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Scientific Research

Effects of mRNA, Protein Expression and Activity for Myocardial SOD2 by a Single Bout of or Long-Term Strenuous Endurance Exercise in Rats

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Abstract

Objective: To explore the effects of myocardial SOD2 by strenuous endurance exercise. Methods: 27 grown male SD rats were randomly divided into control group (C), a single bout of strenuous endurance exercise group (E1) and seventh-week strenuous endurance exercise group (E2). Real-time PCR was used to observe the changes of mRNA expression for myocardial SOD2. Western bolt was used to observe the changes of SOD2 protein expression. In addition, SOD2, T-SOD and SOD1 activity changes were observed. Results: Myocardial SOD2 expression level at mRNA and protein of Group E1, E2 was significantly higher than that in group C, and SOD2 and T-SOD activity in group E2 were significantly higher than those in group C. Those changes were more obvious in group E2. Conclusions: Strenuous endurance exercise can improve level of myocardial SOD2 expression at mRNA and protein, and enhance the activity for SOD2, thus increasing the activity for T-SOD. Effect of long-term strenuous endurance exercise was better than a single bout of one.

Keywords

SOD2, Strenuous Endurance Exercise, Myocardia

1. Introduction

In recent years, with the development of economy and society, people's diet and lifestyle change, and heart

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health is also seriously threatened, *i.e*., the incidence of ischemic heart disease was on the rise "year by year". Therefore, heart health maintenance has always been an important issue. According to the researches, the damage of Reactive Oxygen Species (ROS) plays a considerable role in the process of myocardial pathological changes. It is of great significance to maintain the heart health, such as the prevention of ischemic cardiomyopathy, to increase the ability of heart's antioxidant as well as to effectively remove ROS. Numerous studies have indicated that appropriate exercises can promote heart health and resist to myocardial ischemia-reperfusion injury [1]-[4]. What's more, according to powers and other researchers, the effect of exercise on heart health is closely related to the improved myocardial antioxidant capacity [5]. However, it has yet to explore the improved myocardial antioxidant capacity caused by exercise and cardioprotective effects.

As it's known to all, enzymatic defensive system acts as a considerable part in body issue's antioxidant and ROS removal, among which Superoxide Dismutase (SOD) is regarded as the first protective barrier to defense ROS. Therefore, the improvement of SOD activity marks the myocardial antioxidant capacity enhancement. A great number of researches have shown that exercise can improve the activity of myocardial Total Superoxide Dismutase (T-SOD). However it is not difficult to find that exercise models in studies concerned focus on the high-intensity intermittent exercise or moderate-intensity endurance exercise model; other possibly existed and targeted suitable exercise models needed further exploration. On the other hand, SOD has two types in higher animal vivo, one being Cu, Zn Superoxide Dismutase (SOD1) existed in cytoplasm, another Manganese Superoxide Dismutase (SOD2) in mitochondria; therefore, strengthening in-depth study of SOD types will contribute a further understanding of protective effects of exercise on the heart and its possible mechanisms. Since mitochondria play a crucial role in the maintenance of cardiomyocytes normal function and structural integrity. In addition, they are main sites of ROS production. Theoretically, the increase of SOD2 function has a significant effect on ROS removal as well as myocardial protection. Taking the above reasons into consideration, this study is to observe whether strenuous endurance exercise can accelerate the improvement of myocardial SOD2 expression at mRNA and protein as well as their activity level or not, trying to prove that this effect can be enhanced by long-term endurance exercise. It's also expected to rich the theory of exercise's protective effect on the heart, and to improve practical applications of sports and fitness on cardioprotective effects.

2. Materials and Methods

2.1. Research Objects

27 grown male Sprague Dawley (SD) rats (SPF grade), weighting 146 ± 15 g, are bought from Guangxi Medical University Laboratory Animal Center. They are raised in standard rodent cages, feeding freely. Favorable ventilated conditions, controlling room temperature at about 27˚C and natural light are provided.

2.2. Experiment Methods

2.2.1. Groups and Exercise Modes

After one week adaptive feeding, 27 grown male SD rats were randomly divided into control group (group C, n = 9), a single bout of strenuous endurance exercise group (group E1, n = 9) and seventh-week strenuous endurance exercise group (group E2, n = 9). Group E1 and E2 trained endurance running on treadmills (Type DSPT 202 made by Hangzhou Litai S & T Co. Ltd.). Before formal training, the both groups firstly conducted three-day adaptive treadmill exercise (0˚, 15 m/min), and then strenuous endurance running at a speed of 22 m/min and gradient of +10˚ (85% $VO2_{max}$) for 30 min daily referred to Bedford and other researchers standards [6]. Group E1 only exercised one day while group E2 exercised 6 days/week (Sunday off) for continuous 7 weeks.

2.2.2. Drawing Materials

Intraperitoneal anesthesia with urethane (1.2 g/kg) were performed when group E1 after exercise within 24 h - 48 h, group E2 after the last exercise within 24 h - 48 h together with group C, and then hearts were quickly taken and ventricular myocardia were cut, washing with PBS. Ventricular myocardia were cut into small pieces during an ice-bath. Most ventricular myocardial pieces, placed immediately into the −80˚C ultra-low temperature refrigerator after liquid nitrogen flash freeze, were used for determining SOD2 expression at mRNA and protein; that remaining small portion was used for the observation of SOD activity after weighing (about 100 mg) and recording.

2.2.3. Real-Time Fluorescent Quantitative PCR

SYBR Green I method was adopted, with necessary reagent provided by American Life Technologies Co. Ltd. Real-time PCR primers were synthesized by Invitrogen Corporation Shanghai Representative Office.

Upstream of SOD2: AAGGAGAGTTGCTGGAGGCTATC

Downstream: CTCCTTATTGAAGCCAAGCCAG

Upstream of *β*-actin: CATTGTCACCAACTGGGACGATA

Downstream: GGATGGCTACGTACATGGCTG

1) RNA extraction

A portion of frozen myocardial tissue was taken, weighted (100 mg) and recorded. Grind into powder after adding liquid nitrogen, and then the total myocardial RNA were immediately extracted under the guidance of Trizol kit instructions after the liquid nitrogen were evaporated.

2) Synthesize cDNA by Reverse Transcription (RT)

5 μl (5 μg) of extracted myocardial RNA samples, together with 0.5 μl of Oligo (dt) primer (50 uM), 0.5 μl of random primer, 1 μl of dNTP Mix (10 mM), and 5 μl of DEPC-treated water added one after another, gets a Mix I of 12 μl, 5 min warm bath at 65˚C and then 1 min ice bath at once. Afterwards, 20 μl of Mix II, received by adding 4 μl of 5× First-Strand buffer, 2 μl of 0.1 M dTT, 1 μl of RNaseout (40 U/μl), and 1 μl of SuperScrip III RT (200 U/μl) into Mix I, was treated with 5 min bath at 25˚C, 60 min bath at 50˚C and 15 min bath at 70˚C. The resulting cDNA was placed on ice immediately.

3) Real-time PCR

PCR solution was carried out PCR amplification reaction in real-time PCR instrument (Type 7500 Fast, Life technologies' product). The real-time PCR reaction system is: 14 μl of ultrapure water, 2 μl of 10× PCR buffer solution, 1 μl of magnesian ion (50 mM), 0.5 μl of upstream primer (10 uM), 0.3 μl of SYBR (20×) fluorescent dye, 0.5 μl of downstream primer (10 uM), 0.2 μl of Taq DNA Polymerase, 1 μl of template, accounting for 20 μl. Reaction parameters are: initial denaturation for 2 min at 95˚C; 40 PCR cycles (95˚C, 10 S; 60˚C, 30 S; 70˚C, 45 S). During this course, Ct values (cycled threshold values) of tested products were recorded when fluorescence signal intensity was significantly enhanced; three wells were made in each test sample, calculating the average of each sample's Ct value. Continue to slowly heat from 70˚C to 95˚C after completion, and then melting curves of PCR products were established in order to observe the specificity of amplified products.

After that, the Relative Expression (RE) of target gene SOD2 mRNA was calculated by the $2^{-\Delta\Delta Ct}$ Method [7], using housekeeping gene *β*-actin as reference gene. Average value of each sample was taken as Ct, $\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})$, $\Delta\Delta Ct = (Ct_{\text{experimental group}} - Ct_{\text{control group}})$, $2^{-\Delta\Delta Ct}$ was RE of mRNA for SOD2.

2.2.4. Western Bolt for Myocardia SOD2

The remaining frozen myocardial tissues, weighted and recorded, were speedily ground into powder with liquid nitrogen. They were immediately placed in EP tube after evaporation, and then RIPA lysate (a concentration of 1 mM) formulated from PMSF buffer was added to fully lyse in the proportion of 20 mg of myocardial tissues to 200 μl of lysate (protein extraction reagent); supernatant was collected by hypothermic centrifugal machine (centrifugal force 10,000 - 14,000 ×g, 3 - 5 min). BSA (Bicinchonininc acid) Method was adopted to determine the total protein concentration.

50 μg of myocardial total protein, using prestained maker as protein molecular weight marker, were carried out 12% SDS-PAGE protein electrophoresis (constant pressure electrophoresis with separating gel at 80 V and stacking gel at 100 V) until the front edge of color marker-bromophenol blue stopped electrophoresis when went down to the end of gel. The separated protein bands (0.20 μm of SOD2 and 0.45 μm of i-actin) were transferred to a PVDF membrane by wet cell transmembrane method (250 mA constant current for 1 h). 5% BSA buffer was closed at room temperature for 2 h. After that, SOD2 primary antibodies (diluted rabbit anti-human polyclonal antibody at the ratio of 1 to 1000) and *β*-actin primary antibodies (diluted mouse anti-human monoclonal antibody at the ratio of 1 to 1000) were added into these two membranes correspondingly. Incubate them overnight at 4˚C, and then wash the membrane with TBST three times (10 min/time). Secondary antibodies marked by rabbit anti-horseradish peroxidase (dilute at the ratio of 1 to 2000) and by mouse anti-horseradish peroxidase (dilute at the ratio of 1 to 2000) were mixed in correspondingly. The two mixtures were incubated on the shaking table at room temperature for 1 h, and then washed with TBST three times (10 min/time) as well as developed with chemiluminescence. Electrophoretic bands were treated by Gel-pro analyzer software to analyze the gray value of each band area, the ratio of target band (SOD2)'s grey value to reference band (*β*-actin)'s grey

value representing the expression level at target protein.

For the main reagents used in experiments, RIRA lysate, BCA kit as well as ECL kit were bought from Shanghai Beyotime Biotechnology Co., Ltd., PMSF buffer is provided by Amresco Company, BSA by Sigma-Aldrich Co. LLC; β-actin primary antibodies, secondary antibodies labeled rabbit anti-horseradish peroxidase as well as secondary antibodies labeled mouse anti-horseradish peroxidase were bought from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. while SOD2 primary antibodies from Epitomics Biotechnology Co., Ltd.; TBST from Beijing Solarbio Science & Technology Co., Ltd. Electrophoresis was Powerpac Basic Electrophoresis bought from American Bio-Rad Laboratories, Inc.

2.2.5. Determination of Myocardial SOD2 Activity

10% homogenates were made and then supernatant was collected by centrifuge (4000 rpm, 30 min). SOD1 and T-SOD activity were detected with hydroxylamine method, and finally SOD2 activity was obtained by T-SOD activity minus SOD1 activity. SOD kits were bought from Nanjing Jiancheng Biotechnology Co., Ltd.

2.2.6. Statistical Analysis

Experimental data were presented as "mean ± standard deviation" ($\overline{X} \pm S$). Statistical analysis was completed by SPSS for windows version 17.0 software. The experimental results were all analyzed with One-Way Analysis of Variance (ANOVA). After-effect test was conducted by Least-Significant Difference (LSD) Method in the case of homogeneity of variance, otherwise by Tamhane's T2 Method. $P < 0.05$ was regarded as the standard of significant differences.

3. Results

3.1. Comparisons of RE of mRNA for Myocardia SOD2 in Rats

Melting curve of amplification products in SYBR Green I real-time PCR reflects that solubility changes with temperature, with temperatures as ordinate against solubility as abscissa. In this study, melting curve of both myocardia SOD2 gene and myocardia β-actin gene in rats only appeared a single peak; besides, the melting temperature (Tm) were about 85.35˚C and 88.43˚C respectively, approximately nearing to the annealing temperature of the corresponding products: amplification products were in preferable specificity.

Real-time PCR amplification curve was used cycles as ordinate against real-time fluorescence intensity as abscissa during PCR. According to statistics, RE of mRNA for myocardia SOD2 in group E1 and E2 was obviously higher than that in group C (control group), marking significant differences; RE in group E2 were more distinct than those in group E1, marking significant differences (**Table 1**; **Figure 1**).

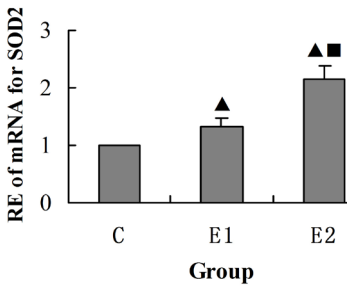

Figure 1. Diagram of changes in RE of mRNA for myocardia SOD2 in rats. ▲Compared with group C, $p < 0.05$; ■Compared with group E1, $p < 0.05$.

Table 1. Changes in RE of mRNA for myocardia SOD2 in rats.

Group	N	SOD2 mRNA
C	9	1.00 ± 0.00
E1	9	1.32 ± 0.16[a]
E2	9	2.16 ± 0.23[ab]

[a]Compared with group C, $p < 0.05$; [b]Compared with group E1, $p < 0.05$.

3.2. Changes in RE of Protein for Myocardia SOD2 in Rats

Group E1 and E2 myocardia SOD2 expression level at protein was clearly improved compared with group C, marking meaningful differences; levels in group E2 were more distinct than those in group E1, marking meaningful differences (**Figure 2**; **Table 2**; **Figure 3**).

3.3. Changes of Activity Level for Myocardia SOD2 in Rats

SOD2 activity was gotten by T-SOD activity subtracting the SOD1. The activity of myocardia SOD2 and T-SOD in group E1 and E2 was clearly improved compared with group C, marking significant differences; improvements in group E2 were more distinct than those in group E1, marking significant differences (**Table 3**; **Figure 4**).

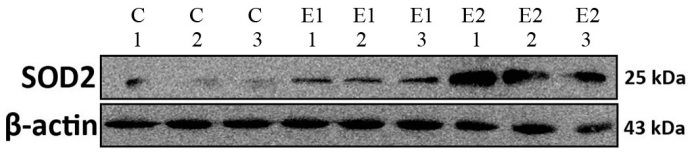

Figure 2. Diagram of western blotting for myocardia SOD2 in rats. 1, 2, 3, three different individual samples in each group rats for C, E1, E2.

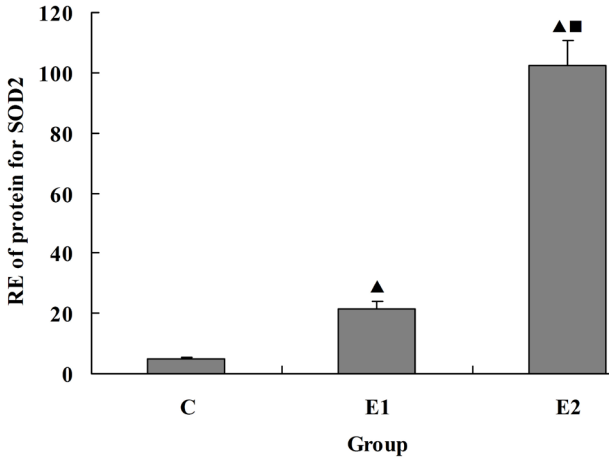

Figure 3. Diagram of changes in RE of protein for myocardia SOD2 in rats. ▲Compared with group C, $p < 0.05$; ■Compared with group E1, $p < 0.05$.

Table 2. Changes in RE of protein for myocardia SOD2 in rats.

Group	n	SOD2
C	9	4.743 ± 0.652
E1	9	21.367 ± 2.787[a]
E2	9	102.424 ± 8.336[ab]

[a]Compared with group C, $p < 0.05$; [b]Compared with group E1, $p < 0.05$.

Table 3. Changes in activity of myocardia SOD2, T-SOD in rats (U/mg·prot).

Group	n	SOD1	SOD2	T-SOD
C	9	77.820 ± 8.955	39.653 ± 6.032	117.473 ± 13.852
E1	9	76.674 ± 9.743	54.274 ± 7.257[a]	131.948 ± 14.285[a]
E2	9	77.221 ± 9.724	79.314 ± 8.456[ab]	156.535 ± 15.755[ab]

[a]Compared with group C, $p < 0.05$; [b]Compared with group E1, $p < 0.05$.

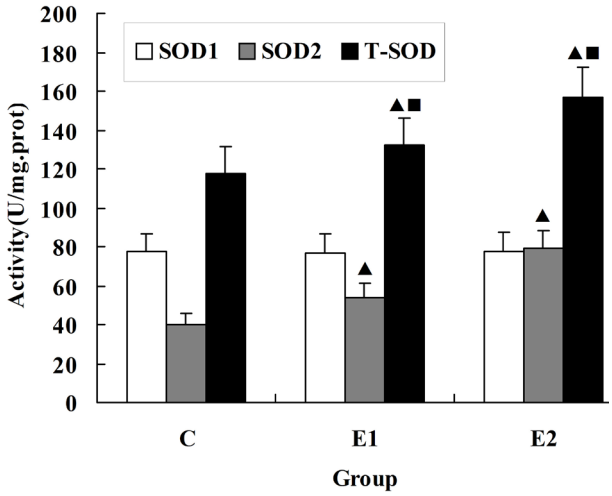

Figure 4. Diagram of changes in activity of myocardia SOD2, T-SOD in rats. ▲Compared with group C, $p < 0.05$; ■Compared with group E1, $p < 0.05$.

4. Discussion

Studies on the impact of exercise on the heart earliest started in an anatomy find, Robinson, an Englishman, pointed out that relative weight of heart in high-intensity physical activity animal was heavier than that in low-intensity physical activity animal in 1748. As humans, Henschen, a Swedish scholar, first discovered the enlargement of skier's heart by percussion in 1899, and then put forward the term of "Athlete's Heart". Afterwards, the field of sports medicine had committed to the study of "effect of exercises on cardiac structure and function", and gradually penetrated into the field of molecular biology. Up to now, it's generally viewed that the exercise is a "double-edged sword" for excessive exercises may be harmful to heart health while moderate exercises are beneficial to it, being able to pre-treat various cardiovascular diseases. In the past reports about "exercise and heart health", most intervention models were moderate-intensity endurance exercise. In recent years, some researches had been conducted by using high-intensity intermittent exercise as intervention model at home, e.g. some references pointed out that intermittent high-intensity exercise can protect the heart and improve myocardial tolerance of ischemia [8] [9]. However, it still needed to build more sports models for reference how to enhance heart health by rationally using physical exercise with targets, such as the improvement of myocardial antioxidant capacity. What's more, this study suggested that excessive exercise, such as intermittent high-intensity exercise, may not be practical for the old and infirm as well as for the crowd urgently needing for sports and fitness, and was somewhat difficult to popularize. That is why endurance exercise was more widespread in physical exercise at present. Taking these conditions into consideration, this study chose the model of "a single bout of or seven-week strenuous endurance exercise" to investigate the effects of myocardial antioxidase SOD by strenuous endurance exercise. It's clear that there were two kinds of SOD in myocardial tissue, namely SOD1 and SOD2.

According to this study, a single bout of or seven-week strenuous endurance exercise can outstandingly improve the level of myocardial SOD2 expression at mRNA and protein. SOD's main function was to remove ROS in vivo, which was mainly produced in mitochondria, where also lay SOD2, a type of SOD. In this study, the increase of myocardial SOD2 expression level may be adaptive changes of ROS increase and oxidative stress stimulate made by mitochondrial. It was found that a single bout of long-term high-intensity endurance exercise can give rise to the increase of myocardial malondialdehyde (MDA) content [10], suggesting the increase of myocardial oxidative stress level. The author considered that the improving myocardial oxidative stress level after exercise resulted from exercise-induced myocardial ischemia and hypoxia. In one recent study, rats were carried out a single bout of strenuous endurance exercise at a speed of 19 - 21 m/min and gradient of +10˚ (80% $VO2_{max}$) to "exhaustion" [11]. And then they found that rats' ECG presented characteristic changes of myocardial ischemia and myocardial stunning, and myocardia had ischemic necrosis, both of which showing that exercise can induce myocardial ischemia [11]. In addition, other similar studies showed that a single bout of exhaustive strenuous endurance exercise can induce myocardial ischemia in rats [12]. The model of a single

bout of "exhaustive" strenuous endurance exercise in the above references, differing from that of a single bout of or long-term strenuous endurance exercise with quantitative loads in the present study, still suggested the existence of myocardial ischemia phenomenon by strenuous endurance exercise. Great amount of studies had proved that effects of exercise-induced myocardial ischemia and hypoxia on myocardial structure and function were bitterly similar to the clinical pathological myocardial ischemia; Large quantity of ROS were generated and oxidative stress levels were significantly increased in myocardium in the process of exercise-induced myocardial ischemia (hypoxia) and reperfusion. Distinguished from clinical pathological myocardial ischemia, exercise-induced myocardial ischemia was relative myocardial ischemia and hypoxia due to the high level of myocardial metabolic as well as initiative ischemia and hypoxia occurred under the protective monitoring system [13]. When cannot tolerate hypoxia, the body will reduce the degree of ischemia and hypoxia by reducing exercise intensity or stopping exercise, thus avoiding myocardial irreversible damage resulted from excessive ischemia and hypoxia [13]. Therefore, combined this study's results and analyses, the author held that relative myocardial ischemia and hypoxia of reasonable duration caused by strenuous endurance exercise gave impetus to a certain amount of ROS and some degree of oxidative stress in mitochondria, which were extremely sensitive to ischemia, as long as exercise will not be too durative. Adaptive changes, occurring after mitochondria were stimulated by oxidative stress, manifested as increased expression at SOD2 mRNA as well as at protein. Their heighten guarantees the increase of activity with the consequences of enhanced mitochondrial elimination ability of ROS. Furthermore, from the comparisons of changes in a single bout of strenuous endurance exercise group (group E1) and seven-week strenuous endurance exercise group (group E2), those changes were more obvious in group E2, which demonstrated that the improving amount of SOD2 mRNA and protein expression of long-term endurance exercise was clearly more than that of a single bout of exercise.

Yamashita and other researchers certified that long-term moderate-intensity endurance exercise increased the activity of myocardial SOD2 by antisense oligonucleotides technology [14]. Hou and other scholars detected that myocardial SOD2 activity in rats can be improved by swimming without load, 1 h per day for three days while SOD1 activity was unchanged [15]. In this study, a single bout of or seven-week strenuous endurance exercise gave rise to distinguished improvement of SOD2 and T-SOD activity; meanwhile, SOD1 activity showed no significant change. Some references discovered that endurance exercise stepped up muscle SOD2 protein expression and activity [16] [17]. Moreover, in this study, myocardial SOD2 activity trends were consistent with those of SOD2 mRNA and protein expression. Therefore, exercise can increase myocardial SOD2 protein expression, enhance its activity as well as improve T-SOD activity by stimulating SOD2 mRNA expression in this study. Moreover, it was found that both the increasing level of myocardial mRNA and protein expression and improvement level of SOD2 and T-SOD activity after seven-week strenuous endurance exercise were much better than those after a single bout of strenuous endurance exercise, which revealed that long-term endurance exercise can improve exercise effects.

5. Conclusions

Strenuous endurance exercise can stimulate myocardial SOD2 expression at mRNA and protein and enhance the activity for SOD2, thus increasing the activity for T-SOD.

The improvements of myocardial mRNA and protein expression as well as the activity for SOD2 by seven-week strenuous endurance exercise were all more obvious than those by a single bout of strenuous endurance exercise, demonstrating that long-term endurance exercise can improve exercise effects.

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List of Abbreviations

Reactive Oxygen Species (ROS); Superoxide Dismutase (SOD); Total Superoxide Dismutase (T-SOD); Cu, Zn Superoxide Dismutase (SOD1); Manganese Superoxide Dismutase (SOD2); Sprague Dawley (SD); Reverse Transcription (RT); Relative Expression (RE); Analysis of Variance (ANOVA); Least-Significant Difference (LSD); Temperature (Tm); Malondialdehyde (MDA).

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