The effects of aerobic exercise training with octopamine supplementation on cardiomyocyte apoptosis induced by deep frying oil: the role of caspase and pro-Caspase 3

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Research

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Abstract

Background Mitophagy is the optional decadence of mitochondria by autophagy and mostly happens to defective mitochondria following damage or stress. Several studies indicated that mitophagy may offer protection against myocyte death induced by cardiac ischemia. On the other hand, the activation of caspase can lead to programmed cell death such as apoptosis and necrosis. The aim of this study was to explore the effect of aerobic exercise and octopamine on cardiomyocytes proliferation and mitophagy and also changes in caspase 3 and pro-caspase 3 expression of rat exposed deep frying oil. Methods 30 mail wistar rats were divided into 5 groups (n=6 in each) including 1) control (CO), 2) deep frying oil (DFO), 3) deep frying oil + exercise (DFO+EXE), 4) deep frying oil + octopamine (DFO+OCT), and 5) deep frying oil + exercise + octopamine (DFO+EXE+OCT). The apoptotic effects of DFO in heart tissue were examined by TUNEL assay. Masson's trichrome stain used to study cardiomyocytic fibers. Moreover, caspase 3 and pro-caspase 3 genes and proteins expression in all groups was evaluated using quantitative real-time PCR and Western blot method, respectively. Results Data showed a significant increase in apoptotic cells in the DFO-treated group ($P<0.05$). In Masson's Trichrome stainanalysis demonstrated more cardiomyocytic fibers degradation and aggregation lymphocytic cells in DFO that exercise and OCT signisicantly improve this degradation. Also, the expression level of caspase 3 and pro-caspase 3 was significantly decrease in deep frying oil + exercise + octopamine group ($P<0.05$). Conclusions According to the result of the current study, it can be assumed that DFO can lead to programmed cell death via the activation of caspase in heart tissue. However it seems that aerobic exercise with octopamine supplementation improve heart tissue by significantly decrease expression of caspase 3 and pro-caspase 3 that inhibit apoptosis.

Background

Cardiomyocytes are the muscle cells that make up the cardiac muscle[1]. Each myocardial cell contains myofibrils, which are specialized organelles consisting of long chains of sarcomeres, the basic contractile units of muscle cells[2]. Cardiovascular disease is a resulting cause of death globally that death of cardiomyocytes is a main contributing factor to these diseases. Cell death in conditions such as heart defeat and myocardial infarction is associated with apoptosis[3]. Cell death has become clear as a basic biological phenomenon substantial for the regulation of tissue homeostasis whose change has an important concept in pathology[4]. Autophagy is an intracellular degradation system that renders cytoplasmic constituents to the lysosome. It has shown that autophagy plays a wide diversity of pathophysiological roles[5]. Mitophagy, the particular autophagic elimination of mitochondria, has been found in yeast, mediated by autophagy-related, and in mammals during red blood cell differentiation[6]. Additionally, mitophagy minister to eliminate the subset of mitochondria producing the most reactive oxygen species[7]. Mitophagy also is degradation of mitochondria via the macroautophagy pathway[8]. Mitochondria play a necessary role in maintaining optimal performance of the heart. All mitochondrial processes participate in the progression of cardiovascular pathologies[9]. Cardiomyocyte apoptosis does occur in several cardiac diseases such as ischemic heart disease, heart failure, and aging[10].

Octopamine is highly optimized in neurons of several invertebrate species. Unlike in mammals, octopaminergic neurons in invertebrates are spatially separated from catecholaminergic neurons[11]. The biosynthesis of octopamine was determined by the administration of amino acid and amine precursors and enzyme inhibitors and measuring the endogenous levels of octopamine[12]. Octopamine leads to increase of amplitude and frequency of the heartbeat. It has proposed that octopamine is not the transmitter of cardio-accelerator neurons[13]. Deep frying is a cooking way in which food is submerged in hot fat, most generally oil [14]. Deep frying oil produces desirable or undesirable flavor compounds and alterations the flavor stability and quality of the oil by hydrolysis, oxidation, and polymerization[15]. It has demonstrated that heating of dietary oils and fats results in lipid oxidation. A direct relationship has reported between CVD risk and consumption of cooking oil polar compounds[16]. Caspases are a family of endoproteases that furnish critical links in cell regulatory networks controlling inflammation and cell death[17]. The activation of Caspases represents a critical step in the pathways resulting in the biochemical and morphological alterations that underlie apoptosis[18]. Among Caspases, Caspase 3 is a frequently activated death protease, catalyzing the special cleavage of plenty of key cellular proteins. However, the particular requirements of Caspase 3 in apoptosis are still unknown[19]. Activation of pro-Caspase 3 is a central event in the execution phase of apoptosis and appears to serve as the convergence point of several apoptotic signaling pathways[20]. Exercise-based cardiac rehabilitation aims to restore people with coronary heart disease to health through either regular exercise alone or a combination of exercise with education and psychological support[21]. Looking for the answer to the question of whether aerobic exercise and octopamine supplement affect cardiomyocytes proliferation and mitophagy, the present study was designed. Thus, the aim of this study was to investigate the impact of aerobic exercise and octopamine supplement affects cardiomyocytes proliferation, mitophagy, and also changes the expression of the Caspase 3 and pro-Caspase 3 in heart tissue of DFO-treated.

Methods

Animals

In this experimental study, overall 30 healthy adult male rats of the Wistar strain (Rattus norvegicus), 8 weeks old and weighing approximately between 200 to 250 g, were obtained from Pasteur Institute of Iran (Tehran, Iran). The rats studied were exclusively housed in plastic cages with sawdust bedding. All rats were fed a standard diet and urban water. The temperature and humidity used for cages were 20 ± 2 ° C and about 60%, respectively. Lighting period was held at 12h light/12h dark cycle. All the experimental methods were performed according to the animal protocols approved by the Ethical Committee for Research on Laboratory Animals of Tehran Azad University, Iran. In this study, the animals were randomly assigned to 5 groups (n=6). Groups were as follows: 1) control (CO), 2) deep frying oil (DFO), 3) deep frying oil + exercise (DFO+EXE), 4) deep frying oil + octopamine (DFO+OCT), and 5) deep frying oil + exercise + octopamine (DFO+EXE+OCT).

Chemicals

DFO

Deep frying oil was made by frying 5 kg catfish 3 times in 2.5 liter cooking palm oil at 200°C (measured with a cooking thermometer) for 15 minutes. After each frying the oil was left to cool for ± 5 hours at room temperature. The 3-times heated DFO preparation will subsequently be referred to as DFO-3. DFO was gavage 5 day per weeks (10 ml/kg).

OCT

OCT was dissolved in distilled water and then diluted with Krebs-bicarbonate solution. OCT was injected intraperitoneally 5 day per weeks (10 ml/kg).

Aerobic exercise protocol

Exercised rats were introduced to treadmill running for 2 week, during which each animal exercised on a motorized rodent treadmill at 9 m/min for 20 min per day (including 10 min at a prescribed speed, a 5-min warm-up, and 5-min cool-down). After the habituation period, rats were subjected to run at moderate exercise intensity for 5 days per week over 4 weeks as adaptation. On the first day of exercise, the training began with the rats exercising at 11 m/min for 10 min per day. The speed gradually accelerated from 11 to 20 m/min over the duration of the experiment. The exercise time was also increased by 2 min per day over the same period until it reached 26 min/day at the end of the second week.

Terminal transferase dUTP nick end labeling (TUNEL) assay

The apoptotic effects of aerobic exercise and octopamine on cardiomyocytes proliferation and mitophagy in DFO treated rats were determined using the transferase-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling assay. The control and experimental groups were fixed with 4 % (w/v) paraformaldehyde and processed using a commercial kit (In Situ Cell Death Detection Kit; Roche, Germany) in accordance with the manufacturer’s instructions. Cells were stained with Hoechst dye and imaged in a fluorescent microscope. The data were expressed as a percentage of the area of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL)-positive cells in ten random fields.

Masson's trichrome staining

Masson's trichrome stain (Poly Scientific, Bay Shore, NY) were performed according to kit directions except as follows: Aniline Blue-Solution I-90 minutes incubation. The prolonged incubation is a standard procedure for trichrome stains of muscle. Briefly, heart tissue slides were placed in staining jar and deparaffinized by submerging into three series of absolute xylene for 4 minutes. After that, the slides were washed with running tap water for 2 minutes. Then, slides were treated with the phosphomolybdic acid solution for another 10 minutes as a mordant and immediately slides were submerged into methyl blue (C.I. 42780, Merck, Germany). Next, slides were washed in running water for 2 minutes and lastly treated with 1% acetic acid solution for 1 minute.

RNA and protein extraction

RNA and protein were extracted from the heart tissue using TRizol reagent (Invitrogen, USA) according to the manufacturer's instruction. The quality of the extracted RNA and protein were determined according to the 260/280 absorbance ratio, measured by NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

cDNA synthesis and quantitative RT-PCR analysis (qRT-PCR)

cDNA synthesis for *Caspase 3* and *pro-Caspase 3* was carried out using a universal cDNA synthesis kit (Exiqon, Denmark) according to manufacturer's instructions. Specific primers for *Caspase 3* and *pro-Caspase 3* and *GAPDH* (as housekeeping gene) genes were designed using primer premier 5 software (Premier Bio-Soft International, Palo Alto, CA, USA). Real-time quantitative PCR reactions were carried out in triplicate by using standard protocols with a Rotor-Gene 6000 system (Corbett Life Science, Mortlake, Australia). Briefly, in a total volume of 10 µl, 20 ng/µl of cDNAs were added to a master mix comprising 10 pmol/µl of each primer (*Caspase 3* and *pro-Caspase 3*) and 5 ml of SYBR premix ExTaq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan). The program for the run was set as follows: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR reaction was followed by a melting curve program (60–95 °C with a temperature transition rate of 1 °C s^{-1} and a continuous fluorescence reading). *GAPDH* was quantified as the reference to normalize differences in total RNA levels. The calculation was performed as follows:

$$\text{Ratio} = \frac{2^{\Delta c_T,\text{target(calibrator}-\text{test)}}}{2^{\Delta c_T,\text{ref(calibrator}-\text{test)}}}$$

Western blot analysis

Cells were harvested and lysed in lysis buffer (RIPA, Beyotime Institute of Biotechnology) supplemented with protease inhibitors (PMSF, Aladdin). The equal amounts of protein were separated by SDS-PAGE with 5%–12% Tris-Glycine gel (Invitrogen) and subjected to standard western blot analysis. Western blotting was performed using *Caspase 3* and pro-*Caspase 3* antibody (1:1000 dilution; Can Ag, Sweden), rabbit anti-HRP enzyme (1:2000 dilution; Can Ag, Sweden) which was then reacted with the corresponding secondary HRP-conjugated antibodies (1:3000 dilution; Abcam, USA). Finally, blots were developed using the ECL Western Blotting Detection system (Amersham Life Sciences Inc., Arlington Heights, IL). The results were subjected to densitometry analysis using the ImageJ software to assist with blind analysis of image data. To ensure equal amounts of protein were loaded; the GAPDH protein was employed as an internal control.

Statistical analysis

The statistical analysis of the obtained data was performed using the Graph Pad Prism statistical software version 5.01 (GraphPad, San Diego, CA, USA). The normality of the data was evaluated using

the Kolmogorov–Smirnov test and one-way ANOVA was used to analyze the data from different groups. Data are expressed as the mean ± SD. The level of significance was set at $P<0.05$.

Results

Analysis of H&E and Masson's trichrome stain

According to H&E and Masson's trichrome stain, it was found that in the control group, cardiomyocytic fibers were normal. Blood cells and lymphocytes were observed very little between muscle fibers. The muscle fiber were arranged in a regular figure with intercalated discs (fig 1). In DFO group with exercise or OCT alone was seen that cardiomyocyte fibers have ruptured in some places. While these injuries were less frequent in the DFO+EXE+OCT group. Also the diameter of muscle fibers in DFO+EXE and DFO+OCT groups were lower than in the control group. But this injures fever in DFO+EXE+OCT group (Figure 1).

Apoptosis

The results showed that DFO lead to increase the apoptotic in heart tissue (Fig 2 A&B). Based on the results, DFO+EXE+OCT significantly decreased the apoptosis in heart tissue. However, DFO+EXE and DFO+OCT alone were not able to inhibit apoptosis (Fig 2 A&B).

Caspase 3 and pro-*Caspase 3* genes expression

The expression of *Caspase 3* and pro-*Caspase 3* genes were significantly different between the five groups. Data analysis demonstrated a significant increase in the expression of *Caspase 3* and *pro-Caspase 3* in DFO group compared with the control group ($p < 0.05$). However, it was observed significantly decrease in expression of both genes studied DFO+EXE+OCT compare to DFO (Figure 3 A&B).

Caspase 3 and pro-*Caspase 3* proteins expression

Western Blot analysis showed that *Caspase 3* and pro-*Caspase 3* protein levels in the DFO group significantly increased compared to the control group. On the other word, our data indicated the highest increase level of both proteins was related to the DFO (Figure 4). However exercise and OCT alone or in combination with together decrease this protein in heart tissue (Figure 4).

Discussion

One of the main questions in this study was to find out how aerobic exercise and octopamine can effect on cardiomyocyte apoptosis. Therefore, present study investigate, the effect of aerobic exercise and octopamine on apoptosis and cardiomyocytes proliferation and mitophagy in DFO treated male Wistar rats. The apoptotic effects of DFO was examined by Tunel assay. We observed that aerobic exercise and octopamine can lead to inhibit the apoptosis induced by DFO. It can be concluded that DFO treatment reduces cell viability and growth and enhances apoptosis in cardiomyocyte by increasing Caspase and

pro-Caspase 3. On the other hand, the results obtained from Masson's trichrome stain showed that in DFO group, more cardiomyocyte fibers were ruptured and some lymphocytic cells were present in some fibers. Gupta et al (2014) consider the effect of different deep fried vegetable oil on cardiovascular system in rats model and show that deep fried vegetable oil cause increase plasma nitric oxide level, and vascular reactivity on blood pressure and aortic structure change[22]. However, in this study we consider the effects of deep frying oil that it has more severe effects than deep fried vegetable oil. Also the results study about the effects of DFO on cardiomyocyte is limited. The results of this study in adverse effects of DFO in heart tissue show that the diameter of muscle fibers in this group (DFO) is significantly less than that of another group. However performing aerobic exercise and OCT supplementation improve cardiomyocyte apoptosis. It has proposed that the cellular adaptation to exercise can be associated with both endogenous and exogenous factors. Exercise induces cardiac growth via hypertrophy and renewal of cardiomyocyte[23]. Also, exercise induces endothelial progenitor cells to proliferate, migrate and differentiate into mature endothelial cells, giving rise to endothelial regeneration and angiogenesis[24]. Exercise reduces the occurrence of atherosclerotic heart disease and can promote the formation of vascular branches[23]. These findings confirm the results of the current study. In an investigation, the effects of aerobic exercise on cell density and apoptosis of hippocampus were examined in adolescent rats. Animals were run on a treadmill for 30 min/session at a speed of 8 m/min and 0° slope, five times a week for 8 weeks. According to the apoptosis results, in an account of TUNEL positive cells, there were no significant differences in any groups. However, it has shown that aerobic exercise-induced increase cell density without altering of apoptosis in the hippocampus rats[25]. However, in this study structure and function of cardiac muscle cells and fibers improved after aerobic exercise and OCT in DFO treated rat.

We also investigated the effects of DFO on the protein and gene expression of Caspase 3 and pro-Caspase 3 in groups studied. We observed a significant increase in the expression of Caspase 3 and pro-Caspase 3 in DFO and DFO + OCT groups compared with the control group. However, the expression level of this protein in DFO group was more than other treated-groups. It can be concluded that combination of aerobic exercise and octopamine may have negative effect on both genes studied (Caspase and pro-Caspase 3) and also these factors can overcome cytotoxicity of DFO (reduce apoptosis induced by DFO). These findings should considering for the future study. In contrast the results of this study it has demonstrated that there is an increase in the activity of caspase-9 and caspase-3 immediately after exercises, whereas the activation of caspase-8 was observed at 3 hours after exercise in prostate tissue[26], however we consider protein and gene of Caspase 3 and pro-Caspase 3 in heart tissue. Also different modality of exercise have different effects on heart tissue. Yang et al. have reported the increases in caspase-3 mRNA expression after resistance-trained [27] and also Kocturk et al have proposed the increase of caspase-9 and caspase-3 activity immediately after strenuous exercise[28]. However we consider the effects of aerobic exercise (moderate intensity) that have better effects on heart tissue. Overall, these findings show that exercise training with herbal supplementation (OCT) by inhibit of Caspase and pro-Caspase 3 can decrease cardiomyocyte apoptosis that improve heart function

Conclusion

In conclusion, our findings indicated apoptosis and cardiomyocytes proliferation were significantly different between the DFO treated-groups in comparison to the control. Moreover, aerobic exercise and octopamine can be considered as factors affecting on inhibit of expression of Caspase 3 and pro-Caspase 3 genes. We assumed that aerobic exercise and octopamine may have a notable impact on DFO and probably apoptotic and mitophagy. However, additional in-vitro and in-vivo experiments are required to further confirm the relevance of aerobic exercise and octopamine to decrease apoptosis in cardiomyocyte.

Abbreviations

DFO: Deep frying oil, CVD: cardio vascular disease

Declarations

Authors' contributions

All author equally contribute in preparation of this manuscript.

Funding

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Availability of data and materials

All data generated or analyzed are included in this paper.

Ethics approval and consent to participate

I promise that all procedures were conducted according to the Guiding Principles in the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 8023, revised 1978) and were approved by the Institutional Ethics Committee of the institution where this research was performed.

Consent for publication

'Not applicable'.

Competing interests

The authors declare that they have no competing interests.

Author Contributions

All author contribute equally in preparation of this manuscript.

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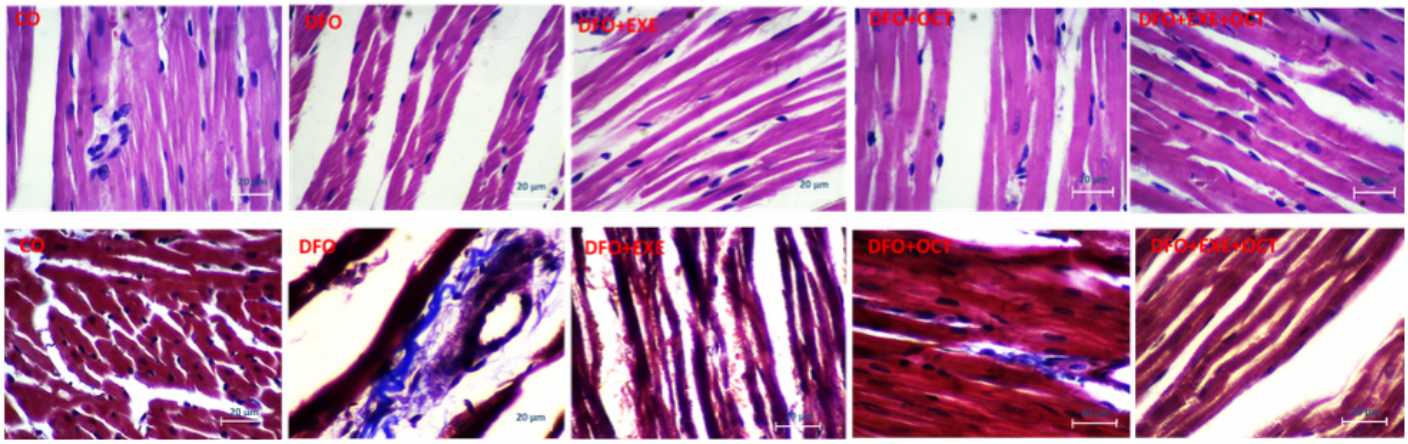
Figures

A)



B)

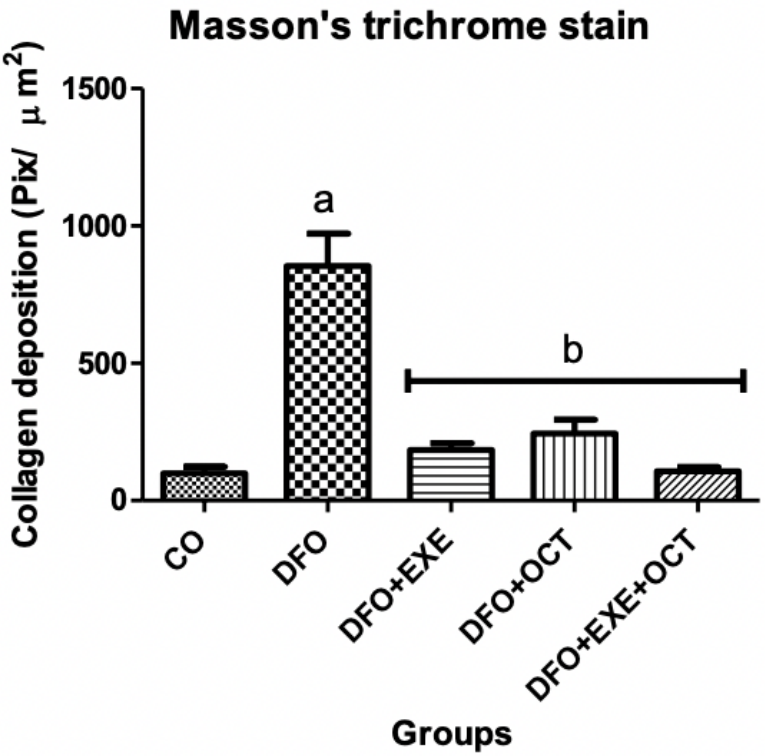
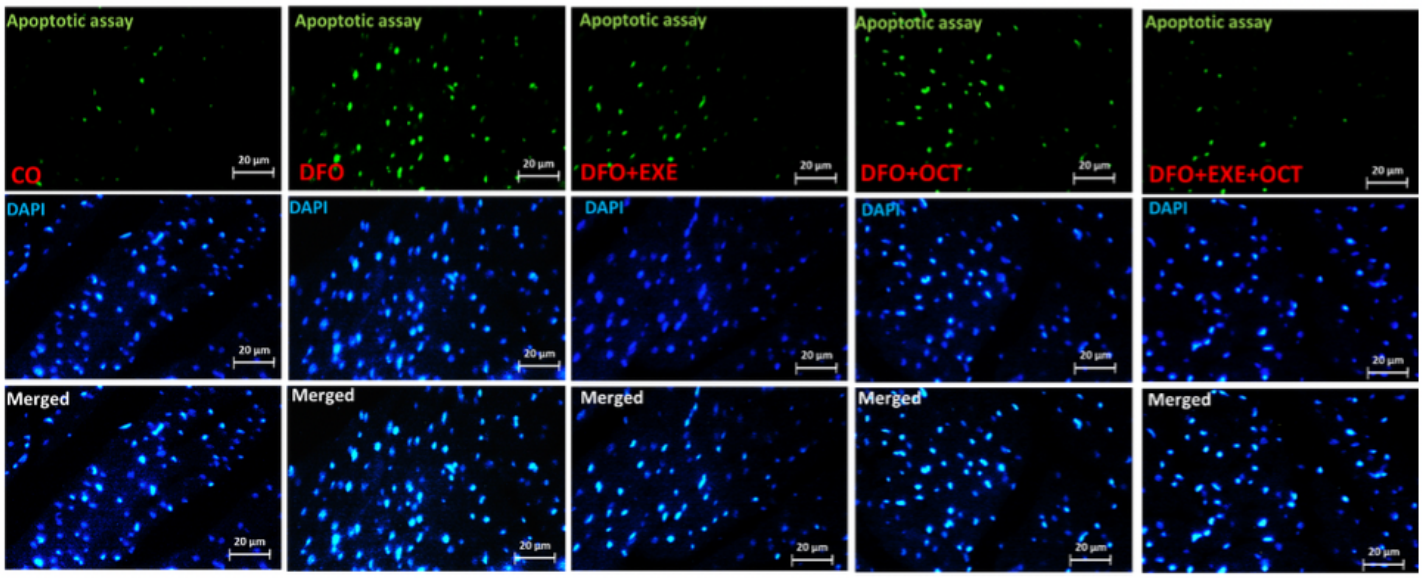


Figure 1

A) Result of H&E (upper) Masson's Trichrome staining (bottom) in studied groups B) the result of Masson's Trichrome staining in studied groups. a: significantly different with the control group (CO) at p ≤ 0.05, b: significantly different with the DFO at p ≤ 0.005. CO: control, DFO: deep frying oil, DFO+EXE: deep frying oil + exercise, DFO+OCT: deep frying oil + octopamine, DFO+EXE+OCT: deep frying oil + exercise + octopamine.

A)



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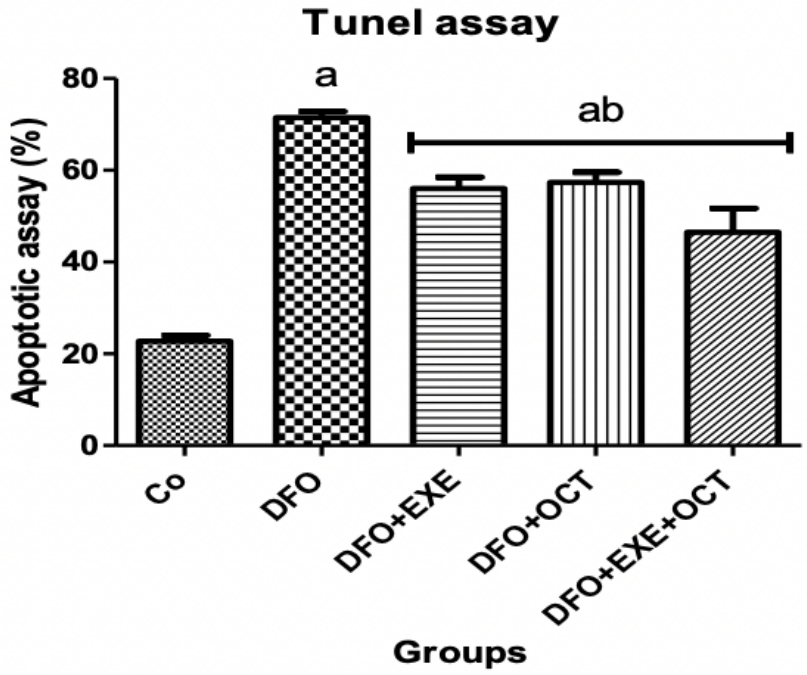
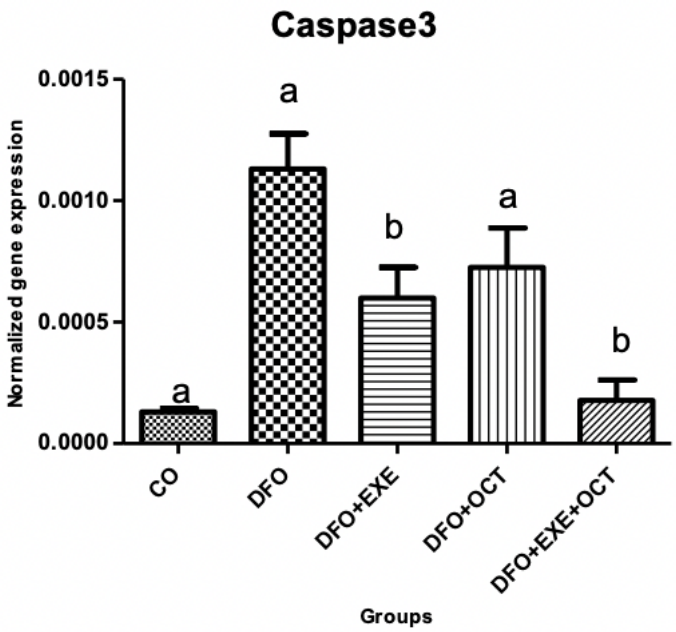


Figure 2

Apoptotic effects of DFO were recognized by TUNEL assay (A & B). a: significantly different with the control group (CO) at p ≤ 0.05, b: significantly different with the DFO at p ≤ 0.005. CO: control, DFO: deep frying oil, DFO+EXE: deep frying oil + exercise, DFO+OCT: deep frying oil + octopamine, DFO+EXE+OCT: deep frying oil + exercise + octopamine.

A)



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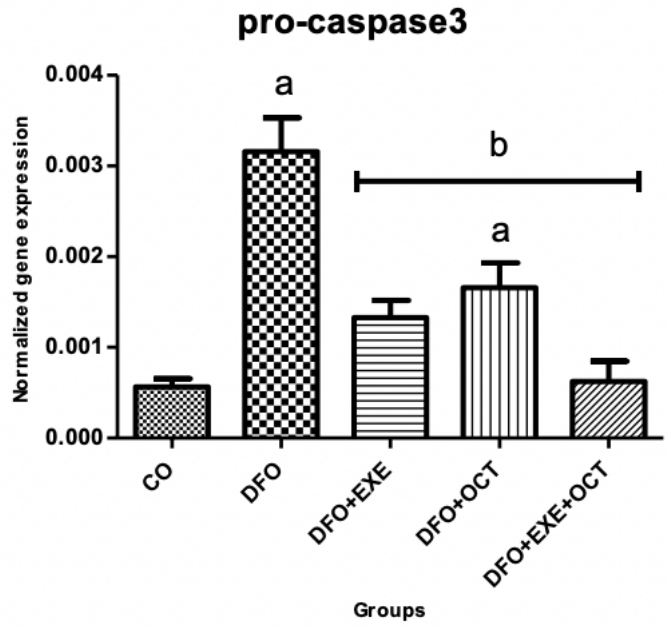

Figure 3

Average relative Caspase 3 expression in the group studied compared to control (A). Relative expression of pro-Caspase 3 in the group studied compared to control (B). Data are expressed in S.E.M. a: significantly different with the control group (CO) at p ≤ 0.05, b: significantly different with the DFO at p ≤ 0.005. CO: control, DFO: deep frying oil, DFO+EXE: deep frying oil + exercise, DFO+OCT: deep frying oil + octopamine, DFO+EXE+OCT: deep frying oil + exercise + octopamine.

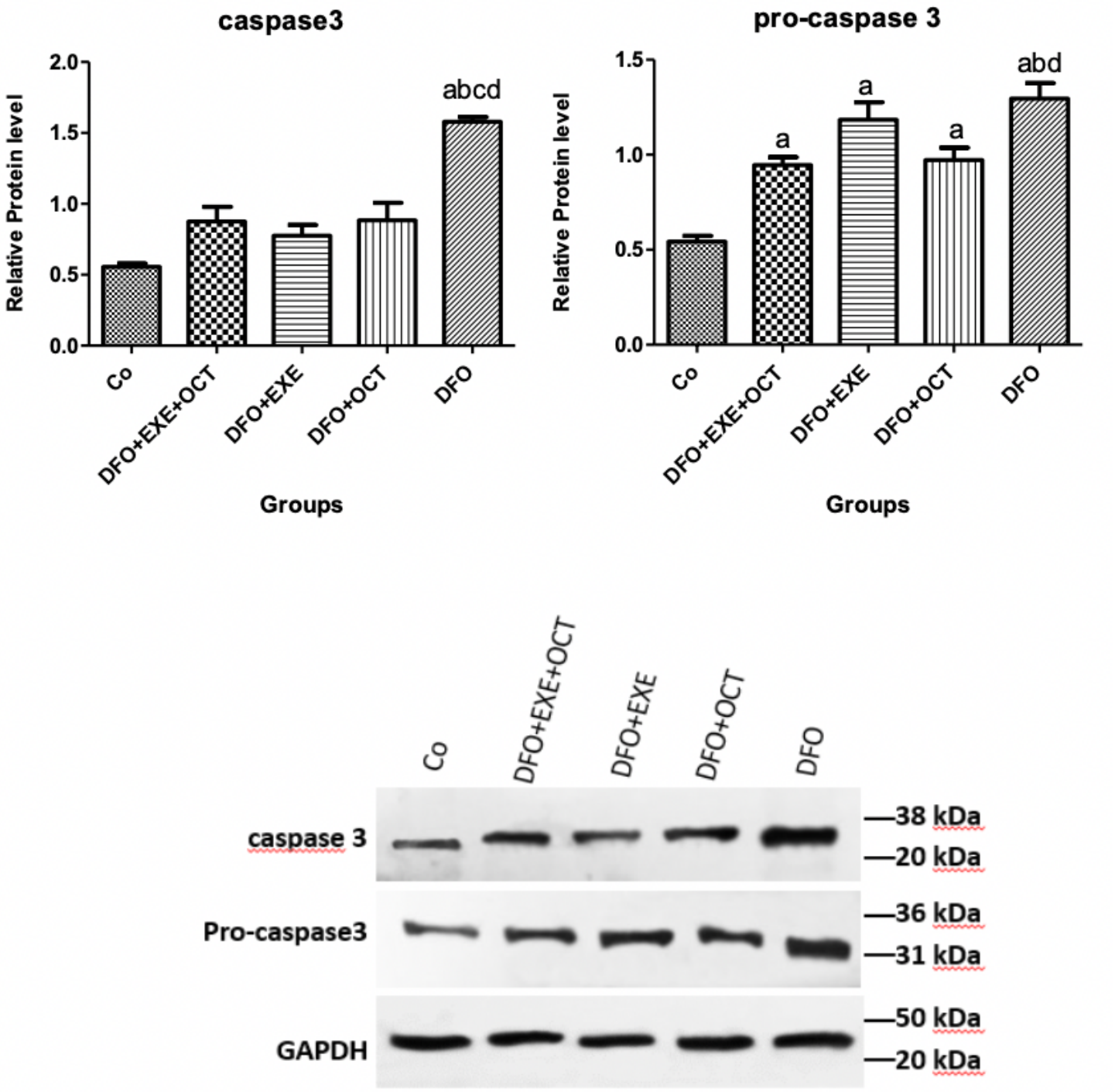

Figure 4

Caspase 3 and pro-Caspase 3 protein expression in groups studied in comparison with control. GAPDH was used to confirm normalization of the protein load. The groups are arranged in order of expression. a: significantly different with the control group (CO) at p ≤ 0.05, b: significantly different with the DFO+EXE+OCT group at p ≤ 0.05, c: significantly different with the DFO+EXE group at p ≤ 0.05, d: significantly different with the DFO +OCT group at p ≤ 0.05, CO: control, DFO: deep frying oil, DFO+EXE:

deep frying oil + exercise, DFO+OCT: deep frying oil + octopamine, DFO+EXE+OCT: deep frying oil + exercise + octopamine.