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Protective effects of olive oil against cardiac aging through mitophagy and apoptosis

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Abstract

Cardiac mitochondrial dysfunction is an important feature of aged heart. However, there is still no potent agent to ameliorate cardiac function abnormalities in aged hosts. Olive oil (OLO), containing monounsaturated fatty acids, has diverse protective effects on the cardiovascular system, including anti-diabetic, anti-inflammatory, and anti-hypertensive effects. We evaluated the beneficial impacts of OLO against aging-related cardiac dysfunction. Wistar rats were randomly allotted into three groups with eight rats, including control, aged rats receiving D-galactose (D-GAL), and aged rats administrated with D-galactose plus OLO (D-GAL + OLO). Aged animals were received D-GAL at a dose of 150.00 mg kg^{-1} daily through intra-peritoneal injection for aging induction. The animals in D-GAL + OLO group were co-administrated with oral OLO at a dose of 1.00 mL kg^{-1} by gavage feeding daily. The administration term was eight weeks. A histological examination of heart tissue was performed. The heart tissues were also harvested to assay the oxidative stress and molecular parameters. The aged animals showed cardiac hypertrophy, increased malondialdehyde level and Bax expression, and reduced mitofusin 2, phosphatase and tensin homologue-induced putative kinase 1, dynamin-related protein 1, and Bcl2 expressions in comparison with the control animals. The OLO treatment ameliorated all these parameters. Overall, OLO could improve cardiac aging through reducing oxidative stress, enhancing genes mediated mitophagy, and improving genes mediated apoptosis in the heart.

Introduction

Cardiac diseases are the important factor of morbidity and mortality worldwide, causing the death of millions people every year. The enhancement of average lifespan and aging are regarded as important agents of cardiac diseases. The molecular mechanisms of aging in the heart include multiple factors. An enhancement of oxidative stress is a critical factor in cardiac aging. Reactive oxygen species (ROS) production contributes to sustain physiological processes; their production is modulated through anti-oxidant enzymes system. In the heart, ROS contribute to cardiac hypertrophy and contractile dysfunction; thereby, affecting cardiac structure and function and involving in cardiac injury and hypertrophy.[1] During normal metabolism, mitochondria produce ROS. In turn, ROS may attack the mitochondrial membranes and DNA, resulting in mitochondrial dysfunctions and more ROS generation.[2] Also, mitochondrial dysfunction contributes to the pathogenesis of aging-induced cardiac hypertrophy. Impaired mitochondria not only generate low adenosine triphosphate but also produce enhanced ROS levels and cause apoptosis. The only demonstrated mechanism whereby mitochondria are removed is mitophagy, a sort of chosen autophagy of mitochondria. This process reduces with increasing age, which may involve in cardiac senescence and age-associated cardio-vascular disease.[3] Mitochondrial fission contributes to mitophagy. It aids to fragment damaged mitochondria, being a requisite for mitophagy.[4] Dynamin-related protein 1 (Drp1) is a critical

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regulator of mitochondria fission.[5] Reduced Drp1-mediated mitophagy increases the progression of heart failure induced by pressure over-load.[6] In addition, a previous study has indicated that down-regulation of Drp1 suppresses mitophagy, leading to mitochondrial dysfunction and finally cell death in the heart.[4] The Drp1 expression is less in the cardiac aging and reduced Drp1 leads to impaired mitophagy, causing cardiac apoptosis in aging. The Drp1 plays a critical role in the crosstalk with phosphatase and tensin homologue (PTEN) -induced putative kinase 1 (PINK1) for mitophagic control.[7] On the other hand, various mitophagy genes have been found, among which the *PINK1* gene is the main one.[8] Increasing impaired mitochondria is associated with the decreased *PINK1* expression. Previous study has indicated that PINK1 protein is markedly decreased in heart failure in human, and PINK1-deficient mitochondria decrease oxidative capacity, being associated with the cardiac hypertrophy progression.[9] Aiming the *PINK1* gene is advantageous for improvement of various cardiac disorders, including myocardial infarction and cardiac hypertrophy. This elevated us to suggest that the *PINK1* gene might be a pharmacological purpose for certain therapeutic factors.[10] Mitofusins are involved in modulating several cellular processes, like mitochondrial morphology and number, and apoptosis.[11,12] Mitofusin 2 (Mfn2) modulates cell proliferation, and autophagy.[13] In addition, Mfn2 contributes to mitophagy in mammalian tissues and PINK1 phosphorylates Mfn2.[14] On the other hand, Mfn2 emerges to be intimately associated with the modulation of PINK1 for mitophagy and Mfn2 knockout in the heart results in impaired mitophagy.[15] Taken together, mitophagy is firmly modulated, being crucial for cellular viability in cardiac aging. Impaired mitochondria and mitophagy reduction often lead to apoptosis. Also, an increase in apoptotic pathways, including pro-apoptotic protein levels such as Bax, Bax/Bcl2 ratio, and the level of apoptosis has been linked to aging in cardiac tissue; cardiac hypertrophy is typically associated with apoptosis.[16]

In terms of selected mitochondrial therapy, diet therapy is beneficial and should be suggested. Moreover, natural agents have major potentials from which we can find better selected mitochondrial medications. Olive oil (OLO) is the basic origin of fat in the Mediterranean diet, being involved in reducing cardiovascular disease.[17] In addition, OLO possessing a high amount of mono-unsaturated fatty acids, includes other ingredients with substantial biological effects.[18] It is also a good origin of polyphenolic compounds, being reported to have cardio-protective effects against cardiac arrhythmias induced ischemia/reperfusion in rats.[19] Numerous researches have reported that the beneficial effect of OLO on cardio vascular disorders may result from its anti-oxidative, anti-inflammatory, anti-apoptotic, inotropic, and anti-hypertensive properties.[20,21] However, investigations are required to indicate its use in improving cardiac hypertrophy in aging. Thus, it was hypothesized that cardiac hypertrophy could be diminished through the protective effect of OLO by improving mitophagy in aged hearts.

Materials and Methods

Chemicals. Extra virgin OLO used in the present study belonged to the Etka (Rudbar, Iran). All chemicals were bought from Sigma-Aldrich Company (St. Louis, USA).

Animals. All experimental protocols were approved by the Animal Care and Experimentation Committee at Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.AEC.1402.019). Three months old male Wistar rats (weighing 300 ± 20.00 g) were given tap water and standard food and kept at a standard temperature of 25.00 ± 2.00 ˚C in a 12-hr light and 12-hr dark cycle. After 7 days of adaptation, animals were randomly allotted into the three groups with eight rats, including control, aged (D-galactose, D-GAL), and aged rats administered D-GAL plus OLO (D-GAL + OLO). An adult person needs 2,000 calories *per* day, which can get 20.00 - 35.00% of these calories from fat, being equal to 44.00 - 78.00 g fat *per* day. If the weight of the rat is 250 g and the daily intake of 250 µL OLO is considered for it, this amount is equal to the consumption of 70.00 g OLO in a 70-year-old person. Aged animals were received D-GAL at a dose of 150 mg kg^{-1} daily through intra-peritoneal (IP) injection for aging induction.[22] The animals in D-GAL + OLO group were co-administrated with oral OLO at a dose of 1.00 mL kg^{-1} by gavage feeding daily. The administration term is 8 weeks. All animals were anesthetized using sodium pentobarbital at a dose of 60.00 mg kg^{-1} via IP injection and hearts were collected.[23] Finally, the heart tissues were kept at – 80.00 ˚C for molecular and biochemical assessments.

Cardiac hypertrophy. The heart weight (HW) *per* body weight (BW) ratio was regarded as a cardiac hypertrophy marker.[24]

Histological examination of the heart tissue. For structural evaluation, the heart tissues were isolated from the animals and then fixed in 10.00% neutral buffered formalin. The fixed tissues were sectioned (4.00-µm) and stained with Hematoxylin and Eosin. The samples were histologically analyzed through photomicroscope imaging by light microscope (Labomed, Los Angeles, USA). The size of cardiomyocytes and cardiomyocytes nuclei diameter were measured by Image J Software (National Institutes of Health, Bethesda, USA). Macroscopic images of the heart wall were also taken.

Cardiac malondialdehyde (MDA) level. Cardiac MDA level was measured through a commercial kit (Zellbio, Berlin, Germany) based on the manufacturer's protocols.

Gene expression levels. Total RNA was isolated from heart tissue through RNX-plus kit (Sinacolon, Tehran, Iran)

based on the manufacturer's protocol. After determination of concentration and purity of RNA through a NanoDrop apparatus, cDNA was synthesized through reverse-transcription kit in a gradient thermal cycler. Real-time polymerase chain reaction was done from 1.00 µg of cDNA using SYBR Green qPCR Master Mix through Light Cycler 96 from Roche Co. (Mannheim, Germany). Beta-actin was used as an internal control for genes mRNA.

Statistical analysis. Statistical analysis was done using SPSS Software (version 26.0; IBM Corp., Armonk, USA). All findings are presented as means ± standard error. The findings were subjected to one-way analysis of variance followed by Tukey's *post hoc* tests. Statistical significance level was $p < 0.05$.

Results

Cardiac hypertrophy. Figure 1 indicates HW *per* BW ratio as a cardiac hypertrophy index in animal groups. It was identified that the aged animals exhibited an increase in cardiac hypertrophy index compared to control animals, while administration of OLO ameliorated aging mediated cardiac hypertrophy index.

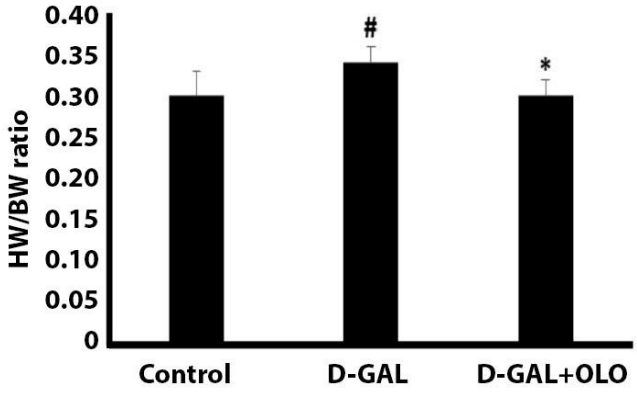

Fig. 1. The heart weight (HW) *per* body weight (BW) of experimental groups. D-GAL: D-galactose; OLO: Olive oil. # $p < 0.05$ compared to the control group; * $p < 0.05$ compared to the D-GAL group.

Cardiac histology. The sections prepared from the control group showed the morphology of cardiomyocytes with oval and bright central nuclei in normal physiological conditions. Examination of different fields of samples from the D-GAL receiving group indicated an irregularity in the arrangement of cardiomyocytes, an increase in the inter-cellular distance, more acidity of the cell cytoplasm, and also importantly the cardiac hypertrophy in D-GAL-induced aging. However, the administration of OLO to the aging rats was able to reduce the irregularity of myofibers, acidification of sarcoplasm, and other pathological conditions being related to the D-GAL-induced cardiac aging. In addition, the sizes of cardiomyocytes were significantly increased in the D-GAL group compared to control group ($p < 0.001$). Also, OLO treatment significantly improved the size of cardiomyocytes in the D-GAL + OLO group in comparison with D-GAL group (Figs. 2 and 3; $p < 0.001$). There was no significant difference in cardio-myocytes nuclei diameter among the groups (Fig. 4). Macroscopic examination of the heart samples showed that the ventricular wall was thickened in the D-GAL group compared to the control group, indicating cardiac hyper-trophy. While, the treatment with OLO reduced the thickness of the ventricles in D-GAL + OLO group (Fig. 5).

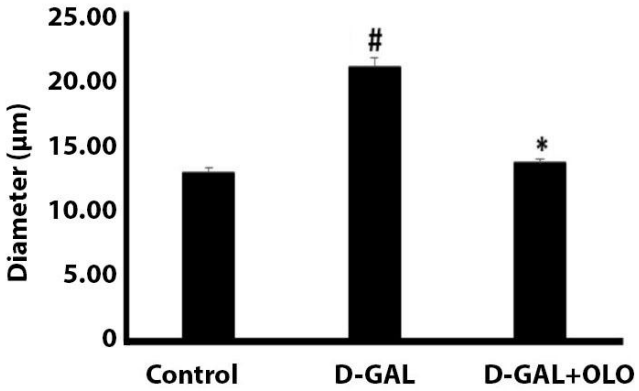

Fig. 3. Cardiomyocytes size in experimental groups. D-GAL: D-galactose; OLO: Olive oil. # $p < 0.001$ compared to the control group; * $p < 0.001$ compared to the D-GAL group.

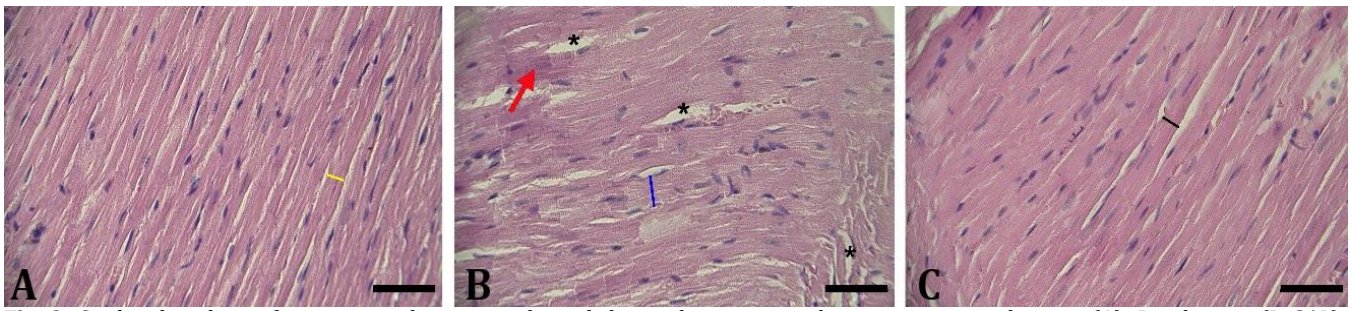

Fig. 2. Cardiac histology of experimental groups indicated the cardiac tissue architecture in control group **(A)**, D-galactose (D-GAL)-induced aging group **(B)**, and D-GAL plus olive oil (OLO) group **(C)**. Asterisks show the disarrangement of cardiomyocytes and the spaces between them. The red arrow shows the acidophilic and altered cytoplasm of cardiomyocytes in the D-GAL group. The yellow, blue, and black lines show the diameter of cardiomyocytes in the control group (14.00 µm), D-GAL group (19.50 µm), and D-GAL + OLO group (14.00 µm), respectively. (Hematoxylin and Eosin staining, Bars = 50.00 µm).

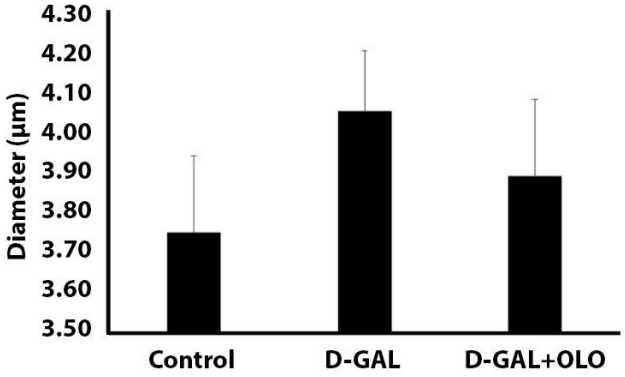

Fig. 4. Cardiomyocytes nuclei diameter in experimental groups. D-GAL: D-galactose; OLO: Olive oil. No significant difference were observed among the groups

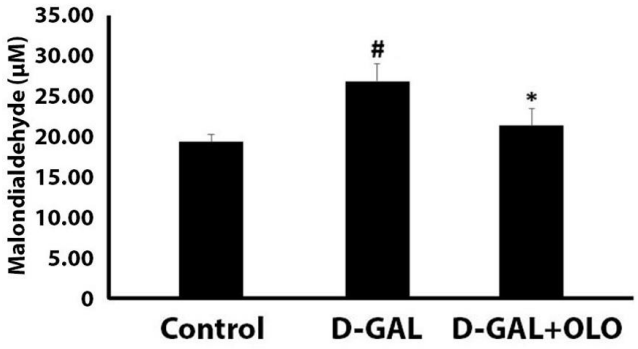

Fig. 6. Malondialdehyde (MDA) levels in the heart tissue of experimental groups. D-GAL: D-galactose; OLO: Olive oil. # $p < 0.001$ compared to the control group; * $p < 0.01$ compared to the D-GAL group.

Cardiac MDA level. Cardiac MDA levels in the experimental groups are displayed in Figure 6. Aged rats showed increased MDA level in the heart tissue compared to the control rats, while treatment with OLO reduced cardiac MDA level. These data indicated that aging plays a critical role in cardiac oxidative stress, and OLO can reverse this parameter.

Gene expression levels. It was observed that there was a remarkable decrease in Drp1, Mfn2, PINK1, and Bcl2 expressions in aged animals compared to the control ones. Moreover, aged animals showed an enhancement in Bax expression compared to the control ones. Also, there was an improvement in the above-mentioned parameters in OLO-treated animals (Table 1).

Discussion

The heart is very sensitive to the aging process. In aging, the heart is prone to become hypertrophic, and cardiac hypertrophy is a major feature of cardiac aging. Interestingly, it was observed in this study that aging induced by D-GAL caused increased HW *per* BW ratio in the aged rats. In addition to the cardiac hypertrophy index, histological alterations in the aged hearts were assessed in the present study. It was found that the D-GAL injections into the peritoneal of rats for eight weeks led to apparent cardiac hypertrophy. These data suggest that D-GAL injection causes aging-mediated cardiac hypertrophy. Previous studies have demonstrated cardiac hypertrophy

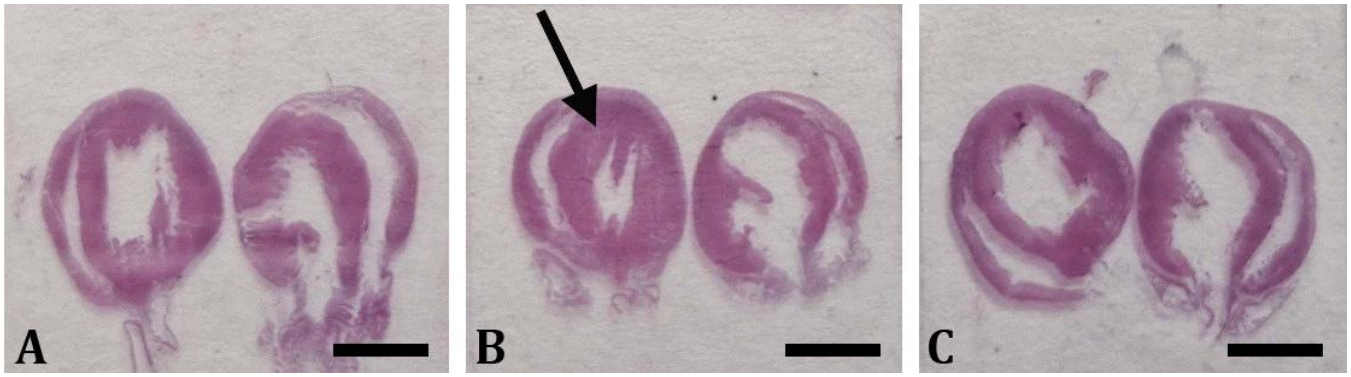

Fig. 5. Macroscopic images of the heart wall section in experimental groups. **A)** Control group; **B)** D-galactose (D-GAL)-induced aging group; and **C)** D-GAL plus olive oil group. The black arrow indicates the thickening of the heart wall in the D-GAL group. (Hematoxylin and Eosin staining, Bars = 5.00 mm).

Table 1. Effect of olive oil treatment on gene expression in aging rats (mean ± SEM).

Genes	Control	D-GAL	D-GAL + OLO
Drp1	1.00 ± 0.02	0.032 ± 0.02#	0.96 ± 0.05***
Mfn2	1.00 ± 0.01	0.025 ± 0.01#	2.00 ± 0.20**
PINK1	1.00 ± 0.25	0.018 ± 0.003#	1.50 ± 0.40***
Bax	1.00 ± 0.40	23.50 ± 5.40#	0.10 ± 0.03**
Bcl2	1.00 ± 0.30	0.04 ± 0.004#	0.35 ± 0.05*

D-GAL: D-galactose; OLO: Olive oil; *Drp1*: Dynamin-related protein 1; *Mfn2*: Mitofusin 2; *PINK1*: Phosphatase and tensin homologue (PTEN)-induced putative kinase 1.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the D-GAL group and # $p < 0.001$ compared to the control group.

in D-GAL-injected rats.[25] However, these cardiac alterations were improved post-long-term treatment with OLO highlighting that OLO has protective effects on D-GAL-induced structural changes in the aged hearts. The accumulation of impaired mitochondria is a major feature of the aging process. Impaired mitophagy contributes to the cardiac hypertrophy induced by aging.[26] In addition, reduced mitochondrial fusion and fission are associated with cardiac hypertrophy. Former study indicated that *Mfn2* gene expression was reduced in a rat model of cardiac hypertrophy.[27] Therefore, it can concluded that cardiac hypertrophy can be suppressed through Mfn2 expression increase.[28] The PINK1-dependent mitophagy is initiated by Mfn2. The Mfn2 is the master modulator of the processes leading to the determination of impaired mitochondria through mitophagy regulator proteins.[29] Moreover, PINK1 contributes to the mitochondrial quality and mitophagy in the heart, and reduced *PINK1* gene expression results in mitochondrial damage in the cells.[30]

Recently, PINK1-dependent mitophagy is the most widely reported mitophagy pathway.[31] The PINK1 knockout results in cardiac hypertrophy associated with oxidative stress, apoptosis, and cardiac dysfunction in mice. The Drp1 expression not only contributes to mitochondrial fission but also significantly plays a major role in PINK1 expression in the heart, increasing mitophagic mitochondrial composition in cardiomyopathy and cardiac hypertrophy.[32] The Drp1-assiociated mitophagy ameliorates mitochondrial dysfunction and cardiac hypertrophy induced by obesity.[33] The integrity of the heart and brain are maintained by mitophagy through Drp1 expression in mammalians.[34] Reduction in *Drp1* gene expression leads to mitophagy inhibition and in turn, mitochondrial damage increase, oxidative stress, and finally apoptosis.[35] Our results showed that PINK1 was markedly reduced in parallel with the reduced cardiac Mfn2 and Drp1 expressions in aged animals. Impaired mitophagy leads to ROS production in aging, enhancing mitochondrial damage and cytochrome C release, further resulting in ROS-evoked injuries, inducing a vicious circuit and in turn, causing cellular apoptosis.[36] This proposes that the enhanced ROS led to the mitochondrial dysfunction and apoptosis increase. Apoptosis is a critical pathway leading to the cardiac hypertrophy and dysfunction.[37] Increasing evidence also shows that a reduction in mitophagy induces apoptosis.[38] Mitochondrial-related apoptosis is modulated through two clusters of Bax and Bcl2 proteins, and the equilibrium between these two clusters controls the apoptosis induction.[39] In the present study, the aged rats showed pronounced increased Bax expression and reduced Bcl2 expression, indicating increased apoptosis in aged animals.

The accumulation of impaired mitochondria is a major feature of the aging process, being related to the increased ROS production and oxidative stress.[40] Experimental studies have reported oxidative stress as a critical factor of cardiac hypertrophy.[41] The oxidative stress results in cell injury by lipid peroxidation of membranes. Cell membranes and other lipid-containing structures are affected by lipid peroxidation.[42] The MDA as a key secondary product of lipid peroxidation is a oxidative stress biomarker in aging process.[43] In the present study, a remarkable increase in the cardiac MDA levels compared to the controls rats was found in the aged rats. It was found that treatment of old rats with OLO resulted in a cardiac hypertrophy reversal, and this decreased HW *per* BW ratio. This structural alteration by OLO treatment was associated with an increase in cardiac gene expression of mitophagic factors, including *Mfn2*, *Drp1*, and *PINK1*, reducing cardiac hypertrophy and *Bax* gene expression, increasing *Bcl2* gene expression, and hence, reducing oxidative stress. Together, these results are consistent with the hypothesis that OLO can improve major structural and molecular features of cardiac aging. It is suggested that OLO regulates cardiac hypertrophy and dysfunction being resulted from myocardial infarction through anti-oxidative and anti-inflammatory effects.[44] The intake of OLO causes a reduction in free radical generation at the mitochondrial level. Moreover, OLO intake results in a better function of the mitochondrial electron transport chain in various models of oxidative stress.[45] Recently, treatment with OLO has led to a reduction in cardiac oxidative stress and tumor necrosis factor-α, and improvement of left ventricular ejection fraction in coronary artery ligation model, suggesting that OLO regulates cardiac hypertrophy and heart failure induced by myocardial infarction.[44] It has been reported that OLO intake improves DNA damage, oxidative injury, anti-oxidative activities, and histological changes of the heart in rats co-exposed to acrylamide and aluminum.[46] Also, it has been shown that treatment with OLO enhances mitochondrial density and function in rats.[47] On the other hand, it can be proposed that OLO regulates apoptosis and oxidative stress through improving mitophagy, ameliorating cardiac hypertrophy in aging models.

In conclusion, the deleterious effects of aging in the heart could be reduced by OLO *via* improving cardiac hypertrophy through regulating apoptosis and oxidative stress by increasing mitophagy in aged rats. These results provide insights into the possible applications and underlying molecular mechanisms of OLO in decelerating of aging process.

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Conflict of interest

None declared.

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