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The Effect of Training and Royal Jelly on the Expression of FoxO3a, MAFbx and AMPK of Cardiomyocytes in Obese Rats

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ABSTRACT

Introduction: Overweight and obesity increase the risk of cardiovascular diseases. Obesity is also known to contribute to heart muscle atrophy. This study investigates the effects of aerobic exercise and royal jelly (RJ) supplementation on indices of heart tissue atrophy and hypertrophy in rats fed a high-fat diet.

Methods: Forty-five male Wistar rats were randomly divided into five groups (n=9 per group): normal diet (ND), high-fat diet (HFD), high-fat diet with training (HFDT), high-fat diet with royal jelly (HFDRJ), and high-fat diet with both training and royal jelly (HFDTRJ). The supplement groups received 100 mg of royal jelly per kg of body weight orally every day. The exercise program involved running on a treadmill at an intensity of 50-60% of VO2max, performed five days a week for eight weeks. Data were analyzed using one-way ANOVA and Tukey's post hoc test with a significance level of $p<0.05$.

Results: In the HFD group, the expression of FoxO3a and MAFbx was significantly increased, while AMPK expression was decreased compared to the ND group (P=0.000). There was a significant decrease in FoxO3a and MAFbx expression in the HFDT (P=0.033 and P=0.027), HFDRJ (P=0.049 and P=0.041), and HFDTRJ (P=0.000 and P=0.000) groups compared to the HFD group. Additionally, the HFDTRJ group showed significant reductions in FoxO3a and MAFbx compared to the HFDT (P=0.045 and P=0.041) and HFDRJ (P=0.030 and P=0.027) groups. A significant increase in AMPK expression was observed in the HFDT (P=0.002), HFDRJ (P=0.007), and HFDTRJ (P=0.000) groups compared to the HFD group. Furthermore, the HFDTRJ group exhibited higher AMPK expression compared to the HFDT (P=0.048) and HFDRJ (P=0.015) groups.

Conclusions: Aerobic training, with or without the intake of royal jelly, leads to a reduction in the expression of FoxO3a and MAFbx and an increase in AMPK. These changes may result in decreased atrophy indices and enhanced hypertrophy of cardiomyocytes in rats fed a high-fat diet.

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Introduction

Obesity is an expensive condition that leads to a diminished quality of life and, ultimately, premature death. It is also a significant risk factor for many cardiovascular diseases (CVD) (1). Several mechanisms, including cytokines, biological mediators, beta-adrenergic signals, and nutritional signaling pathways, link obesity to cardiovascular diseases (2, 3). However, one of the most critical contributors to cardiovascular diseases is the breakdown of cellular proteins and the replacement of lost cells with collagen (3). The primary pathway for protein degradation involves the activation of FoxO. FoxO is the key initiator of protein degradation during the atrophy process. The FoxO3 signaling pathway plays an important role in the pathogenesis of skeletal muscle. This role is carried out by regulating E3 ubiquitin ligases and certain autophagy factors (4). FoxO3a acts as a central mediator in atherogenesis; when activated, it translocates from the cytosol to the nucleus, triggering the activation of two proteins that degrade muscle tissue: MAFbx and MuRF1, thus initiating tissue destruction (5). Relling (2006) demonstrated that obesity increases FoxO3a levels in the cardiac cells of obese rats, contributing to cardiac dysfunction and mitochondrial damage (6). Kandola (2016) also showed that the FoxO family plays a significant role in cardiomyopathy (7). In addition, Torabi et al. (2022) found that the induction of obesity in elderly rats is associated with a decrease in the levels of FoxO3a, MAFbx, and MuRF1 in cardiomyocytes (8). Interestingly, the activation of FoxO regulates the genes for ubiquitin ligases Atrogin-1/MAFbx and MuRF1, leading to cardiac wasting. The upregulation of MAFbx gene

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expression in the heart reduces both physiological and pathological hypertrophy. Furthermore, the upregulation of MuRF1 gene expression in cardiac muscles prevents hypertrophy (9). Inhibiting this process may reduce protein degradation and the incidence of cardiovascular diseases. FoxO inhibition occurs through AKT and PGC1α (10). Moreover, AMPK has been identified as a crucial factor in cardiac hypertrophy. Key processes for the initiation and progression of cardiac hypertrophy include the transcription of hypertrophy-related genes, cell growth, strengthening of the cytoskeleton, protein synthesis, and expansion of the sarcomere. These anabolic processes demand substantial energy, all of which is regulated by AMPK (2). In recent decades, exercise training, alongside diet, has been promoted as an effective non-pharmacological method for managing obesity. Exercise training has beneficial effects on the cardiovascular system by enhancing the condition of myocardial cells. It is thought that chronic exercise leads to an increase in PGC-1α in muscle tissue (11). Research has demonstrated that FoxO, a protein responsible for degrading muscle tissue, interacts with PGC1α, an essential cofactor in mitochondrial biogenesis. Elevated levels of PGC1α can inhibit this muscle protein-degrading factor (12). Liu et al. (2018) showed that moderate-intensity exercise mitigates diabetes-induced muscle atrophy in db/db mice by influencing the SIRT1-AMPKα-PGC1α axis (13). Additionally, aerobic exercise downregulates the expression of MuRF1. Nevertheless, the mechanisms by which exercise training affects atrophy and hypertrophy signaling pathways in obesity remain unclear. Produced by honeybees, royal jelly (RJ) serves as the nutritional source for queen bees and is rich in various nutrients. Certain components in RJ may stimulate muscular adaptation. In studies involving rats, RJ consumption increased serum IGF-1 levels and promoted the regeneration of damaged muscle via the IGF1-Akt pathway in satellite cells (14). Furthermore, the combination of RJ administration and endurance training led to mitochondrial adaptations by activating AMPK in the soleus muscles of rats (15). Clinical trials have also demonstrated that RJ enhances handgrip strength, which tends to decline with age (16). Additionally, RJ treatment has influenced muscle fiber size, as well as markers related to satellite cells and catabolic genes (17). One potential target for protein and/or amino acid supplementation is AMPK, a cellular energy sensor and crucial regulator of mitochondrial biogenesis. Previous studies indicated that leucine and casein peptides can activate AMPK in skeletal muscle. In addition to amino acids and protein, 10-HAD, a fatty acid found in RJ, is also capable of activating AMPK in skeletal muscle (18). Therefore, using royal jelly (RJ) to promote adaptation in skeletal muscles and cardiac tissue can be effective. While the beneficial effects of RJ on various bodily systems have been well documented, there remains insufficient research demonstrating its impact on indices of atrophy and hypertrophy in heart tissue. Conversely, obesity and being overweight may be linked to a decline in cardiac function, which can subsequently damage heart structure. As a result, the implementation of non-pharmacological treatment methods, such as exercise, alongside the use of natural supplements (in this case, RJ), could offer new therapeutic avenues due to their positive effects. This study hypothesizes that the combined application of exercise and RJ may have a more pronounced effect on the indicators of atrophy and hypertrophy in obese rats compared to either treatment alone. Consequently, this study seeks to investigate the simultaneous effects of aerobic exercise and royal jelly on specific indicators of atrophy and hypertrophy in cardiomyocytes of rats fed a high-fat diet (HFD).

Materials and Methods

Forty-five male Wistar rats, eight weeks old with an average weight of 187.51 ± 9.37 grams, were used in this study. The animals were maintained under a 12:12-hour light-dark cycle, at an ambient temperature of 22 ± 1.4°C, with humidity levels at 55.6 ± 4%. After one week of acclimatization to the new environment, the rats were divided into two groups: a normal diet group (ND, n=9) and a high-fat diet group (HFD, n=36). The ND group received a standard diet consisting of 23% protein, 65% carbohydrates, and 12% fat for eight weeks, while the HFD group was fed a high-fat diet comprising 40% fat, 43% carbohydrates, and 17% protein. After eight weeks, the rats were further categorized into five groups: ND, HFD, high-fat diet training (HFDT), high-fat diet with royal jelly (HFDRJ), and high-fat diet training with royal jelly (HFDTRJ). Obesity in the rats was assessed using Lee's

index, with a value of 310 or higher indicating obesity. Rats with Lee index values exceeding 310 were classified as obese (19).

Training Protocol

Before the main training program began, the rats were given five minutes to familiarize themselves with the treadmill. During this familiarization period, they ran at a speed of 8-10 m/min on a flat surface across five sessions over one week. The aerobic exercise protocol consisted of running on a treadmill with a 0% incline, performed five days a week for eight weeks. In the first week, the rats followed an incremental exercise regimen, starting at an intensity of 15 m/min for 30 minutes. Over the following weeks, the intensity progressively increased from 15 m/min to 25 m/min by the seventh week, while the duration of the exercise also increased, reaching 60 minutes by the end of the training period (Table 1) (20).

Table 1. Training protocol

week	1	2	3	4	5	6	7	8
Intensity (m)	15	16	18	20	21	23	25	25
Time (min)	30	35	40	45	50	55	60	60

Royal Jelly Consumption

Royal jelly powder was obtained from Bulk Supplements Co, Ltd (Henderson, USA). In addition, 100mg/kg body weight of royal jelly was consumed daily by the supplement group (21).

Heart Tissue Sampling Method and Gene Expression Measurement

All samples were anesthetized under identical conditions, following standard protocols, using a combined intraperitoneal injection of ketamine and xylazine. After tissue collection and washing, the desired tissues were frozen and stored at -80°C until further analysis. To minimize the influence of diurnal variations, sampling was conducted between 8:00 AM and 11:30 AM. Table 2 presents the primer sequence.

Table 2. Primer pattern of AMPK, MAFbx, FoxO3a

Genes	Sequence (5' → 3')
F AMPK	ACTATCAAAGACATACGAGAGCA
R AMPK	CTTGAGGGTCACCACTGTATAA
F MAFbx	AGGGCAGGTGGATTGGAAGAAGA
R MAFbx	GTTGGGGTGAAAGTGAGACGGAG
F FoxO3a	GCCTCATCTCAAAGCTGGGT
R FoxO3a	TGCTCTGGAGTAGGGATGCT
F β-Actin	AGGAGTACGATGAGTCCGGC
R β-Actin	CGCAGCTCAGTAACAGTCCG

Statistical Analysis

One-way ANOVA and Tukey's post hoc test were employed for statistical analysis at a significance level of $p \leq 0.05$ after confirming that the data followed a normal distribution.

Results

The average weight of the groups before, during, and after the induction of obesity is presented in Table 3.

Table 3. The average weight of the groups before and during the obesity induction period

	before induction of obesity			**Induction of obesity**				
	8-week-old Rat	After adaptation	Grouping	First week	Second week	Fourth week	Sixth week	Eighth week
Age (weeks)	8	9		10	11	12	13	14
Groups	187.51±9.37	200.51±16.26	ND (n=9)	211.33±19.34	216.33±17.66	245.22±16.51	257.22±22.81	270.11±27.55
			HFD(n=36)	209.61±22.74	233.56±13.90	271.89±21.20	310.58±21.68	350.83±41.01

Average weight of the groups after induction of obesity					
Age (weeks)	17	18	20	22	24
ND	270.11 ± 27.55	281.78 ± 24.13	291.55 ± 32.94	297.88 ± 36.06	310.88 ± 38.95
HFD	343.66 ± 50.90	384.33 ± 23.54	410.44 ± 47.65	435.11 ± 81.86	461.11 ± 37.94
HFDT	352.88 ± 42.72	374.22 ± 23.38	389.77 ± 43.33	411.55 ± 37.56	414.00 ± 49.05
HFDRJ	348.44 ± 37.98	377.11 ± 23.12	398.88 ± 51.68	415.88 ± 51.68	423.77 ± 49.11
HFDTRJ	358.33 ± 36.97	368.44 ± 21.48	378.66 ± 45.43	387.22 ± 50.31	390.55 ± 40.12

Data analysis revealed a significant difference in the mean changes of FoxO3a expression in cardiomyocytes (F = 11.553, P = 0.000) (Figure 1). Tukey's test showed a significant increase in FoxO3a expression in the HFD group (P = 0.000) compared to the ND group. Additionally, a significant decrease in FoxO3a levels was observed in the HFDT (P = 0.033), HFDRJ (P = 0.049), and HFDTRJ (P = 0.000) groups compared to the HFD group. Furthermore, a significant reduction was observed in the HFDTRJ group compared to both the HFDT (P = 0.045) and HFDRJ (P = 0.030) groups (Figure 1).

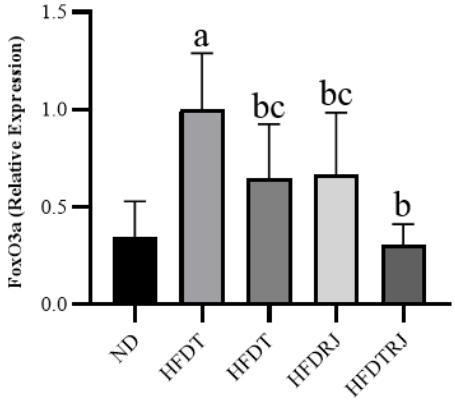

Figure 1. Cardiomyocyte FoxO3a expression by one-way ANOVA test (at P<0.05 level).
a Difference with ND, b Difference with HFD group, c Difference with HFDTRJ group.
ND: normal diet, HFD: high-fat diet, HFDT: high-fat diet-training, HFDRJ: high-fat diet-royal gel, HFDTRJ: high-fat diet-training-royal gel.

Data analysis revealed a significant difference in MAFbx expression changes in cardiomyocytes between the groups (P = 0.000, F = 15.749) (Figure 2). Tukey's test indicated a significant increase in MAFbx expression in the HFD (P = 0.0001), HFDT (P = 0.003), and HFDRJ (P = 0.002) groups compared to the ND group. Furthermore, a significant decrease in MAFbx expression was observed in the HFDT (P = 0.027), HFDRJ (P = 0.041), and HFDTRJ (P = 0.000) groups compared to the HFD group. Additionally, a significant reduction was found in the HFDTRJ group compared to both the HFDT (P = 0.041) and HFDRJ (P = 0.027) groups (Figure 2).

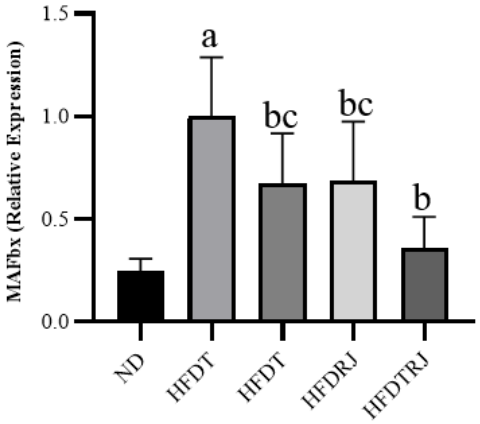

Figure 2. Cardiomyocyte MAFbx expression by one-way ANOVA test (at P<0.05 level).
a Difference with ND, b Difference with HFD group, c Difference with HFDTRJ group.
ND: normal diet, HFD: high-fat diet, HFDT: high-fat diet-training, HFDRJ: high-fat diet-royal gel, HFDTRJ: high-fat diet-training-royal gel.

Finally, data analysis revealed a significant difference in AMPK expression in cardiomyocytes among the different groups (P = 0.0001, F = 18.671) (Figure 3). Tukey's test indicated a significant decrease in AMPK in the HFD (P = 0.000), HFDT (P = 0.007), and HFDRJ (P = 0.002) groups compared to the ND group. Additionally, a significant increase in AMPK expression was observed in the HFDT (P = 0.002), HFDRJ (P = 0.007), and HFDTRJ (P = 0.000) groups compared to the HFD group. Furthermore, a significant increase in AMPK expression was found in the HFDTRJ group compared to both the HFDT (P = 0.048) and HFDRJ (P = 0.015) groups (Figure 3).

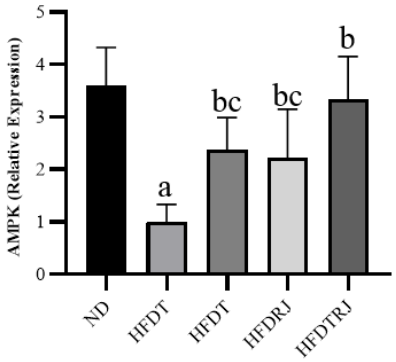

Figure 3. Cardiomyocyte AMPK expression by one-way ANOVA test (at P<0.05 level).
a Difference with ND, b Difference with HFD group, c Difference with HFDTRJ group.
ND: normal diet, HFD: high-fat diet, HFDT: high-fat diet-training, HFDRJ: high-fat diet-royal gel, HFDTRJ: high-fat diet-training-royal gel.

Discussion

In the present study, a high-fat diet (HFD) increased the expression of FoxO3a and MAFbx in cardiomyocytes. This aligns with findings from Hasan et al. (2019), who showed that HFD led to significant increases in FoxO3, Atrogin-1/MAFbx, and MuRF-1, along with a rise in collagen in the soleus muscle of rats (22). Similarly, Abrigo et al. (2016) reported that HFD induced a decrease in muscle weight and muscle fiber diameter, which was attributed to heightened skeletal muscle catabolism (23). Gao et al. (2023) also found that HFD can induce skeletal muscle atrophy by upregulating MuRF1, FbX32, and p53 (24). The elevated expression of FoxO3a has been linked to increased muscle fiber atrophy, with high levels of FoxO3a associated with the upregulation of MAFbx/Atrogin-1 and MuRF-1 (5). Several factors contribute to muscle atrophy, including obesity, excessive caloric intake, insulin resistance, and chronic inflammation (25). Obesity impairs Akt signaling, increases NF-κB and FoxO1, and elevates levels of pro-inflammatory cytokines like TNF-α and IL-6, all of which are associated with muscle atrophy (26). In our study, the increased levels of FoxO3a and MAFbx in HFD rats were accompanied by reduced AMPK expression in cardiomyocytes. AMPK functions as a cellular energy sensor, activated when intracellular AMP levels rise. AMPK activation triggers ATP generation pathways and regulates cell proliferation and biosynthesis. AMPK also plays a role in controlling muscle mass by modulating protein degradation via the ubiquitin-proteasome system and autophagy (27). Previous studies have shown that AMPK activation influences muscle fiber degradation through the FoxO transcription factors. The FoxO family (FoxO1, FoxO3, FoxO4, and FoxO6) regulates cellular processes such as apoptosis, differentiation, metabolism, proliferation, and survival. FoxO transcription factors integrate signals from insulin, growth factors, cytokines, and oxidative stress. AMPK directly phosphorylates FoxO3 (28). Notably, our study showed a decrease in FoxO3a and MAFbx expression, along with an increase in AMPK in the cardiomyocytes of HFD rats following aerobic training. Targeting the inhibition of the FoxO3a pathway may be a promising strategy for preventing muscle atrophy. This is supported by studies showing that aerobic training reduces FoxO3a expression in the cardiomyocytes of diabetic rats (29). Furthermore, Esmaili et al. (2019) demonstrated that aerobic training elevates AMPK expression

while decreasing MAFbx expression in cardiac myocytes (30). Rahimi et al. (2023) found that high-intensity interval training (HIIT) downregulates FoxO1 and Atrogin-1 in the muscles of diabetic rats (31). Aghaei et al. (2021) also showed that HIIT reduces cardiac levels of FoxO3a and FoxO1 in diabetic rats, suggesting that inhibiting these proteins could prevent cardiac autophagy (32). However, Holloway et al. (2015) found that HIIT training did not affect FoxO3a protein levels in the cardiac muscle of rats with heart failure (33). Differences in exercise type, training frequency, and subject characteristics may account for these discrepancies; for instance, previous studies focused on rat models with heart issues, whereas our study focused on HFD rats. Additionally, aerobic training and HIIT differ in intensity and the physiological adaptations they induce. Exercise training inhibits FoxO3a and MAFbx expression in cardiomyocytes by increasing AMPK expression, thereby preventing heart atrophy. One potential mechanism behind these changes is the enhanced muscle levels of PGC1α, which can suppress FoxO3a. FoxO3a is a key mediator in atherogenesis and can activate MAFbx (5).

In our study, royal jelly (RJ) increased the expression of AMPK and decreased the expression of FoxO3a and MAFbx. You et al. (2019) reported that 10-HDA, a fatty acid found in RJ, activates the AMPK pathway and its downstream signaling pathway, PI3K/AKT (34). Furthermore, Okumura et al. (2018) observed that oral intake of RJ in aged mice resulted in a reduction of catabolic genes such as MuRF1 and MAFbx (17). These researchers noted that RJ delays muscle apoptosis by inhibiting the activity of catabolic genes. Additionally, another study demonstrated that RJ enhances the AKT signaling pathway by boosting serum levels of IGF-1. Evidence suggests that RJ (specifically 10-HDA) has beneficial effects on inflammation and autophagy by regulating AMPK, which, in turn, modulates NF-κB and IL-1β inflammatory signaling (35). The molecular mechanism by which RJ inhibits proteolysis involves the interaction of various intracellular messenger pathways. RJ activates mTOR and AKT while inhibiting proteolysis (14). AKT, as a key substrate of mTORC2, is part of the PI3K family. In the presence of growth factors, PI3K activates mTORC1, which regulates key factors in protein synthesis, such as S6K and eIF4E. Additionally, mTORC1 interacts with AMPK, leading to the phosphorylation of Unc-51 Like Autophagy Activating Kinase 1 across various tissues (36). RJ has also been shown to influence FoxO transcription by modulating insulin/IGF-1 signaling (37). FoxO is instrumental in activating the AKT pathway, which regulates multiple stress response pathways, including ROS and DNA repair, as well as translation. Furthermore, the FoxO family directly regulates muscle atrophy genes, such as MUSA1 and SMART (38). Among other findings, we observed a decrease in FoxO3a and MAFbx expression, along with an increase in AMPK expression in the cardiomyocytes of HFD rats in the HFDTRJ group compared to the other groups. To the best of our knowledge, no study has concurrently examined the effects of training and RJ on these indicators in HFD rats. However, Takahashi et al. (2018) reported that oral administration of RJ and 10-HDA, alongside endurance exercise, enhanced mitochondrial function in the soleus muscle and induced AMPK activation (15). Research has shown that aerobic exercise can activate PGC1α while inhibiting FoxO3a (12), thereby facilitating the activation of MAFbx (5) and potentially preventing cardiac atrophy (12). Furthermore, RJ may inhibit the degradation of cardiac muscle by influencing the mTOR and AKT pathways (14). The combination of exercise and RJ resulted in more significant changes in FoxO3a, MAFbx, and AMPK expression due to their synergistic effects. RJ has the potential to enhance the benefits of exercise, despite the differing signaling pathways involved. Therefore, exploring these pathways in future studies could yield valuable insights into the combined effects of exercise and RJ on cardiac muscle hypertrophy and atrophy. The limitations of the current study include its short duration and the forced induction of obesity, which may not fully reflect the long-term effects of obesity, exercise, and RJ supplementation. Additionally, examining the cellular changes in heart tissue, particularly morphology, could provide a clearer understanding of the effects of exercise and supplementation.

Conclusion

In the current study, both aerobic training and royal jelly (RJ) positively impacted cardiac function by improving the indices of atrophy and hypertrophy in rats fed a high-fat diet. Notably,

the combined effect of aerobic training and RJ on these metrics was found to be more significant. Consequently, it is recommended to use both aerobic training and RJ together as an effective approach to mitigate HFD-induced cardiac atrophy in obese individuals.

Declarations

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Conflict of Interest
There is no conflict of interest.

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Ethical Consideration
Approval was granted by the Ethics Committee of the Islamic Azad University, Marvdasht (No. IR.IAU.M.REC.1400.020).

Author Contributions
All authors equally contributed to the design, execution, analysis of results, and writing of the manuscript.

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