P<0.05$).

Conclusion: These results suggest that trehalose has a protective effect on hepatic function, particularly in the aging population, and highlight its potential therapeutic role in age-associated liver dysfunction.

Keywords: Aging, Trehalose, Oxidative stress, Inflammation, Liver dysfunction

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Introduction

The global demographic shift towards an aging population has enforced researchers to investigate the biological processes promoting aging and to develop strategies for reducing age-related pathologies. It was estimated that the number of individuals aged 80 and older will increase significantly, from 108 million in 2015 to over 900 million by 2100 (1). The aging process is characterized by a progressive accumulation of cellular damage and a decline in functional capacity and homeostasis over time, leading to increased susceptibility to disease (2). Chronic diseases, which commonly manifest in older adults, often complicate management and treatment due to their irreversible nature (1).

Liver health is particularly deteriorated with age, resulting in significant liver diseases affecting the quality of life (3). The liver, responsible for numerous metabolic functions, including detoxification and energy metabolism, undergoes critical structural and functional changes with age (3, 4), including endothelial cell thickening in liver sinusoids, collagen deposition, and the depletion of Kupffer cells, leading to vascular remodeling, inflammatory processes, and fibrosis (4). Targeting the molecular mechanisms involved in aging-related liver dysfunction is vital for developing effective therapeutics, particularly those that can ameliorate inflammation and oxidative stress, the hallmarks of aging (2-4).

Trehalose, a naturally occurring non-reducing

disaccharide, is found in organisms such as fungi, honey, and shellfish. Its incorporation into various food products has increased globally due to its beneficial properties (5). Among the potential therapeutic candidates for counteracting age-related effects is trehalose. Recent studies have highlighted trehalose's capacity as oral trehalose administration has multiple beneficial effects on various tissues and organs (6-14). For instance, trehalose has been shown to prevent protein accumulation and exert anti-inflammatory effects by targeting Interleukin-1 (*IL-1*), *IL-6*, *IL-18*, and *TNF-α* in aged mice (11-13). Furthermore, it induces autophagy in hepatocytes, reducing hepatic steatosis and lipid accumulation via inhibition of glucose transporters (6).

Given that the FDA recognizes trehalose as safe for human consumption, its integration into nutrition could offer substantial benefits for the aging population (15). Additionally, recent successes in treating metabolic diseases in animal models have inspired interest in human applications. Trehalose administration results in lower insulin secretion and lipid accumulation compared to glucose (8). Research by Kyryakov et al demonstrated that trehalose extends yeast lifespan by regulating protein folding and oxidative stress responses (9). Recent studies have indicated that dietary trehalose can enhance vasodilation and reduce arterial stiffness in older adults (10) and ameliorate inflammatory signaling in aged mice (11). Oxidative stress and inflammation are interconnected processes; elevated oxidative stress can lead to inflammatory responses, which in turn may exacerbate hepatic damage. This study hypothesizes that trehalose can simultaneously reduce oxidative stress and inflammation, thereby protecting liver function in aging rats (9-11).

Aging is characterized by increased oxidative stress and inflammation, which significantly contribute to liver dysfunction. Trehalose has been identified as a potential therapeutic agent that can counteract these processes, thereby preserving liver function and promoting healthier aging. Aging tissue accumulates senescent cells that, despite losing their capacity for healthy function and replication, resist apoptosis and release senescence-associated secretory phenotype factors, aggravating inflammation, and oxidative stress (16). These senescent cells contribute to an inflammatory environment associated with numerous age-related diseases, including liver disorders, where hepatocytes display significant gene expression changes with age (17). Additionally, the risk of liver diseases increases with aging, resulting in higher mortality from hepatic dysfunction (18, 19). This study aimed to investigate how oral trehalose supplementation affects gene expression related to liver oxidative stress and inflammation in adult and aged rats, with the hypothesis that trehalose administration can improve liver health by modulating these aging-related factors.

Methods

Materials

Bio Basic total RNA extraction kit (BS1361), Parstous cDNA synthesis kit (A101162), and Ampliqon SYBR green (A325402), were utilized in this study.

Experimental protocol

The animal protocol was approved by the guidelines of the Animal Care Committee of the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1400.089). All regulations and methods were performed according to the ARRIVE guidelines 2.0 (20).

Thirty-two male Wistar rats (Weight; Adult Control: 310.4 ± 26.1 g, Aged Control: 429.2 ± 47.5 g, Adult Trehalose: 307.4 ± 15.7 g, Aged Trehalose: 440.6 ± 33.5 g) were acquired from Tehran University of Medical Sciences and acclimatized in the Physiology Research Center. The rats were maintained in groups of four per cage under standard environmental conditions (23 ± 2°C, 12-hour light/dark cycle) with free access to food and water to acclimate to their surroundings. The experimental groups consisted of 1. Adult Control Group: Four-month-old adult with no interventions; 2. Aged Control Group: Twenty-four-month-old with no interventions; 3. Adult Trehalose Group (n = 8): Four-month-old adult rats supplemented with trehalose for one month; 4. Aged Trehalose Group (n = 8): Twenty-four-month-old rats supplemented with trehalose for one month. All groups received an identical standard diet, and the trehalose-treated groups (groups 3 and 4) were given water containing 2% trehalose instead (11, 21).

Tissue Sample Collection

After one month of treatment with Trehalose, the study ended, and following a 10-hour fasting period, the rats were anesthetized via ketamine (80 mg/kg) and xylazine (10 mg/kg) through intraperitoneal injection. Subsequently, the abdominal part was incised, and liver tissue was dissected from each rat. The liver was rinsed with PBS (pH 7.4), placed in microtubes, and immersed in liquid nitrogen (-80°C) to snap freeze and stored at -80°C for future evaluations.

RNA extraction and quantitative Real-Time PCR

We employed Quantitative real-time PCR to evaluate the relative expression of target genes in the liver tissue. Liver total RNA was extracted using the Total RNA Extraction Kit, according to the company's protocol. We used about 15 mg of liver tissue for RNA isolation. To synthesize complementary DNA (cDNA), we used one microgram of RNA. cDNA was produced using a specific Kit under optimized conditions as per the manufacturer's protocol. The synthesized cDNA was then subjected to real-time PCR using the SYBR Green Master Mix on a Step-One Plus Real-Time PCR System (Applied Biosystems). Each

reaction was performed in duplicate with the following components: 10 µL of SYBR Green Master Mix, 1 µL of forward and reverse primers (at a final concentration of 0.5 µM) (Table 1), and 2 µL of cDNA, final volume of 20 µL obtained by nuclease-free water. The thermal cycling conditions include an initial denaturation at 95°C (10 minutes) followed by 40 cycles at 95°C (15 seconds), and the annealing/extension at specific temperatures optimized for each primer set for 45 seconds. To validate the specificity of the amplified products, a melting curve analysis was employed post-cycling by gradually increasing the temperature from 60°C to 95°C while continuously measuring fluorescence. The expression levels of target genes were normalized to 18S rRNA (as housekeeping gene) and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (22-24).

Statistical analysis

Initially, normality tests were performed on the data (Shapiro-Wilk test). Subsequently, one-way analysis of variance (ANOVA) was performed, followed by the Tukey's post-hoc test for further analyses. The data are presented as mean ± SEM. *P* values less than 0.05 were considered statistically significant. The data were analyzed using SPSS software (version 25, SPSS Inc., IL, USA).

Results

The findings of the present study indicated that *PGC-1α*, *NRF2*, and *SOD* expression in the aged control group was decreased compared with the adult control group ($P = 0.01$, $P = 000$, and $P < 0.001$, respectively) (Figures 1-3). On the other hand, trehalose administration (aged trehalose group) compensated for the reductions and increased *PGC-1α* and *NRF2* levels (Figures 1 and 2). Interestingly, *SOD* level was significantly higher in aged trehalose group compared to the other studied groups (*P* values were 0.028 vs adult control, $P < 0.0001$ vs. aged control, and $P = 0.021$ vs. adult trehalose) (Figure 3). The expression of *HO1* did not show a significant change (Figure 4).

Administration of trehalose in the aged rats (aged trehalose group) attenuated the expression of *TNF-α* and *NF-κB* compared to the aged control group (Figures 5 and 6). Additionally, it resulted in a reduction in *IL-1β* and *TGF-β* expression; however, despite this, the levels of *IL-1β* remained significantly higher compared to the adult control group (Figures 7 and 8). Regarding *TGF-β*, although the expression in the aged trehalose group decreased compared to the aged control group, the levels remained significantly elevated compared to the adult control group.

Discussion

In this study, we aimed to evaluate the effect of trehalose on genes related to liver oxidative stress and inflammation in adult and aged Wistar rats. Our results highlighted the beneficial effects of trehalose, particularly in enhancing stress-resistance pathways and reducing pro-inflammatory signaling. We demonstrated that trehalose administration significantly enhances the expression of key antioxidant genes (*PGC-1α*, *NRF2*, and *SOD*) while concurrently reducing inflammatory markers (*NF-κB*, *IL-1β*, and *TNF-α*) in the liver tissues of aged Wistar rats. These results indicate that trehalose not only mitigates oxidative stress but also plays a crucial role in suppressing inflammation, thereby promoting overall liver health in an aging population.

Trehalose has attracted attention for affecting aging (9-13). Research indicates that oxidative stress is intrinsically linked to aging and contributes to age-related diseases, including liver dysfunction (6, 7, 10, 11). Trehalose uniquely activates cellular pathways that counteract oxidative stress and inflammatory responses, potentially offering therapeutic benefits in an aging context (25-29).

The present study established that trehalose administration has a profound effect on liver health by modulating gene expression related to oxidative stress and inflammation in both adult and aged rats. Specifically, the upregulation of *PGC-1α*, *NRF2*, and *SOD* underscores trehalose's role in promoting mitochondrial health and enhancing antioxidant defense mechanisms in the liver (6, 7). *PGC-1α* is a pivotal regulator of mitochondrial biogenesis and oxidative metabolism, while *NRF2* orchestrates the expression of antioxidant genes (30). The increased expression of these genes in both adult and

Table 1. The sequence of target gene primers used in this study

Gene	Reverse	Forward
PGC-1α	GACAAATGCTCTTTGCTTTATTGC	ACCCACAGGATCAGAACAAACC
NRF2	TTTGTTCCACCTCTCCATCAG	TAGCCCATCTCGTACCATCAC
SOD	TCTCCAACATGCCTCTCTTCATC	CCACTGCAGGACCTCATTTTAAT
HO1	CATGGCCTTCTGCGCAATCTTCTT	ACAGCACTACGTAAAGCGTCTCCA
NF-κB	CATCGGCTTGAGAAAAGGAG	AACACTGCCGAGCTCAAGAT
IL-1β	TGAGTGACACTGCCTTCCTG	AGGCTTCCTTGTGCAAGTGT
TNF-α	CCCATTTGGGAACTTCTCCT	AGATGTGGAACTGGCAGAGG
TGF-β	AGCCCTGTATTCCGTTCTCT	ATTCCTGGCGTTACCTTGG
18S rRNA	GGCCTCACTAAACCATCCAA	GCAATTATTCCCCATGAACG

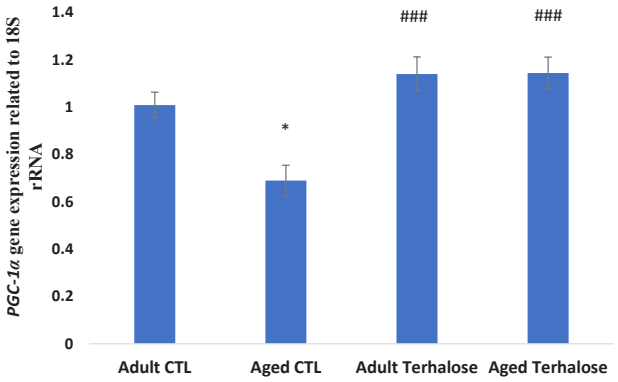

Figure 1. *PGC-1α* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for 1 month), and Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, * $P<0.05$. Statistically significant compared to the Aged CTL, ### $P<0.001$.

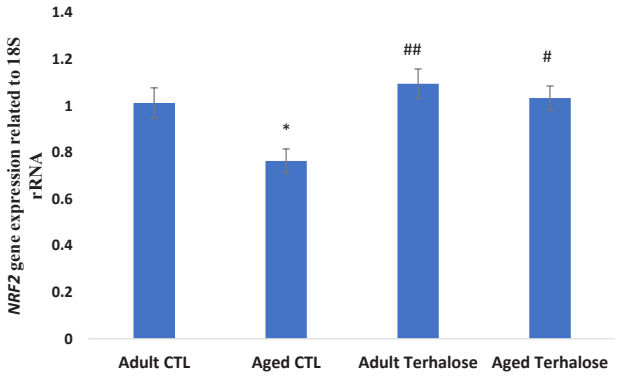

Figure 2. *NRF2* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24 months rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, * $P<0.05$. Statistically significant compared to the Aged CTL, # $P<0.05$, ## $P<0.01$.

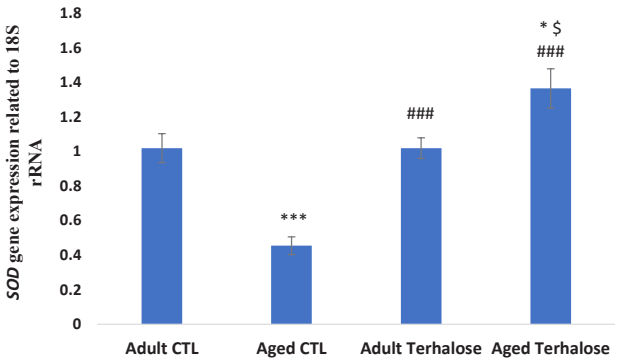

Figure 3. *SOD* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, * $P<0.05$, *** $P<0.001$. Statistically significant compared to the Aged CTL, ### $P<0.001$. Statistically significant compared to the Adult Trehalose, $ $P<0.05$.

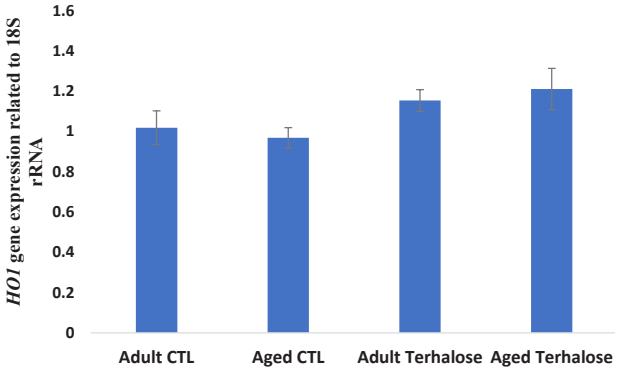

Figure 4. *HO1* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant.

aged rats suggests that trehalose supports mitochondrial function and enhances resistance to oxidative stress across different age groups. The unique finding that only aged rats exhibited elevated *SOD* expression in response to trehalose highlights the compound's potential to recalibrate age-compromised antioxidant defenses, aligning with studies that have reported similar enhancements in older models (12). On the contrary, Hozhabri et al demonstrated that treatment with trehalose did not affect reduced *SOD* activity in the aged rats (25).

In our analysis of inflammatory markers, trehalose exhibited significant anti-inflammatory effects, diminishing the expression of *NF-κB*, *IL-1β*, and *TNF-α*, which are key mediators in the inflammatory response (31). The ability of trehalose to attenuate these pro-inflammatory cytokines is consistent with findings in animal and cellular models that underscore its potential in preventing chronic inflammation associated with aging and liver disease (16, 27). Given that *NF-κB* is central to the inflammatory response, chronic activation can lead to inflammatory conditions commonly observed with aging. The attenuation of pro-inflammatory markers in both adult and aged rats indicates that trehalose not only attenuates age-associated inflammation but may also exert protective effects earlier in life, potentially establishing a foundation for healthier aging (16, 31). Of course, the authors point out that this conclusion is preliminary and needs more studies in experimental models and then clinical studies.

Moreover, the reduction in *TGF-β* gene expression in aged rats' underscores trehalose's role in fibrotic pathways, suggesting the potential to prevent the progression of fibrosis in aging liver tissue (26). These results indicate that trehalose not only ameliorates oxidative stress

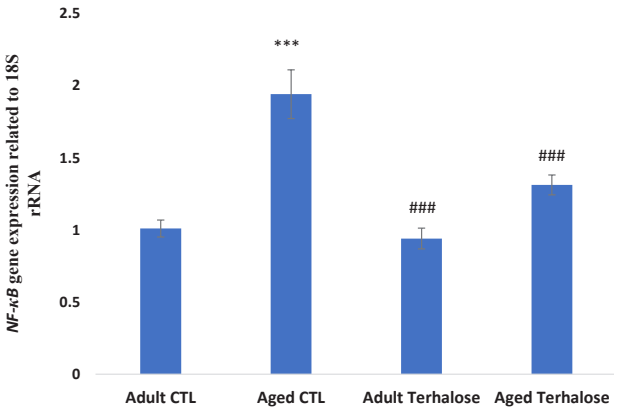

Figure 5. *NF-κB* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24 months rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, *** $P<0.001$. Statistically significant compared to the Aged CTL, ### $P<0.001$.

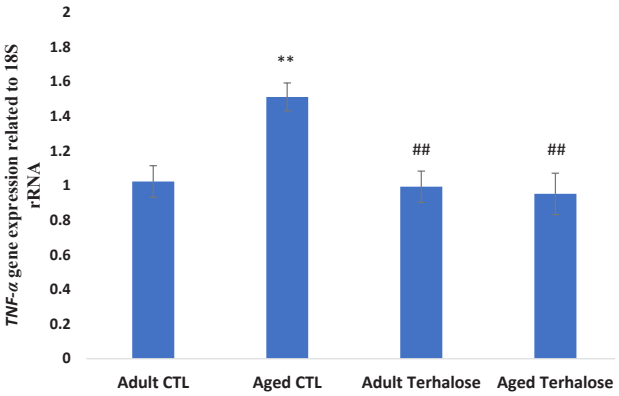

Figure 6. *TNF-α* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4 months rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, ** $P<0.01$. Statistically significant compared to the Aged CTL, ## $P<0.01$.

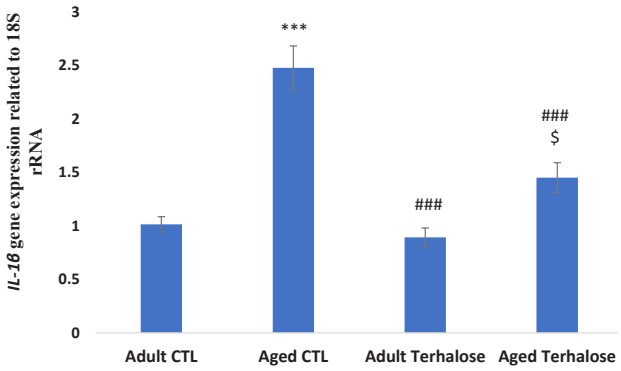

Figure 7. *IL-1β* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24-month-old rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), and Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, *** $P<0.001$. Statistically significant compared to the Aged CTL, ### $P<0.001$. Statistically significant compared to the Adult Trehalose, $ $P<0.05$.

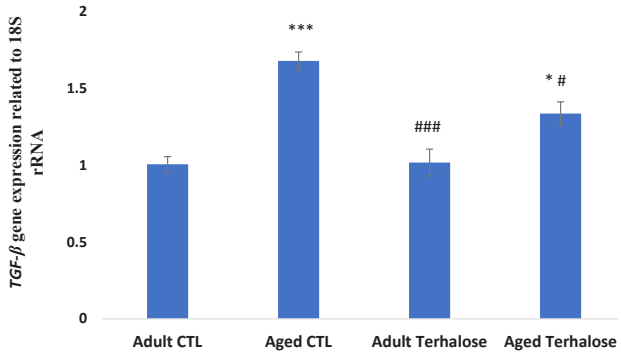

Figure 8. *TGF-β* gene expression quantified by real-time PCR in the liver of study groups, Adult CTL (4-month-old rats, no intervention), Aged CTL (24 months rats, no intervention), Adult Trehalose (4-month-old rats received 2% trehalose in drinking water, for one month), Aged Trehalose (24-month-old rats received 2% trehalose in drinking water, for one month). Data are expressed as Mean ± SEM. $P<0.05$ was considered statistically significant. Statistically significant compared to the Adult CTL, * $P<0.05$, *** $P<0.001$. Statistically significant compared to the Aged CTL, # $P<0.05$, ### $P<0.001$.

but also reduces detrimental inflammatory responses, enhancing liver health. Further, Wu et al reported that trehalose reduced *TGF-β1*-induced fibrosis in human stellate cells by activating autophagy, suggesting a novel perspective for exploring the progression of fibrotic lesions, which supports our findings (26). Similarly, Yu et al demonstrated that trehalose significantly suppresses inflammatory mediators in LPS-induced macrophages, highlighting its role in modulating inflammatory responses (27). In a clinical double-blind trial, Hashemian et al found the anti-inflammatory potential of trehalose in patients with type 2 diabetes (28). These reports also confirmed our finding, as trehalose attenuated inflammation in 24-month-old rats.

Despite the promising findings, our study has some limitations. The use of a specific rat strain may not fully represent the aging processes in humans or other animal models. Additionally, while we analyzed specific gene expressions related to inflammation and oxidative stress, a broader examination (incorporating histological examinations and protein levels) would provide a more comprehensive understanding of trehalose's mechanisms. Also, we did not measure water intake, and we are unaware of the effects of Trehalose on water consumption in rats. Therefore, measuring water intake should be considered in future studies. Furthermore, longer treatment durations could yield insights into the long-term efficacy and potential cumulative effects of trehalose on liver health.

Conclusion

Our findings indicate that trehalose exerted significant protective effects against liver oxidative stress and

inflammation in aged Wistar rats, enhancing the expression of antioxidant defense genes while concurrently suppressing inflammatory markers. The data support the potential of trehalose as a therapeutic agent for decreasing age-related liver dysfunction and improving health outcomes in aging populations. Future studies are warranted to expand on these findings and elucidate the underlying mechanisms, ultimately facilitating clinical applications in combating age-associated pathologies.

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Formal analysis: Beydolah Shahouzehi, Mahdis Rahimi Naiini.
Funding acquisition: Beydolah Shahouzehi.
Investigation: Beydolah Shahouzehi, Mahdis Rahimi Naiini, Mahdieh Nazari-Robati.
Methodology: Beydolah Shahouzehi, Mahdieh Nazari-Robati.
Project administration: Beydolah Shahouzehi, Mahdieh Nazari-Robati.
Supervision: Beydolah Shahouzehi.
Validation: Beydolah Shahouzehi, Mahdis Rahimi Naiini, Mahdieh Nazari-Robati.
Writing–original draft: Beydolah Shahouzehi, Mahdis Rahimi Naiini, Mahdieh Nazari-Robati.

Competing Interests
All the authors declare that they have no conflict of interest.

Ethical Approval
The animal procedure was approved in accordance with the guidelines of the Animal Care Committee of the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.AEC.1402.089). All methods were performed according to the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments) guidelines version 2.0.

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