

Delayed neutrophil apoptosis mediates intermittent hypoxia-induced progressive heart failure in pressure-overloaded rats

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Abstract

Background and objective Obstructive sleep apnea (OSA) and heart failure (HF) are common coexisting diseases. Intermittent hypoxia (IH), caused by repeated apnea/hypopnea events, accompanied by increased systemic inflammation, might contribute to the promotion of HF.

Methods To assess the hypothesis, rats were exposed to IH or normal air condition 4 weeks on the basis of normal heart function or pre-existing HF, which was induced by pressure overload caused by abdominal aortic constriction surgery performed 12 weeks earlier. Echocardiography was performed before and after IH exposure to evaluate left ventricular (LV) function. Serum concentrations of pro-inflammatory cytokines TNF- α and IL-6 were detected by enzyme-linked immunosorbent assay. Flow cytometric analysis was used to

determine the apoptotic rate of polymorphonuclear neutrophils (PMNs).

Results The echocardiographic study showed a significant decrease in LV fractional shortening (FS) and ejection fraction (EF) as well as an increase in the LV relative wall thickness (RWT) index in HF rats, which was aggravated by further exposure to IH compared with single-handed HF-only and sham-IH and sham-control groups. A reduced PMN apoptotic rate was observed in HF-IH rats compared with HF-only, sham-IH, and sham-control rats. Serum concentrations of TNF- α and IL-6 were also increased in HF-IH rats, accompanied by delayed PMN apoptosis, indicating significant systemic inflammation induced by IH.

Conclusion The results of this study demonstrated that IH aggravates LV remodeling and heart dysfunction in rats with pre-existing HF. Delayed neutrophil apoptosis, which was revealed in HF rats following exposure to IH, contributed to the exacerbation of myocardial damage and progression of heart dysfunction.

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Introduction

Obstructive sleep apnea (OSA) is the most common sleep breathing disorder, which is characterized by repeated episodes of complete or partial upper airway collapse, leading to intermittent hypoxia (IH), arousal, and sleep fragmentation. Severe OSA with an apnea hypopnea index exceeding 30 events/h of sleep can induce cardiovascular diseases such as arterial hypertension, arrhythmias, pulmonary hypertension, stroke, and even congestive heart failure (HF) [1–3]. HF developing from different reasons represents abnormal

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myocardial contractility and ventricular remodeling. OSA and HF coexist at high frequency. Data from the Sleep Heart Health Study show that patients with OSA have 2.38 times greater odds of self-reported HF. [2]

Several recent studies also revealed that the prevalence of OSA diagnosis by polysomnography in HF patients ranges from 12 to 53 % higher than the general population [4–6].

Although OSA is highly associated with HF, the pathogenesis of HF in patients with OSA is not completely understood. Precise evidence demonstrates that OSA contributes to progressive HF through IH. Recently, increasing evidence has shown that oxidative stress and systemic inflammation induced by IH are fundamental processes initiating cardiovascular events. Polymorphonuclear neutrophils (PMNs), as an essential part of the systemic inflammatory process, can be activated by IH or other stimulation, producing and releasing reactive oxygen species, inflammatory leukotrienes, and proteolytic lysosomal enzymes, which may directly induce vascular damage. Spontaneous apoptosis and regulation play a key role in maintaining PMN homeostasis and controlling the balance between the potency of inflammatory response and risk of tissue damage. Delayed neutrophil apoptosis has been investigated in both OSA and cardiovascular disease patients [7–9].

PMN apoptosis seems to be critical in control of inflammatory processes and vascular injury in cardiovascular events. To determine whether the progression of HF in LV dysfunction rats is aggravated by IH exposure through delayed PMN apoptosis and an increased inflammatory response, we developed an animal model of HF and OSA. Rats were exposed to additional IH for 4 weeks based on pre-existing HF, which was induced by abdominal aortic constriction surgery performed 12 weeks earlier. Echocardiography was performed before and after IH exposure to evaluate LV function. Pro-inflammatory cytokines TNF- α and IL-6 and PMN apoptosis were detected to identify systemic inflammation. Our overall hypothesis was that IH accelerates the progression of HF in rats that have primary LV dysfunction through enhanced systemic inflammation.

Methods

Rodent animal model

The overall rodent experimental protocol is the addition of IH or normal air exposure for 4 weeks based on normal heart function or pre-existing heart failure that induced by abdominal aortic constriction surgery performed 12 weeks prior to our experiments, see Fig. 1a. The Institutional Review Board of Tianjin Medical University General Hospital approved the ethical and methodological aspects of this animal study (TMU IRB Approving Number: EA-20120002.). Rats were provided

by the Model Animal Center of Radiological Medicine Research Institute, Chinese Academy of Medical Science (License No. SCXK Tianjin 2010–0002). Totally, eighty rats were enrolled and randomly divided into two groups: HF rats which performed abdominal aortic constriction surgery to develop heart dysfunction in the banding-surgery group and non-HF rats which performed sham operation and preserving normal heart function in the sham-operated group.

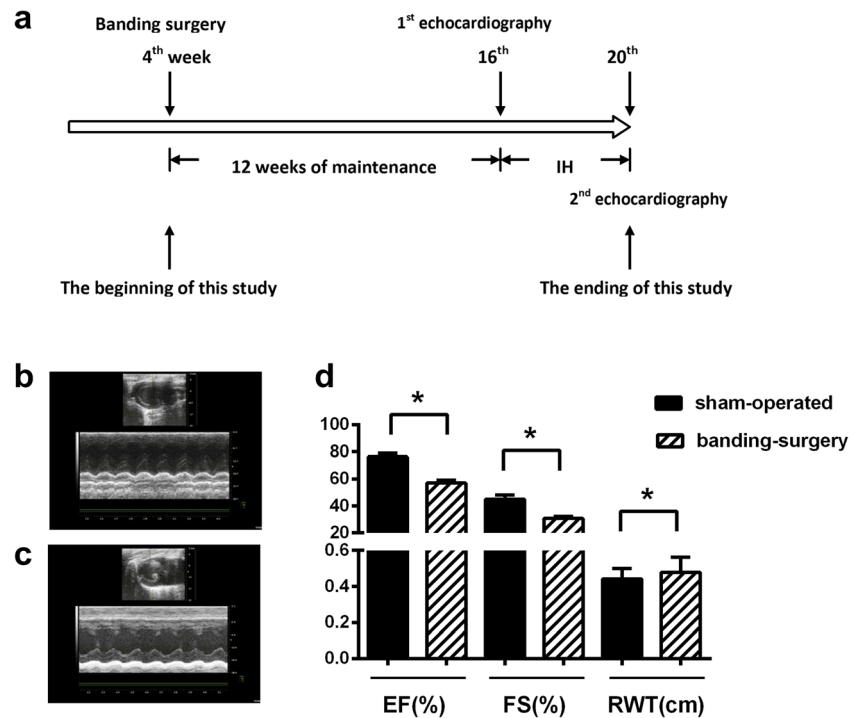
Pressure-overload-induced HF model

Abdominal aortic constriction (banding) surgeries were performed on 4-week-old male Wistar rats weighing 120–150 g. This is a well-established surgical technique for induction of LV chronic pressure overload, hypertrophy, and HF in rodents [10]. The banding initially imposes little or no restriction to aortic flow, but gradually, as the animal grows, the relative severity of the constriction increases, resulting in cardiac hypertrophy. Accordingly, it is characterized by an initial compensatory phase, with concentric LV hypertrophy followed by an enlargement of cardiac chambers associated with a further deterioration of LV function, and HF finally. Suprarenal aortic banding and sham surgery were performed as described [11]. Animals were then housed five per cage and allowed food and water ad libitum during 12 weeks of maintenance [12]. Six rats died in the banding-surgery group (total of 40 rats), and three died in the sham-operated group (total of 40 rats); the mortality rate was 15 and 7.5 %, respectively. Considering an ejection fraction (EF) value less than 60 % of normal controls as a baseline standard for HF in rodents [12], there were 31 rats in the banding-surgery group that developed HF. Thirty of these were then randomly divided into two groups for further exposure of IH (HF-IH group) or normal air (HF-only group). Thirty rats with non-HF coming from sham-operated group were also randomly divided into two groups for further exposure of IH (sham-IH group) or normal air (sham-control group).

IH exposure

Model rats were exposed to IH for 4 weeks, 9 a.m. to 5 p.m. every day, starting at the end of the 12th week after abdominal aortic constriction surgery or sham operation (Fig. 1a). The flow of nitrogen or clean air was regulated by timer-controlled solenoid valves in a customized IH housing chamber (23 cm \times 20 cm \times 12 cm = 5520 cm³ \approx 5.5 L) to maintain a nadir oxygen concentration of 5 %. Each cycle of IH lasted for 2 min, including a 30-s hypoxia phase followed by a 90-s reoxygenation phase [13, 14]. A humidifier, thermostat, and molecular sieve were used to maintain the chamber at a temperature of \sim 22 °C, a humidity of \sim 45 %, and with a relatively germfree environment. An O₂ analyzer (Hamilton, Switzerland) was used to continuously monitor the O₂ and CO₂ concentrations.

Fig. 1 Development of HF animal model. **a** Summary of time course of this study. M-mode and 2D echocardiograms were obtained from rats 12 weeks after abdominal aortic banding surgery (**b**) or sham operation (**c**). **d** Student *t* tests showed significant differences in LV FS, EF, and RWT between banding-surgery and sham-operated rats; *asterisk* indicates comparison to sham-operated group, $p < 0.001$, $n = 31$. LV left ventricle, FS fractional shortening, EF ejection fraction, RWT relative wall thickness



The rats were allowed food and water ad libitum in addition to the experimental time of IH.

Noninvasive evaluation of cardiac function

While lightly anesthetized (10 % chloral hydrate, 0.1 mL/100 g intraperitoneally), all animals underwent baseline echocardiography on the 12th week after abdominal aortic constriction surgery and after 4 weeks of IH exposure. Transthoracic M-mode and 2-dimensional (2D) echocardiography was performed with a Vevo 2100 Imaging System 230 V (VisualSonics Inc., Canada) using a 4 MHz transducer. At the mid-papillary level, long-axis parasternal views and short-axis parasternal 2D views of the LV were obtained. Images were standardized to the short-axis view, and left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), interventricular septal end diastolic thickness (IVSTd), interventricular septal end systolic thickness (IVSTs), left ventricular posterior wall thickness end diastole (LVPWTd), and left ventricular posterior wall thickness end systole (LVPWTs) values were recorded. Three derived parameters, LV fractional shortening (FS), LV ejection fraction (EF), and LV relative wall thickness (RWT), were calculated as described in the literature [15–19].

Isolation of neutrophil granulocytes and lymphocytes

Fifteen animals from each group were anesthetized with 3 % pentobarbital (30 mg/Kg), and blood samples were obtained from the abdominal artery after echocardiography

examination at the end of 4 weeks of IH or normal air exposure. Blood samples were layered on discontinuous gravity gradients consisting of 1.119 g/mL Histopaque-1119 and 1.083 g/mL Histopaque-1083 (Sigma-Aldrich, USA) at 25 °C. After centrifugation at $700 \times g$ and 20 °C for 30 min, the upper band contained the mononuclear leukocytes and platelets, whereas polymorphonuclear leukocytes were identified at the interphase of the Histopaque solutions. Both cell layers were aspirated and washed twice with Hanks' solution (pH 7.4). Cell viability was detected by the trypan blue exclusion test [20] and was at least 98 %.

Assessment of PMN apoptosis

A viability dye containing annexin V and 7-aminoactinomycin D (eBioscience, USA) was used to resolve late-stage apoptotic and necrotic cells from early stage apoptotic cells [21]. After resuspending neutrophils to 10^6 /mL in annexin V binding buffer, 5 μ L of fluorochrome-conjugated annexin V (Annexin V-PE) was added to 100 μ L cell suspension and incubated for 15 min at room temperature. Cells were washed and resuspended in 200 μ L of binding buffer, and then 5 μ L of 7-aminoactinomycin D viability staining solution was added. The final mixtures were stored at 4 °C in the dark and analyzed using a fluorescence-activated cell sorter/scanning flow cytometer (BD Biosciences FACSCalibur) within 4 h to detect apoptotic neutrophils. A total of 10,000 events were measured per sample [22]. The cell survival rate, apoptotic rate, and necrotic rate were obtained based on the number of cells in each quadrant, expressed as percentage of total cell count.

Enzyme-linked immunosorbent assays of pro-inflammatory factors

Enzyme-linked immunosorbent assays were used to detect the concentration of circulating cytokines including TNF- α (Invitrogen, USA) and IL-6 (Thermo Science, USA). The procedure was performed according to the suppliers' protocols, and absorbance was measured at 450 nm in a microplate reader (Labsystems Multiskan, USA).

Statistical analyses

All values were expressed as means \pm SEM. One-way analysis of variance (ANOVA) was used to analyze variations between the means of groups. When ANOVA showed a significant difference, pairwise comparisons between group means were examined by post hoc analysis using Tukey's multiple comparisons test. A Student's *t* test was used to analyze variations between the banding-surgery group and the sham-operated group after abdominal aortic constriction surgery. A *p* value <0.05 was considered statistically significant versus the sham control. Data were analyzed using Prism 6.0 software.

Results

Abdominal aortic constriction surgery leads to development of pressure-overload-induced HF

M-mode and 2D echocardiograms obtained from a sham-operated rat and a banding-surgery rat after 12 weeks of surgery are shown in Fig. 1b, c. Analyses of baseline echocardiography showed indications of decreased LV systolic function and diastolic function in banding-surgery rats compared with sham-operated rats, such as increased LVEDD, LVESD, IVSTd, and LVPWTd and decreased IVSTs and LVPWTs, which contribute to impairment of LV FS and EF and induction of HF. Figure 1d shows a significant decrease in FS and EF, both $p < 0.01$, as well as a significant increase in RWT ($p < 0.05$) in the banding-surgery group compared with the sham-operated group. Considering an EF value less than 60 % of normal controls as a baseline standard for HF in rodents [12], there were 31 rats in the banding-surgery group that developed HF.

IH aggravates LV dysfunction and remodeling in pressure-overload-induced HF rats

Additional echocardiography results obtained after 4 weeks of IH exposure in the sham-control (Fig. 2a), sham-IH (Fig. 2b), HF-only (Fig. 2c), and HF-IH (Fig. 2d) groups showed a further increase in LVEDD, LVESD, IVSTd, and LVPWTd and further decrease in IVSTs and LVPWTs in the HF-IH group,

which aggravated the progression of HF. Figure 2e, f show a significant reduction of FS and EF in HF-IH rats compared with HF-only rats, which indicated that HF conditions were exacerbated by IH exposure ($p < 0.01$). There were no remarkable differences in RWT between the HF-IH and HF-only groups ($p > 0.05$, Fig. 2g). A similar trend of impaired myocardial function was observed in the sham-IH group, with decreased FS and EF compared with the sham-control group ($p < 0.01$).

Delayed neutrophil apoptosis in HF rats after exposure to IH

Flow cytometric assays showed the distribution of PMN cells in the sham-control (Fig. 3a), sham-IH (Fig. 3b), HF-only (Fig. 3c), and HF-IH (Fig. 3d) groups, from which apoptotic cells can be distinguished from healthy and dead cells. The lowest apoptotic rate was found in the HF-IH group, which was lower than the HF-only, sham-IH and sham-control groups ($p < 0.01$); see Fig. 3e. A remarkable decrease in apoptotic rate was also observed in the HF-only and the sham-IH groups compared with the sham-control group ($p < 0.01$). The survival rate of neutrophils was significantly higher in the HF-IH group than the other groups (Fig. 3f).

Serum concentrations of TNF- α and IL-6 higher in HF rats after exposure to IH

The serum concentration of TNF- α was significantly increased in the HF-IH group compared with the HF-only, sham-IH, and sham-control groups ($p < 0.01$). Figure 3h shows a similar increase in IL-6 level in HF-IH rats compared with the HF-only, sham-IH, and sham-control groups ($p < 0.01$). The serum concentration of TNF- α was also increased in the HF-only and sham-IH group compared with the sham-control group ($p < 0.01$). A similar response was also observed in IL-6 level. There were no differences in TNF- α or IL-6 between the HF-only and sham-IH groups ($p > 0.05$). In addition, a negative correlation was observed between the serum concentrations of TNF- α and IL-6 with PMN apoptotic rate ($r = 0.6562$, $p < 0.01$ and $r = 0.5531$, $p < 0.05$, respectively).

Discussion

In this study, we demonstrated that delayed apoptosis of neutrophils arises in HF rats and is significantly exacerbated by IH exposure. HF rodent models were produced through abdominal aortic constriction surgery, which induced pressure overload to the heart, and were then exposed to IH. The baseline echocardiograph obtained on the 12th week after abdominal aortic constriction surgery showed a significant reduction in LV FS and EF and increase in RWT compared with the

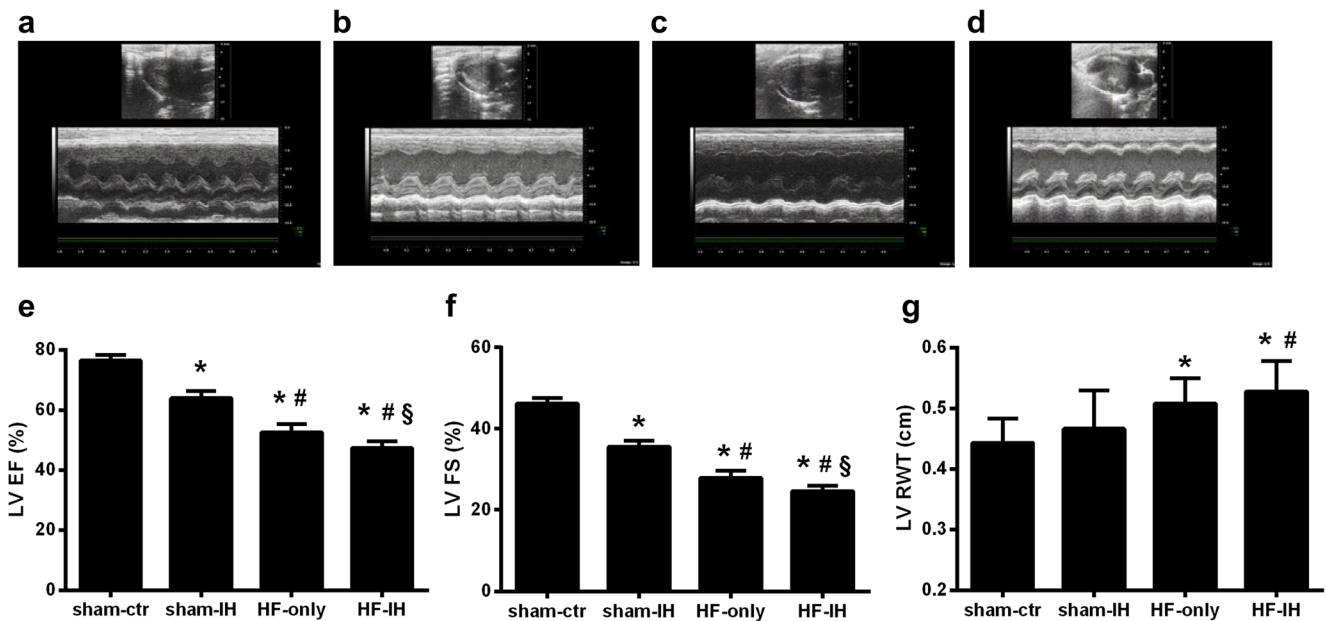


Fig. 2 Changes in myocardial function after IH exposure. M-mode and 2D echocardiograms were obtained at the end of the study in sham-control (a), sham-IH (b), HF-only (c), and HF-IH (d) rats. One-way analysis of variance showed a significant decrease in FS (e) and EF (f) and increase in RWT (g) in the HF-IH group. Asterisk indicates

comparison to sham-control group, $p < 0.01$; number sign indicates comparison to sham-IH group, $p < 0.01$; section sign indicates comparison to HF-only group, $p < 0.01$; $n = 15$. FS fractional shortening, EF ejection fraction, RWT relative wall thickness

sham-operated group, which indicated impaired LV systolic function and remodeling.

IH-induced animal models have been widely used to simulate the cyclical pattern of the hypoxia characteristic of OSA. We previously reported that chronic IH exposure with FiO₂ nadir to 5 % for 30 s in rats results in an oxyhemoglobin desaturation of about 65 % [23]. After 4 weeks of IH exposure, rats exhibit pathological changes that mimic those found in OSA patients, such as systemic hypertension, endothelial dysfunction, and excess sympathetic activity.

Based on our results, LV FS and EF, which serve as measures of LV systolic function, declined significantly after a 4-week IH exposure in HF rats, and RWT, which serves as an LV remodeling index, increased compared with the other study groups. Data from the second echocardiography study demonstrated that further development of HF was induced by IH in rats with pre-existing HF. Impaired heart function and LV remodeling were also observed in sham-IH rats, which demonstrated that IH induces LV remodeling and myocardial dysfunction independent of other risk factors. These findings present strong evidence to support clinical research that reported a significant decrease in LV EF in OSA patients and remarkable improvement after treatment with continuous positive airway pressure [24, 25]. Cioffi et al. also reported that LV RWT was significantly higher in OSA patients and showed a positive association between RWT and severity of OSA [26].

Much evidence suggests that increased systemic inflammation is associated with both cardiovascular diseases and OSA.

In the past decade, delayed PMN apoptosis has been described in acute coronary syndromes, unstable angina, HF and OSA patients [8–10], and the percentage of apoptotic PMNs is negatively correlated with severity based on the apnea hypopnea index. Our findings provide the first evidence that delayed apoptosis of neutrophils occurs in HF rats and is significantly exacerbated by IH exposure. Serum analysis of cytokines also revealed enhanced levels of TNF- α and IL-6 in HF-IH rats, which supports an increase in systemic inflammation of HF rats after exposure to IH. We also found a negative correlation between spontaneous PMN apoptosis and TNF- α and IL-6 concentrations, suggesting that these cytokines might also play a role in determining PMN survival. Therefore, this study showed a prolonged delay of neutrophil apoptosis accompanied by enhanced concentrations of pro-inflammatory factors.

Similar results have been reported in both in vivo and in vitro studies. A body of evidence now demonstrates that delayed apoptosis of neutrophils is strictly correlated with increased concentrations of IL-6 and IL-8 in systemic inflammatory response syndrome patients and in cell cultures [27, 28]. Neutrophils activated by stimulation produce pro-inflammatory cytokines such as TNF- α , IL-6, and IL-8; on the other hand, IL-6 and IL-8 facilitate survival of neutrophils by inhibiting apoptosis [29]. In our previous study, we reported that serum concentrations of pro-inflammatory factors including TNF- α , IL-6, IL-8, and nuclear accumulation of activated NF- κ B P65 were increased in rats exposed to IH and were dependent on the level of hypoxia [23]. In addition, some studies have shown that the late survival effect of TNF- α on

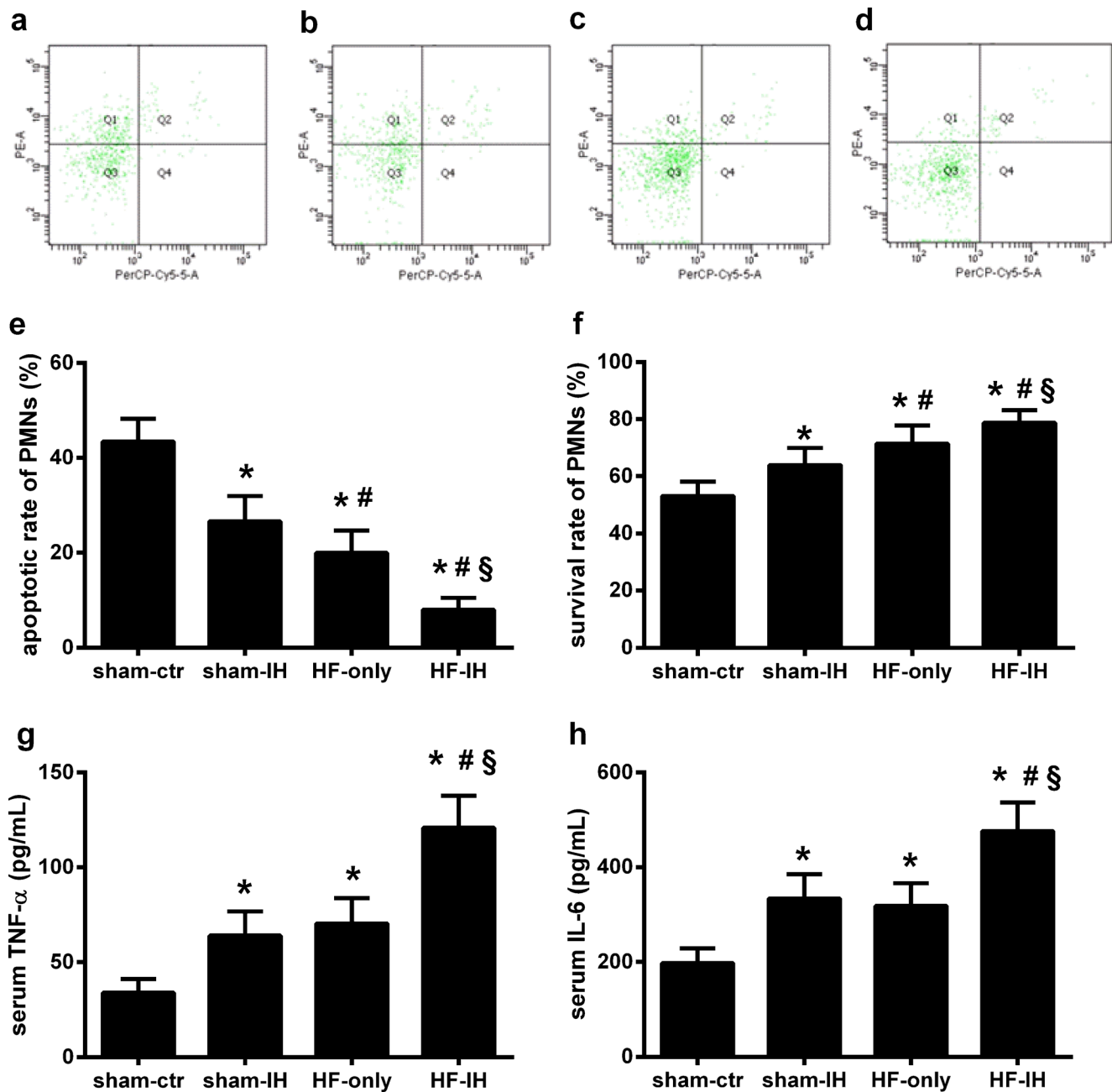


Fig. 3 Systemic inflammatory response following exposure to IH. Flow cytometric assay shows the distribution of PMN cells in sham-control (**a**), sham-IH (**b**), HF-only (**c**), and HF-IH rats (**d**). The *left lower quadrant* (Q3) contains healthy neutrophils (annexin V⁻ and 7-AAD⁻). Apoptotic cells (annexin V⁺ and 7-AAD⁻ neutrophils) are displayed in the *upper left quadrant* (Q1). The *upper right quadrant* (Q2) contains late apoptotic and necrotic neutrophils (annexin V⁺ and 7-AAD⁺). Damaged

neutrophils (annexin V⁻ and 7-AAD⁺) are located in the right lower quadrant (Q4). **e** The apoptotic rate of PMNs was decreased in HF-IH rats, whereas the survival rate was increased (**f**). The serum concentrations of TNF- α (**g**) and IL-6 (**h**) were enhanced in the HF-IH group. Asterisk indicates comparison to sham-control group, $p < 0.01$; number sign indicates comparison to sham-IH group, $p < 0.01$; section sign indicates comparison to HF-only group, $p < 0.01$; $n = 15$

human neutrophils involves activation of both the NF- κ B and phosphoinositide 3-kinase (PI3K) pathways. Activation of both pathways was found to contribute to Toll-like receptor-induced PMN survival, suggesting that NF- κ B regulation might play a key role in PMN survival [30]. Beg et al. indicated a role for the activation of NF- κ B in the active inhibition of apoptosis [31]. Walmsley et al. then reported that TNF- α -

induced survival in human neutrophils depends on the effects of IL-8 generated as a direct response to I κ B/NF- κ B activation and also confirmed that hypoxia-induced neutrophil survival is mediated by a HIF-1 α -dependent NF- κ B activity [32]. Abundant evidence from both clinical and experimental studies has implied that the NF- κ B pathway is constitutively activated in primary neutrophil cells and that this activity is

important in suppressing the constitutive rate of apoptosis [33].

This study has some limitation. First, we did not detect apoptosis of cardio myocytes and the pathological imagination of cardiovascular system. That will provide more strong evidences of myocardial injury and heart remodeling. Second, NF- κ B and PI3K were not tested in this study. Although previous studies have reported the role of NF- κ B and PI3K in regulation of PMN survival, it is important to make clear the pathway involved in the apoptosis of PMN in IH condition for future intervention.

In summary, our results have revealed delayed neutrophil apoptosis accompanied by increased pro-inflammatory factors in HF rats following exposure to IH, which exacerbated myocardial damage and progression of heart dysfunction. Regulation of PMN apoptosis and inhibition of pro-inflammatory cytokines might be a new therapeutic strategy in patients with OSA by prolonging the progression of HF.

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Conflict of interest None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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