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

Pantea kianmehr

Islamic Azad University

Mohammad Ali Azarbayjani (✉ m_azarbayjani@iauctb.ac.ir)

Islamic Azad University <https://orcid.org/0000-0002-3502-7487>

Maghsoud Peeri

Islamic Azad University

Parvin Farzanegi

Islamic Azad University

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Abstract

Background: Deep frying is a common cooking method, in which cooking is accompanied by carcinogenic by-products such as acrolein. Acrolein is a toxic byproduct of lipid peroxidation, which has been implicated in pulmonary, cardiac, and neurodegenerative diseases. The aim of this study was to explore the effect of aerobic exercise and octopamine on caspase 3 and pro-caspase 3 expression level in heart tissue of rat exposed deep frying oil.

Methods: 30 male 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 blotting method, respectively.

Results: Data showed a significant increase in apoptotic cells in the DFO-treated group ($P < 0.05$). In Masson's Trichrome stain analysis demonstrated more cardiomyocytic fibers degradation and aggregation lymphocytic cells in DFO that exercise and OCT significantly 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 (CVD) 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]. Cardiomyocyte apoptosis does occur in several cardiac diseases such as ischemic heart disease, heart failure, and aging[6].

A direct relationship has reported between CVD risk and consumption of cooking oil polar compounds[7]. Deep frying is a cooking way in which food is submerged in hot fat, most generally oil[8]. Deep frying oil produces desirable or undesirable flavor compounds and alterations the flavor stability and quality of the

oil by hydrolysis, oxidation, and polymerization[9]. It has demonstrated that heating of dietary oils and fats results in lipid oxidation and apoptotic induction. Caspases are a family of end proteases that furnish critical links in cell regulatory networks controlling inflammation and cell death[10]. The activation of Caspases represents a critical step in the pathways resulting in the biochemical and morphological alterations that underlie apoptosis[11]. 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[12]. 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[13].

Octopamine is an endogenous biogenic amine that is closely related to norepinephrine, and affects adrenergic and dopaminergic systems. It is naturally present in many plants such as oranges and is derived from the synthesis of D- (-) - enantiomer octopamine by β -hydroxylation of tyramine and by the enzyme dopamine β -hydroxylase and is marketed under the trade names Epirenor, Norden and Norfen (14). Octopamine leads to increase of amplitude and frequency of the heartbeat. It has proposed that octopamine is not the transmitter of cardio-accelerator neurons[14]. Octopamine was studied as a therapeutic agent to treat hypotensive disorders, with doses of 450–600 mg day⁻¹ resulting in mild increases in blood pressure without the presence of adverse effects[15]. However, studies about the supplementation of Octopamine on heart tissue and cardiac apoptosis in cardiovascular disease is limit. Ross et al (2017) show that acute supplementation with a low dose of octopamine did not influence endurance cycle performance, substrate oxidation or circulating hormonal concentrations, which could be due to the low serum octopamine concentrations observed[16]. 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[17]. Looking for the answer to the question of whether aerobic exercise and octopamine supplement affect cardiomyocytes viability, the present study was designed. Thus, the aim of this study was to investigate the impact of aerobic exercise and octopamine supplement affects cardiomyocytes histopathology and also changes the expression of the *Caspase 3* and *pro-Caspase 3* in heart tissue of DFO-treated.

Materials And 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 procedures were approved and monitored by the ethics committee of laboratory animals of Islamic Azad University (Tehran, Iran) and the procedures followed the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) (ethical cod: IR.MUK.REC.1398.242). 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 L cooking palm oil at 200°C (measured with a cooking thermometer) for 15 min[18-20]. After each frying the oil was left to cool for 5 hours at room temperature[21]. The 3-times heated DFO preparation will subsequently be referred to as DFO. DFO was gavage 5 days per week (10 mL/kg) for 4 weeks.

Octopamine

OCT (Sigma, UK) was dissolved in distilled water and then diluted with Krebs-bicarbonate solution. OCT (100 µL in each injection) was injected intraperitoneally 5 days per weeks (10 mL/kg) for 4 weeks.

Aerobic exercise protocol

Exercised rats were introduced to treadmill running for a habituation period, 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 (total duration of the exercise protocol and OCT supplementation). 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.

Animal tissue sampling

48 hours after the last intervention, all rats were sacrificed and heart tissue was removed immediately and washed in saline normal 0.9%. The tissues were divided to two parts for freezing and fixing. Body weight and heart weight also measure at the end of protocol (table 1).

Table 1. body weight and heart weight in different groups of study (mean $\pm$ SD).

Groups	(Body weight (g	(Heart weight (g
<i>Co</i>	256 ± 7	0.03 ± 1.05
<i>DFO</i>	4 ± 265	0.05 ± 1.03
<i>DFO+EXE</i>	8 ± 249	0.07 ± 1.11
<i>DFO+OCT</i>	3 ± 261	0.03 ± 0.99
<i>DFO+EXE+OCT</i>	5 ± 260	0.07 ± 1.08

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.

Terminal transferase dUTP nick end labeling (TUNEL) assay

The apoptotic effects of aerobic exercise and octopamine on heart tissue samples 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. The number of TUNEL positive cells was measured carefully in 3 sections of the heart tissue per animal. The percentage of positively stained cells against total nuclei in the area was analyzed with Image J software v1.8 (NIH, USA).

Masson's trichrome staining

Masson's trichrome stain (Histogenotech. Co., Iran) were performed according to kit directions except as follows: Aniline Blue-Solution I-90 min 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 min. After that, the slides were washed with running tap water for 2 min. Then, slides were treated with the phosphomolybdic acid solution for another 10 min as a mordant and immediately slides were submerged into methyl blue (Merck, Germany). Next, slides were washed in running water for 2 min and lastly treated with 1% acetic acid solution for 1 min.

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, 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* and *GAPDH* (as housekeeping gene) genes were designed using primer premier 5 software (Premier Bio-Soft International, Palo Alto, CA, USA) and summarized in table 2. Real-time quantitative PCR reactions were carried out in triplicate by using standard protocols with a ABI step one system (ABI, USA). 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 5 mL of SYBR premix ExTaq II (Thermo Scientific, USA). 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: (see Equation 1 in the Supplementary Files)

Table 2. Primer sequences for real time PCR assessment

Gene name	Primer sequences	()TM	(Product length (bp
<i>Gapdh</i>	F: 5'- CAT ACT CAG CAC CAG CAT CAC C -3' R: 5'- CATACTCAGCACCAGCATCACC -3'	81.1	121
<i>Caspase 3</i>	F:5'- GCACAAAGTGACTGGATGAAC-3' R:5'- TGTATTCTTACTCTACCGCAC-3'	80.5	82

Western blotting analysis

Heart tissue were 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 blotting analysis. Western blotting was performed using *Caspase 3* and *pro-Caspase 3* antibody (1:1000 dilution; Abcam), which was then reacted with the corresponding secondary HRP-conjugated antibodies (1:2000 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, two-way ANOVA and Tukey post-Hoc test 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

Hsitopatological assessment and fibrosis assessment by Masson's trichrome stain

According to the results in histopathological part, it was found that muscle tissue was cohesive in the control and DFO + exercise + octapamine groups and had fewer vacuole cells compared to other experimental groups. On the other hand, the myofibrils were closely spaced near to each other. In addition, the number of myofibrils in the control and DFO + exercise + octapamine groups had significant difference ($P \leq 0.01$) compared to DFO, DFO + exercise and DFO + octapamine treatment groups (Figure 1). Also, the results showed that the diameter of these myofibrils in the DFO + exercise + octapamine group was higher compared to DFO group significantly ($P = 0.031$).

The results showed that in DFO group the amount of collagen fiber increased significantly ($P = 0.003$) compare to control group. In consider of treatment intervention the results show that, aerobic exercise significantly reduced collagen percentage ($F = 38.10$, $P = 0.001$, $\eta^2 = 0.826$). Also, octopamine treatment was able to significantly reduce collagen levels in heart tissue ($F = 27.62$, $P = 0.001$, $\eta^2 = 0.775$). The effect of aerobic exercise and ectopamine treatment simultaneously was able to significantly reduce the amount of collagen fibers ($F = 16.48$, $P = 0.004$, $\eta^2 = 0.673$) (Figure 2).

Apoptosis

As a result of receiving DFO, the number of apoptotic cells in the tissue increased significantly ($P = 0.001$). Although, aerobic exercise significantly reduced the number of apoptotic cells ($F = 38.10$, $P = 0.001$, $\eta^2 = 0.826$). On the other hand, octopamine treatment was able to significantly reduce the number of apoptotic cells ($F = 14.24$, $P = 0.005$, $\eta^2 = 0.640$) in cardiac tissue. The effect of aerobic exercise and simultaneous octopamine treatment was able to significantly reduce the number of apoptotic cells ($F = 0.547$, $P = 0.481$, $\eta^2 = 0.064$) (Fig 3 A&B).

Caspase 3 gene expression

As a result of receiving DFO, the expression of *Caspase-3* gene ($P = 0.001$) increased significantly in the heart tissue. Although, aerobic exercise significantly reduced the expression of *Caspase 3* gene ($F = 9.16$, $P = 0.007$, $\eta^2 = 0.375$). On the other hand, octopamine treatment was able to significantly reduce the expression of *Caspase 3* gene ($F = 16.39$, $P = 0.001$, $\eta^2 = 0.506$) in cardiac tissue. The effect of aerobic exercise and octapamine treatment simultaneously was able to significantly reduce the expression of *Caspase 3* gene ($F = 0.003$, $P = 0.954$, $\eta^2 = 0.003$) (Figure 4).

Caspase 3 and pro-Caspase 3 proteins expression

As a result of receiving DFO, the expression of *Pro-Caspase 3* protein increased significantly in the heart tissue ($P = 0.001$). It should be noted that the expression ratio of *Caspase 3* protein to *Pro-Caspase 3* ($P = 0.053$) did not change significantly ($P = 0.221$). Aerobic exercise did not have a significant effect on the expression of *Pro-Caspase 3* protein ($F = 0.903$, $P = 0.370$, $\eta^2 = 0.101$). On the other hand, octapamine reduced the expression of *Pro-Caspase 3* protein ($F = 14.19$, $P = 0.005$, $\eta^2 = 0.651$). However, it did not have a significant effect on the ratio of *Caspase 3* to *Pro-Caspase 3* ($F = 0.143$, $P = 0.715$, $\eta^2 = 0.018$). In the exercise and supplement group, *Caspase-3* protein ($F = 19.32$, $P = 0.002$, $\eta^2 = 0.707$) and the ratio of

Caspase 3 to *Pro-Caspase 3* protein expression decreased significantly ($F = 14.32$, $P = 0.005$, $\eta^2 = 0.641$) [Figure 5].

Discussion

Today, due to the importance of cooking and the taste of food, various techniques have been proposed for cooking food around the world. One of the techniques that is welcomed in some countries is frying food at high temperatures with heated oils, which gives a special taste to the food and it makes the food crispy and delicious. However, apart from the good taste of this type of food, it can be very dangerous for human health. One of the body's organ that is affected by diet is the cardiovascular system. As well as high fat intake, is a high risk factor for cardiovascular disease, increasing the use of DFO due to the disruption of their fat structure can leave cardiovascular risks. Among the therapeutic modalities for improving heart disease due to bad diet, is improving lifestyle by doing exercise or using herbal and pharmacological supplements. Therefore, the aim of this study was to investigate the effect of exercise training with octopamine supplementation on apoptosis of cardiomyocytes rats exposed to DFO.

The results of this study show that exposed to DFO for 4 weeks can disruption regularity of cardiomyocytes and increase collagen deposition in heart tissue. In consider apoptosis the result of tunnel assay show that apoptotic cell significantly increased with DFO in animal model. This result also consists with upregulation of caspase 3 and procaspase 3. It seems that one of the reasons for these destructions caused by DFO is related to its changes in structural oil with boiling and frying. During the frying process, various chemical reactions occur, such as thermal oxidation, hydrolysis, and polymerization, due to the exposure of the oil to high temperatures in the presence of air and moisture. All of this change have a risk factor for CVD. It is stated that the repeated heating of oil at high temperatures (180°C) results in the thermal oxidation of the oil, which causes the configuration of the fatty acid to change from the cis isomer to the trans isomer. However, in this study use DFO with 200 °C. This configuration change causes the polyunsaturated fatty acids to acquire undesirable properties associated with saturated fatty acids, such as their correlation with increased serum cholesterol levels and higher low-density lipoprotein (LDL) cholesterol in particular[22]. Also during the frying process, the oil is aged and becomes more oxidized when the natural antioxidants are depleted; thus, after repeated heating, the antioxidants can no longer prevent the oxidation of the fatty acids in the oil. All of this agent can cusses increase the reactive oxygen spice (ROS) in cardiomyocytes. Increase in ROS in cellular level is start point in beginning apoptosis. In this study although the oxidative stress marker wasn't mesure, however apoptosis marker (caspase 3 and procaspase 3) measured and increased with DFO for 4 weeks. 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[23]. However, in this study type of oil and cellular marker was difference. 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, treatment with exercise and OCT improve this distraction.

The result showed that 4 weeks of aerobic exercise and octopamine supplementation improve heart tissue integration and downregulate caspase and procaspase 3 in cardiac cell. Regular aerobic exercise can increase the antioxidant enzymes in various tissues, especially the heart[24, 25]. In this study, although the antioxidant capacity of cardiac tissue was not measured, declining changes in apoptotic cells could indicate control of apoptotic markers by reducing oxidative stress. A part from this mechanism, it has been show 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[26]. Also it is stated that exercise induces endothelial progenitor cells to proliferate, migrate and differentiate into mature endothelial cells, giving rise to endothelial regeneration and angiogenesis in heart tissue [27], that all of this agent show cardio protective effects of exercise, and seems all of this mechanism improve apoptosis in heart. In this study we also use COT with exercise. It seems that part of better effects of combined groups of this study attribute to OCT.

The result of this study show 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). Studies demonstrated the ability of octopamine to activate B3 adrenoreceptors and stimulate lipolysis[28], suggesting octopamine could influence fat metabolism. There for OCT supplement can reduce some CVD risk factor. It seems, OCT with controlling harmful fats caused by DFO, has a role in reducing the cellular damage caused by apoptosis, this positive effect is doubled along with exercise.

There were several potential limitations in the present study. First, don't use characteristic analysis of DFO. Second, don't use of control oil group in this study. Third, in this study, we don't measure lipid peroxidation acid value, peroxide value, smoke point, and ultraviolet absorbance at 232 nm.

Conclusion

Based on results of this study, aerobic exercise training for 4 weeks with octopamine can inhibit apoptosis factors in rats exposed to DFO. It seems that performing exercise training with OCT supplementation can improve cardiomyocyte homeostatic disturbances due to apoptosis-induced by DFO. However, more studies (*in-vitro* and *in-vivo*) are needed, especially on the human sample.

Abbreviations

DFO: Deep frying oil, CVD: cardio vascular disease.

Declarations

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Islamic Azad University, Tehran, Iran (Code: IR.MUK.REC.1398.242).

Consent for publication

'Not applicable'.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All author equally contributes in preparation of this manuscript.

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Figures

Figure 1

A) Caspase 3 and pro-Caspase 3 proteins expression in different groups. GAPDH was used to confirm normalization of the protein load. In western blotting bands, the groups are arranged in order of expression (low to high). a: significantly different with Co group at $P \leq 0.01$, b: significantly different with the DFO group at $P \leq 0.01$, 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. The results represent mean±SD with 5 repeats.

Figure 2

Average relative Caspase 3 expression in the group studied compared to Gapdh. a: significantly different with the control group (CO) at $P \leq 0.002$, b: significantly different with the DFO at $P \leq 0.01$. 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. The results represent mean±SD with 5 repeats.

A)

B)

Figure 3

Apoptotic effects of DFO were recognized by TUNEL assay (A & B). a: significantly different with the control group (CO) at $P \leq 0.001$, b: significantly different with the DFO at $P \leq 0.0042$. 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. The results represent mean $\pm$ SD with 5 repeats.

Figure 4

Fibrotic tissue and collage deposition in cardiac muscle tissue after treatment with DFO in the experimental groups. a: significantly different with the control group (CO) at $P \leq 0.0032$, b: significantly different with the DFO at $P \leq 0.005$. 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. The results represent mean $\pm$ SD with 5 repeats.

Figure 5

Histopathological examination of cardiac muscle tissue after treatment with DFO in the experimental groups. Images of longitudinal sections show tissue integrity, and the images of transverse sections show the diameter and number of muscle fibers. a: significant differences with the control group at $P \leq 0.01$, b: significant differences with the DFO group at $P \leq 0.01$. The results represent mean $\pm$ SD with 5 repeats.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Equation1.pdf](#)