

# Intermittent hypoxia induces beneficial cardiovascular remodeling in left ventricular function of type 1 diabetic rat

Fırat Akat, Hakan Fıçıcılar, Ayşegül Durak\*, Erkan Tuncay\*, Ali Doğan Dursun, Ferda Topal Çelikkan\*\*, Bizden Sabuncuoğlu\*\*, Belma Turan\*, Metin Baştuğ

Departments of Physiology, Biophysics\*, Histology and Embryology\*\*, Faculty of Medicine, Ankara University; Ankara-Turkey

## ABSTRACT

**Objective:** Depressed mechanical activity is a marked complication in diabetics. Hypoxia has properties for novel diagnostic and therapeutic strategies, while intermittent hypoxia (IH) provides early functional and histologic remodeling, including some cardio benefits in early hemodynamic alterations with histologic remodeling and delayed changes in peripheral vasoreactivity. Therefore, we aimed to examine whether IH application presents a cardioprotective effect, via stabilization of hypoxia-inducible factor (HIF) in streptozotocin (STZ)-induced diabetic rat heart.

**Methods:** Male 10-week-old Wistar rats were randomly assigned as control group (C), IH group, (STZ)-induced diabetic group (DM) and IH applied DM group (DM+IH). Diabetes duration was kept 6 weeks and IH groups were exposed to hypobaric hypoxia at about 70 kPa (including ~14% PO<sub>2</sub>; 6 h/day for 6-weeks).

**Results:** Depressed left ventricular developed pressure (LVDP) and prolonged contraction and relaxation of Langendorff-perfused hearts, as well as increased total oxidative status from streptozotocin (STZ)-induced diabetic rats were markedly prevented with IH application. IH application induced significant increase in protein expression levels of both HIF-1 $\alpha$  and vascular endothelial growth factor (VEGF), in both control and diabetic rat hearts, whereas there were significant decreases in the protein levels of prolyl-4 hydroxylase domain enzymes, PHD2, and PHD3 in diabetic hearts. Furthermore, IH application induced marked increases in protein levels of matrix metalloproteinases, MMP-2 and MMP-9 and capillary density in left ventricle of diabetic rats.

**Conclusion:** Overall, we presented how IH application has a beneficial cardiovascular remodeling effect in left ventricular function of diabetic rats, at most, via affecting increased oxidative stress and HIF-VEGF related angiogenesis, providing information on hyperglycemia associated new targets and therapeutic strategies. (*Anatol J Cardiol* 2018; 19: 259-66)

**Keywords:** diabetic cardiomyopathy, intermittent hypoxia, hypoxia-inducible factor (HIF), vascular endothelial growth factor (VEGF), angiogenesis

## Introduction

Cardiovascular complications are important causes of morbidity and mortality in diabetic patients (1). Diabetic cardiomyopathy (DCMP) is defined as “effect of diabetic medium to myocardium in cellular basis” and is considered to be a distinct disease (2). DCMP was identified for the first time by Rubler et al. (3) and defined as ventricular dysfunction developing independently from coronary artery disease and hypertension. Basic pathological changes observed in the heart biopsies are interstitial fibrosis, myocyte hypertrophy, and elevated contractile protein glycosylation (4). Hyperglycemia, which is caused by the deterioration in glucose metabolism in diabetic individuals, is mainly responsible for the formation and development of DCMP (5).

Studies have suggested that intermittent hypoxia (IH) can decrease ischemic damage to the heart and improves cardiac

functional recovery during reperfusion (6-10). At the cellular level, intracellular effects of hypoxia are mainly regulated by HIF signaling pathway (11, 12). HIF is present in almost all types of cells and has three distinct isoforms named as HIF-1, HIF-2, and HIF-3 and is also a heterodimeric transcription factor with  $\alpha$  and  $\beta$  subunits (13). While HIF-1 $\beta$  is abundantly expressed in a hypoxia independent manner, HIF-1 $\alpha$  undergoes a rapid degradation process in the presence of O<sub>2</sub> in the medium, catalyzed by prolyl-4 hydroxylase domain (PHD) enzymes (14). PHDs use O<sub>2</sub> as a cofactor and have three isoforms viz., PHD1, PHD2, and PHD3. In hypoxic conditions PHDs become inactive, and then the HIF-1 $\alpha$  subunit skips the degradation, following which the HIF-1 $\alpha$  subunit merges with the  $\beta$  subunit and forms the HIF-1 molecule. This phenomenon is called “HIF stabilization”. The stabilized HIF molecule affects hypoxia response elements (HRE) in the genome and modifies the transcription of several proteins (15, 16).

This manuscript has been presented as oral presentation in “Joint Meeting of the Federation of European Physiological Societies and the Austrian Physiological Society (FEPS 2017)” at 13-15 September 2017 in Austria Vienna.

**Address for correspondence:** Dr. Fırat Akat, Ankara Üniversitesi Tıp Fakültesi, Fizyoloji Anabilim Dalı, Ankara-Türkiye  
Phone: +90 507 688 46 21 Fax: +90 312 310 63 70 E-mail: akatfirat@gmail.com

**Accepted Date:** 20.02.2018 **Available Online Date:** 29.03.2018

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DOI:10.14744/AnatolJCardiol.2018.00236



All PHD isoforms are present in the myocardium but PHD2 and PHD3 are dominant (17, 18).

VEGF, a downstream target of HIF, plays an important role in the linkage between HIF signaling and angiogenesis while being a major element in the control of angiogenesis (19, 20). In hypoxic conditions, transcription factor VEGF is elevated, thus the HIF-VEGF signaling axis has an important role in vascularization driven by  $O_2$  demand. Matrix metalloproteinases (MMPs), another downstream target of HIF, are zinc-dependent endopeptidases, which have an important role in the induction of extracellular matrix degradation. Histological investigation of heart tissue from either diabetic animals or diabetic humans showed that there was close correlation between increase in interstitial and perivascular fibrosis and increase in collagen deposition (21). It has been demonstrated that the increased fibrosis observed in diabetic cardiomyopathy is related to decrease in the expression/activation of several MMPs (22). Furthermore, it has been also mentioned that these changes in MMPs can be associated with increased oxidative stress and impairment in HIF signaling in diabetes (23). The increased oxidative stress deteriorates angiogenesis via the PHD-HIF-VEGF pathway and accelerates fibrosis development, at most, because of alterations in activity/expression of MMPs (24).

Developing preventive and/or therapeutic strategies against DCMP is very important in terms of public health. Several studies have investigated the cardioprotective effects of intermittent hypoxia application; however, no study has suggested that intermittent hypoxia exhibits a cardioprotective effect in DCMP. Therefore, taking into consideration the potential of hypoxic preconditioning, we aimed to examine whether IH exposure exhibits a cardioprotective effect on heart dysfunction in DCMP. Furthermore, if so, we also aimed to demonstrate its related mechanisms in the diabetic heart. Thus, the hypothesis has a novel contribution potential to the understanding of DCMP.

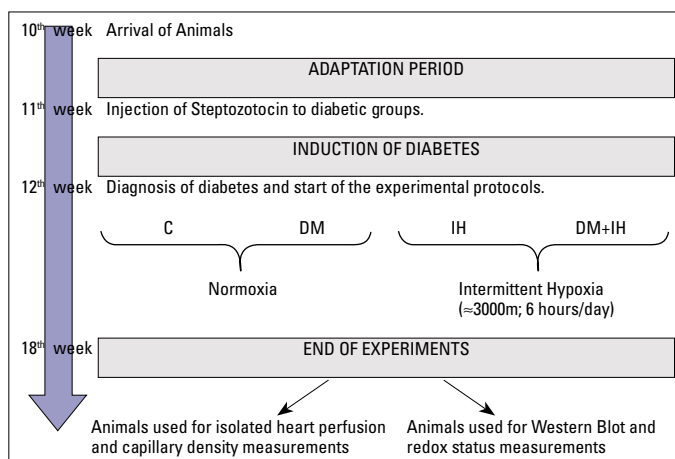
## Methods

### Ethical approval

All the animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The protocol is approved by the Committee on the Local Ethics of Animal Experiments of Ankara University (No: 2012-11-79).

### Animals and induction of diabetes

Type 1 diabetes was induced by injecting streptozotocin (STZ, 50 mg/kg dissolved in 0.1 M citrate buffer at pH 4.5; intraperitoneal; Sigma-Aldrich, Missouri, USA S0130-1G) into male Wistar rats (10-week-old, weighing 200-250 g) as described previously (25). Blood glucose level was 3-fold that of the age-matched control at 7 d following STZ-injection. For hypoxic group, animals were put in a hypobaric chamber (simulates 3,000 m al-



**Figure 1.** Design of the experimental procedure

titude, 520 mm Hg, ~14%  $PO_2$ ) for 6 h/day during 6 weeks. Other groups were kept in their cages at the same time period (Fig. 1).

### Langendorff-perfused hearts

The rats were anesthetized with (i.p.) sodium thiopental injection (50 mg/kg). The heart was rapidly excised, placed in a ice-cold perfusion medium and weighed. The isolated heart was attached to Langendorff apparatus (Biopac Systems Inc., California, USA) and retrogradely perfused at a constant perfusion rate (7 mL/min). Preheated (37°C) Krebs-Henseleit buffer (mM: 119 NaCl, 4.8 KCl, 1.8  $CaCl_2$ , 1.2  $MgSO_4$ , 1.2  $KH_2PO_4$ , 20  $NaHCO_3$ , and 10 Glucose, at pH 7.4) continuously gassed with carbogen (95%  $O_2$  + 5%  $CO_2$ ) was used for retrograde perfusion. A water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve, and the volume was adjusted to achieve a stable end-diastolic pressure (8-12 mm Hg). Isolated hearts were electrically stimulated (DCS, Harvard Instruments Massachusetts, USA) at 300 beats/min by a square wave of twice the threshold voltage of 1.5 ms duration. After 30 min of stabilization, the left ventricular developed pressure (LVDP), and the rates of changes in developed pressure ( $\pm dP/dt$ ) of isolated hearts were measured. The data were recorded online through an analog-digital interface (BSL MP 35 System; Biopac Systems Inc., California, USA).

### Western blot analysis

After sacrifice of the animals, the left ventricular tissue samples were snap frozen in liquid nitrogen and stored at  $-80^\circ C$  for further analysis. After samples were homogenized in lysis buffer, protein concentrations were determined according to Bradford's method. Equal amounts of protein extracts were loaded and separated on 10% SDS-PAGE. After gel electrophoresis (200 V for 2 h) the proteins were transferred (150 V for 1.5 h at  $+4^\circ C$ ) onto high-capacity protein binding nitrocellulose membrane (GVS North America, Maine, USA 0.45  $\mu m$ , 12313225). After blocking of nonspecific binding with 3% BSA in Tris-buffered

**Table 1. Initial and terminal body weights, body weight changes**

Parameters	C Group (n=19)	IH Group (n=19)	DM Group (n=18)	DM + IH Group (n=18)
Body weight (g)				
Initial	215.00±41.43	203.75±44.36	234.56±36.90	229.85±38.12
Terminal	282.90±69.55	291.11±88.20	255.63±43.46	262.37±47.28
Body weight (%)	39.60±14.30 <sup>#</sup>	44.91±17.94 <sup>#†</sup>	7.23±12.26*	7.97±13.42*
Blood glucose (mg/dL)	103.35±12.40 <sup>#</sup>	99.05±9.44 <sup>#†</sup>	474.26±74.62*	464.30±88.60*
HW/BW ratio (mg/g)	4.42±0.32	4.76±0.31	4.76±0.47	4.91±0.58

HW/BW - ratio of heart weight to body weight, data is presented as mean±SD. \*P<0.05 vs. C group, <sup>#</sup>P<0.05 vs. DM, and <sup>†</sup>P<0.05 vs. DM+IH group  
Additional Information: Different animals have been used for functional and molecular experiments. Body weight parameters and blood glucose is measured in all animals and given in the table. HW/BW was only measured in the functional group

saline containing 0.2% Tween-20 for 1 h at room temperature, the membranes were incubated with primary antibodies such as anti-HIF-1 $\alpha$  (1:500), anti-PHD2 (1:500), and anti-PHD3 (1:1000) (Novus Biologicals, Colorado, USA; NB100-105; NB100-2219; NB100-139), anti-MMP-2 (1:500) and anti-MMP-9 (1:500) (Santa Cruz, sc-13594 and sc-6840, respectively) antibodies, anti-VEGFA (1:250) (Abcam, Cambridge UK; ab46154) antibody. After horseradish peroxidase (HRP)-conjugated secondary antibody incubation for 1 h at room temperature, ECL reagent (GE Healthcare Life Sciences Pennsylvania, USA, RPN2106) was added for chemiluminescence reaction. Specific bands were transferred to a sensitive photographic film;  $\alpha$ -tubulin (1:2000) (Cell Signaling Massachusetts, USA 3873S) and GAPDH (1:1000) (Santa Cruz sc-365062) were used as housekeeping proteins. Films were scanned at 600 dpi and bands were analyzed using Image J software.

#### Measurements of total oxidative status and total antioxidant status

Left ventricular tissues lysed in 140 mM KCl buffer (at pH 7.4); 9 mL buffer per 1 gram of tissue was used. The homogenates were centrifuged at 3000 rpm for 5 min; the pellet was discarded and supernatant was used for colorimetric assays, which were performed according to the manufacturer's instructions (Rel Assay Diagnostics, Gaziantep, Turkey).

#### Examination of capillary density

For histological examination with light microscopy, the tissues from the apex of the heart were placed in 10% formalin buffered for 7-10 days. After routine tissue processing, paraffin blocks were sectioned at 5- $\mu$ m slices (Leica RM 2125RT) and stained with Hematoxylin-Eosin (HE). The slides were observed under bright field microscope (Carl Zeiss Axioskop Göttingen, Germany). For immunohistochemistry (IHC) analysis, the slides sectioned at 5- $\mu$ m slices were deparaffinized and rehydrated. Antigen retrieval was performed by boiling in 10 mmol/L sodium citrate buffer at pH 6.0, for 4 min every three times. Following incubation of blocking solution for 30 min, the rabbit polyclonal anti-caveolin-1 antibody (Cell signaling; 3238S) was applied for 1

h at a dilution of 1:250. After washing with phosphate buffer, the sections were incubated with secondary antibody for 1 h (Histo-stain plus kit; 858943). For negative controls, the tissue sections were incubated with PBS in the absence of antibody. Following treatment with HRP, immunoreactivity was detected using DAB (diamino benzidine). Slides were counterstained with hematoxylin. Digital images were acquired using Carl Zeiss Axioskop coupled camera. We regarded cells as immunoreactive when the staining signal was clearly observed in their cytoplasm. Stained cells were enumerated at a magnification of 40 $\times$  by examine examining 3 fields of six sections in every group.

#### Statistical analysis

Test of normality (Shapiro-Wilk) was applied to all data sets. Considering normality analysis and other parametric test assumptions we decided to use parametric tests. Data involving more than two groups were assessed using one-way ANOVA with post-hoc analysis (Tukey and Bonferonni tests). If needed, differences between two significant groups were also assessed by using the Independent Samples t-test. We used SPSS 15.0 software for all analysis. For all comparisons, p<0.05 was considered to be significant.

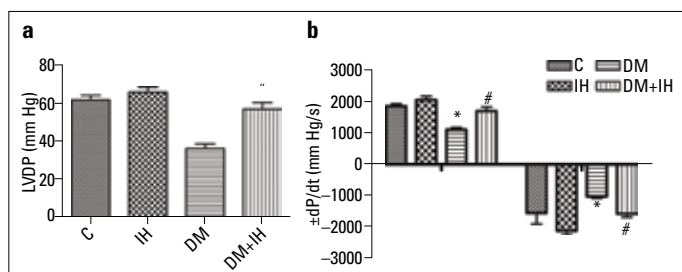
## Results

#### Results of animal follow-up

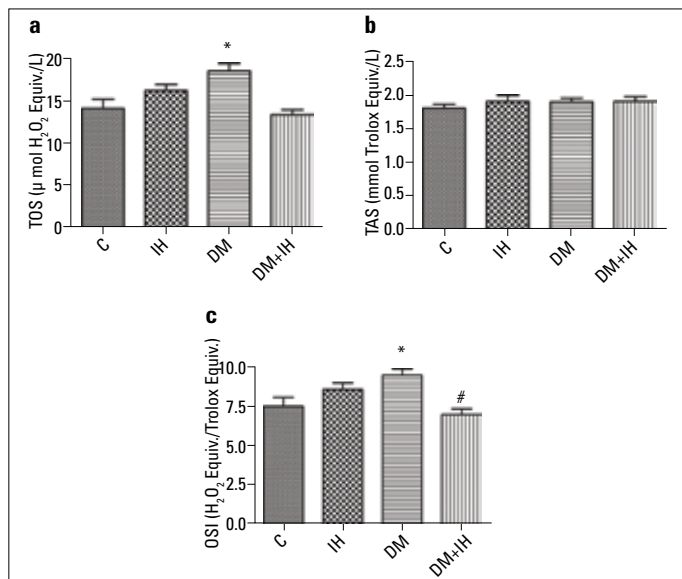
Initial and final body weights, body weight change (%), blood glucose levels, and the ratio of heart weight (wet)/terminal body weight (HW/BW) of animals is given in Table 1. There were significant differences in the body weight change (%) compared to the control (C) and diabetic (DM) groups. Intermittent hypoxia application to DM group slightly but non-significantly induced increase in body weight change.

#### Effect of intermittent hypoxia on left ventricular function of diabetic rats

As expected, the LVDP and the rate of changes in LVDP (+dP/dt and -dP/dt) in diabetic rat heart was depressed, significantly



**Figure 2.** Effects of intermittent hypoxia application on left ventricular developed pressure of diabetic rats. (a) Langendorff-perfused hearts stimulated electrically with 300 beats/min square waves with 1.5 ms duration and twice the threshold voltage and measured left ventricular developed pressure (LVDP). (b) The rate of changes of LVDP (+dP/dt, left and -dP/dt, right). Groups: C, control; IH, intermittent hypoxia; DM, STZ-diabetics; DM+IH, intermittent hypoxia applied diabetics. Data are presented as mean ( $\pm$ SEM) values. The total number of rats in each group;  $n_c=10$ ,  $n_{IH}=10$ ,  $n_{DM}=10$ ,  $n_{DM+IH}=10$ . Significance levels: \* $P<0.05$  vs. C group, and # $P<0.05$  vs. DM tested with ANOVA followed with post-hoc analysis. Exact  $P$  values: (a) C vs. DM  $P<0.0001$ ; IH vs. DM  $P<0.0001$ ; DM vs. DM+IH  $P<0.0001$  (b) +dP/dt C vs. DM  $P<0.0001$ ; IH vs. DM  $P<0.0001$ ; DM vs. DM+IH  $P<0.01$  (b) -dP/dt C vs. DM  $P<0.0001$ ; IH vs. DM  $P<0.0001$ ; IH vs. DM+IH  $P<0.01$ ; DM vs. DM+IH  $P<0.01$

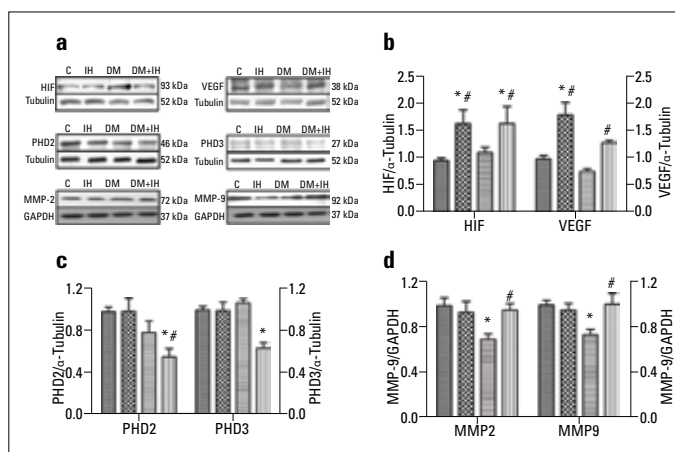


**Figure 3.** Oxidative stress status measured in the heart tissue. Total oxidant status, TOS (a), total antioxidant status, TAS (b) and Oxidative Status Index, OSI (c) measured in the heart of rats. Groups are presented in Figure 1 legend. Data are presenting mean ( $\pm$ SEM) values. The total number of rats in each group;  $n_c=9$ ,  $n_{IH}=10$ ,  $n_{DM}=9$ ,  $n_{DM+IH}=10$ . Significance levels: \* $P<0.05$  vs. C group, and # $P<0.05$  vs. DM, tested with ANOVA followed with post-hoc analysis. Exact  $P$  values: (a) C vs. DM  $P<0.05$ ; DM vs. DM+IH  $P<0.01$  (c) C vs. DM  $P<0.05$ ; DM vs. DM+IH  $P<0.05$

(Fig. 2a and 2b). IH application to diabetic rats for 7 weeks exhibited significant cardioprotective effects in these parameters.

### Effect of intermittent hypoxia on total oxidant status in diabetic rat heart

Serum TAS and TOS levels were not different significantly between control and diabetic rat groups. IH application did not



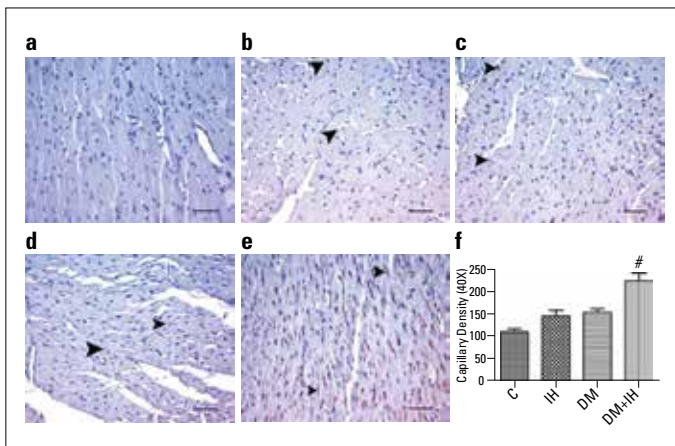
**Figure 4.** Intermittent hypoxia induced normalization in the protein expression levels of biochemical parameters in the heart tissue from diabetic rats. Representative Western Blotting images (a). Left ventricular levels of HIF-1 $\alpha$  (b, left), VEGF (b, right), PHD2 and PHD3 (c, left and right, respectively), and matrix metalloproteinases, MMP-2 and MMP-9 (d, left and right, respectively). Groups are given in Figure 1 legend (C is represented by dotted, IH is represented by checkered, DM is represented by horizontally striped and DM+IH is represented by vertically striped bars). Data are presenting mean ( $\pm$ SEM) values. The total number of rats in each group;  $n_c=7-10$ ,  $n_{IH}=5-9$ ,  $n_{DM}=5-8$ ,  $n_{DM+IH}=5-8$ . Significance levels: \* $P<0.05$  vs. C group, and # $P<0.05$  vs. DM, tested with ANOVA followed with post-hoc analysis. Exact  $P$  values: (a) HIF: C vs. IH  $P<0.05$ ; C vs. DM+IH  $P<0.05$ ; DM vs. DM+IH  $P<0.05$  (a) VEGF C vs. IH  $P<0.001$ ; IH vs. DM+IH  $P<0.05$ ; DM vs. DM+IH  $P<0.05$  (b) PHD2: C vs. DM+IH  $P<0.05$ ; IH vs. DM+IH  $P<0.05$  (b) PHD3: C vs. DM+IH  $P<0.001$ ; IH vs. DM+IH  $P<0.01$ ; DM vs. DM+IH  $P<0.0001$  (c) MMP-2: C vs. DM  $P<0.05$ ; DM vs. DM+IH  $P<0.05$  (c) MMP-9: C vs. DM  $P<0.05$ ; DM vs. DM+IH  $P<0.05$

alter TAS and TOS levels (data not shown). We also examined heart tissue TAS and TOS levels. As shown in Figure 3a and 3b, the TOS level in DM groups significantly increased and IH application to diabetic animals (DM+IH) lowered the TOS levels to control values. However, we could not detect any significant change in TAS level in any group.

### Effect of intermittent hypoxia on the biochemical parameters of left ventricle from diabetic rats

The HIF-1 $\alpha$  protein expression level in the heart of the DM group is slightly but not significantly higher than that of the control group. However, IH application significantly increased the HIF-1 $\alpha$  protein expression level of hearts of both control and DM groups. Similarly, the protein expression level of VEGF in the DM group is not significantly different from that of control group. However, IH application significantly increased the protein expression level of VEGF in the hearts of both control and DM group (Fig. 4a, left and right, respectively).

In another set of experiments, we examined the protein expression levels of PHD2 and PHD3 in the hearts of DM group compared with those of the control group. As seen in Figure 4b (left and right), there are significant differences between these



**Figure 5.** Light microscopic investigation of left ventricular tissue by immunohistochemistry. Tissues staining with Anti-caveolin 1 (a-e). Arrow: capillary endothelial cells (Bar: 50  $\mu$ m). Groups: Negative Control (a), Control (b), Diabetes (c), Intermittent Hypoxia (d), Diabetes + Intermittent Hypoxia (e). Left ventricular capillary density (40 $\times$ ). Data are presenting mean ( $\pm$ SEM) values (f). The total number of rats in each group;  $n_c=6$ ,  $n_{IH}=5$ ,  $n_{DM}=5$ ,  $n_{DM+IH}=5$ . Significance levels: \* $P<0.05$  vs. C group, and # $P<0.05$  vs. DM, tested with ANOVA followed with post-hoc analysis. Exact  $P$  values: (f) C vs. DM+IH  $P<0.0001$ ; IH vs. DM+IH  $P<0.001$ ; DM vs. DM+IH  $P<0.001$

two groups. IH application induced a significant decrease in both PHD2 and PHD3 levels in DM+IH group but we could not observe the same effect in the IH group.

In the last set of experiments for this section, we examined the protein expression levels of MMP-2 and MMP-9 in heart tissue. These were decreased in DM group compared to the control group, significantly. IH application normalized these depressed levels of MMP-2 and MMP-9 in the hearts from DM group (Fig. 4c).

#### Effect of intermittent hypoxia on myocardial capillary density measured with light microscopy

Most interestingly, caveolin-1 stained cells increased in the DM + IH group compared to the other groups. Positive staining was also observed in the diabetes and hypoxia groups but was less than in the DM + IH group. The lowest caveolin-1 expression of endothelial cell was noticeably present in the control group (Fig. 5b-5e). No staining was detected when the primary antibody was omitted (Fig. 5a). The average capillary density for each group is calculated and presented as bar graphs. As can be seen in Figure 5f, IH application in the DM group induced a greater increase in capillary density compared to that in control group.

## Discussion

In the present study, our results have demonstrated that intermittent hypoxia induces beneficial cardiovascular remodeling in left ventricular function of type 1 diabetic rat via increased depressed left ventricular developed pressure and normalized heart tissue total oxidant status. Seven-week IH application in diabetic and control rats induced significant increase in protein expres-

sion levels of both HIF-1 $\alpha$  and, VEGF in the heart tissues, whereas there was a significant decrease in the protein levels of PHD2 and PHD3 in diabetic rat hearts. Furthermore, IH application induced marked increases in the protein expression levels of matrix metalloproteinases, MMP2 and MMP-9 and capillary density in the left ventricle of diabetic rats. Overall, our data presented that IH application has a beneficial cardiovascular remodeling effect in left ventricular function of type 1 diabetic rat, at most, via controlling increased oxidative stress and HIF-VEGF related angiogenesis, providing information related with hyperglycemia associated new targets and therapeutic strategies. In this regard, it has been previously demonstrated that hypoxia has properties for novel diagnostic and therapeutic strategies (26, 27), while IH induces early functional and histologic remodeling in the heart of experimental animals including early hemodynamic alterations with proximal histologic remodeling and delayed changes in peripheral vaso-reactivity (28). Therefore, taking into consideration the sequential cardio benefits of IH, one can suggest that low dose IH may represent a safe and effective treatment to restore hyperglycemia associated functional alterations in diverse clinical disorders such as left ventricular dysfunction in diabetics.

In our present study, functional impairment in the left ventricle was not accompanied by a decrease in HIF-1 $\alpha$ , VEGF protein or capillary density levels. Although there are some reports showing HIF-1 $\alpha$ , VEGF and/or capillary density decrement in 6 weeks or shorter diabetic periods, some studies report that a longer diabetic period is needed for impairment in angiogenesis homeostasis (29-32). In other words, there is a consensus that angiogenesis homeostasis is impaired by diabetes but its initiation time is controversial. Our data show that the duration of diabetes was not sufficient for angiogenesis impairment so cardiac functional deterioration should be discussed independent of the HIF-VEGF-angiogenesis axis.

Our study is the first to examine the effect of hypoxic preconditioning and cardiac remodeling on diabetic heart function. In contrast, unlike the studies reporting hypoxic preconditioning ameliorates metabolic parameters in diabetes (33, 34), IH application could not cause a significant change in blood glucose levels. In this case the cardioprotective effect observed in our study is not related to the control of hyperglycemia with IH application.

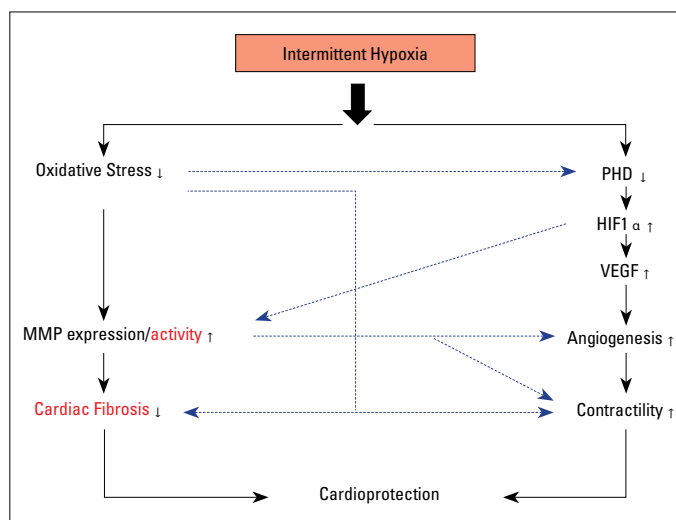
Although there are no significant alterations in the expression levels of HIF-1 $\alpha$ , VEGF and capillary density in diabetic rat heart, IH application significantly upregulated these parameters. Taken into the fact of positive correlation between myocardial contractility and VEGF-angiogenesis (31), the functional recovery induced by IH application may be related with the improvement in HIF-VEGF-angiogenesis pathway. In diabetes, myocardial substrate utilization is significantly changed and  $\beta$ -oxidation of fatty acids is increased while glucose metabolism is decreased (35). In fatty acid oxidation more oxygen (12%) is needed per unit ATP generation compared to glycolytic pathway (36). This change in myocardial substrate utilization increases myocardial oxygen consumption significantly and the mentioned decline in contractility in diabet-

ic heart may be related to the imbalance between oxygen supply and demand. The increase of capillarization provided by IH increases the oxygen supply of diabetic tissues which demands more oxygen; thus, one may speculate that increased angiogenesis and oxygen supply may be related to the restoration of cardiac contractility in diabetic animals. VEGF is key regulator of hypoxia induced angiogenesis in both physiological and pathological conditions (37). Inducing angiogenesis by increasing HIF stabilization and VEGF levels is an important cardioprotection strategy. Kido et al. (38) reported that myocardial infarct size was smaller after MI procedure in HIF-1 $\alpha$  overexpressing mice compared to wild type along with the increased VEGF, capillary density levels and cardiac performance. Cai et al. (39) reported that IH improved functional recovery and reduced infarct size after I/R protocol. In addition, they did not observe this beneficial effect in HIF heterozygous (HIF-1 $\alpha$ <sup>+/-</sup>) mice and might speculate that cardioprotective effect of IH was HIF dependent.

Furthermore, in the present study, IH application caused a decrement in PHD2 (non-significant) and PHD3 levels in diabetic hearts. This data is original because there is no other study which examines effects of IH on PHD levels in diabetic heart. Xia et al. (40) reported that the left ventricle PHD3 level in type II diabetic rats was significantly higher than that of the control animals, and PHD3 silencing restored the functional and structural anomalies caused by diabetes itself. These results indicate that PHD3 inhibition has a cardioprotective effect. Authors also stated that HIF-1 $\alpha$  levels decreased in diabetic rats and with PHD3 silencing procedure HIF-1 $\alpha$  levels returned to control values.

In addition, we have shown that IH application could normalize the increased oxidative status in diabetic rat heart. It has been shown that ROS production and PHD3 expression were increased in hyperglycemic cardiomyoblasts, while they were normalized with N-acetylcystein exposure (40). Our PHD data in diabetic rat heart is not in line with the results of Xia et al. (40), but the restoration of heart function induced by hypoxia in our study may be related to the increase in PHD, which results in HIF stabilization, as reported previously. Conversely, IH application decreased PHD levels along with oxidative stress. These concomitant results emphasize that ROS has a role in PHD regulation.

We found that MMP-2 and MMP-9 levels of the test groups were significantly lower compared to those of control animals, and IH prevented decrement in MMP levels, which was seen in diabetic hearts. Our study is the first to examine the effect of IH on ventricular MMP protein levels. Collagen accumulates in the cardiac tissue in diabetic cardiomyopathy. This accumulation causes interstitial and perivascular fibrosis and is correlated with left ventricular systolic and diastolic functional impairments (41). Bollano et al. (42) reported that in type I diabetic rats (diabetes duration: 12 weeks) left ventricle MMP-2 protein levels decreased and myocardial collagen increased significantly, and cardiac hypertrophy is accompanied by left ventricular functional impairment. Given the fact that there is a negative correlation between MMP-2 activity and collagen deposition (43, 44) fibrotic



**Figure 6.** Schematic illustration of possible mechanisms underlying the cardioprotective effect of intermittent hypoxia in diabetic heart. The roles of HIF-VEGF-angiogenesis pathway and oxidative stress contributing to cardioprotection via intermittent hypoxia are summarized under the light of our current data and literature data. The blue lines correspond to possible cross-relations and red colors indicate the limitations of the study. Upward and downward arrows represent increases and decreases, respectively

changes may commence without a change in the heart weight. In this regard, Faramoushi et al. (34) reported that left ventricular collagen levels increased in type II diabetic rats, and this increment was restored by IH. Cardiac fibrosis, which causes left ventricular stiffness and decreased ventricular compliance contributes the development of both systolic and diastolic dysfunctions. Therefore, IH application induced upregulation in heart tissue levels of both MMP-2 and MMP-9 may be associated with decreased collagen deposition, and this antifibrotic effect may have promoted the functional recovery observed in IH exposed diabetic group. Interestingly, Taraboletti et al. (45) showed that inhibition of MMPs blunts capillary formation via suppression of collagen degradation.

In addition, one can interpret that the MMP downregulation in the diabetic group may be related to the redox status deterioration and IH application to diabetic animals (DM + IH) may have normalized MMP levels via restoration of the redox status. We suggest that normalization of MMP activity may have induced amelioration in fibrotic process and prevented cardiac dysfunction. In contrast, MMP-2 and MMP-9 are downstream targets of the HIF signaling pathway. It was shown that increase in HIF stabilization induces MMP-2 and MMP-9 expression (46, 47). Hence, HIF has an effect on MMP regulation apart from redox status. Taking into account MMP's key regulatory roles in cardiac remodeling, myocardial contractility and angiogenesis, IH may have restored cardiac function via MMP upregulation in addition to HIF stabilization.

#### Study limitations

We could not measure cardiac MMP activity, TIMP (Tissue Inhibitor of Matrix Metalloproteinases) protein levels, and collagen deposition, so our comments on cardiac hypertrophy are weak-

ened. Also, measuring insulin levels or study with a new diabetic group which receives insulin treatment during experiments would reveal the effects of insulin on our experimental model. Last but not least, measuring heart function in vivo (e.g., animal echocardiography) would yield continuous data so we could have followed the prognosis of DCMP in 6 weeks.

## Conclusion

In conclusion, our present results strengthen the need for early identification and treatment of diabetic individuals at risk for cardiovascular complications. Furthermore, IH induces early alterations before overt cardiovascular disease strengthens the need for identifying at-risk individuals for systematic treatment (Fig. 6). Primarily, the therapeutic consequences of hypoxia have been considered in the treatment of cancer. However, it seems to be important for the treatment of diabetics because of its effects on diabetic vasculopathy, as well.

**Acknowledgments:** The authors would like to acknowledge to Yusuf Olgar for his assistance in Western Blot assay. This article is produced from Firat Akat's PhD thesis and was supported by the Ankara University Scientific Research Projects (AU BAP 13B3330002 and AU BAP 15L0230004). Experiments were performed in research laboratories found in Ankara University School of Medicine Physiology, Biophysics, and Histology Departments.

**Funding:** This study was supported by the Ankara University Scientific Research Projects (AU BAP 13B3330002 and AU BAP 15L0230004).

**Conflict of interest:** None declared.

**Peer-review:** Externally peer-reviewed.

**Authorship contributions:** Concept – F.A., H.F., A.D.D., B.T., M.B.; Design – F.A., A.D., E.T., A.D.D.; Supervision – H.F., B.T., M.B.; Fundings – H.F., M.B.; Materials – A.D.D., F.T.Ç.; Data collection &/or processing – F.A., A.D., E.T., F.T.Ç., B.S.; Analysis &/or interpretation – F.A., A.D., E.T., F.T.Ç., B.S.; Literature search – F.A., H.F., B.S.; Writing – F.A., H.F., B.T., M.B.; Critical review – H.F., B.T., M.B.

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