ORIGINAL RESEARCH ARTICLE

# The Effect of Eight Weeks of Aerobic Training and Resveratrol Consumption on the Expression of p21 and p16 in the Liver Tissue of Rats with NAFLD

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## Abstract

**Background** Exercise training, resveratrol supplementation, and cellular aging are important issues in nonalcoholic fatty liver disease. The present study aimed to investigate the effect of 8 weeks of aerobic training and resveratrol consumption on the expression of some indicators of cellular aging in the liver tissue of a nonalcoholic fatty liver disease rat model.

**Methods** In the present experimental study, 35 eight-week-old male Wistar rats were randomly divided into two groups: patients with nonalcoholic fatty liver disease and a control group. Then, the rats in the control group were placed on a standard diet for 6 weeks, while to induce nonalcoholic fatty liver disease in the rats, the animals were placed on a high-fat diet for 6 weeks. The rats in the patient group were further divided into four experimental subgroups: non-alcoholic fatty liver, non-alcoholic fatty liver-exercise, non-alcoholic fatty liver-resveratrol, and non-alcoholic fatty liver-exercise-resveratrol. The rats in the exercise group underwent an eight-week (5 days/week) aerobic interval-training program. Rats in the supplement group were injected intraperitoneally with 20 mg/kg of resveratrol per day for 8 weeks.

**Results** The results showed a significant increase in the rate of changes in p21 expression in the non-alcoholic fatty liver ($p = 0.0001$), non-alcoholic fatty liver-exercise ($p = 0.002$), and non-alcoholic fatty liver-resveratrol ($p = 0.002$) groups compared to healthy controls, and also a significant increase in the rate of changes in p16 expression in the non-alcoholic fatty liver ($p = 0.0001$), non-alcoholic fatty liver-exercise ($p = 0.006$), and non-alcoholic fatty liver-resveratrol ($p = 0.003$) groups compared to healthy controls.

**Conclusion** Treating nonalcoholic fatty liver disease with exercise and resveratrol improves markers of liver aging in rats. Eliminating or reducing cellular markers using exercise and resveratrol may be an effective strategy for improving liver damage associated with nonalcoholic fatty liver disease.

**Keywords** Aerobic Training, Cyclin-dependent kinase inhibitor p16, Cyclin-dependent kinase inhibitor p21, Non-alcoholic Fatty Liver Disease, Resveratrol

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## 1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the excessive accumulation of fat in hepatocytes and can progress to severe liver disorders, including non-alcoholic steatohepatitis and cirrhosis.[1] Notably, both aerobic and resistance exercises have demonstrated efficacy in improving liver health by reducing hepatic fat and enhancing metabolic function, while resveratrol, a natural compound known for its antioxidant and anti-inflammatory properties, has shown promise in attenuating cellular aging by modulating key molecular pathways.[1]

Established risk factors for disease progression in NAFLD include advanced age and the presence of metabolic syndrome features such as obesity, insulin resistance, and hypertension.[2] However, the advancement of liver disease to cirrhosis is generally limited to a subgroup of patients with non-alcoholic steatohepatitis.[2] Genetic factors have also been shown to influence the progression of NAFLD.[3] The condition is often observed in patients with telomeropathies, suggesting that hepatic steatosis may be a consequence of hepatocellular aging. As demonstrated in animal models, NAFLD acts as a trigger for liver disease progression.[3] The stage of fibrosis and the overall progression of liver disease are strongly associated with cellular senescence. Moreover, the expression of hepatocytic p21, a key regulator of the induction and maintenance of cellular senescence, correlates with fibrosis stage in NAFLD and with the increased risk of hepatic complications and mortality.[4]

Sports activity plays a vital role in improving NAFLD by affecting telomeres and markers of cellular aging; however, the underlying mechanisms remain poorly understood. It has been shown that regular running attenuates age-related telomere shortening in hepatic and cardiac cells of mice aged 1 year or older. Furthermore, chronic exercise can counteract the downregulation of shelterin complex components, thereby contributing to telomere stabilization.[5] Studies examining the impact of physical training on NAFLD have reported that aerobic exercise improves hepatic structural damage, pathological changes, and lipid deposition in NAFLD mice.[6,7]

Werner et al. observed that six months of daily running in mice increased the levels of telomere-stabilizing cardiac proteins telomeric repeat-binding factor 2 and telomerase reverse transcriptase, while reducing the expression of aging-related proteins (Chk2, p53, and p16).[8] In recent years, several polyphenols with anti-aging properties, including tea polyphenols, curcumin, flavonoids, silymarin, and grape-derived resveratrol, have been extensively studied.[9] The antioxidant function of polyphenols is related to the mitigation of collagen degradation, which is associated with inflammation due to the release of matrix metalloproteinases, cytokines, and specific signaling pathways such as Nrf2, NF-κB, and Mitogen-Activated Protein Kinase.[10]

Resveratrol, a natural polyphenol found in plants such as Vitis vinifera, Cassia tora, and Polygonum Cuspidatum, has attracted considerable attention for its hepatoprotective effects in NAFLD in both experimental and clinical settings.[11] It may exert beneficial effects in patients with liver disorders[12] and has the potential to prevent and improve NAFLD.[13,14] However, whether resveratrol produces consistent effects on NAFLD in vivo and in randomized clinical trials, and the precise mechanisms underlying these effects, remain critical questions that warrant further investigation.

Based on these considerations, the present study hypothesizes that combining aerobic exercise with resveratrol supplementation may exert greater effects on aging-related indices than either intervention alone. Furthermore, the interaction between NAFLD and aging processes may have a significant impact on telomere dynamics and lifespan, yet few studies have examined the influence of NAFLD on factors affecting telomere alterations. Understanding the effects of aerobic exercise and supplements, such as resveratrol, on the aging process may provide new insights into the cellular mechanisms underlying NAFLD-related disorders and could contribute to the prevention and management of these diseases. Therefore, this study aims to investigate the effects of aerobic exercise and resveratrol on the expression of key modulators of cellular senescence in the hepatic tissue of NAFLD-induced rats.

## 2 Methods

### Study Design

In the present experimental study, 35 eight-week-old male Wistar rats were selected as samples and transferred to the research center. The sample size was determined based on previous studies, using a significance level of 5% (Type I error), a statistical power of 80% (Type II error), and MedCalc software version 18.2.1, resulting in seven rats per group.

Participants were included if they were healthy men who did not take any medications. Participants were excluded if they failed to complete the training program, did not receive the supplement, were female, or sustained an injury during training.

Rats were housed in transparent, autoclavable polycarbonate cages. Sterile wood shavings were used for bedding to absorb urine and feces and to provide comfort. Cages were cleaned and bedding replaced every other day. The animal room was maintained at a temperature of 20–24°C and a relative humidity of 55–

65%. A 12-hour light/dark cycle was precisely controlled by an automatic lighting system.

After transfer to the laboratory, NAFLD induction and treadmill familiarization were performed. Rats were then randomly divided into two main groups: the NAFLD group and the control (CN) group. Randomization was carried out using a random-number allocation method, in which each animal received a unique number, and group assignment was determined using a random-number table. This procedure minimized researcher bias and ensured group homogeneity.

Rats in the control group were fed a standard diet for 6 weeks (12% fat, 57% carbohydrates, 28% protein, and 3% other components). To induce NAFLD, rats in the disease group received a high-fat diet (HFD) for 6 weeks (22% fat, 50% carbohydrates, 24% protein, and 4% other components).[15] Following the induction phase, rats in the NAFLD group were further subdivided into four experimental subgroups:

Participants were divided into four groups: the Non-Alcoholic Fatty Liver Disease (NAFLD) group, the NAFLD with exercise training (NAFLDT) group, the NAFLD with resveratrol supplementation (NAFLDRSV) group, and the NAFLD with combined exercise training and resveratrol supplementation (NAFLDTRSV) group. Rats in the exercise groups performed an eight-week intermittent aerobic training program, 5 days per week, while the remaining animals did not participate in any exercise regimen.

### Exercise Protocol

Before the start of the main training program, rats in the exercise and exercise-supplement groups underwent a one-week familiarization period to adapt to treadmill running. During this phase, the animals ran for five consecutive sessions (one per day), each lasting 5 minutes at a speed of 8–10 m/min with a 0° incline.

The main training protocol was implemented for 8 weeks (Table 1). During the first week, running began at a speed of 15 m/min for 5 minutes. Each subsequent week, both the speed and duration of running were progressively increased by approximately 1–2 m/min and 1–2 min, respectively, so that by the fourth week, the rats reached a running speed of 20 m/min for 60 minutes per session. The exercise intensity and duration were maintained constant from week 4 through week 8. In addition, a five-minute warm-up and cool-down period was provided before and after each exercise session to prevent fatigue and ensure adaptation.[16]

### Supplement Administration

For resveratrol supplementation, 1 g of resveratrol powder (Nutrabio, USA; pharmacological grade, purity 99.87%) was prepared as a stock solution with a concentration of 10 mg/mL. For each administration, 100 µL of 7% ethanol or 10% DMSO in water was prepared for each rat, and resveratrol was suspended in this solution before injection. The mixture was administered intraperitoneally at a dose of 20 mg/kg body weight (at 9:00 a.m.) for 8 weeks in the NAFLDRSV and NAFLDTRSV.[17]

### Sampling and Processing

Forty-eight hours after the final training session and following 12–14 hours of fasting, the rats were anesthetized via intraperitoneal injection of a combination of ketamine (60 mg/kg body weight) and xylazine (60 mg/kg body weight). The liver tissue was immediately excised, rinsed with saline, and promptly placed into tubes containing RNA later to prevent RNA degradation. Samples were then transferred to liquid nitrogen and stored at –80°C until further analysis. To minimize circadian rhythm effects, all sampling was conducted between 8:00 a.m. and 11:30 a.m.

Gene expression analysis for each group was performed using the Real-Time PCR technique. First, primer design was completed, followed by total RNA extraction from the liver tissue. The extracted RNA was then reverse-transcribed into cDNA, which was subsequently amplified using PCR and analyzed for the expression of target genes (Table 2).

### Statistical Analysis

Descriptive statistics (mean ± standard deviation) and inferential statistics, including the Shapiro–Wilk test, Levene's test, one-way analysis of variance (ANOVA), and Tukey's post hoc test, were used to analyze the data. All analyses were performed using SPSS version 26, and statistical significance was set at $p \leq 0.05$.

**Table 1** Aerobic exercise protocol for rats with NAFLD

| Week | First | Second | Third | Fourth | Fifth | Sixth | Seventh | Eighth |
|---|---|---|---|---|---|---|---|---|
| Duration (min) | 5 | 20 | 40 | 60 | 60 | 60 | 60 | 60 |
| Intensity (m/min) | 15 | 17 | 19 | 20 | 20 | 20 | 20 | 20 |

**Table 2** Primer sequences

| Genes | Forward primers | Reverse primers |
|---|---|---|
| P21 | 5'- GTCTTGCACTCTGGTGTCTC-3' | 5'-ATAGAAATCTGTTAGGCTGGTCTG-3' |
| P16 | 5'-CGTACCCCGATACAGGTGATG-3' | 5'-ATACCGCAAATACCGCACGA -3' |

## 3 Results

To analyze the body condition of the rats during the study period, weight measurements were used as one of the main indicators of health. These data allow for accurate comparisons between the experimental and control groups. Table 3 presents the recorded weight values of the rats during the study period.

Data analysis revealed a significant difference in the changes in p21 expression (F = 14.900, P = 0.0001) in the liver tissue among the different groups (Table 4).

Furthermore, the post hoc analysis revealed a significant increase in p21 expression in the NAFLD (p = 0.0001), NAFLDT (p = 0.002), and NAFLDRSV (p = 0.002) groups compared to the CN group. A significant decrease in p21 expression was also observed in the NAFLDT (p = 0.030), NAFLDRSV (p = 0.037), and NAFLDTRSV (p = 0.0001) groups compared to the NAFLD group. Additionally, the NAFLDTRSV group showed a significant decrease compared to the NAFLDT (p = 0.045) and NAFLDRSV (p = 0.036) groups (Figure 1).

The data analysis showed a significant difference in hepatic p16 expression changes among the different groups (F = 15.536, p = 0.0001) (Table 5).

The results of the post hoc analysis showed a significant increase in hepatic p16 expression in the NAFLD (p = 0.0001), NAFLDT (p = 0.006), and NAFLDRSV (p = 0.003) groups compared to the CN group. Moreover, a significant decrease was observed in the NAFLDT (p = 0.021), NAFLDRSV (p = 0.044), and NAFLDTRSV (p = 0.0001) groups compared to the NAFLD group, as well as in the NAFLDTRSV group compared to the NAFLDT (p = 0.042) and NAFLDRSV (p = 0.020) groups (Figure 2).

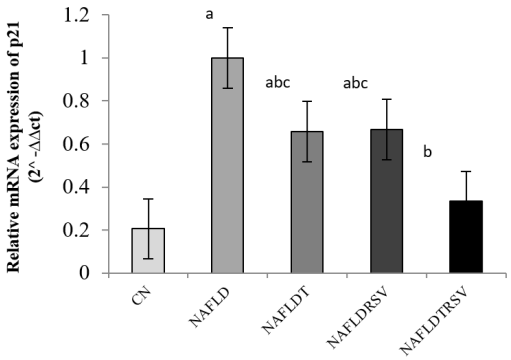


**Figure 1** Changes in p21 expression in the liver tissue across different groups analyzed by one-way ANOVA ($p < 0.05$).
a: Significant difference compared with the CN group; b: Significant difference compared with the NAFLD group; c: Significant difference compared with the NAFLDTRSV group.
CN: Control ، NAFLD: Non-alcoholic fatty liver disease ، NAFLDT: NAFLD – Exercise ، NAFLDRSV: NAFLD – Resveratrol ، NAFLDTRSV: NAFLD – Exercise + Resveratrol

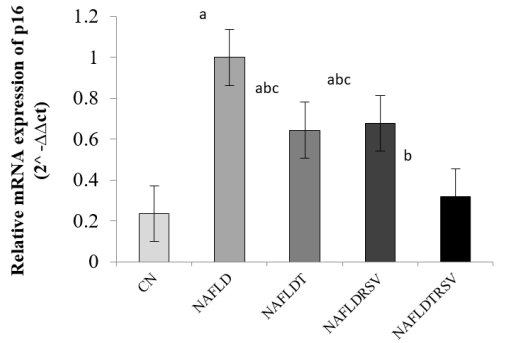


**Figure 2** Changes in p16 expression in the liver tissue across different groups analyzed by one-way ANOVA ($P < 0.05$).
a: Significant difference compared with the CN group; b: Significant difference compared with the NAFLD group; c: Significant difference compared with the NAFLDTRSV group.
CN: Control ، NAFLD: Non-alcoholic fatty liver disease ، NAFLDT: NAFLD – Exercise ، NAFLDRSV: NAFLD – Resveratrol ، NAFLDTRSV: NAFLD – Exercise + Resveratrol

**Table 3** Mean weight of different experimental groups

| Groups | CN (n = 7) | NAFLD (n = 7) | NAFLDT (n = 7) | NAFLDRSV (n = 7) | NAFLDTRSV (n = 7) |
|---|---|---|---|---|---|
| Weight (g) | 88/17 ± 13/223 | 16/16 ± 256 | 99/18 ± 5/259 | 06/14 ± 5/265 | 53/25 ± 75/249 |

CN: Control ،NAFLD: Non-alcoholic fatty liver disease، NAFLDT: NAFLD – Exercise، NAFLDRSV: NAFLD – Resveratrol، NAFLDTRSV: NAFLD – Exercise + Resveratrol

**Table 4** One-way ANOVA results for p21 expression in the liver tissue across different experimental groups

| | Sum of squares | Degrees of freedom (df) | Mean square | F-value | Significance level (p-value) |
|---|---|---|---|---|---|
| Between groups | 3/121 | 4 | 0/780 | 14/900 | 0/0001 |
| Within groups | 1/718 | 35 | 0/049 | | |
| Total | 4/839 | 39 | | | |

**Table 5** Results of one-way ANOVA for hepatic p16 expression among the different experimental groups Table 5 Results of one-way ANOVA for hepatic p16 expression among the different experimental groups

| | Sum of squares | Degrees of freedom (df) | Mean square | F-value | Significance level (p-value) |
|---|---|---|---|---|---|
| Between groups | 3/017 | 4 | 0/754 | 15/536 | 0/0001 |
| Within groups | 1/699 | 35 | 0/049 | | |
| Total | 4/716 | 39 | | | |

## 4 Discussion

The findings of the present study demonstrated that the induction of NAFLD led to a significant increase in hepatic expression of p16 and p21 in rats. Similarly, Kim et al. reported that obesity induced by HFD in mice caused renal cellular senescence, characterized by increased expression of p16 and p21, leading to impaired renal function and cortical oxygenation.[18] Previous studies have also shown that HFD and obesity promote the accumulation of senescent cells in adipose tissue, the pancreas, the liver, and the kidney.[19] Elevated cholesterol levels, lipid peroxidation, oxidative stress, and mitochondrial dysfunction are key triggers of cellular senescence associated with HFD and obesity.[20] Reactive oxygen species (ROS) may induce cellular senescence either by activating p21 via p53 or by upregulating p16INK4a through mitochondrial dysfunction mediated by the p38-MAPK pathway.[21] Additionally, adipokines that increase in response to obesity may directly induce cellular senescence.[22]

However, Yu et al. reported that HFD had no significant effect on cardiac senescence markers p21 and p53 in mice.[23] Such discrepancies might result from differences in tissue type, HFD induction protocols, or animal models used. Despite these inconsistencies, the current study revealed that aerobic exercise significantly reduced hepatic expression of p16 and p21 in HFD rats. In alignment with this, Schafer et al. demonstrated that exercise reversed the elevation of senescence markers induced by a fast-food diet, including p16, EGFP, β-galactosidase, and SASP, in adipose tissue.[24] Similarly, Rossman et al. reported that physical activity protects vascular function in older adults by modulating p53, p21, and p16.[25]

The beneficial effects of exercise on longevity are well established; however, the underlying biological mechanisms are not yet fully understood. Our findings confirm that aerobic exercise improves liver health and, more importantly, counteracts the deleterious effects of HFD. Three potential mechanisms may explain the observed reduction in senescence markers following exercise. First, the increased energy demand during exercise reduces fat storage, leading to smaller adipose depots and lower inflammation, which may subsequently downregulate p53, p21, and p16. Second, exercise may promote the clearance of senescent cells. In certain diseases, senescent cells can persist for decades;[26] however, exercise has been shown to decrease p16 expression concomitant with reduced obesity.[23] Third, exercise may exert protective responses against cellular aging factors, including DNA damage, telomerase shortening, oxidative stress, and mitochondrial dysfunction, across various cell types.[27] Collectively, these findings suggest that exercise prevents hepatic cellular senescence through multiple mechanisms.

Another key finding of this study was the significant reduction in hepatic p16 and p21 expression in NAFLD rats treated with resveratrol. It has been reported that most anti-aging dietary interventions target growth-promoting pathways such as IGF-1 and mTOR-S6K.[28] Resveratrol is thought to extend lifespan by modulating insulin signaling. Moreover, resveratrol activates FoxO and promotes its nuclear translocation, interacting with transcription-related proteins such as SIR-2.1, HCF-1, and FTT-2. This process enhances the expression of proteins associated with longevity and regulates nutrient signaling by decreasing food intake and suppressing the mTOR pathway. The interaction of multiple signaling routes, such as insulin receptor and mTOR pathways, enhances cellular processes related to lifespan extension, including DNA repair, autophagy, antioxidant and anti-inflammatory activities, stress resistance, and cellular proliferation.[29] The present study indicates that the combination of exercise and resveratrol exerts a synergistic and significant effect on cellular senescence markers.

This study had several limitations, including the inability to sample all tissues simultaneously, the use of forced exercise and resveratrol administration, and the relatively short duration of the intervention (eight weeks), which may not fully reflect long-term outcomes. Measuring oxidative stress markers could have further clarified the effects of exercise and resveratrol on p16 and p21 expression; however, this was not possible due to financial constraints. Future studies should include more diverse populations and improved methodologies to assess long-term outcomes. Such approaches would help ensure that findings are generalizable across different populations.

## 5 Conclusion

Our study indicates that NAFLD in rats leads to cellular dysfunction and an increase in markers of cellular senescence, suggesting a potentially important role in the progression of liver injury. Treatment with exercise and resveratrol improved metabolic and cellular function as well as hepatic senescence markers in rats. Therefore, eliminating or reducing cellular senescence markers through exercise and resveratrol supplementation may represent an effective strategy for alleviating NAFLD-related liver injury.

## Declarations

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**Artificial Intelligence Disclosure**
No artificial intelligence tools were used in the preparation and editing of this article.

**Authors' Contributions**
All the authors participated in all stages of the study, reviewed, and approved the final version of the manuscript. There are no disagreements regarding any part of this work.

**Availability of Data and Materials**
The data and materials used in this study are available from the corresponding author.

**Conflict of Interest**
The authors declare no conflicts of interest related to the publication of this article.

**Consent for Publication**
Not applicable.

**Ethical Considerations**
This study is derived from a Master's thesis in Exercise Physiology at the Islamic Azad University, Qaemshahr Branch (Tracking ID: 162977078). Moreover, the study was approved by the Ethics Committee of the Faculty of Medical Sciences, Islamic Azad University, Sari Branch, under the Code of Ethics IR.IAU.SARI.REC.1404.155.

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