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The Simultaneous Effect of Endurance Training and Octopamine Supplementation on Octopamine and its Receptors in the Visceral Fat Tissue of Rats Treated with Deep Fried Oil (DFO)

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ABSTRACT

Introduction: The role of exercise and some supplements in lipolysis has been reported, but given limited information on the simultaneous effect of endurance training (ET) and octopamine (Oct), the present study aimed to investigate the interactive effect of these two interventions on adipose tissue lipolysis with emphasis on octopamine receptors in rats treated with deep fried oil (DFO).

Methods: Thirty male Wistar rats (20-18 weeks old and 280-320 g) were divided into five groups, including (1) healthy control (C), (2) DFO, (3) Oct+DFO, (4) ET+DFO and (5) DFO+Oct+ET. Aerobic training was performed for four weeks, five sessions per week with an intensity of 16-26 m / min and equivalent to 50-65% VO_{2max}; also, 81 µmol/kg octopamine supplementation was intraperitonally injected to rats 5 days a week. Two-way analysis of variance and Bonferroni's *post hoc* test were used to analyze the data.

Results: ET increases *Octβ-R* expression (P=0.02) and Oct protein concentration (P=0.001) in the visceral adipose tissue of rats exposed to DFO. Oct supplementation increases *Octα-R* (P=0.01) expression in the visceral adipose tissue of rats exposed to DFO. Also, ET and Oct do not have a synergistic effect on *Octβ-R* (P=0.91), *Octα-R* (P=0.65) and Oct protein concentration (P=0.16) in the visceral adipose tissue of rats exposed to DFO.

Conclusion: It seems that although training and octopamine supplementation alone play a role in increasing the protein concentration of octopamine and its receptors, these two interventions do not have a synergistic effect on lipolysis by emphasizing the octopamine receptor pathway.

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Introduction

Today, due to the individuals' busy everyday activities and industrialization of societies, a large number of people have turned to fast foods and fried foods. Because a lot of oil is used to cook such foods and these oils are not cost-effective, they may be continuously exposed to heat and have harmful effects on people (1). In other words, high and long-term heat in frying oils leads to an increase in free radicals in the cooked foods and at the same time with the destruction of the structure of vitamin E due to deep heating in these oils, inflammation and vascular dysfunction increase (2). In addition, this style of diet due to the increase of ample unsaturated fatty acids leads to increased fat cell hypertrophy, increased fat mass, obesity and finally related diseases (3).

Researchers believe that increased fat mass is associated with inhibition of lipolysis pathways and its mechanisms. Octopamine (Oct) is a mammalian norepinephrine analog that binds to adrenoceptor receptors (ARs) such as beta-adrenoceptors (4), octopamine receptors (*Oct.R*), beta-adrenergic receptors *Octβ1R*, *Octβ2R* and *Octβ2R*, and α-adrenergic octopamine receptor (Octα.R), and exerts its lipolysis effects (5); therefore, the addition of this dietary supplement, which has long been used by researchers, is recommended to take advantage of its beneficial effects.

In addition, the researchers believe that the octopamine pathway and its receptors act like adrenergic pathways; in other words, the Octβ1R receptor is similar to beta-adrenergic, and similar to it, it phosphorylates and activates the

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downstream pathways such as cAMP/AMPK/HSL (5).

In this vein, in previous studies, the use of honey-derived Oct resulted in an increase in Octβ1R receptors (6); also, in our previous study, Oct consumption increased tyramin-R receptor, increased hormone-sensitive lipase (HSL) and decreased G protein-coupled receptors (GPCR) in adipose tissue (7). Other studies have also shown an improvement in fat profile in rats fed with deep-fried food (DFO) (8).

Also, octopamine supplementation led to an increase in HSL activity and lipolysis pathways in visceral adipose tissue (7). In addition, in another study, researchers showed that the consumption of octopamine led to an increase in β1- or β2-ARs proteins in fat cells derived from mammals (4).

On the other hand, regarding reduced physical activity due to lifestyle changes and its effect on weight gain and obesity, sports scientists believe that the most appropriate and non-invasive way to lose weight is regular exercise (9). In other words, endurance training leads to a reduction in fat mass by activating androgenic pathways, activating HSL and activating the Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) pathway (10). Past studies have also shown that endurance training improves *Octβ1R* expression in heart and skeletal muscle tissue of Dorsophila (5).

In this regard, the researchers showed that Octβ1R expression decreases after aging, however endurance exercise in Dorsophila increases the expression of Octβ1R and OAMB in the heart and skeletal muscle tissue, and this causes changes in body fat tissue (5). Also, in a previously-conducted study, endurance training increased the expression of Octβ1R, Octβ2R and Octβ2R, and Octα.R receptors in the visceral fat tissue of rats exposed to DFO (7).

In addition, researchers' attention has recently been drawn to the interactive effects of training and Oct supplementation on animal models; for example, the study of Kianmehr et al. showed that aerobic training (ET), Oct and interaction of ET and Oct increased the expression of PGC-1α and uncoupled protein-1 (UCP-1) (11), and augmented inhibition of apoptotic factors (12) in the heart tissue of rats exposed to DFO. In a study, the results showed that the interaction of the two (ET and Oct) increased heat shock protein-70 (HSP70) and decreased caspase-3 in the brown adipose tissue of rats exposed to DFO (13).

Although the individual and favorable role of aerobic training and octopamine supplementation on lipolysis pathways has been reported, the lipotic mechanism dependent on different receptors such as Oct-α and Oct-β in the visceral adipose tissue of rats exposed to DFO is still not well understood.

In addition, the simultaneous effect of training and octopamine supplementation on lipolysis pathways is a very interesting topic for researchers; therefore, considering the increase in weight and obesity, the increase in deaths caused by it, and the globalized spread of this problem, conducting basic research that can increase the understanding of researchers to provide more practical solutions and prevent diseases caused by obesity seems necessary.

Therefore, the aim of this study was to investigate the interactive effects of ET and Oct on the octopamine receptor pathway in the visceral adipose tissue of rats exposed to DFO.

Materials and Methods

Preparation and Maintenance of Animals

In this basic and experimental study, 30 male Wistar rats with an age range of 20-18 weeks and a weight range of 280-320 g were prepared from the Histogen Research Center.

The selection of the sample size in this study was based on analogous animal model studies. In this vein, given the ethical codes of research, the researchers sought to use the lowest number of animals in the sample and obtain the optimal results (14,15).

Samples were kept in the laboratory for one week for compatibility. It should also be noted that during the course of research, the ethical principles of working with laboratory animals were observed in accordance with the Helsinki Agreement. In this study, rats were kept in the standard conditions of 22-24° C, relative humidity of 55%, 12-12 hours of light-darkness cycle, in special washable cages, with *ad libitum* access to water and food. Subsequently, after adaptation, they were randomly divided into five groups of six animals, including: (1) healthy control (C), (2) DFO, (3) Oct + DFO, (4) ET + DFO and (5) DFO + Oc + ET.

Preparation of Deep Fried Oil (DFO)

To prepare the deep fried oil, 8 liters of sunflower oil was heated at 190 to 200 ° C for 8 hours for 4 days, and according to the literature review, every 30 minutes some food including chicken

nuggets, potatoes, chicken and protein products (sausages) were dipped in oil. On the fourth day, the deep fried oil was fed orally to rats (0.1 cc per 100 grams of body weight by gavage) for 4 weeks as a poisoning intervention (16).

Consumption of Octopamine

In this study, octopamine supplement prepared by Sigma Aldrich Company was first dissolved in 9% normal saline and then, each time, 81 µmol / kg of it was injected peritoneally into rats for four weeks, five days a week (16).

Endurance Training Protocol

To perform endurance training, first, three introductory sessions were performed with a treadmill. For this purpose, rats were placed on the treadmill for 20 minutes at a speed of 9 m / min for 5 days. The main endurance training protocol was performed for 4 weeks. In this training protocol, the intensity of training was considered 50% VO_{2max} in the first week and 65% VO_{2max} in the last week. In order to comply with the principle of training overload, the intensity of training started at 16 m / min on the first day and reached 26 m/min on the last day. Also, in addition to the main training, a duration of 5 minutes at a speed of 7 meters per minute were considered for warming up and cooling down (11).

Dissection and Sampling

48 hours after the last training session and supplementation, rats were anesthetized with a combination of ketamine (55 mg / kg) and xylazine (18 mg / kg) following 12 hours of fasting. After experts' confirming complete anesthesia and ensuring analgesia and unconsciousness, rats' abdominal cavity was cut with a razor blade size 20 and visceral adipose tissue was carefully extracted. For physiological measurements, 500 mg of tissue was placed in a tissue storage microtube and immediately frozen at -70° C. Also, for pathological examinations, the required amount of visceral adipose tissue was placed in 15% formalin solution to examine tissue incisions and hematoxylin-eosin and trichrome methods in the future.

Assessment of Octopamine by Western Blotting

Octopamine levels in this study were measured by Western blotting. For this purpose, 40 mg of the extracted octopamine protein were boiled for 5 to 10 minutes and then placed on polyacrylamide gel and run for 100 to 2 hours at a voltage of 100. Then the electrophoresis gel was prepared for transfer and the nitrocellulose paper, device pads and sponges were sandwiched inside the transfer buffer and electrophoresed at 350 amps for one hour. The nitrocellulose paper was then washed using TBS buffer for 5 to 10 minutes. The nitrocellulose paper was then left at room temperature for one hour and immersed and shaken in a TBS (or blocking) buffer. This operation was repeated several times and then nitrocellulose paper was diluted with secondary antibody (dilution of 1/3000 antibody) at room temperature for 1 to 2 hours and then incubated in TBS buffer. This operation was repeated twice to ensure stabilization. The nitrocellulose paper then appeared on the photographic film in a dark room by means of the exposure solution and the stabilizer using ECL, and after the bands appeared, the nitrocellulose paper was washed with distilled water. At the end, the paper with B actine antibody was placed on the paper and incubated with secondary antibody. After B actine control appeared as an internal control in the radiology film, the images banded by Image J program were densiometered to obtain quantitative values.

Assessment of Oct-α and Oct-β Gene Expression Levels

In order to evaluate the expression levels of *Oct-α* and *Oct-β* in qReal Time PCR, initially 20 mg of adipose tissue was used to extract RNA.

RNA extraction was performed using the manufacturer's protocol (Trizole, manufactured by Kiagen Company, Made in the United States with catalog number 79306). Then, to determine the quality of RNA extraction, the light absorption property at 260 nm wavelength was used. After ensuring the proper concentration of RNA, cDNA synthesis was performed according to the RevertAid First Strand cDNA Synthesis protocol manufactured by Thermo Scientific Company in the USA with catalog number K1622. The synthesized cDNA was then used to perform the reverse transcription reaction. First, the designed primers were added to the cDNA according to the instructions on the PUBMED site. It should be noted that before performing the test, the researcher made sure that the primer worked using the software available on the PUBMED site. The GAPDH internal control gene was then inserted into the qReal Time PCR

together with the target gene, and the transcription reaction was allowed to continue transcribing in different cycles to reach the threshold cycle (CT). Then, after completing the operation of the device, the formula $2^{-\Delta\Delta CT}$ was used to quantify the data. The sequence of the primers used in the study is presented in Table 1.

Table 1. Sequence of gene primers measured in the research

Genes	Primer Sequences	Size (bp)
GAPDH	Forward: *5'- AAGTTCAACGGCACAGTCAAGG -3'* Reverse: *5'- CATACTCAGCACCAGCATCACC -3'*	112
Oct-α	Forward: *5'- GGTACTTTGGTAAGGTGTGGTG -3'* Reverse: *5'- AGTGGCGGGAAGGAGATGA -3'*	126
Oct-β	Forward: *5' GGTGGAGCAGGATGGGAGGA -3'* Reverse: *5' GTAGCCAGCAGAGGGTGAAG -3'*	118

Histological Examination by Hematoxylin-Eosin Method

Hematoxylin-eosin method was used to evaluate changes in visceral adipose tissue in terms of cell volume and size, cell density and size of fat vacuoles. For this purpose, some adipose tissue was fixed in 15% formalin solution; the tissue was then immersed in an alcohol solution (80, 96, and 99%, respectively) to dehydrate. After clarification and impregnation with paraffin and alcohol, the samples were molded. Blocks of tissue were prepared for molding. Then molten paraffin was poured on the blocks, and then a BASCET was placed on the samples and some molten paraffin was poured on them again. The samples were then placed in the ambient temperature to solidify. The tissue was then prepared for incision and the tissue was cut with a microtome with a diameter of 1 micrometer and after placement on a laboratory slide, it was fixed with alcohol. The samples were then stained with hematoxylin and eosin dyes for three minutes and kept at room temperature. Finally, the tissues were examined by light microscope after dehydration.

Statistical Procedures

The Shapiro-Wilk test was used to evaluate the normality of the distribution of findings. To evaluate the effect of DFO on the levels of research variables, the DFO and HC groups were compared by independent samples t-test.

Given that the present design had two factors of training and octopamine supplementation, a two-factor design of two-way analysis of variance was used.

Two-way analysis of variance was used to evaluate the main effect of training, the main effect of octopamine supplementation as well as the interactive effect of training and octopamine supplementation. Also, Bonferroni's *post hoc* test was used to evaluate the effect of each intervention. The findings of the present study were analyzed in SPSS software version 22 and the significance level was set at 0.05.

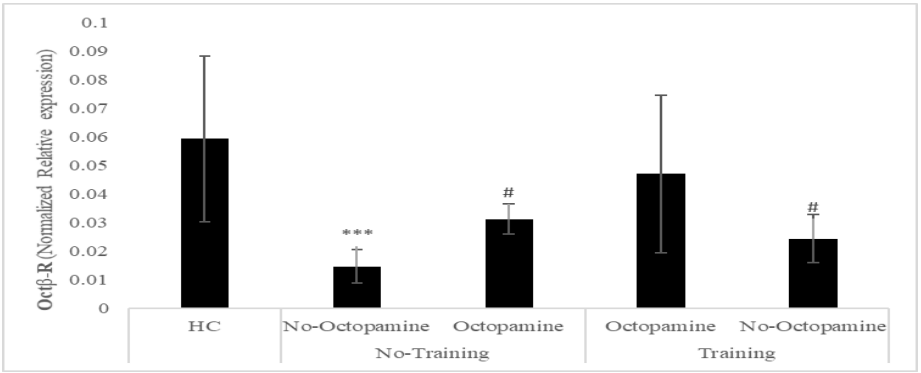

Figure 1. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on *Octβ-R* receptor levels in the visceral adipose tissue of rats exposed to DFO

**(P = 0.01) Significant decrease in the DFO group compared to the HC group

#(P = 0.05) Significant increase in the Training and Oct variables compared to the DFO group

The results show that although *Octβ-R* levels are higher in the ET + Oct group, but these two variables alone increase *Octβ-R* receptor expression and their effect is not synergistic

Results

The results of independent samples t-test showed that *Octβ-R* (P = 0.002), *Octα-R* (P = 0.01) and Oct (P = 0.001) in the HC group were significantly higher than the DFO group.

The results of two-way analysis of variance showed that ET (P = 0.02, F = 6.46 and effect size 0.28) and Oct (P = 0.01, F = 8.18 and effect size 0.33) had a significant effect on changes in *Octβ-R* gene expression levels in the visceral adipose tissue of rats exposed to DFO; but the interaction of ET and Oct on changes in *Octβ-R* receptor levels was not significant (P = 0.91, F = 0.011 and effect size 0.001). Also, the results of Bonferroni's *post hoc* test showed that *Octβ-R* receptor levels in the ET (P = 0.022) and Oct (P = 0.011) groups were significantly higher than the DFO group (Figure 1).

ET has no significant effect on Octα-R changes in the visceral adipose tissue of DFO-poisoned rats (P = 0.075, F = 3.64 and effect size 0.18), but Oct has a significant effect on changes in *Octα-R* levels in the visceral adipose tissue of DFO-poisoned rats (P = 0.01, F = 8.64 and effect size 0.35). Also, the interaction of ET and Oct on changes in *Octα-R* gene expression levels is not significant (P = 0.65, F = 0.21 and effect size 0.013); in other words, Octα-R levels in the Oct groups are significantly higher than the DFO group (P = 0.01) (Figure 2).

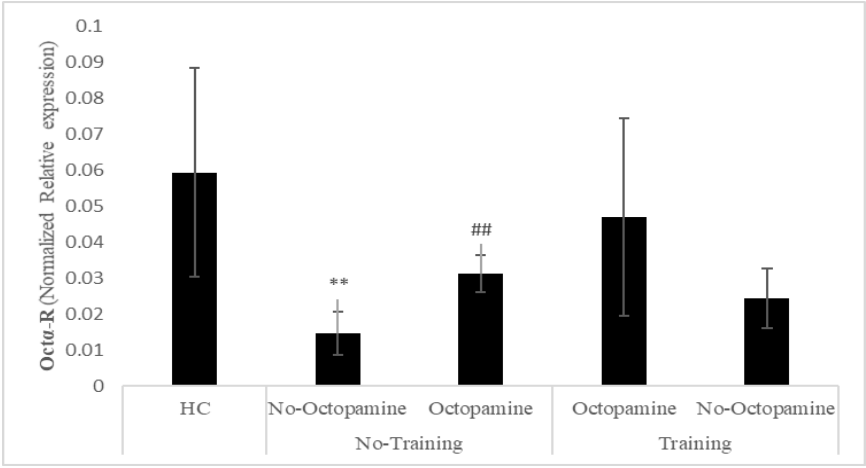

Figure 2. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on *Octα-R* receptor levels in the visceral adipose tissue of rats exposed to DFO

**(P = 0.01) Significant decrease in the DFO group compared to HC group

##(P = 0.01) Significant increase in the Oct variable compared to the DFO group

The results show that although Octα-R levels are higher in the ET + Oct group, only the Oct supplement variable is effective in increasing the expression of Octα-R receptor and the training variable modulates the effect of Oct supplement.

ET has a significant effect on changes in Oct expression levels in the visceral adipose tissue of rats exposed to DFO (P = 0.001, F = 61.38 and effect size 0.83), but Oct supplementation has no significant effect on changes in gene expression levels (P = 0.055, F = 4.51 and effect size 0.27). Also, the interaction of ET and Oct on changes in Oct expression in the visceral adipose tissue of rats exposed to DFO is not significant (P = 0.16, F = 2.18 and effect size 0.15). Oct levels in the ET groups are significantly higher than the DFO group (P = 0.001) (Figure 3).

Examination of images obtained from different groups of visceral adipose tissue show that the number of fat cells in the healthy control group (CH) is significantly lower than the other groups, while the diameter of each fat vacuole in this group is larger than the other groups. Also, the number of these cells in the training + octopamine group is low and there has no significant difference with the normal group. In groups such as the training + octopamine, the number fat cells increase compared to the healthy control (CH) group. In the DFO group, the number of these cells increase significantly compared to the other groups, while the diameter of each fat vacuole is smaller than the other groups. The results of histological images showed that the higher the amount of oil intake in the groups, the number of fat cells initially increases and then the cells begin to become obese and hypertrophic, which indicates a gradual increase in adipose tissue.

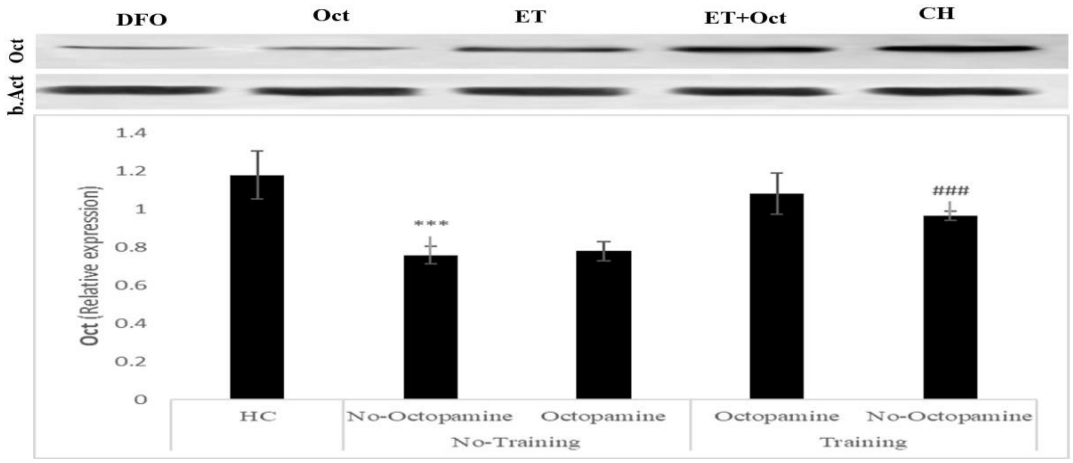

Figure 3. Results of two-way analysis of variance to evaluate the interactive effect of ET and Oct on Oct protein levels in the visceral adipose tissue of rats exposed to DFO
***(P = 0.001) Significant decrease in the DFO group compared to the HC group
###(P = 0.001) Significant increase in the Training variable compared to the DFO group The results show that training and octopamine have a synergistic effect on Oct; However, octopamine supplementation somewhat modulates the effect of training in increasing Oct in the visceral adipose tissue of DFO-exposed rats.

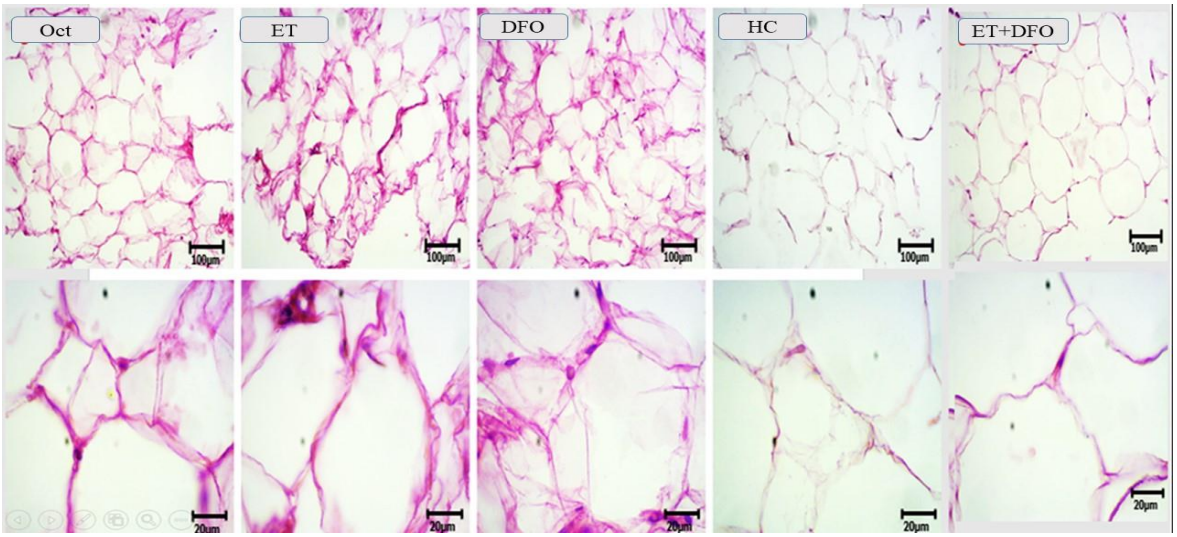

Figure 4. Results of microscopic examination of visceral adipose tissue cells in the research groups

Discussion

According to the previous studies, it seems that improper diet, especially oils that have been heated at high temperatures and for a long time, increases the amount of TG and cholesterol in plasma and liver, and this leads to the activation of the lipogenesis pathway in the visceral fat tissue, because in this case, the amount of energy received from high-fat food increases compared to the calories consumed with physical activities and leads to an increase in body fat percentage (17). In addition, DFO seems to lead to the activation of lipogenic pathways by inhibiting GPCR and tyramine receptors as well as inhibiting beta-adrenergic receptors (7). Furthermore, studies show that octopamine signaling is one of the vital pathways of metabolism, thus the present study aimed to investigate the synergistic effect of endurance training and octopamine supplementation on

protein concentration and two types of octopamine receptor subunits in the visceral adipose tissue of DFO-poisoned rats. The results showed that ET increased *Octβ-R* gene expression levels and Oct protein concentration in the visceral adipose tissue of rats exposed to DFO. Studies show that octopamine, especially *Octβ-R* subunits, is a beta-adrenergic receptor analog such as norepithephrine, and as the beta-adrenergic pathway is activated following training, Octβ-R signaling is activated as well (5). Nonetheless, it appears that the effect of training on lipolysis activation is through emphasis on the octopamine pathway of PKA/cAMP and Ca2+/IP3/CaMK, which ultimately, by activating gene transcription pathways, leads to increased expression of octopamine receptors, especially with *Octβ1R* and *Octβ3R* subunits, and is effective in lipolysis (18). In this regard, Sujkowski's study (2020) showed that endurance training increased Octβ1R in heart tissue, improved physical function in Dorsophila (5). In another study, the researcher increased octopamine levels following endurance training, which improved neuronal function and the octopaminergic pathway in Dorsophila (18).

In addition, in a study it was shown that endurance training leads to the inhibition of myocardial apoptosis in rats exposed to DFO (12). Still, in another study, researchers showed that aerobic training leads to an increase in HDL/LDL ratio as well as in acetyl co-enzyme A, and through this pathway increases lipogenic proteins such as malonyl co-A (8).

Also, in a researchers' previously-conducted study, endurance training increased tyramine receptor levels and expression of hormone-sensitive lipase in the visceral adipose tissue of rats exposed to DFO (7). Therefore, in line with previous studies, our study also showed that different types of training increase lipolysis by activating beta-adrenergic subunits and inhibiting alpha-adrenoceptors.

The results showed that Oct increased *Octβ-R* and *Octα-R* gene expression levels in the visceral adipose tissue of rats exposed to DFO. According to studies, octopamine is known as a biogenic amino acid that has a high affinity for *Octβ3R*; it can also activate the protein by activating beta G subunits and lead to phosphorylation of cAMP by activating adenylate cyclase, thereby activating the transcription pathway of metabolic genes including *Octβ-R* and *Octα-R* receptors as well as lipolysis in adipose tissue (7,19). In this regard, researchers have stated that the use of octopamine as a beta-adrenergic analogue led to the modulation of nervous system function and improved dopamine type 3 receptor levels (20). Furthermore, the consumption of octopamine leads to the inhibition of myocardial apoptosis (12) and an increase in HDL/LDL ratio as well as in acetyl coenzyme A (8) in rats exposed to DFO. In confirmation of these results, another study stated that honey-derived octopamine supplementation increased the expression of Octβ-R and Octα-R receptors; Octopamine supplementation has also been shown to be beneficial in lipolysis and glucose metabolism pathways, and improved genes associated with these thermogenesis pathways (6). In a study, researchers also showed that taking octopamine increased β-3 adrenergic expression and improved glucose metabolism (21); in another study, octopamine increased epinephrine and norepinephrine (22); therefore, octopamine seems to play a role in activating gene transcription from beta-adrenergic pathway, and further transcribes both of its receptors through this pathway.

The results also showed that implementing ET and Oct individually increases the expression of *Octβ-R*, but only Oct supplement is effective in increasing the expression of the *Octα-R* receptor and training modulates the effect of Oct supplement. Octopamine supplementation also partially modulates the effect of training on increasing Oct in the visceral adipose tissue of DFO-exposed rats. It seems that the effect of octopamine supplementation along with training is dose-dependent, so that in a study, researchers showed that low dose (150 mg) of octopamine before training had no significant effect on improving performance in active men (23). In addition, training and octopamine had an interactive effect in reducing G protein-coupled receptor (GPCR) in visceral adipose tissue (7).

Considering the review of literature, few studies have been performed on the synergistic effect of training and octopamine supplementation on its receptor pathways that cause lipolysis; however, it appears that endurance training by increasing catecholamines (epinephrine and norepinephrine) and gene expression transcription pathways increase the expression of octopamine receptors (5); also, octopamine supplementation plays a role genes transcription

through the *octβ3R* and cAMP pathways, and hence both variables are involved in improving fat metabolism by similar mechanisms. Therefore, as can be seen in the above Figures, the training and octopamine supplementation group had a better performance in *Octβ-R* and *Octα-R* gene expression levels than either alone, however non-significance of synergistic effect of these two variables could be attributed to thr intensity of endurance training in lipolysis activation (24,25) and the difference in the dose of octopamine in the lipolysis pathway along with training (23) which can sporadically moderate each other.

It seems that aerobic training plays a role in improving lipolysis by increasing catecholamines as well as increasing angiogenesis and mitochondrial biogenesis, while Oct supplementation plays a role in fat catalysis by increasing HSL activity in the visceral adipose tissue; Therefore, although the interactive effect of the two interventions on the lipolysis pathway was not observed, it appears that both interventions can work together and affect each other mutually.

Given the role of the proteins responsible for transcription in the downstream octopamine-dependent lipolysis pathways, the lack of measurement of these proteins is one of the limitations of the present study. Therefore, it is suggested that NRF1/ 2 and PGC1-a be measured in adipose tissue in future studies.

Conclusion

Even though training and octopamine supplementation alone seem to play a role in increasing the protein concentration of octopamine and its receptors, these two interventions do not have a synergistic effect on lipolysis by focusing on the octopamine receptor pathway.

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Conflicts of Interest

The authors have not declared any conflict of interest.

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