RESEARCH ARTICLE / *Araştırma Makalesi*

Effect of Intermittent Hypoxia on Cardiac Muscle Calcium Homeostasis in Experimental Type 1 Diabetes Mellitus

Deneysel Tip 1 Diabetes Mellitusta Aralıklı Hipoksinin Kardiyak Kas Kalsiyum Homeostazisine Etkisi

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Geliş Tarihi / Received : **12.08.2019** Kabul Tarihi / Accepted : **06.09.2019**

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Abstract

Objective — In this study; it was investigated the effect of intermittent hypoxia on cardiac phospholamban and Ca2+-calmodulin-dependent protein kinase II (CaMKII) levels in experimental diabetic cardiomyopathy. **(Sakarya Med J 2019, 9(3):536-543)**.

Materials and Methods — Wistar albino male rats (n=34) were randomized to four groups: control (C), intermittent hypoxia (IH), diabetes mellitus (DM) and diabetes mellitus + intermittent hypoxia (DM+IH). Injection of streptozotocin (50 mg/kg, i.p.) followed by 250 mg/dL and above blood glucose levels, was accepted as diabetes mellitus. The IH and DM+IH groups were subjected to 6 hours/day hypoxia for 42 days at a pressure corresponding to a height of 3000 m. Kruskal Wallis test, multiple comparisons tests, and Wilcoxon tests were used for evaluating.

Results — Rats were weighed routinely to demonstrate weight loss in diabetes and to monitor metabolic health status of rats. The weight increase in the IH group was at most and the DM group was at least. The differences between C and DM (p=0.003), C to DM+IH (p=0.024), IH to DM (p=0.001), IH to DM+IH (p=0.006) groups were statistically meaningful at the end of the experiment. It has not been detected any meaningful difference among the groups of Phospholamban/glyceraldehyde-3 phosphate dehydrogenase (PLB/GAPDH) (p=0.294). In terms of CaMKII/GAPDH, a statistically significant difference was found between C and DM; C and DM+IH and IH and DM+IH groups (p<0.05).

Conclusion — It was found that CaMKII mRNA levels decreased in DM and DM + IH groups. However, changes in the phospholamban have not been detected, but are important in the effects of translational and/ or posttranslational levels and in the changes that may occur in protein levels and/ or activations.

Keywords — Diabetic cardiomyopathy; Intermittent hypoxia; PLB; CaMKII

Öz

Amaç — *Bu çalışmada; Deneysel diyabetik kardiyomiyopatide aralıklı hipoksinin kardiyak fosfolamban ve Ca+2- kalmodulin bağımlı protein kinaz II (CaMKII) düzeylerine etkisi araştırıldı.* ***(Sakarya Tıp Dergisi 2019, 9(3):536-543)***

Gereç ve Yöntemler — *Wistar albino erkek sıçanlar (n = 34) dört gruba randomize edildi: kontrol (C), aralıklı hipoksi (AH), diabetes mellitus (DM) ve diabetes mellitus + aralıklı hipoksi (DM + AH). Streptozotosin (50 mg/kg, i.p.) uygulandı ve 250 mg/dL ve üzeri kan glukoz seviyeleri diabetes mellitus olarak kabul edildi. AH ve DM+ AH grupları, 3000 m yüksekliğe karşılık gelen bir basınçta 42 gün boyunca 6 saat/ gün hipoksiye tabi tutuldu. Değerlendirmede, Kruskal Wallis testi, çoklu karşılaştırma testleri ve Wilcoxon testleri kullanıldı.*

Bulgular — *Diyabetteki kilo kaybını göstermek ve ratların metabolik sağlık durumlarının takibi için rutin olarak ratlar tartıldı. AH grubundaki ağırlık artışı en fazla idi ve DM grubu en azdı. C ve DM (p= 0.003), C- DM + AH (p= 0.024), AH- DM (p= 0.001), AH- DM+ IH (p= 0.006) arasındaki farklar istatistiksel olarak anlamlı bulundu. Fosfolamban/ gliseraldehit-3 fosfat dehidrogenaz (PLB/ GAPDH) grupları arasında anlamlı bir fark bulunamamıştır (p= 0.294). CaMKII/ GAPDH açısından, C ve DM; C ve DM+ AH ile AH ve DM+ AH grupları arasında istatistiksel olarak anlamlı bir fark bulundu (p <0.05).*

Sonuç — *CaMKII mRNA düzeylerinin DM ve DM+IH gruplarında azaldığı bulundu. Bununla birlikte, fosfolambanda değişiklik tespit edilmemiştir, ancak fosfolambanda meydana gelecek değişiklikler translasyon ve/veya posttranslasyonal seviyelerin etkilerinde ve protein seviyelerinde ve/ veya aktivasyonlarında meydana gelebilecek değişikliklerde önemlidir.*

Anahtar Kelimeler — *Diyabetik kardiyomiyopati; Aralıklı hipoksi; PLB; CaMKII*

INTRODUCTION

Diabetes mellitus (DM), one of the major health problems in the world, has acute and chronic complications.[1,2] One of the major complications, diabetic cardiomyopathy (DC), is independent of coronary artery disease (CAD) or hypertension and is characterized by diastolic dysfunction that occurs prior to the development of cystolic disfunctionality.[1-3] Cardiac insufficiency develops 2 in the later stage of DC.

One of the mechanisms that causes DC formation is degradation in calcium ion (Ca2+) homeostasis. During the relaxation phase, the cytosolic Ca2+ is largely removed by pumping into sarkoplasmic reticulum (SR) via sarco/endoplasmic reticulum Ca2+-ATPase (SERCA). Phospholamban (PLB) is an important regulator of SERCA. Changes in the mRNA and protein levels of myocardium SERCA, PLB and CaMKII (Ca2+-calmodulin-dependent protein kinase II) have been reported in the DM model created with streptozotosin (STZ). In DC, different results regarding PLB and SERCA mRNA and protein levels are found. Some studies have shown that PLB mRNA and protein levels increase[4,5] in diabetic hearts, different studies have shown that PLB protein levels decrease,[6,7] while SERCA protein levels decrease more significantly and the PLB/SERCA ratio increase.[5,7] Activity of CaMKII, one of the essential proteins that enables PLB to be phosphosphorylated, has been reported to increase in diabetic hearts.[6-8] Despite the increase in CaMKII activity, it has been suggested that the decrease in PLB's phosphosphorylation may be due to an increase in protein phosphatase (PP), which reduces PLB's phosphorylation, or a decrease in PLB protein.[6,7] Decreased PLB phosphorylation also causes a decrease in SERCA activation.[9,10] A 30 % reduction in SERCA2a mRNA and protein levels in DM [5-7,11] has been shown to result in the development of diastolic disfunctionality by inadequate pumping of Ca2+ to SR and excessive accumulation in cytosol.[12]

Hypoxia and metabolic and cardiac adaptive responses to hypoxia are one of today's crucial research topics. In a study conducted in Peru, the prevalence of DM was found to be 1.6 % in Lima at an altitude of 150 m above sea level and 0.4 % in Cuzco at an altitude of 4.360 m.[13] Other studies investigating the effects of hypoxia on the cardiac have shown that intermittent and chronic hypoxia reduces the site of infarction in the heart and improves cardiac function after ischemia.[14] It has also been reported in other studies that intermittent hypoxia (IH) increases left ventricle contractility[10], protecting it from myocardial reperfusion damage.[4,5] In studies of cardiac dysfunction induced by ischemia-reperfusion (I/R), IH application has been shown to increase CaMKII activity, PLB phosphorylation[6] and Ca+2 pumping activity into SR.[7] These results suggest that CaMKII is mediated by phosphorylating PLB to the cardioprotective effect of IH.

In addition, it is thought that intermittent hypoxia application in weight reduction in diabetic group improves the metabolic effects of diabetes. It was proven in our previous study that the negative effects of diabetes on the heart were eliminated by increasing angiogenesis through hypoxia inducible factor-1α/vascular endotelial growth factor (HIF-1α/VEGF).[15] It has been shown that weight loss caused by DC decreases with intermittent hypoxia as a result of heart healing.[15] Further studies are needed.

With this study, we investigated CaMKII and PLB mRNA changes in DC, important comorbidity in DM that is common today, and the therapeutic efficacy of IH through these molecules.

MATERIALS AND METHODS

Experimental groups and protocols

The present study is experimental and has been conducted in rats. Adult male rats of the genus "Wistar albino rattus norvegicus" were supplied from the Ankara University Experimental Animal Breeding and Supply Laboratory. The 10-week experiment animals brought a week before launching experiments with the aim of their average ad-

aptations to the animal laboratory in department of Physiology of medicine faculty of Ankara University, were fed freely with standard rat food (ad libitum) and tap water at +22 °C, 12-hour light-dark cycle. All animal experiments were carried out in accordance with the guidelines on human's animal use and care for laboratory animals for biomedical research published by the National Institutes of Health (8th education, 2011) and the Helsinki Declaration was followed. The approval of the Ethics Committee was obtained by Ankara University animal experiments local Ethics Committee with decision number 2012-11-79 dated 23.05.2012.

34 male rats (10 weeks old, weighing 217.9±18.3 g) were randomized to four groups: control (n=7), intermittent hypoxia (n=9), diabetes mellitus (n=8) and diabetes mellitus + intermittent hypoxia (n=10). No intervention was applied to group C. IH group animals were exposed to hypoxia at 6 hours/day for 6 weeks in the hypobaric hypoxia chamber (APCU-01, Bethlehem). The pressure was adjusted to 69.3 kPa (520 mmHg), corresponding to a height of 3000 m. This hypoxia level is considered high altitude with possible pathophysiological effects [8]. The tissues were removed 24 hours after the animals were subjected to the last 6 hours of hypoxia. To create experimental DM, 50 mg/kg single dose of STZ prepared in citrate buffer (0.05 M, pH=4.5) was administered as intraperitoneally (i.p.) to DM and DM+IH groups.[9,10] A week after the STZ injection (12. week), tail venous blood glucose levels of animals were calculated using a glucometer device (OptiumXceed, Abbott). Animals with 250 mg/dL and above blood sugar levels were accepted as diabetic.[12] While DM+IH group exposed to hypoxia along with IH group; DM group along with the Group C were held in normoxia. All subjects were monitored for weight and blood glucose.

Total Ribonucleic Acid (RNA) isolation

Total RNA samples from the left ventricle were isolated via commercial isolation kit (Fibrous tissue Mini Kit K 74704, Qiagen) for fibrous tissues. Mechanochemical tissue homogenization was carried out in liquid nitrogen and some buffers using the tissue homogenizer system (GlasCol, 099C-K5424). Total RNA of the samples following the protease incubation of homogenate was decomposed in spin columns through several centrifugation and washing steps. Right after, the concentration and quality of total RNA samples were measured at 230 nm, 260 nm, and 280 nm (NanoDrop, ND-1000). Rates 260/280 and 260/230 were evaluated for RNA purity and quality, and samples were repeated until a 1.8-2 ratio was obtained. All samples of total RNA were carried out in 1% agarose gel to control their integrity.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (2 µg) per sample was converted to total cDNA with reverse transcriptase using a commercially available reverse transcription kit (RevertAid first StrandcDNASynthesis Kit, Fermentas, Life Sciences, EuropeanUnion). Total cDNAs were amplified by PCR using rat PLB, CaMKII, and GAPDH (housekeeping gene) specific primers to obtain specific mRNAs [16,17]. The gene regions corresponding to these primers were controlled by NCBI, the nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide) and Ensemble genome browser database (http://useast.ensembl.org). The number of base-pair (bp) of PCR products was measured and optimal PCR conditions were arranged due to base sequences (Table 1).

Table 1. Primary sequences for PLB, CaMKII, GAPDH, target DNA sequence, number of nucleotides

Target DNA	Primary directories (5'-3')	Target DNA Index The number of nucleotide
PLB	Forward: TACCTACTCGCTCGGCTATC Reverse: CAGAAGCAT CACAAT GATGCAG	141
CaMKII	Forward: TCAGATGTTTTGCCACAAAGAGGT GCCTCCT Reverse: CCGGATGGGGTAAAG GAGTCA ACTGAGAGCT	531
GAPDH	Forward: TCCACCACCCTGTTGCTGTA Reverse: ACCACAGTCCATGCCATCAC	531

CaMKII-Ca2+/calmodulin-dependent protein kinase II; GAPDH-glyceraldehyde 3-phosphate dehydrogenase; PLB-Phospholamban

Agarose gel electrophoresis and mRNA analysis

PCR products (15 µL) with DNA markers were run on a 2% agarose gel with ethidium bromide at 100 volts for 1 hour. mRNA bands painted with ethidium bromide in the gel were displayed under UV light with a digital camera (Cleaver Scientific, DIHD) and their pictures were transferred to the computer. To ratify whether the resulting cDNA bands correspond to specific genes, localizations of the samples' bands were compared with those of a standard base pair known DNA marker [PhiX174 DNA/BsuRI (Haelll) Marker, 11]. PLB and CaMKII band densities were measured using the software program (Image J 1.38 X, Wayne Rasband, NIH, USA) (http://imagej.nih.gov/ij/). PLB and CaMKII mRNA content was evaluated as the ratio of GAPDH to mRNA density for each sample (PLB/GAPDH and CaMKII/GAPDH). All measurements were repeated three times (Figure 1, 2).

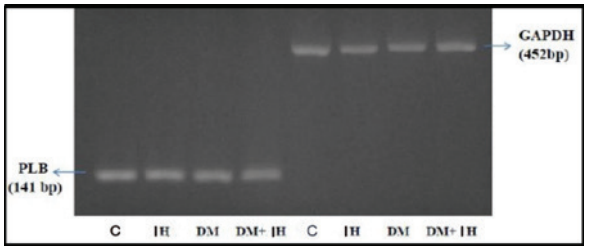

Figure 1. 2% agarose gel samples showing PLB mRNA expression in the left ventricles of hearts obtained from experimental groups

C-control; IH-intermittent hypoxia; DM-diabetes mellitus; GAPDH-glyceraldehyde 3-phosphate dehydrogenase, PLB-Phospholamban

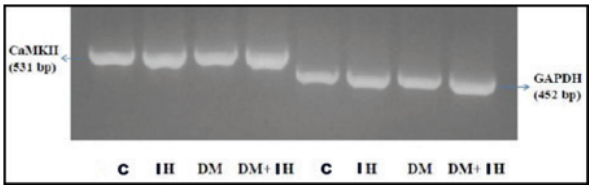

Figure 2. 2% agarose gel samples showing CaMKII mRNA expression in the left ventricles of hearts obtained from experimental groups

C-control; IH-intermittent hypoxia; DM-diabetes mellitus; CaMKII-Ca2+/calmodulin-dependent protein kinase II; GAPDH-glyceraldehyde 3-phosphate dehydrogenase

Statistical Analysis

In statistical evaluations, since the subject numbers in all groups were low, the Kruskal Wallis test, which was a non-parametric test, was used in group comparisons. When the p-value was found to be significant in the Kruskal Wallis test, multiple comparison tests were performed to determine the groups that made the difference. For each group, the measurement values at the beginning and end of the experiment in terms of blood glucose and weight were compared with the Wilcoxon test. Statistical analyses were fullfiled with the SPSS 15.0 program. P <0.05 was evaluated statistically meaningful. The values were given as mean ± standard deviation (X±SD).

RESULTS

It has been found no statistically meaningful differences among the four groups in terms of initial weights (p=0.056). The difference between DM and IH groups in terms of end-of-experiment weights was statistically meaningful (p=0.007). When the weight increase rates of the experimental animals were examined, it was observed that the weight increase was the highest in the IH group and the least in the DM group, and the difference between these two groups was statistically meaningful (p<0.05) (Table 2).

Table 2. Weight values and weight increase rates of experimental animals at the beginning and end of the experiment

Group	Initial Weights (X±SD)	Final Weights (X±SD)	Weight Increase Rate (%)
C	209.71±11.01	298.43±18.42	43.64
IH	210.11±16.27	327±49.11	55.72
DM	232.25±19.41	259±22.35#	27.09*#
DM+IH	219.20±17.75	279±27.53	35.61#
C-control; IH-intermittent hypoxia; DM-diabetes mellitus			

X±SD Mean±Standart Deviation
*Significant difference with C group (p < 0.05)
#Significant difference with IH group (p < 0.05)

It has been found no statistically meaningful difference among the groups in terms of blood glucose levels at the start of the experiments (p=0.618). Respectively, the differences between C and DM (p=0.003), C and DM+IH (p=0.024), IH and DM (p=0.001), IH and DM+IH (p=0.006) groups were statistically significant at the end of the experiment in terms of blood glucose levels (Table 3). It was observed statistically significant differences among C and DM+IH; C and DM; IH and DM and IH and DM+IH groups in terms of increased blood glucose levels (Table 3).

Table 3. Levels of blood glucose and increase rates at the beginning and end of the experiment animals

Group	Initial Blood Glucose Levels (X±SD)	Final Blood Glucose Levels (X±SD)	Rate Of Increase In Blood Glucose Levels (X±SD)
C	95.40±16.13	81.20±4.76	-9.47
IH	92.22±13.54	82.33±6.75	-12.79
DM	96.63±17.32	379.75±86.28*#	329.62*#
DM+IH	103.90±16.66	328.30±71.78*#	217.15*#

C-control; IH-intermittent hypoxia; DM-diabetes mellitus

X±SD Mean±Standart Deviation
*Significant difference with C Group (p < 0.05)
#Significant difference with IH group (p < 0.05)

It has not been detected any statistically significant difference among the groups in terms of PLB/GAPDH (p=0.294). Statistically meaningful difference was found between the groups in terms of CaMKII/GAPDH (p<0.001) (Table 4). It has been evaluated as statistically meaningful difference among C and DM, C and DM+IH and IH and DM+IH groups on CaMKII/GAPDH values (Table 4).

Table 4. PLB/GAPDH and CaMKII/GAPDH results

	C (X±SD)	IH (X±SD)	DM (X±SD)	DM+IH (X±SD)
PLB/GAPDH	0.75±0.15	0.68±0.08	0.72±0.11	0.63±0.13
CaMKII/GAPDH	1.18±0.92	1.14±0.10	1.00±0.05*	0.93±0.11*#

CaMKII-Ca2+/calmodulin-dependent protein kinase II; GAPDH-glyceraldehyde 3-phosphate dehydrogenase; PLB-Phospholamban

*Significant difference with C Group (p < 0.05)
#Significant difference with IH group (p < 0.05)

DISCUSSION

Studies have shown that DC develops after 6 weeks in STZ-induced diabetic rats.[18-20] In our study, the hearts of the experimental animals were taken 7 weeks after the application of STZ. Post-mortem macroscopic examination also identified DC-compatible growth and expansions in rat hearts. An experimental study found that in left ventricle diastolic dysfunction associated with STZ-induced type 1 DM, IH mediated recovery in DM groups (34th National Congress of Physiology, page:147). One of the mechanisms that cause DC formation is the degradation in Ca2+ homeostasis. In our study, no changes in PLB-mRNA level were observed with IH application in STZ-induced DC, while CaMKII mRNA levels were decreased. Studies in diabetic hearts contain conflicting results. Some studies show that PLB-mRNA and protein levels increase[21,22], while other studies show that PLB protein levels decrease, especially the amount of phosphorylated PLB, which is the active form.[23-25] Studies have reported that the activity of CaMKII, one of the essential proteins that phosphorylate PLB, increases in diabetic hearts.[23,24,26] Despite the increase in CaMKII activity, a decrease in phosphorylation of PLB has been shown. It was thought that this decrease may be due to an increase in protein phosphatase that reduces the phosphorylation of PLB, or a decrease in PLB protein.[23,24] There is also a decrease in the activation of SERCA with decreased expression due to a decrease in PLB phosphorylation.[27,28] On the other hand, a new form of reactive oxygen species (ROS)-mediated CaMKII activation has also

been described. It is well known that there was an increase in ROS in DM.[29] CaMKII, formed through ROS, has been described as oxidized CaMKII. Oxidized CaMKII has been shown to increase, and PLB phosphorylation through CaMKII has also increased.[30-32] In our study, no parameters related to the effects of oxidized CaMKII levels and ROS were examined. Reduction in CaMKII mRNA level may have been caused by an increase in oxidized CaMKII or ROS with negative feedback or other mechanisms.

In one study, the protective effect of chronic IH application against I/R damage in ventricular myocytes was shown.[33] In chronic (7 days) IH-administered rat ventricular myocytes, infarction area was shown to be less than those in the normoxic group, parameters related to Ca2+ transport was shown to be better than those in the normoxic group. SERCA, Na+-Ca2+ exchanger (NCX), ryanodine (RyR) protein levels were also measured; no significant changes were found. However, Na+-Ca2+ exchange rate and Ca2+ secretion from SR deposit by RyR are significantly higher in the hypoxic group and protein kinase A and C (PKA and PKC) inhibitors have been observed to eliminate these effects. It has been suggested that increasing RyR and NCX activities, respectively, by phosphorylation of PKA and PKC, has an important role in the cardioprotective effect induced by chronic IH.[33] When these findings and the results from our study are interpreted together, PKC-mediated NCX activity may be responsible for the removal of Ca2+ from the cell as a result of IH application, rather than SERCA. RyR activity is also expected to increase as a result of increased PKA. The decrease in CaMKII level may have been offset by the increase in kinases such as PKA and PKC.

The SERCA gene has been shown to have multiple hypoxia response element (HRE) regions in the promoter region, and hypoxia-induced factor (HIF) provides stimulation of the SERCA.[34] IH has been reported to provide Ca2+ homeostasis by balancing decreased SERCA2 activity and functions during I/R.[35] When our results were evaluated in the light of these data, the increase of HIF-1α transcriptional activity with IH application increased the stimulation of SERCA containing the HRE region and the Ca2+ was taken into SR; since intracellular Ca2+ balance is achieved, the PLB-mRNA level may not have changed.

In a study by Yuan et al, IH was applied to culture cells.[36] CaMKII, whose activity increased 5 times in IH-administered cells; has been observed to phosphorylate HIF-1α.[36,37] KN93, which is also an inhibitor of CaMKII, has been shown to inhibit IH-induced HIF-1α transactivation.[36] These results suggest that IH induces the transcriptional activity of HIF-1α through a new medical signal pathway, including CaMKII. However, in our study; the decrease of CaMKII-mRNA level in DM and DM+IH groups, the increase of HIF-1α transcriptional activity regardless of CaMKII as a result of the IH we applied, may have also increased the stimulation of SERCA to receive Ca2+ into SR.

In cardiac dysfunction induced by I/R, IH administration has been observed to increase CaMKII activity as well as PLB phosphorylation.[7] In cardiac I/R models, Ca2+ pumping activity to SR has been shown to decrease, whereas in IH-administered hearts, this decrease has been prevented.[6] These results suggest that CaMKII mediates the cardioprotective effect of IH in the I/R model by phosphorylating PLB. In our study, we evaluated CaMKII mRNA levels after 6 weeks of IH application. PLB activity may also have increased with the possible CaMKII increase, although we did not evaluate it in the first weeks of IH application. In the following process, the negative feedback mechanism of the SERCA increase may have resulted in a decrease in CaMKII and PLB.

I/R application, has been shown to reduce CaMKII activity, phosphorylated PLB (Thr17 region) and Ca2+ pumping to SR (Ca2+-calmodulin-dependent) in the rat cardia, while IH application has prevented these changes. In I/R groups, the effect was reported to occur with a decrease in cytosolic CaMKII and SR protein phosphatase 1 (PP1) activities, and there was no decrease in the activity of these proteins in the IH applied group.[6] In our study, the PP1 increase through PKC may have reduced PLB by dephosphorylating in the DM+IH group. On the other hand, PKA increase may be induced by stimulation of the β adrenergic system in hypoxic conditions and PLB may be phosphorylated by PP1 suppression. Since the effects of PKA and PKC on PLB are in the opposite direction, it can be interpreted that the PLB level is unchanged. A study has found that IH completely inhibited Ser16 phosphorylation of increased PLB in the cardiac via PKA inhibition.[7] This finding also suggests that IH may have a role over PKA.

In our study, only PLB and CaMKII mRNA levels were determined and the protein levels of these molecules and left ventricular function parameters were not evaluated. In addition, the absence of mRNA and protein levels of SERCA, one of the key proteins in the removal of cytosolic Ca2+, and the phosphorylated form of PLB responsible for the activity of SERCA can also be considered as important constraints.

In addition, rats were weighed routinely to demonstrate weight loss in diabetes and to monitor metabolic health status of rats. It is thought that intermittent hypoxia application in weight reduction in diabetic group improves the metabolic effects of diabetes. It was proven in our previous study that the negative effects of diabetes on the heart were eliminated by increasing angiogenesis through hypoxia inducible factor-1α/vascular endotelial growth factor (HIF-1α/VEGF).[15] It has been shown that weight loss caused by DC decreases with intermittent hypoxia as a result of heart healing.[15] Further studies are needed.

Conclusions: It is not known in which metabolic pathways that IH is acting exactly. In the literature surveys we conducted, there were no studies examining the relationship between IH application and Ca2+ homeostasis in the DM model. This is an original work in this direction and will shed light on other work to be done. However, changes in mRNA levels may not always explain the effects of DM and IH alone. The effects on translational and/or posttranslational levels, as well as changes in protein levels and/or activations, are also important. In addition, it has been shown that weight loss caused by DC decreases with intermittent hypoxia as a result of heart healing. Further studies should evaluate the mRNA levels of the above-mentioned molecules, as well as protein levels and phosphorylated forms, and the parameters related to heart function should also be included in the study.

Conflict of interest statement
None declared.
Ackowledgement

We would like to thank all participants for contributing in the present study. This study was supported by Ankara University Scientific Research Projects with the project number 13B3330002. The current study was obtained from the thesis. This study was presented as an oral presentation at 39th National Physiology Congress in Hacettepe University.

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