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Physiological aspect of apoptosis-regulating microRNAs expressions during fasting

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Abstract. – OBJECTIVE: Fasting is an activity that requires a certain calorie restriction without consuming food or drinks for a certain period of daytime. However, fasting triggers many complex events, including activating cellular stress response pathways, autophagy promotion, apoptosis pathways, and a change in hormonal balance. Among the many events affecting the regulation of apoptosis, the expression of microRNAs (miRNAs) plays an important role. Therefore, we aimed to investigate the levels and importance of miRNA expression in fasting.

PATIENTS AND METHODS: The expressions of 19 miRNAs regulating different pathways from saliva samples, isolated by matching healthy university students (n = 34) as group 1 (fasting for 17 consecutive hours) and group 2 (testing 70 minutes after meal consumption), were examined using the real-time PCR method.

RESULTS: In fasting, modulation of apoptotic pathways by miRNAs triggers anti-pathogenic effects, and the adaptation of abnormal cells in the body decreases. For this reason, vital diseases, such as cancer, can be treated by preventing the proliferation and growth of cancerous cells by increasing programmed cell death due to the down-regulation expression mechanism of miRNAs.

CONCLUSIONS: Our study aims to improve the knowledge about the mechanisms and functions of miRNAs in various apoptosis pathways during fasting and may be a model for further future physiological and pathological studies.

Key Words:
MicroRNAs, Fasting, Polymerase chain reaction, Apoptotic pathways, MiR/221-222.

Abbreviations
MicroRNAs (miRNAs), Polymerase Chain Reaction (PCR), Confidence Interval (CI), Cycling threshold (Ct).

Introduction

Fasting is an activity that requires a specific calorie restriction without consuming food or drink at a specific time of the day. In general, health, religious worship, strikes, and poverty are among the causes of hunger. Moreover, fasting has been proposed as a medical treatment since ancient times. It is known[1-3] that fasting causes hormonal, immunological, and physiological changes in metabolism as well as various effects in terms of mineral and vitamin balance. In addition, the energy required for metabolic events during fasting (up to the 12th hour of fasting) is supplied by glycogen stores[4].

Fasting triggers many complex events, including apoptosis, autophagy, activation of cellular stress response pathways, and a change in hormonal balance. One of these phenomena, apoptosis, is an active process that is genetically or epigenetically regulated in both physiological and pathological phenomena[5,6]. Recently, the effects of fasting on apoptotic pathways in the body have begun to be studied. Experimental animal studies[7] conducted on this subject have supported the view that fasting influences apoptosis. Indeed, prolonged periods of starvation were responsi-

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ble for the increase in apoptosis by suppressing cell proliferation among different cells. In recent years, evidence[8] has been accumulating those miRNAs (such as post-transcriptional control of gene expression as well as small non-coding RNA molecules) are associated with many events, such as apoptosis. In one of these relationships, the binding of various miRNAs to the 3'UTR of the mRNA inhibits protein synthesis partially or completely, and therefore, the role of events such as apoptosis in the regulation of gene expression has been investigated[8]. In addition, it has been discovered by other studies[9] that most miRNAs are found not only inside the cell, but also in various body fluids.

Based on all this information, induction and regulation of apoptosis by miRNAs during starvation might be considered as a defense mechanism against malignancies in the context of the cause-and-effect relationship. Further studies might also be needed to support our statement.

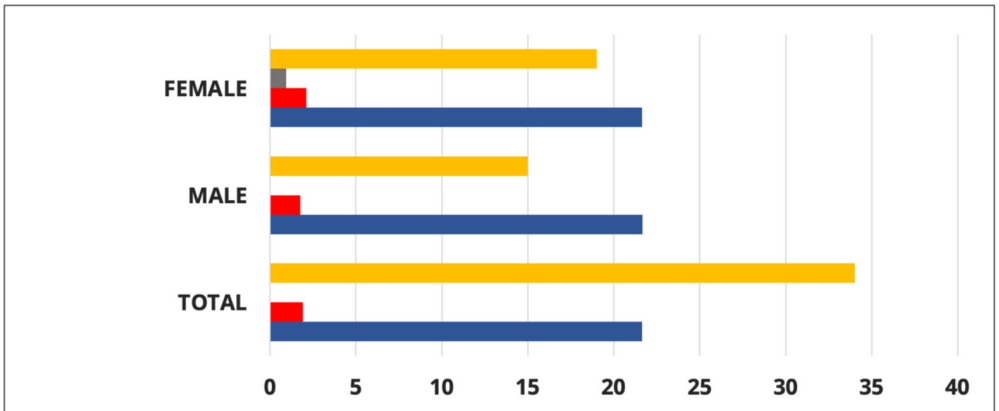
Patients and Methods

Collection of Saliva Samples

Saliva samples were collected from thirty-four healthy university students of all genders and ages who were divided in two different groups (mean age was 21.7 ± 1.92 and at least 18, at most 26 years) and analyzed by PCR test. In the first group, a PCR test was performed after 17 hours of fasting. Group 2, also known as the control group, consisted of a PCR performed 70 minutes after food consumption. To carry out our study, 19 miRNAs (miR-141, miR-146a-5p, miR-331, miR-181b, miR-21, miR-155, miR-133a, miR-221, miR-222-3p, miR-106b, miR-let- 7c, miR-let-7f, miR-31, miR-125b, miR-143, miR-183, miR-210) were isolated from the salivary that were obtained from our candidates. The detailed demographic features are given in Figure 1. The subjects fasting and post-prandial saliva samples were collected in accordance with the approval (2017/0126) and consent obtained by our Local Ethical Committee.

Complementary DNA (cDNA) Synthesis

Complementary DNA (cDNA) was randomly synthesized from 5 µg of miRNA-enriched total RNA using miScript II Reverse Transcription (RT) Kit (Qiagen, Hilden, Germany). A total of 20 µl reaction volume was set for each RT-PCR reaction with 4 µl of 5 × miScript HiSpec Buffer, 2 µl of 10 × Nucleic Mix, 1 µl of miScript Reverse Transcriptase Mixture, 8 µl of RNase-free water, and 5µl of template RNA. The reverse transcription was performed at 37°C for 60 minutes and following 95°C for 5 minutes. The cDNA was diluted with 200 µl of nuclease-free water for the qRT-PCR (Real-Time PCR) reaction. Similar detailed protocols were followed as previously reported by our group in the study published by Aslan et al and Tuncturk et al[10].



	TOTAL	MALE	FEMALE
n	34	15	19
P-Value	0	0	0.953
Standard Deviation	1.924	1.759	2.125
Mean Age	21.65	21.67	21.63

Figure 1. The demographic features of subjects.

RT-PCR

qRT-PCR was performed on Rotor-Gene® Q instrument with software 2.1.0.9 (available at: https://www.qiagen.com/kr/resources/resourcedetail?id=8435805b-2c5d-4fa9-948c-a43de75a7ee1&lang=en) using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). qRT-PCR was performed repeatedly after optimization, and adjusted with slight changes in the manufacturer's instructions as follows: 5 µL 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 1 µL 10x miScript Universal primer, 1 µL 10x miScript primer assay and 1 µL RNase-free water. 19 miRNAs containing various miRNA sequence features, including Ce miR-39, were selected, and *SNORD68* and *SNORD95* were used as housekeeping genes. Shortly after the hot start Taq polymerase activation at 95°C for 10 minutes, the reaction was carried out with 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 15 seconds. Then the melting reaction was carried with the ramps from 55°C-95°C at the acquisition of Melt A on Green.

Statistical Analysis

Statistical threshold was calculated as 0.025 manually in all the reactions, and the standards were imported from the previous study by Aslan et al[10] and Tuncturk et al[11] as conc=10 (-0.328 × Ct+11.903) with cycle threshold (Ct) values of -3.050 × log(conc)+36.302 and R2 of 0.99974[10,11]. The Ct values of the miRNA expressions were exported to Excel from the software after normalization encountered with dynamic tube and slope correction. The average Ct value was converted into quantities for the SABiosciences software (Qiagen, Germany). The quality of extended mature miRNAs was controlled by melt analyses on Green. Subsequently, the expression levels of miRNAs in saliva were analyzed using the fold change [$2^{\Delta\Delta CT}$] method. A p-value <0.05 was considered statistically significant.

Results

The expression patterns of miR-21_2, miR-221_1, miR-222_2, miR-let-7c_1 and miR-let-7f_1 were significantly upregulated whereas miR-141, miR-146a_1, miR-181b_1, miR- 331_1, miR-133a_2 and miR-155_2 were significantly downregulated compared to control group (p<0.05) (Table I-IV and Figures 2-3). Table I, III and IV demonstrated no statistical difference in miRNA expression regarding the fasting phase between the genders of our selected group. We can most likely consider the downregulation of the miRNAs as mentioned above as a compensatory mechanism to maintain progressive apoptosis at a certain level.

Table I. Comparison of miRNA expressions of Group 1 (fasting for 17 consecutive hours) and Group 2 (performing a test 70 minutes after meal consumption). The p-value in blue indicates significant downregulation, while the p-value in red indicates significant upregulation. (CI: December Confidence Interval).

miRNA	Fold Regulation	p-value	95% CI
miR 21	28.9146	0.000003	(7.37, 50.46)
miR-133a	-11.2524	0.000001	(0.05, 0.13)
miR-141	-5.4467	0.000332	(0.08, 0.29)
miR-146a	-3.3727	0.000848	(0.15, 0.44)
miR-155	-9.3242	0.000001	(0.06, 0.16)
miR-181b	-15.9718	0.000389	(0.03, 0.09)
miR-221	4.5451	0.003015	(2.00, 7.09)
miR-222	6.1621	0.004457	(2.49, 9.83)
let-7c	2.8549	0.002032	(1.16, 4.55)
let-7f	4.4761	0.000053	(2.34, 6.61)
miR-331	-13.9981	0	(0.04, 0.10)

Discussion

MiRNA Expression and Therapeutic Usage

In recent years, scientists have turned to various RNA-based technologies for potential therapeutic uses, including cancer treatment[12]. This is due to the fact that miRNAs are responsible for many cellular phenomena (apoptosis, autophagy and cell proliferation)[12]. In our opinion, fasting or intermittent fasting can activate many miRNA-based cellular mechanisms. Thus, as a result of the downregulation of miRNA expression, increased programmed cell death can be used in the

Table II. Comparison of fasting miRNA expressions between gender. While the p-value in blue indicates significant downregulation, the p-value in red indicates substantial upregulation.

miRNA	Fold Change (Group 1/Control)	p-value
miR_31	1.48	0.020
miR_125b	0.42	0.008
miR_210	-1.71	0.007

Table III. Comparison of miRNA expressions of female in Group 1 (fasting for 17 consecutive hours) and Group 2 (testing 70 minutes after meal consumption). The *p*-value in red indicates a significant upregulation.

miRNA	Fold Change (Group 1/Control)	*p*-value
miR 21_2	49.72	0.017
miR_106b_1	3.74	0.009
miR_133a_2	0.06	0.000
miR_141_1	0.10	0.001
miR_146a_1	0.24	0.010
miR_155_2	0.07	0.000
miR_181b_1	0.05	0.014
miR_183_2	0.16	0.000
miR_221_1	5.97	0.015
miR_222_1	11.53	0.001
let7c_1	4.69	0.011
let7f_1	7.39	0.000
miR_331_1	0.04	0.000

Table IV. Comparison of miRNA expressions of men in Group 1 (fasting for 17 consecutive hours) and Group 2 (testing 70 minutes after meal consumption). The *p*-value in red indicates a significant upregulation.

miRNA	Fold Change (Group 1/Control)	*p*-value
miR_21_2	13.20	0.002
miR_106b_1	3.79	0.009
miR_133a_2	0.15	0.003
miR_146a_1	0.36	0.012
miR_155_2	0.17	0.004
miR_181b	0.08	0.003
miR_331_1	0.12	0.003

treatment of diseases such as cancer. In our study, miRNAs (miR-141, miR-146a-1, miR-181b-1, miR-331-1, miR-133a-2, and miR-155-2) were shown to increase the effect of apoptosis by showing downregulation. Perhaps this is due to a decrease in the amount of glycogen and triacylglycerol in the liver after a one-day fast, which increases the rate of apoptosis and affects cellular proliferation[13]. Apart from this view, this apoptosis-reducing effect of miRNAs on various pathways can be considered as a compensatory mechanism against increased apoptosis during prolonged fasting. In our study, it was shown that miRNAs (miR-21-2, miR-221-1, miR -222-2, let-7c-1, and let-7f-1) reduce the effect of apoptosis by upregulating. For example, abnormal miRNA expressions in non-physiological events such as starvation can cause an excess of intracellular molecular signal transduction, resulting in a large number of different gene variations. Therefore, abnormalities in miRNA may have an important role in the pathogenesis of certain diseases[14]. Wang[8] compared with healthy individuals the levels of miRNA in the circulation of a few cancer patients (lung cancer, miR-146, miR-155, miR-331, breast cancer, myeloid leukemia, miR181, and miR-141) which were reported to be low[15,16]. In a parallel study, Ji et al[17] have shown that the low expression of miR-133a-3p may be associated with prostate cancer progression and the development of bone metastases. According to all this information, the idea of future miRNA expression technologies that interfere with the cellular cycle within the neoplastic microenvironment will be the center of interest for many studies.

Expression of MiR-let-7c

In our study, the rate of expression of miR-let-7c in healthy fasting subjects decisively increased in those who fasted for 17 consecutive hours compared to the control group (tested 70

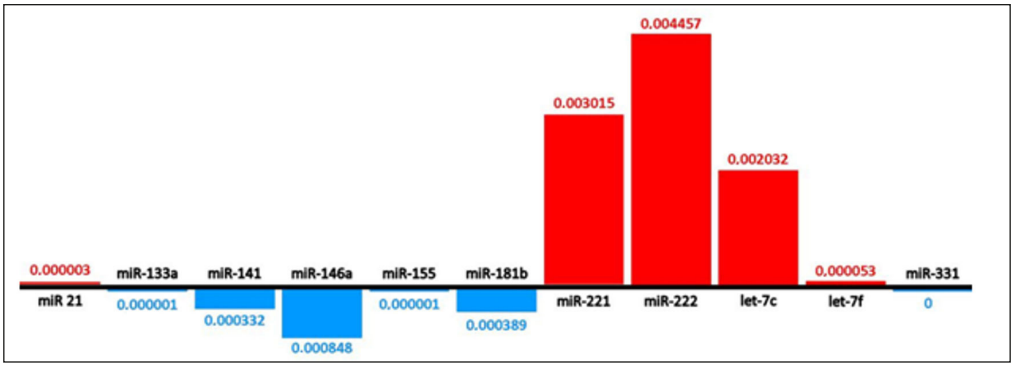


Figure 2. Graphic images of the *p*-values of miRNAs upregulation and downregulation in the groups. The expressions of 19 miRNAs regulating different pathways were examined using the real-time PCR method after isolation from saliva samples of healthy university students (n = 34) paired as a 17-hour fast (Group 1) and the subsequent postprandial 70 minutes (Group 2).

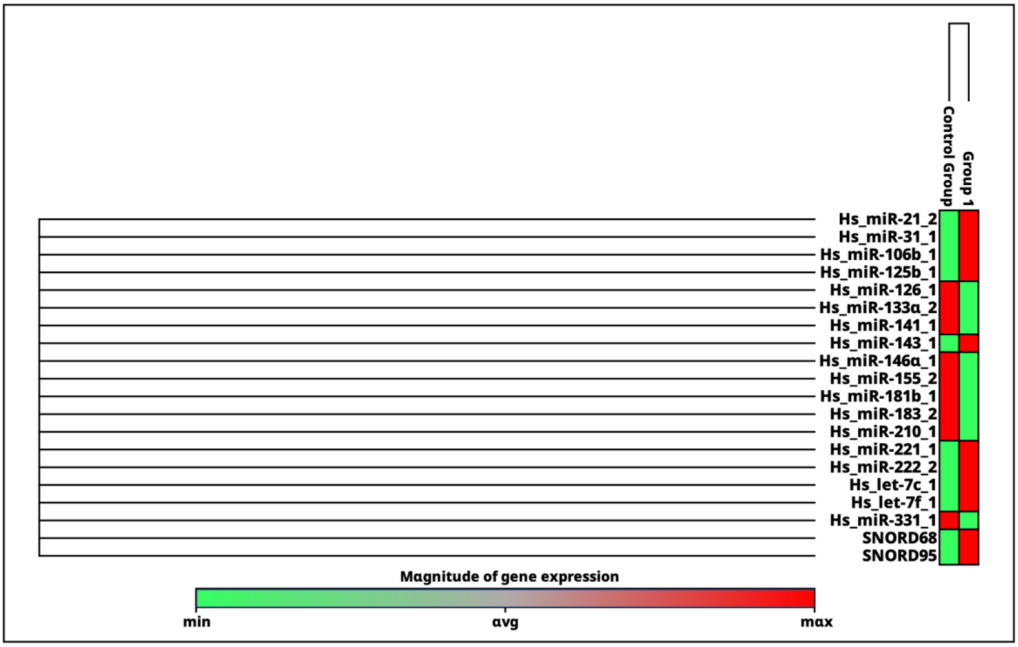


Figure 3. Clustergram of miRNAs expressions of Group 1 compared to Group 2. (This clustergram was created with online Qiagen miScript Primer Assay Data Analysis AAAOSW2 Center; available at: www.qiagen.com). Accordingly, 9 miRNA expression levels were decreased (green) compared to phase 2 (red). 7 miRNA levels were decreased: miR-126_1 (p=0.000), miR-141 (p=0.000), miR 146a_1(p=0.000), miR- 181b_1 (p=0.000), miR-331_1 (p=0.000), miR-133a_2 (p=0.000) and miR-155_2 (p=0.000) were statistically significant. 11 miRNA levels were increased (yellow) compared to control phase (blue). The increased expression level of miR-21_2 (p=0.000), miR-221_1 (p=0.003), miR-222_2 (p=0.004), let-7c_1 (p=0.002) and let7f_1 (p=0.000) were statistically significant.

minutes after meal consumption). Therefore, in long-term fasting studies, healthy individuals can be used as a control group to know the level of increase in miR-let-7c expression in sick subjects. Overexpression of miR-let-7c in cancer patients, has been proved to be a cellular cycle inhibitor in both *in vivo* and *in vitro* experiments, as shown in the study conducted by Nadiminty et al[18]. In another study, it was reported by Zhu et al[19] that an increase in miR-let-7cnin levels suppresses the overexpression of the *CDC25A* gene, which has been confirmed to be one of the cell regulators associated with the progression of cancer. Therefore, fasting's regulation of MIR-let-7c overexpression can be considered as an alternative treatment method in the understanding and treatment of many diseases, especially in addition to the current treatment of oncological diseases.

MiR-221/222 Implications

Ning et al[20] stated that increase in miR-221 and miR-222 levels may be associated with malignancies or other diseases, and an increase in these levels alter the cell cycle and cellular growth by targeting the *p27* and *p57* genes involved in cellular proliferation. In other studies[21,22], it was reported that overexpression of miR-21 inhibited apoptotic cell pathways, thereby reducing the progression of damage to damaged kidney cells. In our study, increased miR-221/222 expression was observed in our candidates after 17 hours of fasting. Based on this information, we hypothesize that this fasting-induced increase similarly prevents apoptosis and, therefore, may act as a protective mechanism in many diseases.

Conclusions

In this study, it has been shown that miRNA expression is responsible for cellular turnover and proliferation. Moreover, the presence of such miRNAs can be considered as an indicator of a disease. This study strengthened the relationship between fasting and miRNA expression in the cell cycle and revealed the idea of how fasting could be used as a new treatment modality in oncological patients by influencing the neoplastic microenvironment. However, in addition to elucidating its epigenetic effects on apoptosis regulation, it paved the way for more controlled clinical trials that will be needed to determine the timing and duration of the fasting period.

Conflict of Interest
The authors declare that they have no conflict of interests.

Ethics Approval
This study was performed in line with the principles of the Declaration of Helsinki. The research protocol was approved by the Medical Ethics Committee of Istanbul Medeniyet University Göztepe Training and Research Hospital (Date: 2017/No. 0126).

Informed Consent
Informed consent was obtained from all participants included in the study.

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