THE IMPACT OF HIGH FAT DIET AND CHRONIC INTERMITTENT HYPOXIA ON GLUCOSE CONTROL AND CARDIORESPIRATORY FUNCTION

By

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ABSTRACT

Previous studies have demonstrated carotid body (CB) activity is increased in a high fat diet (HF) induced model of insulin resistance (IR), and carotid sinus nerve resection prevents this and the associated hypertension (HT). The chronic intermittent hypoxia (CIH) of obstructive sleep apnoea also results in HT. We therefore hypothesised the combination of HF induced IR and CIH would result in further increase of CB activity and HT. Effects of HF and CIH on cardiorespiratory parameters, IR, CB activity and sympathetic output were assessed in anaesthetised Wistar rats. HF rats developed obesity, IR and ventricular hypertrophy, with CIH reducing weight gain, but not affecting IR or hypertrophy. Mean arterial blood pressure (MABP) in HF rats tended to be increased by 12±3mmHg, with systolic BP significantly increased. In combination with CIH, MABP tended to be increased by 10±3mmHg, with systolic BP not significantly increased. Treatment groups showed no increased ventilatory drive or sympathetic activity. In conclusion, HF induces obesity, IR and ventricular hypertrophy, however did not result in significant MABP increase. In combination with CIH, obesity is reduced however IR, hypertrophy and MABP do not appear to be affected. Also neither HF diet nor CIH cause increased CB or sympathetic activity.

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CONTENTS LISTINGS

Table of Contents

Abbreviations

AHI	Apnoea Hypopnea Index
AUC	Area Under the Curve
BP	Blood Pressure
CB	Carotid Body
CIH	Chronic Intermittent Hypoxia
CO	Cardiac Output
CSN	Carotid Sinus Nerve
DBP	Diastolic Blood Pressure
EWAT	Epididymal White Adipose Tissue
FVR	Femoral Vascular Resistance
GLUT	Glucose Transporter
GTT	Glucose Tolerance Test
HF	High Fat
HIF-1α	Hypoxia Inducible Factor-1α
HPV	Hypoxic Pulmonary Vasoconstriction
HR	Heart Rate
HT	Hypertension
HVR	Hypoxic Ventilator Response
IH	Intermittent Hypoxia
IR	Insulin Resistance
LFW	Left Free Wall
LTF	Long Term Facilitation
LV	Left Ventricle
MABP	Mean Arterial Blood Pressure
NA	Nerve Activity
NO	Nitric Oxide
NOX	NADPH Oxidase
NS	Nervous System
OSA	Obstructive Sleep Apnoea
PWAT	Perirenal White Adipose Tissue
RAAS	Renin Angiotensin Aldosterone System
R_f	Respiratory Frequency
RFW	Right Free Wall
ROS	Reactive Oxygen Species
RV	Right Ventricle
SBP	Systolic Blood Pressure
SEM	Standard Error of the Mean
TPR	Total Peripheral Resistance
V_T	Tidal Volume

Tables

Figures

INTRODUCTION

Obstructive Sleep Apnoea Syndrome

Obstructive sleep apnoea syndrome (OSA) is a common disorder affecting approximately 2% middle aged women and 4% middle aged men (Cooper et al 2004). OSA is characterised by apnoeic episodes due to recurrent upper airway collapse of the pharynx during sleep, resulting in arousals, sleep fragmentation and oxyhaemoglobin desaturation (Ong et al 2013, Malhotra and White 2002, and Cooper et al 2004). The pharyngeal dilator muscles and surrounding soft tissues are important in maintaining pharyngeal patency as there is a lack of bony support in the region behind the tongue and soft palate. Airway collapsibility (see fig.1) is promoted by a combination of intraluminal negative pressures produced upon inspiration by the diaphragm and extra-luminal forces applied by the bony structures and tissue surrounding the airway (Ong et al 2013). Opposing the collapsing forces are the pharyngeal dilator muscles action and the longitudinal traction on the airway from lung inflation (Ong et al 2013). Change in activation of these dilator muscles with the onset of sleep is thought to be the pivotal event in susceptible individuals.

The apnoeic events caused by the obstructed inspiratory flow are associated with hypoxia and hypercapnia, which stimulate the carotid body (CB) chemoreceptors, and can vary in frequency from 5-60+ an hour (Cooper et al 2004, Polotsky et al 2003). Once an apnoea develops arousal and strong inspiratory efforts are necessary to stop the event, as pharyngeal patency is only restored by sympathetic nervous system (NS) activation upon arousal (Cooper et al 2004). Patients therefore repeatedly arouse from sleep throughout the night (Polotsky et al 2003, Nacher et al 2007). The stimulus of arousal is debated with

it suggested to be increased respiratory effort alone or some combination with hypoxia or hypercapnia as the cause (Malhotra and White 2002).

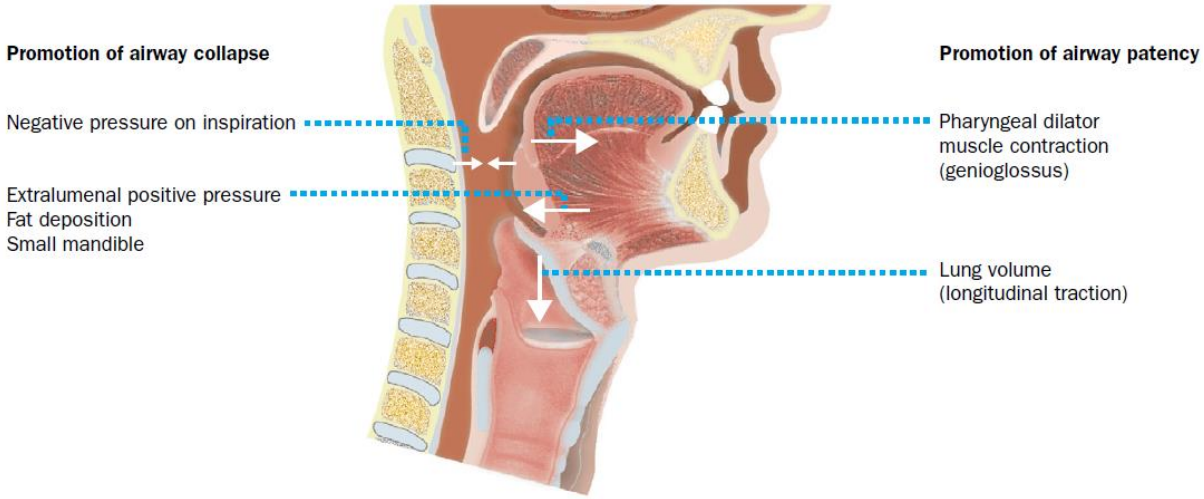

Figure 1: The balance of forces. Pharyngeal collapse is promoted by inspiratory negative pressure and extraluminal positive pressure. Airway patency is maintained by upper airway dilator muscles and increased lung volume. Figure adapted from Malhotra and White 2002.

The ongoing respiratory effort leads to considerable decrease in intrathoracic pressure, these negative pressures contribute to cardiac preload and left ventricular (LV) afterload. At the end of an apopnea, the increased stroke volume (preload) along with the vasoconstricted circulation (due to sympathetic activation) can lead to repetitive intense acute increases in systemic blood pressure (BP).

Chronic consequences of OSA are sustained periods of high BP. This observation is supported by animal investigations and the mechanisms probably include sustained sympathetic excitation, oxidative stress and release of endothelin-1 (Thomas 2011). This sustained hypertension (HT) occurs in 40-60% OSA patients and is independent of common risk factors such as obesity, and may also be linked to subsequent myocardial infarction, cerebrovascular events and congestive heart failure (Cooper et al 2004).

The apnoea-hypopnea index (AHI) is a measure of the severity of OSA. Apnoeas are defined as a minimum 10s cessation of airflow, and based on the absence or presence of respiratory effort, are classified as central or obstructive respectively. Hypopneas are defined as including one of three features: substantial (>50%) airflow reduction, moderate (<50%) airflow reduction with desaturation (>3%) and moderate (<50%) airflow reduction with electroencephalographic evidence of arousal (Malhotra and White 2002). The statistics about prevalence vary depending on the definition used. Previously OSA was defined as increased AHI and symptoms such as excessive daytime sleepiness, but because of the increased cardiovascular risk many classify non-sleepy patients with high AHI as having the disease (Malhotra and White 2002).

The most important risk factor for OSA is obesity as 70% patients with the disorder are obese. OSA prevalence is rising as a result of obesity reaching epidemic proportions in developed countries. This undoubtedly makes OSA an increasingly important public health problem, with obesity the only major reversible risk factor. Despite this relationship with obesity the underlying mechanism is unclear with it probably due to a reduction in pharyngeal airway size with increased weight (Arias et al 2005, Malhotra and White 2002).

Obesity

OSA and obesity both share multiple pathophysiological mechanisms such as endothelial dysfunction, insulin resistance (IR), hyperleptinaemia, systemic inflammation and impairment of baroreflex or hyperactivity of sympathetic NS (Arias et al 2005).

Obesity's role in OSA pathogenesis is thought to include fat deposition altering upper airway structure and function that promotes collapsibility, reduction in resting lung volume and disturbance of central breathing control mechanisms (Ong et al 2013, Arias et al 2005).

Lung inflation applies longitudinal traction on the larynx and trachea reducing collapsibility by increasing airway rigidity. Obesity, particularly central, lessens this effect as fat deposition in the thorax decreases lung compliance and increases the airways passive collapsibility and predisposes to respiratory control system instability (Ong et al 2013). Most obese subjects have elevated leptin levels, due to adipocyte mass, which may also have a pathogenic role. Leptin acts as a neurohumoral modulator of central respiratory control mechanisms and the development of leptin resistance, due to hyperleptinaemia, is associated with a reduction in respiratory drive and hypercapnic response (Campo et al 2007, Malli et al 2010, O'Donnell et al 2000). OSA itself is also associated with raised leptin levels and has been hypothesised as a leptin resistant state (Malli et al 2010, Phillips et al 2000).

Obesity is also linked to a number of other comorbidities such as those which make up the metabolic syndrome. A group of interrelated disorders metabolic syndrome includes obesity, glucose intolerance, IR and HT, which amongst others significantly increase cardiovascular risk (Eckel et al 2005, Guo 2014). The association of IR and HT with obesity is thought to be due to increased CB activity causing elevation of sympathetic NA, as cartotid sinus nerve (CSN) resection has been shown to prevent their development (Ribeiro et al 2013).

Glucoregulation and Insulin Resistance

For most tissues, glucose is the primary source of energy, and plasma glucose needs to be regulated closely as hyper and hypoglycaemia can have severe consequences (Yeo and Sawdon 2013). Many factors can affect glucose delivery and removal from the blood,

including dietary carbohydrate and fat, circulating free fatty acids, exercise, hypoxia and hormone action (Casey 2004.) Hormone action is the main regulator of plasma glucose (see fig.2), with a number of hormones responding to changes in plasma glucose concentration. The most important hormone is insulin which promotes plasma glucose clearance, whereas a number of other hormones, glucagon, growth hormone and catecholamines, stimulate the release of glucose into the plasma (Casey 2004).

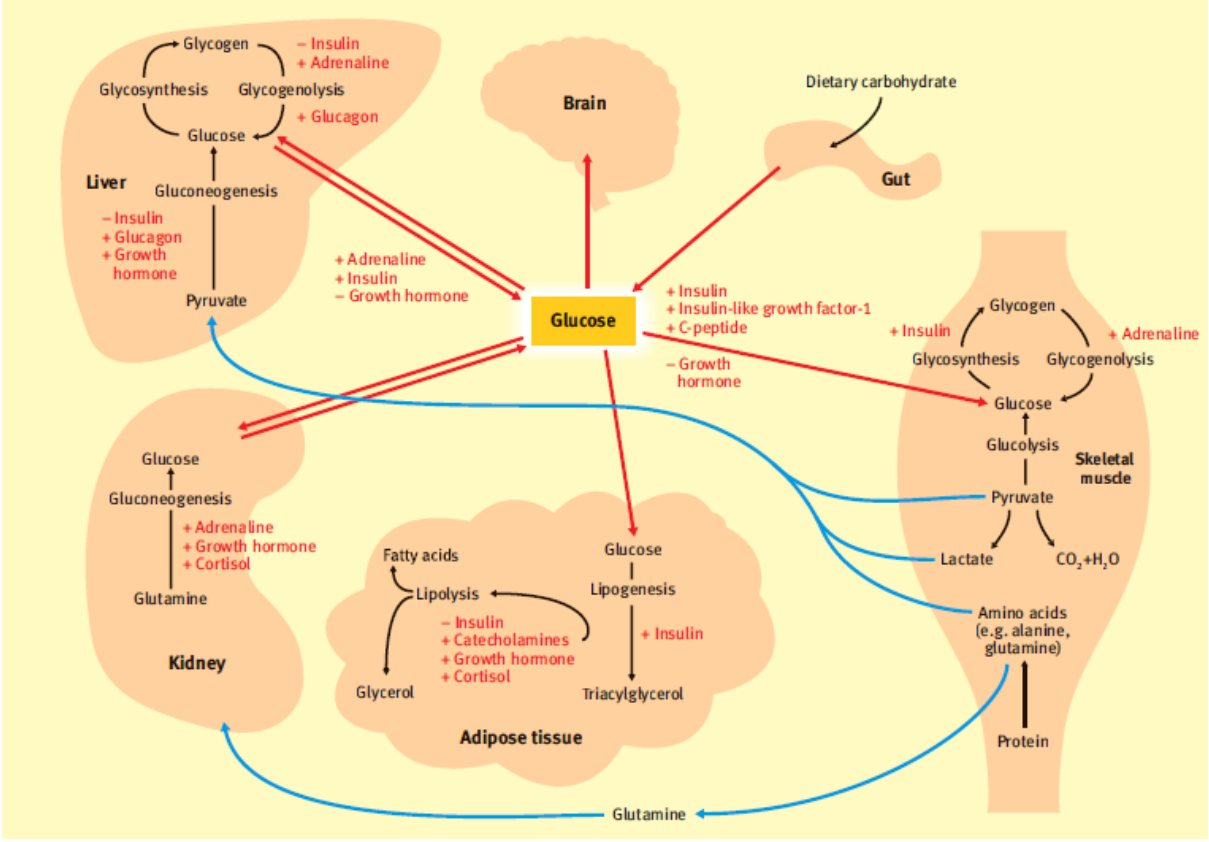

Figure 2: Hormonal control of plasma glucose. Figure adapted form Casey 2004

Insulin is secreted from β cells of the islets of Langerhans in the pancreas in response to elevated blood glucose (Casey 2004, Yeo and Sawdon 2013). It acts to promote glucose uptake into skeletal muscle, liver and adipose tissue while at the same time promoting glucoses conversion to glycogen, the major storage form of glucose. Insulin also dampens

down gluconeogenesis activity (the metabolic pathways leading to the production of glucose). The net result of these actions is a decrease in plasma glucose (Casey 2004, Yeo and Sawdon 2013).

In response to low plasma glucose levels glucagon is secreted from the α cells of the pancreas. Glucagon increases glycogen breakdown into glucose (glycogenolysis), which can then be directly exported to the blood from the liver. Glucagon also acts to reduce glucose demand by inhibition of metabolic pathways (Yeo and Sawdon 2013).

Catecholamine hormones (adrenaline and noradrenaline) are secreted from the adrenal medulla and inhibit insulin secretion and help to preserve plasma glucose levels. Adrenaline secretion leads to activation of glycogenolysis in muscle and liver. Hepatic gluconeogenesis, the conversion of hepatic glucose-6-phosphate to glucose and its release into the circulation is also enhanced by increased catecholamine levels (Casey 2004, Yeo and Sawdon 2013). The Pancreatic α cells are additionally under the control of the sympathoadrenal response, with adrenaline secreted after a fall in glucose stimulating an increase in levels of glucagon release (Yeo and Sawdon 2013).

Glucose transport across membranes is facilitated by the GLUT family of glucose transporters present on the surface of cells. GLUT-1 is constitutively expressed and responsible for basal glucose level, and its expression is not affected by hormone action. GLUT-4 is however affected, with high insulin concentrations up regulating its expression in skeletal muscle and adipose tissue, resulting in increased glucose uptake by tissues (Yeo and Sawdon 2013). This action is insulin's principle method of promoting glucose clearance from the blood (Yeo and Sawdon 2013).

Skeletal muscle is the most important store for glucose in the fed state, with its sensitivity important for maintaining glycaemic homeostasis (Casey 2004, Yeo and Sawdon 2013). Insulin resistance is when insulin induced glucose uptake is impaired in insulin-sensitive tissue, meaning a higher than normal insulin concentration is needed to achieve a normal metabolic response (El Zayadi 2010, Ye 2013). Insulin resistance can be peripheral or hepatic, with peripheral referring to diminished insulin-mediated uptake by skeletal muscle, chiefly due to impairment of GLUT-4 expression (El Zayadi 2010).

Wistar rats fed hyper-caloric diets produce similar changes in body weight, BP and insulin de-sensitivity as those seen in humans (Ballal et al 2010, Conde et al 2012, Ribeiro et al 2013), and as such are a model of diet-induced IR and HT. In this high fat model increased CB chemoreceptor activity is observed. The CB is known to activate the sympathetic NS, a well-known pathophysiological mechanism of HT (Ribiero et al 2013). Elevated sympathetic NA also contributes to skeletal muscle IR and impaired glucose tolerance by sympathetic mediated lipolysis and increased arterial pressure (Ribiero et al 2013). In rats carotid sinus nerve (CSN) resection completely prevents diet induced HT and IR, suggesting obesity is a related factor of CB stimulation, and CB activation is an underlying mechanism of dietary induced HT (Ribeiro et al 2013).

Recognised OSA subjects have central obesity and other features of the metabolic syndrome which include IR, glucose intolerance and HT (Ip et al 2002). However findings suggest OSA is independently associated with IR and HT (Ip et al 2002). Both HT and IR are developed by lean-sleep apnoea patients demonstrating their association with CIH in the absence of visceral obesity and the fundamental role it is proposed to play in the pathophysiology (Ribiero et al 2013).

Intravenous glucose tolerance test (GTT) is a robust and reproducible technique allowing the assessment of insulin release to a glucose load (Frangiousdakis et al 2008). Animals are given a set glucose load intravenously, with blood samples taken over time points, to allow blood glucose and plasma insulin level monitoring.

Hypertension

A dose-response relationship between OSA and HT has been demonstrated, with greater OSA severity resulting in increased risk HT and a greater elevation in BP (Baguet et al 2009). The exact mechanism of OSA induced HT is not clear, with multiple mechanisms thought to contribute to the elevation of BP (see fig.3) (Schulz et al 2014, Qian et al 2012).

OSA patients present haemodynamic oscillations during the night; heart rate (HR), BP and cardiac output (CO) vary due to respiratory events and changes in alertness. These cardiovascular responses are the outcome of four stimuli: hypoxaemia, hypercapnia, changes in intrathoracic pressure and microarousals (Baguet et al 2009). The dipping phenomenon, a fall in BP of approximately 10%, that occurs at night is also absent in apnoeic patients (Baguet et al 2009). With episodic nightly hypoxia observed to result in elevation of diurnal BP (Fletcher et al 1992a). The sustained HT can be attributed to the hypoxia and not changes in arousal as in the canine model upper airway obstruction leads to sustained increase in BP; however sleep fragmentation causes only acute HT peaks (Baguet et al 2009). It has also been put forward that repetitive intermittent hypoxia (IH) may be a major cause of hypertension even in some patients labelled essential hypertensive (Fletcher 2001).

Human and animal studies suggest sympathetic over-activity is the major pathophysiological mechanism behind the chronic arterial HT associated with OSA (Baguet

et al 2009, Schulz et al 2014). Chemical sympathectomy, using a neurotoxin to poison peripheral sympathetic synapses, resulted in no change in BP in IH exposed rats (Fletcher 2001). Also increased sympathetic activity causes elevated peripheral arterial resistance and explains the predominately diastolic nature of OSA HT (Baguet et al 2009).

The sympathetic NS has a dominant role in cardiovascular control. Sympathetic discharge can involve multiple regional effectors and cause dispersed responses (Thomas 2011). Its effects increase cardiac rate, contractility, vasoconstriction, release of adrenal catecholamines and activation of the renin angiotensin aldosterone system (RAAS) (Thomas 2011). Sympathetic control of arteriolar resistance regulates regional blood flow. Arterioles are chief contributors to total peripheral resistance (TPR) and as such sympathetic control is a principle regulator systemic BP (BP = CO x TPR) (Thomas 2011).

Sympathetic NS also innervates the adrenal medulla, causing synthesis and release of adrenaline and noradrenaline. These circulating catecholamines have vascular effects; noradrenaline causes vasoconstriction and adrenaline at physiological levels induces vasodilation in skeletal muscle and the liver (Thomas 2011).

Chronic intermittent hypoxia (CIH) induced HT is prevented by CB denervation, which suggests intact chemoreceptors are needed for arterial pressure to increase (Kohler and Stradling 2013). Hypercapnia is itself a sympathetic stimulation factor (Baguet et al 2009) and studies show hyperoxia suppresses sympathetic stimulation and BP variations during apnoeas (Baguet et al 2009).

In addition to the sympathetic over-activity, endothelial dysfunction and oxidative stress are thought to play a role. During OSA there are changes in endothelial function

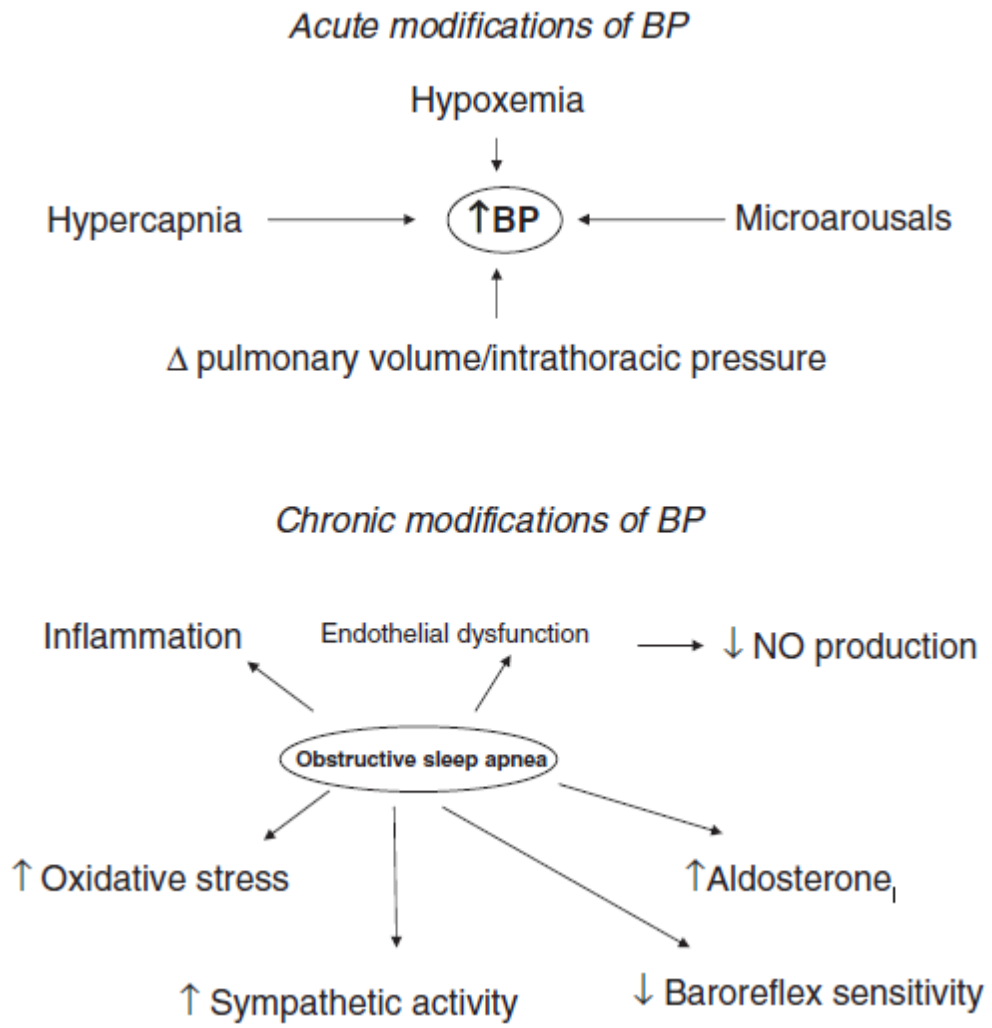

Figure 3: OSA related mechanisms of hypertension. Figure adapted from Baguet et al 2009.

(independent of obesity or atherosclerosis risk factors) that cause decreased vasodilation and vasoconstriction. This dysfunction is caused by sympathetic activation, systemic inflammation and oxidative stress usually present in apnoeic patients (Baguet et al 2009).

The desaturation re-oxygenation that occurs during CIH leads to the production of reactive oxygen species (ROS). Increased ROS activate leukocytes producing systemic inflammation, generating vascular endothelium damage and dysfunction promoting arterial HT (Schulz et al 2014, Baguet et al 2009, Qian et al 2012). Oxidative stress causes

vasoconstriction by multiple mechanisms; activation of angiotensin II, increased generation of endothelin-1 (Baguet et al 2009) and also by reducing the bioavailability of the endothelial-derived vasodilator nitric oxide (NO) (Schulz et al 2014). The NADPH oxidases (NOX) are major ROS generating enzymes. NOX derived ROS decrease NO bioavailability causing loss of vasodilator tone and arterial hypertension (Schulz et al 2014). The glomus cells of the CB also have enhanced NOX2 activity under CIH, the transcription factor HIF-1α activates NOX2 and causes a subsequent ROS release and increased sympathetic activity, elevating BP (see fig.4) (Schulz et al 2014). It has been shown in animals and humans that IH increases circulating endothelin-1 levels, a powerful vasoconstrictor (Baguet et al 2009).

Rats exposed to IH also show over activity of the adrenergic and renin-angiotensin systems contribute to the chronic BP elevations (Fletcher 2001). The increase in BP caused by CIH can be prevented by CB denervation, sympathetic nerve ablation, renal sympathectomy and angiotensin II receptor blockade (Fletcher 2001).

Sympathetic and Parasympathetic Nervous System Cardiovascular Control

Obesity related HT and IR and OSA related HT are all thought to be caused by increased sympathetic activity. Subjects with OSA have altered chemoreceptor reflexes and baroreflex control, and the periodic nightly sympathetic activation evolves into a rise in daytime level sympathetic NS activity when subjects are breathing normally (Thomas 2011).

The arterial baroreflex is a negative feedback system for BP around an internal set point. This sympathoinhibitory reflex is stimulated by acute changes in arterial BP sensed by baroreceptors in the carotid sinus and aortic arch, which adjust CO and vascular resistance

to return BP to its set point (Thomas 2011). Increased BP causes an increase in baroreceptor discharge, resulting in reflex inhibition of sympathetic outflow to the heart and vasculature and activation of vagal parasympathetic outflow to the heart. This causes decreases in vascular resistance, stroke volume and HR and therefore reduces BP (Thomas 2011). A decrease in BP has the opposite effect evoking reflex increases in peripheral resistance, stroke volume and HR by increasing sympathetic outflow, to the heart and vasculature, and reducing vagal parasympathetic activity to the heart, to restore BP (Thomas 2011). Chronic resetting can occur, with the reflex operating around a new set point (Thomas 2011). Asphyxic gas breathing has been shown to reset the vascular resistance component of carotid baroreflex at a higher pressure (Cooper et al 2004).

The reason for HT development may be related to baroreceptor resetting occurring as result of repeated nightly HT (Cooper et al 2004). Resetting of the sympathetic baroreflex control, not impairment of sensitivity is associated with the onset of CIH induced hypertension (Kohler and Stradling 2013). Rats exposed to CIH showed increases in BP before any alteration in sensitivity is observed (Kohler and Stradling 2013).

The Carotid Body Chemoreceptors

Overstimulation of the carotid body is thought to be the chief mechanism involved in the increase in sympathetic activity with obesity and OSA (Ribeiro et al 2013). Located bilaterally in the neck CBs are classically activated by hypoxia and hypercapnia increasing their sensory nerves, the CSN, action potential frequency (Kumar and Prabhakar 2012). Integration of the CSN occurs in the brain stem, where it induces respiratory reflexes directed at normalising altered blood gases. This is mainly achieved by hyperventilation, and BP and cardiac performance regulation via sympathetic NA (Ribeiro et al 2013).

Recurrent apnoea patients exhibit augmented hypoxic ventilator response (HVR) which is attributed to enhanced sensitivity of peripheral chemoreceptors (see fig 4). This is consistent with the finding of a more pronounced ventilatory depression to hyperoxic challenge (Dejours test) in OSA patients compared to controls (Kumar and Prabhakar 2012). The Dejours test is a measure of peripheral chemoreceptor sensitivity (Kumar and Prabhakar 2012) and involves breathing 100% O_2 for a short period. During the early stages of apnoea syndrome carotid body sensitization may be beneficial in aiding to maintain oxygenation via HVR augmentation and BP increases. If untreated however this augmented hypoxic sensitivity may result in unstable breathing and an increased number of apnoeas (Kumar and Prabhakar 2012).

Table 1. Stimuli causing carotid body discharge and reflex consequences (adapted from Kumar and Bin-Jaliah 2007)

Adequate Stimuli of the Carotid Body	Reflexes Produced by Carotid Body Activation
Hypoxia	Hyperventilation
Hypercapnia	Cardiovascular Responses :
Acidosis	Primary: Bradycardia ↓ Cardiac Output ↓ Stroke Volume Peripheral Vasoconstriction
Hyperkalaemia	
Hyperthermia	
Catecholamines	Secondary (to Hyperventilation): Tachycardia Renal Vasoconstriction Peripheral Vasodilation
Angiotensin II	
ADH	
Adenosine	Other Sympathoexcitatory Responses:
Endothelins	↑ Renal Sympathetic NA ↑ Muscle Sympathetic NA Augmentation Pituitary-Adrenocortical Axis Augmentation Adrenomedullary Catecholamine Secretion

Glomectomised sleep apnoea patients do not develop HT. Also IH exposed rats develop HT similar to recurrent apnea patients with sectioning of sinus nerve preventing the response, both provide indirect evidence of altered CB function in humans and rodents exposed IH (Lesseke et al 1997, Kumar and Prabhakar 2012).

Activation of the carotid body reflexly activates the sympathetic nervous system. CIH-induced sensory long-term facilitation (LTF) contributes to the continuing sympathetic NA elevation observed in recurrent apnoea patients (Kumar and Prabhakar 2012).

Hypoxia is still considered the most significant peripheral chemostimulus, but it is becoming increasingly evident the CB can transduce many other stimuli: arterial PCO_2 and pH, also blood potassium concentration, temperature and osmolarity, as well as potentially blood glucose levels (see table 1) (Kumar and Bin-Jaliah 2007). However the question remains, is there one transduction process which senses all the stimuli or distinct stimuli sensors with common downstream transduction pathways (Kumar and Bin-Jaliah 2007). Osmolarity and hypoglycaemia appear to be sensed independently of the hypoxia sensing mechanism but both mechanisms converge at type 1 cell membrane depolarisation or Ca^{2+}-dependent neurosecretion (Kumar and Bin-Jaliah 2007). The CB's afferent neural output does not appear to distinguish between stimuli but rather only grades for intensity to induce cardiorespiratory and endocrine reflexes (Kumar and Bin-Jaliah 2007).

In addition to its role in control of ventilation the CB has been proposed as being able to sense glucose and thus implicated in the control of energy homeostasis (Ribiero et al 2013). Although some central neurons can sense glucose concentration, peripheral glucose sensors outside the brain are needed for the counter-regulatory responses. The

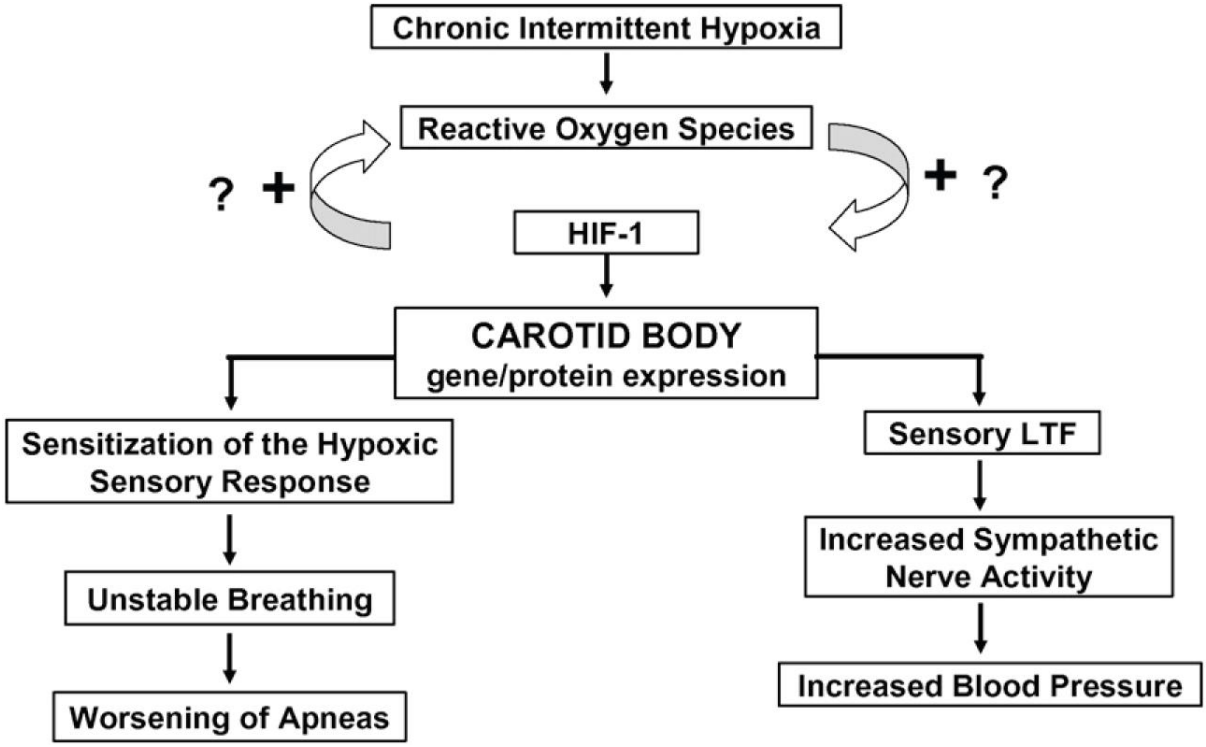

Figure 4: Effect of CIH on the cardiorespiratory system. Mechanisms and consequences of CIH induced changes in carotid body on the cardiorespiratory system. Figure adapted from Kumar and Prahbakar 2012.

CB's location and characteristics (high blood flow and metabolic rate) would enable it to detect rapid glycaemic changes (Lopez-Barneo 2003). However the CBs role in glucose sensing is yet to be established definitively, with different preparations observing different results. In CB thin slices hypoglycaemia has been shown to induce a secretory response similar to hypoxia (Pardel et al 2002). However Bin-Jaliah (2004) reported insulin induced hypoglycaemia resulted in increased ventilation in vivo, but in vitro hypoglycaemia had no effect on CB discharge frequency.

Though there is increasing evidence that suggests it may play a part in initiation of the reflex counter-regulatory responses along with the pancreas, liver and hepatic portal vein (Kumar and Bin-Jaliah 2007). It has been shown that CB resection in dogs impairs the

counter regulatory responses to insulin-induced mild hypoglycaemia (Lopez-Barneo 2003). CSN resection in rats also showed decreased insulin sensitivity (Ribeiro et al 2013). This suggests CBs contribute to the maintenance of glucose homeostasis.

A new role for hyperinsulinaemia as a stimulus of CB over activation has also been proposed (Ribiero et al 2013). Increased insulin levels trigger the CB activating the sympathetic NS and initiating a cycle that worsens peripheral insulin action, impairs B-cell function and causes systemic HT (Ribiero et al 2013).

Animal Models of Obstructive Sleep Apnoea

To advance the understanding of sleep apnoea and target patients who are at greatest risk of morbidities with treatment, it is necessary to identify the interactions between physiological variables. However it is difficult to undertake in-depth study into the chronic effects and pathophysiological mechanisms of the various factors (fluctuations in O_2, CO_2, behavioural state, respiratory drive, ventilatory effort and upper airway intraluminal pressures) influencing the course of apnoeas in humans (Farre et al 2007, Nacher et al 2007). The difficulty arises due to a number of confounding variables, such as those embodying the metabolic syndrome, that are associated with OSA and also because the chronic cardiovascular changes take many years to manifest (Fletcher 2001).

Due to these difficulties animal models mimicking the characteristic recurrent upper airway obstructions of OSA are useful in eliciting the pathophysiological mechanisms of the syndrome (Nacher et al 2007). Animal models avoid the associated comorbidities, and develop OSA's chronic pathophysiology on a much shorter time scale, as well as enable the use of invasive techniques and measurements under well-controlled experimental conditions that are not possible in humans (Farre et al 2007).

There are many different animal models available for the study of OSA. Dog models of OSA have been used, as well as miniature pigs which have been used as an obesity induced model (Lonergan et al 1998, Kimoff et al 1997). Mice and rats are also used in research as rodents have many similarities in BP control and cardiovascular responses with humans (Fletcher 2001). Rodents are a relatively cheap model of OSA and allow the different properties to be easily copied artificially.

Airway obstruction can be applied indirectly avoiding the need to manipulate the animal's airway by an occluding head chamber. As the animal's airway is not actually occluded it is able to breathe a small airflow associated with pressure changes induced by muscle effort exerted on low compliance gas chamber (Farre et al 2007).

Another model involves performing a tracheotomy with a cannula containing an inflatable balloon inside a rigid Teflon tube implanted in trachea. Inflation of the balloon allows the induction of an apnoea without inducing pain (Schoorlemmer et al 2011). The apnoea can last up to 16 seconds during REM sleep without causing the rat to wake. The use of the balloon also allows apnoeas to be induced at a fixed point in the respiratory cycle and elicit cardiorespiratory responses similar to humans (Schoorlemmer et al 2011).

However the downside of these models is the rats' movement has to be restricted, which even after their familiarisation with the setup is a source of stress. This stress can cause reduced weight gain and elevated plasma corticosterone levels (Farre et al 2007).

Commonly the mechanisms of sleep apnoea are studied in conscious rats or mice exposed to IH, as it avoids movement restriction (Schoorlemmer et al 2011). Hypoxic chambers can contain single or multiple animals, with pure nitrogen gas used to lower the O_2

concentration to 3-5%. The IH can be delivered in various cycle length durations (hours) and number of days or weeks (Fletcher 2001).

IH replicates many features of OSA but not all. IH differs to OSA as it does not provoke the characteristic respiratory effort, therefore not permitting study of the potential cardiovascular consequences of strenuous breathing against an obstructed airway (Farre et al 2007). Strenuous breathing causes alteration of cardiac pre and afterload which may contribute to OSA pathology (Schoorlemmer et al 2011) and the mechanical stimulus acts as an immune challenge causing inflammation and metabolic responses that may be significant (Farre et al 2007). IH also does not induce the hypercapnia observed during apnoea, although it can be replicated by creating a hypoxic, hypercapnic atmosphere to breathe (Schoorlemmer et al 2011). Lastly respiration usually becomes blocked during sleep in OSA and then resumes on awakening, this is not easily replicated with the IH model (Schoorlemmer et al 2011). However hypoxia is the most important factor in the development of HT, with it demonstrated that CIH alone can cause persistent elevation of MABP (Fletcher et al 1992a, Fletcher 2001).

CIH models allow the manipulation of the number of apnoeas per hour as well as the duration of each apnoea. This ability to alter the severity of CIH is something experimenters have taken full advantage of with cycle lengths used varying from <10 to >60 per hour, and apnoea duration from <5 to >15 seconds. The severity of the induced apnoea also varies with some using oxygen concentrations as low as 3% and others as high as 10% (Crossland et al 2013). The total duration used also differs with some studies using as little as 3 hours and others 6 or 8 hours a day (Nacher et al 2007, Farre et al 2007). All

these variations in the CIH model may actually be beneficial as it can be used to reflect the various severities of OSA found in the human population.

The model of CIH used in this study has not been used before, it was chosen with the aim of mimicking the early stages of OSA. The protocol involves a low number of apnoeas, 6 per hour, which is above the level for diagnosis with mild OSA, ≥5 per hour, but below the level recommended for treatment, ≥15 per hour. During the protocol the oxygen concentration decreases to 5-6% over 90 seconds before increasing back to normoxic levels over a further 90 seconds. Rats were placed in the hypoxic chamber for 8 hours every day during their normal sleep cycle for 2 weeks. This duration was chosen as the normal sleep period for humans and rodents is between 6-8 hours every a day (Fletcher 2001).

Aims and Hypotheses

Given evidence that CB activity is increased in a HF diet induced model of IR and that CSN resection prevents this and the associated increases in BP, and evidence that the CIH of OSA results in HT that can be prevented by CSN resection, It is hypothesised that the combination of HF diet induced IR and CIH will result in greater up regulation of CB activity and result in a more severe HT.

The aims of this study are:

1. To assess the effect of HF and HF+CIH on baseline cardiovascular and respiratory variables.
2. To assess the effect of HF and HF+CIH on glucose tolerance and insulin sensitivity.
3. To assess the effect of HF and HF+CIH on CB function with use of the Dejours test.

4. To assess the effect of HF and HF+CIH on vascular response to sympathetic stimulation.

METHODS

All experiments were approved under the Animals (Scientific Procedures) Act 1986.

Animals

All experiments were carried out on 9 week old male Wistar rats. Animals were obtained from Charles River Laboratories and housed 3 animals per cage, in the Biomedical Services Unit, University of Birmingham. Animals were allowed 1 week acclimatisation before experiments. Animals' weight, food and water intake were all monitored twice weekly following acclimatisation. Food and water intake was calculated as a third of the total cage consumption, with all animals assumed to consume the same amount.

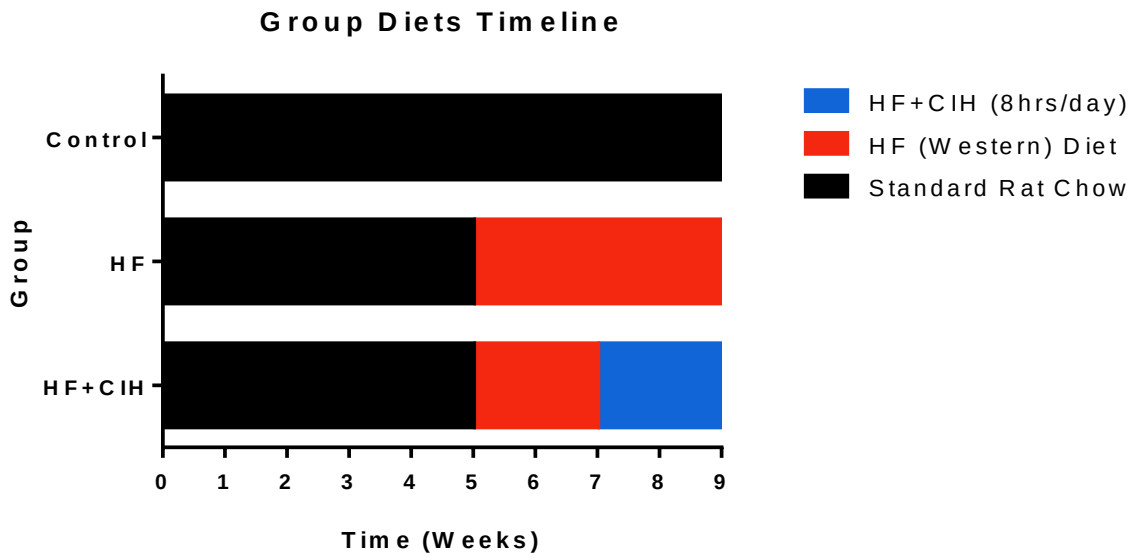

Figure 5: Diet Timeline for control, HF and HF+CIH groups.

Three groups of animals were used in this study (see fig.5). Control animals (weight 353±7g) were fed a normal diet of standard laboratory rat chow (3.9Kcal.g^{-1}) containing 14% protein, 2.5% fat and 6% fibre (EURdent Diet 14%, LabDiet). The high fat group (HF group) animals were fed on the Western diet (4.63Kcal.g^{-1}) containing 17.5% protein, 21%

fat and 3.5% fibre (Special Diet Services, LBS Biotech) for 4 weeks (weight 440±14g). The high fat intermittent hypoxia group (HF+CIH group) animals were fed Western diet for 4 weeks with the final 2 weeks spent in a hypoxia chamber (weight 418±8g).

HF+CIH animals spent 8 hours a day in the hypoxia chamber (OxyCycler, BioSpherix, see fig.6A). The chamber undergoes 6 cycles every hour, with O_2 decreasing from 20-21% down to between 5-6% over 90 seconds and then increases back up to 20-21% over a further 90 seconds. There is a then a 7 minute break between cycles (see fig.6B). The decrease in O_2 is achieved by the introduction of nitrogen gas into the chamber, with the increase in O_2 due to the reintroduction of O_2.

All animals were fasted with water and restricted food overnight before surgery. 10g of food was given the day before experiments to ensure animals were fasted from approximately midnight until surgery the following morning.

Surgery

All experiments were conducted in vivo with animals under terminal anaesthesia. Anaesthesia was induced by inhalation of IsoFlo (100% isoflurane, National Ventinary Supplies) 3-4% with an oxygen flow of 4L.min^{-1} using a Fluovac anaesthesia system. The animal was placed in the induction chamber until anaesthetised, then moved to the surgical bench. Inhalation anaesthesia was continued through a tube placed over the animals head. The depth of anaesthesia was monitored by looking at the animal's ventilation and the pedal withdrawal reflex test. Once anaesthesia was deep enough the right jugular vein was cannulated to allow anaesthesia to be switched to and maintained by intravenous (IV) infusion of Alfaxan anaesthetic. The Alfaxan (10mg.ml^{-1} alfaxalone, National Vetinary Supplies) was diluted 1:1 with saline before infusion. The inhalation

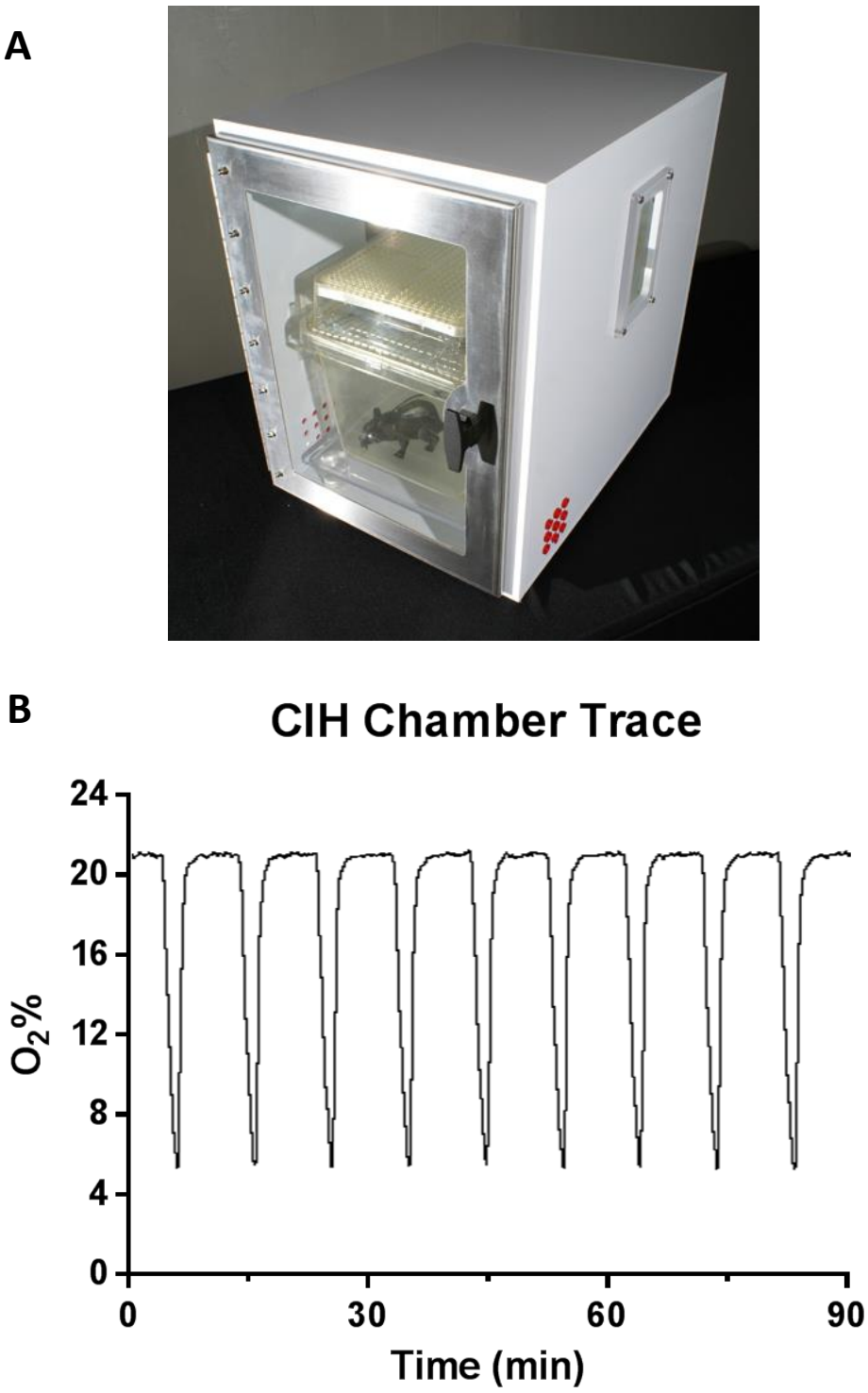

Figure 6: A: Picture of hypoxic chamber (taken from website; http://www.biotech-europe.eu/files/pdf/Animal%20Chamber.pdf) and B: Example of CIH chamber trace

anaesthetic was stopped once the jugular vein was cannulated, the animal was then allowed to breath off the inhalation anaesthetic while small boluses of Alfaxan were given, this was to prevent an anaesthetic overdose.

Once the inhalation anaesthetic had been breathed off Alfaxan was given by automated infusion pump (Perfusor, Secura). Infusion rate was set between 1.5-2.5 $ml.hr^{-1}$ and could be raised or lowered as required; IV boluses could also be given when needed.

The trachea was then isolated and a trachea tube was inserted through an incision made between the cartilage rings. This tube allowed the airway to remain clear and the animal's respiratory parameters to be monitored throughout the experiment. Cannulae filled with heparinised saline (0.1ml heparin in 25ml 0.9% saline) were also inserted in the left brachial artery, left femoral vein and left femoral artery for BP measurement, IV infusion and taking blood samples respectively. The right femoral artery was also isolated to allow measurement of blood flow.

Following the cannulations a laparotomy was performed on the animal. Once inside the abdominal cavity the internal organs were gently moved aside and the tissue blunt dissected to expose the vena cava. The vena cava was then freed from the surrounding connective tissue and moved to expose the sympathetic chain underneath. The sympathetic chain was then freed and silver-wire electrodes were then placed around the right lumbar sympathetic chain, between L3 and L4, and fixed in place using silicone adhesive (Kwik-Sil, World Precision Instruments) to mechanically fix and electrically isolate them. The animal's abdominal cavity was then closed using suture.

Animal's core body temperature was monitored and maintained at 37^{o}C throughout the experiment using an internal temperature probe and homoeothermic monitor connected to a thermostatic blanket (Harvard Apparatus).

Recording Set Up

Experimental setup is shown in fig.7. All signal recording was done using a PowerLab 4/30 and LabChart software (both AD Instruments). LabChart was set to sample at 100Hz (100 measurements per second) to ensure accurate data collection.

A pressure transducer was attached to the brachial artery cannula to allow arterial blood pressure (ABP) to be measured. A 2-point calibration (0 mmHg and 200 mmHg) was performed before being attached. The two points are outside but close to the expected range of recordings to prevent inaccurate extrapolation. From the raw cyclic ABP signal mean arterial BP (MABP) was derived on-line as the average measurement of a cycle. Systolic (SBP) and diastolic BP (DBP) were also on-line derived as the maximum and minimum of cycles respectively. Similarly HR was derived as 1 beat per cycle peak from the ABP signal.

A perivascular flow probe (0.7V; Transonic Systems Inc. Ithaca, NY, USA) was placed around the right femoral artery and used to record blood flow (FBF). The probe was attached to the PowerLab via a blood flow meter (T206, Transonic Systems Inc.). Mean blood flow (MFBF) was derived as an average of the raw FBF signal. Femoral vascular resistance (FVR) was calculated on-line from the division of ABP by FBF.

A pneumotac spirometer was attached to the trachea tube allowing the airflow signal to be recorded. From the raw cycle trace the tidal volume (V_T) was calculated from the area

under the curve, and respiratory frequency derived as one breath per cycle peak. The spirometer was calibrated for tidal volumes between 0 and 5ml before it was attached to ensure V_T are correctly derived. A three lead ECG was taken with electrodes attached to the animals' left and right forelimbs and right hind limbs.

The sympathetic chain electrodes were attached to the PowerLab and stimulated at a constant voltage of 2 volts (V). The chain was stimulated with 60 impulses at two different frequencies, 20Hz and 2Hz.

Glucose infusion was made via the left femoral vein cannula, with all blood samples taken from the left femoral artery.

Protocol

Glucose Tolerance Test

Following surgery the anaesthesia was adjusted and the animal was allowed to stabilise for approximately 30 minutes. Baseline blood samples were taken once the recordings had stabilized for blood glucose, insulin (~150µl) and haematocrit (20-30 µl). $1ml.kg^{-1}$ animal weight of $0.5g.ml^{-1}$ of glucose was given as an IV bolus followed by 0.5ml of saline with arterial blood samples taken at 2, 5, 10, 15 and 30 minute intervals to monitor blood glucose and insulin levels.

Blood glucose was measured using blood glucose strips and a blood glucose monitor (Roche Contour XT, Accu-Check Aviva Nano and FreeStyle Optimum) with values given in $mMol.L^{-1}$. Insulin arterial blood samples were spun in a centrifuge at 10,000RPM for 5min to separate out the plasma. The plasma was then removed and frozen in liquid nitrogen and then stored in a $-80^{o}C$ freezer until analysed for insulin content. Haematocrit was measured in duplicate; with arterial blood samples collected in small 40µl heparinised

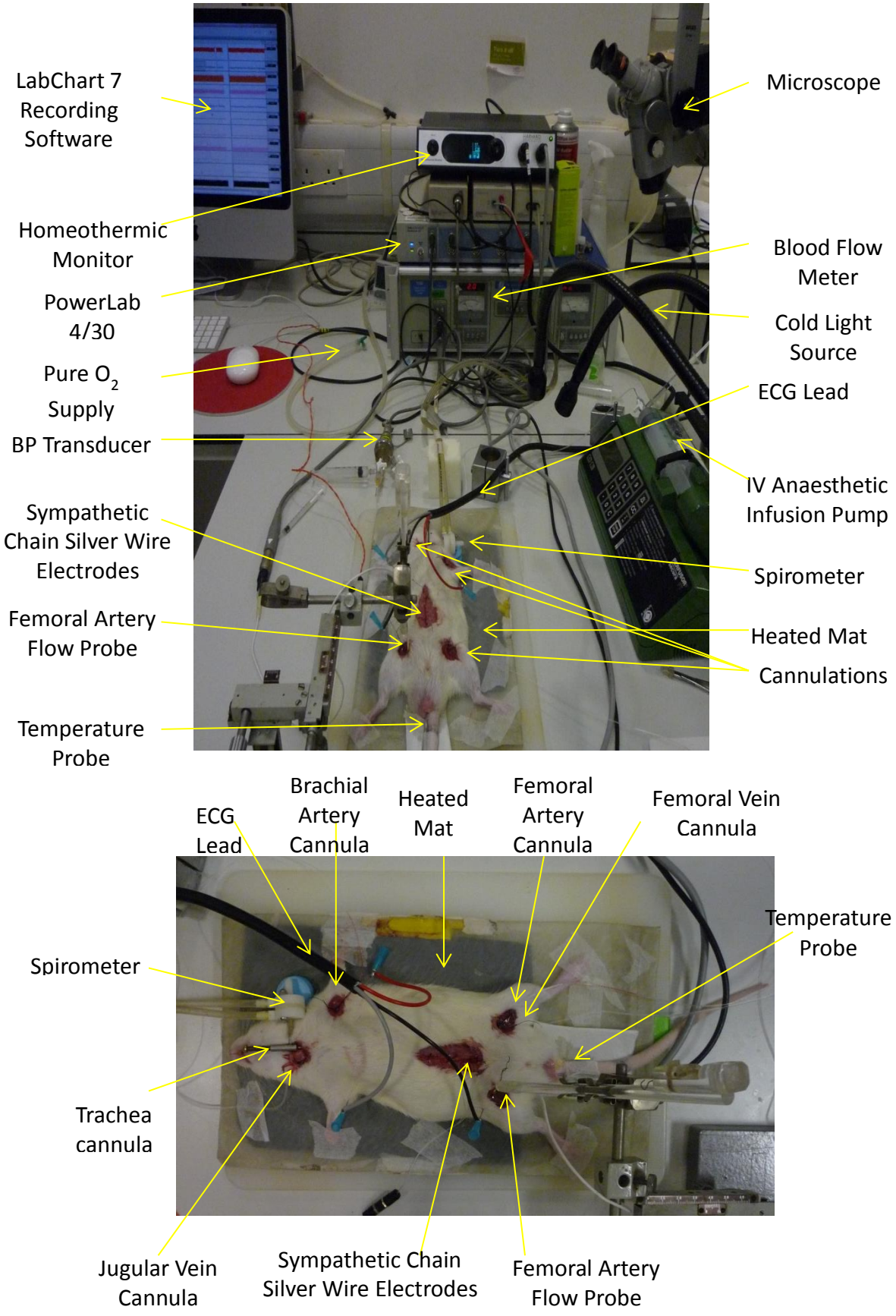

Figure 7: Experimental setup

capillary tubes. The samples were then centrifuged for 5 minutes at 10,000RPM to separate the plasma and red blood cells. The length of the packed cell volume was then measured and calculated as a proportion of the whole sample length.

Dejours Test

Dejours tests were performed. The Dejours test involved giving the animal 100% O_2 to breathe for 30 seconds, and was repeated five times with suitable time for recovery between repeats.

Sympathetic Stimulation

The vascular responses to sympathetic stimulation were evoked by stimulation of the sympathetic chain at two different frequencies, 20Hz and 2Hz. The stimulation patterns were chosen to compromise the same number of 0.1ms impulses, in a 30 second period. The protocol patterns were adapted from previous work by Coney and Marshall (2003). Both stimulation protocols consisted of 60 impulses, at a constant voltage of 2V. The 20Hz stimulation consisted of 3 times 20 impulse bursts spaced 10 seconds apart, with the 2Hz stimulation consisting of 1 set of 60 impulses.

At the end of the protocol animals were euthanased using euthatal, with death confirmed by cervical dislocation.

Insulin Quantification

Plasma samples taken during the GTT at 0, 2 and 30 minutes for control, and 0, 2 and 60 minutes for HF and HF+CIH groups were analysed for insulin. Insulin concentration was determined using Mercodia Rat Insulin ELISA (10-1250-01, Mercodia AB, Sweden). Absorbance values were read at 450nm with insulin concentration ($\mu g.L^{-1}$) determined from a calibration curve of known insulin concentrations.

Tissue Samples

Tissue samples were obtained from HF and HF+CIH animal groups. Normal tissue samples were obtained from a previous study, with animals fed standard rat chow. Tissues were removed, weighed and frozen using liquid nitrogen. Samples were then stored at -80^{o}C.

Skeletal muscle samples were obtained from animal groups, with the right leg tibialis anterior (TA), extensor digitorium long (EDL) and soleus muscles all removed, weighed, attached to corkboard using OCT and frozen in liquid nitrogen cooled 2-methylbutane (sigma).

The heart was removed, weighed and split into the right free wall, left free wall and septum which were weighed and frozen separately in the same way as above, as were sections of blood vessels from the aorta and right femoral artery.

The liver and kidney were also removed along with samples of the perirenal white adipose tissue (PWAT) and epididymal white adipose tissue (EWAT). Samples were weighed, wrapped in foil and frozen directly in liquid nitrogen. Lung and diaphragm tissue samples were also frozen in this way.

Analysis

Data was taken from LabChart traces and put into a spread sheet before being graphed and analysed. Statistical analysis was carried out using GraphPad Prism software. All values are given as mean ±SEM. During GTT and insulin quantification values that return to baseline before 60 minutes were assumed to continue at that level. Statistical significance was determined by use of Students t test and one way ANOVA with Bonferonni post-hoc analysis. Comparisons were considered significant at P values < 0.05.

RESULTS

Blood Pressure

Baseline mean arterial pressure (MABP) in the control group was 110±4mmHg. In both the HF and HF+CIH groups MABP was ~10mmHg higher (122±3 and 120±3mmHg respectively), however these increases were not significant (see fig.8B and table 2). There was no significant difference between diastolic blood pressure (fig.8D) between control and treatment groups however systolic blood pressure (fig.8C) was significantly higher in the HF group (control 136±4 and HF 153±4mmHg, $P<0.05$) but was not in the HF+CIH group (149±4mmHg).

MABP was relatively constant over the protocol with a comparison of MABPs at the start and end of the protocol showing no significant difference in control and HF+CIH groups. A significant decrease in MABP was found in the HF group (start 122±3 and end 117±3mmHg), however this is not a very large or physiologically relevant decrease.

Heart Rate

Baseline HR in control group was 427±13BPM (table 2). HR in the HF and HF+CIH groups was ~10 BPM higher (438±13 and 437±9BPM respectively), this increase is however not significant (see fig.9).

Haematocrit

Haematocrit value for control group was 0.46±0.01. Both HF and HF+CIH groups also had similar values (0.45±0.01 and 0.45±0.01); with no significant difference found between the groups (see fig.10).

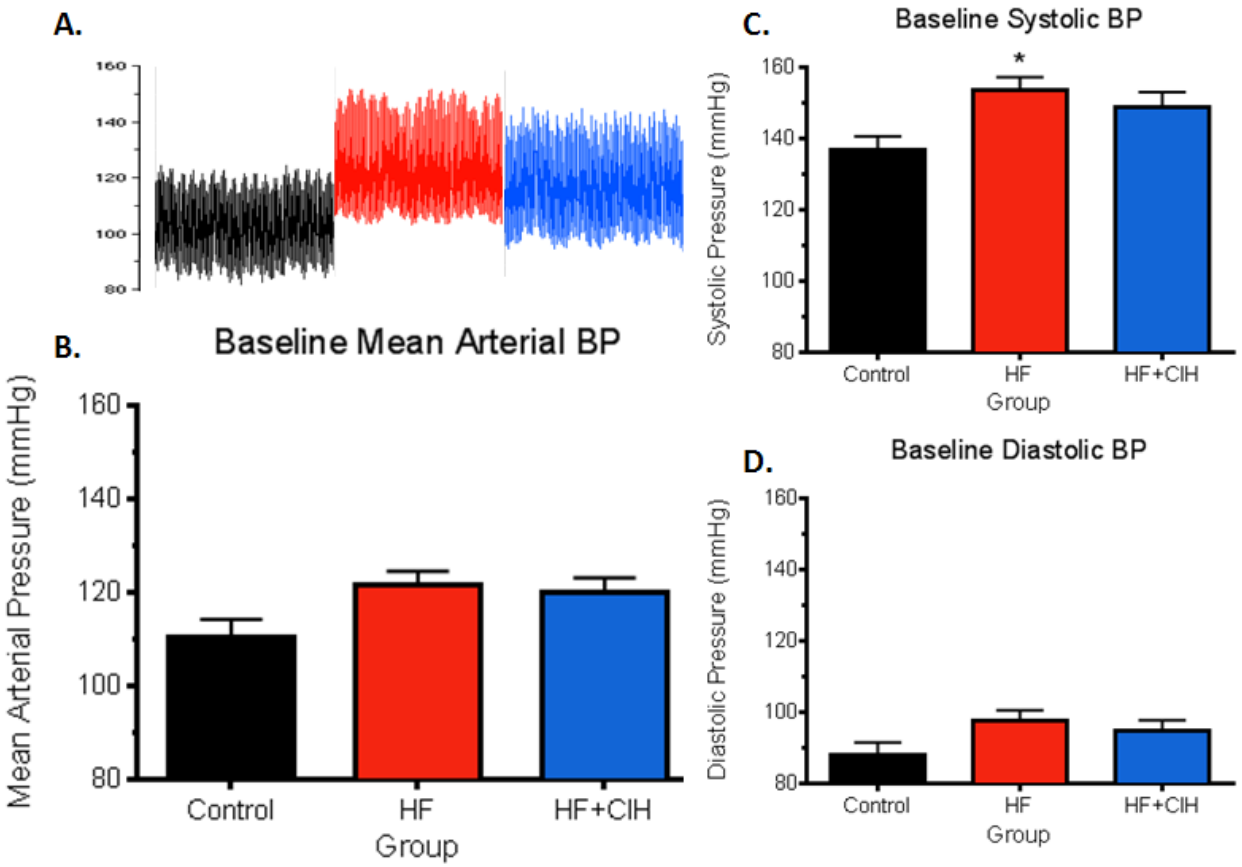

Figure 8: Effects of HF diet and CIH on baseline arterial blood pressures (mmHg) in control (n=9), HF (n=8) and HF+CIH (n=8) groups. A: Raw BP trace, B: Mean arterial blood pressure, C: Systolic blood pressure and D: Diastolic blood pressure. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison test; *$P < 0.05$ vs. control values.

Table 2. The effect of HF diet and CIH on baseline cardiorespiratory variables in control (n=9), HF (n=8) and HF+CIH groups (n=8).			
	Control	HF	HF+CIH
MABP (mmHg)	110±4	122 ± 3	120±3
SBP (mmHg)	137±4	153±4 *	149±4
DBP (mmHg)	88±4	97±3	95±3
HR (bpm)	427±13	438±13	437±9
R_f (BPM)	89±3	92±4	89±3
V_T (ml)	1.74±0.08	1.95±0.06	1.93±0.08
Minute Ventilation ($ml.min^{-1}$)	154±6	180±10 *	171±3
FBF ($ml.min^{-1}$)	2.3(1.4-3)	1.7(0.6-3.6)	3.4(2.2-5.8) ##
FVR ($mmHg.(ml.min^{-1})^{-1}$)	51.7(34.8-88.2)	92.1(33.4-231.7)	38.7(20.1-56.1) #
Values represent mean ± SEM or (range). One-way ANOVA with Bonferonni multicomparison tests,* *P* <0.05 vs. control and # *P*<0.05 and ## *P* <0.01 HF vs. HF+CIH.			

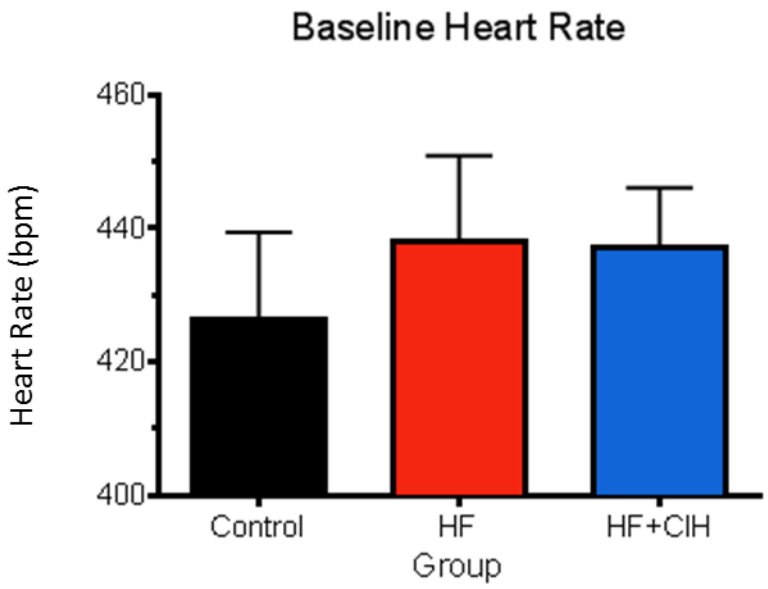

Figure 9: Effects of HF diet and CIH on baseline heart rate (bpm) in control (n=9), HF (n=8) and HF+CIH (n=8) groups. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests.

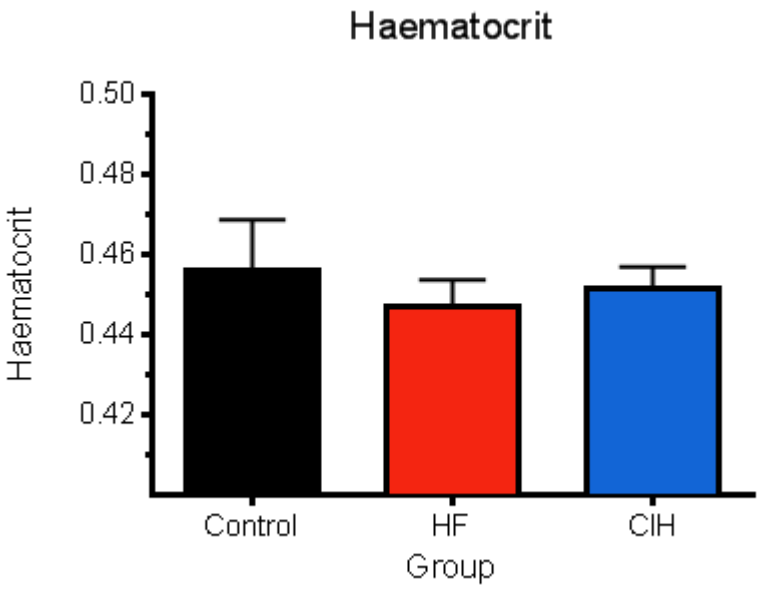

Figure 10 Effects of HF diet and CIH on haematocrit in control (n=9), HF (n=8) and HF+CIH (n=8) groups. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests.

Baseline Femoral Blood Flow and Femoral Vascular Resistance

Baseline FBF for control group was 2.3(1.4-3)ml.min^{-1} (table 2). HF group's FBF was decreased but not significantly (1.7(0.6-3.6)ml.min^{-1}), while HF+CIH groups FBF was increased (3.4(2.2-5.8)ml.min^{-1}) but not significantly compared to control group. HF+CIH group's FBF was significantly increased when compared to HF group.

Baseline FVR for control was 51.7(34.8-88.2)mmHg.(ml.min^{-1})$^{-1}$ (table 2). HF group's baseline FVR was increased (92.1(33.4-231.7)mmHg.(ml.min^{-1})$^{-1}$) but not significantly compared to control. Baseline FVR for the HF+CIH group was decreased (38.7(20.1-56.1)mmHg.(ml.min^{-1})$^{-1}$) but not significantly compared to control group, however it was significantly decreased compared to HF group.

Baseline Respiratory Parameters

Control group had a R_f of 89±3BPM (table 2), with HF and HF+CIH groups also having similar frequencies (92±4 and 89±3BPM respectively), no significant difference was found between groups (see fig.11A). V_T for control group was 1.74±0.08ml (table 2). The HF and HF+CIH groups V_T was ~0.2ml larger (1.95±0.06 and 1.93±0.08ml respectively), but this increase is not significant (see fig.11B).

Control groups minute ventilation was 154±6ml.min^{-1} (table 2). HF and HF+CIH both have increased minute ventilation (180±10 and 171±3ml.min^{-1} respectively). Interestingly the increase is significant for the HF (P<0.05), but not HF+CIH group (see fig.11C).

Normalised minute ventilation was 436±15ml.min^{-1}.kg^{-1} for control group (table 2). Both HF and HF+CIH groups had minute ventilation per kg ~25ml.min^{-1}.kg^{-1} lower (408±14 and 409±9ml.min^{-1}.kg^{-1}), this decrease is not however significant (see fig.11D).

Growth Curves and Body Mass

Five week old animals start weights for HF and HF+CIH groups were 222±2 and 222±4g respectively. The HF group showed a linear increase in weight over the 4 weeks HF diet (fig.12 A). The HF+CIH group's growth curve however shows a change in growth with exposure to CIH (fig.12B). The first 2 weeks on the diet the HF+CIH group's growth was similar to the HF group, but during the final 2 weeks, when exposed to CIH, growth was significantly reduced (P < 0.05). The result of this change in growth meant the HF group showed a larger mean increase in weight than the HF+CIH group during the final 2 weeks of the diet (see fig.12C).

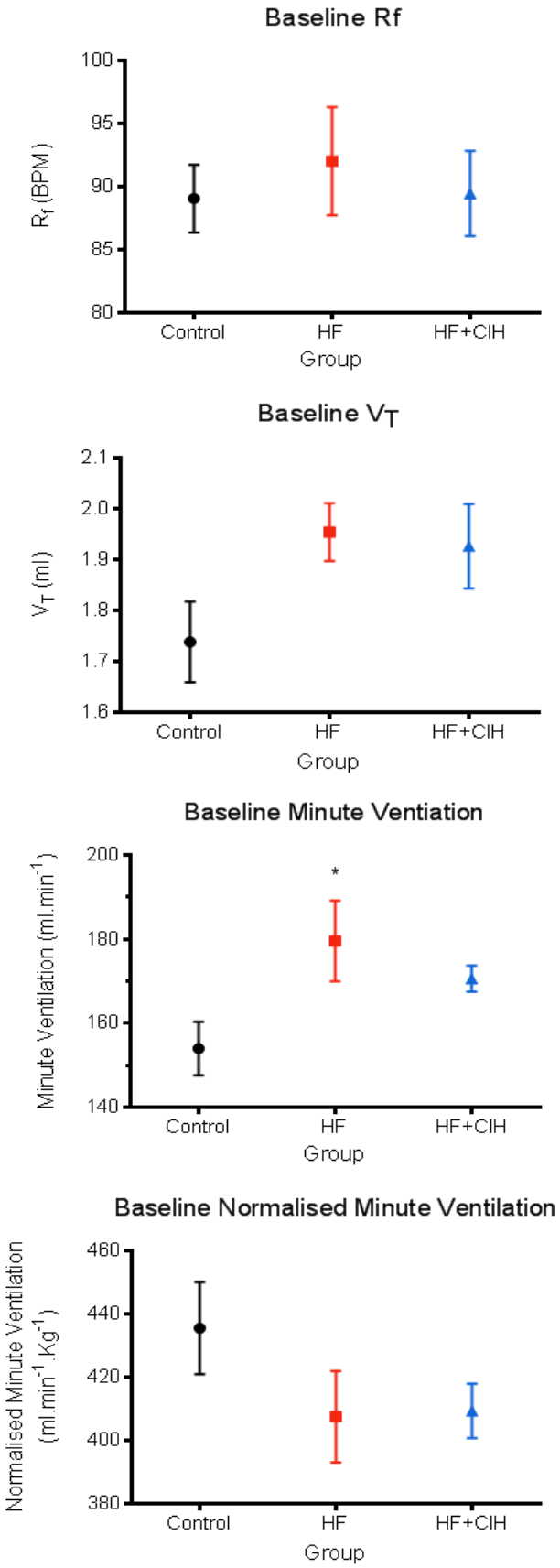

Figure 11: Baseline ventilation values for control (n=9), HF (n=8) and HF+CIH (n=8) groups. A: Baseline R_f (BPM), B: Baseline V_T (ml), C: Baseline minute ventilation (ml.min^{-1}) and D: Baseline normalised minute ventilation (ml.min^{-1}.Kg^{-1}). Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests. *P < 0.05 vs. control.

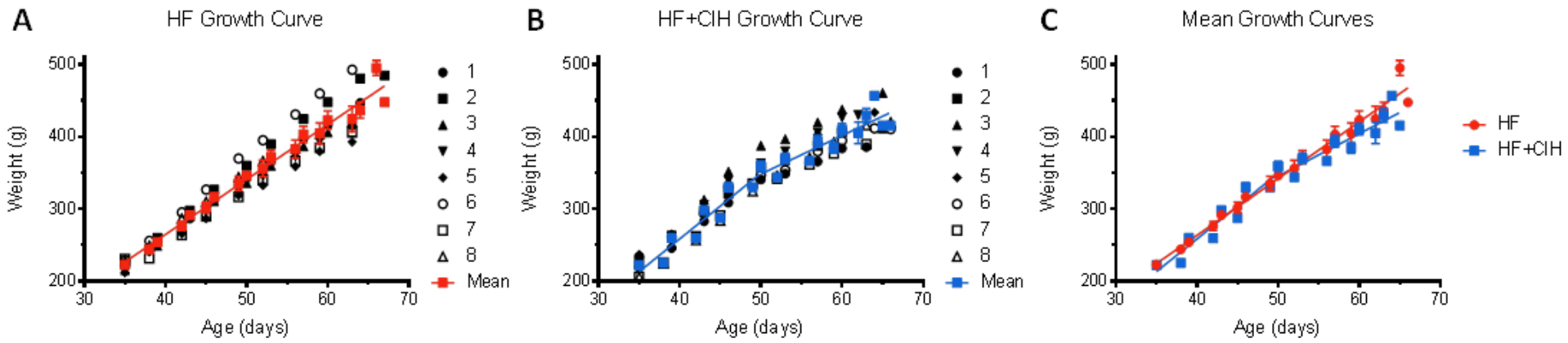

Figure 12: Growth curves (weight, g) from weeks 5-9 for HF (n=8) and HF+CIH (n=8) groups. A: HF growth curve, B: HF+CIH growth curve and C: Mean growth curves for HF and HF+CIH groups. Values represent individual rat weights with mean ± SEM.

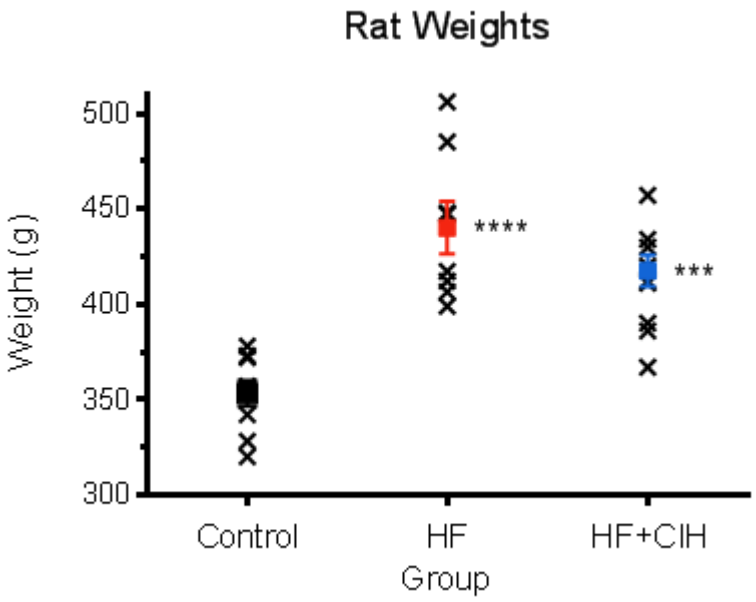

Figure 13: Final rat weights (g) for control (n=9), HF (n=8) and HF+CIH (n=8) groups. Individual rat weights with mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests; ***$P < 0.001$, and ****$P < 0.001$ vs. control.

Control group animals had a final weight of 353±7g. The HF and HF+CIH group animals' final weights were significantly heavier than control (440±14 and 418±8g respectively, see fig.13). No significance was found between the HF and HF+CIH group weights.

Food Consumption

Food consumption was monitored throughout the HF diet and while animals were in the hypoxic chamber. Rats in the HF group consumed a total of 1887±61g per cage and the HF+CIH group 1769±38g per cage. HF groups mean food consumption over the 4 weeks HF diet was 22.5±0.7g.day^{-1} (see fig 14). HF+CIH group's food intake was split into the first 2 weeks HF diet only, and final 2 weeks HF diet with CIH exposure. The HF+CIH group's food consumption over the first 2 weeks of the diet was 21.8±0.2g.day^{-1}. During CIH exposure HF+CIH group's food consumption decreased, but not significantly, compared to the first 2 weeks of the diet.

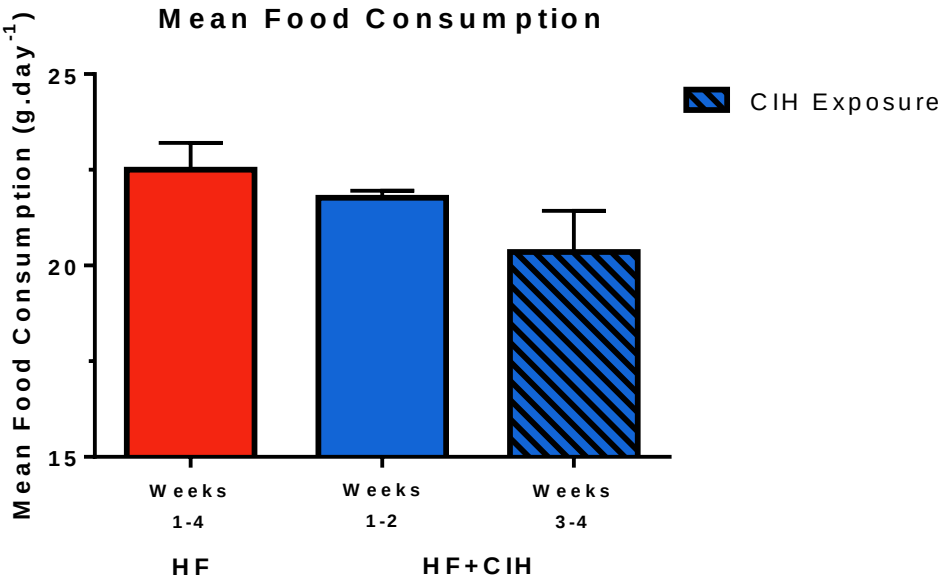

Figure 14: Mean food consumption (g.day^{-1}) during high fat diet and CIH exposure for HF (n=8) and HF+CIH (n=8) groups. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests.

Tissue Weights

All tissues were weighed and normalised per g body weight (table 3). Tissue weights for normal animals were obtained from a previous experimental protocol. The normalised whole heart weight in the normal group was 2.67±0.11mg.g^{-1}. HF and HF+CIH groups normalised whole hearts weights were heavier (3.02±0.06 and 2.78±0.34 mg.g^{-1} respectively), though the differences were not significant (see fig.15A).

Normalised right free wall (RFW) and left free wall (LFW) weights in the normal group were 0.33±0.07 and 1.11±0.17mg.g^{-1} respectively (see fig.15B). The RFW in HF and HF+CIH groups were significantly heavier (0.52±0.02 and 0.57±0.02mg.g^{-1} respectively). Similarly the LFW was also significantly heavier in treatment groups than normal (1.58±0.03 and 1.50±0.06 mg.g^{-1} respectively). No data was available for septum weight in the normal group. Normalised septum weight in the HF group was 0.45±0.03mg.g^{-1} (see fig.15B). A

Table 3. Effect of diet and CIH on normalised tissue weight (mg.g^{-1}) in normal (n=6), HF (n=8) and HF+CIH groups (n=8).			
	Normal	HF	HF+CIH
Whole Heart	2.67±0.10	3.02±0.06	2.78±0.38
LFW	1.11±0.17	1.58±0.03 **	1.50±0.06 *
RFW	0.33±0.72	0.52±0.02 *	0.57±0.02 **
Septum	N/A	0.45±0.03	0.53±0.03
Liver	34.38±0.96	38.83±0.89 *	38.71±1.16 *
PWAT	13.94±2.30	23.38±1.35 **	22.93±1.87 **
EWAT	12.64±0.55	24.41±1.59 ****	20.97±0.82 ***
Kidney	3.10±0.60	3.77±0.12	4.12±0.24
EDL	0.41±0.02	0.40±0.02	0.41±0.02
TA	1.81±0.04	1.62±0.05 *	1.73±0.03
Soleus	0.46±0.02	0.41±0.01	0.39±0.02
Values represent mean ± SEM. One-way ANOVA with Bonferonni multicomparison tests,* *P* <0.05, ** *P* <0.01, *** *P* <0.001 and **** *P* <0.0001 vs. control. Normal rat tissue weights were obtained from a previous study.			

larger septum weight was observed in the HF+CIH group (0.53±0.03 mg.g^{-1}), although no significant difference between groups was found.

Normal normalised liver weight was 34.38±0.96mg.g^{-1} (see fig.15C). HF and HF+CIH groups had significantly increased liver tissue weights (38.83±0.89 and 38.71±1.16 mg.g^{-1} respectively). PWAT and EWAT normalised tissue weight in the normal group was 13.94±2.30 and 12.64±0.55mg.g^{-1} respectively (see fig.15C). Normalised PWAT weight for treated groups were ~ 10mg.g^{-1} heavier (23.38±1.35 and 22.93±1.87mg.g^{-1} respectively) and were significantly different to normal. Likewise treated groups normalised EWAT weight was significantly larger than normal (24.42±1.59 and 20.97±2.33 mg.g^{-1} respectively). No significant differences were found between HF and HF+CIH treated

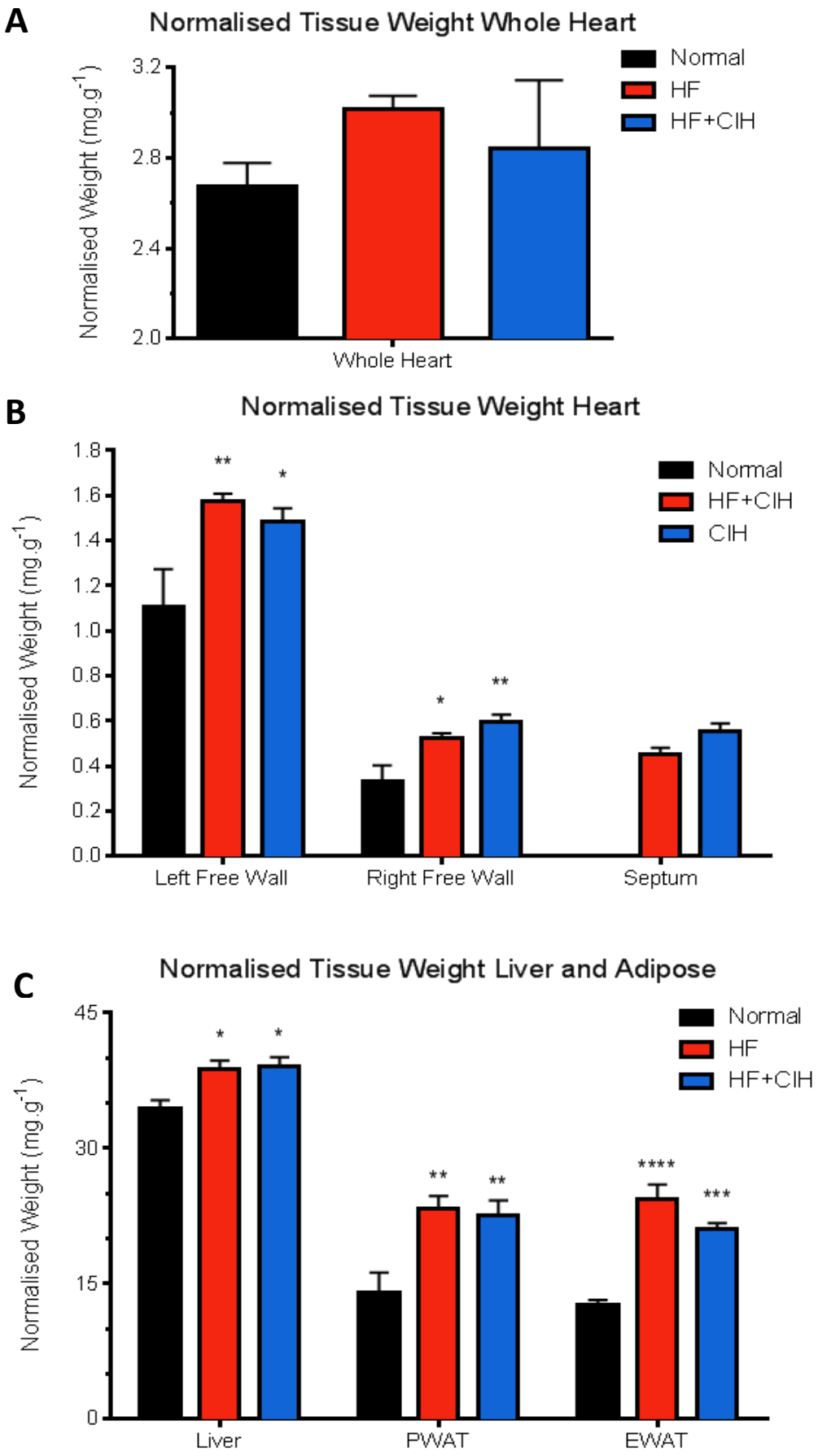

Figure 15: Effect of HF diet and CIH on tissue weight in normal (n=6), HF (n=8) and HF+CIH (n=8) groups. Tissue weights normalised for body weight (mg.g^{-1}). A: Whole heart weight, B: Heart weights (normal group septum weights were not available and HF group only includes 7) and C: Liver and adipose tissue weights. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. control. Normal rat tissue weights were obtained from a previous study.

groups. Normal groups normalised kidney weight was 3.1±0.60mg.g^{-1}. HF and HF+CIH treatment groups were 3.77±0.12 and 4.12±0.24mg.g^{-1} respectively. No significant difference was found between groups.

Normalised EDL weight was 0.41±0.02mg.g^{-1} for normal group with similar weights observed in HF and HF+CIH groups (0.40±0.02 and 0.41±0.02mg.g^{-1} respectively). Normal group's normalised TA weight was 1.81±0.04mg.g^{-1}. Both HF and HF+CIH treatment groups' normalised TA weights were lower than the normal group's (1.62±0.05mg.g^{-1} and 1.73±0.03mg.g^{-1} respectively), although only the HF group was significantly different. Normalised Soleus weight was 0.46±0.02mg.g^{-1} in the normal group, 0.41±0.01mg.g^{-1} in the HF group and 0.39±0.02mg.g^{-1} in the HF+CIH group. No significant difference was found between groups.

Glucose Tolerance Test

Baseline blood glucose in the control group was 7.9±0.5mMol.L^{-1}. In treatment groups the baseline glucose was lower but not significantly in the HF group (6.8±0.3mMol.L^{-1}) and significantly lower ($P < 0.05$) in the HF+CIH groups (6.5±0.3 mMol.L^{-1}).

All groups had the same size increase in blood glucose at 2 minutes. The blood glucose levels were returned back to baseline levels within 30 minutes of the glucose load in the control group (see fig.16A). The HF and HF+CIH groups both had prolonged elevated glucose, and were still above baseline levels 60 minutes after being given the glucose load.

The total area under the curve (AUC) of the control groups GTT was 140±16. The two treatment groups HF and HF+CIH both had much larger AUCs (183±30 and 206±16 respectively); however no significance was found between groups (see fig.16B).

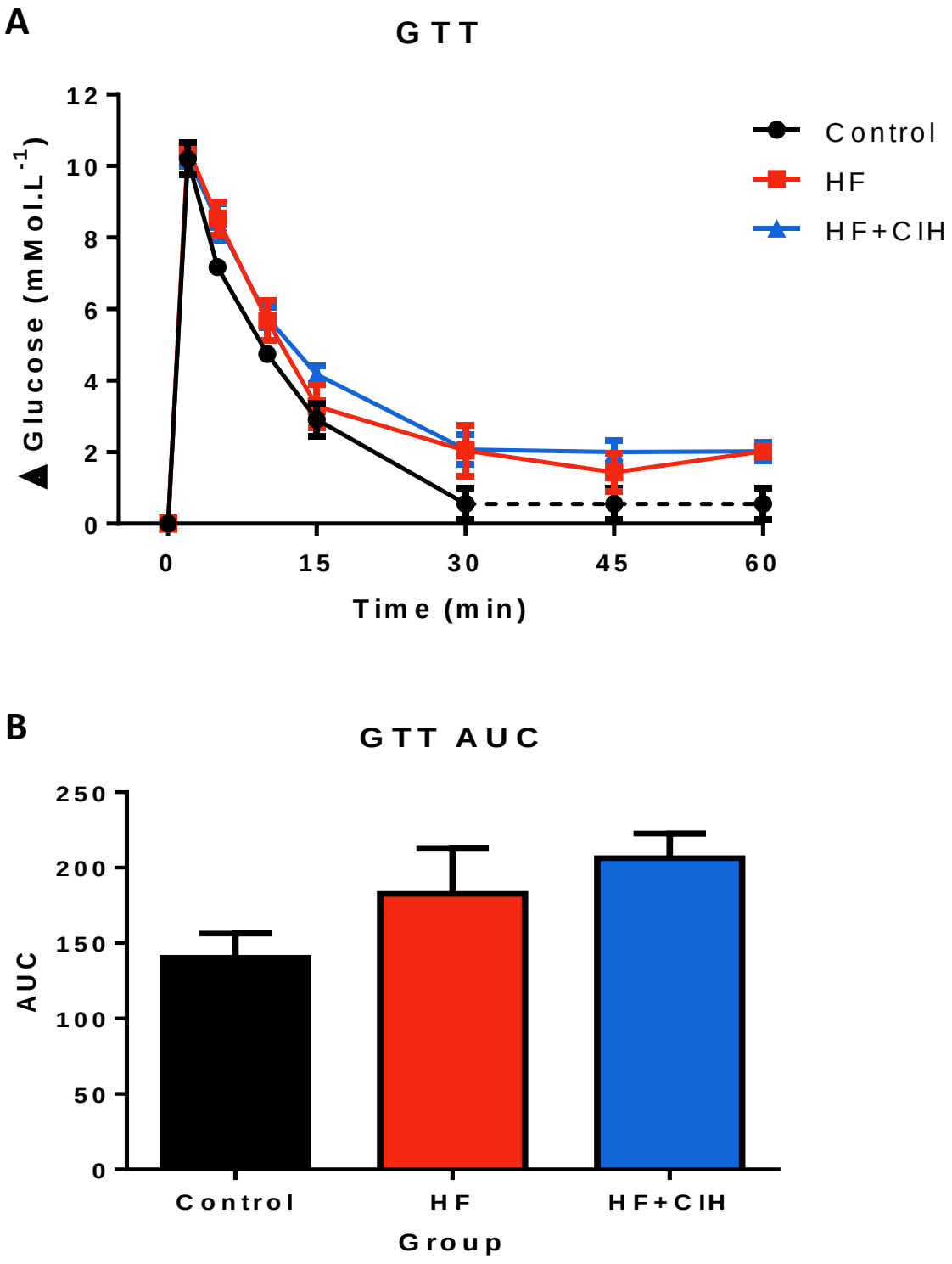

Figure 16: Effect of an intravenous glucose load (0.5g.Kg^{-1}) on blood glucose levels in control (n=7), HF (n=6) and HF+CIH (n=8) groups. A: GTT change in blood glucose from baseline (mMol.L^{-1}). B: GTT area under the curve. Points represent mean ±SEM One-way ANOVA with Bonferroni multicomparison tests.

Insulin Quantification

Baseline insulin concentration for control group was 1.2±0.1µg.L^{-1}. HF and HF+CIH groups both had much higher baseline levels (4.8±0.8 and 2.6±0.6µg.L^{-1} respectively), however only the HF group was significant (see fig.18A). The peak insulin level for control was 7.58±0.41, both HF and HF+CIH groups had increased values (8.95±0.07 and 8.23±0.37 µg.L^{-1} respectively), however only the HF group was significant (see fig.18B). Control group's insulin returned to baseline level within 30 minutes (see fig.17 and fig.18C), conversely HF and HF+CIH groups insulin was still elevated after 60 minutes. The AUC for control groups was 176±5 (see fig.18D); significantly larger AUCs were found in the HF and HF+CIH groups (437±24 and 360±20 respectively).

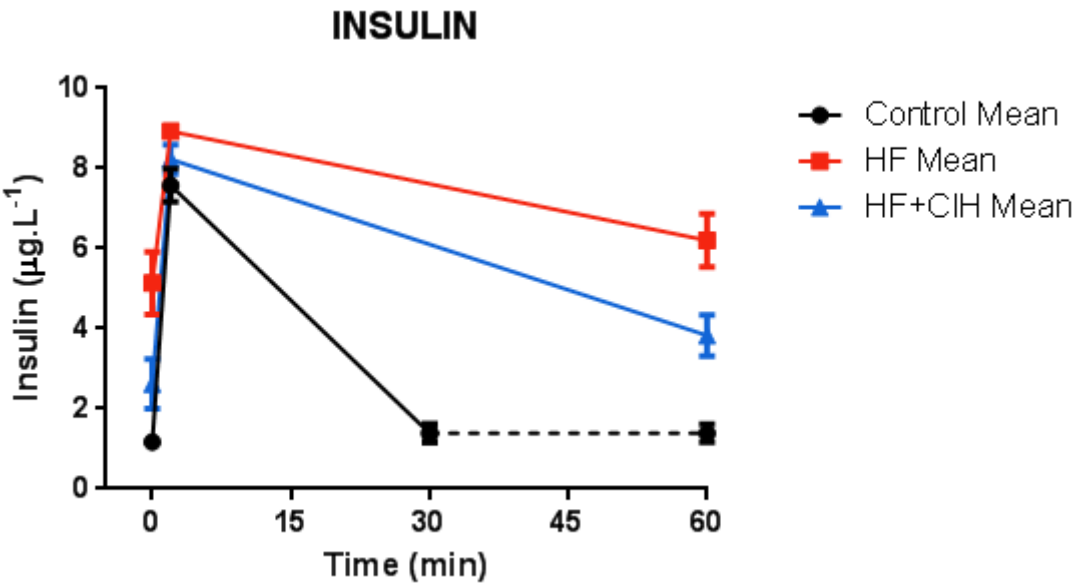

Figure 17: Effect of an intravenous glucose load (0.5g.kg^{-1}) on serum insulin levels in control (n=4), HF (n=5) and HF+CIH groups (n=3). Points represent mean ± SEM.

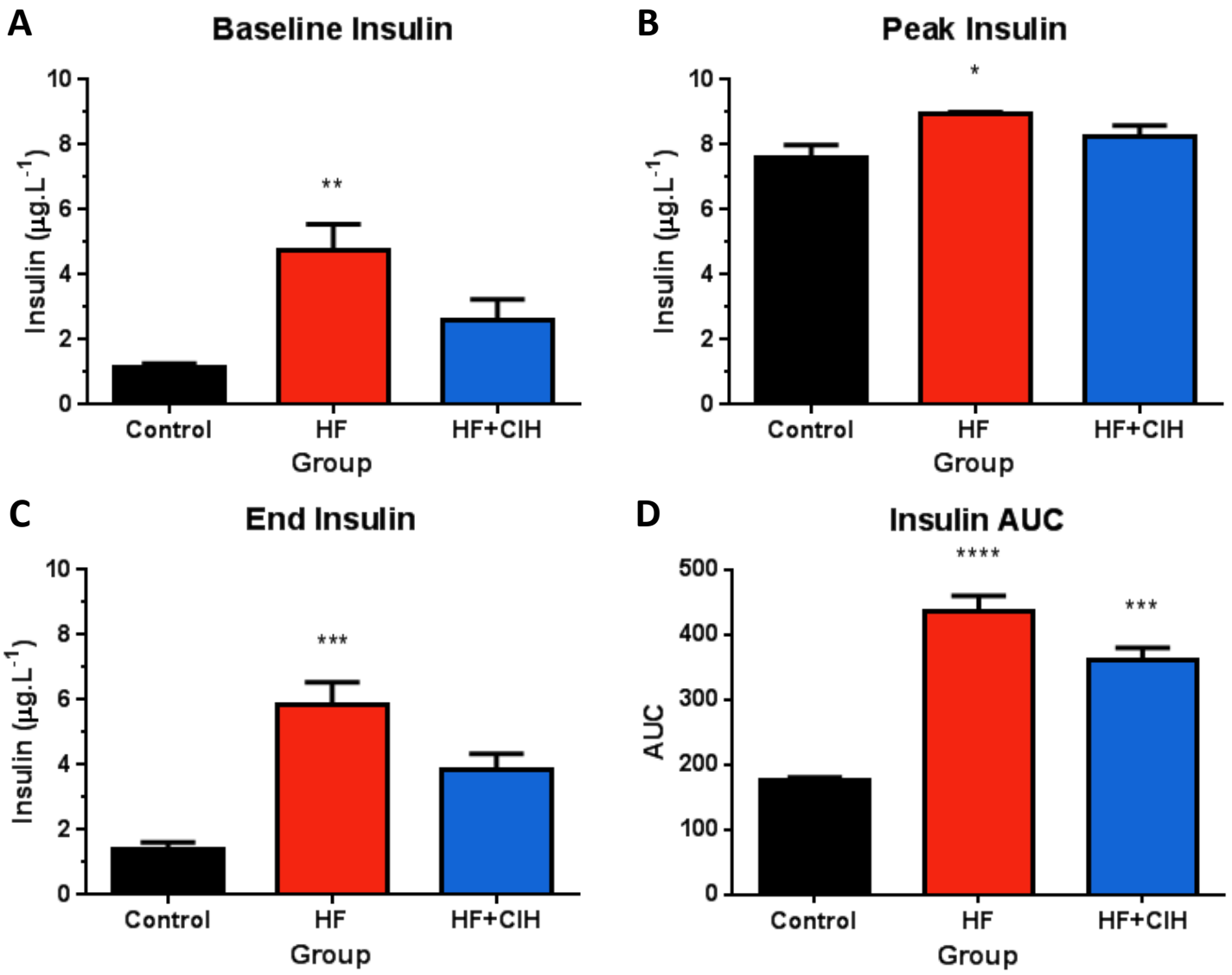

Figure 18: Effect of GTT on serum insulin levels for control (n=4), HF (n=5) and HF+CIH (n=3). A: Baseline insulin (µg.L^{-1}), B: Peak insulin (µg.L^{-1}), C: End insulin (µg.L^{-1}) and D: Insulin AUC. Bars represent mean±SEM. One-way ANOVA with Bonferroni multicomparison tests; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs control.

GTT Respiratory Parameters

Control groups baseline R_f was 91±6BPM. HF and HF+CIH both had similar baseline R_f 93±5 and 89±3BPM respectively. All groups R_f was increased at 2 minutes, although only the HF+CIH group was significant. Control and HF+CIH groups R_f returned back to baseline by 30 minutes, however the HF groups R_f remained elevated (see fig.19A).

Baseline V_T in the control group was 1.82±0.06ml and did not change throughout the GTT. HF and HF+CIH groups' baseline V_T were 2.00±0.08 and 1.96±0.08ml respectively, with both groups V_T increasing over the test. The HF group was significantly increased at 60 minutes and the HF+CIH group was significantly at 30 and 60 minutes (see fig.19B).

Baseline minute ventilation in control group was 165±10ml.min^{-1}. HF and HF+CIH both had slightly higher baseline minute ventilations 185±11 and 173±3 ml.min^{-1} respectively. All groups minute ventilation was increased at 2 minutes but only the HF+CIH groups increase was significant (see fig.19C). Control groups minute ventilation then returned to baseline, whereas the treatment groups remained elevated with both significantly increased at 60 minutes (see fig.19C).

Dejours Test Respiratory Parameters

The control groups R_f decreased significantly from baseline during the Dejours test. The HF and HF+CIH groups also decreased significantly from their baselines during the test, with all three groups decrease ~20BPM in magnitude (see fig.21A). No significant difference was found between groups R_f at baseline (see fig.20A).

V_T was not altered from baseline during the Dejours test for the control group. The HF and HF+CIH groups V_T also did not appear to be affected by application of the Dejours test. No significant difference was found between individual groups' baseline and Dejours V_T

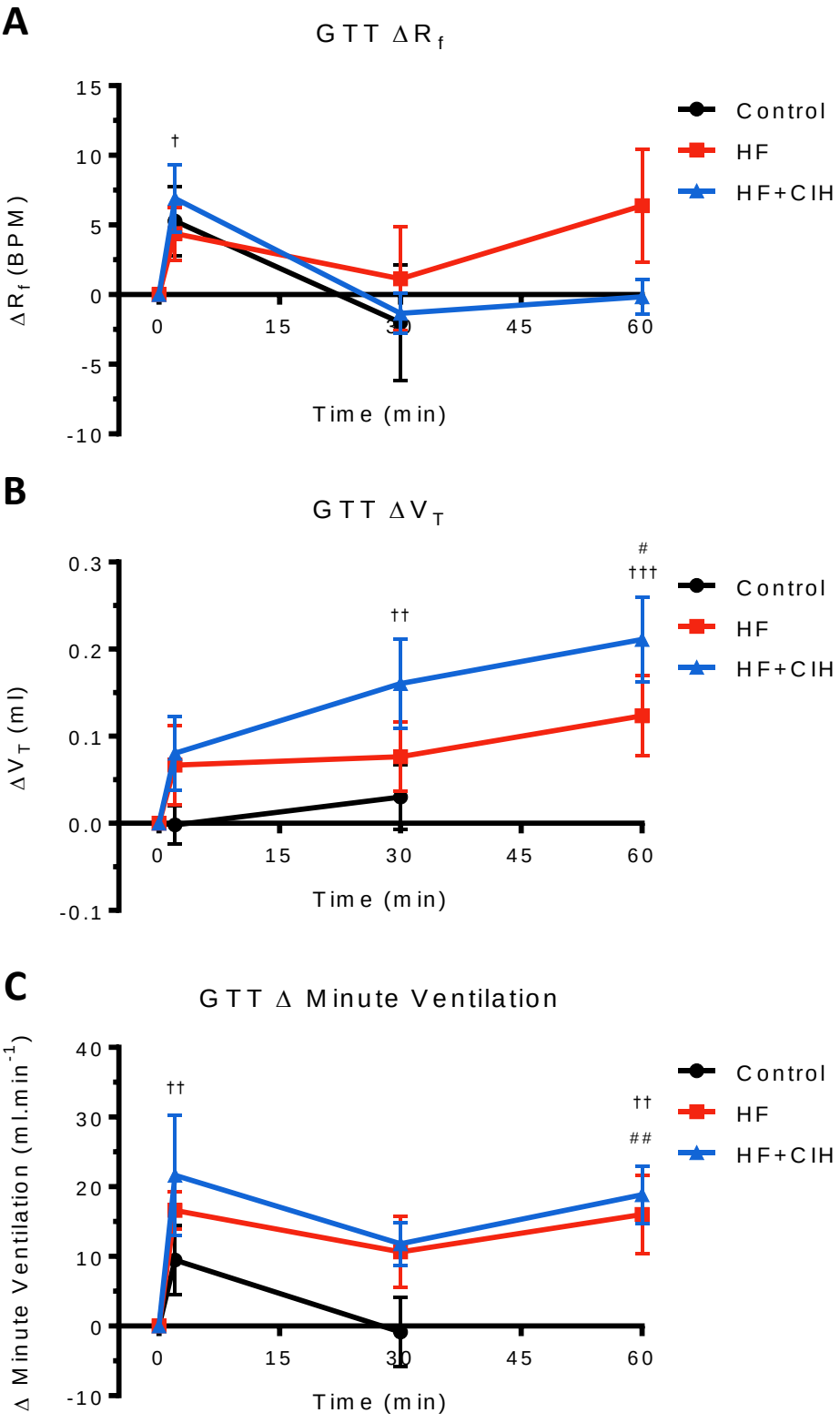

Figure 19: Effect of intravenous glucose load (0.5g.Kg^{-1}) on ventilation in control (n=7), HF (n=6) and HF+CIH (n=8) groups. A: ΔR_f (BPM), B: ΔV_T (ml) and C: Δ minute ventilation (ml.min^{-1}). Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests; # P < 0.05, ## P < 0.01 HF vs HF baseline, † *P* < 0.05, †† *P* < 0.01 and ††† *P* < 0.001 HF+CIH vs HF+CIH baseline.

values, there was however a significant difference between the control and HF+CIH groups baseline V_T (see fig.20B).

The control group's minute ventilation at baseline was lower than the HF and HF+CIH group's minute ventilation. However only the HF+CIH groups baseline minute ventilation was significantly increased (see fig.20C). The control group's minute ventilation significantly decreased with application of the Dejours test. Significant decreases were also observed in the treatment groups during test application (see fig.21B). The decrease in minute ventilation in the control group was 36±5ml.min^{-1}. HF and HF+CIH both had larger decreases in minute ventilation (42±5 and 49±3ml.min^{-1} respectively), however neither decrease was significant compared to control (see fig.21B).

Normalised minute ventilation decreased significantly with application of the Dejours test in all groups ($P < 0.0001$, $P < 0.001$ and $P < 0.0001$ respectively). No significant difference was found between groups' baseline normalised minute ventilations (see fig.20D).

Sympathetic Chain Stimulation

Sympathetic chain stimulation at 20Hz produced an increase in control group FVR of 30±4 mmHg.(ml.min^{-1})$^{-1}$. The increase in HF and HF+CIH groups FVR during 20Hz stimulation was slightly less (25±7 and 27±7mmHg.(ml.min^{-1})$^{-1}$respectively), with no significance found between groups (see fig.22A).

2Hz stimulation caused a much smaller increase in FVR with control group only increasing by 5.6±0.9 mmHg.ml^{-1}.min. A slightly smaller increase was also seen in the HF group (3.7±1.7 mmHg.(ml.min^{-1})$^{-1}$). The increase produced in the HF+CIH group however is twice

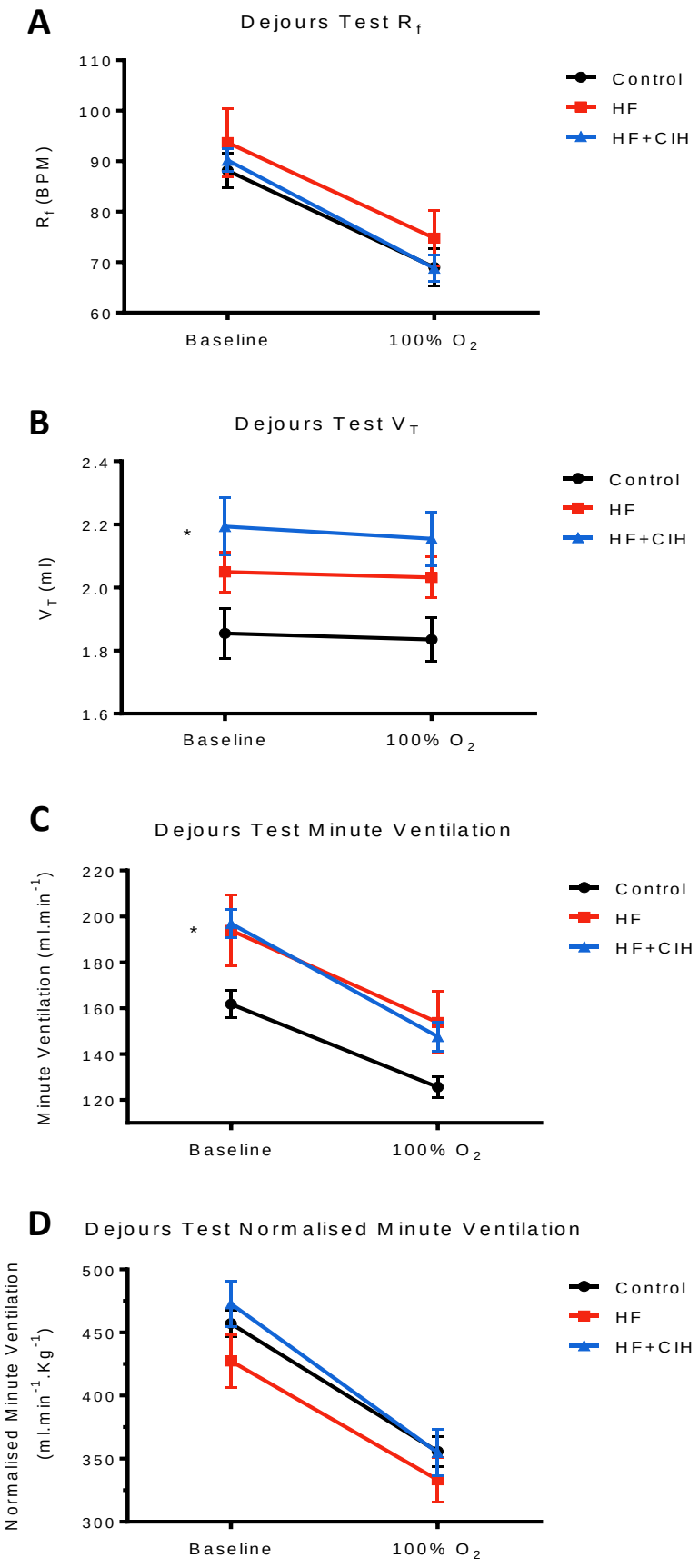

Figure 20: Effects of the Dejours test (100% O2) on ventilation in control (n=9), HF (n=8) and HF+CIH (n=8) groups. A: R_f (BPM), B: V_T (ml), C: Minute Ventilation (ml.min^{-1}) and D: Normalised minute ventilation (ml.min^{-1}.Kg^{-1}). Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests compare baseline values; *P < 0.05 vs control.

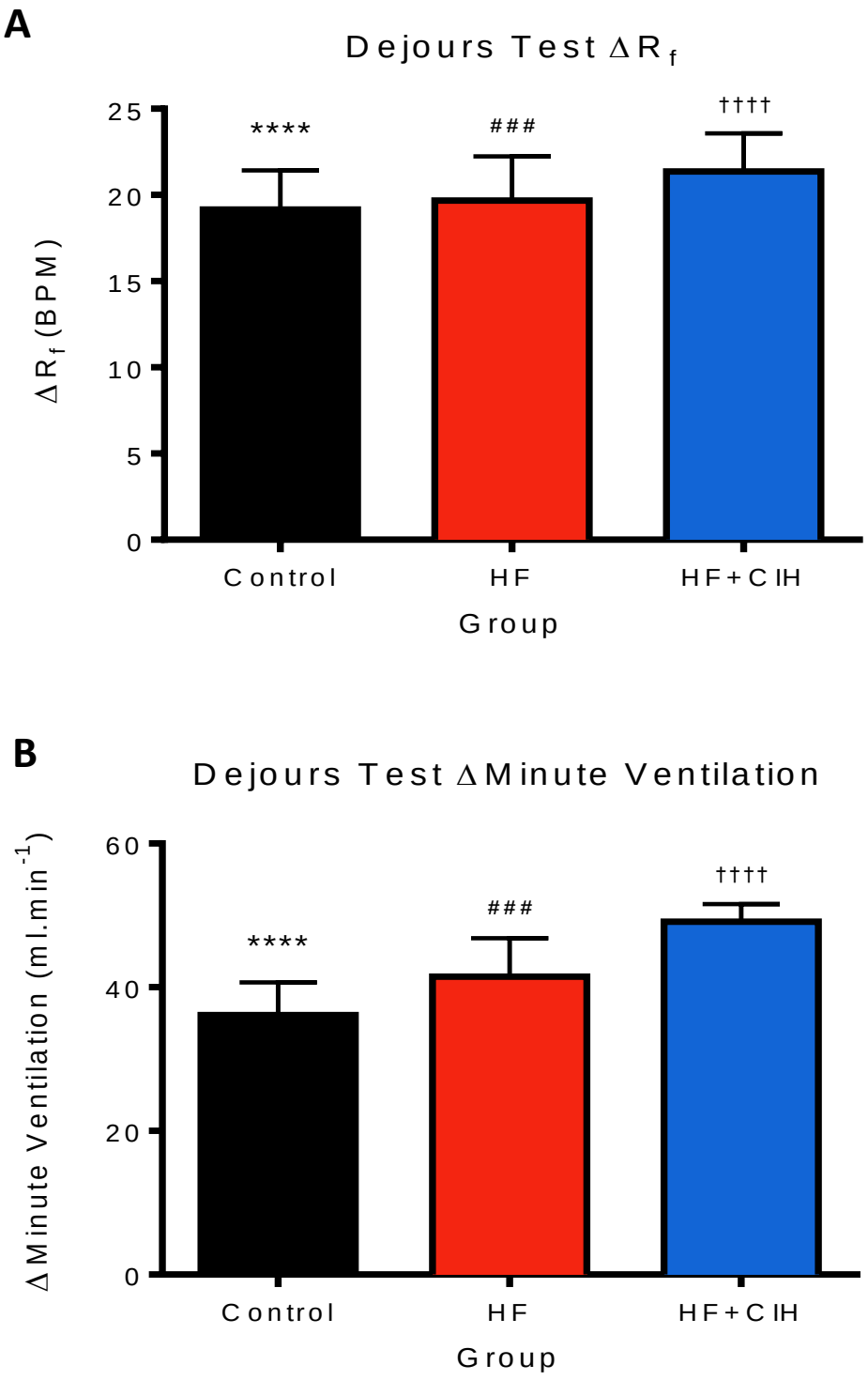

Figure 21: Effects of the Dejours test (100% O_2) on ventilation in control (n=9), HF (n=8) and HF+CIH (n=8) groups. A: Change in R_f from baseline (BPM) and B: Change in minute ventilation from baseline ($ml.min^{-1}$). Bars represent mean ± SEM. Students t test between baseline and 100% O_2 values; ***P < 0.001, ****P < 0.0001 control, ###P < 0.001 HF and ††††P < 0.0001 HF+CIH groups. One-way ANOVA with Bonferroni multicomparison tests.

as large (10.5±2.1 mmHg.(ml.min^{-1})$^{-1}$) but was only significantly different to the HF group (see fig.22B).

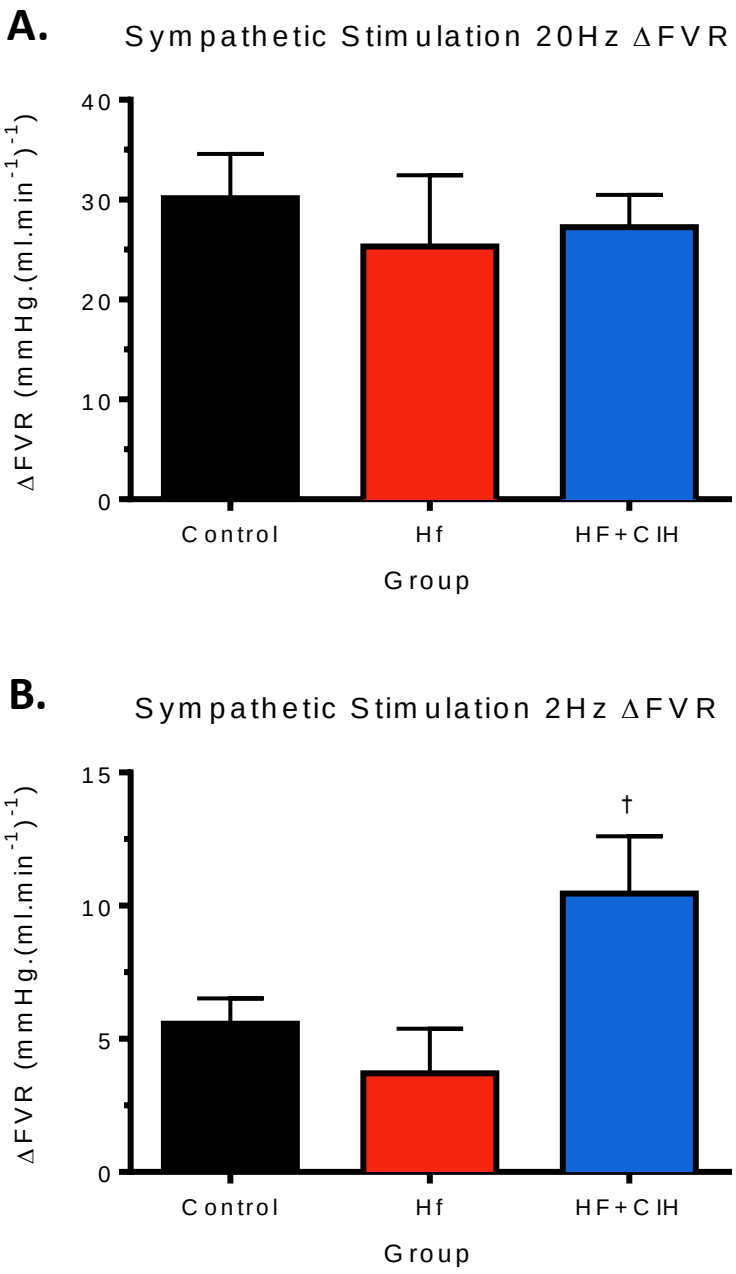

Figure 22: Effects of sympathetic chain stimulation on femoral vascular resistance (mmHg.(ml.min^{-1})$^{-1}$)in control (n=5), HF (n=4) and HF+CIH (n=5) groups: A. Sympathetic Stimulation 20Hz; B. Sympathetic Stimulation 2Hz. Bars represent mean ± SEM. One-way ANOVA with Bonferroni multicomparison tests; † $P < 0.05$ HF vs. HF+CIH.

DISCUSSION

Overview

We aimed to examine the combined effects of HF diet and CIH on cardiorespiratory parameters, glucoregulation, CB activity and sympathetic tone. Our findings show HF diet generates obesity as increases in body weight; PWAT and EWAT were observed, with CIH tending to reduce weight gain on HF diet. HF diet also caused a significant increase in SBP which contributed to the tendency for MABP to be increased by 12±3mmHg. The addition of CIH resulted in MABP tending to be increased by 10±3mmHg, with SBP not significantly increased. IR and ventricular hypertrophy were both caused by HF diet, with CIH not affecting IR or hypertrophy. Findings present no evidence of increased CB activity or sympathetic tone which do not appear to be altered by HF diet or CIH.

Blood Pressure and Heart Rate

As both HF diet and CIH have been reported to cause HT we hypothesised that HF diet and CIH would result in a more severe HT than HF diet alone. Baseline MABP showed a tendency to be increased in treatment groups although not significantly. SBP and DBP also showed a tendency to be increased in treatment groups; with the HF groups SBP significantly higher.

Comparing these finding to the literature, showed that other studies which used the HF diet model reported MABP significantly greater than their controls. Ribeiro et al (2013) reported MABPs of 96±3mmHg in control and 142±2mmHg in animals fed a high fat diet. These values differ considerably from the findings of this study. Ribeiro et al (2013) attribute the HF diet induced HT to increased CB activity as CSN resection prevented arterial pressure increase. They dismissed any baroreceptor mediated effect, which also

transmits information via the CSN, stating rats would have become hypertensive after CSN denervation (Ribeiro et al 2013).

Firstly Ribeiro's control groups MABP is much lower than the 110±4mmHg found in this study. A possible explanation of this may be the difference in anaesthesia used in the studies. Most anaesthetics cause a reduction of BP in rats, with the haemodynamic changes dependent on the type of anaesthetic used (Bencze et al 2013). Ribeiro used intraperitoneal injection of sodium pentobarbital whereas IV Alfaxan (alfaxalone) was used in this study. Pentobarbital anaesthesia is known to lower CO and decrease systemic vascular resistance resulting in BP decrease (Bencze et al 2013). Alfaxan has been shown to cause minimal changes in cardiovascular variables, with no change in CO and only a small decrease in systemic vascular resistance resulting in minor decreases in BP (Muir et al 2009).

Secondly the difference observed between MABP in HF diet fed rats, may be down to a number of reasons. Time spent on the diet may affect MABP. Ribeiro fed rats HF diet for 21 days, which was less than the 4 weeks diet used in this study. As HF diet is a model of HT, it would be expected the longer spent on the diet to result in a greater increase in MABP, although this was not the case. Therefore a possible explanation is the actual composition of the HF diet itself. Ribeiro states the HF diet used was 45% fat, 35% carbohydrate and 20% protein, however it was not clear if this was the diet composition or where animal's calories are derived from. The diet used in this study contained 21% fat by composition but 42% of calories were derived from fat. This potentially considerable difference in dietary fat content could be an explanation of why MABP was so much higher in Ribeiro's study even though they spend less time on the diet. Rat's age is also a possible explanation for

differences in MABP. The rats in our study were 9 weeks old when experiments were performed. In Ribeiro's study rats were 3 months old, 3 weeks older than this study. Older rats may be more susceptible to HF diet induced HT than younger rats.

An explanation for the increased SBP could be increased sympathetic activity to the heart. The sympathetic NS innervates the heart at the SA and AV nodes as well as innervating throughout the myocardium. Increased sympathetic activity to the ventricular myocardium would increase their contractility and thereby explain the observed increases in SBP.

In agreement with this was the observed tendency for HR to be increased, although not significantly, in treatment groups. As already stated the sympathetic NS acts at the SA node, the hearts pacemaker. An increase in sympathetic activity results in an increased rate of SA node discharge, increasing HR.

The tendency for DBP to be increased could also be explained by increased sympathetic activity. DBP is increased with a sympathetic mediated increase in HR, as well as any increase in sympathetic NA causing increased tonic constriction of blood vessels (Fisher and Paton 2012).

Although these results did not indicate HF diet induced HT, they do appear to suggest animals BP was moving in that direction, as they showed a tendency to be increased. It is possible that increasing the length of time on the HF diet or increasing the diet's fat content further may result in animals becoming hypertensive.

The findings did not support what was initially proposed. The addition of CIH on top of HF diet did not result in a larger BP increase. If anything the addition of CIH had a tendency to lessen the BP increase, however difference between the HF and HF+CIH group were not

significant. The absence of a CIH only group means determining the effect of this CIH protocol on BP is difficult. Fletcher et al (1992a) reported elevated BP from CIH alone, accrediting the elevation to increased CB activity as the elevation was prevented by CSN resection. In a further experiment by Fletcher (1992b), he showed that the peripheral sympathetic NS is necessary for the persistent BP increase associated with CIH.

As both HF and CIH appear to elevate BP by activating the CB, It poses the question why the effects were not additive. It is possible that as the CB was already activated by HF diet the addition of the CIH on top did not result in any greater stimulation, therefore did not cause an additional increase in BP.

Another potential explanation of why treatment group's BP lacks significance could be due to the study possibly being underpowered and failing to detect an effect that actually existed. The time constraints of an 18 week project meant it was not possible to get equal numbers of animals in each group therefore control (9 animals) has more than the treatment groups (8 animals each). As increasing the sample size is the easiest way to boost statistical power. If more time was available increasing these treatment group numbers could possibly result in the data becoming statistically significant as BPs are already close to being significantly increased.

Heart Tissue Weights

Animal hearts were removed and weighed (including atria), before being separated into the LFW, RFW and septum. Whole heart weights were not significantly altered in treatment groups although there was a tendency to be increased, with the effect more pronounced in the HF group.

Although whole heart weights were not significantly increased, treatment groups LFW and RFW weights were, suggesting hypertrophy of the ventricles. These findings agree with what was expected and are associated to the tendency of BP and HR to be increased. The increase in BP is potentially caused by sympathetic mediated increase in TPR, which has been correlated to increased LV wall thickness (Devereux et al 1987). Increased TPR causes the heart to pump against an increased pressure load and results in LV hypertrophy, as an adaptation to maintain normal wall stress levels , as increased pressure can be offset by an increase in wall thickness(Devereux et al 1987, Ganau et al 1992, Lorell and Carabello 2000).

The LV hypertrophy could also be explained by increased sympathetic NA to the heart, which would agree with the tendency for HR to be elevated in treatment groups. Increased sympathetic activity would increase HR and CO, consequently the increased workload would cause ventricular hypertrophy.

Increased RV mass associated with obesity is thought to be due to increased CO and increased pulmonary vascular resistance and artery pressure which lead to increased RV afterload and hypertrophy (Her et al 2006). OSA has also been associated to RV hypertrophy due to hypoxic pulmonary vasoconstriction (HPV) and pulmonary vascular remodelling. In the presence of hypoxia, pulmonary arteries constrict, redirecting blood flow to alveoli with higher oxygen content, causing pulmonary HT (Welsh and Peacock 2013). OSA patients have pulmonary hypertension in the daytime as well when they are not apnoeic suggesting other complex physiological and structural factors are involved and it is not just down to HPV (Welsh and Peacock 2013). Hypoxia is also known to cause an increase in pulmonary vascular cell proliferation, by inhibiting antimitogenic factors and

production and release of mitogenic stimuli such as endothelin-1, VEGF and inflammatory mediators (Welsh and Peacock 2013). The increased cell proliferation results in pulmonary vascular remodelling increasing resistance due to vascular wall thickening that reduces the size of the vessel lumen. Both HPV and pulmonary vascular remodelling would cause increased vascular resistance forcing the heart to work harder, and result in hypertrophy of the right ventricle.

Treatment group septum weights were not different from each other; however normal group weights were unavailable. It is therefore not possible to conclude if mass was increased in treatment groups although it would be expected. The septum splits the left and right ventricles but functionally is more part of the left ventricle (Myslinski et al 2007). As left ventricle mass was increased it would be expected the same mechanisms would increase septum mass.

The addition of CIH to HF diet did not seem to have any effect on heart tissue mass, this agrees with what was reported by Niroumaud et al (2001) that LV mass is increased in OSA only due to patient's associated obesity, aging and HT. However the lack of a CIH only group means we cannot confirm that this CIH protocol had no effect on heart tissue mass.

Haematocrit

No alterations in haematocrit were observed, with treatment groups having nearly identical haematocrit values as control. This observation suggests neither HF diet nor the addition of CIH had any effect on the volume of red blood cells in the blood.

Obesity is a known inflammatory condition. Inflammation is known to have a role in suppressing erythropoiesis, so haematocrit value could be expected to decrease with HF diet (Vuong et al 2014). However in humans haematocrit has been shown to be positively

correlated to waist circumference, a known correlate of obesity (Vuong et al 2014). This is also supported by increased erythropoiesis in spite of the effects of inflammation in metabolic syndrome patients. The possible explanation being increased insulin levels, as there is evidence insulin and insulin like growth factors enhance erythropoiesis (Vuong et al 2014).

Previous studies have reported mixed findings with regard to the effect of CIH on haematocrit. Studies by Fletcher et al (1992a and 2000) found CIH exposure (2 per minute, 8 hours per day over 40 days) caused no change in haematocrit or only a tendency to increase without being significant. McGuire and Bradford (2001) however report significant increase in haematocrit with exposure to CIH (2 per minute, 8 hours per day over 25 days). Comparing these studies with the CIH treatment we used, showed a similar duration (8 hours) and a similar length (28 days) as that of McGuire's study. This study however used a much lower frequency of hypoxias 6 per hour. Therefore if increasing haematocrit is one of the body's mechanisms for dealing with CIH, as it is known to be for chronic hypoxia (McGuire and Bradford 2001), then this adaptation would likely be occurring at a much greater rate with the increased frequency of hypoxias in Fletcher and McGuire's studies. The CIH model used in this study is over the threshold for diagnosis yet under the level required for treatment so can be thought of as an early stage development model, so would not necessarily show this adaptation.

As haematocrit was not altered throughout this study we expected normal O_2 delivery was taking place during normoxic conditions. However the absence of increased red blood cell numbers may be detrimental during periods of IH, and animals may not have be able to maintain the same level of O_2 delivery.

Baseline Respiratory Parameters

Based on the literature which shows both HF diet and CIH stimulate the CBs, we hypothesised that baseline respiratory parameters would be increased in HF diet fed animals, and the addition of CIH would further augment the increase.

No difference in baseline R_f was observed between groups, therefore providing no evidence of increased CB drive in treatment groups. This finding does not match what has been previously reported in the literature. Ribeiro et al (2013) observed increases in all spontaneous ventilatory parameters (R_f, V_T and minute ventilation) with application of a HF diet. They proposed the effect was mediated by overstimulation of the CBs, as surgical cut of CSN completely abolished the diet induced increase in spontaneous ventilation. Again these differences may be explained by differences, as previously stated, in HF diet composition with Ribeiro's study possibly using a diet containing more than twice as much fat as the diet used in this study.

Minute ventilation is the product of R_f and V_T and was increased in both treatment groups, significantly in the case of the HF group. These increases in minute ventilation can be put down to V_T, as it was increased but not significantly in treatment groups, and R_f remained unaltered. Ventilation is matched to metabolism, so an increased V_T indicates an increase in metabolism and CO_2 production in treatment groups.

When body weight was taken into account with minute ventilation, it showed the control group's minute ventilation was higher than the treatment groups. The disappearance of the significance between control and HF group's normalised minute ventilation may suggest that the significantly larger minute ventilation was purely down to the size of the animals, with HF group rats considerably heavier than the control group. No additional

increase in ventilation with application of CIH is observed and provides no evidence of augmented CB activity.

Growth, Body Mass and Food Consumption

The final weights of treatment groups were significantly larger than control animals, which was expected as HF diets are a model of obesity and previous studies have shown increased weight gain (Ribeiro et al 2013).

The addition of CIH on to a HF diet caused a significant reduction in animal's growth which ultimately explains the difference observed in final weights. This difference was attributed to CIH as during the two weeks prior to the introduction of CIH animal's growth, on HF diet alone, was very similar to the HF group. Growth was only significantly altered between groups during the CIH exposure in the final two weeks of HF diet.

Animals' food consumption data showed the HF+CIH groups food intake was decreased, although not significantly, during CIH exposure by approximately 1.5g.day^{-1}. This reduced intake explains the observed reduction in growth during CIH exposure, and ~20g difference in final weights between HF and HF+CIH groups.

Crossland et al (2013) found no effect of CIH on growth or food intake. However Fletcher et al (1992b) and also Olea et al (2014) report CIH decreased growth in normal and HF diet fed rats, which agree with our findings. The different results may be explained by the different rat strains used; Crossland used Long Evans rats which may respond to CIH differently to the Wistar used in the other studies. Length of CIH exposure may also explain the different results, with rats exposed much longer than the 4 weeks Crossland used. Although this study only used 2 weeks CIH exposure, the results could be explained by the

HF diet used in this study, which may have exacerbated growth differences due to increased weight gain.

Tissue weights

Feeding rats a HF diet is a model of obesity, we therefore hypothesised that there would be increased amounts of fat in rats fed HF diet. There were significant increases in liver, PWAT and EWAT tissue weights in treatment groups, which were expected as a result of the HF diet.

The PWAT, EWAT and liver are known sites of fat deposition; however the kidney is not, so can be used as an estimate of lean body mass. Comparing kidney weights showed no significant increases in treatment groups, indicating HF diet has not affected lean body mass. From this the assumption can be made that the increased final weights of treatment groups' was predominantly down to the increases in fat stores, therefore indicating obesity was present.

The addition of CIH to HF diet did not seem to have any effect on adipose and liver tissue weights as no differences were observed when compared to the HF diet only group. These results agreed with Olea et al (2014), who reported significantly larger EWAT and PWAT weights in HF diet fed animals and found no difference in fat pad weight in animals also exposed to CIH (Olea et al 2014). The observation that treatment group livers had a discoloured appearance and the significant increase in mass suggest HF diet may have resulted in development of non-alcoholic fatty liver disease.

The HF group showed a significant decrease in normalised TA muscle mass and a tendency for normalised soleus muscle mass to be decreased. The HF+CIH group also showed a tendency for decreased normalised TA and soleus muscle masses. No change in normalised

EDL mass was observed between groups. Looking at groups' actual muscle mass values (appendix table 4) revealed there are no significant differences between groups, suggesting animals' have the same lean body mass. Therefore the decrease in normalised muscle mass is due to the extra body mass, which is most likely to be fat, of treatment groups and not a reduction in the actual muscle mass.

This tendency for decreased normalised muscle mass and same lean body mass suggests treatment group animals do not exercise as much. This however cannot be confirmed as no measures of animals' activity were made. The HF+CIH group did not show the same decrease in normalised mass as the HF group, this possibly could be explained by the reduced obesity of the HF+CIH group compared to the HF group. Alternatively it could be explained by exposure to hypoxia, which may cause excitation of animals and an increase in exercise.

GTT and Insulin Levels

IR is known to be associated with obesity and it has also been linked to IH. We therefore hypothesised that feeding rats a HF diet would result in IR and altered blood glucose profiles, with the addition of CIH causing these effects to a greater degree.

Baseline fasting blood glucose showed a tendency to be higher in control group than HF group and was significantly higher than HF+CIH group. Baseline fasting glucose levels appear high as previous results from this lab have found baseline levels to be around 4.6mmol.L^{-1}, which is similar to the 4.1mmol.L^{-1} reported by Olea et al (2014). Other studies report slightly higher control group glucose levels ~6mmol.L^{-1} (Nagarajan et al 2013, Yokoe et al 2008), but they are still lower than found in this study. A simple explanation for this difference could be due to surgical trauma. The control group

experiments were the first set to be completed and as such were done during a learning period of surgical skills and technique. This resulted in control group animals being anaesthetised for a much longer periods and undergoing more traumatic surgery than HF and HF+CIH groups. Unfortunately due to the time pressures associated with an 18 week project it was not possible to repeat the control group to definitively determine if the higher baseline was a result of traumatic surgery. To avoid the effect of the traumatic surgery in future experiments a tail prick sample immediately before surgery would allow accurate measurement of baseline glucose.

Two studies (Nagarajan et al 2013, Olea et al 2014) both reports HF diet increases fasting glucose levels. This does not agree with our results, but may be due to the elevated appearance of control group glucose levels.

Olea (2014) and He (2014) both showed hypoxia increased blood glucose and insulin levels. Olea (2014) also found the combination of CIH and HF diet to significantly increase blood glucose, but it was not significantly higher than HF diet alone. Again these results do not agree with our findings but may be due to increased glucose levels in control group.

As expected the blood glucose levels increased with administration of the IV glucose dose and evoked the same size change in all groups. However that was where similarities between control and treatment groups blood glucose profile stopped. Control group blood glucose was returned to baseline levels by 30 minutes, whereas HF and HF+CIH groups had not recovered and blood glucose was still elevated after 60 minutes. The differences in these profiles was clearly shown by the AUC with both treatment groups having a tendency to be increased although not significantly.

Baseline insulin levels were significantly elevated in HF group and HF+CIH also showed a tendency to be increased. This suggests altered glucose handling, with larger quantities of insulin required to maintain blood glucose at resting levels due to IR. The significant increase in baseline insulin with HF diet agrees with Olea et al (2014), however they also showed when combined with IH it causes a further increase in baseline levels.

IR was expected with the HF diet known to result in altered insulin sensitivity. The insulin curve matched the GTT curve, with both increased at 2 minutes and lower at 60 minutes. Although groups were given the same glucose dose each group produced different changes in insulin. The peak insulin levels were at similar values, even though the HF group baseline was significantly higher statistically than control. This points to a maximum insulin output with HF and HF+CIH basal insulin levels reducing the amount of increase possible.

The insulin AUC was significantly larger than control showing insulin levels were raised for longer. This agrees with the GTT, as glucose levels were still raised after an hour. Failure to return to baseline levels within an hour points towards altered glucose handling ability and IR, which was expected to occur with the HF diet. The absence of any significant difference between HF and HF+CIH groups suggests no additional detrimental effects on glucose handling or increased IR were caused by CIH.

GTT Respiratory Parameters

Glycaemic level and insulin have both been proposed as CB stimulators; therefore alterations in ventilation during GTT due to CB activation were expected. All groups showed a trend of increased ventilation at 2 minutes of the GTT; however treatment groups remained elevated over the rest of the GTT and control returned to baseline. This

effect could possibly be explained by increased CB activity, as change in R_f was the cause of the increased ventilation. The effect may be driven by glucose or insulin. As both were increased at 2 minutes and returned to baseline with control group's ventilation. Both also stayed elevated in treatment groups past 30 minutes where ventilation remained elevated.

Glucose has been put forward as a stimulator of the CB, with both Hypo- and hyperglycaemia showing increases in ventilation and augmentation of HVR (Limberg et al 2014, Ward et al 2007). Insulin has also been proposed as a direct trigger of the CBs, capable of initiating a neurosecretory response that is transduced into an increase in ventilation (Ribeiro et al 2013). The continued elevation of ventilation in treatments groups as glucose and insulin levels were falling points towards an increased sensitivity rather than just an increase in tonic baseline activity. This agrees with the observed slow increase in HVR with hyperglycaemia, and its persistence even with return to euglycaemia could imply a slow modification of hypoxic sensing mechanisms by glucose (Ward et al 2007). However due to the lack of blood gas data, hypoxia and hypercapnia cannot be ruled out as causes of the ventilatory changes.

Dejours Test

Inhalation of hyperoxia (Dejours test) suppresses CB drive, therefore the magnitude of ventilatory depression caused by O_2 can be used as an index of CB activation. HF diet and CIH have been shown to stimulate the CB. We therefore hypothesised CB activity would be increased in HF diet, with further augmentation when combined with CIH.

The significant baseline minute ventilation increase in HF+CIH and elevated tendency in HF groups can be explained by the increased V_T in treatment groups, as no difference in R_f was seen between groups. No significant differences between minute ventilation were

observed when it is normalised for body weight suggesting these increases may be due to increased animal size.

All respiratory parameters except V_T significantly decreased with application of 100% O_2 as expected. The size of the decreases in R_f was the same for all groups and there was no significant difference in minute ventilation decreases. This along with the similar R_f baseline values indicates no CB over activity in treatment groups. This was not what was expected, with increased baseline R_f and larger ventilatory depressions in HF group with further augmentations when combined with CIH.

Baseline FBF and FVR

Baseline FBF showed a tendency to be decreased in the HF group, although not significantly. The HF group also had a significantly reduced normalised TA mass, and a tendency for soleus normalised mass to be decreased. No change in normalised EDL mass was observed. This suggests that the differences in muscle mass can explain the observed differences in FBF, as a higher blood flow would be expected to larger muscles.

The HF+CIH group data did not agree with the HF group. The HF+CIH group showed a non-significant tendency for increased FBF, but tendency for decreased TA and soleus normalised muscle masses, and no change in normalised EDL mass. Therefore FBF differences cannot be explained by differences in leg muscle masses.

One possible explanation for the tendency for raised BP would be an increase in TPR and the trend in FVR is consistent with this**.** The tendency for FVR to be decreased in the HF+CIH group however does not agree with this as HF+CIH group also showed a tendency for BP to be increased. Instead the HF+CIH groups BP may be explained by a specific

increase in cardiac sympathetic activity, with no change in muscle sympathetic activity causing the tendency for BP to be elevated.

There are however concerns over the baseline FVR values, due to variation in baseline FBF within groups. The instrument is the likely cause of variation observed within groups and therefore comparisons between groups baseline values cannot be confidently made. The linearity of the changes from baseline however did appear to be consistent and so can be used for comparison. This means it is not possible to conclude definitively that TPR is altered in treatment groups.

Sympathetic Stimulation

Vasoconstriction is a major mechanism in the development of HT, which could be mediated by sympathetic NS. Alternatively it may be due to increased endothelial vasoconstrictors or decreased vasodilators, but due to time constraints we cannot assess all these effects. Therefore we hypothesised that animals fed HF diet would show increased levels of sympathetic output, which would be further increased by the addition of CIH.

Sympathetic chain stimulation at 20Hz resulted in larger FVR increases than stimulation at 2Hz. The 20Hz stimulation evoked similar increases in FVR in all groups; 2Hz stimulation evoked similar FVR increases in control and HF groups but caused a larger increase in the HF+CIH group. The HF+CIH group FVR increase was significantly larger than the HF group; however this is not an accurate representation as the difference was not actually significant. In the HF group the 2Hz stimulation did not work on one animal, yet the 20Hz stimulation did. Unfortunately this is a limitation in the nature of this type of stimulation. Therefore in order to keep the 20Hz stimulation in the results, as n numbers were quite

low, the animal was not excluded. Due to the inclusion of this result it has reduced the mean FVR and also resulted in a statistical significance between the HF and HF+CIH groups. Exclusion of this value (see appendix fig 23) increased the HF group mean to 5.2±1.2mmHg.(ml.min^{-1})$^{-1}$ and resulted in loss of significance when compared to the HF+CIH group.

The same FVR response was evoked by 20Hz stimulation in all groups and no real significant difference in 2Hz stimulation responses point towards no increase in tonic sympathetic activity in HF or HF+CIH groups. This does not agree with the literature. Olea et al (2014) measured plasma catecholamine levels, another indirect measure of sympathetic activity, showing increases in noradrenaline and adrenaline levels with HF diet and CIH. Concluding sympathetic activity is increased and proposed it was mediated by the CB in CIH, and obesity causing activation by leptin with the CBs also contributing.

Concerns over the variability observed in baseline FBF and FVR values would not have affected the sympathetic evoked changes in FVR results. Peak values from the baseline immediately before each stimulation were taken, therefore variations in the baseline level within groups would have no effect on the magnitude of the FVR change, which appeared consistent within groups.

Study Limitations

Insulin quantification was done using a rat specific insulin ELISA, however absorbance readings given were outside the calibration curve range. Insulin values therefore had to be extrapolated beyond the calibration curve using a non-linear regression curve. Although this curve did fit it was not the curve recommended by the manufacturer for interpolation of insulin content. Cubic spline regression was recommended however this did not allow

for extrapolation of results beyond the calibration curve range. Values that fell within the calibration curve were interpolated and compared using both non-linear and cubic spline regression which showed similar values. However extrapolated values may not be as accurate due to insulin's absorbance not continuing exactly on the extended curve. Repetition of insulin quantification was not however possible due to time and ELISA cost. In future extrapolations may be avoided by dilutions of serum samples before insulin quantification.

A limitation in the design of this study was the absence of a CIH only group. Unfortunately due to the time constraints it was not possible to fit an additional group fed standard rat chow and exposed to CIH for 2 weeks. In future studies the inclusion of a CIH only group would make the effects of the CIH protocol clearer than when it is in combination with other treatments.

The lack of blood gas analysis is also a limitation of this study. Inclusion in future projects would enable hypoxia and hypercapnia to be ruled out as the cause of any ventilatory changes, as well as provide information about the activity of the metabolic system (Burns 2014).

The food and water intake of HF and HF+CIH groups was monitored throughout the 4 week diets. However there were some issues with the monitoring. Animals were housed in BMSU with staff checking them every day. As such if rat's water was getting low they would refill the bottle, however would not record the amount of water left before refilling, only the amount they had filled to. Another issue was they would not always record the date they had refilled which caused problems when calculating intake. These problems

could be avoided by monitoring the animals on a daily basis, instead of the twice weekly, as this would ensure water does not become low and consumption recorded properly.

Another limitation is that control group animal's growth and intake over the 4 weeks on standard diet was not monitored. Animals were bought in at the correct age for experiments due to time implications. Ideally all animals would have been obtained at the same age (5 weeks) and kept in BMSU over the duration of the diets.

There are various limitations involved with the method of sympathetic stimulation used. Firstly the response elicited by this technique is not always an increase in FVR, no response or occasionally a negative response are observed, and because of this n numbers are lower than other parts of the protocol. The absence of some responses may be explained by placement of electrodes. Electrodes were fixed in place with silicon, however if they moved during set up and touched or became surrounded by fluid it could cause them to short out. Constant voltage stimulation was used in this study, this method results in more variable FVR responses. Alternatively constant current stimulation could be used as it evokes more consistent responses; however a constant current stimulator was not available for this project. Substitute methods that could be used instead of sympathetic stimulation are plasma and urine catecholamine level quantification or muscle sympathetic nerve activity recording both of which would provide accurate measures of sympathetic activity.

A major unavoidable limitation of this project was the surgery itself. At the start of the project there was a major learning curve of surgical skills. As such animals that were experimented on at the start of the project underwent a lot more trauma and spent longer under anaesthesia than those at the end of the project. Control animals were the first

group experimented on; therefore any effects of the surgery may have effects on the significance of findings when comparing with the treatment groups. This could be avoided in future by surgery being conducted by a competent experimenter, where surgery trauma is not going to vary dramatically. Alternatively it could be avoided by mixing up the order animals are experimented on. This was not possible due to the time restraints of this project, as the HF and HF+CIH animals take longer to develop than control group. If more time was available further control group experiments could be done to determine the effects of traumatic surgery on results such as baseline glucose levels.

Conclusions

In conclusion, 4 weeks HF diet induced obesity, IR and ventricular hypertrophy. HF diet also caused a significant increase in SBP, which contributed to the tendency of MABP to be increased by 12±3mmHg. In combination with CIH, there was a reduction in weight gain, but no effect on IR or hypertrophy. MABP tended to be increased by 10±3mmHg, and SBP was not significantly increased. The absence of altered ventilation and augmented response to hyperoxic challenge both suggest no increase in CB activity was caused by HF diet or CIH. Also results provide no evidence HF diet or CIH caused increased sympathetic output.

The tendency for increased BP and the ventricular hypertrophy suggest this low AHI model did produce cardiovascular changes. However these changes may be on different time scales and with longer exposure rats may have developed significant increase in MABP. Therefore this could possibly be thought of as an early stage development model of OSA. As alteration of glucose homeostasis and cardiovascular changes are observed with this model it may indicate that OSA should be targeted for treatment earlier. Allowing the use

of less radical treatment methods, such as weight loss and glycaemic control, to prevent OSA developing further.

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APPENDIX

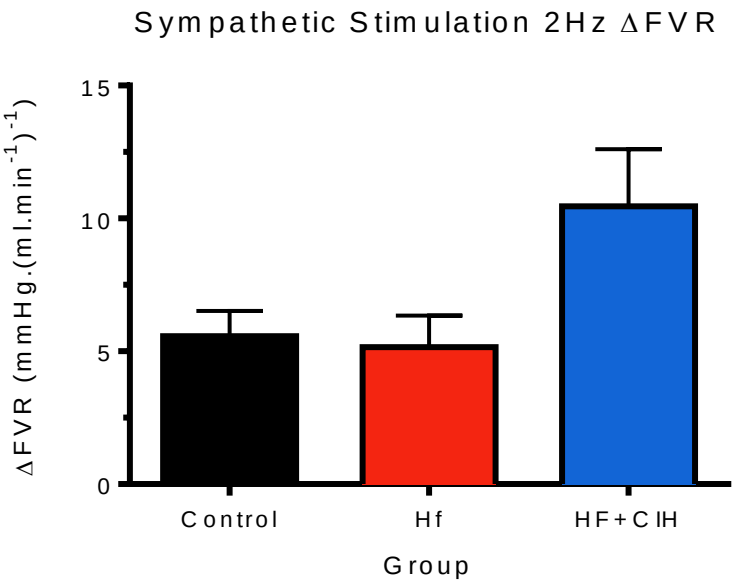

Figure 23: Effect of 2Hz sympathetic chain stimulation on femoral vascular resistance (mmHg.(ml.min-1)-1) in control (n=5), HF (n=3) and HF+CIH (n=5) groups. Bars represent mean ±SEM. One-way ANOVA with Bonferroni multicomparison tests.

Table 4: Effect of diet and CIH on muscle tissue weight (mg) in normal (n=6), HF (n=8) and HF+CIH groups (n=8).

	Normal	HF	HF+CIH
EDL	147±8	175±10	171±9
TA	653±19	714±36	719±9
Soleus	166±7	182±9	162±8

Values represent mean ± SEM. One-way ANOVA with Bonferonni multicomparison tests. Normal rat tissue weights were obtained from a previous study.