The Simultaneous Effects of Aerobic Training and Octopamine on the Mitophagy of the Brown Adipose Tissue after the Induction of Intoxication by Deeply Heated Oil in Male Wistar Rats

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Introduction: Deep heated oils produce toxins that pose significant risk to the human health. The present study aims to investigate the simultaneous effects of aerobic training and octopamine consumption on the mitophagy of the brown adipose tissue after the induction of intoxication by deeply heated oil in male Wistar rats.

Methods: This study was conducted on 40 male Wistar rats after four weeks of feeding with heated oil. The animals were randomly assigned to the groups of control-intoxication (CI; n=8), exercise training-intoxication (ETI; n=8), supplement-intoxication (SI; n=8), supplement-exercise-intoxication (SEI; n=8), and healthy control (HC; n=8). Exercise training was performed for four weeks at the intensity of 50-65% VO2_peak for 20 minutes per session. Octopamine was used as the supplement for four weeks. In addition, intraperitoneal injection was carried out with the dose of 81 µmol/kg five days per week in the CI, SI, and SEI groups. Real-time polymerase chain reaction (RT-PCR) was applied to evaluate the expression of the PTEN-induced kinase 1 (PINK1) and lysosome-associated membrane protein 2 (LAMP2A) genes.

Results: No significant difference was observed in the PINK1 gene expression between the CI, ETI, and SEI groups (P>0.05), while a significant difference was observed between the CI, ETI, and SEI groups with the HC in this regard (P≤0.05). The LAMP2 gene expression significantly was higher in the HC group compared to the other groups (P≤0.05). In addition, the LAMP2 gene expression in the SEI group was significantly higher than the HC group (P=0.001).

Conclusion: According to the results, the combination of aerobic training and octopamine supplementation led to mitophagy induction through intoxication with heated oils by increasing the expression of the LAMP2A gene and induction of chaperones by the activation of the chaperones with binding to the LAMP2 receptor protein in the lysosome.

Introduction

Obesity is associated with several metabolic diseases, such as type II diabetes, dyslipidemia, hypertension, cardiovascular diseases, and some cancers(1). Lifestyle changes are a consequence of modernity that has caused obesity among the younger population (2). Obesity is associated with several metabolic diseases, and some cancers (3). While cooking with heated oils, acrylamide is released from starch at higher doses than the recommended limits (4). Heterocyclic amine is another toxin that is produced in this process, which is formed through the heating and cooking process form the protein substances when amino acids and creatine simultaneously react at high cooking temperatures (barbecued/fried foods) (5). The findings in this regard have indicated that acrolein-induced oxidative stress directly influences the cardiovascular health of rats due to apoptosis in the cardiomyocyte cells. Furthermore, acrolein affects the mitochondrial ability as a component of cellular respiration (6). Other reports have shown that acrolein may...
exert inhibitory effects on some cellular respiratory components, such as complex I and II, pyruvate dehydrogenase, and Alpha-Ketoglutaric dehydrogenase in the liver cells of rats.(5).
Recently, attempts have been made to diminish the adverse effects of acrolein in the heated oils through exercise training and its synergistic effects with supplementations(6). Today, herbal supplements are considered highly effective in the reduction of oxidative stress and inflammation (7).
Octopamine supplementation has been reported to have antioxidant properties. Octopamine is used as a food ingredient, as well as medicinal and dietary supplementation (8). Octopamine is an adrenergic substance with a sympathetic function. Weight loss and adrenergic actions are associated with metabolic changes (9). Some of the main functions of octopamine are in antioxidant and anti-inflammatory processes, weight loss, fat burning, and anticancer treatments(10). On the other hand, recent findings have indicated that exercise training could decrease oxidative stress and inflammation and improve antioxidant enzymes (11,12). Meanwhile, some studies have reported that exercise training has no significant impact on inflammation and oxidative stress (13). In this study, we used an antioxidant supplement during exercise training in order to reduce inflammation and control diseases (14).
According to the literature, the increased production of reactive oxygen species (ROS) and respiratory chain dysfunction might be associated with decreased PTEN-induced kinase 1(PINK1) activity (15). The reduction of PINK1 expression leads to higher oxidative stress (16), which in turn could lead to apoptosis. The PINK1 protein is composed of a serine/threonine kinase activity, which is located in the mitochondrial membrane and protects cells from ectopic apoptosis (17). On the other hand, the lysosome-associated membrane protein 2 (LAMP2) is a lysosome-associated membrane glycoprotein, which plays a key role in the incidence of cancer and is expressed on the surface of cancerous tumors, especially the cells of highly metastatic cancer, such as colon cancer and melanoma(18). A mitophagy mechanism involves functioning through PINK1 and LAMP2α(10).
Data is scarce regarding the effects of octopamine consumption in the form of an herbal extract on the induction of the mitophagy caused by the use of heated oils as the use of such oils has been on the rise in various food products. The present study aimed to determine whether aerobic exercise training and octopamine supplementation could affect the mitophagy of the brown adipose tissue after the induction of intoxication by deeply heated oil.

Materials and Methods

Experimental Animals
This study was conducted on 40 male Wistar rats (age: 20 weeks, weight: 300-350 g), which were provided by Pasteur Institute of Iran. The rats were randomly assigned to the groups of control-intoxication (CI; n=8), exercise training-intoxication (ETI; n=8), supplement-intoxication (SI; n=8), supplement-exercise-intoxication (SEI; n=8), and healthy control (HC; n=8). The animals were kept in standard laboratory conditions at the approximate temperature of 25°C and relative humidity of 45% within a 12-hour dark-light cycle until completing the experiment and exercise training. All the animals had free access to water and feed standard for laboratory animals.
The study protocol was in accordance with the Principles of Laboratory Animals and approved by Kurdistan University of Medical Sciences (code: IR.MUK.REC.1398/5006).

Preparation of the Rations with Hot Oil
Initially, eight liters of sunflower oil was heated for four consecutive days eight hours per day at the temperature of 190-200°C (3), and every 30 minutes, foods such as chicken nuggets, potatoes, chicken, and protein products (e.g., sausages), were immersed in the oil. On the fourth day, the oil was preserved until the start of the experiment to be used as the intoxication agent and administered orally to the animals via gavage as the feed for four weeks.

Aerobic Exercise Training
The exercise training program was conducted for four weeks at moderate intensity every other day. The intensity of exercise training in the first week reached 50% of the maximum consumed oxygen, while it reached 65% of the maximum consumed oxygen in the last week. In order for the animals to adapt to the exercise protocol, an adaptation exercise training session was implemented at the speed of nine meters per
minute for 20 minutes during one week before the main exercise training program. The duration of exercise training was 20 minutes, with the intensity set at 16 meters per minute on the first day and 26 meters per minute on the last day of the intervention. To initiate the exercise training program, the rats warmed-up for five minutes at the speed of seven meters per minute, and after the main workout, the animals cooled-down at the speed of five meters per minute.

**Supplementation and Blood and Tissue Sampling**

Octopamine (Sigma-Aldrich, USA) was used as the supplement for four weeks and was administered via intraperitoneal injection at the dose of 81 µmol/kg five days per week (IP solution with 9% normal saline). At the next stage and 48 hours after the intervention, all the rats had breakfast for 10-8 hours and were weighed before tissue biopsy. Afterwards, anesthesia was performed via inhalation using chloroform. After completing the anesthesia, pain test, and assurance of unconsciousness, blood sampling was carried out from the left ventricle of the heart. Following that, the small intestinal tissues were immediately removed and washed with mucosal PBS. Blood and additional materials were also removed, and the tissues were encoded in two-milliliter microtubes, which were transferred into a nitrogen tank and stored at the temperature of -80°C until cell analysis.

**Biochemical Analysis**

After blood sampling, the serum containing normal saline entered the left ventricle, and the corner of the right atrium was cut with thin scissors to prevent the return of the blood to the heart. After approximately 20 minutes and based on the weight of the rats, complete whitening of the eyes, and returned blood from the organs, the fixative serum (4% aldehyde paraffin) was replaced with normal saline. After 20-30 minutes and the complete fixation of the organs, the serum was removed from the heart, and the brown adipose tissue was excrated from the body, and the tissues were transferred to a secondary fixative container (10% formalin). After 3-5 days, the tissues were transferred to the tissue process apparatus for dehydration and paraffin molding. In this process, the real-time polymerase chain reaction RT-PCR was used to evaluate the expression of the *PINK1* and *LAMP2* genes.

**RT-PCR**

RT-PCR was used to investigate the gene expression in the brown adipose tissue. For the molecular investigations at the gene expression level, RNA was initially extracted from the tissues of the animals in all the groups in accordance with the instructions of the manufacturer (Qiagen, Germany). To this end, 200 Landa of Qiazole was added to the samples, and the samples were incubated at the temperature of -80°C for 24 hours. The plaque available on the cryotube was crushed in a semi-frozen state to lubricate the samples, and 100 Landa of chloroform was added to the samples for one minute. The resulting solution was centrifuged at 12,000 rpm for 10 minutes. The transparent liquid on top of the tube containing the RNA was slowly removed and placed in a DEPC microtubule. Afterwards, 1 cc of isopropanol was poured on the transparent RNA and stirred manually for one minute.

At the next stage, the samples were centrifuged at 12,000 rpm for 10 minutes, the top liquid was discarded, and 1 cc of 70% alcohol was added to the sediment. After vortexing, the mixture was centrifuged at 7,500 rpm for 10 minutes. Following that, the liquid was drained, and the plaque dried inside the microtubule. In addition, 20 Landa of distilled water (60°C) was poured on the plaque and placed on a plaque (60°C) for five minutes. After RNA extraction from all the studied samples with high purity and concentration, cDNA synthesis was performed in accordance with the instructions of the manufacturer (Fermentas, USA), and the synthesized cDNA was used for the reverse transcription reaction. The *PINK1* expression was measured using the quantitative model of RT-PCR. The primers were designed based on the data of *PINK1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) available in the NCBI gene bank, as well as the data obtained by Macrogen Company. The *GAPDH* gene was used as the control gene, and its expression rate was calculated using the ΔΔCT-2 formula. Table 1 shows the sequence of the applied primers.
**Table 1. Primer Sequence**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligo Sequence 5ˈ-3ˈ</th>
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<tr>
<td>PINK1</td>
<td>F5ˈ CAAAGGGAGTAGATGGGGAG3ˈ</td>
</tr>
<tr>
<td></td>
<td>R5ˈ GATGGATGGGAATAGTAGTG3ˈ</td>
</tr>
<tr>
<td>LAMP2</td>
<td>F5ˈ GCAGTGAGATGAAGACAC3ˈ</td>
</tr>
<tr>
<td></td>
<td>R5ˈ AGTATGATGGGCTTGAGAC3ˈ</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F5ˈ CAT ACT CAG CAC CAC CAC Cˈ</td>
</tr>
<tr>
<td></td>
<td>R5ˈ AAG TTC AAC GGC ACA GTC AAG G 3ˈ</td>
</tr>
</tbody>
</table>

**Immunohistochemical (IHC) Assay**

At this stage, 20 grams of paraformaldehyde (Merck, Germany) was added to 500 cc of distilled water, and the mixture was placed on a hot plate until its temperature reached 60°C. Afterwards, NaOH was added to the solution until the pH of the solution reached the range of 7.2-7.4. Finally, aPBS tablet was added to the solution. In the next step, 2 cc of 37.5% hydrochloric acid was added to 100 cc of distilled water. In addition, 100 Landa of fetal bovine serum was added to 900 Landa of PBS, and 10% bovine serum was obtained. Following that, 3 Landa of Triton 100x was added to 997 Landa of PBS, and 0.3% Triton was obtained.

The samples were washed with PBS in four steps at five-minute intervals between each step. In order to restore the antigens, normal hydrochloric acid was poured on the samples for 30 minutes. In order to neutralize the acid, Borate buffers were added for five minutes, and the cells were washed with PBS. In addition, 0.3% Triton was used for 30 minutes to make the cell membranes permeable, and the cells were washed with PBS. In order to block the reaction of the secondary antibody, the 10% bovine serum was added in the form of an additional background color for 30 minutes. Following that, the primary antibody (1-100) was diluted with PBS and added to the samples. After creating a moist environment for the prevention of the tissues from drying, the samples were placed in a refrigerator at the temperature of 2-8°C overnight. On the following day, the tissue container was taken out of the refrigerator and washed four times with PBS (five minutes each time). In the next step, the secondary antibodies with the dilutions of 1-150 were added to the samples and incubated at the temperature of 37°C for one hour and 30 minutes in the dark using an incubator. Afterwards, the samples were transferred from the incubator to a dark room, and after they were washed four times, DAPI was added, immediately removed, and poured on the PBS samples. Finally, the samples were observed using the Olympus fluorescence microscope with a 400 lens to confirm the markers.

**Statistical Analysis**

Data analysis was performed in SPSS version 22, and the data were expressed as mean and standard deviation. In order to determine the effects of intoxication by heated oil, t-test was used for the comparison of the findings between the independent groups of HC and intoxication-control. Furthermore, two-way analysis of variance (ANOVA) was used to assess the main effects of exercise training and octopamine, as well as the simultaneous effects of octopamine. Bonferroni's trial test was also applied in case significant differences were observed. In all the statistical analyses, the significance level was considered at P≤0.05.

**Results**

The findings indicated that the expression of the PINK gene significantly increased due to intoxication by deeply heated oil (P=0.001). On the other hand, exercise training could significantly reduce the PINK gene expression (F=11.49; P=0.003; μ=0.365). Octopamine supplementation had no significant effect on the PINK gene expression (F=2.13; P=0.160; μ=0.096). Although the lowest PINK gene expression was observed in the octopamine and exercise-training group, the interactive effects between exercise and octopamine supplementation on the PINK gene expression were not considered significant (F=0.42; P=0.514; μ=0.022) (Figure 2).
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Figure 1. Comparison of PINK Gene Expression in Healthy Controls and Controls Poisoned with Deeply Heated Oil (*Significant changes compared to healthy control)

Figure 2. Expression of PINK Gene in Study Groups (Data expressed as mean and standard deviation)

According to the obtained results, the expression of the LAMP2A gene significantly decreased (P=0.001) due to intoxication by deeply heated oil. On the other hand, exercise training significantly increased the LAMP2A gene expression (F=9.63; P=0.006; μ=0.325). Octopamine supplementation also significantly increased the LAMP2A gene expression (F=21.25; P=0.001; μ=0.515). The interaction of exercise training and octopamine supplementation had a significant effect on the LAMP2A gene expression (F=4.80; P=0.040; μ=0.194). Considering the amount of LAMP2A gene expression in the exercise-training and supplementation groups, it was expected that the expression of the LAMP2A gene would be higher than the other interventions. This was confirmed by the findings as the expression of the LAMP2A gene was higher in the mentioned groups compared to the other interventions (Figure 4).
Discussion
The present study aimed to investigate the simultaneous effects of aerobic exercise training and octopamine consumption on the mitophagy of the brown adipose tissue. The obtained results indicated a significant difference in the PINK1 gene expression between the HC and intervention groups, which could be due to the fact that heated oils increase the expression of the PINK gene. According to the literature, the consumption of heated oils significantly increases the formation of metastatic lung tumors (19). Moreover, the toxic compounds produced in burnt oils and assimilation of oil in the Wistar rats increased the lipid peroxidation and liver enzymes (20).

The findings of the current research indicated no significant difference between the SI, SEI, and EIT groups in terms of the PINK1 gene expression. In other words, the combination of octopamine with exercise training has not more advantages than exercise training or octopamine supplementation alone. Although the difference in this regard was not considered significant, octopamine supplementation resulted in the reduction of the PINK1 gene expression. Therefore, it could be concluded that as an emulator of adrenergic compounds, octopamine could exert antioxidant effects on the brown adipose tissue of rats. These findings support a new model for the elimination of damaged mitochondria, where PINK1 utilizes parkin for malfunctioning mitochondria to stimulate
mitochondrial deletion by mitophagy (21). PINK1 reduction is associated with increased mitophagy (22). Through increasing the total antioxidant capacity and activity of the red blood cells in the body, this interaction could prevent oxidative stress and inappropriate production of the creatine kinase indicator of muscle damage and malondialdehyde and carbonylated protein indicators of lipid peroxidation. According to the results of the present study, exercise training and octopamine supplementation had significant effects on the expression of the LAMP2A gene in the Wistar rats intoxicated by deeply heated oil, and the maximum effect could be achieved when the two interventions are combined. In this regard, Han et al. (2017) reported that aerobic exercise training could change the gene expression of LAMP2A through a high-fat diet, thereby modulating oxidative stress and improving the oxidative indicators of the brown adipose tissue in obese rats (23).

In another study, Park et al. (2016) observed a significant difference in the antioxidant indicators after a period of aerobic exercise training (24). Furthermore, the mentioned study indicated that intoxication by heated oils blocked the regulation of the LAMP2A gene expression by affecting its receptors and moving the mitophagy regulation in a negative direction, thereby decreasing the LAMP2A gene expression. LAMP2A is a protein receptor located in the lysosomal membrane, inside which the lysosomes break down with the autophagosome, causing the inner membrane to become lubricious and the inner components of the autophagosome to decompose. The toxicities that are induced by ROS could reduce the expression of the LAMP2A gene through inactivating the hsc70 or mutagen receptors in these receptors, which in turn increases the production of ROS in damaged mitochondria. Therefore, the continued consumption of burned fats may adversely affect the content of lipid mutagens, cause cellular damage to the liver and kidneys, increase urinary mutagenicity, and increase the cell proliferation of the esophagus (19).

The findings of the current research demonstrated that exercise training, octopamine supplementation, and their interactive effects increased the expression of the LAMP2A gene, thereby activating oxidative stress as a result of intoxication with oil through inducing mitophagy by the chaperones (25). Furthermore, they are moved to the lysosome surface by hsc70 with the help of the chaperones, and after binding to the 2A-LAMP receptor protein on the lysosome surface, the substrates cross the membrane width through the 2A-LAMP-dependent displacement complex and rapidly decompose into the lumen (22). According to the results of the present study, it seems that the interactive effects of aerobic exercise training and octopamine consumption could induce mitophagy and decrease the ROS produced through intoxication by the heated oils.

Conclusion
According to the results, exercise training and octopamine supplementation might have significant effects on the improvement of mitophagy and prevention of the damages caused by the use of heated oils in the brown adipose tissue. Given the scarcity of data in this regard and limitations of our study regarding the loss of the laboratory rats, it is recommended that the current study be replicated using more samples in order to obtain more generalizable findings in the future.

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References
4. Eliz E, Oz P. The effects of different frying oils on the formation of heterocyclic aromatic amines in meatballs and the changes in fatty acid compositions.