Effects of Environmentally Relevant Secondhand Smoke, High-Fat Diet, and Their Co-Exposure on Cardiovascular Regulation in Mice

By

SHIYUE PAN

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Approved:

Chao-Yin Chen, Chair

Manuel F. Navedo

Karen Ryan

Committee in Charge

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Abstract

While the incidences of cardiovascular diseases declined by 27% over the past few decades, the number of deaths related to cardiovascular diseases increased by 42%, owing to the population growth and increased life expectancy. Consequently, cardiovascular diseases are the leading cause of death worldwide. Cardiovascular diseases are complex, multifactorial diseases that cannot be ascribed to a single risk factor. Secondhand smoke (SHS) and high-fat diet (HFD) are two preventable risk factors for the development and progression of cardiovascular diseases. Interestingly, epidemiological studies have correlated SHS exposure with poor dietary habits including higher total fat intakes, suggesting an underestimated incidences of co-exposure of SHS and HFD worldwide. In this study, we sought to characterize the effects of SHS, at an environmentally relevant concentration, on cardiovascular regulation, and the time-course of SHS-induced cardiovascular changes using a mouse model (chapter 2). We also assessed the effects of long-term consumption of HFD on heart rate (HR) and blood pressure (BP) regulation in mice (chapter 3). Moreover, this work aimed to investigate the effects of SHS and HFD co-exposure on cardiovascular function (chapter 3).

To test these aims, mice implanted with BP/ electrocardiogram (ECG) telemetry devices were fed with either standard rodent chow (13.6 kcal% fat) or HFD (60 kcal% fat) and exposed to either filtered air (FA) or SHS (3 mg/m^3, 6 hours/day, 5 days/week). BP, HR, HR variability (HRV), BP variability, and baroreflex sensitivity (BRS) were obtained to evaluate the cardiovascular function.

We found that long-term exposure of SHS decreased measures of autonomic regulation of HR (HRV) and BP (BRS), as well as decreased pulse pressure (PP) in a time-dependent manner. The effects of SHS on PP and HRV had a faster onset that observed after four weeks of exposure, while the effects of SHS on BRS had a slower onset and accumulated with longer exposure time. However, all SHS-induced cardiovascular dysfunctions recovered after removal from SHS exposure for four weeks. Long-term exposure of HFD alone increased weight gain, increased BP, and reduced BRS and HRV. Strikingly, we found that co-exposure of SHS and HFD significantly increased BP variability that was not observed with SHS or HFD alone, suggesting a co-exposure-induced BP dysregulation.

Overall, this study demonstrated that chronic exposure to SHS and HFD both reduces the autonomic regulation of the cardiovascular function. Co-exposure of SHS and HFD can interactively induce BP dysregulation, which may put individuals under even higher risks of cardiovascular morbidity and mortality. This study provides insights into the importance of understanding the interplay between multiple risk factors in inducing cardiovascular dysfunctions when studying the multifactorial cardiovascular diseases.

Chapter 1:

Overall introduction

1.1 Cardiovascular diseases are multifactorial diseases

Cardiovascular diseases, such as coronary heart disease and heart attack, are the leading cause of death worldwide. From 1990 to 2019, the number of cases of cardiovascular diseases has increased from 271 million to 523 million (Roth *et al.*, 2020). In 2019, an estimated 17.9 million population died from cardiovascular diseases, representing about one-third of deaths globally (Roth *et al.*, 2020). An increase in the number of cases and deaths from cardiovascular diseases is expected to continue in both developing and developed countries, owing to the population growth and longevity. As described in a special issue of the Science (Kiberstis and Roberts, 2002), common modern diseases such as cardiovascular diseases are hard to prevent and treat because they are “complex” and “multifactorial” in nature. They do not have a single cause, but are influenced by many contributing factors, including genetic components and environmental factors such as physical exercise, air pollution exposure, and diet.

In today’s world, the rapid growth of economy, industrialization, and urbanization have led to severe air pollution problems (Li *et al.*, 2016; Power *et al.*, 2018). In the United States, along with the increase in population and gross domestic product over the past few decades, there were increases in industrial manufacturing, vehicle transportation, and energy consumption, which all are sources for the air pollutants (U.S. EPA, 2016).

Epidemiological studies showed that both chronic and short-term exposure to air pollution is associated with the development and progression of cardiovascular diseases, such as stroke, ischemic heart disease, heart failure, and increased arrhythmia susceptibility (Franchini and Mannucci, 2012).

Along with the industrialization and urbanization, the dietary pattern has been changed drastically worldwide as well, from diets with a high intake of fiber and grains towards diets with high level of carbohydrate and fats (Popkin, Adair and Ng, 2012; Sproesser *et al.*, 2019). Suboptimal diets, such as diets with low intake of whole grains, fruit and vegetables, high intake of sodium, sugar and fat, are significant risk factors for cardiovascular diseases such as coronary heart disease, ischemic heart disease, and stroke (Micha *et al.*, 2017; Dong *et al.*, 2021; Lichtenstein *et al.*, 2021). According to the global burden of disease study, cardiovascular diseases are the leading cause of diet-related deaths (Afshin *et al.*, 2019). Moreover, a recent systematic analysis from Dong *et al.* found that from 1990 to 2019, the number of diet-related deaths from cardiovascular diseases have dramatically increased by 43.8% (Dong *et al.*, 2021).

While air pollution and dietary risks are two well-established risk factors for cardiovascular diseases, we have limited information on the interplay of these two cardiovascular risk factors. There are studies showed that Mediterranean diet, which rich in fruits, vegetables, fish, and whole grains, can reduce air pollution-related risks of cardiovascular morbidity and mortality (Lim *et al.*, 2019), suggesting that changes in dietary pattern can modify the effects of air pollution on cardiovascular functions. On the other hand, there are studies

indicate that exposure to air pollution may induce metabolic abnormalities leading to obesity (An *et al.*, 2018). Air pollution can directly induce metabolic abnormalities through increased oxidative stress, visceral inflammation, hepatic lipid accumulation, and insulin resistance (Sun *et al.*, 2009; Xu *et al.*, 2011; Liu *et al.*, 2014). Additionally, air pollution may also result in metabolic abnormalities and obesity indirectly through increasing risks of other chronic diseases, such as cardiovascular diseases (An *et al.*, 2018).

The major goal of this project is to investigate the interplay between air pollution exposure and suboptimal dietary on cardiovascular functions. This could provide additional information of revealing population groups that are more susceptible to single cardiovascular risk factors.

1.2 Secondhand smoke (SHS) and high-fat diet (HFD) as the co-exposure paradigm

Over the past few decades, dining out and eating takeout food has become increasingly popular all over the world. It has been shown that eating out was associated with higher intake of total fat, especially saturated fat, and lower intake of fiber (Lachat *et al.*, 2012). In the United States, from year 1977 to 2005, people had significantly increased their caloric intake from food prepared away from home by ~15%, with most of them coming from table-service and fast-food restaurants, which are also major sources of SHS exposure outside of households (Lin and Guthrie, 2013). Co-exposure to SHS and HFD

may be more common than we realized, especially in cities without the implementation of smoking bans.

Intriguingly, a few epidemiological studies showed that women exposed to SHS tend to have unhealthy diet habits and life style (Koo, 1988; Thompson and Warburton, 1992). Koo *et al.* showed that never-smoked wives with smoking husbands had a significantly higher total fat intakes than never-smoked wives without smoking husbands (Koo *et al.*, 1997). Similarly, Thompson and Warburton found that non-smokers living in smoking households ate more fried food and had a higher intake of fat than non-smokers living in non-smoking households (Thompson and Warburton, 1992). These data further suggest that incidences of co-exposure of SHS and HFD may be underestimated worldwide.

1.3 SHS as the major indoor air pollution

SHS, also known as environmental tobacco smoke, is a major source of indoor air pollution. SHS consists of the mainstream smoke exhaled by smokers, and the sidestream smoke from the cigarette burning end. More than 4000 harmful chemicals are found in SHS, including known carcinogens (e.g.: benzene, 1,3-butadiene, formaldehyde, and acetaldehyde), and toxins (e.g.: hydrogen cyanide, carbon monoxide, and butane) that have been shown to lead to various health consequences (Center for Disease Control and U.S. Department of Health and Human Services, 2006; U.S.DHSS, 2010). In fact, the U.S. Environmental Protection Agency (EPA) has classified SHS as a human

carcinogen. The National Institute for Occupational Safety and Health (NIOSH) also classified SHS as an occupational carcinogen.

Worldwide, about 30% of non-smokers are exposed to SHS (Öberg *et al.*, 2011). Evidence of exposing to SHS can be determined by several tracers, including nicotine and its metabolite cotinine, and the particulate matter (PM) level (Institute of Medicine (US) Committee on Secondhand Smoke Exposure and Acute Coronary Events, 2010). PM is a major harmful component that could be found in SHS. In this regard, SHS is also known as a substantial contributor to the level of particulate indoor air pollution (Mueller *et al.*, 2011). The size of the particulates is directly linked to their potential health risks. Particles with aerodynamic diameters smaller than 2.5 μm ($PM_{2.5}$) poses the greatest health risks as it can get deep into the lungs and even get into the systemic circulation, while exposure to coarse particles (aerodynamic diameters of less than 10 μm and greater than 2.5 μm) are respectively of less concern (Kaiser, 2005). Compared to a nonsmoking place, the concentrations of respirable suspended particles in smoking areas could be threefold higher and exceed the U.S. EPA-proposed maximal level for fine particles in outdoor ambient air (IARC, 2004). There is no risk-free level for SHS exposure (Potera, 2010), therefore, smoke-free laws that prohibit smoking in public places become extremely crucial to protect non-smokers from such exposure.

Besides the PM, another harmful component found in SHS is carbon monoxide (CO). In an indoor environment where people smoke, such as offices, restaurants, bars, and public

transportation, the measured mean concentrations reported for CO ranged from 0.2 to 33 ppm, while the standard for CO concentration in indoor air is 9 ppm (IARC, 2004).

1.4 Cardiovascular consequences of SHS and HFD

1.4.1 SHS-related cardiovascular consequences

Although the relative impacts of SHS exposure are greater on respiratory system rather than on cardiovascular system, the actual number of deaths from SHS-induced cardiovascular diseases is greater than that from respiratory diseases (Brook, Brook and Rajagopalan, 2003). Epidemiological studies showed that 70-80% of deaths attributable to SHS are caused by cardiovascular diseases, suggesting the cardiovascular system is extremely vulnerable to SHS (Glantz and Parmley, 1991). While the exposure level of SHS is much lower than that of active smoking, the effects of SHS exposure on the cardiovascular system have been shown to be as large as 80-100% of those related to active smoking (Barnoya and Glantz, 2005).

SHS exposure can have an immediate direct effect of triggering acute cardiac consequences, such as acute myocardial infarction (one of the leading causes of morbidity and mortality worldwide), hospital admissions for cardiac symptoms, and sudden cardiac death (Glantz and Parmley, 1991; Center for Disease Control and U.S. Department of Health and Human Services, 2006; Jayaraj *et al.*, 2018). It has been shown

that 20 min of SHS exposure was associated with an increased platelet activation, a pivotal event in thrombosis and plays a key role in the progression of acute coronary syndrome, in male non-smokers (Davis *et al.*, 1989). Adult non-smokers exposed to 30 min of SHS showed a persistent elevation of both endothelial microparticles and vascular endothelial growth factor levels at 24 hr post-exposure, suggesting a functional endothelial impairment upon acute exposure to SHS (Heiss *et al.*, 2008). Similarly, another study found a significant reduction in coronary flow velocity reserve, which is a surrogate marker of endothelial-dependent vasodilation, in male non-smokers after 30 min exposure to SHS (Otsuka *et al.*, 2001).

Chronic exposure to SHS can worsen and promote the development of cardiovascular and cardiometabolic diseases, such as hypertension, atherosclerosis, coronary heart diseases, insulin resistance, and diabetes (Glantz and Parmley, 1991; Raupach *et al.*, 2006). A population-based cohort study reported that exposure to SHS for about 10 hr per week for 3 years was associated with a 20% increase in the progression of atherosclerosis (Howard *et al.*, 1998). Similar findings have been observed in mice and rabbit models as well (Zhu *et al.*, 1993; Knight-Lozano *et al.*, 2002). In a cross-sectional epidemiological study, adult non-smokers chronically exposed to SHS were reported to have a significant increase in carotid arterial stiffness index (Mack *et al.*, 2003).

Although there's growing evidence link SHS exposure to cardiovascular dysfunctions, we have limited information on the time course of SHS-induced cardiovascular changes. We also don't know the effects of SHS exposure on baroreflex function, which is a key

regulatory mechanism for short-term blood pressure (BP) regulation. To fill these gaps, chapter 2 was designed to assess the time-dependent changes in cardiovascular functions under SHS exposure using a mouse model.

1.4.2 HFD-related cardiovascular consequences

Numerous studies in both human and animal models have shown a strong association between HFD exposure and the development of obesity and type 2 diabetes (Winzell and Ahrén, 2004; Astrup, 2005; Dirkx *et al.*, 2011; Speakman, 2019). Surprisingly, while obesity and type 2 diabetes themselves are two well-known risk factors of cardiovascular morbidity and mortality (Hubert *et al.*, 1983; Sowers, 2003; Fox, 2010), the existed epidemiological evidence on the direct association between HFD and cardiovascular diseases remained controversial (Forouhi *et al.*, 2018).

The Seven Countries Study was the first epidemiological evidence that showed an association between high-fat intake, especially increased consumption of saturated fatty acids which are mostly found in animal products such as meat, milk, and butter, and increased risk of cardiovascular morbidity and mortality (Forman and Bulwer, 2006). Following this study, growing evidence have reported a link between dietary fat intake and cardiovascular diseases, such as coronary heart disease, ischemic heart disease, atherosclerosis, and stroke (Forouhi *et al.*, 2018; Korakas *et al.*, 2018). Hu *et al.* showed that higher intakes of saturated and *trans* fats are related to an increase in the risk of

coronary heart disease (Hu *et al.*, 1999). In the Northern Manhattan Study, Boden-Albala and colleagues found that a daily intake of total dietary fat greater than 65 g, which is approximately 37 kcal% from fat, is associated with an increased risk of ischemic stroke by 60% (Boden-Albala *et al.*, 2009).

While these evidence emphasized the association between higher fat intake and increased incidence of cardiovascular diseases, several randomized trials and epidemiological studies showed inconsistent and contraindicated findings (Forouhi *et al.*, 2018). A few meta-analysis of data from cohort studies and clinical trials suggested that there's no association between increased fat intake and higher risk of coronary heart disease (Siri-Tarino *et al.*, 2010; Chowdhury *et al.*, 2014). Similarly, results from the recent Prospective Urban Rural Epidemiology (PURE) study demonstrated that total fat intake as well as types of fat is not related to cardiovascular risks (Dehghan *et al.*, 2017).

There are remained controversies and discrepancies in understanding the effects of increased dietary fat intake on risks of cardiovascular diseases. With the interests of investigating the direct association between higher fat intake and cardiovascular morbidity and mortality, chapter 3 was designed to assess cardiovascular function under HFD exposure using a mouse model.

1.5 Potential mechanisms

1.5.1 SHS-induced cardiovascular dysfunctions

Several non-mutually exclusive mechanisms have been proposed to explain SHS exposure-related cardiovascular morbidity and mortality, including activation of inflammatory cascades, oxidative stress, atherosclerosis, endothelial dysfunction, and autonomic dysfunction (Barnoya and Glantz, 2005). For example, non-smokers that exposed to SHS for more than 3 days per week have been demonstrated to have a higher level of C-reactive protein, homocysteine, oxidized low-density lipoprotein cholesterol, and white blood cell counts, suggesting an increased oxidative stress and inflammatory response under chronic exposure to SHS (Panagiotakos *et al.*, 2004).

Among these potential pathways, dysfunction of autonomic regulation of the cardiovascular system may be particularly important in mediating both acute and chronic effects of SHS exposure. SHS exposure has been shown to reduce heart rate variability (HRV) and impair BP regulation in both human (Pope *et al.*, 2001; Pijanowska and Zajączkowska, 2004) and animal studies (Gentner and Weber, 2012). A reduced HRV, an index of cardiac autonomic imbalance, is arrhythmogenic and may act as a trigger of the acute cardiovascular consequences in responses to SHS exposure (Zipes and Wellens, 1998; Pope *et al.*, 2001; Raupach *et al.*, 2006). In this regard, the most detrimental SHS exposure-related cardiovascular morbidity and mortality is sudden cardiac death (Glantz and Parmley, 1995; Thun, Henley and Apicella, 1999). The first

direct evidence of SHS-induced reduction in HRV came from a study performed by Pope and colleagues, in which subjects moved between smoking and non-smoking areas in an airport. Two hours in the smoking area significantly reduced measures of HRV in all subjects - an effect that recovered when the subjects moved back to the non-smoking area (Pope *et al.*, 2001).

There is also evidence showing that SHS exposure increased BP in adult non-smokers (Pijanowska and Zajączkowska, 2004; Alshaarawy, Xiao and Shankar, 2013). While elevated BP is a well-known risk factor for cardiovascular morbidity and mortality, increased BP variability and impaired baroreflex function have been shown to cause more cardiovascular end-organ damage than increased BP (Lanfranchi and Somers, 2002; Stevens *et al.*, 2016). Baroreflex plays a key role in both short-term and long-term regulation of BP through the autonomic nervous system (Lohmeier and Iliescu, 2015). It has also been shown to be one of the contributors in regulating the BP variability (Mancia and Grassi, 2000). Impairment of baroreflex function, which is indexed by a reduced baroreflex sensitivity (BRS), suggests an autonomic dysfunction and is associated with increased cardiovascular morbidity and mortality (La Rovere, Pinna and Raczak, 2008).

While epidemiological studies have correlated SHS exposure with cardiovascular morbidity and mortality, and several experimental studies have shown SHS-induced changes in cardiovascular system, we have no information on the effects of SHS on BP variability and baroreflex function. We also don’t know the time course of SHS-induced changes in HRV and BP. Moreover, since most experimental studies used a high dose of

SHS which is irrelevant to the real-world exposure, it is important to use an environmentally relevant concentration of SHS in investigating the effects of SHS exposure on cardiovascular system. Overall, this dissertation project (chapter 2) aims to fill the above gaps in our knowledge.

1.5.2 HFD-induced cardiovascular dysfunctions

HFD exposure can induce cardiovascular dysfunctions through several potential mechanisms, and many of them share similarities with the previously described SHS-induced cardiovascular dysfunctions, such as increased systemic inflammation, oxidative stress, endothelial dysfunction, and autonomic imbalance (Dow *et al.*, 2015; Wali *et al.*, 2020; Garcia *et al.*, 2021).

Guillemot-Legris and colleagues showed that mice fed with 60% HFD for 16 weeks had an increased inflammation in the liver, adipose tissue, intestine, and central nervous system compared to mice fed on a normal diet (Guillemot-Legris *et al.*, 2016). Moreover, Dow *et al.* found that in healthy adults who have habitual dietary fat intake $\geqslant$35% of total calories, there was an impaired endothelium-dependent vasodilation compared to adults with habitual dietary fat intake meets the American Heart Association's (AHA) recommendation (< 30 kcal% from fat) (Dow *et al.*, 2015). An impaired endothelial function is associated with the progression of atherosclerosis and can lead to an increased risk of cardiovascular diseases (Vanhoutte *et al.*, 2009).

There is also evidence showing that HFD exposure can induce impaired autonomic regulation of BP (Speretta *et al.*, 2016, 2019; Garcia *et al.*, 2021). Garcia and colleagues showed that rats fed on HFD for 8 weeks had a significantly higher BP and splanchnic sympathetic nerve activity (Garcia *et al.*, 2021). Similarly, a study from Speretta *et al.* found that HFD-exposed rats displayed an elevated BP (Speretta *et al.*, 2019). They also found that exposure to HFD led to a higher systolic BP variability and a decreased baroreflex response to drug-induced changes in BP, suggesting an impairment in baroreflex function (Speretta *et al.*, 2016, 2019). As we previously mentioned, increased BP variability and impaired baroreflex function can cause more cardiovascular end-organ damage than the elevated BP itself, and is two significant risk factors of cardiovascular morbidity and mortality (Lanfranchi and Somers, 2002; Stevens *et al.*, 2016).

Moreover, HFD exposure can induce an autonomic dysregulation of the heart. Harenett and colleagues showed that mice fed on HFD for 14 weeks displayed a reduced bradycardia responses to the vagus nerve stimulation, suggesting a suppressed parasympathetic regulation of the heart (Hartnett *et al.*, 2015). Similarly, Verwaerde and colleagues used spectral analysis to assess HRV in HFD-exposed dogs. They found a reduction in the high frequency band of HRV, suggesting a decreased parasympathetic activity (Verwaerde *et al.*, 1999). A reduction in parasympathetic regulation has been shown to be associated with various cardiovascular diseases such as heart failure (Olshansky *et al.*, 2008).

While many experimental studies have demonstrated HFD-induced changes in the autonomic regulation of the cardiovascular system, most of them are looking these in the context of HFD-induced obesity and at prolonged exposure timepoint. We have limited information on HFD-induced changes in HRV and baroreflex function during early exposure stage. We also don't know the time course of HFD-induced cardiovascular dysfunctions. In this regard, one aim of chapter 3 is to understand the independent effects of HFD on cardiovascular function.

1.6 Potential SHS-HFD interactions

As described in the previous sections, SHS and HFD showed a few similarities in the underlying mechanisms of exposure-related cardiovascular morbidity and mortality. Exposure to SHS- and HFD-alone had been shown to lead to increased BP, increased sympathetic nerve activity, and a reduced HRV (reduced cardiac parasympathetic regulation) (Pope *et al.*, 2001; Pijanowska and Zajączkowska, 2004; Hartnett *et al.*, 2015; Speretta *et al.*, 2016). These similarities in SHS- and HFD-induced cardiovascular changes suggest that the two exposures may share some of the same pathways in inducing cardiovascular dysfunctions.

Although we have limited information on the effects of co-exposure of SHS and HFD on cardiovascular consequences, studies investigating the co-exposure effects of air pollution and HFD, or air pollution and obesity may provide us with some clues. For

instance, a prospective cohort study reported that the incidence of cardiovascular diseases caused by long-term exposure of $PM_{2.5}$ was related to the increase in body-mass index, suggesting the obese population was more susceptible to $PM_{2.5}$ exposure (Miller *et al.*, 2007). A recent study published by Jiang *et al.* found that HFD exposure aggravated $PM_{2.5}$-induced cardiac fibrosis in mice, suggesting co-exposure of HFD and $PM_{2.5}$ induced cardiovascular dysfunctions in a synergistic manner (Jiang *et al.*, 2020). Similarly, there are studies reported that HFD can exaggerate $PM_{2.5}$-induced vascular dysfunction and accelerated the progress of atherosclerosis in mice (Sun *et al.*, 2005; Xu *et al.*, 2010).

Furthermore, Martin *et al*. assessed the effects of a brief peat smoke exposure on cardiopulmonary response after a high-fat meal intake in rats. They found that consumption of a HFD after exposure to peat smoke could serve as a trigger for more extensive subsequent adverse cardiovascular responses, including an increase in isovolumic relaxation time, which indicates a poor myocardial relaxation and an impaired diastolic function (Martin *et al.*, 2018). These data suggest that with the co-exposure of HFD, one may reveal effects of air pollution that would otherwise be imperceptible. With these clues, the second aim of chapter 3 is to investigate how SHS and HFD may interplay with each other in altering the cardiovascular function.

To summarize, this study aims to understand the effects of SHS and HFD on cardiovascular function, as well as the potential interactions between these two risk factors under co-exposure. We hypothesized that while exposure to SHS- and HFD-alone

can independently induce cardiovascular dysfunctions, co-exposure of SHS and HFD can interactively exaggerate the exposure-related cardiovascular consequences.

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Chapter 2:

Environmentally relevant concentration of secondhand smoke exposure induced cardiovascular dysfunctions

Introduction

Worldwide, about 30-40% of non-smokers (Öberg *et al.*, 2011; Veeranki *et al.*, 2015) are exposed to secondhand smoke (SHS), a major indoor air pollutant and leading cause of preventable death (U.S. Department of Health and Human Services, 2014). SHS consists of the mainstream smoke that exhaled by smokers, and the sidestream smoke from the cigarette burning end. While the concentration of smoke that non-smokers are exposed during SHS exposure is about 100 times lower than that of active smokers, the effects of SHS exposure could be as large as 80-100% of those associated with active smoking (Barnoya and Glantz, 2005). Furthermore, even though the relative risk of SHS exposure is greater on the respiratory system, 70-80% of SHS-related deaths are due to cardiovascular causes (Brook, Brook and Rajagopalan, 2003). These data suggest that the cardiovascular system is extremely vulnerable to SHS.

It is well recognized that SHS exposure is related to increased incidences of ischemic heart disease (Lee, Chamberlain and Aldersont, 1986; Hole *et al.*, 1989), coronary heart disease (McElduff *et al.*, 1998), and myocardial infarction (Attard *et al.*, 2017). Exposure to SHS has also been shown to increase the risk of stroke (Malek *et al.*, 2015) and sudden cardiac death (Aune *et al.*, 2018). These data suggest that cardiovascular consequences

of SHS exposure are significant health concerns, and further emphasize the importance of understanding how SHS could affect cardiovascular function.

Heart rate variability (HRV), a measurement of variations in heartbeat intervals, is a commonly used measure to assess autonomic function. Intrinsic heart rate (HR) is set by the sinoatrial (SA) node pacemaker cells. The variability of HR (HRV) is dually regulated in the central nervous system (CNS) by the cardio-inhibitory parasympathetic (vagal) and the cardio-excitatory sympathetic branches of the autonomic nervous system. The autonomic nervous system can adjust both sympathetic and parasympathetic outputs to change HR over a wide dynamic range to maintain cardiac output to meet the physical and mental stresses of daily living while ensuring the favorable rapid slowing of heart rate after acute stresses. Loss of autonomic regulation of HR manifests a reduced HRV. Reduction of such variability indicates a loss of complexity and impaired ability to adapt to physiological stress (Malik *et al.*, 1996; Varadhan *et al.*, 2009). A reduced HRV is a well-established independent risk factor associated with increased susceptibility to ventricular arrhythmias and cardiovascular-related sudden death (Villareal, Liu and Massumi, 2002; La Rovere, Pinna and Raczak, 2008).

Growing evidence showed a correlation between SHS exposure and reduced HRV (Dinas, Koutedakis and Flouris, 2013). Pope *et al.* showed that 2-hr of SHS exposure (up to 0.15 mg/m^3) in an airport setting was sufficient to temporarily attenuate HRV during the exposure period (Pope *et al.*, 2001). Epidemiological studies found that chronic exposure of SHS at home or at work place is associated with lower HRV in adult non-smokers

(Dietrich *et al.*, 2007; Dinas, Koutedakis and Flouris, 2013). Key to resolving mechanisms underlying the SHS exposure-induced autonomic dysfunction is suitable animal models. We previously showed, in a mouse model of SHS exposure, that exposure to 30 mg/m^3 of sidestream cigarette smoke for three days significantly reduced HRV and increased arrhythmia susceptibility the day after exposure (Chen *et al.*, 2008). However, this effect was not observed with three days of exposure to a lower smoke concentration (3 mg/m^3). One drawback in most animal research is that a higher exposure concentration with a shorter exposure duration is often used, assuming same mechanisms underly both the high concentration/ short duration and low concentration/ long duration exposure conditions. In this regard, the first goal of this study was to determine the time-dependent changes in SHS exposure-induced decrease in HRV, using an environmentally relevant SHS concentration (3 mg/m^3).

Blood pressure (BP) is another measure commonly used to evaluate SHS exposure-induced changes in cardiovascular function. Epidemiological studies showed a correlation between chronic SHS exposure and significantly higher systolic BP (SBP) (Panagiotakos *et al.*, 2004). Hausberg and colleagues showed that acute exposure to an hour of SHS significantly increased BP in human subjects (Hausberg *et al.*, 1997). These data suggest that SHS exposure can increase cardiovascular morbidity and mortality risks by raising BP. Of relevance is that impaired baroreflex function and increased BP variability have been shown to cause more cardiovascular end-organ damage than the elevated BP per se (Lanfranchi and Somers, 2002; Stevens *et al.*, 2016).

Baroreflex is a key regulatory mechanism for moment-to-moment BP regulation. An increase in BP activates baroreceptors located in carotid sinus and aortic arch, resulting in a decrease in HR to restore BP back to control level. Conversely, a decrease in BP will trigger a reflex-mediated increase in HR to bring BP back to control level. The slope of this input-output relationship (changes in HR per until changes in BP) is referred to as baroreflex sensitivity (BRS) and is a measure of baroreflex function. A reduction in BRS suggests an impaired baroreflex function, and has been consistently reported in many pathological conditions, such as hypertension, diabetes, coronary artery disease, myocardial infarction, and heart failure (La Rovere, Pinna and Raczak, 2008). A reduced BRS has also been shown to be correlated with a higher risk of sudden cardiac death (Airaksinen *et al.*, 1998). In addition, baroreflex function is one of the contributors of BP variability (Rosei, Chiarini and Rizzoni, 2020). Therefore, the second goal of this study was to determine the time-dependent changes in SHS exposure-induced changes in BRS.

Materials and Methods

All protocols were approved by the University of California, Davis Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and Public Health Service Policy on Humane Care and Use of Laboratory Animals. All animals were housed individually on a 12-hr dark-light cycle (6:00 am – 6:00 pm) with standard rodent chow and water available *ad libitum* (temperature 69 ± 4°F, 60 ± 15% humidity, means ± SD).

BP and electrocardiogram (ECG) telemetry implants: Male C57/BL/6J mice (from The Jackson Lab, Sacramento, CA) arrived at UC Davis housing facility at 8 weeks old. Two weeks after their arrival, mice were anesthetized with isoflurane (5% induction, 1.5-3% maintenance). The criteria for adequacy of anesthesia include the following: 1) lack of eye blink reflex, 2) no whisker movement, 3) lack of paw pinch withdraw, and 4) no irregular or sudden changes in breathing frequency. A BP + ECG telemetry device (HD-X11, Data Sciences International, St Paul, MN, USA) was implanted subcutaneously in the left side of body via a small midline incision at the ventral neck region. The tip of the arterial catheter was placed into the aortic arch through the left common carotid artery. The two ECG leads were tunneled subcutaneously to obtain the lead II configuration. The negative ECG lead was sutured to the upper right pectoral muscle near the shoulder, and the positive ECG leas was sutured to the left lateral side of the xiphoid process. Mice were given Buprenex (0.05 mg/kg) subcutaneously prior to the end of surgery and twice daily post-operation for 2 days for pain control. Mice were allowed to recover from the

surgery for 2 weeks before the exposure regimen began. During the 2 weeks of recovery, mice were checked daily for signs of pain, such as excessive grooming and inactivity.

SHS exposure: This study used 3R4F cigarettes from the University of Kentucky Tobacco and Health Research Institute (Lexington, KY). Two cigarettes at a time were smoked in a staggered fashion under Federal Trade Commission conditions at a rate of 1 puff/min (35 ml/puff, 2 s duration). The smoke was diluted with filtered air (FA) in a mixing chamber and then passed into a stainless steel-and-glass Hinners-type exposure chamber that was 0.44 m^3 in size. Nicotine was sampled daily for 15 min during the exposure period. Mice were exposed (whole body exposure) in their home cage with wire lids, standard rodent chow, and water *ad libitum*. Total suspended particulate (TSP) concentration was sampled gravimetrically taken in the morning and again in the afternoon daily. Carbon monoxide concentration was measured every 30 min during the exposure period using a carbon monoxide analyzer (X-STREAM Gas Analyzer, Rosemount Analytical, Orrville, OH). The SHS exposure condition was: TSP 3.0 ± 0.2 mg/m^3, nicotine 0.2 ± 0.1 mg/m^3, and carbon monoxide 15.6 ± 1.8 ppm (means ± SD).

Restraint test: A 2-hr restraint test was performed: 1) to evaluate whether those SHS-induced cardiovascular changes at rest would be exaggerated under stress; and 2) to assess whether SHS altered the cardiovascular recovery from stress. Mice were restrained by placing them in flat bottomed restrainers. The restrainers were placed with animals in a prone position in their individual cage. Continuous BP and ECG were

recorded for 2-hr baseline before restraint, 2-hr restraint, and 6-hr recovery from restraint stress.

Recording protocols: Mice were randomly assigned to either FA- (n = 20) or SHS-exposed (n = 20) group. Mice were exposed to FA or SHS (6 hr/d, Monday through Friday) for 12 weeks followed by four weeks of recovery from SHS exposure (wk 16) (**Figure 2.1**). At week 4, 8, 12, and 16, right after Friday's exposure, continuous 36-hr BP and ECG recordings were performed from Friday 6:00 pm to Sunday 6:00 am. Then, a 2-hr restraint test was performed on Sunday morning. ECG signals were sampled at 4 kHz and BP signals were sampled at 500 Hz with Ponemah software (Data Sciences International, St Paul, MN, USA).

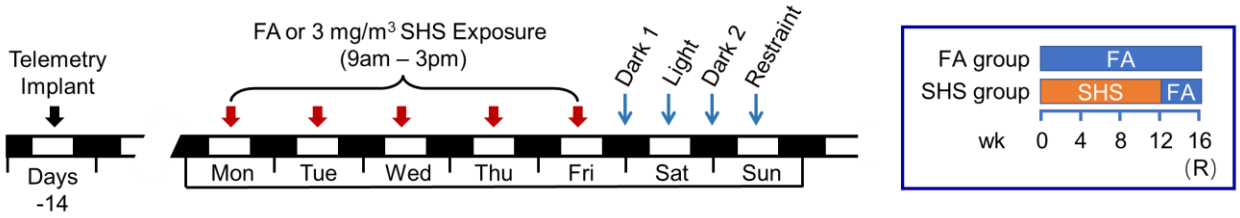

***Figure 2.1**: Schematic diagram for exposure regimen.*

Data analysis: Weekly body weight gained were calculated by subtracting week 1 body weight from each week's body weight.

R waves from the ECG recording were marked with Ponemah analysis attributes (**Table 2.1**). The following settings provided most optimal R wave detection in mice: 1) QRS detection threshold set at 25%, which means 25% of the largest rectified derivative signal within a QRS segment is used for identifying potential R waves; 2) Minimum R deflection set at 0.03 – 0.25 mV, which was the voltage threshold for R wave detection; 3) Peak

bias set at 20% to favor positive R wave. For HRV analysis, it is crucial that abnormal RR intervals (from ectopic beats, missing beats, noise, artifacts, arrhythmias etc,.) are excluded in the analysis for accurate HRV measures (Malik *et al.*, 1996). The following settings were included to minimize errors in R wave detection: 1) Maximum heart rate set at 1500 bpm, excluding all RR intervals corresponding to HR over 1500 bpm (from either non-physiological tachycardia or noise marked as R wave by the program); 2) Minimum heart rate set at 400 bpm, which means there could be an R wave that the program failed to mark and the software tried to find the missing R wave if R-R interval (RRI) was longer than 150 ms (corresponding to HR < 400 bpm).

After the initial R wave detection with the Ponemah software, two additional criteria were used to further exclude abnormal RRIs using Data Insights software (Data Sciences International, St Paul, MN, USA): 1) any RRI longer than 400 ms (HR < 150 bpm) were excluded (from either non-physiological bradycardia, AV block or the program failed to identify an R wave; 2) any RRI that differed by ≥ 20% from either adjacent RRIs were excluded (mostly from pre-matured ventricular complexs or first degree AV block) (Karey *et al.*, 2019). This 20% exclusion approach have been shown to have the best sensitivity in correctly including normal RRIs and specificity in eliminating abnormal RRIs and, thus, providing reliable estimates of time domain HRV measures from long-term continuous recordings in mice (Karey *et al.*, 2019).

Table 2.1: *Ponemah Analysis Attribute settings used for marking R waves in ECG.*

Attribute	Setting
QRS Detection Threshold	25%
Minimum R Deflection	0.03 – 0.25 mV
Peak Bias	20%
Maximum Heart Rate	1500 bpm
Minimum Heart rate	400 bpm

Standard HRV parameters (Malik *et al.*, 1996) were calculated from beat-to-beat RRI using Ponemah and excel (**Table 2.2**). The overall HRV (SDNN) and SDANN are indicators of influence from the autonomic regulation (sympathetic and parasympathetic) and humoral/ behavioral factors (Cowan, 1995). The intermediate HRV (SDNNIDX) is an index of influence within 2-min, mostly from the autonomic regulation (sympathetic and parasympathetic). The short-term HRV (rMSSD) is an index of HR change from one beat to another, dominated by parasympathetic regulation.

Table 2.2: *Standard time domain HRV measures and definitions.*

Parameter	Definition	Physiological Representation
SDNN (ms)	SD of all RRI	autonomic + humoral/ behavioral
SDANN (ms)	SD of all 2-min RRI averages	autonomic + humoral/ behavioral
SDNNIDX (ms)	Averages of SD of all 2-min RRI	autonomic (sympathetic + parasympathetic)
rMSSD (ms)	Root mean square of successive difference	autonomic (parasympathetic)

Systolic BP (SBP), diastolic BP (DBP), mean BP (MBP), and pulse pressure (PP) were averaged from each 12-hr light cycle. BP variability was obtained by taking standard deviation of SBP (SBP-SD), DBP (DBP-SD), and MBP (MBP-SD). BRS was evaluated by the sequence method (Bertinieri *et al.*, 1988). Using Data Insights software, beat-by-beat time series of SBP and RRI were scanned for sequences that consists of three or more consecutive beats that meet one of the following two criteria: 1) an increase in SBP with an increase in RRI, or 2) a decrease in SBP with a decrease in RRI. The threshold for changes in beat-to-beat SBP was set at 0.5 mmHg and the threshold for changes in beat-to-beat RRI was set at 5 ms. A three beats delay was used between SBP and RRI (Laude, Baudrie and Elghozi, 2009). A linear regression was applied to each baroreflex sequence and only r^2 values > 0.85 were accepted. The spontaneous BRS was obtained by averaging slopes of all accepted baroreflex sequences.

Statistical analysis: For the 36-hr monthly recordings, the data were divided into three 12-hr sections based on the dark-light cycle: the dark 1 cycle (from Friday 6:00 pm to Saturday 6:00 am), the light cycle (from Saturday 6:00 am to Saturday 6:00 pm), and the dark 2 cycle (from Saturday 6:00 pm to Sunday 6:00 am). All data are expressed as means ± SEM unless otherwise indicated. A two-way repeated measures ANOVA was used for analyzing the difference in weekly body weight and body weight gained with exposure (FA vs. SHS) as the between factor and time (weeks) as the within factor. A two-way ANOVA was used for analyzing the difference in BP, HR, BP variability, BRS, and HRV with exposure (FA vs. SHS) as one between factor and time (weeks 4, 8, and 12) as the other between factor, followed by Fisher's least significant differences tests

when appropriate. A *t*-test was used for analyzing the difference between the FA- and SHS-exposed mice after four weeks of recovery from SHS (wk 16). For restraint test, a two-way ANOVA was used for analyzing the difference in stress-induced changes in BP, HR, SDNN, and rMSSD with exposure (FA vs. SHS) as one between factor and time (weeks 4, 8, and 12) as the other between factor. A *t*-test was used for analyzing the difference in stress-induced changes in BP, HR, SDNN, and rMSSD between the two groups after four weeks of recovery from SHS (wk 16). $p < 0.05$ was considered statistically significant.

Results

There were 40 mice (20 FA and 20 SHS) in this study. Seven mice did not have BP signals and one mouse did not have ECG signals. Although mice in the SHS-exposed group had slightly lower body weight gained than those in the FA-exposed group (**Figure 2.2 B**), there was no significant difference in total body weight over time between the two groups (**Figure 2.2 A**), suggesting that SHS exposure, at this environmentally relevant concentration (3 mg/m^3), had no significant effect on overall body weight.

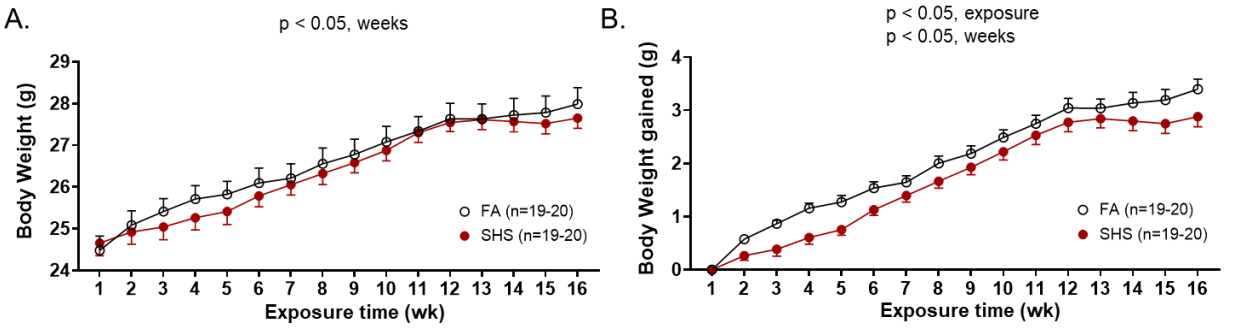

***Figure 2.2**: Group data of weekly body weight (**A**) and body weight gained (**B**).* Body weight increased over time in both groups. SHS-exposed group had lower body weight gained than FA-exposed group. However, there was no difference in total body weight between the two groups over time. SHS group were exposed to FA in weeks 13-16, a period of recovery from SHS exposure. Data were analyzed with a two-way repeated measures ANOVA.

The DSI telemetry system estimates the animal's activity level based on the strength of telemetry signal transmitted to the receiver antennas. Both the orientation of the animal relative to the receiver and the distance from the animal to the receiver antennas can change the signal strength. An activity count was generated when the signal strength changes by a certain amount and the recording system reports activity in counts per minute.

The mice decreased their nighttime activity levels over the 12-weeks FA/SHS period. There was an overall exposure effect on activity levels across all three light cycles. (**Figure 2.3**). Post-hoc test results showed that SHS exposure significantly increased activity levels across all three light cycles after four weeks of exposure and during the first dark cycle after 12 weeks of exposure (**Figure 2.3**). These data suggest that exposure to SHS increased activity levels during dark cycles when the animals were presumably more active. Four weeks after exposure stopped, higher activity levels during dark cycles persisted in the SHS-exposed group ($p < 0.05$, *t*-test).

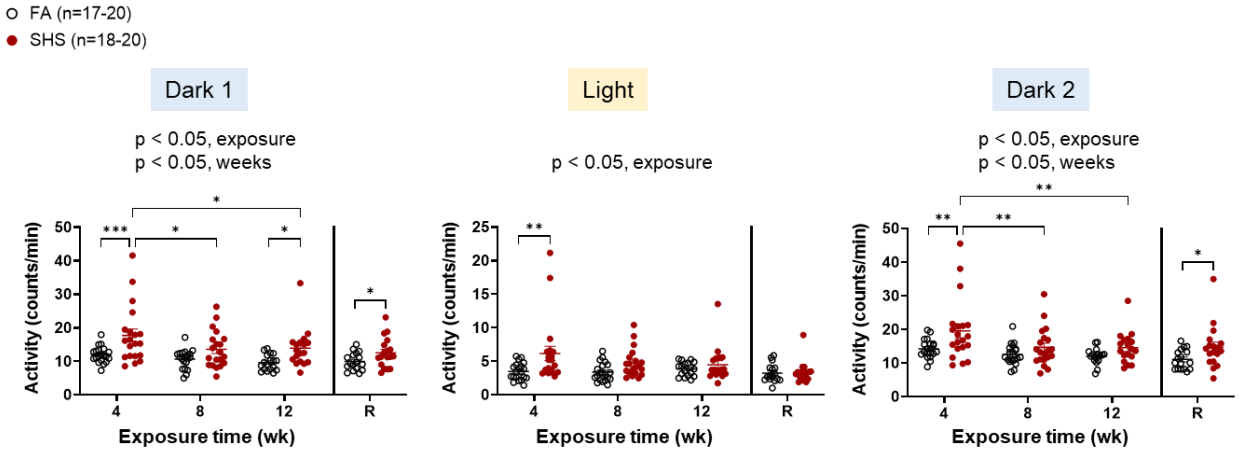

***Figure 2.3**: Group data of activity level after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. Data obtained during recovery period were compared with a *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Effects of SHS exposure on HR and HRV regulation

There was a gradual decrease in RRI over the 12-weeks exposure period. However, no significant effect of SHS exposure on RRI was observed throughout the 12 weeks of exposure (**Figure 2.4**).

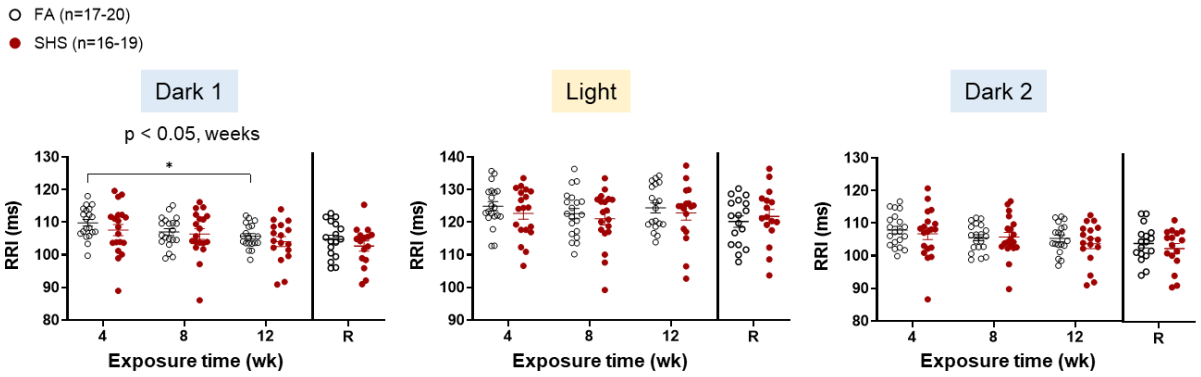

Figure 2.4*: Group data of RRI after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. Data obtained during recovery period were compared with a *t*-test. *$p < 0.05$

Overall HRV (SDNN) decreased over time in both FA- and SHS-exposed groups (**Figure 2.5 A**). This reduction in HRV was unlikely due to the decrease in RRI, since normalized SDNN for changes in baseline RRI did not change the results (**Figure 2.5 B**). For exposure effects, SHS significantly decreased the overall HRV (SDNN) during the dark 1 cycle (**Figure 2.5 A**). This SHS-induced reduction in SDNN was greater with longer exposure time (decreased by 4.7%, 4.1%, and 13.2%, weeks 4, 8, and 12, respectively). Since there was no difference in baseline RRI between FA- and SHS-exposed group, the data suggest that 12 weeks of SHS exposure decreased overall HRV.

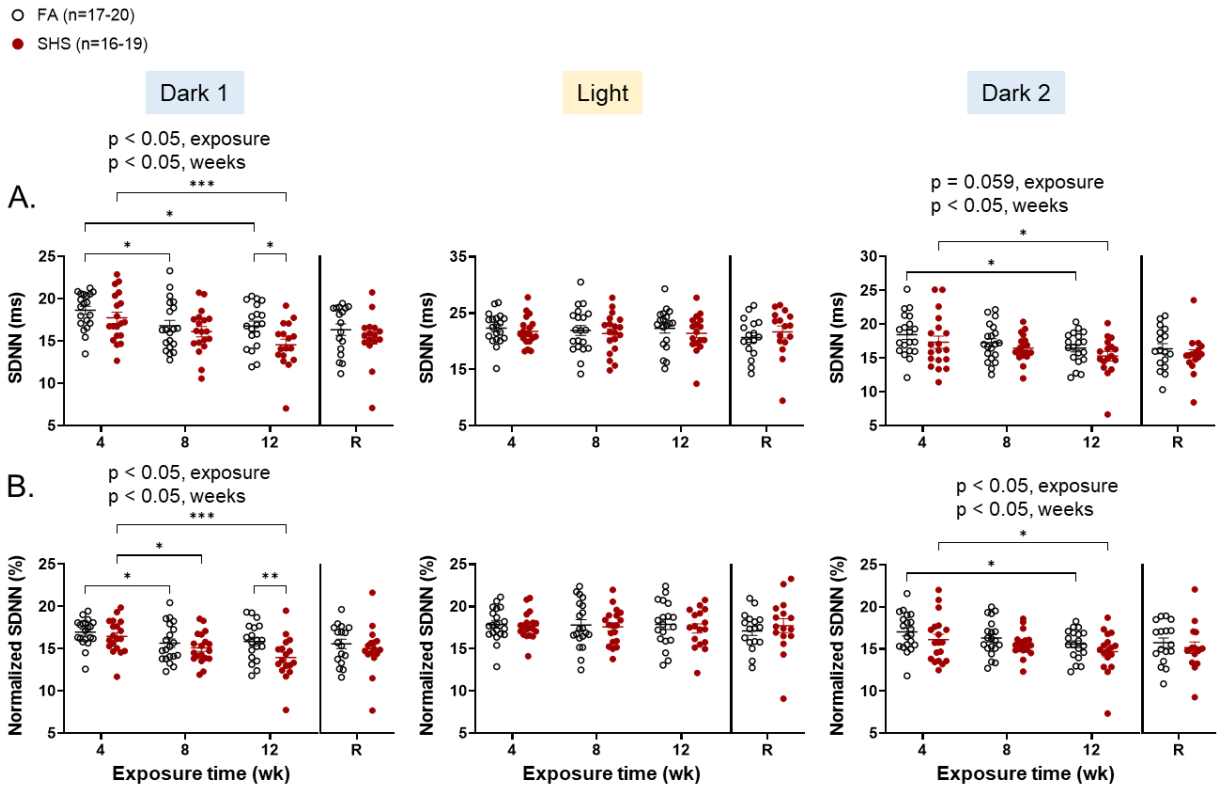

Figure 2.5*: Group data of SDNN (**A**) and normalized SDNN for changes in baseline RRI (**B**) after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. Data obtained during recovery period were compared with a *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

For HRV regulation between 2-min segments (SDANN), SHS significantly decreased SDANN during dark cycles with the magnitude of effect became greater with longer exposure duration (**Dark 1 cycle:** decreased by 5.6%, 8.0%, 21.4%, weeks 4, 8, and 12, respectively; **Dark 2 cycle:** decreased by 6.4%, 7.5%, 13.0%, weeks 4, 8, and 12, respectively) (**Figure 2.6**). Given the similarity in the effects of SHS on SDNN and SDANN (**Figure 2.5 & 2.6**), these data suggest that SDANN may be regulated by factors that also modified the overall HRV (SDNN).

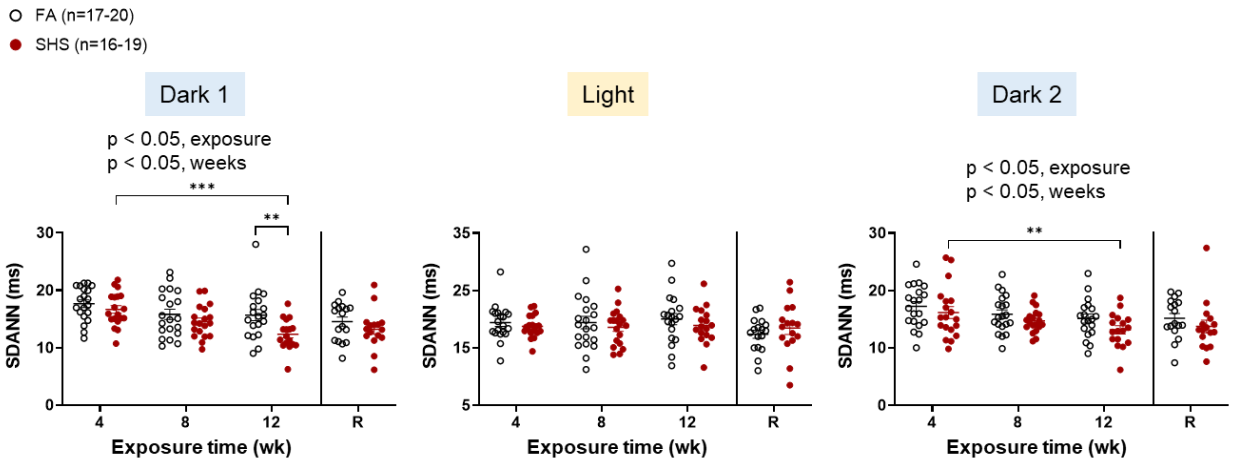

***Figure 2.6**: Group data of SDANN after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. Data obtained during recovery period were compared with a *t*-test. ***p* < 0.01, ****p* < 0.001

For HRV regulation within 2-min segments, SHS exposure significantly decreased SDNNIDX during the light cycle when the parasympathetic regulation was expected to be higher in mice (**Figure 2.7 A**).

For beat-to-beat HRV regulation (rMSSD), there was an overall SHS exposure effect (**Figure 2.7 B**). SHS-exposed mice had significantly lower rMSSD. Because the beat-to-beat changes in HR reflect the parasympathetic regulation, these data suggest that SHS exposure decreased cardiac parasympathetic regulation of HRV for the first 12-hr of post-exposure period.

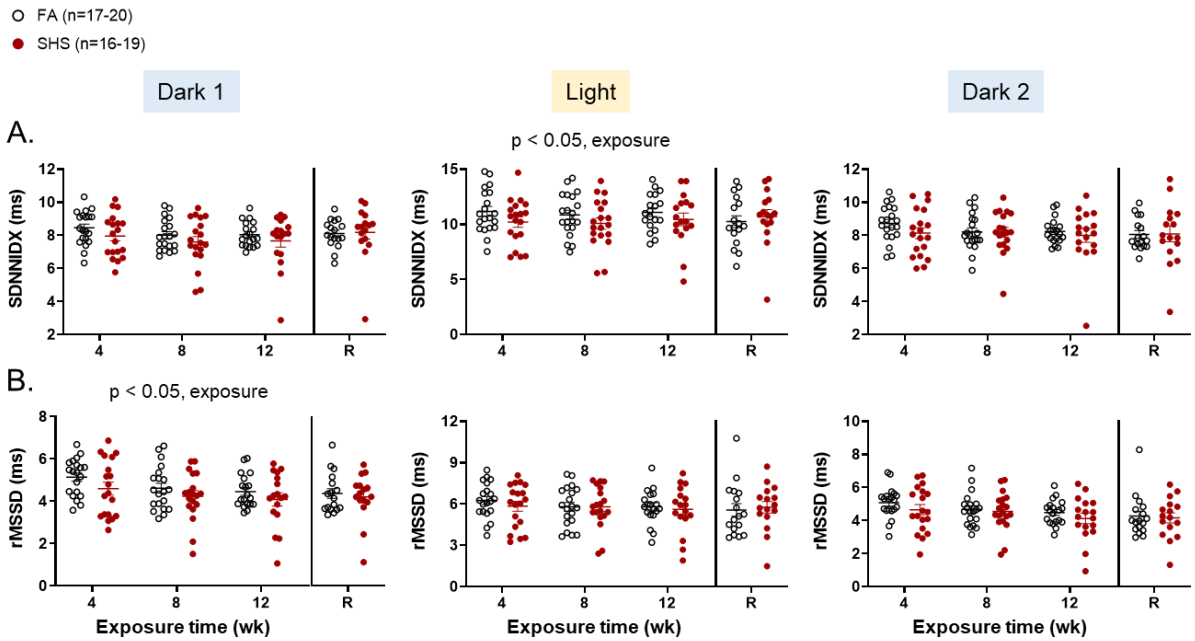

Figure 2.7*: Group data of SDNNIDX (**A**) and rMSSD (**B**) after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. Data obtained during recovery period were compared with a *t*-test.

After removal from SHS exposure for four weeks, no difference in any of the HRV measures was observed ($p > 0.05$, *t*-test) (**Figure 2.5, 2.6, and 2.7**). These data suggest that the effects of SHS on autonomic regulation of HRV did not persist and four weeks of exposure cessation was sufficient for a complete recovery from SHS exposure.

Effects of SHS exposure on BP and BP regulation

BP was recorded from 33 mice (16 FA and 17 SHS). There was a significant overall exposure effect on daytime SBP when animals were less active (**Figure 2.8 A, light cycle**). After 12 weeks of exposure, SHS significantly decreased SBP by 3.3 mmHg during the light cycle. This SHS-induced decrease in SBP recovered after removal from

SHS exposure for four weeks ($p > 0.05$, *t*-test). In contrast, SHS-exposed group showed a significantly higher DBP (~3 mmHg) after four weeks of exposure and a subsequent trend for lower DBP after 12 weeks of exposure (**Figure 2.8 B**). However, the MBP was not different between SHS- and FA-exposed groups in all three light cycles (**Figure 2.8 C**). Overall, these data suggest a biphasic BP response to SHS exposure: an increased DBP at earlier time point (4 weeks) and a decreased SBP with more prolonged exposure (12 weeks).

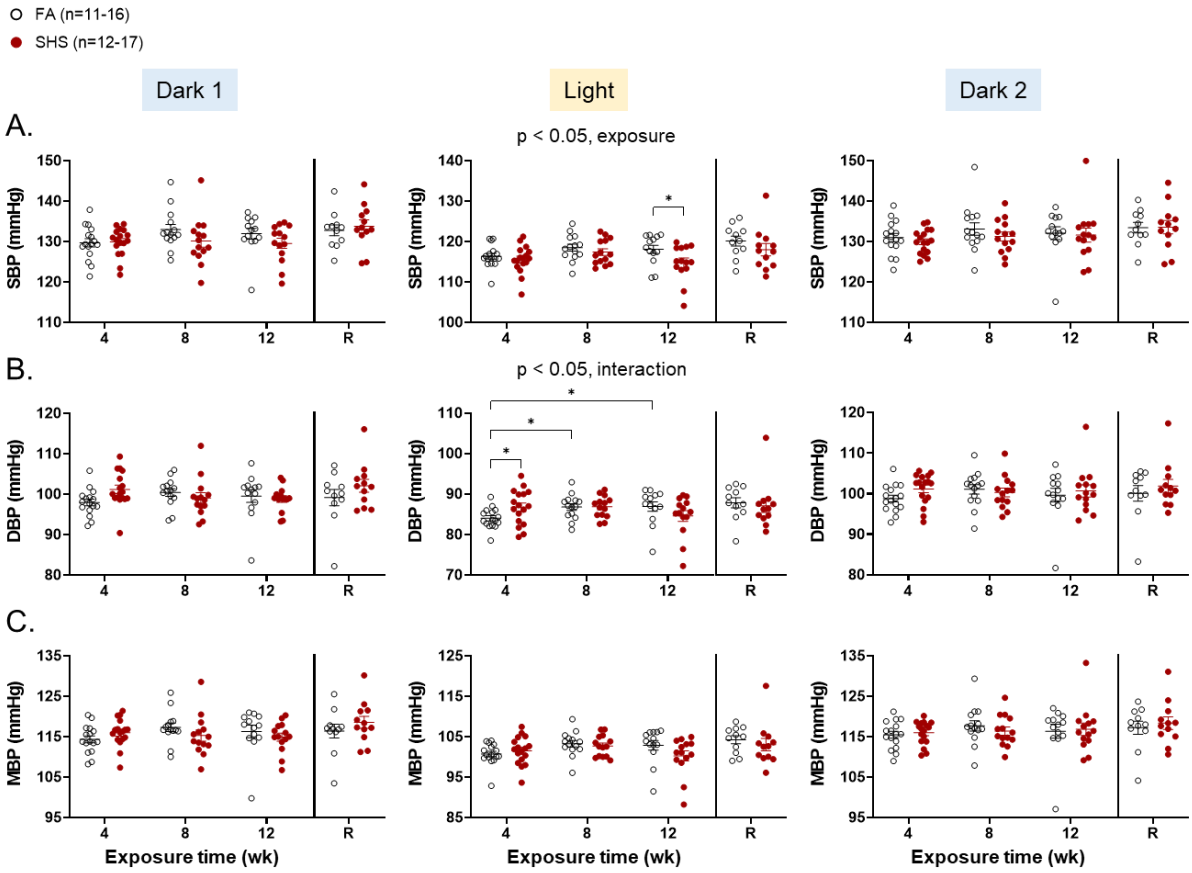

Figure 2.8*: Group data of SBP (**A**), DBP (**B**), and MBP (**C**) after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests. Data obtained during recovery period were compared with a *t*-test. *$p < 0.05$

There was an overall exposure effect on PP across all three light cycles (**Figure 2.9**). Mice from the SHS-exposed group showed a lower PP. This SHS-induced decreased PP was the most significant after four weeks of exposure. However, the SHS-induced decrease in PP recovered after the cessation of SHS exposure ($p > 0.05$, *t*-test). Together with the findings on SBP and DBP, these data suggest that the reduced SBP seen at week 12 may be due to the reduction in PP.

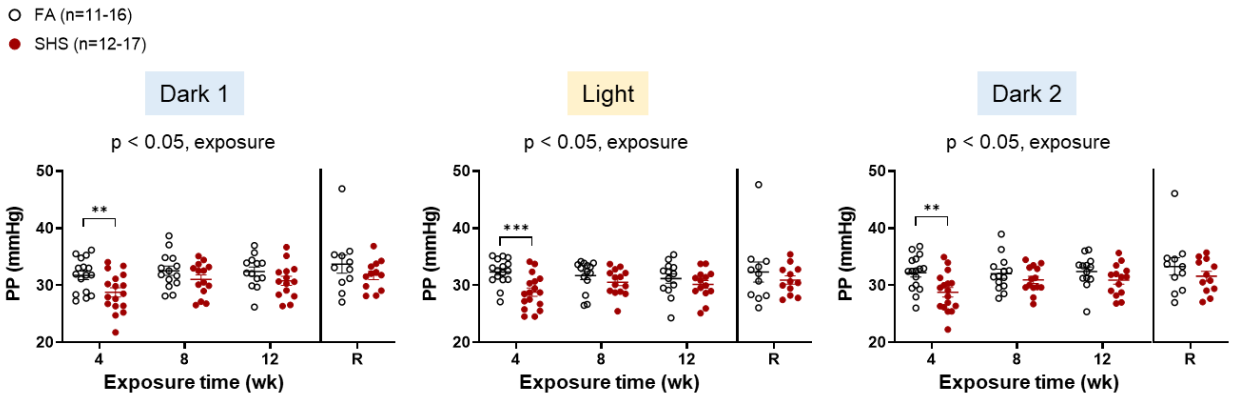

***Figure 2.9**: Group data of PP after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests. Data obtained during recovery period were compared with a *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$

Thirty-two mice (16 FA and 16 SHS) that had both BP and ECG signals were used for BRS analysis. During the dark cycles, both FA- and SHS-exposed groups showed an increase in BRS over the 12-weeks exposure period (**Figure 2.10**). There was an overall exposure effect on BRS during the dark 1 cycle. The BRS in the SHS-exposed group was 2.3%, 6.4%, and 10.0% lower than the FA control group (weeks 4, 8, and 12, respectively). These data suggest that SHS exposure attenuated BRS. There was no exposure effect during the subsequent light and dark 2 cycle, suggesting that the exposure effect has a faster recovery rate than the effect on PP. Not surprisingly, after removal from SHS

exposure for four weeks, there was no significant difference in BRS between SHS- and FA-exposed groups ($p > 0.05$, *t*-test).

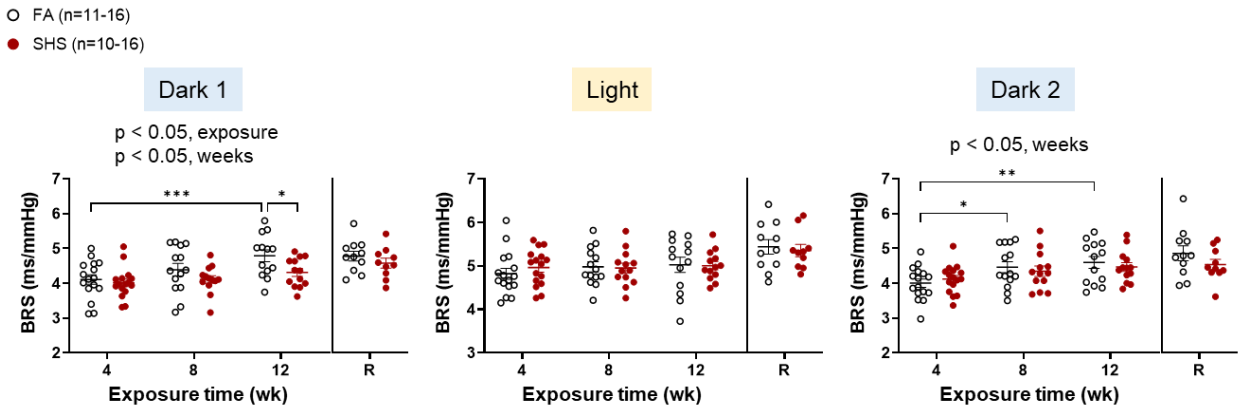

Figure 2.10*: Group data of BRS after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA followed by Fisher's LSD tests. Data obtained during recovery period were compared with a *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

There was no detectable difference in standard deviation of SBP, DBP, and MBP throughout the 12 weeks of exposure (**Figure 2.11**). These data suggest that despite a SHS-induced attenuated BRS during exposure period, BP variability was not elevated. However, after removal from SHS exposure for four weeks, SHS-exposed group showed a lower DBP-SD and MBP-SD during the dark 1 cycle ($p < 0.05$, *t*-test) (**Figure 2.11 B-C**), raising the possibility that factors other than baroreflex function may contribute to the changes in BP variability.

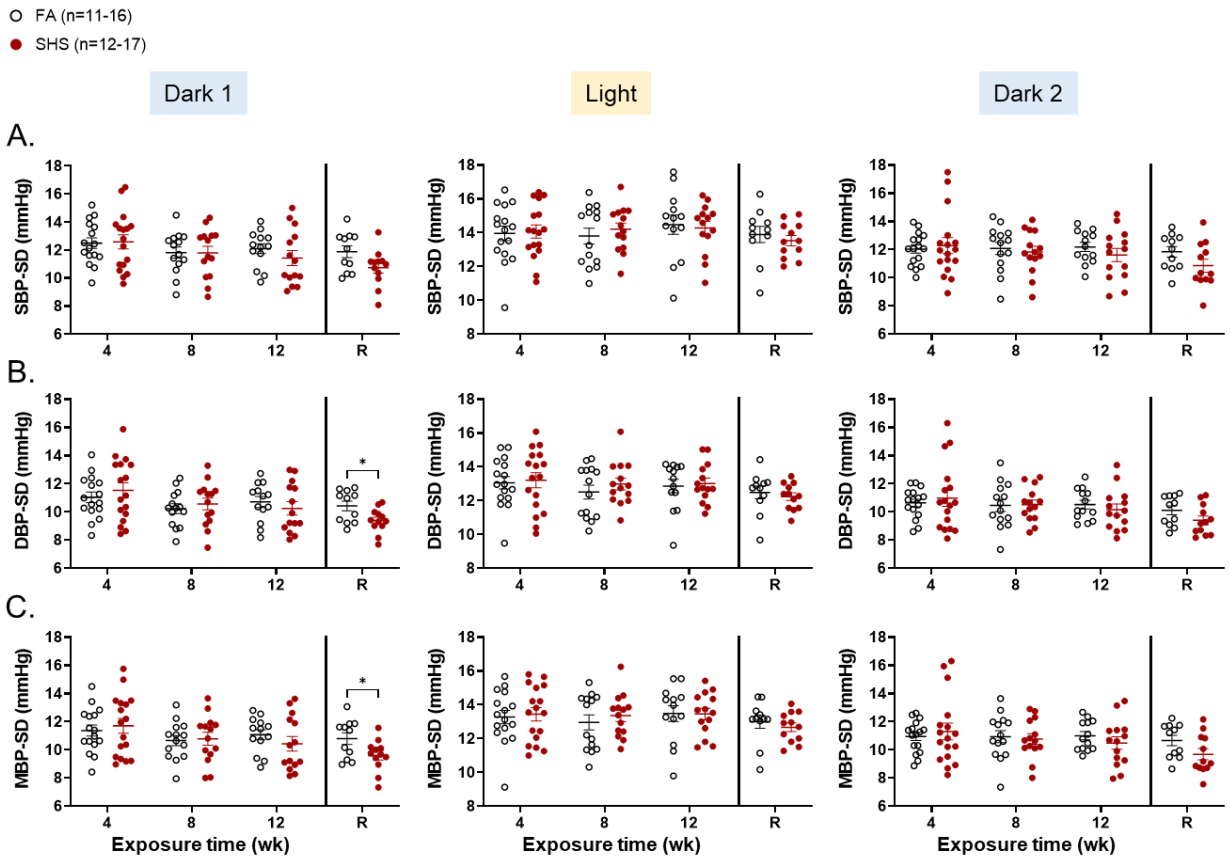

Figure 2.11*: Group data of SBP-SD (**A**), DBP-SD (**B**), and MBP-SD (**C**) after 4, 8, and 12 weeks of SHS or FA exposure followed by four weeks of recovery from SHS exposure (R).* Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA. Data obtained during recovery period were compared with a *t*-test. *$p < 0.05$

Effects of SHS exposure on cardiovascular responses to stress

Figure 2.12 (left) shows 30-min averages of MBP, HR, SDNN, and rMSSD during the 2-hr baseline, 2-hr restraint stress, and 6-hr recovery period after four weeks of FA or SHS exposure. All animals showed increased MBP/ HR and decreased SDNN/ rMSSD during the 2-hr restraint period compared to the baseline. After removed from the restraint, all animals had their MBP, HR, SDNN, and rMSSD gradually returned to baseline level. The magnitudes of restraint stress-induced changes (delta changes) in MBP, HR, SDNN, and rMSSD are presented in the right panels of **Figure 2.12**. SHS-exposed mice had similar

changes of MBP, HR, SDNN, and rMSSD in response to stress as the FA-exposed mice. This was also true after four weeks of recovery from SHS exposure.

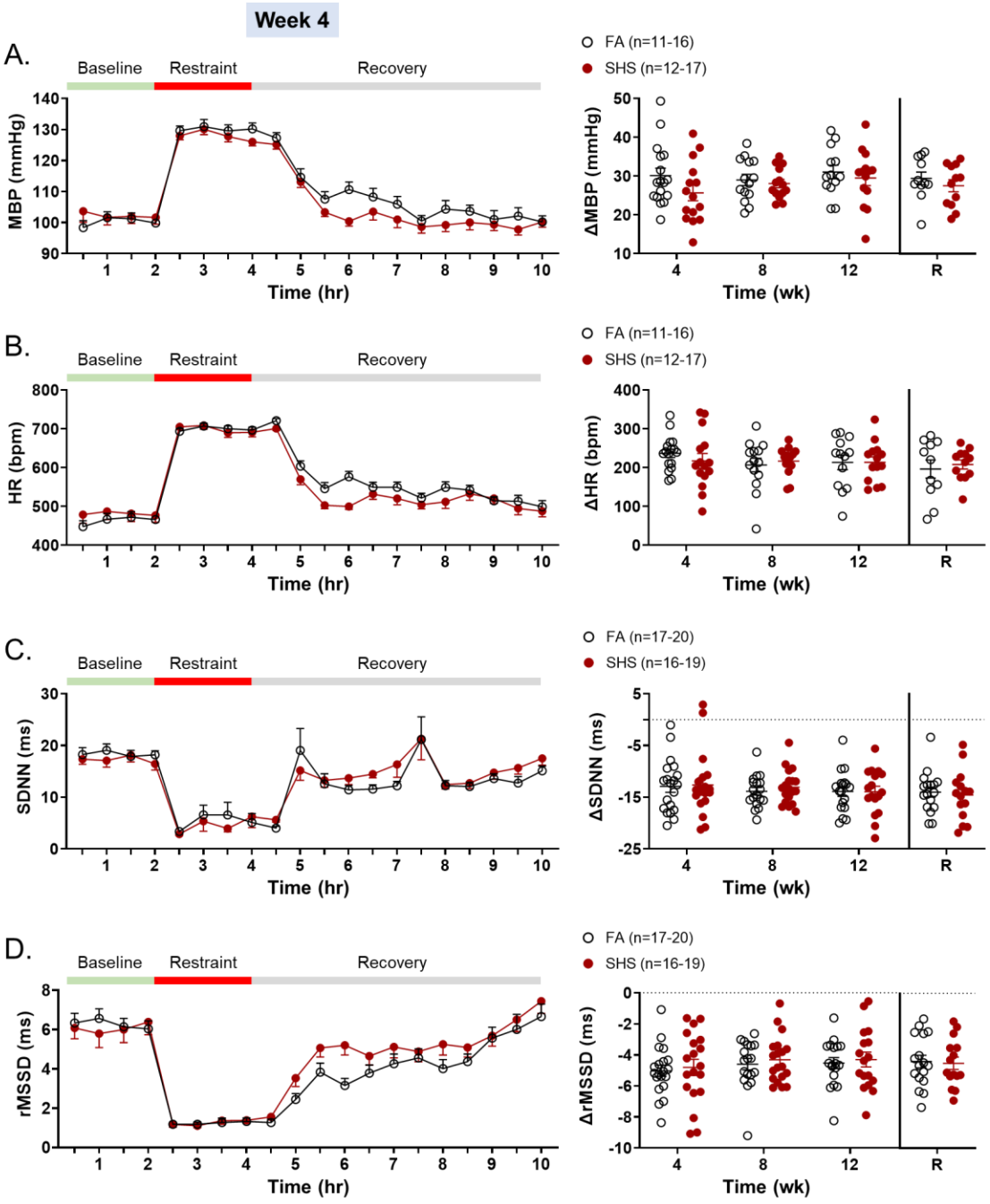

***Figure 2.12**: Group data of MBP (**A**), HR (**B**), SDNN (**C**), and rMSSD (**D**) during the restraint test.* Left panels: 30-min averaged values during the 2-hr baseline, 2-hr restraint, and 6-hr recovery period. Right panels: stress-induced delta changes from the 2-hr baseline. Data obtained during exposure period (weeks 4-12) were analyzed with a two-way ANOVA. Data obtained during recovery period were compared with a *t*-test.

Discussion

The major finding of this study is that 12 weeks of exposure to an environmentally relevant concentration of SHS (similar to a smoky bar) decreased measures of autonomic regulation of HR (HRV) and BP (BRS) as well as PP, presumably due to a decrease in contractile function of the heart. All these SHS-induced cardiovascular changes were reversible after removal from SHS for four weeks. The SHS-induced changes in cardiovascular function showed a time-dependent manner in which the effects of SHS on PP and HRV had a faster onset (wk 4), while the decrease in BRS was observed with a slower onset and accumulated with longer exposure time (wk 12). These data demonstrated that chronic exposure to an environmentally relevant concentration of SHS can result in cardiovascular dysfunctions that is reversible.

SHS exposure on BP

The most striking and unexpected finding of this study is the SHS-induced decrease in PP. Such decrease in PP was first observed after four weeks of SHS exposure and persisted throughout the 12 weeks exposure period across all three light cycles (**Figure 2.9**). Similar to our results, a study in dog showed that acute exposure to fine particulate matter ($PM_{2.5}$) for five hours led to a significantly lower PP (Bartoli *et al.*, 2009). Additionally, Chen *et al.* showed that, in humans, short-term exposure to air pollution resulted in a reduced PP in adult nonsmokers (Chen *et al.*, 2012). As PP is proportional to the volume of blood ejected from the ventricle (stroke volume) during each cardiac cycle and inversely proportional to arterial compliance (Chaudhry, Miao and Rehman,

2020), the SHS-induced decrease in PP may suggest a decrease in the stroke volume and/or an increase in arterial compliance.

Several studies have demonstrated that exposure to SHS is associated with an increase in arterial stiffness – in other words, a decrease in arterial compliance (Mack *et al.*, 2003; Doonan *et al.*, 2010). Mack and colleagues evaluate the association between SHS exposure and arterial stiffness using ultrasonographic methods. They found that in adult non-smokers, SHS increased the arterial stiffness in a dose-dependent manner, in which the carotid stiffness index beta increased with the number of hours of SHS exposure increased (Mack *et al.*, 2003). Similarly, Argacha *et al.* showed that an hour of acute exposure of SHS led to an increased augmentation index, which suggest a decreased aortic compliance, in male nonsmokers (Argacha *et al.*, 2008). Taken together, these data suggest that the decrease in PP observed in this study is unlikely due to an increased vascular compliance, raising the possibility of SHS-induced reduction in cardiac contractility.

Epidemiological studies have shown a correlation between chronic SHS exposure and elevated BP (Panagiotakos *et al.*, 2004). Le and colleagues (Le *et al.*, 2021) showed that mice exposed to 12 weeks of SHS had increased myogenic tone in mesenteric arteries. These data suggest that an increased arterial myogenic tone may contribute, in part, to the elevated BP. In the present study, even though DBP was elevated after four weeks of SHS exposure, it was transient. After 12 weeks of exposure, SBP was lower in the SHS-exposed group (**Figure 2.8 A**). The reason for this SHS-induced decrease in SBP

is not entirely clear, but the significant reduction in PP after SHS exposure, which presumably due to a decreased cardiac contractility and stroke volume, may explain this lower SBP with prolonged exposure. As PP is an indirect measure of cardiac function, to better understand how SHS may induce cardiac structural and functional changes that can lead to the SHS-related decrease in SBP, further studies are needed for accurate assessment of cardiac function, such as using echocardiography. In relation to this, several studies showed that chronic exposure to SHS leads to an increase in left ventricular end systolic and diastolic diameters, an increase in left ventricular mass, and inhibited fractional shortening in mouse hearts using echocardiography, suggesting a SHS-induced impairment of cardiac function (Hu *et al.*, 2013; Wang *et al.*, 2020).

SHS exposure on autonomic regulation of BP

Our results showed that 12 weeks of SHS exposure significantly reduced BRS (**Figure 2.10**). A reduced BRS has been shown to be associated with increased cardiovascular morbidity and mortality (La Rovere, Pinna and Raczak, 2008). Many studies showed that reduced BRS was linked to increased risks of end-organ damage, progression and development of cardiovascular diseases including hypertension, coronary artery disease, myocardial infarction, and heart failure (La Rovere *et al.*, 1998; La Rovere, Pinna and Raczak, 2008). Our results provide evidence that long-term exposure of SHS impairs the baroreflex function that can result in sympatho-vagal imbalance and increase the risks for cardiovascular morbidity and mortality.

In the present study, spontaneous BRS was determined using the sequence method. The

sequence method was first described in unanesthetized cats to study the baroreflex function without pharmacological or mechanical interventions (Bertinieri *et al.*, 1988). Subsequently, this method was validated pharmacologically and surgically (via sinoaortic denervation). First, spontaneous BRS obtained from the sequence method is comparable to the BRS obtained from classic drug-induced changes in BP (Parlow *et al.*, 1995; Pitzalis *et al.*, 1998). Second, removal of baroreceptor input with sinoaortic denervation dramatically reduced the number of spontaneous baroreflex sequences (Bertinieri *et al.*, 1988). Furthermore, Frankel and colleagues found that, in conscious dogs, the spontaneous BRS obtained by the sequence method was abolished after bilateral carotid sinus denervation or the administration of a ganglionic blocker, hexamethonium (Frankel, Metting and Britton, 1993). Lastly, surrogate data analysis in mice showed that scrambled and randomized data had a lower sequence number and BRS than the original data (Laude, Baudrie and Elghozi, 2008). These studies suggest that the sequence method is a validated approach for the BRS assessment.

Several researches have used pharmacological blockades to evaluate the autonomic relevance of spontaneous BRS. Frankel *et al.* showed that atropine (parasympathetic blocker) abolished spontaneous BRS (Frankel, Metting and Britton, 1993). Similarily, Laude *et al.* showed that while administration of atropine significantly reduced BRS, administration of atenolol (sympathetic blocker) did not affect BRS in mice (Laude, Baudrie and Elghozi, 2008). These data suggest that the BRS obtained from the sequence method is prodominantly under the parasympathetic regulation. Taken together, the SHS-induced reduction in spontaneous BRS suggests a reduced parasympathetic

regulation of the cardiovascular system.

SHS exposure on autonomic regulation of HRV

Standard measures of time-domain HRV were used in this study. Our results showed that 12 weeks of SHS exposure significantly reduced overall HRV (SDNN) and SDANN (**Figure 2.5 & 2.6**). These two measures estimate the long-term components of HRV that is regulated by factors including the autonomic nervous system and humoral/ behavioral alternations (Malik *et al.*, 1996). Therefore, the SHS-induced decrease in SDNN and SDANN indicating an overall impaired HRV regulation of the cardiovascular system. To underscore the autonomic components of this SHS-induced impaired HRV regulation, we looked into measures that estimate the short-term components of HRV (**Figure 2.7**). Our results showed that exposure of SHS significantly reduced beat-to-beat HRV regulation (rMSSD), which predominantly reflects a decreased parasympathetic regulation of the heart (Malik *et al.*, 1996). Similarly, SDNNIDX measures variability of HR within 2-min segments and is primarily regulated by both sympathetic and parasympathetic nervous system. We found that 12 weeks of SHS exposure decreased SDNNIDX during the light cycle when mice were less active and had higher parasympathetic activity. Taken together, these data suggest that 12 weeks of SHS exposure impaired the autonomic regulation of HRV, particularly decreased parasympathetic regulation of the heart and, thus, increase the risks of cardiovascular morbidity and mortality.

The parasympathetic regulation of HR and beat-to-beat HRV is innervated by the cardiac vagal neurons (CNVs) located in *nucleus ambiguus* (McAllen and Spyer, 1978; Geis and

Wurster, 1980). CNVs do not have pacemaker-like activity, thus their firing is totally dependent on synaptic inputs from other region such like the *nucleus tractus solitarius* (Mendelowitz, 1999). Our previous study showed that four weeks of SHS exposure, at an environmentally relevant concentration, resulted in a decreased neuronal input-output relationship, which is attributable to the higher voltage/ current threshold required for action potential generation and lower spiking response to depolarizing stimuli, of these CVNs (Sun *et al.*, 2021). In this regard, the SHS-induced decreased intrinsic excitability of CVNs can lead to attenuated parasympathetic output to the heart, which may explain the decreased HRV observed after SHS exposure.

Recovery from SHS exposure

Four weeks after removal from SHS exposure, all SHS-induced changes in BP, BRS, and HRV were recovered. These results further underscore the importance of implementing smoke-free policies. Growing evidence showed that implementation of smoke-free laws resulted in a reduction in hospital admissions for acute myocardial infarction, coronary syndrome, and heart attack (Juster *et al.*, 2007; Pell *et al.*, 2008; Herman and Walsh, 2011). In 2003, New York State have implemented a statewide comprehensive smoke-free law to restrict smoking in workplaces, bars, and restaurants. It has been shown that, upon the implementation of the smoke-free policy, there was a 50% decrease in population that exposed to SHS. Juster and colleagues showed that this was associated with a reduction in hospital admissions for acute myocardial infarction by 8% in the following year (Juster *et al.*, 2007). Similarly, Herman and Walsh showed that the implementation of smoking ban in Arizona State resulted in a significant reduction in

hospital admissions for acute myocardial infarction, stroke, asthma, and angina (Herman and Walsh, 2011). Thus, a smoke-free environment not only help preventing non-smokers from being exposed to harmful SHS, but also help improving health of those who previously exposed to SHS.

Conclusions

Chronic SHS exposure reduces autonomic regulation of the cardiovascular functions, particularly the regulation of the heart. These cardiovascular effects were transient, and reversed themselves in the absence of SHS exposure, underscoring the importance and efficacy of public smoking bans.

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Chapter 3:

Effects of co-exposure to secondhand smoke and high-fat diet on cardiovascular function

Introduction

Over the past few decades, cardiovascular disease incidence significantly decreased by 27.3% (Amini, Zayeri and Salehi, 2021). Paradoxically, the number of deaths from cardiovascular diseases increased by 42.4% over the same period, owing to population growth and longevity (Amini, Zayeri and Salehi, 2021). Consequently, cardiovascular diseases became the leading cause of death worldwide. As described in chapter 1, cardiovascular diseases are complex, multifactorial diseases that cannot be ascribed to a single factor (Kiberstis and Roberts, 2002). High-fat diet (HFD) and secondhand smoke (SHS) are two well-known preventable risk factors for the development and progression of cardiovascular diseases (Center for Disease Control and U.S. Department of Health and Human Services, 2006; Roth *et al.*, 2020; Wali *et al.*, 2020).

HFD can lead to obesity and increase cardiovascular risks through several mechanisms including impaired endothelial function, atherosclerosis, increased inflammation and oxidative stress, and impaired autonomic regulation of the cardiovascular system (Li, Dai and Jia, 2009; Dow *et al.*, 2015; Chaar *et al.*, 2016; Kesh, Sarkar and Manna, 2016; Duan *et al.*, 2018). Plotnick and colleagues found that intake of a high-fat meal resulted in an acute decrease in brachial artery vasoactivity for up to 4 hours, suggesting an impaired

endothelial function (Vogel, Corretti and Plotnick, 1997; Plotnick *et al.*, 2003). Another study from Dow *et al.* showed that habitual consumption of HFD leaded to an impaired nitric oxide-mediated endothelium-dependent vasodilation in healthy adults (Dow *et al.*, 2015), and such vascular endothelial dysfunction is a major contributor to the progression of atherosclerosis (Kawashima and Yokoyama, 2004; Weil *et al.*, 2011). A growing body of evidence demonstrated that HFD induces systemic inflammation and oxidative stress, both are established risk factors for cardiovascular diseases (Kesh, Sarkar and Manna, 2016; Duan *et al.*, 2018).

Similarly, adult non-smokers exposed to 30 min of SHS had a significantly decrease in coronary flow velocity reserve, suggesting an impaired endothelial-dependent vasodilation (Otsuka *et al.*, 2001). Chronic exposure to SHS also has been shown to increase oxidative stress and inflammatory response in adult non-smokers (Panagiotakos *et al.*, 2004). Furthermore, both SHS and HFD are associated with increase blood pressure (BP) with elevated sympathetic nerve activity and vascular myogenic tone (Hausberg *et al.*, 1997; Mahmud and Feely, 2004; Armitage *et al.*, 2012; Muntzel *et al.*, 2012). These similarities in SHS- and HFD-induced changes suggest that the two exposures may share some of the same pathways in inducing cardiovascular dysfunctions. However, whether co-exposure to SHS and HFD induces additive, overlapping, or synergistic effects is not entirely clear.

Interestingly, several epidemiological studies showed that women exposed to SHS tend to have unhealthy diet habits and life style (Koo, 1988; Thompson and Warburton, 1992).

Koo *et al.* (Koo *et al.*, 1997) showed that never-smoked wives with smoking husbands had a significantly higher total fat intakes than never-smoked wives without smoking husbands. Similarly, Thompson and Warburton showed that non-smokers living in smoking households ate more fried food and had a higher intake of fat than non-smokers living in non-smoking households (Thompson and Warburton, 1992). Furthermore, people who consumed food at restaurants and bars, a major source of SHS exposure outside of households, have been shown to had higher intakes of saturated or total fat, sugars, and sodium (Lin and Guthrie, 2013; Wellard-Cole, Davies and Allman-Farinelli, 2021).

Moreover, researchers found that the obese population was more susceptible to $PM_{2.5}$ exposure and was under a higher risk of $PM_{2.5}$-related cardiovascular morbidity and mortality (Miller *et al.*, 2007). Although there are limited studies looking at the co-exposure effect of SHS and obesity on cardiovascular function, there are some evidences suggested that SHS exposure and obesity can synergistically affect subject's health. For instance, Kurniasari and colleagues showed a synergistic effect of SHS exposure and overweight on increased risk of gouty arthritis in humans (Kurniasari *et al.*, 2021). Similarly, Wu *et al.* showed that exposure to SHS was associated with worsened symptoms of asthma in overweight and obese children than lean children, suggesting overweight subjects might be more susceptible to SHS exposure (Wu *et al.*, 2018).

These findings further emphasize the need to understand the cardiovascular effects of co-exposure of SHS and HFD. However, we have limited information on how HFD may

interact with SHS in changing cardiovascular function. To fill this gap, this study aimed to investigate the effects of HFD and SHS co-exposure on cardiovascular function. Mice were exposed to an environmentally relevant concentration of SHS (3 mg/m^3) and fed on a diet that contained 60 kcal% from fat. BP, heart rate (HR), heart rate variability (HRV) and baroreflex sensitivity (BRS) were obtained to evaluate cardiovascular function.

Materials and Methods

All protocols were approved by the University of California, Davis Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and Public Health Service Policy on Humane Care and Use of Laboratory Animals. All animals were housed individually on a 12-hr dark-light cycle (6:00 am – 6:00 pm) in a temperature-controlled room (71 ± 2°F, 33 ± 15% humidity, means ± SD).

BP and electrocardiogram (ECG) telemetry implants: Twenty-four male C57/BL/6J mice (from The Jackson Lab, Sacramento, CA) arrived at UC Davis housing facility at 8 weeks old. Two weeks after their arrival, mice were implanted with BP/ECG telemetry devices as described in chapter 2. Mice were fed with standard rodent chow and water available *ad libitum* upon arrival and through two weeks of recovery from surgery.

SHS exposure: SHS exposure was performed as described in chapter 2. The SHS exposure condition was: TSP 3.0 ± 0.1 mg/m^3, nicotine 0.9 ± 0.1 mg/m^3, and carbon monoxide 16.6 ± 1.6 ppm (means ± SD).

Recording protocols: After two weeks of recovery from surgery, all mice were put on a solid HFD (60 kcal% fat, D12492, Research Diets, Inc, New Brunswick, NJ, USA) throughout the exposure regimen. Mice were randomly assigned to either a filtered air- (FA, n = 12) or SHS-exposed (n = 12) group. The original plan was to expose mice to FA or SHS (6 hr/d, Monday through Friday) for 12 weeks followed by four weeks of recovery

from SHS exposure (same exposure regimen as in chapter 2). However, there was a fire at the exposure facility at week 12 and mice were exposed to smoke from the fire. Thus, only 11 weeks of exposure data were collected without recovery from SHS period. At week 4, 8, and 11, right after Friday's exposure, continuous 36-hr BP and ECG recordings were performed from Friday 6:00 pm to Sunday 6:00 am. A 2-hr restraint test was performed on Sunday morning at weeks 4 and 8 as described in chapter 2. Continuous BP and ECG were recorded for 2-hr baseline before restraint, 2-hr restraint, and 6-hr recovery from restraint stress. ECG signals were sampled at 4 kHz and BP signals were sampled at 1000 Hz with Ponemah software (Data Sciences International, St Paul, MN, USA).

Data analysis: As described in chapter 2, data were divided into three 12-hr sections: the dark 1 cycle (from Friday 6:00 pm to Saturday 6:00 am), the light cycle (from Saturday 6:00 am to Saturday 6:00 pm), and the dark 2 cycle (from Saturday 6:00 pm to Sunday 6:00 am). Weekly body weight gained were calculated by subtracting week 1 body weight from each week's body weight.

Systolic BP (SBP), diastolic BP (DBP), mean BP (MBP), and pulse pressure (PP) were averaged from each 12-hr light cycle. BP variability was obtained by taking standard deviation of SBP (SBP-SD), DBP (DBP-SD), and MBP (MBP-SD) during each 12-hr light cycle. Spontaneous BRS was evaluated by the sequence method. Standard time-domain HRV parameters were obtained from normal-to-normal RRIs as described in chapter 2.

Statistical analysis: All data are expressed as means ± SEM unless otherwise indicated. Mice exposed to FA and fed on normal diet (ND) from chapter 2 were used to compare with the HFD+FA group after 11 weeks of exposure for examining the effects of diet alone. For effects of 11 weeks of HFD without SHS exposure: 1) A two-way repeated measures ANOVA was used for analyzing the difference in weekly body weight with diet (ND vs. HFD) as the between factor and time (weeks) as the within factor, followed by Fisher's least significant differences test when appropriate; 2) A two-way repeated measures ANOVA was used for analyzing the difference in BP, BP variability, BRS, RRI, and HRV between the ND- and HFD-fed FA-exposed mice at weeks 11 with diet (ND vs. HFD) as the between factor and light cycle (dark vs. light, values from dark 1 and dark 2 cycles were averaged) as the within factor, followed by Fisher's least significant difference test when appropriate. $p < 0.05$ was considered statistically significant.

Day-night differences in activity, BP, BP variability, BRS, RRI, and HRV were calculated by subtracting 12-hr averages during light cycle from 12-hr averages during dark cycles (values from dark 1 and dark 2 cycles were averaged). A *t*-test was used for analyzing the difference in day-night differences between ND+FA and HFD+FA groups after 11 weeks of exposure. $p < 0.05$ was considered statistically significant.

For effects of SHS exposure in HFD mice: A two-way repeated measures ANOVA was used for analyzing the difference in weekly body weight and body weight gained with exposure (FA vs. SHS) as the between factor and time (weeks) as the within factor. A two-way ANOVA was used for analyzing the difference in BP, BP variability, BRS, RRI,

and HRV with exposure (FA vs. SHS) as one between factor and time (weeks 4, 8, and 11) as the other between factor, followed by Fisher's least significant differences test when appropriate. For restraint test, a two-way ANOVA was used for analyzing the difference in stress-induced changes in BP, HR, and HRV with exposure (FA vs. SHS) as one between factor and time (weeks 4 and 8) as the other between factor. $p < 0.05$ was considered statistically significant.

Results

Mice on HFD had significantly higher body weight than those fed with ND over 11 weeks of exposure period (+15.7 ± 0.8 g and 2.9 ± 0.3 g, HFD and ND, respectively) (**Figure 3.1**).

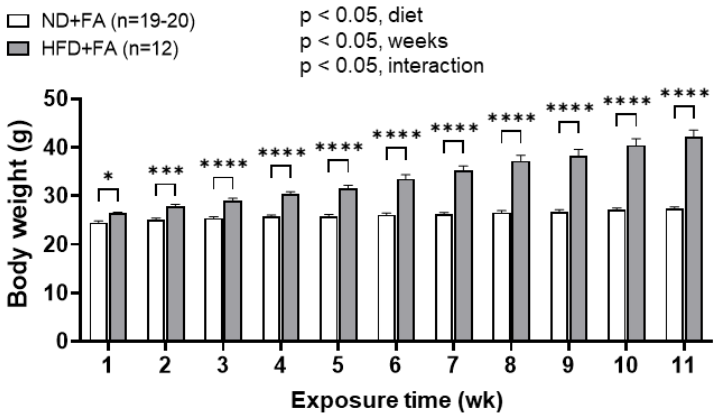

***Figure 3.1**: Group data of weekly body weight in ND+FA and HFD+FA mice over 11 weeks of exposure.* Body weight was significantly higher in HFD mice. Data obtained over 11 weeks were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, HFD vs. ND

SHS exposure had no effects on body weight and body weight gained over time in these HFD mice (**Figure 3.2**).

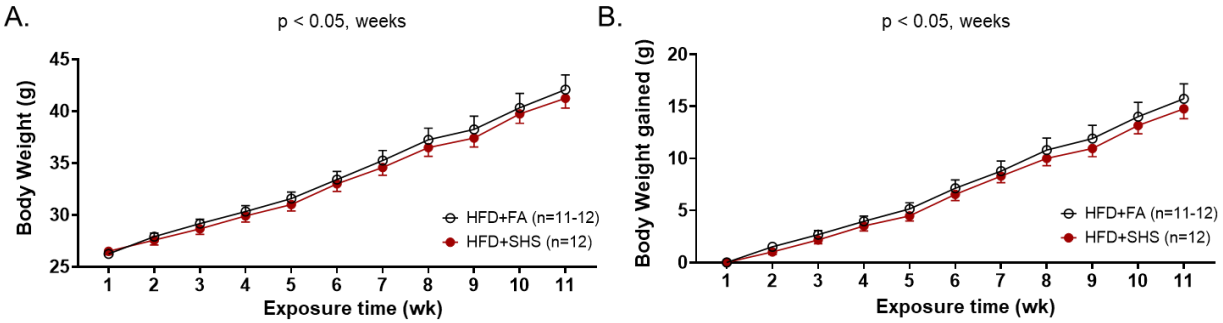

***Figure 3.2**: Group data of weekly body weight (**A**) and body weight gained (**B**) in HFD mice over 11 weeks of exposure.* There was no difference between FA- and SHS-exposed groups among HFD mice. Data obtained over 11 weeks were analyzed with a two-way repeated measures ANOVA.

HFD significantly reduced the activity level in dark cycle when animals are more active, compared to the ND mice (**Figure 3.3 A**), resulting in a smaller day-night difference (7.8 ± 0.5 and 4.1 ± 0.4 counts/min for ND and HFD, respectively, $p < 0.05$).

Similar to ND mice, activity level gradually decreased in these HFD-fed mice over the 11 weeks of exposure in dark cycles (**Figure 3.3 B**). As observed in ND mice (**Figure 2.3**), SHS exposure also increased overall activity level in HFD mice.

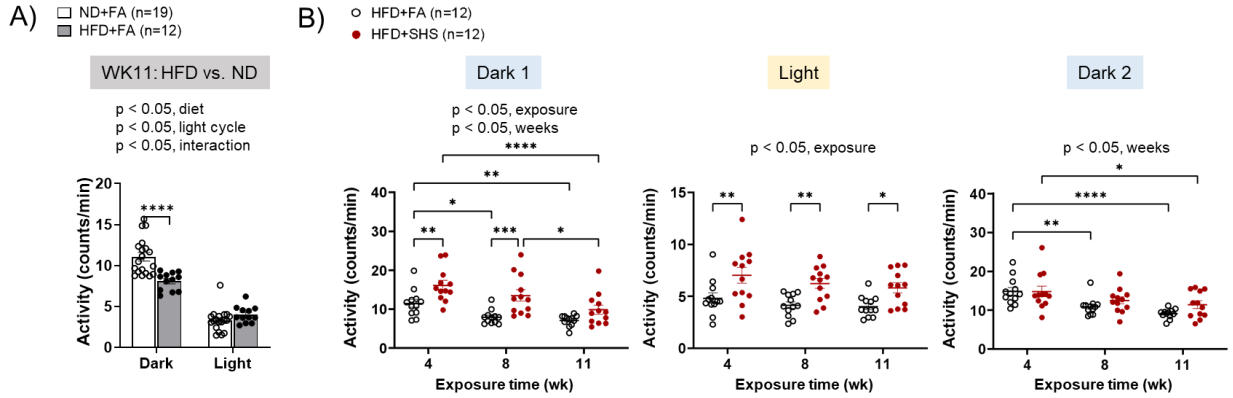

***Figure 3.3**: (**A**) Group data of activity level in ND+FA and HFD+FA mice at weeks 11.* Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. ****$p <$ 0.0001, HFD vs. ND *(**B**) Group data of activity level in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ****$p < 0.0001$

Effects of HFD on HR and HRV regulation

After 11 weeks of exposure, HFD-fed mice had shorter RRI (-10.0 ± 1.2 ms, and -20.3 ± 1.4 ms, during dark and light, respectively) than ND mice in both dark and light cycles (**Figure 3.4**). The shorter RRI was not due to the difference in activity as HFD mice had lower activity levels in dark cycles in compared to ND mice.

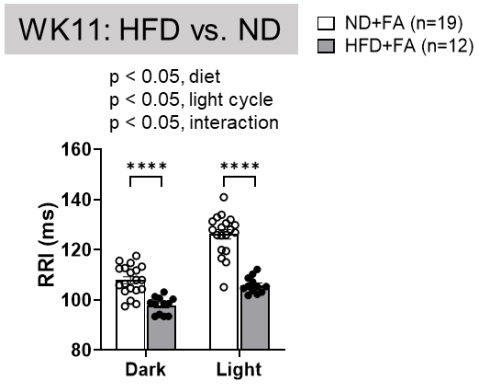

Figure 3.4*: Group data of RRI in ND+FA and HFD+FA mice at weeks 11.* Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. ****p < 0.0001, HFD vs. ND

Eleven weeks of HFD significantly reduced longer-term HRV (SDNN and SDANN) in both dark and light cycles (**Figure 3.5 A & B**). Similar to the longer-term HRV, 11 weeks of HFD decreased shorter-term HRV (rMSSD and SDNNIDX) in both dark and light cycles (**Figure 3.5 C & D**).

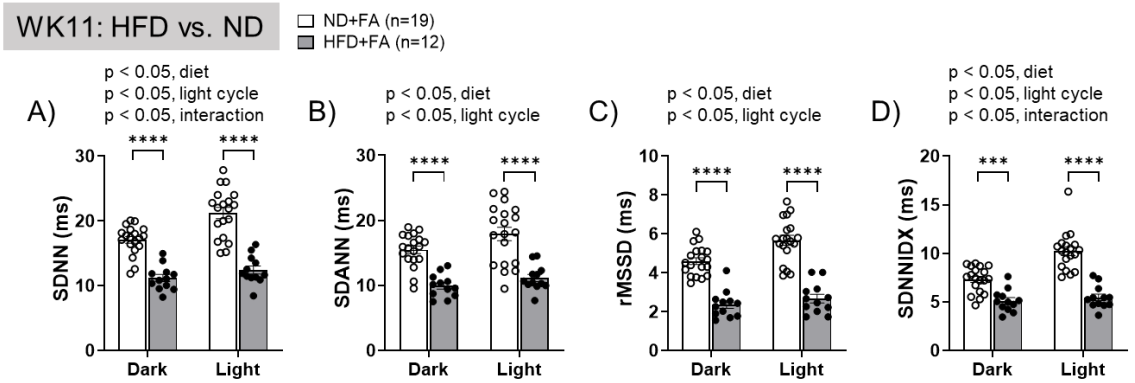

Figure 3.5*: Group data of SDNN (**A**), SDANN (**B**), rMSSD (**C**), and SDNNIDX (**D**) in ND+FA and HFD+FA mice at weeks 11.* Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. ***p<0.001, ****p < 0.0001, HFD vs. ND

These data indicate that 11 weeks of HFD alone significantly and dramatically attenuated autonomic regulation of HRV (~35.7%, 46.7%, 35.3%, and 34.5% reduction in SDNN, SDANN, rMSSD, and SDNNIDX, respectively) in both dark and light cycles. Since a shorter RRI in the HFD group can artificially result in lower HRV, HRV parameters were normalized to baseline RRI. Normalizing HRV parameters to baseline RRI did not change the results (**Figure 3.6**).

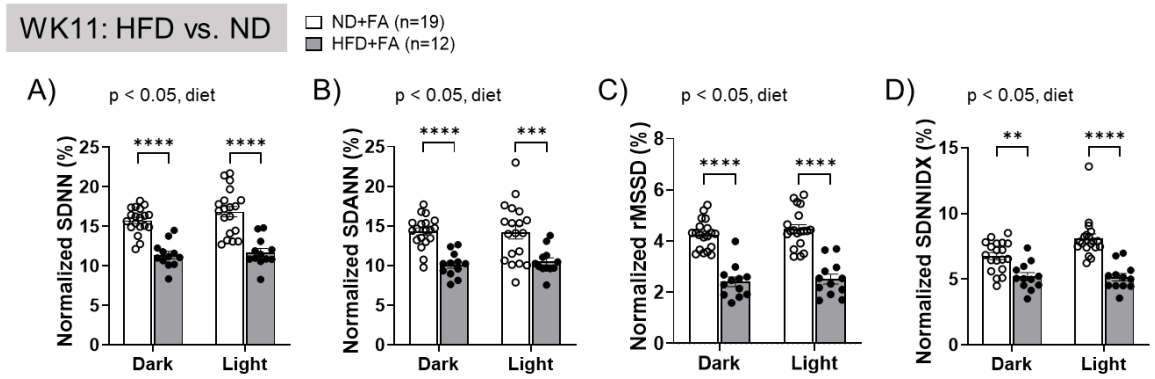

Figure 3.6*: Group data of normalized HRV for changes in baseline RRI: SDNN (**A**), SDANN (**B**), rMSSD (**C**), and SDNNIDX (**D**) in ND+FA and HFD+FA mice at weeks 11.* HRV measures were normalized for changes in baseline RRI. Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. ** $p < 0.01$, *** $p<0.001$, **** $p < 0.0001$, HFD vs. ND

In addition to the overall reduction in HRV, HFD group had a smaller day-night difference in all HRV parameters except for SDANN (**Table 3.1**).

Table 3.1: *Comparison of day-night differences (dark cycles minus light cycle) of HRV measures in ND+FA and HFD+FA mice at weeks 11.*

	ND+FA	HFD+FA	*p* value
ΔRRI (ms)	-18.3 ± 1.1	-8.0 ± 0.9	****
ΔSDNN (ms)	-4.2 ± 0.6	-1.3 ± 0.5	**
ΔSDANN (ms)	-2.4 ± 0.8	-1.2 ± 0.4	0.26
ΔrMSSD (ms)	-1.1 ± 0.2	-0.3 ± 0.1	**
ΔSDNNIDX (ms)	-2.9 ± 0.4	-0.4 ± 0.2	***

***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

Effects of HFD on BP and BP regulation

Mice fed with HFD had significantly higher SBP (+6.5 ± 0.9 mmHg), DBP (+6.1 ± 0.9 mmHg), and MBP (+6.7 ± 1.0 mmHg) during the light cycle compared to the ND group (**Figure 3.7 A-C**). HFD have no effects on PP (**Figure 3.7 D**).

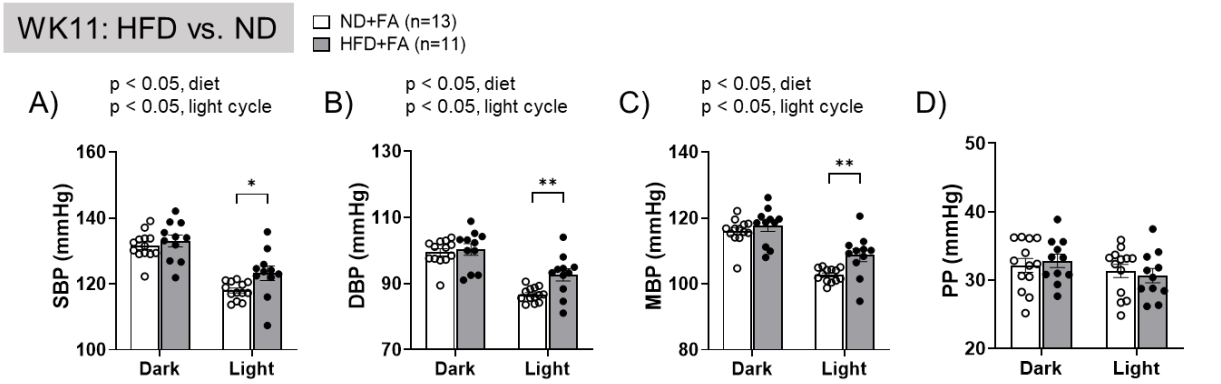

Figure 3.7*: Group data of SBP (**A**), DBP (**B**), MBP (**C**), and PP (**D**) in ND+FA and HFD+FA mice at weeks 11.* Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ***p* < 0.01, HFD vs. ND

HFD resulted in a blunted day-night difference in BP (**Table 3.2**) compared to the ND mice. This blunted day-night difference in BP was due to the HFD-induced elevated BP during the light cycle (**Table 3.2** & **Figure 3.7 A-C**).

Table 3.2: *Comparison of day-night differences (dark cycle minus light cycle) in BP in ND+FA and HFD+FA mice at weeks 11.*

	ND+FA	HFD+FA	p value
ΔSBP (mmHg)	13.6 ± 0.9	9.8 ± 0.9	**
ΔDBP (mmHg)	12.8 ± 0.8	7.7 ± 0.8	****
ΔMBP (mmHg)	13.2 ± 0.8	8.9 ± 0.9	**

***p* < 0.01, ****p* < 0.0001

Mice fed with HFD for 11 weeks had a lower BRS compared to those fed with ND (-15.6%, -20.8%, and -18.4% during dark 1, light, and dark 2 cycles, respectively) (**Figure 3.8 A**). Despite a lower BRS, eleven weeks of HFD had no significant effect on BP variability (**Figure 3.8 B-D**)

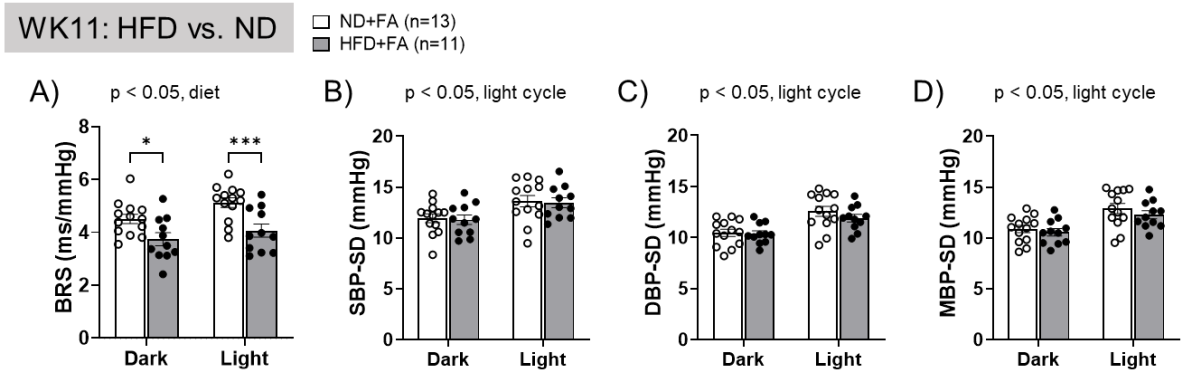

Figure 3.8*: Group data of BRS (**A**), SBP-SD (**B**), DBP-SD (**C**), and MBP-SD (**D**) in ND+FA and HFD+FA mice at weeks 11.* Data from dark 1 and dark 2 were averaged as dark cycle. Data obtained at weeks 11 were analyzed with a two-way repeated measures ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ****p* < 0.0001, HFD vs. ND

HFD group and ND group showed similar day-night differences in BRS and BP variability, in which BP variability was lower during the dark cycle (**Table 3.3**).

Table 3.3: *Day-night differences (dark cycle minus light cycle) of BRS and BP variability in ND+FA and HFD+FA mice at week 11.*

	ND+FA	HFD+FA	p value
ΔBRS (ms/mmHg)	-0.6 ± 0.1	-0.3 ± 0.2	0.17
ΔSBP-SD (mmHg)	-1.7 ± 0.4	-1.7 ± 0.3	0.98
ΔDBP-SD (mmHg)	-2.1 ± 0.3	-1.5 ± 0.3	0.22
ΔMBP-SD (mmHg)	-2.0 ± 0.4	-1.7 ± 0.3	0.55

Effects of SHS and HFD co-exposure on HR and HRV regulation

In HFD-fed mice, SHS exposure significantly decreased RRI during the light cycle (- 2.8% and 3.0% at week 8 and 11, respectively) (**Figure 3.9**). This was different from the ND-fed mice in which SHS exposure had no effects on RRI (**Figure 2.4**).

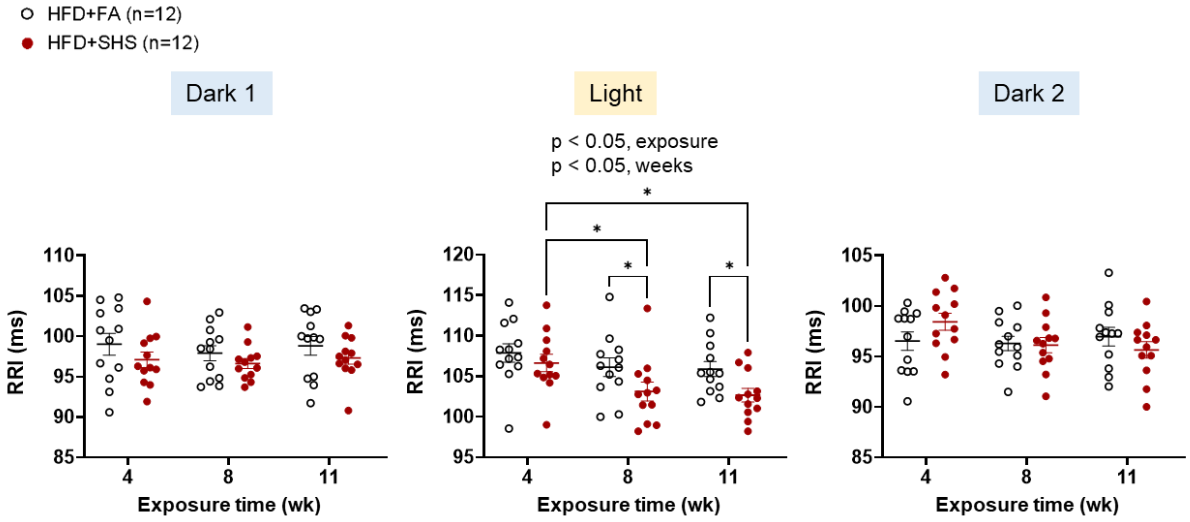

Figure 3.9*: Group data of RRI in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. *$p < 0.05$

In HFD-fed mice, overall HRV (SDNN) decreased over time, and this pattern was true for both dark and light cycles – an observation that is similar to the ND mice (**Figure 3.10 A & 2.5 A**). Similar to the ND group, this reduction in HRV over time was unlikely due to the decrease in RRI, since normalized SDNN for changes in baseline RRI did not change the results (**Figure 3.10 B & 2.5 B**).

However, SHS exposure did not further decrease SDNN in these HFD-fed mice (**Figure 3.10 A**). This was in contrast with ND mice, in which SHS exposure decreased overall HRV (SDNN) by ~13.2% (**Figure 2.5**). These results were also true after normalized SDNN to the baseline RRI (**Figure 3.10 B**).

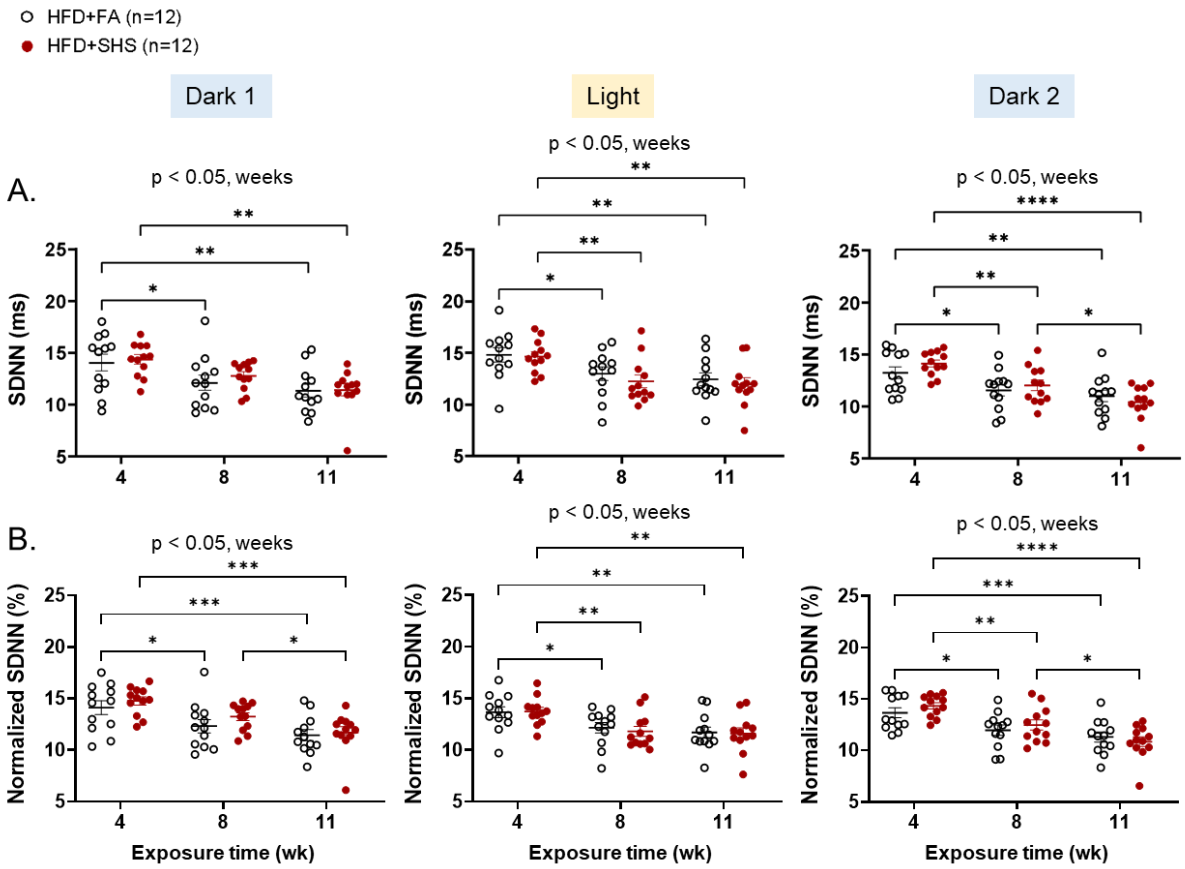

Figure 3.10*: Group data of SDNN (**A**) and normalized SDNN for changes in baseline RRI (**B**) in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

For HRV regulation between 2-min segments (SDANN), the results were similar to that of SDNN, in which SDANN decreased over time in both dark and light cycles in these HFD-fed mice. Also, SHS exposure did not further decrease SDANN in HFD group (**Figure 3.11**).

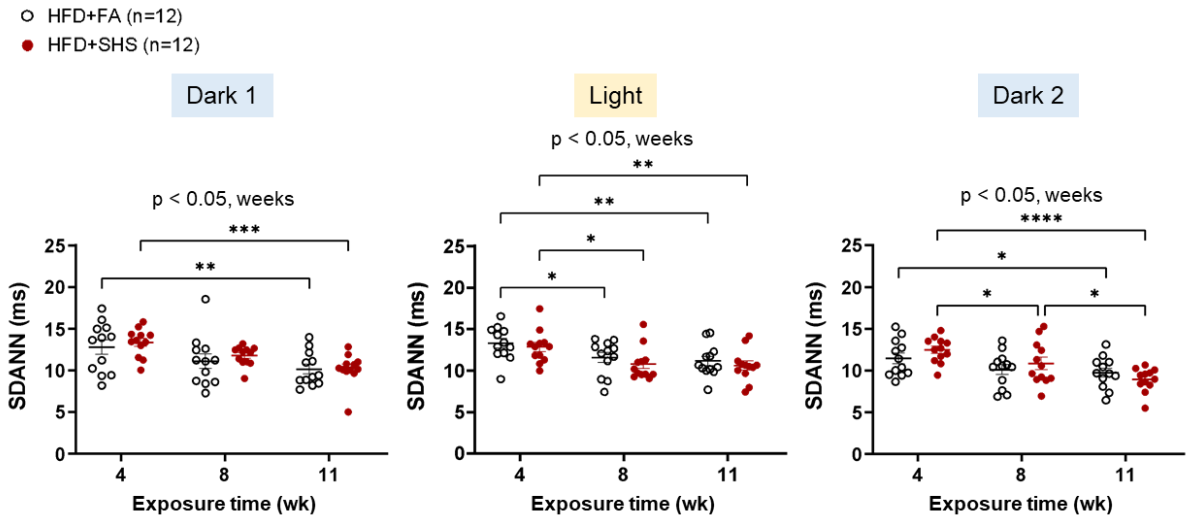

***Figure 3.11**: Group data of SDANN in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

Similarly, there was a significant time effect on rMSSD and SDNNIDX in HFD-fed mice. Eleven weeks of SHS exposure had no effects on shorter-term HRV (rMSSD and SDNNIDX) in HFD-fed mice (**Figure 3.12**). This was different to the ND groups, in which SHS reduced rMSSD and SDNNIDX by ~10% (**Figure 2.7**).

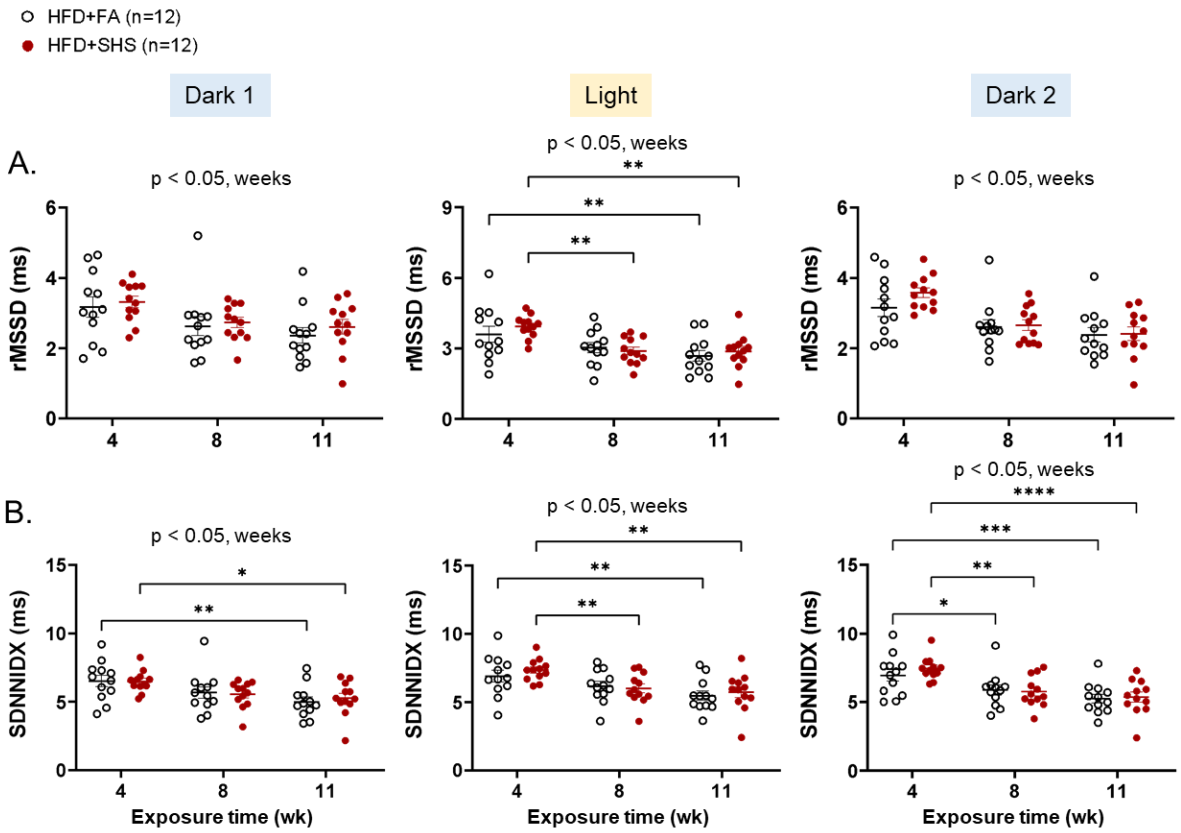

***Figure 3.12**: Group data of rMSSD (**A**) and SDNNIDX (**B**) in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Taken together, the overall small reduction in HRV over time in both SHS- and FA-exposed groups may reflect aging-related changes in HRV regulation. In light of a significant reduction in HRV (~35%, **Figure 3.5**) from HFD alone, these data suggest that HFD alone had a significantly larger effect on HRV regulation than SHS alone such that additional SHS exposure imposes no further changes in HRV.

Effects of SHS and HFD co-exposure on BP and BP regulation

SHS exposure did not change BP in HFD-fed mice (**Figure 3.13**). This was in contrast with the ND-fed mice in which SHS increased DBP after four weeks of exposure and decreased SBP during the light cycle with longer exposure time (**Figure 2.8**). Since 11 weeks of HFD alone increased BP (~6 mmHg, **Figure 3.7**), these data further suggest that HFD alone had a greater effect on BP than SHS alone, and the effects of SHS on BP might be masked by HFD-induced changes under co-exposure.

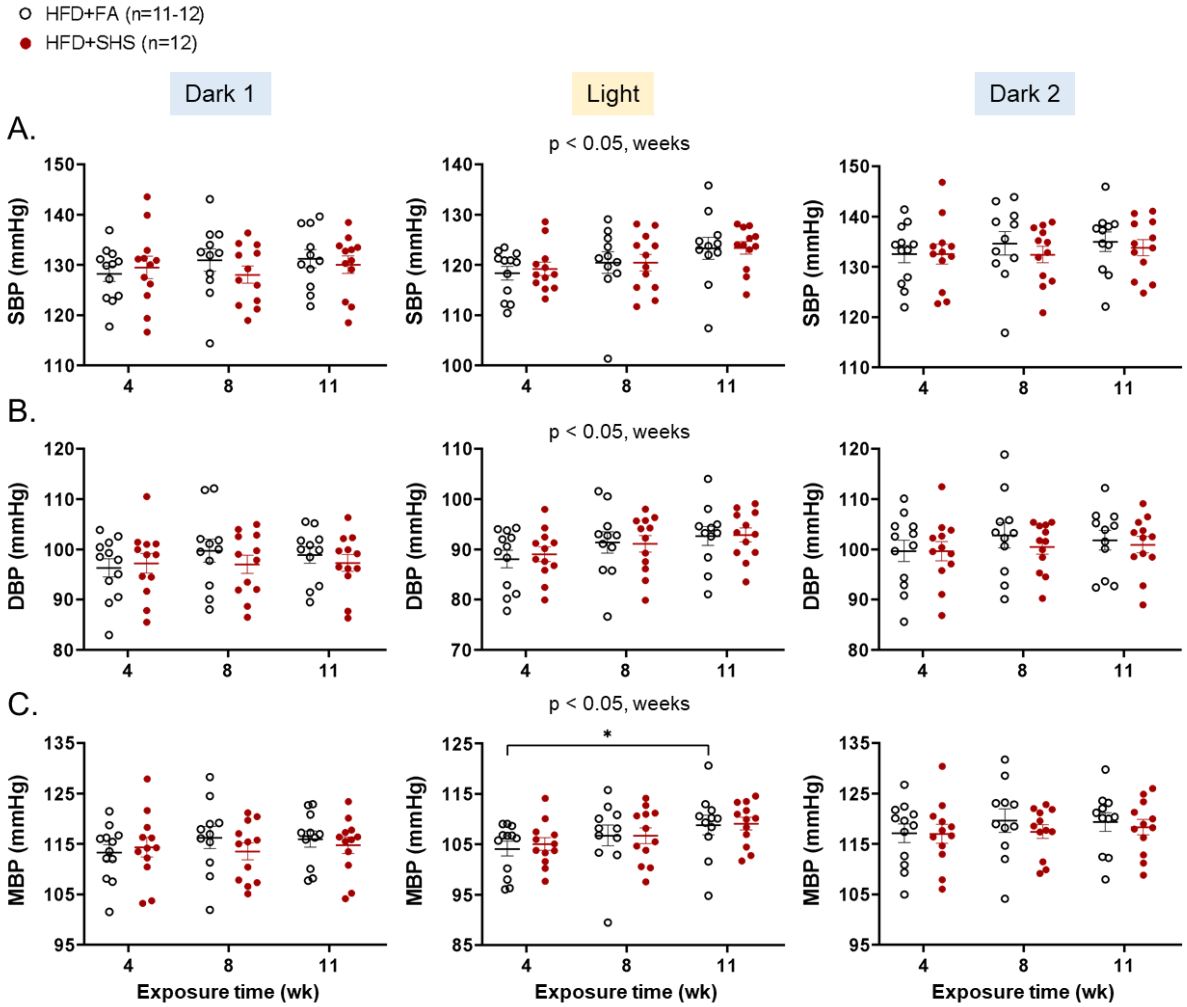

Figure 3.13*: Group data of SBP (**A**), DBP (**B**), and MBP (**C**) in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05

For PP, 11 weeks of SHS exposure did not change PP in HFD mice (**Figure 3.14**). This was different from the ND group in which SHS significantly decreased PP across all three light cycles (**Figure 2.9**).

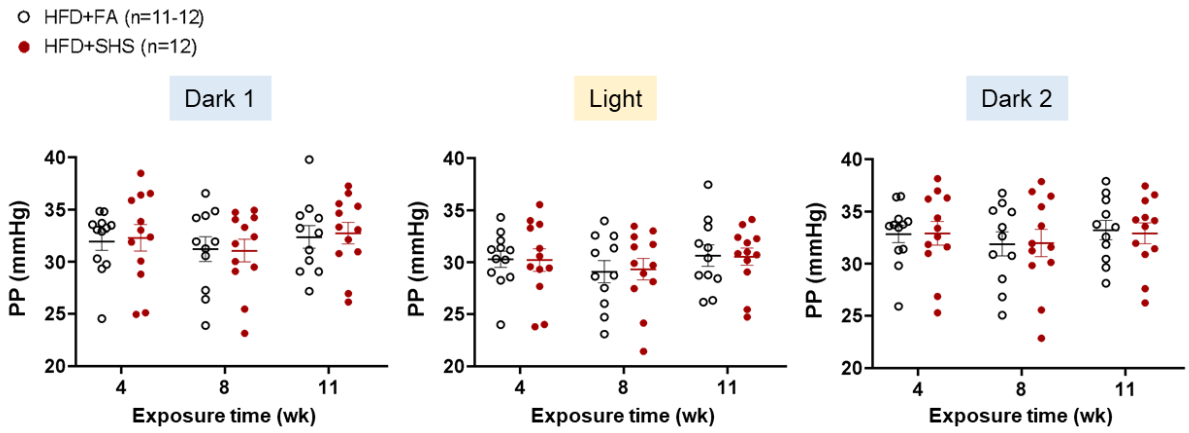

***Figure 3.14**: Group data of PP in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA.

In HFD-fed mice, there was an overall higher BRS in SHS-exposed group across all three light cycles (**Figure 3.15**). Post-hoc test results showed that the effect of SHS exposure on BRS was the most significant after 4 and 11 weeks of exposure. During the dark 1 cycle, SHS significantly increased BRS in HFD-fed mice by 13.8%, 12.6%, and 21.3% after 4, 8, and 11 weeks of exposure, respectively, compared to HFD+FA group. These findings were the opposite of what we've observed in the ND groups (**Figure 2.10**) in which SHS decreased BRS during the dark 1 cycle.

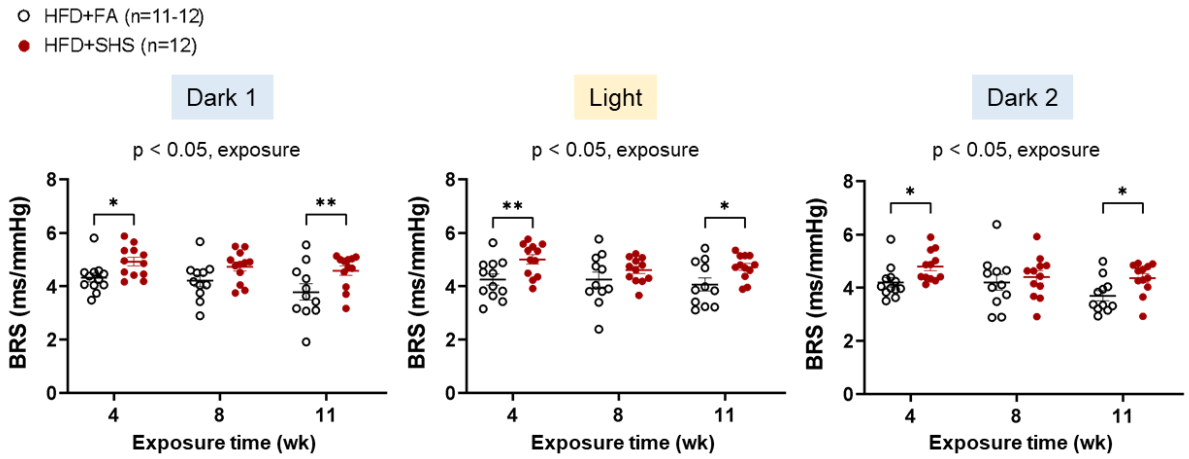

***Figure 3.15**: Group data of BRS in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ***p* < 0.01

In HFD-fed mice, there was an overall SHS exposure effect on the standard deviation of SBP and DBP during the dark cycles, as well as on the standard deviation of MBP across all three light cycles (**Figure 3.16**). Post-hoc test results showed that, after four weeks of exposure, the BP variability was significantly higher (16.8%, 15.6%, and 17.3% for SBP-SD, DBP-SD, and MBP-SD, respectively) in SHS-exposed HFD-fed mice than that in FA-exposed HFD-fed mice during the dark 1 cycle. This was different from the ND group in which SHS exposure alone did not affect BP variability (**Figure 2.11**).

In light of an increased BP variability despite a higher BRS, these results suggested that co-exposure of SHS and HFD induced an impaired BP regulation that was not observed under SHS or HFD alone. The higher BRS in SHS+HFD mice might be triggered by this co-exposure-induced greater BP variability.

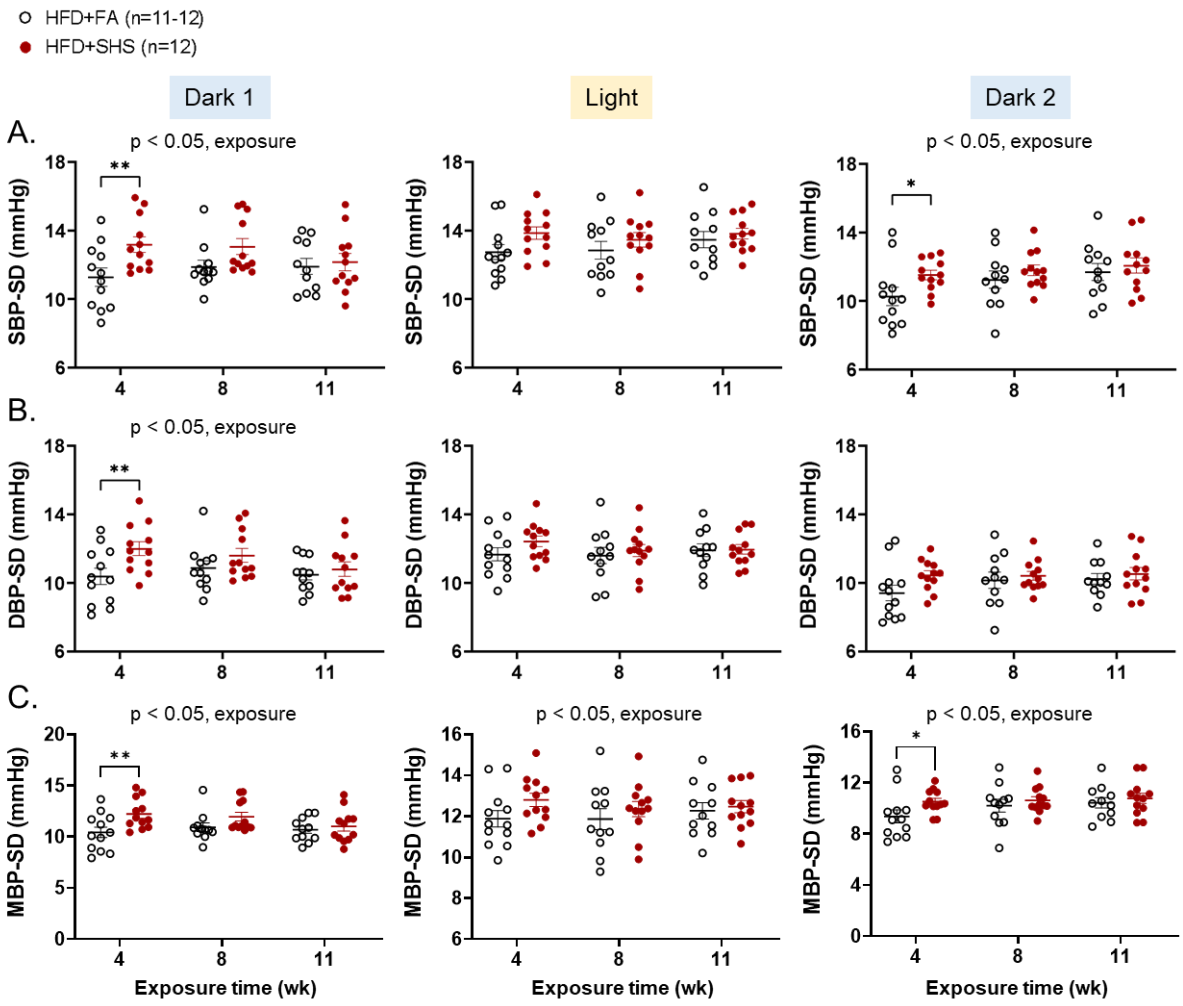

***Figure 3.16**: Group data of SBP-SD (**A**), DBP-SD (**B**), and MBP-SD (**C**) in HFD-fed mice after 4, 8, and 11 weeks of SHS or FA exposure.* Data obtained at weeks 4, 8, and 11 were analyzed with a two-way ANOVA followed by Fisher's LSD tests when appropriate. **p* < 0.05, ***p* < 0.01

SHS and HFD co-exposure on cardiovascular responses to stress

Figure 3.17 (left) shows 30-min averaged MBP, HR, SDNN, and rMSSD during the 2-hr baseline, 2-hr restraint stress, and 6-hr recovery period after four weeks of FA or SHS exposure in HFD-fed mice. Similar to the stress response observed in ND groups (**Figure 2.12**), all HFD-fed mice had increased MBP and HR, while decreased overall HRV (SDNN) and short-term HRV (rMSSD) during the 2-hr restraint period compared to the 2-hr baseline. After removed from the restraint stress, all HFD-fed mice had their MBP, HR, overall and short-term HRV gradually returned to baseline level.

Figure 3.17 (right) presents the magnitudes of restraint stress-induced changes (delta changes) in MBP, HR, SDNN, and rMSSD. In HFD mice, SHS-exposed group had similar restraint stress-induced changes in MBP, HR, overall and short-term HRV as the FA control group.

Overall, these results were similar to what we've observed in ND group (**Figure 2.12**), suggesting that SHS exposure did not alter cardiovascular response to stress, even with 11 weeks of co-exposure to HFD.

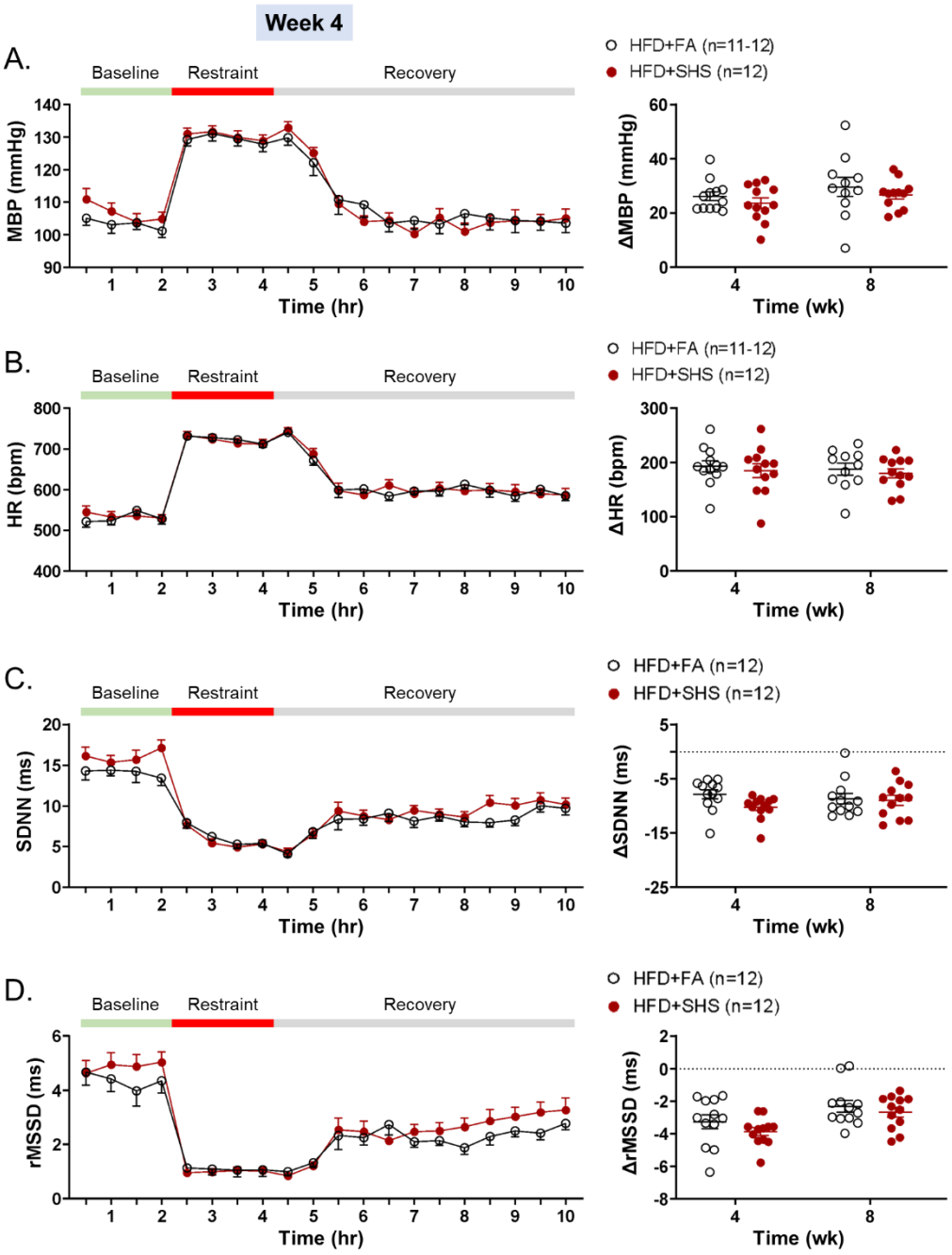

***Figure 3.17**: Group data of MBP (**A**), HR (**B**), SDNN (**C**), and rMSSD (**D**) in HFD-fed mice during the restraint test.* Left panels: 30-min averaged values during the 2-hr baseline, 2-hr restraint, and 6-hr recovery period. Right panels: stress-induced delta changes from the 2-hr baseline. Data obtained during restraint test were analyzed with a two-way ANOVA.

Discussion

We found that HFD alone significantly increased BP, and decreased BRS and HRV, suggesting an impaired BP and HR regulation. In HFD mice, exposure of SHS increased BP variability that was not observed with HFD or SHS exposure alone. These data suggest an interactive effect of SHS and HFD co-exposure on BP dysregulation. Moreover, exposure of SHS significantly increased BRS in HFD-fed mice, which was the opposite of the effect of SHS or HFD alone. These results further emphasize that the SHS-induced cardiovascular changes could be altered under different dietary conditions, and it is important to consider the differences in diet when studying the cardiovascular effects of SHS in future studies.

HFD alone on BP

Many studies demonstrated that long-term intake of HFD is associated with elevated BP (Wilde *et al.*, 2000; Barnes *et al.*, 2003). In the present study, we found that 11 weeks of HFD increased BP compared to the ND group; however, this HFD-induced elevated BP was only observed during the light cycle (**Figure 3.7 A-C**). Just like humans, mice also showed circadian pattern of BP, in which the BP is higher during dark cycle when they are most active, and lower during light cycle when they spend most time in sleep (Li *et al.*, 1999). While the nighttime BP did not change in HFD-fed group, the increased BP during daytime resulted in a blunted day-night difference in BP. In HFD-fed mice, the daytime BP was about 6% lower than their nighttime BP after 11 weeks of exposure, indicating a non-dipper BP pattern (**Figure 3.7 A-C & Table 3.2**). As in humans, a non-

dipper BP pattern is defined as a BP drop of less than 10% from daytime (more active) to nighttime (less active). It has been shown that individuals with a non-dipper BP pattern had a higher risk of hypertension-induced organ damage, such as left ventricular hypertrophy and arterial stiffness (Redon and Lurbe, 2008). However, studies reported that even within the normotensive range, a blunted day-night difference in BP is associated with increased risk of cardiovascular morbidity and mortality (Hermida *et al.*, 2013). Here, our results showed a decreased day-night difference in BP in HFD-fed mice, suggesting HFD-induced BP dysregulation. Moreover, HFD-fed mice showed an increase in BP over time, which is not observed in the ND group. HFD-fed mice had SBP, DBP, and MBP increased significantly (~6.5 mmHg, 6.1 mmHg, 6.7 mmHg, respectively) over the 11 weeks experimental regimen during the light cycle (**Figure 3.7 A-C**). These results further suggested the adverse effects of HFD alone on elevated BP.

PP is used as an indicator of stroke volume and an indirect marker of arterial stiffness (Safar, 2001; Chaudhry, Miao and Rehman, 2020). Several studies have demonstrated an association between HFD and increased arterial stiffness (Lithander *et al.*, 2013; Santana *et al.*, 2014; Fryer *et al.*, 2021). For instance, Santana and colleagues showed that mice fed on 8 weeks of HFD had increased deposition and disorganization of collagen fibers, which led to arterial stiffening and decreased arterial compliance (Santana *et al.*, 2014). On the other hand, there's evidence showing that consumption of HFD did not affect the stroke volume (Jakulj *et al.*, 2007; Kwiatkowski *et al.*, 2021; Yu *et al.*, 2021). Yu *et al.* found that in mice chronically exposed to HFD (60 kcal% from fat), there was no change in stroke volume and cardiac output compared to mice fed on a regular diet (11.9

kcal% from fat) (Yu *et al.*, 2021). Consistent with these findings, a study from Tófolo *et al.* showed that rats fed on HFD for 30 days had a higher PP than those on normal chow, and there was no difference in HR (Tófolo *et al.*, 2019).

However, our data showed that 11 weeks of HFD alone did not change PP, while induced a shorter RRI (higher HR) (**Figure 3.7 & 3.4**). A higher HR has often been reported with other cardiovascular risk factors, including hypertension and overweight (Palatini and Julius, 1999; Fox *et al.*, 2007). The discrepancy between our study and Tófolo *et al.* raise a question that could the HFD-induced changes in RRI/ HR interfere with what we've observed in PP? A study from Wilkinson *et al.* measured central PP during right atrial pacing in healthy adults (Wilkinson *et al.*, 2002). They found that within a physiological range of heart rates, incremental right atrial pacing (from baseline 65 bpm to 80, 100, and 120 bpm) was associated with a significant reduction in central pulse pressure, while there's no change in arterial stiffness as indicated by the aortic pulse wave velocity (PWV) (Wilkinson *et al.*, 2002). In line with these results, Koskela and colleagues found an inverse association between resting HR and central PP (Koskela *et al.*, 2013). In this study, our data showed that HFD induced a decrease in resting RRI by 10% and 16%, which was equivalent to an increase in HR by ~57 bpm and ~91 bpm, during the dark and light cycles, respectively, compared to the ND mice (**Figure 3.4**). In light of these findings, PP might not be a reliable marker for comparing differences in arterial stiffness between the ND and HFD mice in this study. Future studies are needed to obtain accurate measures of arterial stiffness and stroke volume, such as using noninvasive methods like aortic PWV and echocardiography (Safar, 2001; Tournoux *et al.*, 2011).

Co-exposure of SHS and HFD on BP

We have previously shown in chapter 2 that SHS alone induced a decrease in SBP with prolonged exposure which was presumably due to a decreased cardiac function (**Figure 2.8**). However, with co-exposure of SHS and HFD, changes in BP were the same as those with HFD alone (**Figure 3.13**), suggesting the effects of HFD on BP were great enough that masked the effects of SHS under co-exposure.

On the other hand, while exposure of SHS decreased PP which presumably suggesting a decreased stroke volume (**Figure 2.9**), with co-exposure of HFD, this SHS-induced decreased PP was no longer detected (**Figure 3.14**). As HFD alone has no effect on PP, these data raise a possibility of the co-exposure effects of SHS and HFD on PP. One potential explanation is that SHS and HFD may additively, or even synergistically increase arterial stiffness, and therefore, the effect of co-exposure is great enough to mask the potential SHS-induced reduction in PP. On the other hand, as we previously discussed that central PP can be inversely associated with resting HR, the SHS-induced increased HR in these HFD-fed mice may potentially interfere with what we've observed in the co-exposure-related changes in PP. Again, since PP is an indirect measure of arterial stiffness and stroke volume, future studies are needed to elucidate the effects as well as the effect size of co-exposure on arterial stiffness and stroke volume with validated methods.

HFD alone on BP regulation

A study from Fardin *et al.* group reported that in rats exposed to a HFD for 20 weeks, there was a significant reduction in BRS, which suggests a baroreflex dysfunction (Fardin, Oyama and Campos, 2012). Consistent with these results, our data showed that without SHS exposure, 11 weeks of HFD alone decreased BRS by ~18% during both dark and light cycles (**Figure 3.8 A**), suggesting for an HFD-induced impaired baroreflex function, which can contribute to the increased risk of cardiovascular morbidity and mortality.

Co-exposure of SHS and HFD on BP regulation

It is well acknowledged that a higher BP variability is an independent risk factor for end-organ damage and increased cardiovascular morbidity and mortality (Su and Miao, 2005; Chadachan *et al.*, 2018). Our results showed that 11 weeks of SHS significantly increased BP variability in HFD-fed mice (**Figure 3.16**). Since such change in BP variability was not observed in mice exposed to SHS or HFD alone, our results suggest that co-exposure of SHS and HFD may lead more severe impact on BP regulation when compared to their individual effects, which make individuals under co-exposure of SHS and HFD at a higher risk of cardiovascular morbidity and mortality.

Several factors contribute to BP variability including reflexes, behavioral, neural, and hormonal changes (Mancia and Grassi, 2000; Grassi *et al.*, 2012; Rosei, Chiarini and Rizzoni, 2020). In this study, spontaneous BRS was used to assess baroreflex function. The BRS obtained from the sequence method is predominantly an indicator of beat-to-beat regulation of BP. Although BRS was significantly reduced with SHS or HFD exposure

alone, there was no detectable change in BP variability. Our data showed that SHS and HFD co-exposed mice had higher BRS than each exposure alone, resulting in BRS similar to no exposure level (ND+FA group) with an elevated BP variability. These data suggested that the increased BP variability in co-exposed mice might be attributed to factors other than the baroreflex function, such as the behavioral alterations and humoral factors. These data also raised the possibility that a higher BRS in co-exposure may be triggered by a higher BP variability. In this regard, Hesse *et al.*, showed that BRS was positively related to BP variability in healthy normotensive humans (Hesse *et al.*, 2007). Further investigations are needed to understand the correlation between BRS and BP variability under different conditions, as well as examine the potential underlying contributors to these co-exposure-induced BP dysregulation.

HFD alone, and co-exposure of SHS and HFD on HR regulation

Our results showed that 11 weeks of exposure to HFD alone led to decreases in longer-term HRV (SDNN and SDANN) and shorter-term HRV (rMSSD and SDNNIDX) by ~35% (**Figure 3.5**), suggesting an impaired HR regulation, particularly a decreased parasympathetic regulation of the heart. Several studies have shown that consumption of HFD was linked to a decreased cardiac parasympathetic regulation (Soares-Miranda *et al.*, 2012; Young and Benton, 2018). For instance, mice fed on HFD for 14 weeks had a reduced bradycardia response to vagus nerve stimulation, suggesting a HFD-induced decreased parasympathetic regulation of the heart (Hartnett *et al.*, 2015). Verwaerde *et al.* also showed that dogs exposed to 21-weeks of HFD displayed a reduction in the high frequency band of HRV, suggesting a reduced parasympathetic nerve activity (Verwaerde

et al., 1999). However, there is limited information on the association between HFD and time-domain measures of HRV.

The effect size of HFD exposure on HRV was much greater than that from SHS exposure. In comparison, SHS alone resulted in a decrease in SDNN by ~13.2% and a decrease in rMSSD by ~10% during the first dark cycle right after the exposure (**Figure 2.5 & 2.7**), which was consistent with human studies in literature (Zhang *et al.*, 2013; Garza *et al.*, 2016).

Our data showed that mice co-exposed to HFD and SHS had similar HRV as mice exposed to HFD alone (**Figure 3.9, 3.10, and 3.11**). The lack of additional SHS exposure effect on HRV in HFD mice suggests that HFD has induced a maximal decrease in HRV, such that additional SHS exposure failed to impose extra effects on HRV.

Conclusions

This study suggested that the effects of SHS on cardiovascular functions can be altered by co-exposure to different diets. Moreover, we found that co-exposure of HFD and SHS resulted in BP dysregulation that did not observe in HFD or SHS alone, suggesting individuals co-exposed to these two risk factors might be at a higher risk of cardiovascular morbidity and mortality. This study emphasizes the importance of understanding how multiple risk factors may interplay with each other in inducing cardiovascular changes when studying the multifactorial cardiovascular diseases.

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Chapter 4:

Perspective and future directions

Major findings

The overall objective of this study was to investigate the effects of secondhand smoke (SHS) and high-fat diet (HFD) – which are two significant risk factors for cardiovascular morbidity and mortality, as well as their co-exposure effects on cardiovascular functions. The major findings reported in this study were summarized in **Table 4.1**. These findings suggest that exposure to SHS or HFD alone can both lead to impaired regulation of blood pressure (BP) and heart rate (HR). Co-exposure of SHS and HFD induced an increase in BP variability that was not observed with SHS or HFD alone, suggesting SHS and HFD can interactively induce a different pattern of BP dysregulation than exposure to SHS or HFD alone. This finding further demonstrates that co-exposure of SHS and HFD may put individuals under higher risks of cardiovascular morbidity and mortality.

Table 4.1: *Summary of major findings from Chapter 2 and 3.*

	Exposure Effect		
	SHS alone [a]	**HFD alone [b]**	**SHS+HFD [b]**
BP	↓	↑	↑
Pulse pressure	↓	-	-
Baroreflex sensitivity	↓	↓	-
BP variability	-	-	↑
HR variability	↓	↓	↓

[a] Exposure effect was compared to no exposure (filtered air + normal diet exposed) group after 12 weeks of exposure.
[b] Exposure effect was compared to no exposure (filtered air + normal diet exposed) group after 11 weeks of exposure.

Studying real-world SHS exposure

To investigate the adverse effects of SHS on cardiovascular functions as well as the underlying mechanisms, it is important to use environmentally relevant exposure levels so that the experimental results can reflect the human experience. One limitation in most animal studies of SHS exposure is that a higher exposure level was used, sometimes 10 folds or more than people may encounter in their daily lives. This might potentially explain some of the discrepancies in SHS-induced changes between animal studies and epidemiological studies. For instance, a study showed that mice exposed to 30 mg/m^3 SHS for 10 months displayed a reduced bodyweight, while there's lack of epidemiological evidence that link SHS exposure to weight loss (Raber *et al.*, 2021). Therefore, designing experimental studies with an environmentally relevant level of SHS can help us to obtain a better understanding of the exposure effects as well as link experimental conclusions to the real-world exposure problems.

One significance of this study is that we used an environmentally relevant concentration of SHS that mimics the higher end of SHS exposure humans might encounter in smoky bars (3 mg/m^3). Our results showed that mice exposed to 3 mg/m^3 SHS for 12 weeks displayed a reduced HRV that was similar to what have observed in humans exposed to SHS (Zhang *et al.*, 2013; Garza *et al.*, 2016). This study provides a valid mouse model that could be used in future studies to dissect the underlying mechanisms of SHS-related cardiovascular morbidity and mortality.

Insights from our studies

In our study, we showed that exposure to SHS induced cardiovascular dysfunctions in a time-dependent manner (**Figure 4.1**). The SHS-induced decrease in pulse pressure (PP) and HR variability (HRV) had a faster onset that was first observed after four weeks of exposure and sustained with prolonged exposure, indicating a compromised cardiac function, and reduced parasympathetic regulation of the heart start at the early stage of exposure. The decreased cardiac function may then contribute to the lower BP with prolonged exposure. In contrast, SHS-induced decrease in baroreflex sensitivity (BRS) was accumulated and observed towards the end of exposure regimen (weeks 12), suggesting a SHS-induced impaired baroreflex function with longer exposure.

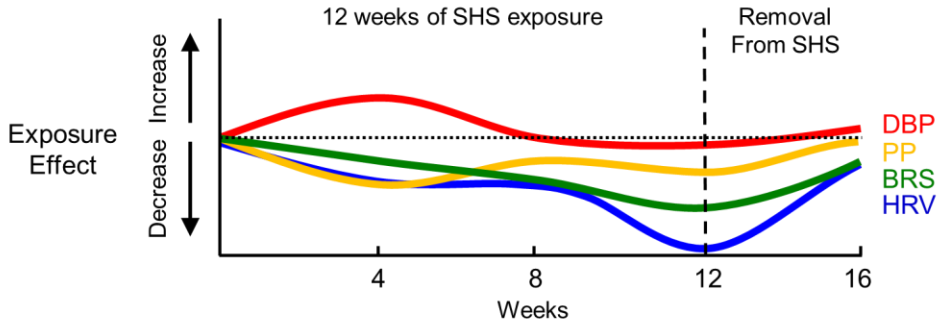

Figure 4.1*: Schematic diagram for the time course of SHS-induced cardiovascular dysfunctions.*

HRV and BRS are two commonly used indices for assessing the autonomic function of the cardiovascular system. An attenuated HRV has been associated with increased susceptibility to arrhythmia and higher risks of sudden cardiac death (Schwartz, La Rovere and Vanoli, 1992; Villareal, Liu and Massumi, 2002; Agarwal *et al.*, 2017). A reduction in BRS has been reported to accompany the development and progression of cardiovascular diseases including atherosclerosis, hypertension, and heart failure (Verwaerde *et al.*, 1999; La Rovere, Pinna and Raczak, 2008; Lohmeier and Iliescu, 2015). To our knowledge, this is the first study that presenting the time-course of SHS-

induced alterations in HRV and providing experimental evidence of SHS-induced baroreflex dysfunction. These findings improve our understandings on the potential underlying mechanisms, particularly through the SHS-induced autonomic dysregulation of the cardiovascular system, of SHS exposure-related cardiovascular diseases such as stroke, sudden cardiac death, and the development of hypertension (Malek *et al.*, 2015; Aune *et al.*, 2018; Skipina, Soliman and Upadhya, 2020).

In this study, we were also able to show that long-term intake of HFD increased BP, and decreased BRS and HRV, indicating an impaired baroreflex function and reduced cardiac parasympathetic activity, which are consistent with the literature review (Barnes *et al.*, 2003; Fardin, Oyama and Campos, 2012; Young and Benton, 2018). Our data further showed that the HFD-induced increase in BP was the greatest during the light cycle, which resulted in a blunted day-night difference in BP and led to a non-dipper BP pattern. Individuals with a non-dipper BP pattern have been reported to be associated with deteriorated vascular functions and higher risks of cardiovascular diseases compared to individuals with normal day-night difference in BP (Yano and Kario, 2012; Kuzeytemiz *et al.*, 2013). Most studies used 24-hr ambulatory BP when studying the effects of HFD on cardiovascular function; here, our findings showed evidence and give insights of the importance of looking at 12-hr day-night changes in BP, which could provide additional information on diet-related cardiovascular risks other than the elevated BP itself.

Our results showed that co-exposure of SHS and HFD increased BP variability that was not observed with either SHS or HFD exposure alone, suggesting that co-exposure of

SHS and HFD may induce different impacts on BP regulation than their individual effects. Increased BP variability has been shown to be independently associated with higher risks of cardiovascular end-organ damage and increased cardiovascular morbidity and mortality, over and above the effects of elevated mean BP (Lanfranchi and Somers, 2002; Stevens *et al.*, 2016). In this regard, individuals exposed to both SHS and HFD in their daily lives are potentially under higher risks of the development of cardiovascular diseases, as well as at higher risks of worsening their existing health conditions. This provides insights into taking dietary factors into account when considering the cardiovascular effects of SHS exposure. As of September 30, 2021, only 27 states in the United States have implemented 100% smoking bans for workplace, bars, and restaurants (CDC, 2021). Foods at restaurants and bars are associated with higher content of sugar and fats (Brindal *et al.*, 2008; Lin and Guthrie, 2013). Our study further emphasizes the importance of implementing smoking bans in bars and restaurants, which not only aim to protect non-smokers from the effects of SHS exposure, but also to reduce/ eliminate the risks from co-exposure of SHS and fat consumption.

Recovery from exposure

In this study, we found that despite of the decrease in PP, SHS-induced changes in BP, BRS, and HRV were reversible and can be recovered after the cessation of SHS exposure. Unfortunately, we did not have the chance to investigate the recovery period from HFD exposure. However, studies from other research groups provide insights that HFD-induced cardiovascular changes that observed in this study is likely to recover if mice were switched back to the normal diet. For instance, Littlejohns and colleagues showed

that in mice, after switched the diet from high-fat back to normal diet, the previously HFD-induced increased vulnerability to ischemia and reperfusion injury were partially recovered (Littlejohns *et al.*, 2014).

The remaining questions and curiosity are whether the co-exposure-induced cardiovascular dysfunctions are also reversible, or whether the recovery rate from co-exposure is the same or slower than that from SHS or HFD exposure alone. In addition, if one exposure was removed and the other exposure remained, will we see a partial recovery from this? Investigating these questions can provide valuable information on the implementation of health guidelines. Instead of a general health guideline, specific and targeted guidelines should be considered for different population groups in accordance with the potential risks factors they may encounter.

Future directions

Characterization of the time course of SHS- and HFD-induced cardiovascular changes is only a beginning. Future studies are needed to underscore the underlying mechanisms of these exposure-related cardiovascular dysfunctions, as well as to investigate the potentially shared or independent pathways under the co-exposure of SHS and HFD. For example, does SHS have greater impacts on parasympathetic nervous system while HFD having greater impacts on sympathetic nervous system? Whether and how SHS-induced lung inflammatory response may have effects on HFD-induced systemic inflammation?

Exposure to SHS and HFD can induce cardiovascular dysfunctions via several proposed mechanisms, including increased inflammatory response, oxidative stress, endothelial dysfunction, atherosclerosis, and autonomic imbalance (Barnoya and Glantz, 2005; Li, Dai and Jia, 2009; Dow *et al.*, 2015; Chaar *et al.*, 2016; Kesh, Sarkar and Manna, 2016; Duan *et al.*, 2018). Among these potential pathways, autonomic imbalance may be particularly important. In our study, we showed that exposure of SHS and HFD led to a decreased HRV and BRS, which both are indicators of a decreased parasympathetic regulation of the heart (Malik *et al.*, 1996; Laude, Baudrie and Elghozi, 2009), suggesting a SHS- and HFD-induced autonomic dysfunction. However, the role of sympathetic nervous system in these exposure-induced cardiovascular changes was unclear in this study. Several researches have shown that chronic exposure of SHS and HFD are associated with increased sympathetic nerve activity and BP, suggesting that SHS and HFD may both induce sympathoexcitation to increase the BP (Hausberg *et al.*, 1997; Armitage *et al.*, 2012). Here, our results showed that 11 weeks of HFD led to an elevated BP, which may presumably be due to an increased sympathetic nerve activity. However, SHS-induced increase in BP was transient and only observed after four weeks of exposure. Additionally, the co-exposure induced changes in BP were similar to that from HFD exposure alone. Therefore, in order to acquire a better understanding of how sympathetic nervous system may contribute to these exposure-related changes in BP, further studies are required to measure sympathetic nerve activity in response to SHS and/or HFD exposure and incorporate it into the presented time course.

Furthermore, our previous study have suggested a potential neuronal mechanism underlying the SHS-induced decrease in HRV (Sun *et al.*, 2021). We found that four weeks of SHS exposure, at an environmentally relevant concentration, resulted in a decreased intrinsic excitability of cardiac vagal neurons located in *nucleus ambiguus*, which can in turns lead to attenuated parasympathetic output to the heart and, thus, a decreased HRV (Sun *et al.*, 2021). In the light of these findings, the question was raised whether HFD exposure share a similar pathway in decreasing the parasympathetic regulation of the heart. On the other hand, the central neuronal mechanisms underlying the SHS- and HFD-induced impaired baroreflex function are unknown. Future studies are needed to investigate the contribution of central neuroplasticity to these exposure-related autonomic dysfunctions of the cardiovascular system.

In closing, our study contributes to the understandings of how exposure of SHS and HFD can lead to cardiovascular morbidity and mortality. Studying the co-exposure effects of SHS and HFD also uncover a group of population that is more vulnerable to exposure and is at higher risk of the development of cardiovascular diseases. Moreover, the knowledge into how two independent risk factors can interplay with each other in inducing health outcomes provide valuable information and inspiration in exploring the development and progression of complex, multifactorial diseases.

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