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ABSTRACT

Title of Thesis: The Effects of Stress and Nicotine on Heart Histopathology Differ in Male and Female Sprague-Dawley and Long-Evans Rats

Brenda M. Elliott, Master of Science, 2001

Thesis directed by: Neil E. Grunberg, Ph.D.

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The present experiment investigated the effects of nicotine for 14 days (0, 6, or 12 mg/kg/day) and immobilization stress on heart histopathology in 120 male and 120 female rats of two strains (Sprague-Dawley and Long-Evans). Both nicotine and stress affected heart tissue, including heart mass and wall thickness. These effects differed between males and females. Females were more sensitive than males to the effects of nicotine on heart histopathology. In contrast, males were more sensitive than females to the effects of stress. The effects of nicotine also differed between Sprague-Dawleys and Long-Evans. The hearts of Long-Evans rats were more affected by both nicotine and stress than were Sprague-Dawleys. These findings have important implications for understanding the cardiotoxic effects of both nicotine and stress and could be used to further elucidate the mechanisms by which stress and nicotine separately contribute to heart disease.

The Effects of Stress and Nicotine on Heart Histopathology

Differ in Male and Female

Sprague-Dawley and Long-Evans Rats

by

Brenda M. Elliott

Master's Thesis submitted to the faculty of the

Department of Medical and Clinical Psychology

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INTRODUCTION

Heart disease is the leading cause of death in the United States. Every year over half-a-million Americans die from heart disease and related causes. Although non-modifiable factors such as age and gender can explain a proportion of these deaths, modifiable behaviors such as cigarette-smoking account for almost half of heart disease-related morbidity and mortality (Baum, Gatchel, & Krantz, 1997). Exposure to stressors also is known to play a role in the pathophysiology of heart disease and individuals who smoke smoke more when under stress (Shiffman, 1985; Wills & Shiffman, 1985; Shiffman, 1982; USDHHS, 1988).

Because the role of smoking in heart disease is well-established, nicotine replacement products have been developed to increase smoking cessation by suppressing the adverse effects of withdrawal. Nicotine, however, is the addictive and primary vasoactive substance in cigarettes and may actually be responsible for many of smoking's deleterious cardiovascular effects (Benowitz, 1997). Consequently, it is imperative to examine the specific contributions of nicotine to heart disease apart from the effects of cigarette smoking.

In addition, because little is known about the extent to which nicotine alone contributes to heart disease-related morbidity and mortality, determining nicotine's specific role in heart disease is useful to better assess the potential risks associated with nicotine replacement therapies used in the treatment of smoking cessation. A few preliminary studies have examined the implications of the use of nicotine replacement therapies for cardiovascular patients (Benowitz & Gorulay, 1997; MacDougall,

Dembrowski, Slaats, Herd, & Eliot, 1983); however, the effects of sustained nicotine on heart tissue (i.e., heart mass, wall thickness) have not been examined. Consequently, the extent to which nicotine replacement therapies may contribute to heart disease is not known. Understanding the specific effects of nicotine on the heart both alone and in the context of other factors that separately contribute to heart disease (i.e., stress) may illuminate both the mechanisms of heart disease and the limits of the effectiveness of nicotine replacement products.

Because heart disease is not typically the result of a single factor, such as tobacco smoking, it is important to examine how other factors may interact with nicotine exposure to alter heart tissue. The effect of stress, for example, may be particularly relevant given that smoking increases when individuals are stressed. Furthermore, because behavioral and biochemical responses to nicotine and stress may depend on individuals' gender and genotype, these factors also should be considered in the context of nicotine replacement therapies.

The present work examined the effects of nicotine administration with and without stress on heart histopathology using an animal model. The purpose of the study was to determine the specific effects of nicotine on heart tissue as it might be administered via tobacco or as it might be administered via nicotine replacement therapy. Because stress separately contributes to heart disease and because smoking and stress have been found to co-occur, the effects of stress on heart tissue and the combined effects of nicotine and stress also were examined. Finally, because individuals may respond differently to the effects of stress and nicotine, the present study included several

different genotypes (i.e., rat sex and strain) to determine if such factors mediate the effects of nicotine, stress, or the combined effects of nicotine and stress on the heart.

Background material pertinent to this work is presented in the sections that follow. These sections include information regarding the heart and heart disease, smoking and heart disease, the effects of nicotine and nicotine replacement products, stress and heart disease, the relationship between stress and smoking, and the influence of individual differences. Then, a complete review of the literature related to the current work is presented.

Heart and Heart Disease

From the moment it starts beating until the end of life, the heart works tirelessly to supply life-sustaining power to the organism. The heart is a hollow organ that in humans is roughly the size of a clenched fist. It is made up of four chambers enclosed by four muscular walls. The chambers at the top of the heart are called the atria (right and left). They serve as temporary storage places for blood waiting to enter into the ventricles. The lower chambers are the ventricles (right and left). Unlike the atria, the ventricles serve as powerful pumps that forcefully propel the blood to the lungs (right ventricle) or to the rest of the body (left ventricle). They are vital to the working of the heart.

Essentially, the ventricles serve as the power source for the heart. The right ventricle is thin, irregular in shape, and appears to wrap itself around the left ventricle (see Appendix). In contrast, the left ventricle is circular or ellipsoidal in shape, resembling a cavity. The left ventricle is the most important chamber of the heart, functioning to propel oxygenated blood to the systemic circulation. Bordering the left

ventricle and contributing to its essential functioning are four muscular walls: 1) the inter-ventricular septum (IVS) serves as a partition between the right and left ventricle; 2) the anterior wall separates the left ventricle from the apex of the heart; 3) the posterior wall separates the left ventricle from the bottom of the heart; 4) the lateral wall lies immediately adjacent to the left ventricle. Together, these muscular walls work in concert to provide the ventricle with the strength needed to carry out its forceful pumping actions.

Because the heart plays such a vital role in sustaining life, it is important that it is adequately nourished and free from undue strain. The heart receives its supply of blood through the coronary arteries. If these arteries become clogged because of the accumulation of fats or atherosclerotic plaque, then blood flow is interrupted and the heart is not able to function effectively. Specifically, decreased blood flow to the heart forces the heart to work harder to meet oxygen demands. If the heart is repeatedly forced to increase its workload to meet such demands or overcome the increased peripheral resistance resulting from arterial obstructions, then damage to the heart can ensue.

The most common definition of heart disease is the inability of the heart to provide adequate nutrient supply to metabolizing tissue (Doggrell & Brown, 1998). Disruption of the heart's source of nourishment forces the heart to work harder, resulting in eventual damage to the heart. Among humans, the development of heart disease is multiply determined; therefore, it is often difficult to disentangle the factor or factor(s) that contribute most significantly to disease onset. Animal models, in contrast, allow specific variables to be isolated and examined for their role in heart disease.

Over the past decade, significant advances have been made to understand the many factors that contribute to heart disease. Studies with animals, in particular, have provided useful models to examine human physiology and biology. In cardiovascular and heart disease research, for example, animals allow the study of the early stages of heart disease as well as the pathogenesis of disease and disease outcomes following pharmacological intervention (see Doggrell & Brown, 1998, for review).

In contrast to studies with humans, animal models provide an efficient and inexpensive way to determine the specific effects of isolated factors on heart disease and function. Additionally, animal models provide opportunities to advance the understanding of human disease when ethical considerations preclude studies with humans. Although no one animal model perfectly mimics patterns of heart failure in humans, different models can be used to complement clinical experience and extend our current understanding of heart disease (Doggrell & Brown, 1998). More specifically, animal models of heart disease have been used to determine whether the administration of a specific drug or pharmaceutical agent is protective or toxic to the heart. Additionally, how stress contributes causally to heart disease has been examined using animal models.

The Bowman-Gray monkey studies conducted by Kaplan and colleagues (1982), for example, provide excellent examples of how animals can be used successfully to unravel the links between stress and heart disease. Animal models, therefore, may offer promise in determining just how behaviors such as cigarette smoking and stress

contribute to the pathogenesis and progression of heart disease.

Cigarette Smoking Relevant to Heart Disease and Function

Cigarette smoking accounts for as many as 30% of all coronary heart disease (CHD) related deaths in the United States each year (USDHHS, 1988; 1989). Epidemiological data collected from the United States, Canada, and the United Kingdom indicate that smoking increases the risk of fatal and nonfatal myocardial infarction and sudden cardiac death in men and women (Baum, Gatchel, & Krantz, 1997). Fielding (1985), for example, found a higher incidence of myocardial infarction, CHD, and sudden death among smokers, averaging 70% above non-smokers. In a separate study, male smokers, ages 40-59, had a relative risk factor of 2.5 when compared to nonsmokers . (Pooling, 1978). These risks substantially decrease soon after cessation of smoking.

Although such studies offer convincing support for the relationship between smoking and heart disease, they do not specifically identify the components of cigarette smoke that are responsible for these effects. Nicotine has been identified as the most clinically relevant component to understand the effects of nicotine replacement therapies. It is, however, only one of the many potentially toxic components of cigarette smoke.

Nicotine and Tobacco Smoke

Cigarette smoke is composed of volatile and particulate phases (Zevin, Goourlay, & Benowitz, 1998). The volatile phase, which comprises about 95% of the total cigarette weight, contains 500 gaseous compounds (Hoffman & Hoffman, 1997). Conversely, the particulate phase, which accounts for the remaining 5%, contains over 3500 compounds, the major one being nicotine alkaloid (Hoffman & Hoffman, 1997). Nicotine has been

identified as the addictive substance in tobacco and is the primary pharmacologic reason why people smoke (Benowitz, 1988).

Nicotine acts by binding to nicotinic acetylcholine receptors in both the central and the peripheral nervous system (USDHHS, 1988). By acting on specific binding sites or receptors throughout the sympathetic (SNS) and parasympathetic (PNS) nervous systems, nicotine triggers the release of catecholamines, serotonin, corticosteroids, and pituitary hormones that together contribute to the cardiovascular effects of smoking.

In addition to triggering the release of a cascade of hormones, the binding of exogenous nicotine to the receptors typically lasts longer than the endogenous neurotransmitters, producing a secondary blockade of these receptors (Okuyemi, Ahluwalia, & Harris, 2000). Nicotine dependence is central to smoking behavior and serves as the rationale for the pharmacological replacement of nicotine for treatment of smoking cessation. Because the specific effects of nicotine are of primary importance with respect to clinically relevant risks of nicotine replacement therapies, it is important to examine the effects of nicotine on the heart apart from those obtained via cigarette smoke.

Nicotine and Heart Disease

Recent investigation into nicotine's role in the pathological processes of heart disease has focused largely on nicotine's potential to exacerbate the effects of other cardiotoxic substances, such as carbon monoxide, which are released during smoking (McDougall, et al., 1983). Additionally, nicotine's activation of sympathetic ganglia result in increased cardiac output, myocardial contractility, increased metabolic demand,

increased vasoconstriction, and increased blood viscosity. Together, these changes contribute to endothelial injury that is thought to be the antecedent to atherosclerosis.

Nicotine administration at concentrations similar to those of cigarette smoke modulates structural and functional characteristics of vascular smooth muscle and endothelial cells (Csonka, Somogyi, Haberbosch, Schettler, & Jellinek, 1985; Thyberg, 1986). Although the mechanism by which nicotine exerts its effects on the endothelium is not clear, evidence that it occurs has been obtained in animal studies. Krupski (1987), for example, found that oral nicotine administered to rats to achieve blood levels comparable to those in humans produced greater myointimal thickening of the aorta after experimental injury than in control animals.

Although nicotine is known to mediate the hemodynamic effects of smoking, thereby contributing to heart disease, it is unclear whether nicotine alone has the potential to cause acute or chronic heart disease. Knowledge of nicotine's role in heart disease is crucial not only for illuminating the risks associated with smoking, but also for aiding in the risk-benefit analysis of using nicotine analogues as pharmacological treatment strategies. Careful assessment of the effects of nicotine medications using animal models can provide the context in which the actions of nicotine on the heart can be isolated and compared to the effects observed with cigarette smoking.

Examination of Nicotine's Effects using an Animal Model

Although information about the effects of nicotine in man can be inferred by studying smokeless tobacco users, the pure effects of nicotine can be examined more appropriately using animal models. Specifically, by using animal models, the

investigator can isolate variables that are not confounded by the introduction of human variables such as attitudes, beliefs, and opinion (Grunberg, Faraday, & Rahman, 2000). Additionally, animal models allow the experimenter to conduct true experiments by using random assignment and manipulation of variables as well as to use invasive procedures. To date, several animal models have been used to investigate the neurobiological, physiological, and behavioral effects of nicotine (Grunberg, et al., 2000).

It is difficult to precisely mimic nicotine delivery via smoking without using smoke as a vehicle; however, a paradigm that uses the osmostic mini-pump provides an acceptable model of human smoking. Although self-administration models are more face-valid models of human smoking, these models do not provide for the complete control of the amount of drug being administered. In contrast, the osmotic mini-pump (see Appendix) administers nicotine through a small, pill-like device (Alzet mini-pumps: Alza Corporation, California) that is implanted subcutaneously between the animal's withers. The device slowly releases about 0.5 ul/hour of the solution (saline, nicotine) for up to twenty-one days depending on the model of the minipump. Such a device provides for the controlled administration of the drug being delivered. The osmotic mini-pump model used to examine the effects of nicotine was developed by Grunberg (1982) and has been used successfully to investigate the effects of nicotine on body weight, appetitive behaviors, activity, and attention that subsequently have been replicated in humans (e.g., Gritz, Klesges, & Meyers, 1989; Grunberg, 1992; Grunberg, Bowen, & Morse, 1984).

In general, this paradigm yields results in rats that have been replicated in human laboratory and clinical studies (Gritz et al., 1989; Grunberg, 1982). In addition, the

minipump eliminates the stress of exposure to repeated injections and the stress associated with indwelling catheters in the self-administration paradigms. Because stress also is an independent variable in this experiment, it is important to eliminate stress as a confound in nicotine's effects. Further, this particular method and rate of administration even more closely models the transdermal nicotine system used in smoking cessation because of their similar pharmacokinetic profiles (Benowitz, 1998).

Pharmacokinetics of Nicotine in Humans and Animals

Nicotine's actions in the body depend largely on route of administration. The rapid dosing obtained through cigarette smoking, for example, produces more intense cardiac effects than those obtained through administration via nicotine replacement products (Benowitz, 1998). After smoking, nicotine is absorbed rapidly from the lungs into the pulmonary venous circulation. From there it travels to the heart and then onto other organs via arterial circulation (Benowitz, 1998). After a cigarette, nicotine levels in the heart may be as high as 200ng/ml-300ng/ml. Venous levels, in contrast, are typically lower (20-30% of arterial levels) because by the time blood reaches the venous circulation, drugs have usually undergone first pass metabolism by the liver (Benowitz, 1998).

The pharmacokinetic profile of nicotine obtained via chewing gum, tobacco, and snuff differs from that seen in smoking. Nicotine from nicotine gum, chewing tobacco, and snuff is absorbed slowly through the oral mucosa. Plasma levels rise slowly, reach a plateau after 30 minutes, and then decline over the next 2 hours (Zevin et al., 1998). Because extraction of nicotine from gum is incomplete, plasma levels obtained from

nicotine gum (two 2-mg pieces) are lower than levels obtained after smoking, averaging between 10-15 ng /ml during exposure. Nicotine delivered via transdermal patches (15-22 mg nicotine per day), averages between 15-25 ng/ml. Nicotine obtained via patch is gradually absorbed and plasma levels rise slowly over 6-10 hours, remaining at steady levels for 7-8 hours, and then declining during the next 6 hours (Benowitz, 1998). Overall, plasma levels obtained via transdermal patch are substantially lower than those obtained through heavy cigarette smoking. Additionally, because the drug is absorbed more slowly, the differences between arterial and venous concentrations are much smaller than that observed during smoking.

In summary, when nicotine is inhaled during cigarette smoking, it is rapidly absorbed. High arterial plasma levels reach the brain and the vasculature almost immediately. In contrast, when nicotine is delivered through the transdermal system, the rise in plasma levels is slow and gradual and peak arterial levels are low. These levels are, on the average, much lower than what is obtained from cigarette smoke. Although the mini-pump provides a model analogous to human smoking because of its constant infusion over the course of a day, the levels obtained through such administration more closely resemble those obtained via transdermal exposure such as that used in nicotine replacement therapy.

Nicotine Replacement Therapy

Use for Smoking Cessation

Nicotine is the addictive component in cigarettes. Deprivation from nicotine as a result of abstinence or a reduction in the number of cigarettes smoked results in

withdrawal symptoms characterized by a number of unpleasant symptoms, including craving, tenseness, irritability, and difficulty concentrating (Gore & Chien, 1998). These abstinence effects are largely responsible for the high relapse rates among smokers attempting to quit (West, 1984). The amount of nicotine obtained through nicotine replacement therapies (NRT), does not provide the same pharmacological effects as are obtained via smoking cigarettes. Instead, nicotine replacement therapies partially alleviate the adverse symptoms associated with withdrawal by administering nicotine systemically and subsequently maintaining chronic low levels of nicotine. In addition, nicotine replacement products are considered a safe alternative to smoking because the toxic substances found in cigarette smoke are eliminated. Although NRTs appear to be a safe alternative to cigarette smoking, smokers with cardiovascular risks have been advised to seek physician counseling before using nicotine products. Despite this warning, the cardiovascular effects of nicotine replacement, *per se*, have not been clearly delineated. It is well known that nicotine exerts deleterious effects on cardiovascular function. The extent to which nicotine may directly affect heart tissue, however, has not been examined.

The Effects of Nicotine Replacement Therapy on Heart Disease/Function

To date, most of the research on the adverse effects of NRT on heart disease and function has focused more broadly on the risks of use among heart disease patients. Some published reports have suggested that nicotine medications may be linked to cardiovascular events; however, this research is sparse and inconclusive. Understanding the link between NRT and cardiovascular disease is important because smokers diagnosed

with cardiovascular disease may be trying to quit and may be prescribed a nicotine patch and because nicotine and nicotine analogues may be used in the treatment of disorders such as Alzheimer's disease, Parkinson's disease, and schizophrenia (Silver & Sandberg, 1993; Smith & Giacobini, 1992).

For those individuals with cardiovascular disease who are prescribed nicotine replacement therapy, establishing a relationship between NRT and cardiovascular events is often complicated by a long history of smoking behavior. Specifically, it is difficult to determine whether cardiovascular effects are a result of underlying effects of smoking or the nicotine medication itself. To date, no published studies exist on the effects of nicotine alone on heart tissue. Preliminary data, however, have suggested that nicotine may affect heart tissue in ways that may compromise heart function and subsequently lead to heart disease.

Preliminary data supporting the effects of nicotine on heart morphology appeared in a footnote in a study by Morse (1984). While examining biological effects of nicotine on rabbits, Morse (1984) serendipitously discovered that the hearts of those animals that had been administered nicotine looked markedly different from the control group. Measurements taken at that time revealed that nicotine reduced both left and right ventricular wall size in a dose-response fashion. A pilot study by Popp and Grunberg (1986) using rats replicated Morse's findings (N.E. Grunberg, personal communication, 1999).

Popp and Grunberg (1986) administered nicotine (6 mg/kg/day, 12 mg/kg/day) or physiological saline via osmotic mini-pump for two weeks to fifty-six male Sprague

Dawley rats. Following the two-week administration period, the rats were sacrificed and their hearts were examined for histopathological changes. Although histopathological findings were minimal and limited to two tissue sections, the changes in ventricle size and heart weight were consistent with those found in the Morse (1985) study. Specifically, nicotine reduced heart weight in a dose-response fashion. Similar trends were found for right and left ventricle wall thickness, although these findings were not statistically significant.

The consistency of these findings suggests that nicotine may exert direct effects on the heart, damaging heart tissue and reducing the efficiency with which the heart is able to function. These studies, however, were not designed to carefully and fully examine nicotine-induced changes in heart morphology. In addition, despite the fact that stress is a separate risk factor for heart disease and is commonly linked to smoking, the combined effects of nicotine and stress on the heart have not been examined.

Stress and Heart Disease

Stress can be defined as the process by which environment demands tax or exceed the adaptive capacity of an organism, resulting in psychological and biological changes that may place a person at risk for disease (Cohen, Kessler, & Gordon, 1997). Stress is a major risk factor for the development of coronary artery disease morbidity and mortality (Krantz, Kop, Santiago, & Gottdiener, 1996). Evidence for this view has been corroborated by anecdotal evidence, case reports, epidemiological studies, and human and animal experiments that examine the pathological and physiological effects of stress on the cardiovascular system (Bellack & Hersen, 1997).

Animal studies

Experimental studies with animals repeatedly have demonstrated that stress exposure increases the risk of heart disease (Manuck, Kaplan, & Matthews, 1986). In a series of studies conducted at the Bowman-Gray School of Medicine repeated exposure to social stress led to the development of atherosclerosis (Kaplan & Bush, 1982). In a study of male monkeys, dominant animals in an unstable social condition developed extensive coronary atherosclerosis. A subsequent study with female monkeys found that subordinate animals developed greater atherosclerosis than dominants. Together, these studies support a causal link between stress and coronary artery disease (CAD). Additionally, however, these studies revealed that the specific effects of stress depend on individual characteristics (i.e., gender and social status) that determine how conditions will affect behavior (Bellack & Hersen, 1993).

The contribution of environmental factors (i.e., stress) to heart disease and the extent to which individual factors such as gender and genotype may moderate these effects also has been examined using rodent models. These models, similar to those used in the Bowman-Gray studies, have provided the opportunity for the straightforward analysis of the behavioral and physiological correlates of heart disease and the context in which causal factors of heart disease can be isolated and investigated. In addition, because of their condensed life span, rodents provide the added advantage of evaluating life span changes (Campbell & Henry, 1987). Knowledge gained from such studies can then be used to generate parallel human models that validate the role of identified risk factors among humans (Campbell & Henry, 1987).

Human Studies

Findings from human epidemiological and laboratory studies have identified stress as a major risk factor in the development of heart disease. Myers and Dewar (1975), for example, reported that stressful life events preceded sudden death in 40% of patients studied. Cottingham, Matthews, Talbott, and Kuller (1980) reported that individuals who had recently suffered a loss were more likely than controls to become victims of sudden cardiac death. Recently, attempts to elucidate the relationship between stress and cardiac morbidity and mortality have focused on measurements of left ventricle mass. Specifically, the enlargement of left ventricle mass repeatedly has been found to correlate with stress reactivity and constitutes a proposed risk factor for heart related diseases. Julius, S., Li, Y., Brant, D., Krause, L., & Buda, A. (1989) found that neurogenic pressor episodes in dogs failed to cause hypertension but did induce cardiac hypertrophy suggesting increased cardiac mass also might be a reliable predictor of heart disease. More recent work by Julius (1998) and colleagues has provided additional evidence to suggest that left ventricular hypertrophy is a potent risk factor of cardiac mortality, congestive heart failure and sudden death.

The measurement of left ventricular mass in humans has documented utility in predicting blood pressure responses in both children and adults. Manuck and colleagues (1994), for example, observed that patients who exhibited the greatest blood pressure changes following exposure to a stressor also had greater left ventricular mass after 4 years of follow-up. More recently, Kop, Gottdiener, Patterson, and Krantz (2000) reported that the magnitude of mental-stress-induced systolic blood pressure response is

significantly related to left ventricular mass (LVM) among healthy individuals. Studies such as these suggest that changes in left ventricular mass following exposure to a stressful event may reflect underlying differences in cardiac reactivity that can be used to predict the potential risks for heart related diseases or, more broadly, help to elucidate in part the mechanisms by which stress exerts its effects on the heart.

In addition to these studies corroborating the findings of earlier animal studies, human studies also have suggested an underlying heterogeneity in an individual's reactions to stress. Specifically responses to a stressor may represent individual-specific response potentials (Manuck, 1994). Individuals may differ, therefore, in how they respond physically and psychologically to a given stressor.

Individual Differences in Response to Stress

In recent years, data collected from epidemiological and laboratory studies have indicated that gender and ethnicity are reliable predictors of CHD. Several studies, for example, have revealed that stress-induced changes in blood pressure occur more often in men, whereas women exhibit more stress-induced changes in heart rate than do men (Baum & Grunberg, 1991). More recently, Marcus and colleagues (1994) reported that increased activation of the sympathetic nervous system is related to left ventricular mass (LVM) in men, but not in women. These differences in reactivity and response type extend to ethnic and racial groups. Anderson, McNeily, and Meyers (1992) reported that blacks, in contrast to whites, demonstrate greater increases in cardiovascular activity in response to laboratory stressors. Furthermore, in the United States, blacks are more likely to develop CHD than are whites (Baum, Gatchel, & Krantz, 1997). In addition to

factors such as age, gender, and race affecting the likelihood of developing heart disease, individual differences also may indirectly affect heart disease risk by influencing sensitivity and tolerance to cardio-toxic drugs. Nicotine is an example of such a drug.

Individual Differences in Tobacco/Nicotine effects

Genotype can be conceptualized as biologically-based individual differences that are a consequence of differences at the level of the genome. Genotypic differences are manifested most broadly in ethnic and racial differences among humans and in strain differences among rats. Differences in genotype may explain differential sensitivity and tolerance to drugs such as cocaine, alcohol, and nicotine (Perkins, 1996), as well as explain physiological vulnerabilities to disease states or tissue damage associated with exposure to these drugs.

Human studies

It has been hypothesized that genetic differences in nicotinic receptors or nicotine metabolism might play a role in smoking behavior. Twin studies indicate that smoking is determined partially by genetic factors. More specifically, there is a mean heritability estimate of 53% for tobacco use (Hughes, 1986). Recent changes in gender rates of smoking further support this hypothesis. From the late 1950s to the early 1990s, the rates of smoking among females were lower than rates among males. More recently, with the introduction of low-tar and low-nicotine cigarettes, rates of smoking among females have equaled rates of male smokers (Grunberg, Winders, & Wewers, 1991). A similar trend has been found among adolescent girls. These patterns suggest that women and girls may be more sensitive to nicotine than men and boys and the availability of lower nicotine-

dose cigarettes increased female smoking rates (Kozlowski, Frecker, & Lei, 1982).

Tobacco use also varies within and among racial/ethnic groups. Whites smoke more than do Hispanics who in turn smoke more than do African Americans. Similar patterns have been found among adolescent smokers (Greisler & Kandel, 1998). These individual differences are consistent with the idea that biologically-based differences, in addition to sociocultural influences, may determine smoking behavior. Animal studies have provided corroborating evidence for this view.

Animal Studies

For several years, the differential effects of nicotine on various aspects of behavior have been examined using classic genetic approaches. Several investigators have conducted studies using inbred and selectively bred strains of mice to gather information on genetic correlates of some behavioral actions of nicotine (Mohammed, 2000). Hatchell and Collins (1977, 1980), for example, have completed extensive work in this area, conducting a comprehensive series of studies to elucidate the role of genotype in nicotine sensitivity. Their studies have revealed significant differences in mouse strains in response to both acute and chronic nicotine administration.

Similar strain specific differences have been found using rats as subjects with nicotine increasing startle and sensory-gating response of Sprague-Dawley rats but decreasing these responses in Long-Evans rats (Faraday, Rahman, Scheufele, & Grunberg, 1998; Faraday, O'Donoghue, & Grunberg, 1993; Acri, Grunberg, & Morse 1991; Acri, Morse, Popke, & Grunberg, 1994; Acri, 1994). Together, these studies support the notion that the effects of nicotine may vary as a function of individual

characteristics. Individual effects alone, however, cannot account for the variability in responding across individuals. Recent studies have suggested that identifying the contexts in which cigarette smoking exerts its effects is also crucial to understanding nicotine's effects (Gilbert, 1995).

Stress and Nicotine

Whether nicotine's effects on the heart depend on the arousal state of the organism is not clear. Many of the studies that examined the relationship between smoking and cardiovascular function were conducted on individuals in a relaxed, controlled situation. The findings, therefore, do not fully account for the interaction of variables, such as stress, that either may attenuate or exacerbate the nicotinic effects alone.

It is well established that stress is positively correlated with the use of addictive substances (Grunberg, et al., 2000). Smokers smoke more under stress than when not under stress (Schachter, Silverstein, & Kozlowski, et al., 1977; Schiffman, 1982; USDHHS, 1988). These reports seem paradoxical given that nicotine, a sympathomimetic, increases physiological (e.g., heart rate, blood pressure) and biochemical stress responses (e.g. cortisol) in humans and in animals (MacDougall, Dembroski, Slaats, Herd & Eliot, 1983; Morse, 1989; USDHHS, 1988). Several explanations have been proposed for this reported dissociation between behavior and biological response (Schachter, 1973): (1) Stress speeds nicotine excretion so that the smoker must self-administer more nicotine in order to obtain desired behavioral and psychological effects similar to those experienced in the non-stressed state; (2) nicotine's

behavioral and psychological effects are different in the stressed state than in the non-stressed state; (3) nicotine alleviates the unpleasant subjective experience of stress; and (4) individuals misattribute the symptoms of stress as symptoms of withdrawal (Schachter, 1977; Grunberg, Morse, & Barrett, 1983; Grunberg & Baum, 1985). These explanations together offer a starting point for why an individual might smoke more under stress; however, they do not characterize the potential adverse consequences associated with combined exposure to nicotine and stress.

Although it appears that nicotine may act to modify the effects of stress on behavior, the interaction of nicotine and stress on physiological parameters has been examined by only a few studies. Given that both stress and cigarette smoking have been linked to various disease processes, one might expect that the combined effect of cigarette smoking and stress would exacerbate the effects of either alone. Lisonkova (1976) reported that individuals who smoked and also had high levels of stress had a higher rate of clinical CHD than either condition alone. Additionally, MacDougall and colleagues (1983) reported that stress and smoking produced marked hemodynamic effects that were twice as large as those produced by either stress or cigarette smoking alone. MacDougall and colleagues (1983) also reported that individuals with increased responses to stress also had heightened responses to nicotine. More recent studies conducted by Perkins (1995) and Rosecrans (1995) have corroborated these findings. The study by Rosecrans (1995), for example, suggested that the administration of nicotine in experimental animals tends to transpose behavior depending on pre-baseline rates of behavior. Specifically, high rates of behavior are reduced, while low rates of

behavior appear to be increased by nicotine.

Together, these findings suggest that the variable effects of nicotine among both humans and animals may be due not only to individual specific factors (i.e., gender and genotype) but may in addition be affected by more transient factors such as stress. Further research in this area, therefore, should focus on the nature of the relationship between stress and nicotine and the extent to which the effects may be moderated by individual differences (i.e., gender and genotype).

Limitations of previous research and purpose of current study

Although a substantial body of research exists on the contribution of nicotine and stress to heart disease, few studies have examined the nature of these effects using histopathological samples. Studies examining the relationship between stress and heart disease have focused largely on the relationship between sympathetic arousal, left ventricular mass, and circulating lipids, and how these factors lead to hypertension and atherosclerosis. The effects of stress on other aspects of heart tissue have not been evaluated using histopathological samples. Studies with humans have suggested that increased left ventricular mass is a risk factor for cardiac morbidity and mortality (Casale, Devereux, & Milner, 1986). These findings, however, have been inconsistent with small effect sizes, have used primarily male subjects, and have examined only transient changes in left-ventricular mass. The chronic effects of stress on left ventricular mass have not been thoroughly examined using histopathological samples.

Similar limitations exist in studies of nicotine. First, there is a substantial literature examining the effects of nicotine on cardiovascular function but few studies

have examined the histopathological consequences of nicotine exposure. Second, it is not yet known whether nicotine replacement therapies may be potential contributors to pathogenesis of heart disease. Third, although nicotine exerts biochemical and physiological effects that are additive with stress, the combined effects on the cardiovascular system have not been fully examined. The extent to which these effects depend on subject's sex and genotype is relevant to understanding the cardiovascular effects of smoking and the possible risks associated with clinical use of nicotine.

The Present Research: Effects of Nicotine and Stress on the Hearts of Rats

The present research examined the effects of nicotine and stress on cardiac histopathology using an animal model. This experiment was conducted with rats as subjects. Use of an animal model to investigate the role of genotype and sex in cardiovascular effects of nicotine, stress, and nicotine and stress together allowed experimental control of additional factors that could potentially confound the findings in the study. For example, the animal's environment was controlled 24-hours a day for the duration of the study. Additionally, because factors such as gender, genotype, nicotine, and stress combine at multiple and complex levels to affect the heart, animals provide a valuable means to identify the extent to which these factors separately affect the pathogenesis of heart disease.

The present experiment used adult male and female rats of the outbred Sprague-Dawley and Long-Evans strains. These strains were selected based on findings in the context of an earlier experiment (Faraday, Rahman, Scheufele, & Grunberg, 1998) that these strains differed in behavioral responses to nicotine. A follow-up study examining

behavioral and biochemical responses to nicotine indicated that these two strains respond differently to nicotine, to stress, and to nicotine and stress, across a range of behaviors and biological indices (Faraday, O'Donoghue, & Grunberg, 1999; Faraday, Scheufele, Rahman, & Grunberg, 1999; Scheufele, Faraday, & Grunberg, 2000). In addition, Henry and colleagues (1993) reported that cardiovascular responses to stress also differed between these two strains. Specifically, Long-Evans rats experienced greater blood pressure changes in response to pychosocial stressors than did Sprague-Dawley rats. Because these two strains differed in their behavioral, biological, and cardiovascular responses to stress and nicotine, it seemed important to determine if these differences extended to changes in heart morphology.

In addition, these strains are widely used for a variety of experimental questions, including studies concerned with stress, immune function, patterns of drug addiction, and the aging process, and unlike other strains they are not bred specifically for particular behavioral or biological responses. Use of these strains is appropriate, therefore, when the goal is to examine differences that may extrapolate to human individual differences in a genotypically variable population.

Finally, use of an animal model allows for the careful control of drug administration and provides a careful examination of the drug-disease and stress-disease relationship. The effects of nicotine replacement therapy (NRT) are difficult to parse out from the cardiovascular effects of smoking that exist prior to NRT in humans. Isolating the specific effects of nicotine on cardiac tissue may illuminate the effects of nicotine on heart tissue as well as offer further information on the mechanisms by which chronic

nicotine exposure contributes to heart disease. Similarly, isolating the effects of stress and carefully examining the effects of stress and nicotine allow for future hypotheses about specific disease mechanisms.

The purpose of the current study was to determine whether nicotine alone or in combination with stress exerts deleterious effects on heart histopathology. The specific hypotheses for this study were based on preliminary findings from earlier studies in which nicotine was found to reduce heart mass. Because stress also has been identified as a major culprit in the pathogenesis of heart disease and because smoking and stress have similar vasoactive and cardiovascular effects, it was hypothesized that the effects of stress and nicotine would be additive, together resulting in more serious deleterious effects than those effects exerted by either factor separately. The specific hypotheses for the current study are discussed in the context of the predicted effects of each independent variable (i.e., drug, stress, strain, and sex).

Specific Hypotheses: Nicotine

Hypothesis 1: Nicotine will decrease heart muscle evidenced by a reduction in overall heart mass, heart length, left ventricle wall thickness, and right ventricle wall thickness.

Rationale: Morse (1984) reported that in male rabbits nicotine reduced left ventricle thickness, right ventricle thickness, and heart weight in a dose response fashion. Popp and Grunberg (1986) replicated these findings using rats.

Hypothesis 2: The effects of nicotine on heart tissue will be greater among females than among males.

Rationale: This hypothesis is based on several behavioral studies, which have reported

that females are more sensitive to the effects of nicotine than are males (Bättig, 1981; Collins, Miner, & Marks, 1988). Nicotine, for example, has been found to stimulate locomotion more in female rats compared to male rats (Bättig, 1981). In addition, several studies have found that the effects of nicotine on feeding and body weight are greater in female rats than in male rats (Grunberg, Bowen, & Winders, 1986; Grunberg, Winders, & Popp, 1987).

Hypothesis 3: The effects of nicotine on heart morphology will differ between strains.

Rationale: These two strains differ in their behavioral and biochemical responses to nicotine (Faraday, Rahman, Scheufele, & Grunberg, 1998; Faraday, O'Donoghue & Grunberg, 1993; Acri, Grunberg, & Morse 1991; Acri, Morse, Popke, & Grunberg, 1994; Acri, 1994).

Specific Hypotheses: Stress

Hypothesis 4: Stress will increase left ventricle width.

Rationale: Manuck and colleagues (1994) observed that patients who exhibited the greatest blood pressure changes following exposure to a stressor also had greater left ventricular mass after 4 years of follow-up.

Hypothesis 5: The effects of stress on heart morphology will be greater among males than among females.

Rationale: Throughout most of their lives, at least until age 55, men are at greater risk for heart disease than women. It was, therefore, predicted that the effects of stress on heart morphology would be greater among males.

Hypothesis 6: The effects of stress on heart morphology will differ between strains.

Rationale: Previous studies have reported that these strains differ on behavioral and biochemical responses to this stressor (Faraday, et al., 1999).

Specific Hypotheses: Stress and Nicotine

Hypothesis 7: The effects of stress and nicotine on the heart will be additive in that stress will exacerbate the effects of nicotine on heart mass and wall thickness. For example, if nicotine decreases heart mass and wall thickness as hypothesized, then those hearts exposed to both nicotine and stress together will be smaller than those hearts exposed to nicotine alone.

Rationale: Lisonkova (1976) reported that those individuals who smoked and also had high levels of stress had a higher rate of clinical CHD than any one condition alone. Recent studies examining the effects of environmental stressors on the pharmacokinetics of nicotine found that on exposure to high temperatures or physical exercise transient increases in plasma concentration of nicotine were noted (Vanakoski, Seppala, Sievi, et al., 1996; Klemsdal, Gjesdal, & Zahlsen, 1995). Therefore, the effects of nicotine were expected to increase under stressful conditions, exacerbating the effects with only nicotine

METHODS

Subjects and Housing

Subjects were 120 Sprague-Dawley (60 male, 60 female) rats and 120 Long-Evans (60 male, 60 female) rats (Charles River Laboratories, Wilmington, MA). All animals were individually-housed throughout the experiment in standard polypropylene shoebox cages (42 x 20.5 x 20 cm) on hardwood chip bedding (Pine-Dri). Throughout the study subjects had continuous access to rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. Housing rooms were maintained at 23° C at 50% relative humidity on a 12-hour reversed light/dark cycle (lights on at 1900). At the beginning of the experiment, subjects were approximately 49 days old. At the beginning of the experiment males weighed approximately 228 g; females weighed approximately 172 g.

Drug Administration and Surgical Procedure

Nicotine (6 mg/kg/day or 12 mg/kg/day) or physiologic saline was administered for 14 days via Alzet osmotic mini-pumps (Model 2002, Alza Corp., Palo Alto, CA). Physiological saline also was used as vehicle for the nicotine solution. Nicotine solution was made from nicotine dihydrochloride. The nicotine concentrations are expressed as nicotine base. Mini-pumps administered nicotine or saline solution at a rate of approximately 0.48 µl/hr. Dosages were calculated based on body weight such that nicotine animals received either 6 mg/kg/day or 12 mg/kg/day depending on experimental group assignment. These dosages have been used extensively in studies with rats that have been replicated in experiments with human smokers (Grunberg et al., 1984; Grunberg, 1986).

Mini-pumps were implanted during a brief surgical procedure. Subjects were anaesthetized using methoxyflurane (Metofane) in a bell jar inside a vented hood. Subjects were removed from the bell jar when tail pinch produced no reflex movement. Then, a 3 x 5 cm area between the withers was shaved and cleaned with Betadine. A 2 cm transverse incision within the shaved region was made with blunt-nosed surgical scissors, a subcutaneous pocket was created by spreading the subcutaneous tissues with the scissor tips, and the mini-pump was inserted with the flow modulator toward the subject's head. The incision was closed with 9 mm stainless steel wound clips. The entire surgical procedure including anesthesia took approximately 4 minutes.

Stress Manipulation

Animals in the stress condition were restrained in commercially available finger-like restraining devices (Centrap Cage, Fisher Scientific) 20 min/day beginning the day after surgery. Subjects were placed in the Centrap cage and the restraining "fingers" were tightened until subjects were immobilized, but not pinched or in pain. Restrained animals were checked every 5 min during the stress procedure to insure the manipulation did not result in pain or undue distress. This restraint procedure has reliably produced elevations in hormones associated with a stress response, including adrenocorticotropin hormone (ACTH) and corticosterone (Kant et al., 1983; Acri, 1992, 1994; Raygada et al., 1992; Klein, 1997; Faraday, 2000).

Procedure

The experiment was conducted in two phases: an acclimation phase and a drug administration/stress phase (see Table 1).

Acclimation Phase. Subjects were gentled 2 minutes a day for three days prior to the start of the study. Gentling reduces the stress associated with repeated handling during the course of the experiment. Subjects then underwent two weeks of acclimation to the animal facility. Body weight also was measured during this period for the purpose of balancing experimental groups.

Drug Administration /Stress Phase. After the completion of the acclimation phase, subjects were assigned within sex and strain to drug (0 mg/kg/day, 6 mg/kg/day, or 12 mg/kg/day nicotine) and stress (stress or no stress) groups in a manner insuring comparable initial body weights (Table 2). This assignment resulted in 24 balanced groups of 10 subjects per group. Attenuated growth and decreased body weight are well-established effects of nicotine administration at these dosages in the dynamic growth phase. Suppression of body weight gain was, therefore, used to validate drug administration.

Beginning on the first post-surgery day, animals in the stress groups underwent 20 minutes a day stress exposure. Following 14 days of drug administration, animals were sacrificed by decapitation. Hearts were removed immediately from the thoracic cavity by making an incision just under the breastbone and peeling away the rib cage. The heart was lifted carefully from the thoracic cavity and the still-beating heart was then gently placed in a glass vial containing neutral buffered formalin. Animals in the stress groups underwent stress exposure approximately one half-hour before sacrifice.

Heart Histopathology. Two hundred thirty-six of the original 240 rats were used for cardiac histopathological examination. Four of the original rats were eliminated from

all analyses: two male Sprague-Dawley rats in the stress plus 12 mg/kg nicotine condition, one male Long-Evans rat in the no-stress saline condition, and one female Sprague-Dawley rat in the no stress plus 12 mg/kg nicotine condition. In each case, a surgical complication resulted in failure of the mini-pump to deliver drug reliably. This failure was evident because these subjects did not lose weight and the site of the minipumps appeared encapsulated or infected.

Histopathological examination proceeded as follows (see Appendix). First, using 10mm calipers (Roboz Instruments, Rockville, MD), the length of each heart was measured from base to apex. Cross-sectional slices of the heart were made at the level of the ventricle (i.e., halfway between the apex and base of the heart). The tissues were examined for variations in the thickness of the myocardial wall of the left ventricle, right ventricle, septum, lateral wall, anterior wall, and posterior wall. Measurements were taken of each section with interrater reliability of .940. Specifically, two raters were used to measure each heart. If the raters' measurements correlated less than .90, a third rater, blind to the previous measurements, was used. The two measurements that were most closely correlated ($\geq$.90) were used. The histopathological procedures used in this study are based on methods used by cardiopathologist, R. Vermani (personal communication, June, 1999). Dr. Vermani personally instructed the principal investigator in the measurements and the correct procedure.

RESULTS

Analytic approach

Overall, two data analytic strategies were used. First, an analysis of variance (ANOVA) was performed to examine the effects of stress, nicotine, sex, and strain on selected heart parameters. Then, the same analyses were run using body weight as a covariate (ANCOVA). The purpose of using this dual approach was to separate the direct effects of stress and nicotine on heart mass from those occurring indirectly via overall changes in body mass because nicotine and stress reduce body weight in rodents. Specifically, this multi-analytic strategy allowed for the examination of whether stress and nicotine's effects on heart mass are fully attributable to changes in body weight or whether further changes in heart mass occur that cannot be explained by a reduction in the overall size of the organism.

At first all animals were analyzed together (N =120). Then, because there were statistically significant differences between sexes, males and females were analyzed separately. Finally, because the two strains are known to exhibit different responses to nicotine and to stress, animals were further analyzed within strain.

Heart histopathology data first were analyzed using a univariate analysis of variance to examine the effects of stress, nicotine, strain, and sex on heart weight. Heart weight was analyzed first as the primary parameter of interest because it provides a useful index of overall changes in heart mass. Tukey's HSD post hoc tests were used to determine which groups differed for nicotine effects. These analyses then were repeated using body weight as a covariate for reasons described above. Next, multivariate tests

were performed on heart length, left ventricle thickness, and right ventricle thickness to examine the effects of stress conditions and nicotine on cardiac wall segments. All effects and tests reported are two-tailed and significant at $p \leq .05$ unless otherwise noted.

Analysis of Heart Weight

When all animals were considered together, main effects for Stress [F (1, 211) = 6.23], Drug [F (2, 211) = 31.489], Sex [F (1, 211) =965.081], and Strain [F (1,211) =5.32)] on heart weight were found. Specifically, both stress (see Figure 1) and nicotine separately reduced heart weight. Post hoc analysis of drug effects revealed that nicotine reduced heart weight in a dose response manner with all groups significantly differing from one another (see Figure 2).

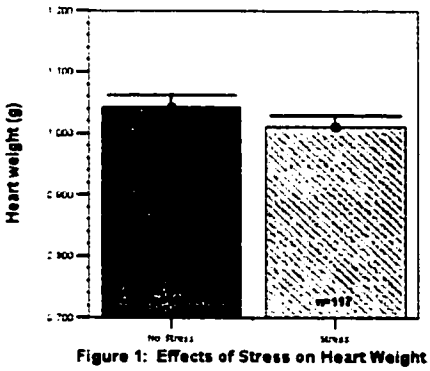

Figure 1: Effects of Stress on Heart Weight

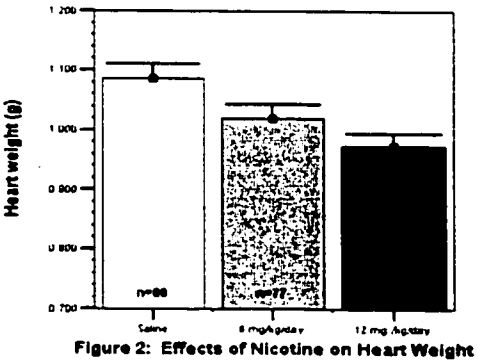

Figure 2: Effects of Nicotine on Heart Weight

In addition, males had larger hearts than females (see Figure 3) and Long-Evans had larger hearts than Sprague-Dawleys (see Figure 4).

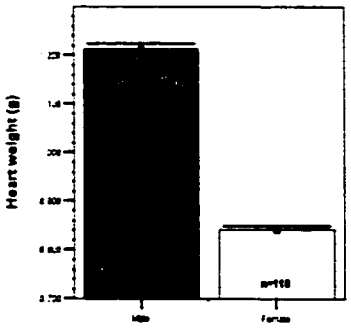

Figure 3: Gender Differences in Heart Weight

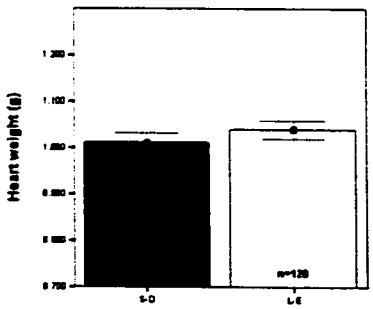

Figure 4: Strain Differences in Heart Weight

Because there was a main effect for sex and because the effects of sex accounted for a significant portion of the variance ($\kappa^2 = .834$), the effects of stress and nicotine were examined separately within sex. Results of this analysis revealed that the effects of stress were present for males only (see Figure 5). Specifically, stress significantly reduced heart weight in males [F (1, 105) =5.554] but not in females [F (1,106) =0.848, p =0.372]. In contrast, nicotine decreased heart weight in both males [F (2, 105) =13.268] and females [F (2, 106) =24.063]. Additionally, within females a Stress X Drug interaction was revealed [F (2,106)=5.281] in which the effects of nicotine and stress were not additive but instead moderated the effects of one another (see Figure 6). Specifically, stress and nicotine together appeared to counter the effects of nicotine alone, suppressing the overall reduction in heart weight.

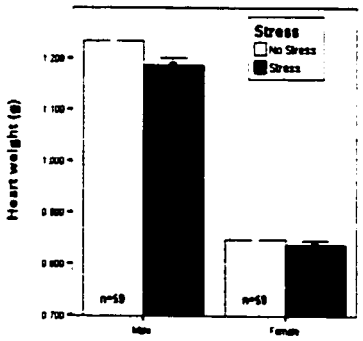

Figure 5: The Effects of Stress Differ within Sex

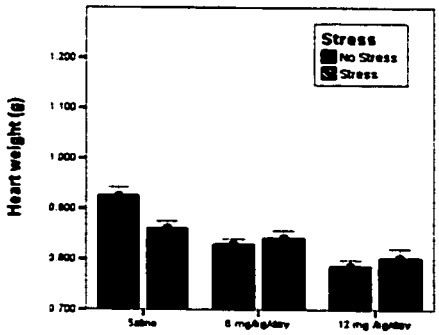

Figure 6: The Combined Effects of Stress and Nicotine Within Females

Next, because there also was a main effect for strain and because strain differences were hypothesized *a priori*, the effects of stress and nicotine were analyzed further within strain. Results of this hypothesis revealed that the effects of stress were

present for Long-Evans only [F (1, 108) =8.570]. Specifically, stress reduced overall heart weight in Long-Evans rats (see Figure 7).

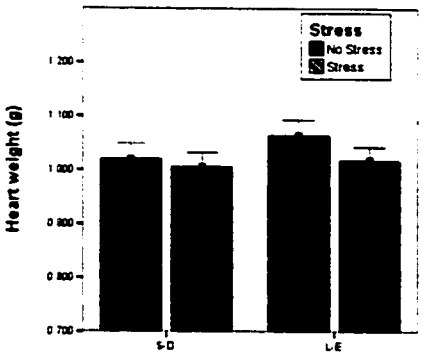

Figure 7: The Effects of Stress on Heart Weight Differ Within Strain

In contrast, nicotine reduced heart weight in both Long-Evans [F (2,108) =16.515] and Sprague-Dawley rats [F (2, 103) =15.121] with all drug groups significantly differing from one another (see Figure 8).

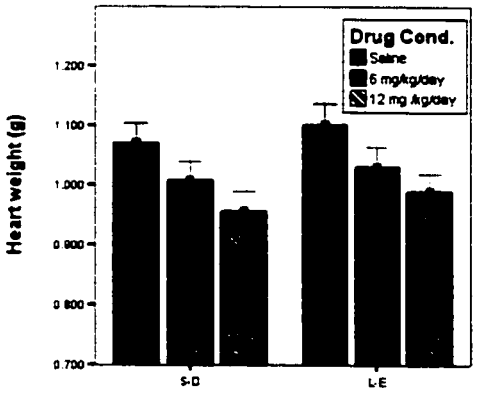

Figure 8: The Effects of Nicotine Within Strain

Finally, the results of stress and nicotine were examined within both sex and strain. Results of this analysis revealed that nicotine reduced heart weight in Sprague-Dawley [F (2, 51) =5.257] and Long-Evans [(2, 54) =8.418] males and Sprague-Dawley [F (2, 54) = 16.947] and Long-Evans females [F (2, 54) =9.331] (see Figure 9).

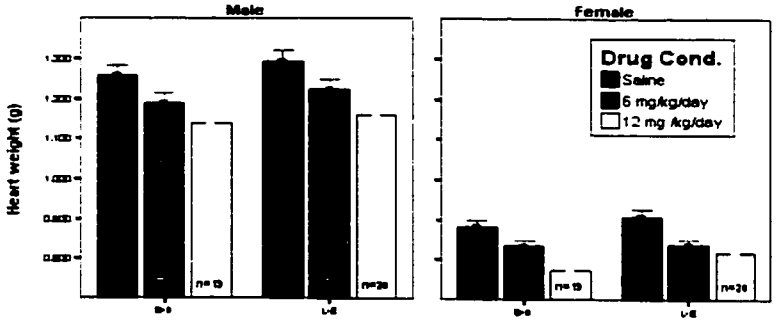

Figure 9: The Effects of Nicotine on Heart Weight Differ Within Strain and Sex

Post hoc analysis of drug effects revealed that within both Sprague-Dawley and Long-Evans males, only the 12 mg/kg groups significantly differed from saline. In contrast, within Sprague-Dawley and Long-Evans females, both the 6 mg/kg and 12 mg/kg groups differed significantly from saline. The effects of stress were present within Long-Evans males only [F (1, 54) = 7.144] (see Figure 10).

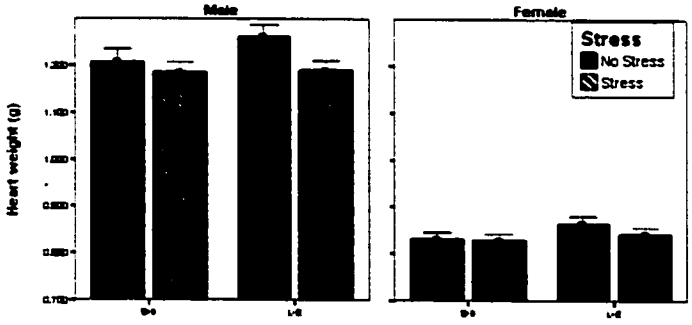

Figure10: The Effects of Stress on Heart Weight Differ Within Sex and Strain

Next, because overall body weight was found to correlate highly with heart weight (r =0.933, p =.0001) (see Table 3), the data were reanalyzed using body weight as a covariate.

Analyses of Heart Weight: Adjustment for Body Weight

Results of this analysis revealed that nicotine reduced overall heart weight [F (2, 210) = 8.273]. The main effect for strain also remained [F (1, 210) =14.982]. A main

effect for stress was not found when overall body weight was used as a covariate. The absence of stress effects following this analysis indicates that the effects of stress on heart weight do not exceed the effects of stress on overall body weight. That is, any reduction in heart mass likely can be attributable to an overall reduction in the size of the organism.

In order to parallel the previous data analytic strategy (with covariate), the results once again were analyzed within sex. When the sexes were considered separately, nicotine significantly reduced heart weight in both male [F (2, 104)= 3.128] and female [F (2, 105) =6.136] rats. In addition, an effect of strain remained within both males [F (1, 105) =8.439] and females [F (1, 105) =6.847].

Next, because a main effect of strain remained, the data were further analyzed within strain. Results of this analysis revealed a main effect of drug for both strains. Specifically, nicotine reduced heart weight within both Sprague-Dawley [F (2, 102) =3.358] and Long-Evans [F (2, 107) =5.131] rats.

Finally, the results of stress and nicotine were further analyzed within both sex and strain, using body weight as a covariate. Results of this analysis revealed that drug reduced heart weight in Sprague-Dawley females [F (2, 51) =7.005] and Long-Evans [(2, 53) =3.328] males only. There were no effects of stress present for males and females of either strain.

Analyses of Additional Heart Parameters

Next, because heart length also was thought to be a reliable indicator of changes in heart shape and because left and right ventricle size previously have been found to be affected by nicotine, the effects of stress, nicotine, sex, and strain were examined

separately on these parameters using a multivariate analysis of variance (MANOVA). Results of this analysis revealed main effects of Sex [F (3, 206) =205.930], Strain [F (3, 206) = 7.119], Stress [F (3, 206) =4.111], and Drug [F (6, 414) =5.884] as well as a Sex X Stress interaction [F (3, 206) =4.645], a Sex X Strain interaction [F (3, 206) =2.633], and a trend toward a Stress X Drug interaction [F (6, 414) =1.843, p. =0.089].

Results of subsequent univariate analyses revealed that stress significantly reduced both heart length [F (1, 208) =3.422] and left ventricle width [F (1, 208) =9.245] (see Figures 11 and 12).

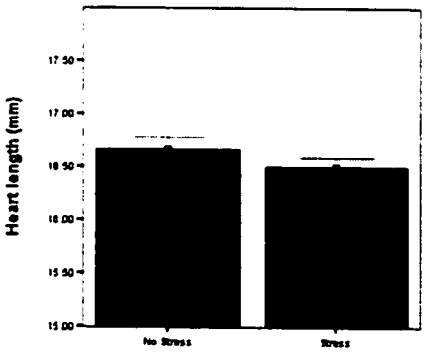

Figure 11: The Effects of Stress on Heart Length

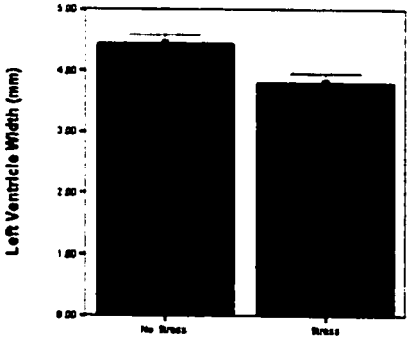

Figure 12: The Effects of Stress on Left Ventricle Width

In contrast, nicotine reduced heart length only [F (1, 208) = 16.328] with both the 6 mg/kg and 12 mg/kg groups differing significantly from saline (see Figure 13).

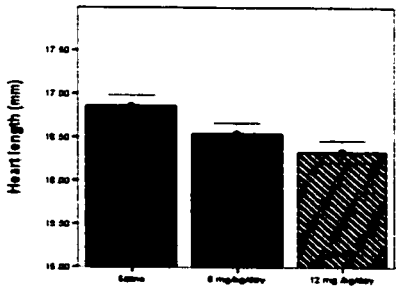

Figure 13: The Effects of Nicotine on Heart Length

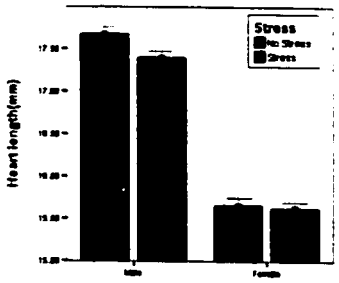

Figure 14: The Effects of Stress on Heart Length Differ Within Sex

Because a Sex X Stress interaction also was found, the effects of stress were

Specifically, there was no effect of stress within Sprague-Dawley rats. Stress, however, reduced left ventricle width in Long-Evans rats [F (1, 108) =10.697]. Nicotine reduced heart length within both Sprague-Dawley and Long-Evans rats. Within Long-Evans, both the 6-mg/kg group and the 12 mg/kg group differed significantly from saline. Within Sprague-Dawley rats, only the 12-mg/kg group differed significantly from saline.

Finally, the results were analyzed within both sex and strain. Results of this analysis revealed that stress reduced left ventricle width in Sprague-Dawley males [F (1, 49) =6.848] and left ventricle width [F (1, 54) =14.809] and heart length [F (1, 54) =14.809] in Long-Evans males (see Figure 20 and 21). There were no effects of stress within females of either strain.

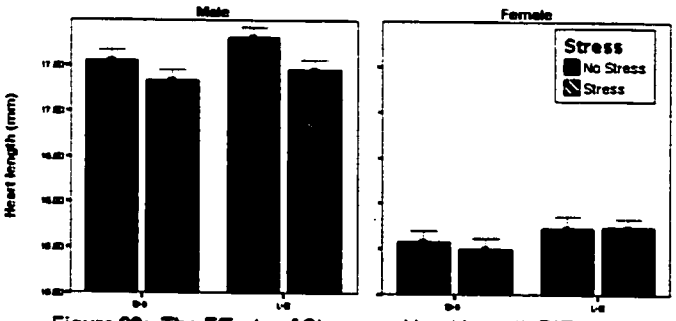

Figure 20: The Effects of Stress on Heart Length Differ Within Strain and Sex

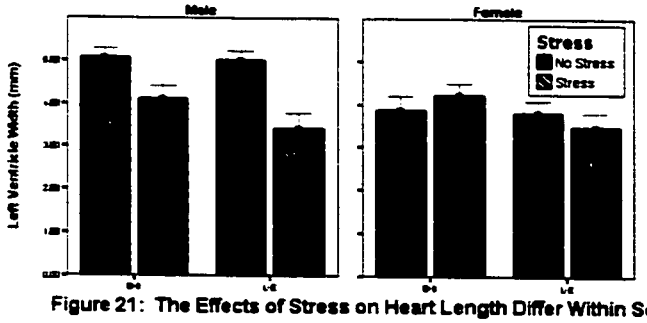

Figure 21: The Effects of Stress on Heart Length Differ Within Sex and Strain

Nicotine, in contrast, reduced heart length in Sprague-Dawley males [2, 49) =4.598]. Sprague-Dawley females [F (2, 51) = 2.787] and Long-Evans females [F (2, 54) =6.965] (see Figure 22). There were no effects of nicotine within Long-Evans males.

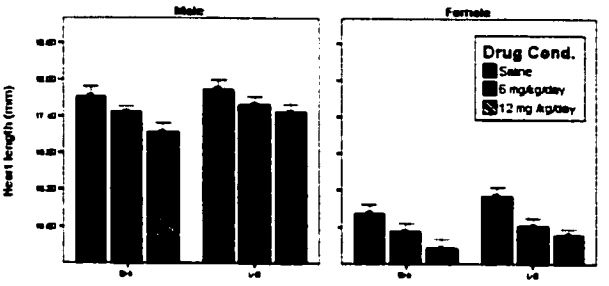

Figure 22: The Effects of Nicotine on Heart Length Differ Within Sex and Strain

Analyses of Additional Heart Parameters: Adjusted for Body Weight

Next, because body weight was found to correlate significantly with both left ventricle (r =0.202, p =0.002) and heart length (r = 0.875, p = .000), body weight was used as a covariate. When controlling for the effects of body weight, there was a main effect of Strain [F (3, 205) =8.679], Stress [F (3, 205) =2.577], and a trend toward a Drug effect [F (6, 412) = 2.007, p. =0.064]. In addition, a Strain X Sex [F (3, 205) = 2.602], and a Sex X Stress interaction remained [F (3, 205) =3.547]. Subsequent univariate analyses revealed that stress reduced left ventricle width [F (1, 207) =7.760] and nicotine reduced heart length [F (2, 207) = 3.628]. In addition, a Sex X Stress interaction was found for left ventricle [F (1, 207) =8.941] and right ventricle width [F (1, 207) =4.074]. A Stress X Drug interaction was found for heart length [F (2, 207) = 3.057].

When these results were analyzed further within sex, using body weight as a covariate, the effects of stress were found within males only. Specifically, stress reduced left ventricle width [F (1, 102) =18.573] and increased right ventricle width [F (1, 102) =

4.103]. In contrast, the effects of nicotine were present in females only. Specifically, nicotine reduced heart length [2, 104) =2.936] in females but nicotine alone did not affect heart length in males. Within males, a Stress X Drug interaction was found for heart length [F (2, 102) =3.201].

Next, because a main effect for strain was found, the results were further analyzed within strain. Within Sprague-Dawley rats, there was no main effect of stress. There was, however, a Stress X Sex interaction on left ventricle width within Sprague-Dawley rats. In contrast, a main effect for stress was revealed for Long-Evans rats. Specifically, stress decreased left ventricle width in Long-Evans rats. There was no main effect of Drug when animals were examined within strain.

Finally, the results were analyzed within both sex and strain, using body weight as a covariate. Results of this analysis revealed that only the effects of stress remained. Specifically, stress reduced left ventricle width in both Sprague-Dawley [F (1, 48) =10.336] and Long-Evans males [F (1, 53) =9.515]. Stress additionally increased right ventricle width in Long-Evans males [F (1, 53) =5.118].

Although additional heart parameters were measured, these measurements correlated significantly with one another and with the variables already examined. Changes in these parameters, therefore, did not occur independently of changes in the size of heart weight, heart length, or left and right ventricle width. Subsequent analyses, therefore, were not performed on additional heart parameters, but means and standard error or the means were calculated and are presented below. These results are reported in Table 4.

Confirmation of Nicotine Hypotheses

Hypothesis 1 that nicotine will affect heart morphology was **confirmed.** Nicotine reduced heart weight and heart length. Nicotine did not, however, affect left or right ventricle wall thickness.

Hypothesis 2 that the effects of nicotine on heart tissue will be greater among females than males was **confirmed**. Nicotine affected heart weight and heart length of both males and females, the size of these effects was greater within females. In addition, within females, the effects of nicotine were present at both the 6 mg/kg and 12 mg/kg dose, whereas within males, the effects were present at the 12 mg/kg dose only.

Hypothesis 3: that the effects of nicotine on heart morphology will differ among strain was **confirmed.** Although, nicotine reduced heart weight and heart length in both Long-Evans and Sprague-Dawley rats, the effect of nicotine on heart length were present at both the 6 mg/kg and 12 mg/kg dose for Long-Evans but only at the 12-mg/kg dose for Sprague-Dawleys, suggesting that these two strains differ in their sensitivity to nicotine's effects.

Confirmation of Stress Hypotheses

Hypothesis 4 that stress will increase left ventricle width was **not confirmed.** Stress decreased left ventricle width, heart weight, and heart length. The specific nature of these effects depended on both sex and strain.

Hypothesis 5: that the effects of stress on heart morphology would be greater among males than females was **confirmed**. When the effects of stress on heart weight, heart length, left ventricle width, and right ventricle width were examined separately

within each sex, the effects were present for males but not for females.

Hypothesis 6 that the effects of stress on heart morphology will differ between strains was **confirmed.** Overall, stress' effects on the heart were greater among Long-Evans rats.

Stress-Nicotine Effects:

Hypothesis 7 that the effects of stress and nicotine on the heart will be additive in that effects will be greater than exposure to any one condition alone was **not confirmed.** Within females, stress appeared to suppress nicotine's effects on heart weight. Specifically, the effects of stress and nicotine on heart weight were less than those found with nicotine alone.

DISCUSSION

The purpose of this experiment was to determine the specific effects of nicotine and stress on heart histopathology. Although the relationship between smoking and heart disease has been well established, this study focused on the specific effects of nicotine on heart tissue. More specifically, the present experiment was designed to evaluate whether exposure to nicotine, the primary vasoactive substance in tobacco smoke, exerts deleterious effects on the heart, apart from those obtained via tobacco smoke. In addition, the present experiment included stress as a variable to determine if stress alone alters heart histopathology and if stress affects the proposed relationship between nicotine and heart disease. Finally, the current study included males and females and two different rat strains (Sprague-Dawley and Long-Evans) to determine whether or not individual differences may account for the relationship between nicotine and heart disease. An animal model was chosen for this study to allow careful control of all the independent variables. The results indicated the effects of stress and nicotine on heart histopathology depend on both strain and gender.

Nicotine Effects

Nicotine decreased heart weight and heart length, thereby reducing overall heart size. In contrast to the Morse (1984) and Popp (1986) studies, nicotine did not affect left or right ventricle width. These findings have serious implications for understanding the mechanisms by which smoking may contribute to heart disease as well as the potential cardiotoxic effects of nicotine replacement therapies. Notably, however, these findings varied as a function of strain and sex, suggesting that individuals actually may possess

differential vulnerabilities to nicotine related heart disease.

The most striking finding regarding nicotine's effects in this experiment was the extent to which males and females differed in their responses. Nicotine decreased heart weight and length in both males and females. The size of these effects was greater in females as indicated by F values that were twice as large as those found in males. Furthermore, these effects were present for females at both the 6 mg-kg dose and 12 mg-dose but only at the 12 mg-kg dose for males. These results suggest that females are more sensitive to the effects of nicotine on heart histopathology. Such findings are consistent with other reports that females are differentially sensitive to the effects of nicotine than are males (Bättig, 1981; Collins, Miner, & Marks, 1988).

There are several explanations that have been offered for this reported differential sensitivity. Beckett, Gorrod, and Jenner (1971) suggested that males and females differ in the rate at which they metabolize nicotine. It is, therefore, possible that gender may influence the induction of nicotine metabolizing enzymes, making more nicotine available or for longer periods of time (Kyerematen & Vesell, 1991). Rosecrans' (1972) finding that injection of nicotine into male and female rats resulted in significantly higher levels of nicotine in the brains of female rats, relative to males further supports this view.

In addition to the striking gender differences, strain differences also were revealed. For example, although the effects of nicotine on heart weight and heart length were present for both strains, Long-Evans appeared slightly more affected than Sprague-Dawley. These findings offer the suggestion that underlying genetic differences (i.e., strain) may mediate the effects of nicotine on heart histopathology. Overall, however, the

differences are minor and therefore cannot be used to draw any firm conclusions about the role of strain in effects of nicotine on the heart.

Stress Effects

Stress was found to have an effect on heart histopathology. Specifically, stress altered heart weight, heart length, right ventricle width and left ventricle width. The specific nature of these effects is complex and differed based on both strain and sex.

The effects of stress on heart histopathology also exhibited striking sex differences. Specifically, when the effects of stress on the selected heart parameters were examined within sex, the effects were present for males only. Stress did not appear to significantly affect the female hearts.

These observed sex differences are consistent with previous findings that the rates of heart disease among men are greater than among women. Throughout most of their lives men are at greater risk for Coronary Heart Disease than women (Lerner & Kannel, 1986). Because, the risk of heart disease among women rises after menopause, female hormones such as estrogen have been thought to play a protective role for women. Whether this same explanation can be offered for the results of this study is not known.

Although the striking gender differences parallel the results of previous studies on heart disease, the finding that stress decreased left ventricle wall thickness contrasts with earlier human and animal studies in which stress increased left ventricle mass. This divergent finding may be explained by the fact that the results of this study were obtained using an acute stressor and, therefore, may not directly parallel the left ventricular hypertrophy that has been found to occur during chronic stress exposure (Manuck, 1994).

Instead, the reduction in left ventricle width found in this study may be fully attributable to stress inducted changes in body weight. That is, stress reduced overall body weight, thereby indirectly affecting the size of the heart. This explanation is supported by the fact that once body weight was used as a covariate, the effects of stress on heart mass disappeared. Further studies should explore this relationship to determine if the effects of stress on left ventricle size changes as a function of time or duration of stressor.

The changes in length that occur in response to chronic nicotine exposure or stress may be explained partially by the concept of cardiac remodeling. Cardiac remodeling has been defined as genome expression, molecular, cellular, and interstitial changes that are manifested clinically as changes in shape, size and function of the heart after cardiac injury (Cohn et al., 2000). The process of remodeling may occur in response to volume overload or pressure overload. As the heart remodels, it becomes less elliptical and more spherical in shape, a change that is consistent with reductions in heart length. Although the initial changes in the heart due to cardiac remodeling are thought to be adaptive, with changes in shape allowing the heart to maintain function in response to pressure or volume overload, over time changes in the size and shape of the heart may have adverse effects leading to progressive decompensation.

Stress and Nicotine

In contrast to the originally stated hypothesis, the effects of nicotine and stress together were not additive across all heart parameters. In fact, a significant Stress X Drug interaction was found on overall measures of heart weight within females only. For females, the effects of nicotine appeared to be cancelled out by the effects of stress at

both the 6 mg/kg and the 12 mg/kg dose. That is the heart weights of those animals receiving both stress and nicotine were larger than those receiving nicotine alone. Although limited to only a portion of the sample, these findings suggest that stress may affect how nicotine is metabolized, attenuating the effects of nicotine even at high doses.

Limitations of Current Study

The findings of the current study have important implications for understanding the pathological effects of both stress and nicotine on heart tissue. There are several limitations of this study, however, that limit the generalizability of the results. For example, the current study included only two dosages of nicotine, 6 mg/kg and 12 mg/kg. To more fully understand the effect of nicotine on heart tissue, it would be important to include a greater range of nicotine doses. Inclusion of more doses would help to delineate the shape of the dose response curve and therefore better understand the extent of nicotine's effects. Additionally, inclusion of lower doses would help to further examine the possibility of a sex difference in sensitivity to nicotine with regard to changes in heart shape and morphology.

A second limitation of the specific dosages selected for this study is the extent to which they parallel levels of nicotine obtained via NRT or cigarette smoking. Winders, Grunberg, Benowitz, and Alvares (1998) reported that the mean nicotine levels for animals receiving 6 mg/kg and 12 mg/kg via osmotic minipump were 148.30(+/- 32.49) ng/ml, and 257.29 (+/- 53.96) ng/ml respectively. Although nicotine levels obtained at the 6 mg/kg dosage are analogous to levels obtained following smoking, the levels obtained at the 12 mg/kg are actually much higher than those found in human smokers.

Because the effects of nicotine on heart histopathology were greater at the 12mg/kg doses, the results of this study must be interpreted with caution. Specifically, it is unlikely that the levels of nicotine obtained via NRT pose any substantial risks to the heart. Heavy smokers, however, are at potential risk.

Another limitation of the current study was that the measures used were not sensitive enough to fully elucidate the mechanisms via which nicotine and stress were exerting their effects. Specifically, the current study took only simple measurements of heart mass and wall thickness. Although these measurements provided evidence that nicotine and stress have direct effects on heart tissue, the factors responsible for these underlying changes could not be determined via current means. More precise measurements of heart tissue including an examination of the cytoarchitecture (myocyte shape and size, myocardium integrity) are needed to fully elucidate the factors that contribute to the observed effects.

Implications of Current Study

Despite the limitations of the current study, the findings have important implications for understanding both the role of stress and nicotine on heart tissue. These implications will be discussed separately for nicotine and stress.

Nicotine

The results of this study suggest that nicotine's effects on the heart exist independent from those obtained through smoking. Specifically, nicotine appears to reduce heart mass and muscle which may decrease the effectiveness of the heart to function effectively. Furthermore, if the results of these studies generalize to humans,

any nicotine exposure (e.g., cigarettes, chewing gum, nicotine replacement therapies) might also have deleterious effects on the heart. Understanding more specifically the extent of these effects is important to determine not only the potential implications associated with the use of nicotine replacement products in the treatment of smoking cessation but also to determine the efficacy of nicotine in the treatment of neurogenerative disorders such as Alzheimer's Disease, Tourette's syndrome, and Parkinson's disease.

In addition to the overall nicotine effects, the robustness of the gender differences in nicotine's effects revealed in this study is particularly relevant given that women tobacco smokers represent a major public health problem (Booze, Welch, Wood, Billings, Apple, & Mactutus, 1999). Specifically, smoking-induced lung cancer has surpassed breast cancer as the leading cause of cancer in U.S. women (Grunberg, Winders, & Wewers, 1991). The results of this study further extend the notion that women are also vulnerable to nicotine's deleterious effects and may be more prone to smoking-related diseases than previously thought.

Understanding the risks associated with nicotine exposure among females is important not only for further delineating the health risks associated with smoking, but has particular implications for the use of nicotine replacement therapies among females. If females are more sensitive to the effects of nicotine, then physicians should exercise caution when prescribing NRT for females interested in smoking cessation, especially if an underlying risk for heart disease is present.

Stress

The finding that stress had an affect on heart morphology is not surprising given the vast literature supporting a link between stress and heart disease. The results of this study overall, therefore, corroborated earlier findings but still did not fully elucidate the specific means by which stress may exert direct effects on the heart. The changes in heart mass that were revealed appeared to be the indirect results of changes in overall body weight. Stress did not appear to alter heart mass or shape directly. Further examination of heart tissue using more sensitive measures would be useful to illuminate the specific changes in heart tissue that occur as a result of chronic stress exposure.

The finding that men are more sensitive to the cardiovascular consequences of stress is again consistent with the findings reported here. Although the protective role of estrogen has been offered as a viable explanation for these observed gender differences, whether this same explanation can be offered for the current findings is not clear. One way to further explore this possibility, however, would be to compare the effects of stress on heart histopathology on ovariectomized females vs. controls. If the effects of stress on heart histopathology were greater among ovariectomized females, then support for the role of estrogen would be provided.

Future Studies

In conclusion, the results of this study have implications to understand the role of nicotine and stress in heart disease. Further studies are needed to elucidate the mechanisms that are responsible for the observed effects. Future studies should attempt to examine the factors that contribute to the heightened sensitivity to nicotine among

females. Additional studies also should examine the effects of nicotine on heart-related changes over time to more fully understand the health risks associated with different exposure periods or if the effects of nicotine on heart tissue may be reversed after cessation. Finally, it may be useful to include a group of adolescent rats to help clarify the changes in heart morphology that occur as a function of age. Further elucidating the role of age in contributing to nicotine's effects on the heart has potential implications for the use of nicotine replacement products to treat Tourette's disorder in young children.

In terms of stress effects, further studies should include more sensitive measures to more fully understand the morphological changes that occur as a result of chronic stress exposure. Information obtained from such studies could then be used to further illuminate the mechanisms via which stress leads to heart disease. In addition, it is equally important to examine the factors that contribute to the striking gender differences observed. The inclusion of ovariectomized animals would be one way in which to examine if estrogen is actually responsible for these effects.

Understanding the specific mechanisms via which nicotine and stress exert their effects on heart tissue can provide information necessary for both the prevention and treatment of heart disease. Further studies that examine heart morphology may provide valuable information relevant to reducing rates of heart disease.

Table 1: Timeline of the experiment

Baseline Phase	
Baseline Days 1-3	**Gentling**
Baseline Days 4-14	**Acclimation to Facility**
Drug Administration Phase	
Drug Administration Day 1:	**Nicotine/Saline Minipump Implant**
Drug Administration Day 2-15	**Nicotine/saline Exposure and Stress Manipulation begins**
Drug Administration Day 15	**All subjects sacrificed, hearts extracted**

Table 2: Design of Experiment

Strain (2)	**x Sex (2)**	**x Stress (2)**	**x Drug (3)**
Long-Evans (n =120)	Male (n =60)	No Stress (n=30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
		Stress (n =30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
	Female (n =60)	No Stress (n=30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
		Stress (n =30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
Sprague-Dawley (n=120)	Male (n =60)	No Stress (n=30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
		Stress (n =30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
	Female (n =60)	No Stress (n=30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)
		Stress (n =30)	0 mg/kg/day (n =10) 6 mg/kg/day (n =10) 12 mg/kg/day (n =10)

Table 3: Correlation of Heart Parameters With Overall Body Weight

		Heart Weight	Heart Length	Anterior	Posterior	Septum	Lateral	Left Ventricle	Rt Ventricle	Body Weight
Heart Weight	Pearson Correlation									
	Sig. (2-tailed)									
	N									
Heart Length	Pearson Correlation	.899 **								
	Sig. (2-tailed)	.000								
	N	234								
Anterior	Pearson Correlation	.422 **	.370 **							
	Sig. (2-tailed)	.000	.000							
	N	235	234							
Posterior	Pearson Correlation	.346 **	.331 **	.632 **						
	Sig. (2-tailed)	.000	.000	.000						
	N	235	234	235						
Septum	Pearson Correlation	.352 **	.250 **	.515 **	.484 **					
	Sig. (2-tailed)	.000	.000	.000	.000					
	N	235	234	235	235					
Lateral	Pearson Correlation	.271 **	.217 **	.303 **	.386 **	.490 **				
	Sig. (2-tailed)	.000	.001	.000	.000	.000				
	N	235	234	235	235	235				
Left Ventricle	Pearson Correlation	.139 *	.165 *	-.328 **	-.405 **	-.465 **	-.382 **			
	Sig. (2-tailed)	.033	.012	.000	.000	.000	.000			
	N	234	233	234	234	234	234			
Right Ventricle	Pearson Correlation	.181 **	.134 *	.253 **	.278 **	.299 **	.204 **	-.239 **		
	Sig. (2-tailed)	.005	.041	.000	.000	.000	.002	.000		
	N	234	233	234	234	234	234	233		
Body Weight	Pearson Correlation	.933 **	.875 **	.342 **	.268 **	.270 **	.206 **	.202 **	.114	
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.001	.002	.081	
	N	235	234	235	235	235	235	234	234	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4: Means and SEM on select parameters not included in overall analysis.

	Sprague-Dawley Males	Sprague-Dawley Females	Long-Evans Males	Long-Evans Females
Anterior Wall Mean; SEM	2.8202; 0.072	2.5953;0.065	3.2250; 0.067	2.7188; 0.058
Posterior Wall Mean; SEM	2.8684; 0.057	2.7168; 0.057	3.3208 ;0.065	2.8792 ;0.060
Septal Wall Mean; SEM	2.7018; 0.068	2.4461; 0.061	2.9583; 0.068	2.6583; 0.052
Lateral Wall Mean; SEM	2.9684; 0.083	2.6509; 0.070	3.1083; 0.082	2.8492; 0.062

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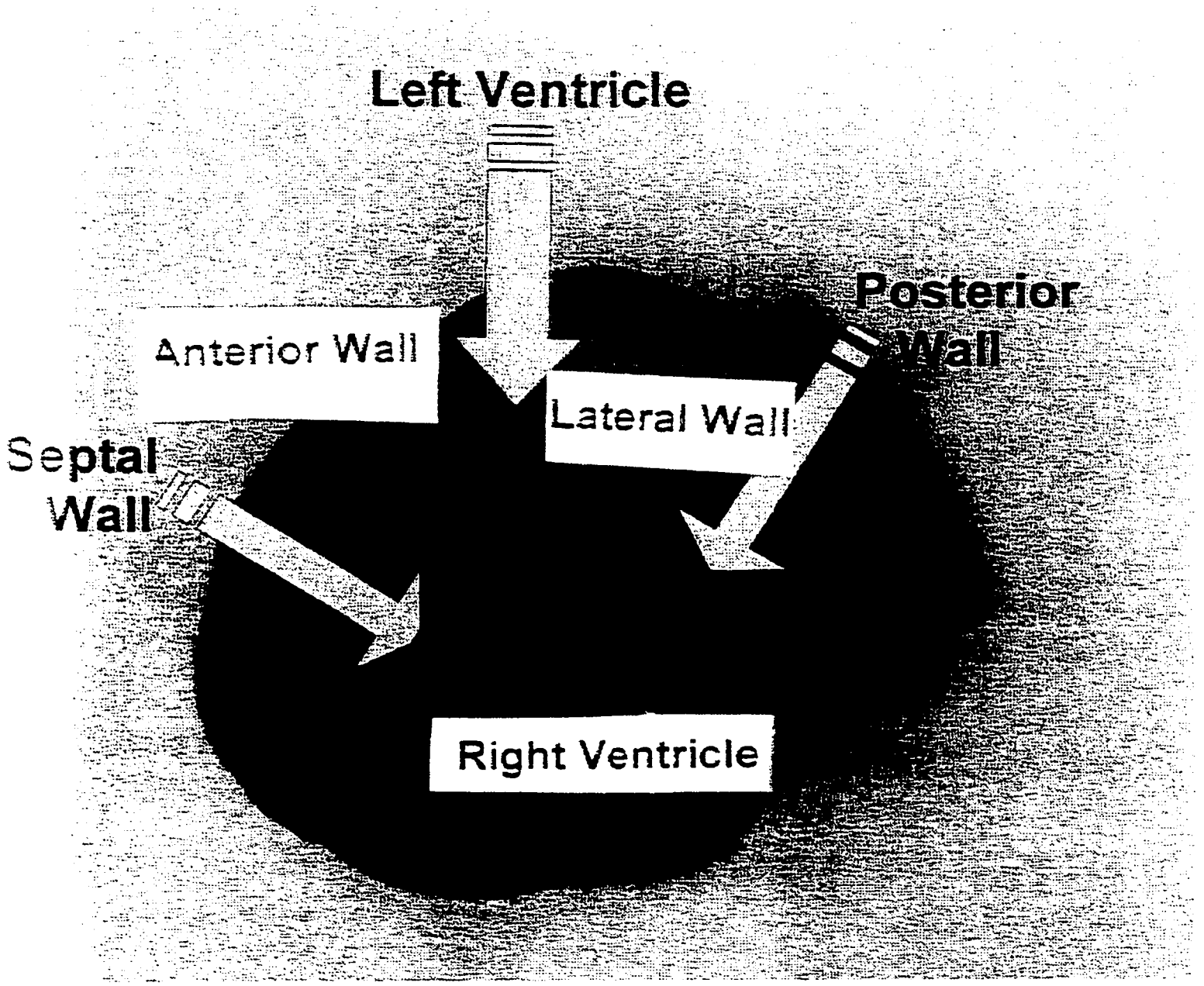

Figure 23. Cross-Sectional Diagram of the Heart

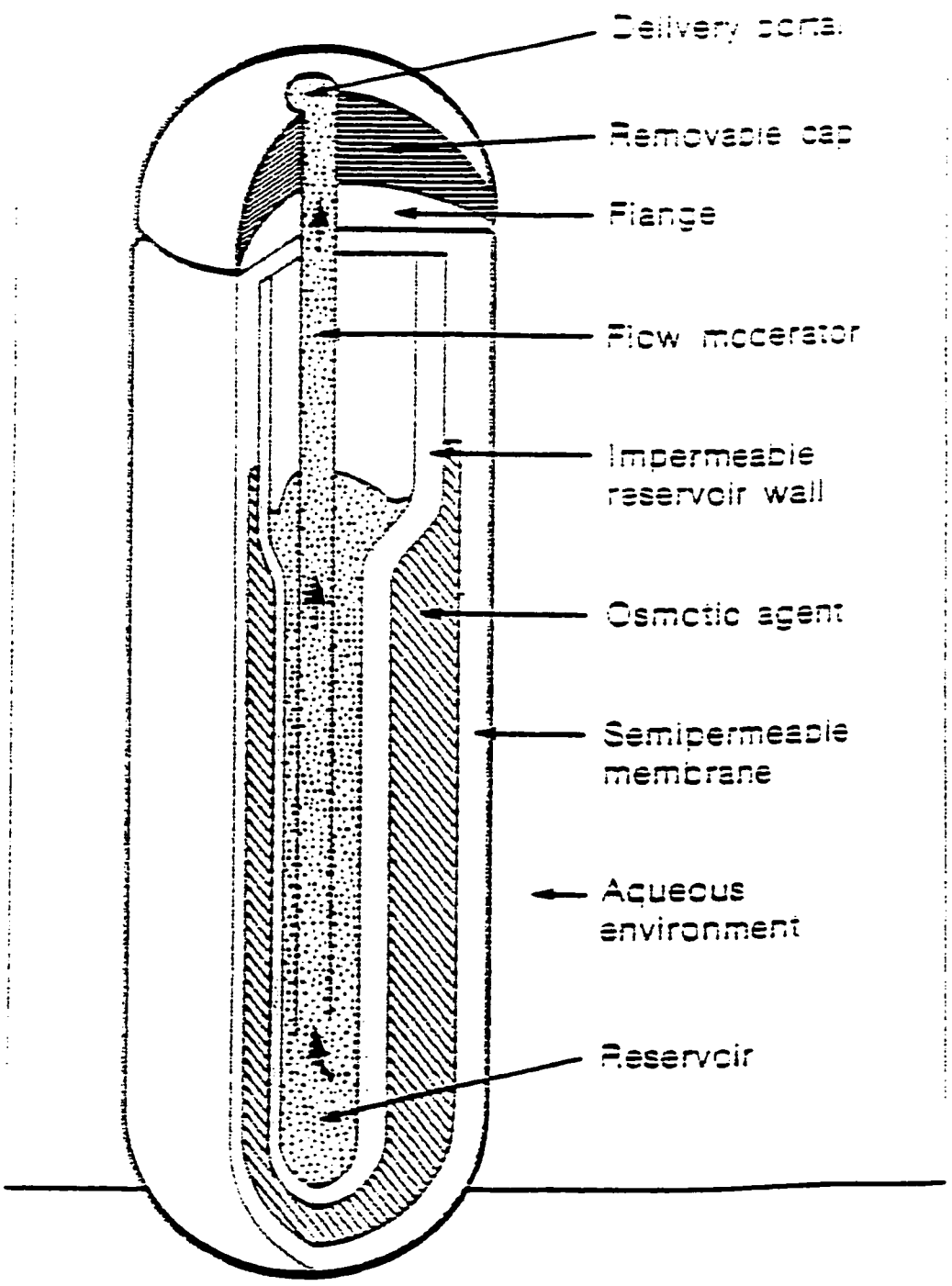

Figure 24: Diagram of Alzet osmotic minipump (Model 2002; Alza Corporation)