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EFFECTS OF NORTH AMERICAN GINSENG ON SEXUAL BEHAVIOR IN MALE RATS

Matthew Joseph Barnes

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Matthew J. Barnes

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CERTIFICATE OF EXAMINATION

Supervisor	Examiners
Dr. Lique Coolen	Dr. Morris Karmazyn
Supervisory Committee	Dr. John Ciriello
Dr. James Hammond	Dr. Kem Rogers
Dr. Edmund Lui	
Dr. Qingping Feng	

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Matthew Joseph Barnes

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Abstract

Two North American ginseng extracts were used to test sexual behavior in healthy rats and pre-diabetic rats, with erectile dysfunction, after four weeks of oral administration. Behavioral tests assessed sexual performance while animal tissues were used to examine eNOS and nNOS protein and mRNA in central and peripheral sites known to control erectile function. Results show an improvement in erectile function during copulation in healthy rats with chronic treatment of ethanol extract of NA ginseng but not aqueous extract. In addition, eNOS and nNOS mRNA was increased in penile tissue and nNOS staining was increased in the paraventricular nucleus of the hypothalamus with acute treatment of ethanol extract. For pre-diabetic rats, ethanol extract improved erectile function during mating and penile reflex tests. Also, nNOS was increased in penile tissue with chronic treatment and eNOS with acute treatment. The results show NA ginseng ethanol extract can improve erectile function.

Keywords: North American ginseng, erection, *ex copula*, erectile dysfunction, diabetes, nitric oxide synthase, pavaventricular nucleus of the hypothalamus.

Co-Authorship Statement

Chapter 2:

Analysis of qPCR for eNOS and nNOS mRNA were conducted by research assistant Xu Wang.

Chapter 3:

Analysis of qPCR for eNOS and nNOS mRNA were conducted by research assistants Xu Wang and Jessica Davie. The testosterone samples were assayed at the Core Assay Facility in the Department of Psychology at the University of Michigan (Ann Arbor, Michigan).

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List of Abbreviations

ABC – avidin horseradish peroxidise complex
BNST – bed nucleus of the stria terminalis
BSA – bovine serum albumin
CE – copulation efficiency
cGMP – cyclic guanosine monophosphate
CPP – conditioned place preference
C_t – cycle threshold
DAB - diaminobenzidine
DPX – dibutyl phthalate xylene
ED – erectile dysfunction
EL – ejaculation latency
eNOS – endothelial nitric oxide synthase
EPM – elevated plus maze
GAPDH – glyceraldehydes-3-phosphate dehydrenase
I IM I – inter-intromission interval
ICP – intracavernous pressure
IIEF – international index of erectile function
IL – intromission latency
IM - intromission
LE – Long Evans
LZ – Lean Zucker
M - mount
MED – minimum effective dose
ML – mount latency
mPOA – medial preoptic area
mRNA – messenger ribonucleic acid
NA – North American
NANC – non-adrenergic non-cholinergic
NCE – non contact erection
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
OLETF – Otsuka Long Evans Tokushima Fatty
OZ – Obese Zucker
PBS – saline-buffered sodium phosphate
PDE5 – phosphodiesterase type 5
PEI – post-ejaculatory interval
PFA - paraformaldehyde
ppd – protopanaxadiol
ppt – protopanaxatriol
PS - polysaccharides
PVN – paraventricular nucleus of the hypothalamus
ROS – reactive oxygen species
s.c. – subcutaneous

sGC – soluble guanylate cyclase
T1DM – type 1 diabetes mellitus
T2DM – type 2 diabetes mellitus

Chapter 1:

1.1 Introduction

1.1.1. Male Rat Sexual Behavior

Complex interactions of hormonal and neurochemical signals regulate the various aspects of male sexual behavior (Pfaus *et al.*, 2001). Rats exhibit discontinuous sexual mating in comparison to the "locked" behavior of humans where physical contact and penetration are maintained until ejaculation (Pfaus, 2009). In summary, olfactory, tactile, and auditory cues from a receptive female stimulate desire and elicit a consummatory response from the male rats. The male will mount the female from the rear and perform several rapid pelvic thrusts; if no vaginal penetration occurs then the action is called a mount (Pfaus, 2009). If during a mount the male detects the female vagina and obtains an erection rigid enough for penetration, he will give one strong thrust and insert his penis; this is known as an intromission (Pfaus, 2009). After either a mount or an intromission the female darts away while the male dismounts and typically grooms his genitals; this gives rise to the discontinuous mating.

After a few seconds, the female will again display proceptive behavior to stimulate desire in the male (Beach, 1976). The male will continue to mount and intromit the female until ejaculation is reached when the male performs rhythmic thrusts and inserts a seminal plug into the vagina followed by a slow dismount (Pfaus, 2009). After

ejaculation, a post-ejaculatory interval (PEI) occurs for a few minutes where the male will not engage in sexual behavior; the PEI ends once the male successfully intromits the female (Clark, 1995).

The male will initiate another copulatory series if presented with a receptive female and will typically repeat five to ten copulatory series until satiation (Clark, 1995). Importantly, previous sexual experience facilitates the initiation and overall performance of male sexual behavior, as evidenced by decreased latencies to initiate sexual behavior and increased sexual performance (Balfour *et al.*, 2004; Pitchers *et al.*, 2010). Successful male sexual behavior is dependent on intact erectile function to maintain a penis rigid enough for vaginal penetration.

1.1.2. Experiments on Erectile Function

Many behavioral tests have been developed to determine the quality and quantity of erections in rats. During copulation, measurements for erectile function are evaluated based on mount and intromission frequency and copulation efficiency (CE) (Clark, 1995). CE is a ratio of the number of total successful intromissions to the number of total mounts and intromissions (Clark, 1995). In essence, CE is a measure of penile rigidity; it compares the amount of successful vaginal penetrations to the total number of attempted vaginal penetrations.

Simulated tests for erectile function have been developed to evaluate erections outside of copulation. Noncontact erections (NCE), also known as psychogenic erections in humans, are erectile responses initiated by sexual arousal (Sachs *et al.*, 1994). In rodents, stimulation from receptive females in the form of olfactory, auditory, and tactile vibrations elicits erectile responses in male rats even without physical contact (Sachs *et al.*, 1994). The male displays hallmark signs of erections based on rhythmic hip contractions and flexions while grooming his genitals (Sachs, 1996). The number or erections and timing to the first erection are measures of erectile function induced by arousal.

An additional method of examining erectile function is a reflexive test known as *ex copula*. During the test, rats are restrained in a supine position, the prepuce is retracted and light pressure is used to hold the penis in place and maintain prepuce retraction. Both tactile stimulation and prepuce retraction elicit erectile reflexes of varying magnitudes that can be analyzed for erectile function (Sachs and Garinello, 1978). In summary, three levels of erections occur. E1 erections occur when the base of the glans swells with blood, E2 erections occur when the tip of the glans swell with blood, and E3 erections occur when the tip of the glans swell so the circumference of the tip is greater than the base (Sachs and Garinello, 1978). Intracavernous pressure increases as the erectile reflex increases in number, indicating an increase in penile rigidity (Bernabe *et al.*, 1995). In addition, penile flexions and clusters of erectile responses, which occur during *ex copula* tests can also be used to help measure erectile reflexes (Sachs and Garinello, 1978).

1.1.3. Central Regulation of Penile Erection

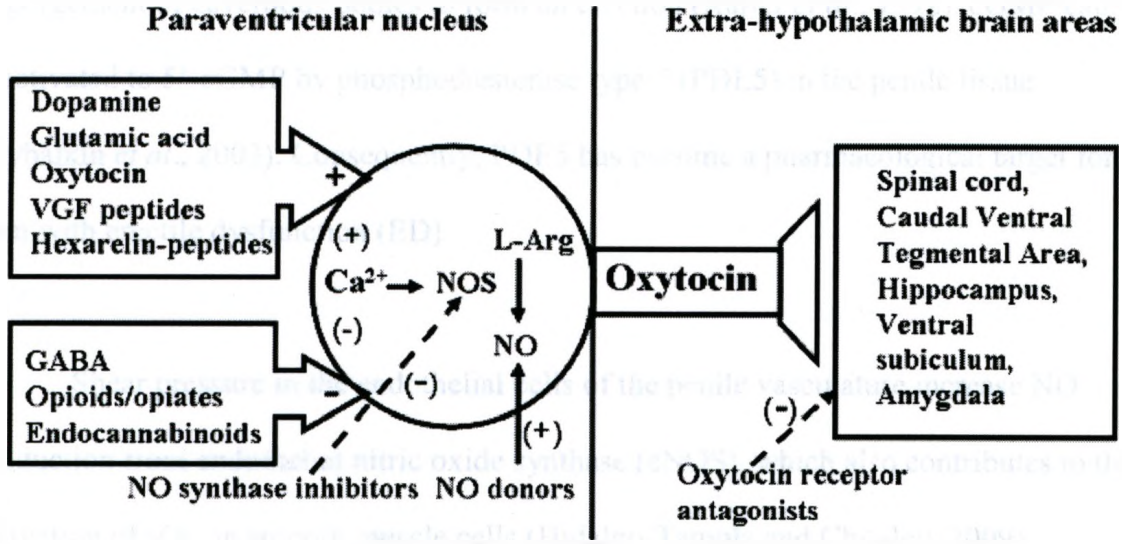
Penile erections involve tumescence of the cavernous bodies by engorging them with blood when penile veno-occlusion occurs in conjunction with dilation of penile arteries (Giuliano and Rampin, 2004; Hidalgo-Tamola and Chitaley, 2009). Control of erection involves a complex interaction between sensory inputs and neural and peripheral systems (Argiolas and Melis, 2005). In humans, tactile, visual, and imaginative stimuli can initiate an erection, whereas in rodents, auditory and olfactory cues can also contribute to inducing erectile responses (Sachs, 2000; Steers, 2000). Although the neural control for erections originate in the spine, erections are under excitatory influences from supraspinal sites (Rosen and Sachs, 2000) such as the paraventricular nucleus of the hypothalamus (PVN; Figure 1).

Paraventricular Nucleus of the Hypothalamus: The PVN receives projections from the areas of the brain involved in male sexual behavior and sends oxytocin projections to extra-hypothalamic brain regions (Argiolas and Melis, 2005) (Figure 1.1). Importantly for modulating erections, the PVN is thought to be the only supraspinal brain region to send projections to the spinal cord to regulate erectile function (Argiolas and Melis, 2005). Stimulation of the PVN induces penile erections in animals (Melis *et al.*, 1994; Chen and Chang, 2002, 2003). Lesions of the PVN have region-specific effects; lesions that incorporate both parvo- and magnocellular subregions of the PVN using radiofrequency show impairment on male copulation as indicated by increased mounts and ejaculatory

latency and a decreased number of NCE (Liu *et al.*, 1997). However, lesions in the PVN using N-methyl-D-aspartic acid, which only destroys the parvocellular subregion, showed no impairment in copulatory performance but decreased the number of NCE (Ackerman *et al.*, 1997; Liu *et al.*, 1997). Activation of the oxytocin neurons in the PVN requires an intracellular increase in nitric oxide (NO) production through the activation of neuronal nitric oxide synthase (nNOS); however, the mechanism through which NO activates oxytocin neurons is currently unknown (Melis *et al.*, 1998; Chen and Chang, 2002; Argiolas and Melis, 2005). In conclusion, the PVN plays a supportive, non-essential role in erectile function and is thought to be the only supraspinal region with connections to the spinal cord that contributes to the regulation of erections.

1.1.4. Peripheral Control of Penile Erection

Upon appropriate appetitive stimulation, projections from the central nervous system increase the parasympathetic tone and decrease the sympathetic output of nerves in the penis (Andersson, 2001). Specifically, parasympathetic stimulation activates nonadrenergic-noncholenergic (NANC) nerves, which increases nNOS activity that further increases NO release into the vasculature. NO is vital for tumescence in erectile tissue (Rajfer, 2008). NO then enters the smooth muscle cells to activate soluble guanylate cyclase (sGC) and increase its production of cyclic guanosine monophosphate (cGMP) (Giuliano and Rampin, 2004). cGMP then causes a cascade of events leading to the hyperpolarization and relaxation of the smooth muscle cells and, consequently, the



Modified from (Melis and Argiolas, 2010)

Figure 1.1. Schematic diagram of the paraventricular nucleus of the hypothalamus with oxytocinergic projections. Oxytocinergic projections to the spinal cord are thought to be the only projections to the spinal cord that regulate erectile function.

engorgement of cavernous bodies to form an erection (Rajfer *et al.*, 1992). cGMP can be inactivated to 5'-cGMP by phosphodiesterase type 5 (PDE5) in the penile tissue (Rybalkin *et al.*, 2003). Consequently, PDE5 has become a pharmacological target for men with erectile dysfunction (ED).

Shear pressure in the endothelial cells of the penile vasculature increase NO production from endothelial nitric oxide synthase (eNOS), which also contributes to the activation of sGC in smooth muscle cells (Hidalgo-Tamola and Chitaley, 2009). Production of NO via nNOS is seen as the initiator of erections while eNOS is viewed as the maintainer of erections since an increase in intracavernous pressure is required before eNOS increases its NO production (Gratzke *et al.*, 2010).

Proper erectile function can deviate from healthy conditions at many areas in the central and peripheral erectile pathways. Moreover, ED can occur through psychological and cardiovascular pathological states; however, there is no consensus on the mechanism of ED development (Rosen *et al.*, 2004). Furthermore, it is likely that a combination of alterations contribute to the development of ED in both healthy and diabetic patients.

1.2.1. Erectile Dysfunction

The prevalence of ED varies based on the population analyzed, the methods of assessing ED, and the threshold used to classify a patient with ED (Prins *et al.*, 2002). Regional variation with identical screening methods of men between the age of 20 to 75

shows a range of ED prevalence between 10 to 22% (Rosen *et al.*, 2004). Overall, approximately 16% of men experience ED; prevalence of ED increases with age, high blood pressure (26%), cholesterolemia (26%), depression or anxiety (26%), and diabetes (39%). Of this population, 58% seek treatment and only 16% who are prescribed PDE5 inhibitors, such as Sildenafil (Viagra), actively use the drug (Rosen *et al.*, 2004). In addition, not all men respond positively to PDE5 inhibitors. Only 56% of men with Type 2 diabetes mellitus (T2DM) respond to treatment as compared to 86% of the general population (Rendell *et al.*, 1999). Also, after a year of PDE5 treatment, men who responded positively to PDE5 inhibitors may revert back to pre-treatment conditions (Penson *et al.*, 2003).

1.2.2. Erectile Dysfunction in Type 2 Diabetes Mellitus

Development of ED can occur when the NO/cGMP pathway in penile tissue is disrupted. Diabetes can negatively affect multiple mechanisms through which normal erectile function occurs: impaired vasodilatory signalling through NANC dysfunction and/or endothelial damage, oxidative stress, hypogonadism, and veno-occlusive dysfunction, to name a few (Hidalgo-Tamola and Chitaley, 2009).

Although NANC dysfunction is established in Type 1 diabetes mellitus (T1DM), the role of NANC dysfunction in T2DM is still unclear due to conflicting results from different studies. In mice, no significance was reported between control and T2DM mice in regards to NANC dysfunction; however, Otsuka Long Evans Tokushima Fatty

(OLETF) rats, as a model of T2DM, presented with decreased nNOS immunofluorescent staining in penile nerves and a 40% reduction in nNOS protein expression (Vernet *et al.*, 1995; Hidalgo-Tamola and Chitaley, 2009). In addition to NANC dysfunction, loss of endothelial NO production may contribute to the pathological state of impaired penile vasodilation. Decreased immunofluorescent staining and protein expression of eNOS was found in OLETF rats (Jesmin *et al.*, 2003). Given the discrepancy in NANC dysfunction and the limited studies on T2DM endothelial function, further experiments are needed to verify any decrease in NO production in T2DM models.

In diabetes, the chronic state of hyperglycemia causes inflammation and an increase in the formation of reactive oxygen species (ROS), such as superoxide anions (Gur *et al.*, 2009). Superoxide anion reacts with NO to form peroxynitrite, which is another ROS. Peroxynitrite is pro-apoptotic and causes vascular damage to small penile resistance arteries causing endothelial apoptosis, which decreases the contribution of NO from eNOS (Jeremy *et al.*, 2007). ROS also contribute to ED by scavenging the vasculature for NO, causing a direct decrease in its bioavailability to cause vasodilation (Hidalgo-Tamola and Chitaley, 2009). In addition, the antioxidant glutathione, which reduces ROS, is decreased in hyperglycaemic conditions (Gur *et al.*, 2009). As such, diabetic populations experience an increase in oxidative stress as well as a decrease in antioxidant levels to combat oxidative stress.

Men with both ED and T2DM have a higher prevalence of hypogonadism; approximately 25% of men with both pathologies experience hypogonadism compared to

less than 13% in men with ED who are not diabetic (Corona *et al.*, 2004; Corona *et al.*, 2006). Adipose tissue converts androgens to estrogens via aromatase, which causes an overall decrease in testosterone levels (Hidalgo-Tamola and Chitaley, 2009). Testosterone has an important role in regulating both erectile function and male sexual libido; severely decreased testosterone levels can lead to a reduction in both parameters (Mills and Lewis, 1999; Gray *et al.*, 2005).

Finally, T2DM rats inadequately maintain intracaveronsal pressure after saline infusion indicating veno-occlusive dysfunction in penile tissue (Kovanecz *et al.*, 2006). Indeed, studies have shown that 39-67% of patients with T2DM experience veno-occlusive dysfunction (Colakoglu *et al.*, 1999; Metro and Broderick, 1999). In conclusion, treatment of diabetic patients with ED may require combination therapies since PDE5 inhibitors alone may not provide adequate pro-erectile treatment and the combined secondary effects of diabetes decreases erectile capacity.

1.2.3. The Obese Zucker Rat: A Model of Pre-Diabetes

Even though 90% of DM patients are categorized with T2DM, previous studies focus on T1DM animals models (Hidalgo-Tamola and Chitaley, 2009). The Obese Zucker (OZ) rat is a model for pre-diabetes with similar pathologies as T2DM. OZ rats have an autosomal recessive mutation in the leptin gene and are glucose intolerant (Kasiske *et al.*, 1992). Also, similar to T2DM patients, OZ rats have hyperinsulinemia, insulin resistance, hyperlipidemia, obesity, hypertension, and impaired vasodilation

(Kasiske *et al.*, 1992). These secondary pathologies of pre-diabetes contribute to ED development in OZ rats (Edmonds and Withyachumnarnkul, 1980; Edmonds *et al.*, 1982; Withyachumnarnkul and Edmonds, 1982; Doherty *et al.*, 1985; Hemmes and Schoch, 1988).

Male OZ rats have a decrease in intromission latency (Withyachumnarnkul and Edmonds, 1982), intromission frequency (Doherty *et al.*, 1985), and ejaculation latency (Doherty *et al.*, 1985). The OZ rat is a good model for testing pharmacological therapies for ED because diabetes is not fully established and the secondary effects of diabetes on erectile function are not completely manifested to potentially cause irreversible ED. However, it is not uncommon for the sexual performance of older OZ rats to be so severely impaired that they do not copulate (Young *et al.*, 1986).

1.3.1. Ginseng Plant

The ginseng genus is composed of a minimum of nine species including *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (North American (NA) ginseng) (Leung and Wong, 2010). Ginseng plants are perennial herbs that have been used for millennia as health tonics either alone or in combination with other medicinal ingredients (Attele *et al.*, 1999) (Figure 1.2). Ginsenosides are considered the main, active, medical constituent in the ginseng genus and were first isolated in 1963 (Shibata *et al.*, 1963). Ginsenosides are triterpene saponins with a dammarane skeleton consisting of multiple sugar moieties; they possess a rigid four *trans*-ring steroid skeleton

Modified from: http://www.wildcrafting.net/forage/plant/129/

Figure 1.2. Photograph of *Panax quinquefolius*

(Shibata *et al.*, 1965; Leung and Wong, 2010). In NA ginseng, ginsenosides Rb1, Re, Rd, Rc, Rg1, and Rb3 constitute more than 70% of the total ginsenoside content and provide a basis to standardize NA ginseng extracts (Lim *et al.*, 2005; Wang *et al.*, 2005). Ginsenoside Rf is found exclusively in Asian ginseng and can be used as a marker to distinguish Asian from NA ginseng (Li *et al.*, 2000). Importantly, differing effects on angiogenesis and immunomodulation have been reported based on the solvent used to create the ginseng extract (Azike *et al.*, 2011).

As a general health tonic, ginseng has traditionally been used to treat multiple pathological states. It is rationalized that the ginseng plant can be used as a health tonic since it contains more than 20 ginsenosides and each ginsenoside may cause differing effects on certain receptors or tissues (Attele *et al.*, 1999). However, the ginsenoside content in ginseng root extracts varies by species, age, cultivation practise, time of harvest, and manufacturing methods (Lim *et al.*, 2005; Schlag and McIntosh, 2006). Thus, developing crops with consistent ginsenoside contents is difficult. In response, creating a composite extract from multiple farming locations can help minimize variability of ginsenosides. In addition, recent studies are indicating polysaccharides in the ginseng root may contribute to some of the medicinal qualities of ginseng (Wang *et al.*, 2010a).

1.3.2. Ginseng Treatment for Enhancing/Rescuing Erectile Function

Among its many uses as a health tonic and "adaptogen", ginseng has been used for millennia as a natural aphrodisiac in men. Recently, the scientific literature has begun to support this claim. Various animal models have been used to examine the effects of ginseng extracts on male sexual performance. Tests on Asian and Korean Red ginseng (the steamed form of Asian ginseng) have used humans (Hong *et al.*, 2002; de Andrade *et al.*, 2007; Jang *et al.*, 2008; Kim *et al.*, 2009), rats (Kim *et al.*, 1976), mice (Yoshimura *et al.*, 1998), and rabbits (Choi *et al.*, 1999). In addition, one study examined the effects of ginsenoside Rg1 on copulatory behavior in mice (Wang *et al.*, 2010b) and another study used NA ginseng on rats (Murphy *et al.*, 1998).

Studies on sexual performance in humans using Asian ginseng show an increase in erectile function as indicated by a self reporting questionnaire called the International Index of Erectile Function (IIEF). Tests indicate that ginseng treatment was able to improve scores on penetration, erection maintenance, and rigidity in men with ED after 12 weeks of treatment (de Andrade *et al.*, 2007). In addition, after 8 weeks of treatment penile rigidity in men with ED was increased upon auditory and visual stimulation, as indicated by the RigiScan test (Hong *et al.*, 2002). However, a systemic review indicates that although chronic ginseng treatment shows promise for treating ED in humans, more studies are required to draw definitive conclusions (Jang *et al.*, 2008).

Animal studies indicate ginseng can be used to increase erectile function (Choi *et al.*, 1999) as well as increase male sexual performance during mating (Kim *et al.*, 1976; Murphy *et al.*, 1998; Yoshimura *et al.*, 1998; Wang *et al.*, 2010b). In summary, electrostimulation of pelvic nerves in rabbits treated for three months with Korean Red ginseng caused an increase in intracavernous pressure, indicating increased penile rigidity (Choi *et al.*, 1999). This occurred with an increase in relaxation of penile muscles to acetylcholine in ginseng-treated rabbits compared to control animals (Choi *et al.*, 1999). Regarding sexual performance, ginseng treatment was able to enhance mating behavior in mice and rats with (Yoshimura *et al.*, 1998) and without (Kim *et al.*, 1976) impaired performance, respectively. Furthermore, sexual arousal was enhanced with NA ginseng treatment in healthy rats as indicated by decreased latencies to mount, intromit, and ejaculate (Murphy *et al.*, 1998). The increase in sexual arousal occurred after 14 days of ginseng treatment and persisted until 28 days of treatment alongside reductions in circulating prolactin levels (Murphy *et al.*, 1998). The authors conclude that increased dopaminergic activity in the central nervous system caused a decrease in prolactin levels, which they correlate to increased male rat copulatory behavior (Murphy *et al.*, 1998). However, this may not translate to humans since Korean Red ginseng treatment has not been shown to influence prolactin levels (de Andrade *et al.*, 2007).

Finally, ginsenoside Rg1 increased male mice copulatory behavior after 20 days of treatment (Wang *et al.*, 2010b). In addition, Rg1 increased plasma testosterone as well as cGMP and NO levels in penile tissue (Wang *et al.*, 2010b). However, testosterone levels were reported to be unchanged with NA ginseng treatment in rats (Murphy *et al.*,

1998) and with Korean Red ginseng treatment in humans (de Andrade *et al.*, 2007). As such, further tests are required to clarify hormone changes in plasma with ginseng treatment regarding male sexual behavior.

With regards to penile erectile function, experiments *in vitro* (Chen and Lee, 1995; Rimar *et al.*, 1996) and *in vivo* (Lee *et al.*, 1981) show the vasodilatory effect of ginseng on the vasculature. In particular, Asian ginseng extract applied to rabbit corpus cavernosum caused a dose-dependent vasodilatory effect, which was enhanced with superoxide dismutase and abolished by a NOS inhibitor (Chen and Lee, 1995). These results indicate a role of NO in the vasodilatory effects of ginseng in the penile vasculature.

In addition, as previously stated, the maintenance of healthy endothelial cells in the vasculature is imperative to proper erectile function. Ginseng has been shown to possess antioxidant properties (Kim *et al.*, 1992; Gillis, 1997) that would protect endothelial cells from oxidative stress in pathological conditions such as diabetes (Hidalgo-Tamola and Chitaley, 2009). Moreover, the use of ginseng may provide diabetic patients with the needed boost in antioxidant levels to protect the endothelial cells from ROS and provide pro-erectile effects by activating the NO/cGMP pathway.

1.4. Objectives and Hypothesis

The overall goal of this study is to determine the effects of NA ginseng extracts on sexual performance and erectile function in healthy (chapter 2) and pre-diabetic (chapter 3) male rats. While ginseng has shown promise in enhancing male sexual function, further studies are needed to validate this claim. In addition, only one study has been published examining the effects of NA ginseng on male sexual performance by analyzing measures for sexual arousal. The study by Murphy and colleagues did not measure effects of NA ginseng on erectile function. As such, further studies are needed to determine potential effects of NA ginseng at enhancing erectile function in healthy models and potential effects of NA ginseng at improving sexual behavior in ED. The mechanisms by which ginseng acts to elicit pro-erectile effects remains largely unexplored, although evidence thus far indicates increased activity in the NO/cGMP pathway and increased ROS scavenging (Kim *et al.*, 1992; Chen and Lee, 1995; Gillis, 1997). We hypothesize that NA ginseng will increase sexual performance in healthy rats and partially recover erectile function in pre-diabetic rats by acting on the NO/cGMP pathway. In addition, we will investigate expression of NOS in pro-erectile brain areas in healthy rats. Indeed, ginseng has been shown to act centrally to produce effects such as memory enhancement (Nah *et al.*, 2007; Radad *et al.*, 2010). Another objective of this study is to examine two common ginseng extract preparations, ethanol and aqueous, to help determine preparation differences and help hypothesize on the active constituents in NA ginseng that enhance male sexual performance.

Finally, effects of NA ginseng administration on anxiety-like behaviors will be conducted to determine if effects on sexual behavior are occurring secondary to negative effects on this parameter. Currently, chronic administration of Asian ginseng is not known to influence anxiety-like behaviors; however, acute administration of Asian ginseng at high doses has been shown to decrease anxiety levels (Carr *et al.*, 2006; Einat, 2007). Also, for the same reasons as testing anxiety levels we will test the rewarding or aversive properties of NA ginseng.

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Chapter 2: North American ginseng improves erectile function and increases nitric oxide synthase in penile tissue and hypothalamus of male rats.

2.1. Introduction

Erectile function is a complex interplay of physiological and psychological factors. Prevalence of erectile dysfunction (ED) varies widely with select sample populations but estimates range from 12% (Martin-Morales *et al.*, 2001) to 22% (Laumann *et al.*, 1999) in men from 20 to 75 years of age. The prevalence of ED greatly increases with co-morbid medical conditions and risk factors such as coronary artery disease and diabetes (Rosen *et al.*, 2004). Even though great advances have been made towards ED treatment, only 58% of ED patients seek treatment and only 16% who are prescribed phosphodiesterase type 5 (PDE5) inhibitors, such as Sildenafil, actively use the drug (Rosen *et al.*, 2004). In addition, not all men respond positively to PDE5 inhibitors, especially those with co-morbidities, and will discontinue its use when expectations are not met (Azadzoi and Saenz de Tejada, 1992; Goldstein *et al.*, 2003; Jiann *et al.*, 2006). Hence, there is continued need for additional ED treatments.

Ginseng has been used for millennia as a natural aphrodisiac. The two most common species of ginseng are *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (North American [NA] ginseng). Both species have unique ginsenoside profiles (Wan *et al.*, 2007; Sun *et al.*, 2011), which are the main active constituents. Several studies have

shown effects of Asian and Korean ginseng on male rat and mouse sexual behavior (Murphy *et al.*, 1998; Yoshimura *et al.*, 1998; Choi *et al.*, 1999). These studies have shown decreases in mount and ejaculation latencies upon ginseng administration and restoration of mating behavior in mice with copulatory disorders. Also, Asian ginseng extracts have been shown to cause vasodilation in canine corpus cavernosum (Kang *et al.*, 2005), rabbit corpus cavernosum (Chen and Lee, 1995), as well as smooth muscle cells in rat aorta (Kim *et al.*, 1999). However, few studies have been conducted using NA ginseng. In one study, chronic NA ginseng treatment facilitated sexual behavior in male rats by decreasing mount, intromission, and ejaculatory latencies (Murphy *et al.*, 1998). The current study expanded on these previous findings by examining the effects of NA ginseng, using two different extraction methods, on male sexual behavior in young adult rats. In addition, effects on male erectile function and sexual arousal were examined in models where an estrus female was physically inaccessible.

Finally, the third goal of the study was to elucidate potential sites of action by which NA ginseng can improve sexual behavior. It has been well established that nitric oxide (NO) is a major cellular mediator of erectile function via vasodilation in the corpus cavernosum in penile tissue (Rajfer, 2008). NO activates soluble guanylate cyclase which increases cyclic guanosine monophosphate (cGMP) levels, causing a cascade of events leading to relaxation of smooth muscle cells and tumescence (Rajfer *et al.*, 1992). It is hypothesized that NA ginseng improves sexual function by increasing NO signalling in erectile tissues. Indeed, ginsenoside Rg1, present in Asian and NA ginseng, increases NO and cGMP levels in corpus cavernosum of male mice treated for 20 days (Wang *et al.*,

2010b), which supports the hypothesis. Hence, effects of NA ginseng on expression of nitric oxide synthase (NOS) in erectile tissue will be determined.

Moreover, NA ginseng may influence sexual behavior by acting on the central nervous system. The central control of male erectile and sexual function derives from a neural circuit including the paraventricular nucleus of the hypothalamus (PVN) (McKenna, 2000; Giuliano and Rampin, 2004). In the PVN neuronal activation of oxytocin neurons has been implicated with male erectile function (Argiolas and Melis, 2005). Neuronal nitric oxide synthase (nNOS) is required for the activation of oxytocin neurons in the PVN (Melis *et al.*, 1998). Effects of NA ginseng on the PVN have not yet been established. Therefore, the final goal of this study was to examine effects of NA ginseng on nNOS expression in the PVN.

2.2. Methods and Materials

2.2.1. Animals

Adult, male Long Evans rats (200-250 g) were delivered from Charles River (Sherbrooke, Quebec, Canada) and housed in pairs in Plexiglas cages. Rats were placed under a 12/12 reversed light-dark cycle with lights off at 10 a.m. Food and water were available *ad libitum*. Female Long Evans rats (Charles River) were bilaterally ovariectomized and subcutaneously implanted with a capsule containing 5% 17-β-estradiol benzoate (Sigma, St. Louis, Missouri, USA) and 95% cholesterol (Sigma, St. Louis, Missouri, USA). To induce sexual receptivity, females were injected (s.c.) with 500 mg progesterone dissolved in 0.1 mL sesame oil (Sigma, St. Louis, Missouri, USA) four hours prior to experiments. All procedures were approved by the Animal Care Committee at the University of Western Ontario and conform to the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

2.2.2. North American Ginseng Extracts and Administration

Ginseng extracts were prepared from the roots of four-year-old NA ginseng plants harvested from five farms in Ontario, Canada, which were provided by the Ontario Ginseng Grower's Association and processed by Naturex (South Hackensack, New Jersey). Roots were ground and soaked at 40°C three times for a total of five hours in either ethanol/water (75/25%) or aqueous solutions. The extracts were filtered and excess

solvent was removed at 45°C until total solid content reached 60%. Ethanol and aqueous NA ginseng root extracts were prepared after lyophilization at the Ontario Ginseng Innovation and Research Consortium (University of Western Ontario, London, Canada). The concentration of the major ginsenosides (Rb1 Re, Rg1, Rb2, Rd, and Rc) for both extracts have previously been determined by high-pressure liquid chromatography (Azike *et al.*, 2011).

Ginseng extracts were suspended in 0.9% saline and were injected daily to the rats via oral gavage two to three hours after lights off under red light illumination. Rats were randomly assigned to experimental groups and received one of two dosages of ethanol extract (125 or 250 mg/kg/day) or aqueous extract (125 or 250 mg/kg/day) or vehicle (0.9% saline). The volumes of all oral injections were adjusted so that less than 1 mL of fluid was administered per day. Average weight of treatment groups did not differ at the start of experiment. Male rats administered daily aqueous or ethanol extracts did not differ in weight gain at any time point compared to control male rats administered saline (data not shown).

2.2.3. Sex Behavior Analysis

Following 28 days of daily NA ginseng (ethanol or aqueous) or vehicle administration, males were tested for sexual behavior every other day during four tests. Males were sexually naïve prior to onset of experiments, thus, effects of NA ginseng on mating were determined in males when sexually naïve and experienced. In addition,

effects of NA ginseng on mating were determined either shortly following NA ginseng administration or one day following last injection. For tests 1 and 3, males received NA ginseng or vehicle 30 minutes prior to mating, while tests 2 and 4 were conducted 24 hours following last injection. During all tests, male rats were placed in a clean test cage (with clean bedding; 60 x 45 x 50 cm^3) 10 minutes prior to introduction of a receptive female, and were allowed to mate to one ejaculation (and completed a post-ejaculatory interval; PEI) or for one hour, whichever came first. All standard parameters of male sexual performance were recorded including: mount latency (ML; time from introduction of a female until the first mount), intromission latency (IL; time from introduction of a female until the first mount with successful vaginal penetration), ejaculation latency (EL; time from the first intromission of a female until ejaculation), PEI (time from ejaculation to first subsequent intromission), number of mounts (M), and intromissions (IM) prior to first ejaculation, and copulation efficiency (CE; ratio of number of intromissions to total number of mounts). Differences between groups were compared using two-way ANOVA (factors: test and treatment) and Student-Newman-Keuls t-test for post hoc analysis with 95% confidence levels (SigmaPlot version 11.0).

2.2.4. Non-Contact Erections

Next, following 36 days of daily NA ginseng or vehicle administration, male rats (sexually experienced) were tested for non-contact erections (NCE); a paradigm for psychogenic erections (Sachs and Garinello, 1978). The NCE apparatus consisted of two same-sized compartments with clear walls, ceiling, and floors separated by a perforated

screen (MED Associates Inc., St. Albans, Vermont, USA). Experimental design was adapted from Sachs (Sachs, 1996). Briefly, males were habituated in the apparatus for five minutes one day prior to testing. On the test day, male rats were injected with NA ginseng 30 minutes prior to the experiment in the second half of the dark phase. Male rats were then placed in one compartment with an estrus female in the other compartment. A fan was placed behind the female to blow odors towards the male. The behavior was recorded for 20 minutes using digital cameras (Samsung Seoul, South Korea; Sony, Tokyo, Japan) and the latency to first erection and the number of erections were measured. Erections were scored if the rat displayed one of three criteria: 1) visible erection, or the male rat displays genital grooming with one of the two following behaviors; 2) hip contractions or; 3) hip flexions. Differences between groups were compared using one-way ANOVA and student t-tests for post hoc analysis with 95% confidence levels.

2.2.5. Additional Behavioral Tests

Although the main goal of the study was to test the effects of NA ginseng on sexual behavior and erectile function, additional tests were performed to determine potential effects of NA ginseng on anxiety-like behaviors and to determine rewarding or aversive properties. Moreover, ethanol or aqueous-treated animals were tested in conditioned place preference (CPP) paradigm, elevated plus maze (EPM), and open field tests between 2 to 3 weeks of NA ginseng or vehicle administration (prior to sex behavior tests).

2.2.5.1. Conditioned Place Preference Paradigm

Fourteen days after daily NA ginseng or vehicle injections, rats were tested for rewarding or aversive properties of NA ginseng using the CPP paradigm as described by Di Sebastiano *et al.* (Di Sebastiano *et al.*, 2011). Briefly, the CPP apparatus (MED Associates Inc., St. Albans, Vermont, USA), consisted of three chambers that differ in visual and tactile cues: two test chambers (28 x 22 x 21 cm) with either black walls and parallel bar flooring or white walls and metal grid flooring, and a central, neutral compartment (13 x 22 x 21 cm) with grey walls and a smooth Plexiglas floor. Chambers were connected by solid guillotine doors and equipped with photobeam arrays to record time in each chamber. During the pre-test, male rats were allowed to freely roam the CPP apparatus for 15 minutes to establish individual chamber preferences for each rat. The apparatus is unbiased and, as a group, rats do not display a preference for any chamber. Subsequently, during two conditioning days, rats received either NA ginseng or saline and were placed in the paired (initially non-preferred) or unpaired (initially preferred) chambers, respectively, for 30 minutes in a counter-balanced manner. During the post-test, rats were allowed to freely roam the apparatus for 15 minutes to determine a place preference. A preference score (percentage of time spent in the ginseng-paired chamber) and difference score (time in paired chamber minus time in unpaired chamber) was calculated for each animal. Differences between pre- and post-tests scores were analyzed using paired t-tests with 95% confidence levels.

2.2.5.2. Elevated Plus Maze (EMP)

After 24 days of daily NA ginseng or vehicle administration, male rats were tested on the EPM (MED Associates Inc., St. Albans, Vermont, USA), 24 hours after last NA ginseng or vehicle injection and during the second half of the light phase. The EPM apparatus consisted of four arms elevated 75 cm from ground level, each 50 cm in length extending from a central junction, equipped with photobeam arrays to record activity. Two arms were enclosed by black siding 40 cm high, while the other two arms were unenclosed. Male rats were placed in the center junction and allowed to roam the apparatus for 5 minutes. Differences between groups were compared using one-way ANOVA and student t-tests for post hoc analysis with 95% confidence levels.

2.2.5.3. Open Field

One day after the EPM test, rats were tested in an open field apparatus 24 hours after last NA ginseng or vehicle injection and during the second half of the light phase. Rats were placed in the open filed apparatus (40.5 × 40.5 cm; equipped with 16×16 photobeam arrays; MED Associates Inc., St. Albans, Vermont, USA) for 20 minutes. Total ambulatory distance and resting time were recorded along the perimeter and in the center of the chamber. Differences between groups were compared using one-way ANOVA and Dunn's Method for post hoc analysis with 95% confidence levels.

2.2.6. Tissue Collection

After completion of all behavioral tests, brains, blood, and penile tissues were collected to determine effects of NA ginseng on cellular mediators of erectile function. Effects of aqueous extract were not determined, based on observed lack of effects of this extract on sexual function (see results). Male rats chronically treated with NA ginseng or vehicle received a final administration of vehicle or NA ginseng (250 mg/kg), creating four groups. One hour later, males were anaesthetised with sodium pentobarbital (270 mg/mL/kg), and blood samples (collected in centrifuge tubes coated with heparin; Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) and penile tissue were collected and immediately frozen and stored at -80 °C. Rats were then transcardially perfused with 500 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and post-fixed for one hour in PFA and then subsequently transferred to 20% sucrose solution. Brains were cut coronally at 35 μm on a freezing microtome (Thermo Fisher Scientific, Walldorf, Germany) and collected in four parallel series in cryoprotectant solution (30% sucrose in 0.1 M PB with 30% ethylene glycol and 0.01% sodium azide) and stored at -20°C.

2.2.7. Immunohistochemistry

All procedures were performed under gentle agitation at room temperature. Sections were washed in 0.1 M saline-buffered sodium phosphate (PBS) between all incubation steps. Sections were incubated in H_2O_2 (1% in PBS; 10 minutes), incubation solution (PBS solution containing 0.1% bovine serum albumin (BSA; Fisher Scientific,

Fair Lawn, New Jersey, USA) and 0.4% Triton-X-100 (Fisher Scientific, Fair Lawn, New Jersey, USA); 1 hour), and then in primary antibody recognizing nNOS (rabbit anti-nNOS; Lot #24287; 1:40,000, DiaSorin, Saluggia, Italy) dissolved in incubation solution for 17 hours.

Next, sections were incubated with biotinylated goat anti-rabbit (1:500 in incubation solution, Vector Laboratories, Burlingame, CA, USA; 1 hour) and avidin horseradish peroxidase complex (ABC; 1:1,000 in PBS, Vector Laboratories; 1 hour). Reaction product was visualized with nickel-enhanced diaminobenzidine (DAB; 0.02% in 0.1 M PB containing 0.012% H_2O_2 and 0.08% nickel sulfate; Sigma, St. Louis, MO; 10 minutes). Finally, sections were mounted on plus-charged glass slides (Fisher Scientific, Fair Lawn, New Jersey, USA) and coverslipped with dibutyl phthalate xylene (DPX; Electron Microscopy Science, Hatfield, PA, USA).

2.2.8. Densitometry analysis for nNOS

nNOS immunoreactivity was analyzed bilaterally in the PVN (Bregma -1.80mm, nNOS: 1248 x 946 pixels). All images were taken under brightfield illumination using Optronics camera (Optronics Goleta, California, USA) and Neurolucida software. All images were taken together under the same magnification (using 20x objectives) and camera settings. Each area of analysis was defined using unique landmarks specific for the region (Paxinos and Watson, 1998). Density analyses of immunoreactive neurons were measured by calculating a threshold based on a mean gray value of all the images

and then determining the number of pixels above the threshold, expressed as the percentage of the analyzed area using ImageJ (National Institute of Health, Bethesda, MD, USA). For each brain region analysed, two images were analyzed and averaged per animal. Differences between groups were compared using two-way ANOVA (factors: chronic treatment and last injection) and Student-Newman-Keuls for post hoc analysis with 95% confidence levels.

2.2.9. RNA isolation and real time PCR

Penile tissues from Long Evans male rats were homogenized in TriZol reagent (Invitrogen, Burlington, Ontario, Canada) for total RNA isolation. To establish the 260/280nm absorbance ratio between 1.8 and 2.0, RNA quantity and purity were assessed using a NanoDrop spectrophotometer (ND 1000 v3.3.0; NanoDrop Technologies, Inc., Wilmington, DE). High Capacity cDNA Reverse Transcription Kit from Applied Biosystems Inc. was used to perform cDNA synthesis according to manufacturer's instructions (Foster City, CA). nNOS and eNOS gene expression were then quantified by qRT-PCT. All reactions contained 2µL of cDNA, 10µL of TaqMan Universal PCR Master Mix 2X (Applied Biosystems, Foster City, CA), 0.8µL of 25mM $MgCl_2$ (Invitrogen, Burlington, ON), 6.2µL DEPC treated water and 1µL of TaqMan gene expression assay solution for the gene of interest (nNOS Assay ID: Rn00583793_m1; eNOS Assay ID: Rn02132634_s1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay ID: Rn99999916_s1) was used for an endogenous control. Samples were assayed in triplicate using the Rotor Gene RG 6000 Thermocycler and software (Corbett Life Sciences, San Francisco, CA) with cycling conditions of: hold at 50°C for 2

min and 95°C for 10 min, 40 cycles of 95°C for 15 sec and 58°C for 1 min. A standard curve was generated from qRT-PCR data for each gene to quantify gene expression. Penile cDNA reverse transcribed from RNA was used to create a standard curve with 5-log serial dilutions. Optimal standard curves were achieved only when the line of best fit of cycle threshold (C_t) values of dilutions had a $R^2 \geq 0.99$ and an efficiency value between 0.8-1. Each gene's optimal threshold line from the standard curve was used to determine the C_t value of each sample, which were determined from the intersection between the baseline subtracted amplification curve of the sample and the threshold line established by the optimal standard curve. For each experiment sample, the C_t value for each gene of interest was normalized to their respective C_t value of GAPDH, thus, generating the ΔC_t value. In addition, $\Delta\Delta C_t$ values ($= 2^{\wedge}(\Delta C_{t(sample)} - \Delta C_{t(average)})*100$) were calculated using the difference between the ΔC_t value for each individual sample in the treatment groups and the average ΔC_t value of the control group. Two-way ANOVA with Holms Sidak post hoc analysis were conducted to identify significant differences between groups. Values were considered significantly different with 95% confidence levels.

2.3. Results

2.3.1. Sexual Behavior

Administration of ethanol extract of NA ginseng significantly affected expression of sexual behavior. There was a significant effect of treatment on the numbers of mounts and intromissions, copulation efficiency, and inter-intromission interval ($F(2,209)=3.3$-166; $p=0.041$-<0.001) (Figure 2.1). Effects of trials were not detected for these parameters of sex behavior except for number of intromissions ($F(3, 209)=3.8$; $p=0.011$). Post hoc analyses revealed that number of mounts were significantly lower following treatment of either of the two dosages compared to saline in mating trails 1 ($p=0.013$-0.009), 2 ($p= 0.032$-0.038), and 4 ($p=0.03$-<0.001). Number of intromissions were higher in trial 3 in the higher dosage group ($p=0.017$-0.022) and in trial 4 in the lower dosage group ($p=0.019$) compared to saline controls. Ethanol extract of NA ginseng improved the copulation efficiency in both low and high dosage groups compared to saline controls in trials 1 ($p=0.041$-$0,029$), 2 ($p=0.013$-0.01) and 4 ($p=0.048$-0.005). Finally, inter-intromission interval was shorter in both lower and higher dosage groups compared to saline, but only in trial 1 ($p=0.016$-0.005) (Table 2.1). In contrast to the effects observed following chronic administration of the ethanol extract, the aqueous extract of NA ginseng did not affect male sexual behavior. No significant effects of treatment were detected for any of the parameters analyzed (Figure 2.1 and Table 2.1). Thus, ethanol, but not aqueous, extract of NA ginseng improved copulation efficiency by decreasing number of mounts and increasing number of intromissions.

2.3.2. Non Contact Erections

Chronic administration of ethanol or aqueous extracts of NA ginseng did not affect erectile function as analyzed in the NCE test as there were no changes in the total number of erections or the timing to the first erection compared to control (Table 2.2).

2.3.3. Other behavioral tests

The rewarding or aversive properties of NA ginseng were tested with the CPP paradigm. Ethanol extract had rewarding properties as male rats displayed an increased preference during the post-test for the chamber of the CPP apparatus that was paired with the higher dose of NA ginseng ethanol extract as indicated by a increased preference score ($p=0.009$) and decreased difference score ($p=0.005$) (Figure 2.2A,B). The aqueous extract did not affect CPP parameters, hence did not have rewarding nor aversive properties (Figure 2.2C,D).

To determine if chronic NA ginseng administration affects general levels of anxiety, animals were tested for anxiety-like behavior using EPM and open field tests. Neither ethanol nor aqueous extracts of NA ginseng affected EPM parameters (Table 2.3). Similar results were observed in the open field test and neither ethanol nor aqueous extracts affected distance moved or time spent in the center of the open field. The higher dose of ethanol extract did, however, increase active time in the perimeter of the chamber

compared to control ($p<0.05$), suggesting an effect on locomotor activity, rather than anxiety-like behavior (Table 2.4).

2.3.4. Penile eNOS and nNOS mRNA

Administration of ethanol extract of NA ginseng resulted in increased eNOS (Figure 2.3A) and nNOS (Figure 2.3B) mRNA expression in penile tissue. There was no effect of chronic treatment, but rather of the last injection the animals received prior to tissue collection ($F(1, 20)=6.898$; $p=0.018$). Hence, a final administration of NA ginseng in males chronically treated with NA ginseng or saline caused increased eNOS and nNOS mRNA compared to saline controls.

2.3.5. Brain nNOS expression

In the PVN, ethanol extract of NA ginseng increased nNOS immunoreactivity (Figure 2.4). Similar to the effects observed in penile tissue, there was no effect of chronic administration but rather of the final injection of NA ginseng prior to tissue collection ($F(1,19)= 5.448$; $p=0.033$). NA ginseng significantly increased nNOS expression compared to saline controls in animals chronically treated with either NA ginseng or saline.

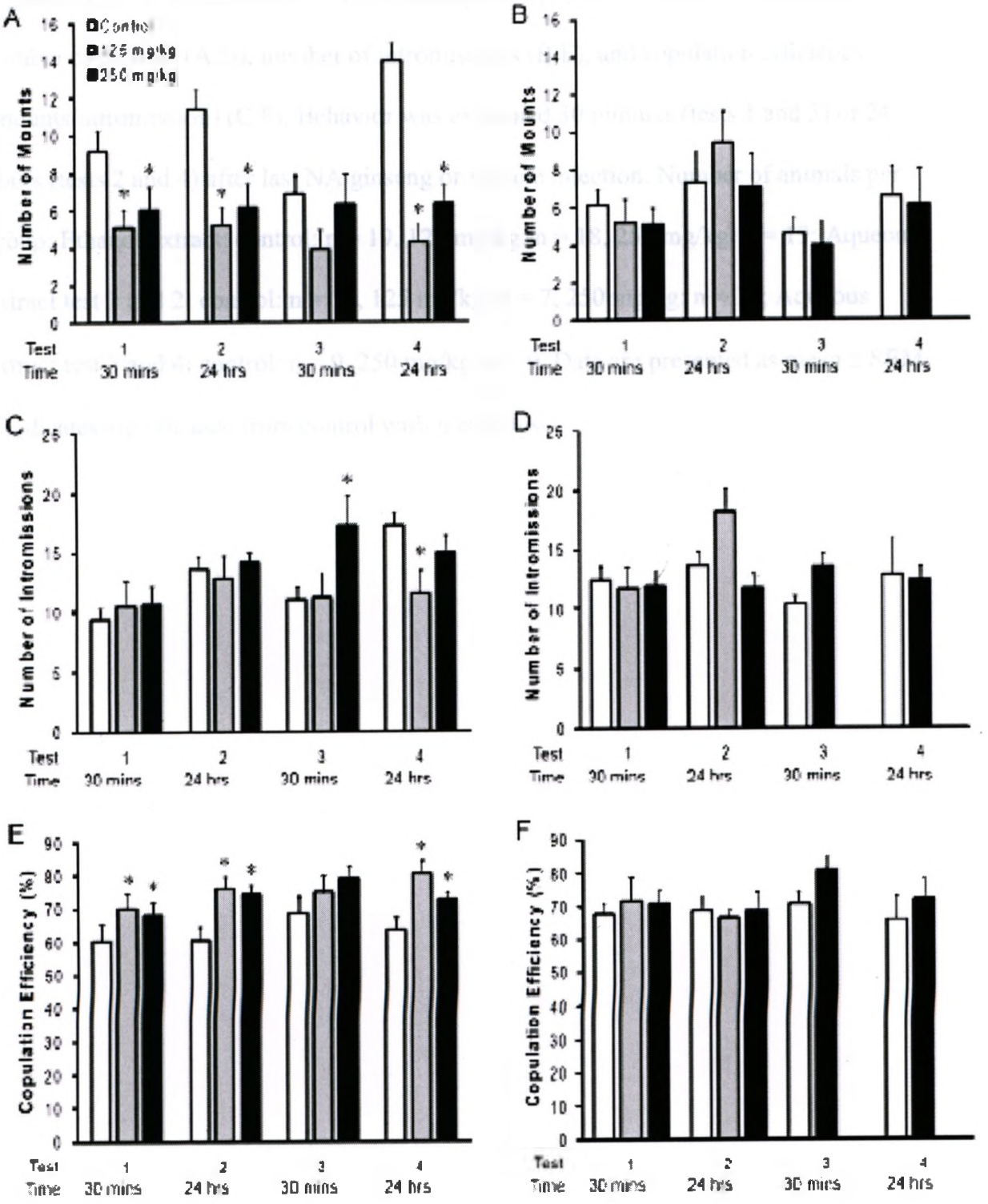
A
Control
125 mg/kg
250 mg/kg
Number of Mounts
Test
Time
1
30 mins
2
24 hrs
3
30 mins
4
24 hrs
B
Number of Mounts
C
Number of Intromissions
D
Number of Intromissions
E
Copulation Efficiency (%)
F
Copulation Efficiency (%)

Figure 2.1. Effects of ethanol (A,B,C) and aqueous (D,E,F) extracts of NA ginseng on number of mounts (A,D), number of intromissions (B,E), and copulation efficiency (mounts/intromission) (C,F). Behavior was examined 30 minutes (tests 1 and 3) or 24 hours (tests 2 and 4) after last NA ginseng or vehicle injection. Number of animals per group: Ethanol extract: control: n = 19, 125 mg/kg: n = 18, 250 mg/kg: n = 19; Aqueous extract test 1 and 2: control: n = 18, 125 mg/kg: n = 7, 250 mg/kg: n = 18; Aqueous extract test 3 and 4: control: n = 9, 250 mg/kg: n = 9. Data are presented as mean ± SEM. * indicates significance from control within each test.

Table 2.1. Effects of ethanol and aqueous extracts of NA ginseng on latencies to mount (ML), intromit (IL), or ejaculate (EL), post-ejaculatory interval (PEI), and inter-intromission interval (I IM I). All data are expressed in seconds. Behavior was examined 30 minutes (test 1 and 3) or 24 hours (test 2 and 4) after last NA ginseng or vehicle injection. Number of animals per group: Ethanol extract: control: n = 19, 125 mg/kg: n = 18, 250 mg/kg: n = 19; Aqueous extract test 1 and 2: control: n = 18, 125 mg/kg: n = 7, 250 mg/kg: n = 18; Aqueous extract test 3 and 4: control: n = 9, 250 mg/kg: n = 9. Data are presented as mean ± SEM. * indicates significance from control within each test.

		Test			
Ethanol		1	2	3	4
Control	ML	157.1 ± 48.8	67.2 ± 21.9	38.1 ± 11.0	21.8 ± 2.9
	IL	158.9 ± 48.7	78.4 ± 22.2	39.5 ± 10.9	26.2 ± 3.9
	EL	412.9 ± 79.7	529.3 ± 75.3	416.1 ± 80.7	638.4 ± 113.2
	PEI	342.8 ± 13.8	330.3 ± 18.2	380.1 ± 30.1	392.5 ± 25.2
	I IM I	40.9 ± 4.0	39.1 ± 5.8	42.2 ± 7.9	34.9 ± 3.7
125 mg/kg	ML	172.6 ± 48.8	108.2 ± 21.9	39.2 ± 21.5	65.2 ± 2.9
	IL	179.3 ± 48.7	122.2 ± 45.9	45.1 ± 21.6	66.1 ± 39.1
	EL	349.9 ± 45.1	469.1 ± 73.5	305.3 ± 45.1	360.2 ± 53.4
	PEI	280.1 ± 20.1	302.3 ± 14.2	328.8 ± 12.3	331.8 ± 15.5
	I IM I	34.1 ± 3.3*	41.5 ± 7.0	28.3 ± 3.1	32.4 ± 3.1
250 mg/kg	ML	68.0 ± 13.6	99.3 ± 47.4	22.1 ± 4.2	25.2 ± 5.4
	IL	75.6 ± 15.1	123.2 ± 50.5	23.8 ± 5.3	29.4 ± 5.5
	EL	357.9 ± 52.2	475.8 ± 72.8	627.4 ± 145.0	349.7 ± 45.4
	PEI	296.5 ± 13.8	324.7 ± 18.2	380.8 ± 30.1	372.6 ± 25.2
	I IM I	34.8 ± 4.0*	33.5 ± 5.8	34.2 ± 7.9	23.6 ± 3.7
Aqueous					
Control	ML	98.6 ± 28.3	93.4 ± 43.7	210.5 ± 149.1	182.6 ± 42.8
	IL	101.9 ± 28.8	93.4 ± 43.7	218.3 ± 148.2	183.1 ± 42.9
	EL	467.5 ± 58.1	480.1 ± 69.1	300.2 ± 46.4	497.1 ± 129.7
	PEI	308.2 ± 16.3	367.2 ± 47.9	326.6 ± 16.6	404.0 ± 78.7
	I IM I	39.4 ± 4.9	37.8 ± 5.9	28.3 ± 3.1	42.1 ± 5.2
125 mg/kg	ML	52.7 ± 23.7	15.1 ± 4.9		
	IL	56.3 ± 23.4	20.7 ± 6.3		
	EL	372.4 ± 50.9	524.7 ± 113.8		
	PEI	309.7 ± 5.9	304.9 ± 22.4		
	I IM I	33.5 ± 3.1	27.8 ± 11.8		
250 mg/kg	ML	156.4 ± 39.4	163.4 ± 72.9	43.3 ± 15.8	52.3 ± 14.9
	IL	234.8 ± 59.4	202.9 ± 73.0	43.7 ± 15.7	74.6 ± 23.4
	EL	478.7 ± 60.5	553.9 ± 160.6	389.6 ± 94.7	395.8 ± 90.4
	PEI	356.3 ± 20.2	334.1 ± 19.7	343.4 ± 19.8	399.0 ± 26.6
	I IM I	43.5 ± 4.9	49.0 ± 10.2	27.6 ± 4.6	30.1 ± 3.9

Table 2.2. Effects of ethanol extract of NA ginseng on number of non-contact erections (NCE) and timing to first NCE over a test period of 20 minutes. Number of animals per group: control: n = 20, 125 mg/kg n = 19, 250 mg/kg n = 20. Data are presented as mean ± SEM. No significant differences between groups were detected.

	Ethanol		Aqueous	
	Number of Erections	Time to First Erection (secs)	Number of Erections	Time to First Erection (secs)
Control	1.20 ± 0.30	713.1 ± 82.1	1.63 ± 0.42	584.1 ± 76.31
125 mg/kg	1.74 ± 0.31	571.5 ± 62.6	1.38 ± 0.50	732.2 ± 136.4
250 mg/kg	1.60 ± 0.32	571.1 ± 55.7	2.00 ± 0.93	532.2 ± 105.6

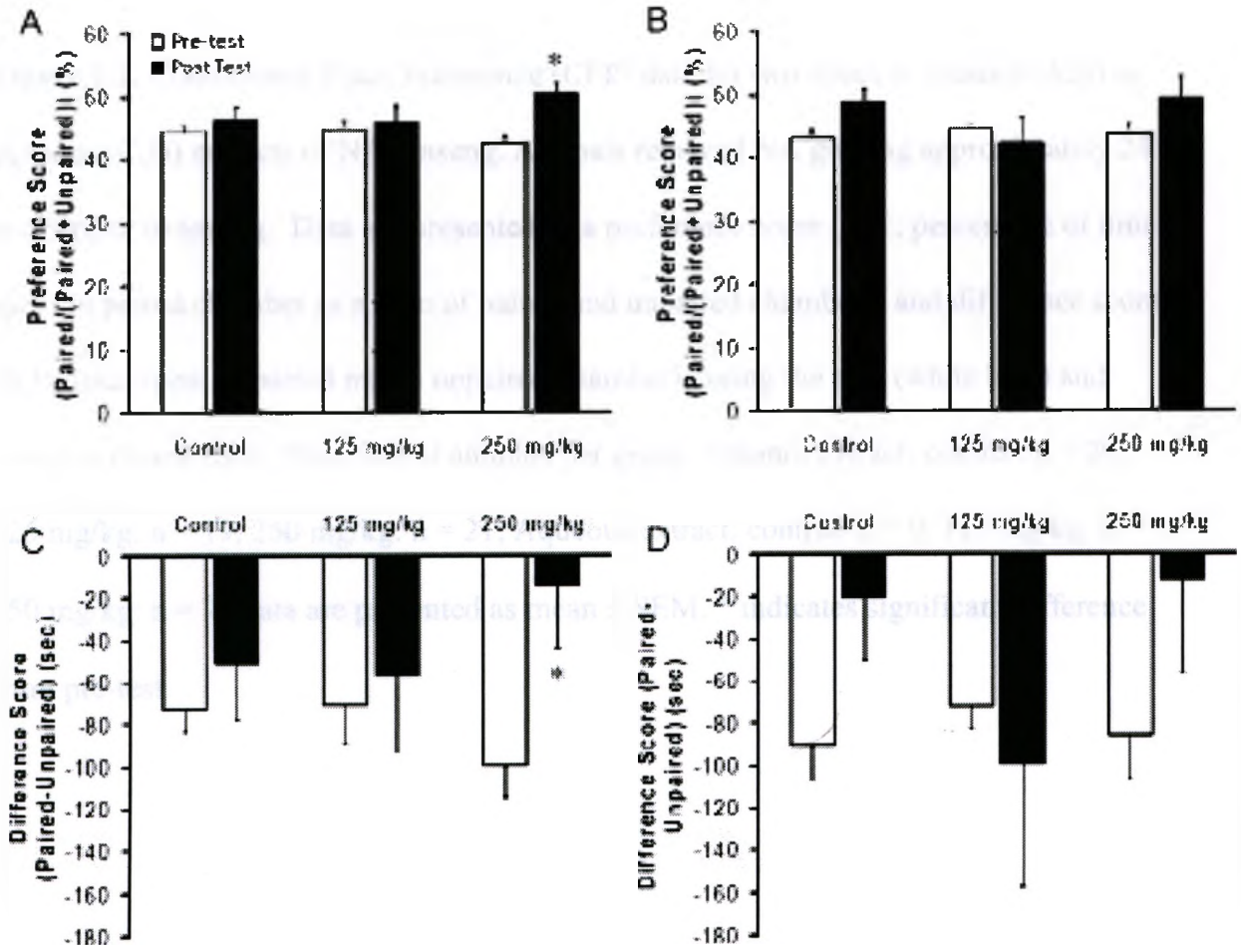
A
Pre-test
Post Test
Preference Score
(Paired/(Paired+Unpaired)) (%)
60
50
40
30
20
10
0
*
Control
125 mg/kg
250 mg/kg
B
Preference Score
(Paired/(Paired+Unpaired)) (%)
60
50
40
30
20
10
0
Control
125 mg/kg
250 mg/kg
C
Control
125 mg/kg
250 mg/kg
Difference Score
(Paired-Unpaired) (sec)
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
*
D
Control
125 mg/kg
250 mg/kg
Difference Score (Paired-
Unpaired) (sec)
0
-20
-40
-60
-80
-100
-120
-140
-160
-180

Figure 2.2. Conditioned Place Preference (CPP) data for two doses of ethanol (A,B) or aqueous (C,D) extracts of NA ginseng. Animals received NA ginseng approximately 24 hours prior to testing. Data are presented as a preference score (A,C; percentage of time spent in paired chamber as a ratio of paired and unpaired chambers) and difference score (B,D; time spent in paired minus unpaired chamber) during the pre- (white bars) and post-test (black bars). Numbers of animals per group: Ethanol extract: control: n = 20, 125 mg/kg: n = 19, 250 mg/kg: n = 21; Aqueous extract: control: n = 9, 125 mg/kg: n = 8, 250 mg/kg: n = 7. Data are presented as mean ± SEM. * indicates significant difference from pre-test.

Table 2.3. Effects of chronic administration of ethanol and aqueous extracts of NA ginseng on behavior in the elevated plus maze (EPM). Data are presented as mean ± SEM of the percent of time spent in open or closed arms and the number of entries into the open or closed arms of the EPM apparatus during a five minute test period. Numbers of animals per group: Ethanol extract: control: n = 19, 125 mg/kg: n = 18, 250 mg/kg: n = 21; Aqueous extract: control: n = 9, 125 mg/kg: n = 8, 250 mg/kg: n = 7. No significant differences between groups were detected.

		Percent of Time		# of Entries	
		Open	Closed	Open	Close
Ethanol	Control	23.00 ± 2.72	64.66 ± 3.50	4.63 ± 1.05	10.42 ± 0.97
	125 mg/kg	28.17 ± 1.56	61.44 ± 1.78	4.06 ± 0.51	9.83 ± 0.78
	250 mg/kg	27.97 ± 2.14	59.66 ± 2.64	4.10 ± 0.50	11.43 ± 0.82
Aqueous	Control	33.74 ± 5.37	57.49 ± 6.21	5.33 ± 1.30	12.44 ± 1.39
	125 mg/kg	30.25 ± 6.61	61.13 ± 7.71	4.63 ± 1.10	11.38 ± 0.75
	250 mg/kg	33.87 ± 3.44	52.61 ± 4.11	7.29 ± 1.02	11.57 ± 1.66

Table 2.4. Effects of chronic administration of ethanol and aqueous extracts of NA ginseng on behavior in an open field test. Data are presented as mean ± SEM of the ambulatory distance and the time the animal was active in the center and perimeter of the apparatus during a twenty minute testing period. Numbers of animals per group: Ethanol extract: control: n = 20, 125 mg/kg: n = 19, 250 mg/kg: n = 22; Aqueous extract: control: n = 9, 125 mg/kg: n = 8, 250 mg/kg: n = 7. * indicates significance from control.

		Ambulatory Distance (cm)		Active Time (secs)	
		Center	Perimeter	Center	Perimeter
Ethanol	Control	277.9 ± 35.6	2355 ± 140	115.6 ± 14.9	875.8 ± 71.6
	125 mg/kg	229.2 ± 25.7	2243 ± 164	92.58 ± 11.7	825.0 ± 69.5
	250 mg/kg	328.0 ± 24.6	2671 ± 97	129.8 ± 10.6	1020 ± 42.3*
Aqueous	Control	300.9 ± 48.4	2942 ± 264	121.1 ±18.1	1106 ± 114.9
	125 mg/kg	280.0 ± 40.2	2501 ± 225	136.6 ± 31.7	957.6 ± 103.5
	250 mg/kg	367.6 ± 60.3	2507 ± 245	137.6 ± 24.4	884.4 ± 113.3

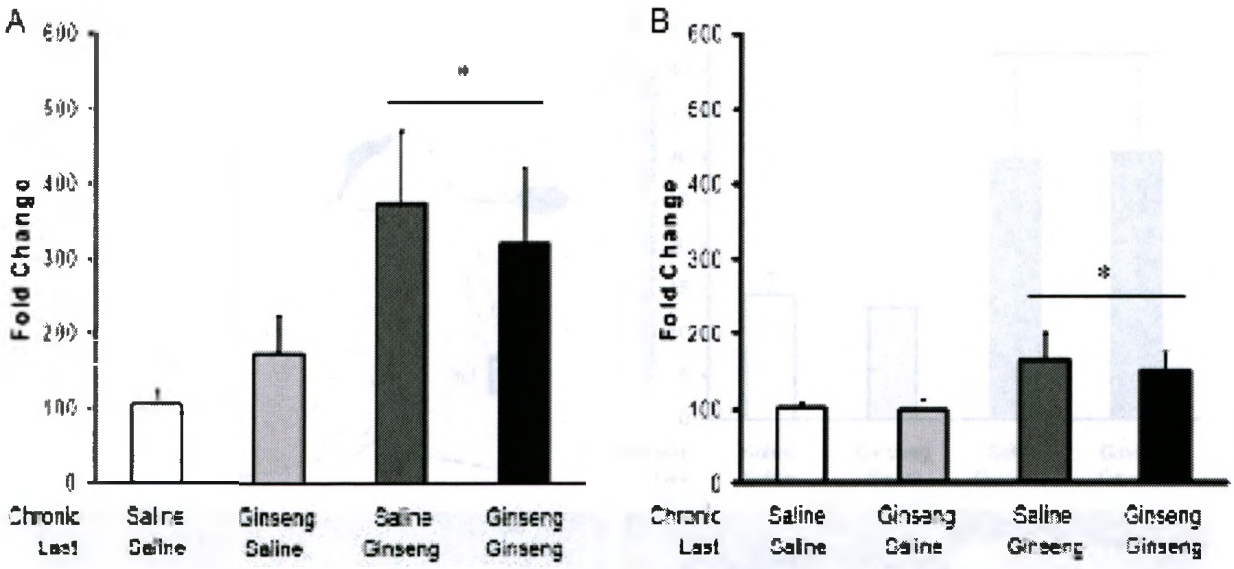

Figure 2.3. Effects of ethanol extract of NA ginseng on mRNA expression of eNOS (A) and nNOS (B) in penile tissue. Males were treated chronically with saline or NA ginseng (250 mg/kg) and received vehicle or NA ginseng (250 mg/kg) 1 hour prior to tissue collection. n = 5 (groups: saline/saline, ginseng/saline, saline/ginseng) or n=6 (ginseng/ginseng group). Data are presented as mean ± SEM. * indicates significant difference between groups last treated with NA ginseng compared to saline.

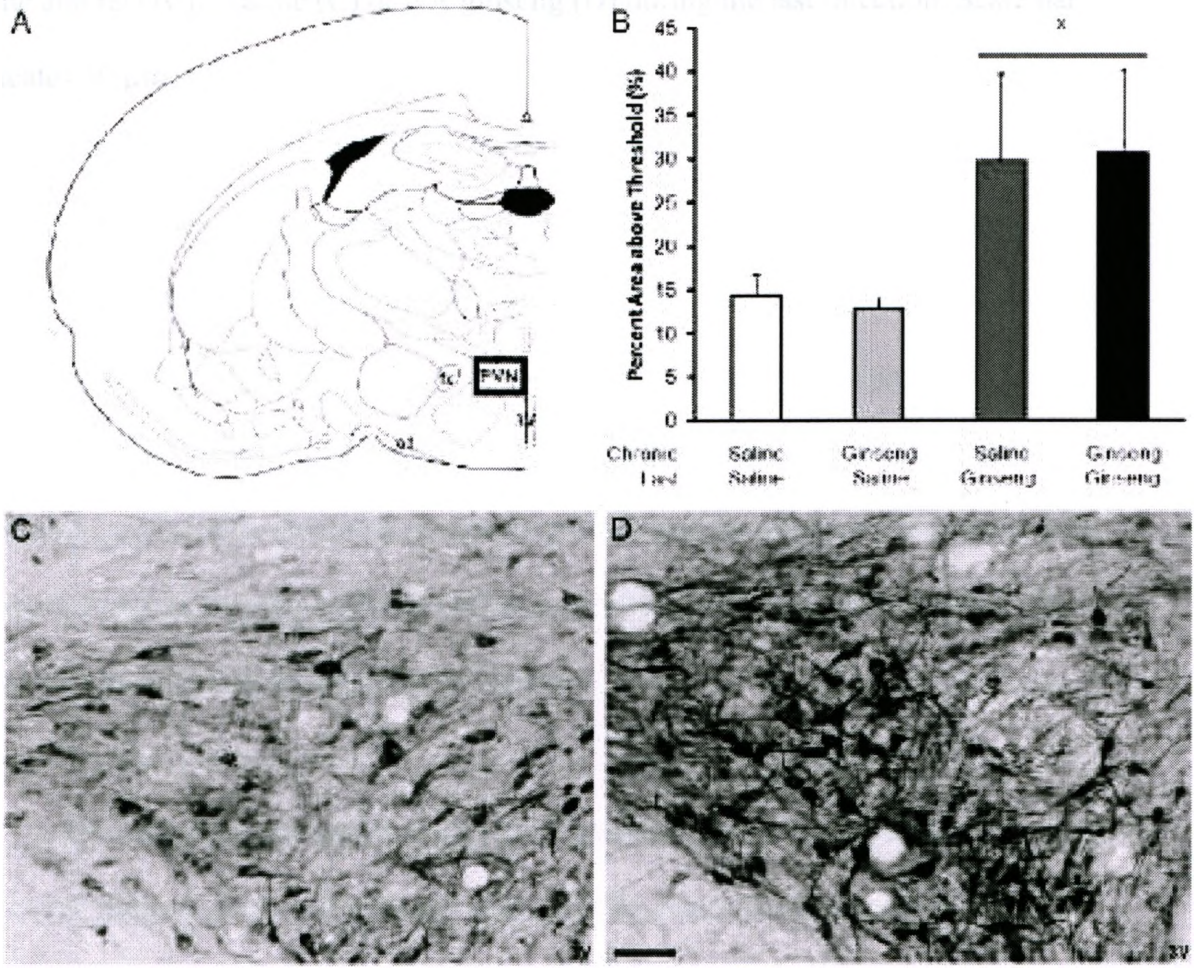

Figure 2.4. Effects of ethanol extract of NA ginseng on nNOS immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN). A is a schematic drawing of area of analysis (Paxinos and Watson, 1998); 3V: third vetricle; fx: fornix; ot: optic tract. B illustrates the quantitative densitometry analysis showing percentage of the area of analysis that is above threshold in males chronically treated with saline or NA ginseng (250 mg/kg) and received vehicle or NA ginseng (250 mg/kg) 1 hour prior to tissue collection. n = 5 for all groups. Data are presented as mean ± SEM. * indicates significant difference between groups last treated with NA ginseng compared to saline. C and D show representative images of nNos immunoreactivity in the PVN of males treated with

saline and receiving saline (C) or NA ginseng (D) during the last injection. Scale bar indicates 50 μm.

2.4. Discussion

The results of this study show that administration of NA ginseng results in facilitation of sexual behavior in adult, male rats. In particular, ethanol extract of NA ginseng improved copulation efficiency, which is indicative of improved erectile function (Clark, 1995). These effects on erectile function were only noted when animals were tested in a mating context and were not observed when males were not in direct contact with an estrus females. NA ginseng ethanol extract may modulate erectile function by increasing expression of nNOS and eNOS in erectile tissues and nNOS in the PVN; however other unknown pathways are involved.

The effects of ginseng on male sexual behavior have been reported in rats (Kim *et al.*, 1976; Murphy *et al.*, 1998; Wang *et al.*, 2010b), mice (Yoshimura *et al.*, 1998), rabbits (Chen and Lee, 1995; Choi *et al.*, 1999) and humans (Hong *et al.*, 2002; de Andrade *et al.*, 2007; Jang *et al.*, 2008; Kim *et al.*, 2009). The majority of these studies were conducted using extracts of Asian ginseng or its processed form known as Korean Red ginseng, which is the steamed root of Asian ginseng. Human studies have shown improved male erectile ability as indicated by physiological tests on penile rigidity and self-reporting scores on penetration, erection maintenance, and rigidity using the International Index of Erectile Function (IIEF) (Hong *et al.*, 2002; de Andrade *et al.*, 2007; Jang *et al.*, 2008; Kim *et al.*, 2009). The animal studies on ginseng have shown a propensity to induce vasodilation in erectile tissue that will cause an erection and improve male sexual performance (Kim *et al.*, 1976; Choi *et al.*, 1999). Other studies have used ginsenoside extracts, rather than full root extracts, to test the effects on sexual

behavior (Yoshimura *et al.*, 1998; Wang *et al.*, 2010b). Ginsenosides are considered the main active constituents of the ginseng genus (Leung and Wong, 2010; Wang *et al.*, 2010a; Tsutsumi *et al.*, 2011; Yin *et al.*, 2011). Previous studies have identified Rg1 as a main contributor to enhancing male sexual behavior with little to no contribution from ginsenosides Rb1 or Rb2 (Yoshimura *et al.*, 1998; Wang *et al.*, 2010b). Much less is known about the effects of other ginseng species, such as NA ginseng, on male sexual behavior. The only previous study on male sexual behavior with NA ginseng demonstrated an increase in sexual performance indicated by decreased latencies to mount, intromission, and ejaculation (Murphy *et al.*, 1998). The current study did not demonstrate effects of NA ginseng on measures of motivation. Rather, it was shown that NA ginseng facilitates male sexual behavior by improving copulation efficiency and reducing number of mounts prior to ejaculation. These parameters reflect erectile function (Clark, 1995). Hence, NA ginseng improved erectile function during mating. Moreover, the effect of NA ginseng was evident in males that were sexually naïve or experienced.

The effects of NA ginseng appeared dependent on the context in which erectile function was observed; i.e. it was improved during mating behavior but effects of NA ginseng were not detected on NCE. NCE are considered a representation for erectile function induced by arousal, which are called psychogenic erections in humans (Sachs and Garinello, 1978). Potential explanations for the difference in facilitation of erection include dosage of NA ginseng; effects with NCE may require higher dosages. In addition, the lack of effects of NA ginseng on NCE may be related to differences in neural control

of erections during mating compared to NCE. For example, lesions of the medial preoptic area (mPOA) caused a large impairment in sexual performance during mating but had minimal to no effects on NCE (Liu *et al.*, 1997a). The mPOA is essential for orchestrating male sexual behavior and studies have shown its activation during sexual behavior (Coolen *et al.*, 1996, 1998). In the same study mentioned above, Liu and colleagues reported the reverse effect in the bed nucleus of the stria terminalis (BNST); lesions mostly abolished NCE but had little effect on copulatory performance. The BNST is a conduit for information between brain regions involved in male sexual behaviour and is activated during appetitive and consummatory phases of copulation (Kondo and Arai, 1995; Coolen *et al.*, 1997). Consequently, NA ginseng may not affect the neural substrates mediating NCE.

An objective of the current study was to differentiate any erectile enhancing effects between two common extracts of ginseng: ethanol and aqueous, since different effects of extract preparations have been reported on immunomodulation and the cardiovascular system (Lee *et al.*, 1981; Azike *et al.*, 2011). Indeed, differing effects of the two extracts were noted; improved erectile function occurred only with the administration of ethanol extract and not with the aqueous extract of NA ginseng. Both extracts have different ginsenoside profiles based on the solubility of the individual ginsenosides (Azike *et al.*, 2011). The ethanol extract of the NA ginseng used in the current study has a higher percentage of ginsenoside content (approximately 28% total ginsenoside content by weight in ethanol extract compared to 14% in aqueous extract). While ginsenoside Rb1 has been shown not to have an effect on preventing male mice

copulatory disorder when testing the latencies and frequencies of sexual behavior parameters (Yoshimura *et al.*, 1998), its ability on enhancing erectile function has not been examined and cannot be ruled out in our study. Ginsenoside Rg1 has been shown to enhance sexual behavior (Yoshimura *et al.*, 1998; Wang *et al.*, 2010b); however, Rg1 concentrations did not significantly differ between extracts. As such, ginsenoside Rg1 is unlikely to account for differences in erectile enhancing effects seen between extracts. Moreover, differences in extract effectiveness may be due to a minimum effective dose; ginsenoside concentration in the aqueous extract may not be sufficient to induce an effect. More studies are needed to determine which ginsenosides of NA ginseng are active at improving erectile function. Finally, differences in extract effects may also be due to polysaccharides (PS), which are found in NA ginseng. Although ginsenosides have traditionally been seen as the principal active constituents of ginseng, studies show that PS have active properties and are found in aqueous, not ethanol, extracts (Azike *et al.*, 2011). However, since aqueous extract was not effective in facilitating erectile function, it is unlikely that PS are major contributors to the effects of ginseng on male sex function.

The mechanisms by which NA ginseng influences sexual behavior are unclear. It has been proposed that ginseng affects the cellular mediators of erectile function in the erectile tissues and/or testosterone synthesis (Chen and Lee, 1995; Choi *et al.*, 1999; Wang *et al.*, 2010b). Ginseng extracts cause vasodilation in the vasculature as shown in *in vivo* (Tsutsumi *et al.*, 2011; Xu *et al.*, 2011) and *in vitro* (Kang *et al.*, 1995; Toda *et al.*, 2001; Tsutsumi *et al.*, 2011) studies. Effects of ginseng on cellular mediators of erectile function (Gratzke *et al.*, 2010) have also been demonstrated. Concentration-

dependent vasodilatory effects of Asian ginseng *in vitro* using rabbit corpus cavernosum (Chen and Lee, 1995) have been reported in addition to increased levels of cGMP in ginseng-treated tissue. Chen and Lee acutely treated penile tissue with ginseng for 10 minutes and discovered an increase in the percent relaxation compared to control. Also, ginseng has been shown to act at the vascular level on the NO/cGMP pathway to induce erections. Chronic administration of Rg1 ginsenoside extract increased cGMP and NO levels in mice corpus cavernosum concomitant to improved sexual performance (Wang *et al.*, 2010b). The current study provides a potential target of an upstream effect of ethanol extract of NA ginseng on the NO/cGMP pathway. NA ginseng ethanol extract increased levels of both eNOS and nNOS in penile tissue. Unexpectedly, levels of nNOS and eNOS mRNA were not up-regulated by chronic, but rather by acute administration of ethanol extract. Previous studies have shown minute concentrations of ginsenosides in circulation within 15 minutes after oral administration (Song *et al.*, 2010). Furthermore, ginseng extracts have been demonstrated to induce NO-mediated relaxation of rabbit corpus cavernosum and increased cGMP levels after only 10 minutes of exposure (Chen and Lee, 1995). Hence, it is possible that NA ginseng increases eNOS and nNOS mRNA in the corpus cavernosum one hour after injection, which can lead to an increase in cGMP content in smooth muscle cells. However, the increase in nNOS and eNOS mRNA does not appear to be required for the facilitative effects of NA ginseng on erectile function, as chronic administration of ginseng altered sexual function without altering mRNA levels for nNOS or eNOS in penile tissue.

Another potential mediator of the effects of ginseng on sexual function is the steroid hormone testosterone. Chronic ginsenoside Rg1 exposure has been shown to result in increased levels of testosterone in plasma of male mice (Wang *et al.*, 2010b). In contrast, another study using NA ginseng detected no effects on testosterone levels (Murphy *et al.*, 1998). As such, further studies are needed to determine if NA ginseng has any effect on hormone levels in animals and in humans.

Ginseng may influence sexual function by acting on the brain. Hence, the current study tested if ethanol extract of NA ginseng influenced brain areas that mediate erectile function such as the PVN (McKenna, 2000). The PVN is an integration center involved in many autonomic processes (Argiolas and Melis, 2005). The role of the PVN in male erectile function has been well established through studies showing stimulation induces penile erection (Melis *et al.*, 1994; Chen and Chang, 2002, 2003) and bilateral lesions greatly reduces the ability to obtain an erection (Argiolas *et al.*, 1987; Liu *et al.*, 1997b). A role for oxytocinergic neurons in the PVN in the control of penile erection in the male rat has been implicated (Argiolas and Melis, 2004). In turn, PVN oxytocin neurons are influenced by endogenous NO production by nNOS to cause release of oxytocin in extra-hypothalamic brain regions and the spinal cord (Melis *et al.*, 1996; Melis and Argiolas, 1997; Melis *et al.*, 1997).

The current study demonstrated an increase in nNOS immunoreactivity in the PVN of rats treated with ethanol extract of NA ginseng one hour before sacrifice. Chronic administration did not influence nNOS immunoreactivity. Similar to the penile

tissue, the increase in nNOS in the PVN does not appear to be required for the facilitative effects of NA ginseng on male sexual behavior. These data suggest that NA ginseng may influence erectile function via increased nNOS expression; in turn possibly activating oxytocin projections from the PVN. However, chronic administration of NA ginseng enhanced sexual behavior during copulation, however, did not influence nNOS in the PVN. As such, alternate mechanisms of action exist for the ethanol extract of NA ginseng to enhance erectile function besides those detected in the current experiment.

Finally, the current study shows that ethanol extract of NA ginseng has rewarding properties since males formed a conditioned place preference, a widely used test for reward-seeking behavior (Tzschentke, 2007). Also, ginseng has also been indicated to be anxiolytic (Carr *et al.*, 2006). In the previous study, anxiety behaviors were tested immediately following Asian ginseng administration at high dosing levels; however, the current study tested animals after approximately 24 hours after last injection and used lower doses of NA ginseng. As such, the lack of anxiolytic effects of our study may be due to timing of injection prior to testing and the doses used. Indeed, studies have shown that chronic Asian ginseng administration at doses similar to those used in our experiment do not have anxiolytic properties (Einat, 2007). Therefore, anxiolytic effects of ginseng may only be detected shortly after consumption and may not be long lasting.

In conclusion, this study shows that ethanol, but not aqueous, extract of NA ginseng increased erectile function in male rats during mating behavior. NA ginseng effects are potentially mediated through eNOS and nNOS mRNA in penile tissue and

nNOS density in the PVN; however, other mechanisms of action contribute to its pro-erectile effects seen during chronic treatment, which are yet to be discovered.

2.5. References

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Chapter 3: North American ginseng improves erectile function in Obese Zucker rats.

3.1. Introduction

Erectile dysfunction (ED) is a serious disorder that decreases the quality of life in men and their partners (Vickers and Wright, 2004). ED may occur by various alterations in physiology. It has been well established that nitric oxide (NO) is the main cellular mediator of erectile function, causing vasodilation in the corpus cavernosum in penile tissue (Awad *et al.*, 2011). Type 2 Diabetes Mellitus (T2DM) patients experience many physiological alterations that predispose them to ED such as decreased neuronal nitric oxide synthase (nNOS) expression (Xie *et al.*, 2007) and increased reactive oxygen species (ROS) that scavenge for NO in vasculature and decrease its availability to vasodilate corpus cavernosum (De Young *et al.*, 2004; Yan *et al.*, 2008). While under 10% of the general male population experiences some form or ED, men with T2DM have a prevalence rate of approximately 28% (Feldman *et al.*, 1994). In addition, only 56% of men with T2DM respond to phosphodiesterase type 5(PDE5) inhibitors as compared to 87% of the general population (Rendell *et al.*, 1999). Moreover, men who responded positively to PDE5 inhibitors may revert back to pre-treatment conditions (Penson *et al.*, 2003). Hence, development of additional pharmacological treatments is needed.

In the current study, North American (NA) ginseng was examined as a potential treatment for ED. The two most common species in the ginseng genus are *Panax ginseng*

(Asian ginseng) and *Panax quinquefolius* (North American ginseng). Extracts of Asian ginseng and Korean Red ginseng, created when Asian ginseng is steamed, have been shown to facilitate male sexual behavior (Yoshimura *et al.*, 1998; Choi *et al.*, 1999; Hong *et al.*, 2002; de Andrade *et al.*, 2007; Kim *et al.*, 2009) and to cause vasodilation in corpus cavernosum (Chen and Lee, 1995; Kang *et al.*, 2005). In addition, NA ginseng has been shown to enhance erectile function and sexual performance in male rats (Murphy *et al.*, 1998; Barnes *et al.*, 2011).

Ginsenosides are triterpenoid glycosides, also called saponins, and are considered the principal active constituents in ginseng (Attele *et al.*, 1999; Leung and Wong, 2010). Ginsenosides have been shown to reduce glucose levels in blood (Ji and Gong, 2007) and increase glutathione and decrease markers of lipid peroxidation (Ryu *et al.*, 2005). Hence, NA ginseng may restore some of the underlying physical alterations associated with T2DM that contribute to ED development.

The current study tested if NA ginseng improves erectile function using Obese Zucker (OZ) rats as a pre-diabetic model for T2DM, as T2DM accounts for 90-95% of diabetic patients (Hidalgo-Tamola and Chitaley, 2009). OZ rats have an autosomal recessive mutation in the leptin receptor gene (Kasiske *et al.*, 1992), and represent a less severe model of ED caused by diabetic complications than a model with complete diabetes such as the Zucker Diabetic rat (Garcia *et al.*, 2009). Also, similar to T2DM patients, OZ rats have hyperinsulinemia, insulin resistance, glucose intolerance, hyperlipidemia, obesity, hypertension, and impaired vasodilation (Kasiske *et al.*, 1992).

This study examines the effects of NA ginseng on mating behavior, penile reflexes, and cellular mediators of erectile function in penile tissue of OZ rats.

3.2. Methods and Materials

3.2.1. Animals

Adult male OZ (250-300 g) and Lean Zucker (LZ) (200-250 g) rats were obtained from Charles River (Sherbrooke, Quebec, Canada). Rats were housed in pairs in standard Plexiglas holding cages, under a 12/12 light-dark cycle (lights off at 10 a.m.) with food and water available at all times. Female Long Evans rats (200-225 g; Charles River) were bilaterally ovariectomized and subcutaneously implanted with a capsule containing 5% 17-β-estradiol benzoate (Sigma, St. Louis, Missouri, USA) and 95% cholesterol (Sigma, St. Louis, Missouri, USA). Sexual receptivity in females was induced on test days with a subcutaneous injection of 500 mg progesterone dissolved in 0.1 mL sesame oil (Sigma, St. Louis, Missouri, USA) four hours prior to experiments. All procedures were approved by the Animal Care Committee at the University of Western Ontario and conform to the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

3.2.2. North American Ginseng Extracts and Administration

Roots of NA ginseng from five farms in Ontario, Canada were harvested after four years of growth by the Ontario Ginseng Grower's Association. Roots were processed by Naturex (South Hackensack, New Jersey) by grinding and soaking them at 40°C, three times for a total of five hours in either 75% ethanol or aqueous solutions. The ethanol or aqueous extracts were filtered and heated at 45°C to remove excess solvent to

produce 60% total solid content. Ethanol and aqueous NA ginseng root extracts were prepared after lyophilization at the Ontario Ginseng Innovation and Research Consortium (University of Western Ontario, London, Canada). Concentration for the major ginsenosides (Rb1 Re, Rg1, Rb2, Rd, and Rc) in both extracts have previously been determined by high-pressure liquid chromatography (Azike *et al.*, 2011).

Saline (0.9%) was used to suspend the powdered NA ginseng ethanol or aqueous extracts and rats were injected daily with either extract (250 mg/kg) or vehicle (saline) via oral gavage two to three hours after lights off under red light illumination unless otherwise stated for test days. Concentration of each injection was adjusted based on the weights of the animals so all injection volumes were less than 1 mL per day.

3.2.3. Experimental design

In the first experiment, untreated OZ and LZ rats (n = 10) were tested for sexual behavior every other day for three tests. In the second experiment, a separate group of OZ rats were treated with ethanol or aqueous NA ginseng extracts or vehicle (n = 12 for saline and aqueous groups, n = 13 for ethanol group) and tested for sexual behavior in a series of four tests every other day after 28 days of daily NA ginseng or vehicle administration. Subsequently, these males were tested for erectile function using *ex copula* tests. Throughout the experiment, males were weighed every two days prior to injection. Average weight of treatment groups did not differ at the start of the experiment. For each animal body weights were expressed as a percentage of the initial body weight at start of the experiment. Differences between groups were analyzed using repeated

measures ANOVA (factors: time and treatment) and Student-Newman-Keuls for post hoc analyses with 95% confidence levels (SigmaPlot version 11.0).

3.2.4. Sex Behavior

Tests occurred every other day for a total of three (untreated OZ and LZ rats; experiment 1) or four (treated OZ rats; experiment 2) tests. Male rats were sexually naïve prior to the start of the tests. As such, effects of treatment on sexual behavior were determined in the rats while naïve and sexually experienced. In experiment 2, at the start of the experiment, weights of the groups were measured for appropriate dosing. In addition effects of NA ginseng treatment were tested either 30 minutes or 24 hours following last drug administration; for tests 1 and 3 rats received NA ginseng 30 minutes prior to mating while during tests 2 and 4 rats received NA ginseng 24 hours prior to testing. As such, testing examined acute and chronic effects of NA ginseng dosing. During all tests, rats were placed in a clean test cage (60 x 45 x 50 cm^3) ten minutes prior to introduction of a receptive female. Male rats were allowed to mate to either one ejaculation (and completed post-ejaculatory interval; PEI) or for one hour, whichever came first. All standard parameters of male sexual performance were recorded including: mount latency (ML; time from introduction of receptive female until the first mount), intromission latency (IL; time from introduction of receptive female until the first mount with successful vaginal penetration), ejaculation latency (EL; time from the first intromission until ejaculation), PEI (time from ejaculation to first subsequent intromission), numbers of mounts and intromissions (M and IM) prior to ejaculation,

copulation efficiency (CE; ratio of number of intromissions to total number of mounts plus intromissions), and inter-intromission interval (I IM I; testing period divided by number of intromission). Because few OZ rats ejaculated within one hour, all animals were included in the analysis if they performed at least one mount (approximately 50% in naïve rats and up to 90% by final test); no values for latencies were recorded unless the rat performed the particular sex behavior parameter. In addition, when calculating I IM I, if the rat intromitted but did not ejaculate then a value of 3600 seconds for EL was used (duration of test). For both experiments, differences between groups were compared using two-way analysis of variance (factors: trial and strain (LZ vs. OZ) or treatment (NA ginseng vs. vehicle)) and all pairwise multiple comparison procedures with Holm-Sidak method for post hoc analysis with 95% confidence levels.

3.2.5. *Ex Copula* Tests

A testing apparatus consisting of a Plexiglas base fixed to a 12 inch tube was used to restrain the rats in a supine position with the rat's head and upper torso inside the tube and lower torso and legs exposed. Rats were habituated to the apparatus and testing position for 10 minutes for four consecutive days prior to testing. On test days, the penile prepuce was retracted to allow measurements of penile reflexes (Sachs and Garinello, 1978) and rats were video recorded for 30 minutes if no erection occurred or for 15 minutes after the first erection. Rats received NA ginseng or saline 30 minutes prior to testing. Videos were scored by an experimenter blinded to treatment groups and the following measures were quantified: E1 erections (base of glans engorges with blood),

E2 erections (both tip and base of glans engorge with blood), E3 erections (cups; an E2 erection with flaring of the tip of the glans so that the diameter of the glans tip surpassed the base), F1 anteroflexions (ventral penile extension from resting position that is less than 90 degrees from ventrum), and F2 anteroflexions (ventral penile extension from resting position that surpasses 90 degrees from ventrum) (Sachs, 1985). Also, the latency to the first erection and clusters of erections within 30 seconds (series of penile responses occurring within a 30 second time period) were recorded (Sachs, 1985). Differences between either the ethanol or aqueous group and control vehicle-treated group were compared using Mann-Whitney Rank Sum tests with 95% confidence levels. Percentages of animals displaying E3 erections were compared between groups using Chi-square analysis.

3.2.6. Tissue Collection

Following completion of behavioral tests, animals received an overdose of pentobarbital (270 mg/mL/kg) and blood, testes, and penile tissues were harvested. In experiment 1, LZ and OZ rats were not treated prior to tissue collection. In experiment 2, rats chronically treated with NA ginseng were administered either a final dose of NA ginseng or vehicle one hour before sacrifice, thus, five treatment groups were created to determine the effects of NA ginseng on cellular mediators of erectile function. Testes were weighed at time of collection. Penile tissues were frozen at -80°C until processing for real time PCR. Blood was collected in centrifuge tubes containing heparin (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) and centrifuged at

3000 rpm for 15min at 0°C (Beckman Model TJ-6 centrifuge attached to a TJ-R Refrigeration Unit). Plasma was removed, aliquoted and stored at -80°C until further processing for steroid assays.

3.2.7. Real time PCR

Penile tissues of untreated LZ and OZ, and NA ginseng or vehicle treated OZ males were homogenized in TriZol reagent (Invitrogen, Burlington, ON) for total RNA isolation. RNA quantity and purity were assessed using a NanoDrop spectrophotometer (ND 1000 v3.3.0; NanoDrop Technologies, Inc., Wilmington, DE) to ensure that the 260/280nm absorbance ratio was between 1.8 and 2.0. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. Following cDNA synthesis, nNOS and eNOS gene expression was quantified by qRT-PCR. Each reaction contained 2µL of cDNA, 10µL of TaqMan Universal PCR Master Mix 2X (Applied Biosystems), 0.8µL of 25mM $MgCl_2$ (Invitrogen), 6.2µL DEPC treated water, and 1µL of TaqMan gene expression assay solution for the gene of interest (nNOS Assay ID: Rn00583793_m1; eNOS Assay ID: Rn02132634_s1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay ID: Rn99999916_s1) was used as an endogenous control. Each sample was assayed in triplicate using the Rotor Gene RG 6000 thermocycler and software (Corbett Life Sciences, San Francisco, CA) at the following cycling conditions: hold at 50°C for 2 min and 95°C for 10 min, 40 cycles of 95°C for 15 sec and 58°C for 1 min. Quantification of gene expression was calculated by use of a

standard curve generated from qRT-PCR for each gene analyzed. cDNA reverse transcribed from RNA isolated from penile tissue was used to create a standard curve with 5-log serial dilutions. Standard curves were deemed optimal when the line of best fit through the cycle threshold (C_t) values of the dilutions had a $R^2 \geq 0.99$ and an efficiency value between 0.8-1. An optimal threshold line generated from the standard curve for each gene was used to determine the C_t value for each sample. The C_t of each experiment sample was determined from the intersection between the baseline subtracted amplification curve of the sample and the threshold line established by the optimal standard curve. For each experiment sample, the C_t value for each gene of interest was normalized to their respective C_t value of the housekeeping gene, GAPDH. This value corresponds to the ΔC_t value. Furthermore, the difference between the ΔC_t value for each individual sample in the treatment groups and the average ΔC_t value of the control group was calculated as the $\Delta\Delta C_t$ value ($= 2^{\wedge}(\Delta C_{t(sample)} - \Delta C_{t(average)})*100$). One-way ANOVA with Holms Sidak post hoc analysis were conducted to identify significant differences between groups. Values were considered significantly different with $p<0.05$.

Testosterone Analysis

All samples were assayed at the Core Assay Facility in the Department of Psychology at the University of Michigan (Ann Arbor, Michigan). ELISA kits specifically designed for measuring testosterone in mouse/rat serum or plasma were used (Cat. # TE187S-100, Calbiotech Inc, USA). The reference for this ELISA ranged from

0.1-18.0 ng/mL and 25 uL of standards, samples, and controls were analyzed in duplicate. Absorbance was read at 450 nm with a microtiter well reader.

3.3. Results

3.3.1. Sexual behavior

Sexual behavior was severely impaired in OZ rats compared to LZ controls (Figure 3.1). Effects of strain (LZ vs. OZ) were detected for latencies to mount ($F(1,52)=5.86$; $p=0.019$) and intromit ($F(1,54)=5.55$; $p=0.023$), numbers of mounts ($F(1,54)= 38.95$; $p=<0.001$) and intromissions ($F(1,54)=41.65$; $p=<0.001$), copulation efficiency ($F(1,52)=159.56$; $p=<0.001$) and inter-intromission intervals ($F(1,49)=56.09$; $p=<0.001$). Impaired behavior was detected during all three trials, indicating that sexual experience did not alleviate the impairments. One of the OZ males reached ejaculation during any of the three tests compared to 100% of LZ rats.

NA ginseng improved sexual behavior in OZ males. Treatment effects were detected on copulation efficiency ($F(2,106)=8.8$; $p=<0.001$) and numbers of intromission ($F(2,108)=4.1$; $p=0.020$). Post hoc analyses revealed facilitative effects of aqueous extract during test 3 and test 4, the latter with a strong trend ($p = 0.062$), and ethanol extract during tests 1 and 4 (Figure 3.2). Hence, both extracts improved copulation efficiency on test days. In addition, treatment effects were found on mount ($F(2, 100)=12.67$; $p=<0.001$) and intromission ($F(2, 85)=13.14$; $p=<0.001$) latencies (Table 3.1). Specifically, aqueous and ethanol extracts of NA ginseng significantly shortened mount and intromission latencies, but only during the first test when males were sexually

naïve. Also, aqueous extract improved intromission latency during the second test, but no effects during the subsequent tests were found.

3.3.2. *Ex copula* test

In addition to improving erectile function during mating behavior, NA ginseng also improved penile reflexes determined by the *ex copula* tests (Figure 3.3). Ethanol, but not aqueous, extract significantly improved total number of erections ($p=0.034$), and the percentage of animals displaying E3 erections ($p=0.044$), with a trend towards significance for the percentage of E3 erections ($p=0.065$). Other parameters were not affected by either extract (Table 3.2).

3.3.3. eNOS and nNOS mRNA

There were no significant differences in either eNOS (OZ: 119 ± 13.5 % fold change compared to LZ) or nNOS (OZ: 92 ± 5.7 % fold change compared to LZ) mRNA content in penile tissue between LZ and OZ rats. Ethanol extract of NA ginseng increased nNOS ($F(2,15)=9.2$; $p=0.002$) and eNOS ($F(2,16)= 4.78$; $p=0.024$) mRNA (Figure 3.4). Post hoc analyses demonstrated that males chronically treated with ethanol NA ginseng and received a final ginseng administration prior to tissue collection (EE) had increased nNOS ($p<0.001$) and eNOS ($p=0.008$) compared to chronic saline treated groups (SS). In contrast, chronically treated ethanol NA ginseng males that received saline on the final day (ES) had increased nNOS ($p=0.015$), but not eNOS. Hence, it appears that in addition

to the chronic treatment, the final administration prior to tissue collection affected levels of eNOS, but not nNOS. Aqueous extract of NA ginseng did not have any effect on levels of nNOS or eNOS (Table 3.3).

3.3.4. Body weight

NA ginseng affected weight gain in OZ males ($F(2, 816)=53.935$; $p<0.001$); significantly lower increases in bodyweight compared to vehicle-treated males were first detected following 25 (aqueous) or 33 days (both extracts) of daily administration (Figure 3.5).

Testes weight and Testosterone Levels

OZ rats had a decrease in testes weight compared to LZ rats ($p=0.004$) (Figure 3.6). Testes weight was increased by treatment with NA ginseng (Figure 3.6) as ethanol, but not aqueous, extract significantly increased testes weight compared to saline-treated controls ($p=0.041$). However, plasma testosterone levels were not affected by NA ginseng as there were no significant differences between control (2.0 ± 0.2 ng/mL) and NA ginseng-treated rats (aqueous: 1.7 ± 0.2 ng/mL, ethanol: 2.6 ± 0.4 ng/mL).

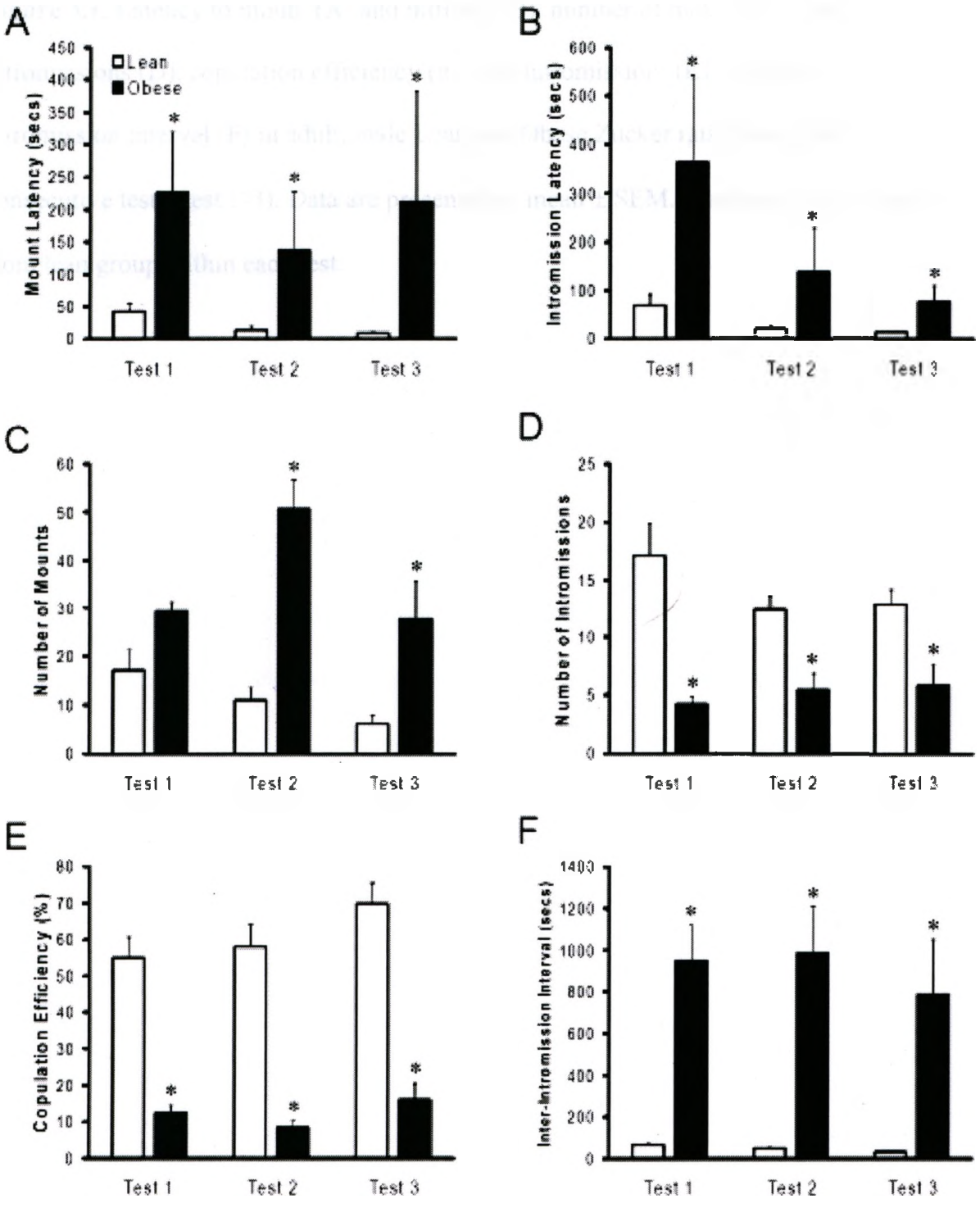
A
Lean
Obese
Mount Latency (secs)
450
400
350
300
250
200
150
100
50
0
Test 1
Test 2
Test 3
B
Intromission Latency (secs)
600
500
400
300
200
100
0
Test 1
Test 2
Test 3
C
Number of Mounts
60
50
40
30
20
10
0
Test 1
Test 2
Test 3
D
Number of Intromissions
25
20
15
10
5
0
Test 1
Test 2
Test 3
E
Copulation Efficiency (%)
80
70
60
50
40
30
20
10
0
Test 1
Test 2
Test 3
F
Inter-Intromission Interval (secs)
1400
1200
1000
800
600
400
200
0
Test 1
Test 2
Test 3

Figure 3.1. Latency to mount (A) and intromit (B), number of mounts (C), and intromissions (D), copulation efficiency (mounts/intromission) (E), and inter-intromission interval (F) in adult, male Lean and Obese Zucker rats during three consecutive tests (test 1-3). Data are presented as mean ± SEM. * indicates significance from lean group within each test.

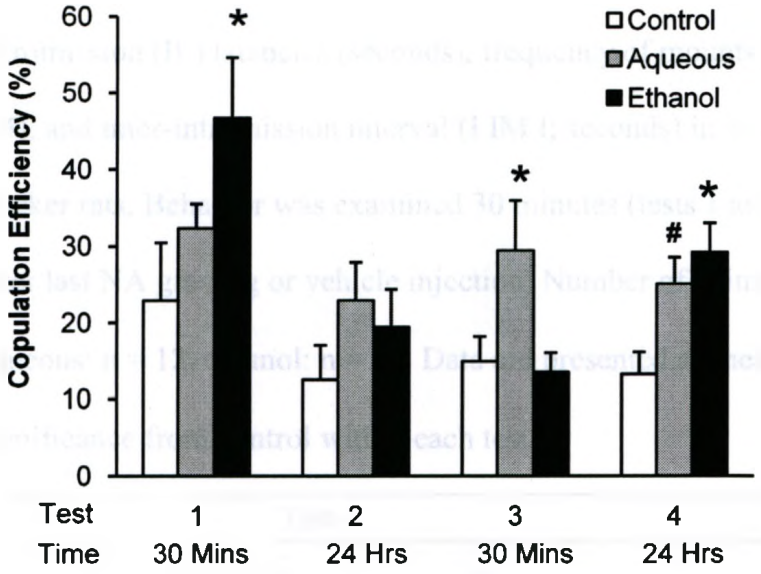

Figure 3.2. Effects of ethanol and aqueous extracts of NA ginseng on copulation efficiency (mounts/intromission) in male Obese Zucker rats after 28 days of oral treatment at 250 mg/kg. Behavior was examined 30 minutes (tests 1 and 3) or 24 hours (tests 2 and 4) after last ginseng or vehicle injection. Number of animals per group: control: n = 11, aqueous: n = 12, ethanol: n = 13. Data are presented as mean ± SEM. * indicates significance from control within each test and # indicates p = 0.062 compared to control.

Table 3.1. Effects of aqueous and ethanol extracts of NA ginseng on mount (ML) and intromission (IL) latencies (seconds), frequency of mounts (# M) and intromissions (# IM), and inter-intromission interval (I IM I; seconds) in sexually naïve male Obese Zucker rats. Behavior was examined 30 minutes (tests 1 and 3) or 24 hours (tests 2 and 4) after last NA ginseng or vehicle injection. Number of animals per group: control: n = 11, aqueous: n = 12, ethanol: n = 13. Data are presented as mean ± SEM. * indicates significance from control within each test.

		Test			
		1	2	3	4
Control	ML	1123 ± 397	415 ± 112	85 ± 27	34 ± 9
	IL	1405 ± 523	657 ± 218	103 ± 37	64 ± 31
	#M	8 ± 4	9 ± 4	34 ± 10	42 ± 10
	#IM	3 ± 2	2 ± 1	7 ± 2	6 ± 2
	I IM I	1288 ± 645	1813 ± 734	476 ± 95	967 ± 355
Aqueous	ML	202 ± 115*	177 ± 72	112 ± 80	26 ± 8
	IL	405 ± 198*	116 ± 50*	171 ± 138	31 ± 6
	#M	9 ± 3	23 ± 9	26 ± 9	50 ± 10
	#IM	5 ± 2	9 ± 3	9 ± 3	15 ± 4
	I IM I	912 ± 454	741 ± 413	717 ± 421	396 ± 96
Ethanol	ML	200 ± 56*	246 ± 115	113 ± 55	32 ± 7
	IL	267 ± 83*	349 ± 137	144 ± 82	110 ± 49
	#M	14 ± 5	12 ± 4	46 ± 9	31 ± 8
	#IM	9 ± 3	3 ± 1	9 ± 2	11 ± 4
	I IM I	360 ± 129	1322 ± 411	510 ± 171	916 ± 407

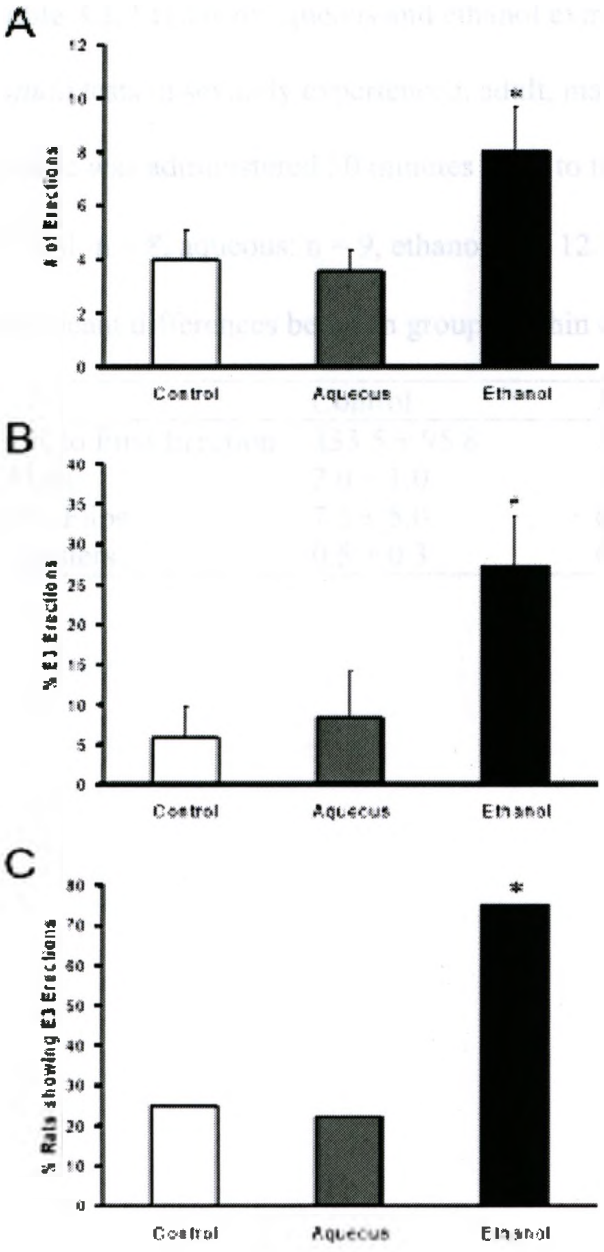

Figure 3.3. Effects of ethanol and aqueous extracts of NA ginseng on number of erections (A), percent of E3 erections (B), and percent or rats displaying E3 erections (C) during the *ex copula* test in sexually experienced male Obese Zucker rats. NA ginseng or vehicle was administered 30 minutes prior to testing. Number of animals per group: control: n = 8, aqueous: n = 9, ethanol: n = 12. Data are presented as mean ± SEM. * indicates significance from control group within each test and # indicates p = 0.065 from control within each group.

Table 3.2. Effects of aqueous and ethanol extracts of NA ginseng on parameters of ex *copula* tests in sexually experienced, adult, male Obese Zucker rats. NA ginseng or vehicle was administered 30 minutes prior to testing. Number of animals per group: control: n = 8, aqueous: n = 9, ethanol: n = 12. Data are presented as mean ± SEM. No significant differences between groups within each test were detected.

	Control	Aqueous	Ethanol
Time to First Erection	333.5 ± 95.8	227.2 ± 54.5	194.0 ± 35.5
# Flips	2.0 ± 1.0	1.3 ± 0.6	3.0 ± 0.9
% F2 Flips	7.5 ± 5.0	6.3 ± 6.3	32.0 ± 11.6
# Clusters	0.5 ± 0.3	0.3 ± 0.2	2.0 ± 0.5

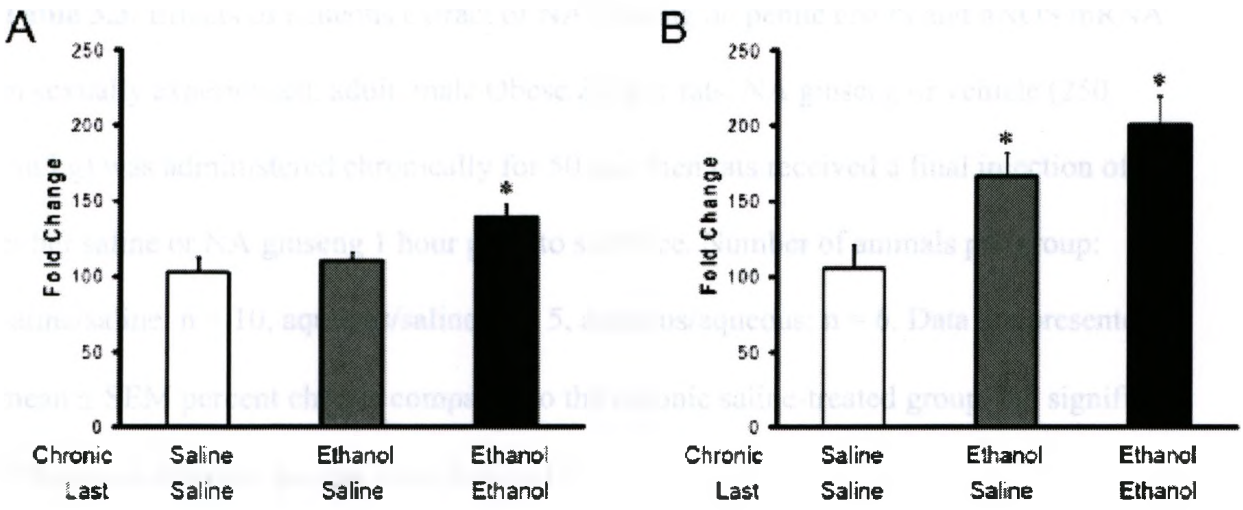

Figure 3.4. Effects of ethanol extract of NA ginseng on mRNA expression of eNOS (A) and nNOS (B) in penile tissue. Males were treated chronically with saline or NA ginseng (250 mg/kg) and received vehicle or ginseng (250 mg/kg) 1 hour prior to tissue collection. Number of animals per group: saline/saline: n = 7, ethanol/saline: n = 6, ethanol/ethanol: n = 6. Data are presented as mean ± SEM fold change compared to the chronic saline treated control group (white bar). * indicates significant compared to chronic saline-treated group.

Table 3.3. Effects of aqueous extract of NA ginseng on penile eNOS and nNOS mRNA in sexually experienced, adult, male Obese Zucker rats. NA ginseng or vehicle (250 mg/kg) was administered chronically for 50 day then rats received a final injection of either saline or NA ginseng 1 hour prior to sacrifice. Number of animals per group: saline/saline: n = 10, aqueous/saline: n = 5, aqueous/aqueous: n = 6. Data are presented as mean ± SEM percent change compared to the chronic saline-treated group. No significant differences between groups were detected.

Chronic Treatment Final Treatment	Saline Saline	Aqueous Saline	Aqueous Aqueous
eNOS	107 ± 14	76 ± 10	87 ± 9
nNOS	106 ± 13	107 ± 4	98 ± 14

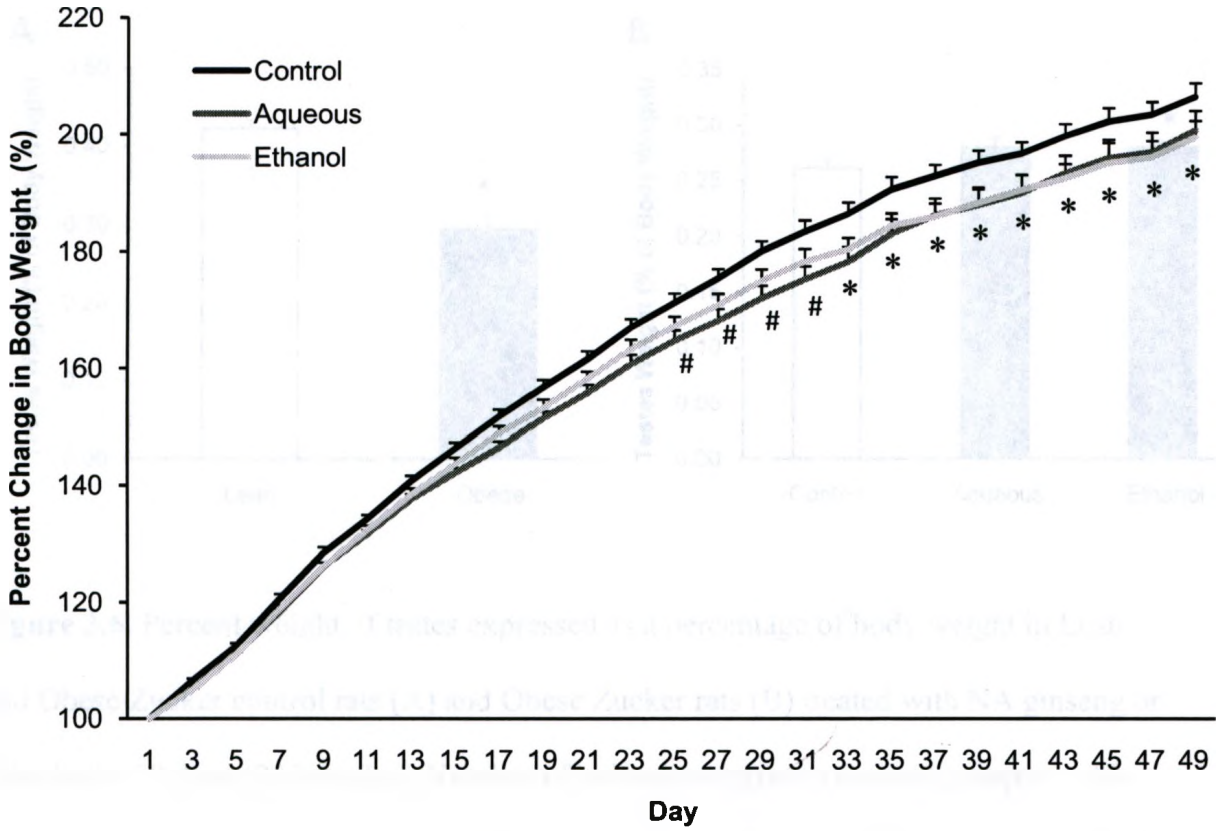

Figure 3.5. Weight of Obese Zucker rats treated with aqueous and ethanol extracts of NA ginseng. Measurements were conducted one to two hours after lights off before daily NA ginseng administration. Number of animals per group: control: n = 12, aqueous: n = 12, ethanol: n = 13. Data are presented as mean ± SEM. * indicates significance from control group within each test and # indicates significance of aqueous treatment from control.

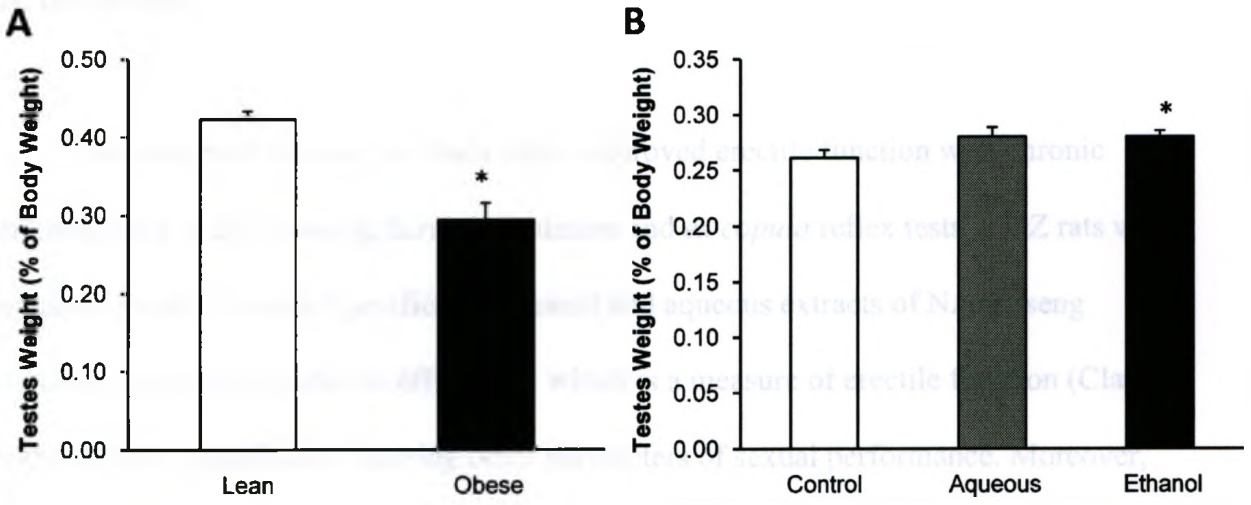

Figure 3.6. Percent weight of testes expressed as a percentage of body weight in Lean and Obese Zucker control rats (A) and Obese Zucker rats (B) treated with NA ginseng or vehicle for 50 days (250 mg/kg). Number of animals per group: Control group: n = 10, Treatment group: control: n = 12, aqueous: n = 12, ethanol: n = 13. Data are presented as mean ± SEM. * indicates significance from control group within each test.

3.4. Discussion

The results of the current study show improved erectile function with chronic administration of NA ginseng during copulation and *ex copula* reflex tests in OZ rats with impaired sexual function. Specifically, ethanol and aqueous extracts of NA ginseng treatment improved copulation efficiency, which is a measure of erectile function (Clark, 1995), without significantly altering other parameters of sexual performance. Moreover, in *ex copula* reflex tests, ethanol extract increased the display of intense erections (cups), which are associated with increased intracavernosal pressure with flaring of the tip of the penile glans (Bernabe *et al.*, 1995). In penile tissue, chronic treatment of ethanol, but not aqueous, extract increased nNOS mRNA while acute treatment increased eNOS mRNA. Finally, both extracts of NA ginseng treatment reduced weight gain after 25-33 days of treatment, which persisted throughout the remainder of the study.

The OZ rat is a well established model of pre-diabetes, and shares similar secondary pathologies as T2DM patients (Kasiske *et al.*, 1992; Hidalgo-Tamola and Chitaley, 2009). These secondary pathologies, such as obesity, insulin resistance, decreased testosterone, and impaired vasodilation, contribute to ED (Edmonds and Withyachumnarnkul, 1980; Withyachumnarnkul and Edmonds, 1982; Doherty *et al.*, 1985; Hemmes and Schoch, 1988). Likewise, male OZ rats display an impaired initiation of sexual behavior (Withyachumnarnkul and Edmonds, 1982), decreased intromission frequency (Doherty *et al.*, 1985), decreased ejaculation latency (Doherty *et al.*, 1985), or were unsuccessful at initiating mating (Young *et al.*, 1986). The current results are in

agreement with these studies. OZ rats were significantly impaired in most parameters of sexual behavior, including initiation of mating and measures of erectile function.

PDE5 inhibitors are currently the main treatment for ED. However, diabetic men have a diminished response to PDE5 inhibitors (Rendell *et al.*, 1999). As such, the decrease in efficacy of PDE5 inhibitors in the diabetic population necessitates alternate treatments. Novel treatments have been tested to improve erectile function using rat models with T2DM (Garcia *et al.*, 2009; Wingard *et al.*, 2009). In particular, Zucker diabetic rats injected with a single dose of adipose-derived stem cells in the penis displayed greater intracavernous pressure (ICP) upon electrostimulation than control rats (Garcia *et al.*, 2009). Also, Rosuvastatin, injected intraperitoneally for three days, improved erectile response to electrostimulation compared to control (Wingard *et al.*, 2009). Although improved erectile function was not observed during mating, these studies provide pharmacological treatment options for restoring erectile function in diabetic populations. The current study demonstrated that NA ginseng can provide an additional pharmacological option for ED treatment, as erectile function during mating as well as in reflex tests was improved.

The current data showed differing effects of NA ginseng extracts on enhancing erectile function in OZ rats. Although both extracts improved copulation efficiency during mating, only ethanol extract improved *ex copula* reflexes. A previous study from our laboratory also detected differing effects of the two extracts and showed that ethanol, but not aqueous, extract improved erectile function during mating behavior in healthy,

young, adult Long Evans rats (Barnes *et al.*, 2011). Other research studies have reported differing effects of ginseng on immunomodulation and cardiovascular effects based on the solvent used to create the extract (Lee *et al.*, 1981; Azike *et al.*, 2011). Ginsenoside profiles differ between extracts due to the solubilities of the ginsenosides in either ethanol or aqueous solutions (Azike *et al.*, 2011). The ethanol extract used in this study has a higher percentage of ginsenoside content compared to the aqueous extract (approximately 28% to 14% dry weight, respectively) (Azike *et al.*, 2011), including slight differences in levels of Rb1. Ginsenoside Rb1 did not prevent male mice copulatory disorder based on measuring latencies and frequencies of sexual performance parameters (Yoshimura *et al.*, 1998); however, effects on erectile function have not been studied and cannot be ruled out. Ginsenoside Rg1 has been shown to improve male sexual performance (Wang *et al.*, 2010) but difference in concentration between extracts did not reach significance, thus, it is unlikely to contribute to pro-erectile effects. The four remaining major ginsenosides of the two extracts used in this study have not been tested for enhancing male sexual function. A final contribution to the differences in effects between extracts may be due to polysaccharides (PS). PS differ been the extracts used in this study; the aqueous extract had detectable levels while the ethanol extract did not (Azike *et al.*, 2011). PS in aqueous extracts are believed to contribute to the pro-inflammatory response that increased NO levels in macrophages while the lack of PS and increased ginsenoside concentrations in ethanol extracts caused an anti-inflammatory response (Azike *et al.*, 2011). As such, the low levels of ginsenosides in the aqueous extract may be partially compensated by its pro-inflammatory response in regards to increasing erectile function while the ethanol

extract increases erectile function through alternate mechanisms possibly involving central and peripheral sites of action (Barnes *et al.*, 2011).

The mechanisms through which the ethanol extract of NA ginseng elicits its effects on erectile function in OZ rats are unknown. A combination of factors contribute to the development of ED in OZ rats, including obesity, impaired vasodilation, and hyperglycemia (Gur *et al.*, 2009; Hidalgo-Tamola and Chitaley, 2009). Villalba *et al.* have shown vascular remodelling of penile arteries in OZ rats alongside increased contractile response to high potassium-saline solutions and decreased endothelium-dependent relaxation to acetylcholine (Villalba *et al.*, 2009). NO levels are reduced in OZ rat arteries concomitant to reduced endothelium-dependent relaxation, which occurred with vascular remodelling (Bouvet *et al.*, 2007). Furthermore, decreased levels of antioxidants and increased production of ROS, which scavenge NO in the vasculature and decrease its bioavailability, are associated with vascular dysfunction in T2DM (Katakam *et al.*, 2005; Hidalgo-Tamola and Chitaley, 2009). Treatment with antioxidants can partially rescue contractile responses in penile arteries of OZ rats (Villalba *et al.*, 2009). In conclusion, the above changes in the vasculature influence the NO/cGMP pathway required for proper erectile function (Rajfer, 2008) and these physiological changes provide mechanisms through which diabetic individuals can develop ED. In agreement with the present results, changes in total penile eNOS have not been detected in OZ rats compared to control (Villalba *et al.*, 2009). Furthermore, our results do not indicate changes in either eNOS or nNOS mRNA in penile tissue of OZ rats compared to LZ rats. Conversely, decreased protein levels of both eNOS and nNOS in penile tissue

were detected in male rats with streptozotocin-induced T2DM (Chiou *et al.*, 2009) or genetic models of T2DM (Jesmin *et al.*, 2003). It appears that changes in eNOS and nNOS mRNA or protein levels may appear with increased severity of T2DM, as in the current study OZ males were pre-diabetic and did not yet require insulin treatment. As such, deregulation of eNOS and nNOS in penile tissue may not yet be established.

The current results demonstrate that ethanol, but not aqueous extract of NA ginseng increased eNOS and nNOS mRNA expression in penile tissues of OZ males. nNOS mRNA content was increased with chronic administration of ethanol extract while eNOS mRNA appears to be increased acutely (one hour). Recent findings from our laboratory show healthy, young, adult Long Evens rats administered ethanol extract of NA ginseng had increased eNOS and nNOS mRNA in penile tissue (Barnes *et al.*, 2011). Interestingly, both eNOS and nNOS mRNA in our previous study were increased only with acute dosing while in this study nNOS is increased with chronic treatment and eNOS by acute treatment. Reasons for the difference can only be speculated. Healthy Long Evans rats may have tighter control of mRNA expression in penile tissue, thus, nNOS mRNA content is rapidly brought to homeostatic levels, whereas in pre-diabetic models, OZ rats may not possess such control. Furthermore, induction of mRNA, such as nNOS, persists over a longer period. Indeed, mRNA levels of genes associated with cardiovascular regulation have been shown to be increased or decreased in diabetic models and may be deregulated compared to healthy controls (Banz *et al.*, 2007; Keller and Attie, 2010).

The mechanism through which eNOS or nNOS mRNA is increased is unknown. Rb1, which is present in higher concentrations in the ethanol extract, and its metabolite (compound K) have both been shown to increase eNOS phosphorylation and activity (Yu *et al.*, 2007; Tsutsumi *et al.*, 2011). As such, Rb1 may enhance the NO/cGMP pathway in penile vasculature, which is required for reversal of ED in diabetes (Angulo *et al.*, 2005). In addition, ginsenoside Rd, among many other ginsenosides, has been shown to increase antioxidant enzymes and decrease oxidative stress (Yokozawa *et al.*, 2004; Ye *et al.*, 2011). As such, Rd may protect the vasculature from the oxidative stress that has been linked to ED in diabetes (Hidalgo-Tamola and Chitaley, 2009). In conclusion, it is possible that NA ginseng treatment caused enhanced vasodilation and protection from oxidative stress, resulting in partial recovery of erectile function in OZ rats.

However, improvement of erectile function during mating behavior appeared to occur independent of changes in eNOS or nNOS, since aqueous extract improved sexual behavior without affecting levels of NOS. Hence, other factors must contribute to the facilitative actions of NA ginseng on erectile function. Such factors do not appear to involve increased production of testosterone. Circulating testosterone levels have been shown to be decreased in OZ rats (Whitaker *et al.*, 1983). However, even though the testicular weight, as a percent of total body weight, was increased with ethanol extract treatment (indicating a potential increase in plasma testosterone levels), plasma testosterone was not significantly affected by NA ginseng treatment. This is in agreement with a study by Murphy and colleagues testing NA ginseng on male sexual behavior; no effect of NA ginseng on plasma testosterone levels was measured (Murphy *et al.*, 1998).

Hence, it is unlikely that NA ginseng improves sexual function by increasing testosterone production. Other factors that may have contributed to the action of NA ginseng in OZ rats include actions on the central nervous system (Barnes et al, 2011) and future studies are needed to test that hypothesis.

In conclusion, this study provides evidence for using the ethanol extract of NA ginseng to partially rescue erectile function in pre-diabetes. The ethanol extract was able to moderately enhance erectile function during mating and in an *ex copula* reflex test. As such, NA ginseng may provide an additional pharmacological treatment for men with diabetic complications that experience ED.

3.5. References

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Chapter 4

4.1. Discussion:

The results of these studies provide evidence of the pro-erectile effects of NA ginseng in healthy and pre-diabetic male rats. Previous studies have shown that Asian ginseng and ginsenoside Rg1 treatment have pro-erectile properties by acting on the NO/cGMP pathway (Chen and Lee, 1995; Wang *et al.*, 2010); however effects of NA ginseng on erectile function have not been investigated. One goal of this study was to characterize two common extracts of NA ginseng. Indeed, different effects were found; the ethanol extract enhances erectile function while the aqueous extract provided little to no effect. An additional goal was to determine potential mechanisms through which NA ginseng produces pro-erectile effects. We have shown a central action of NA ginseng as indicated by an increase of nNOS in the PVN (Chapter 2) as well as a peripheral action as indicated by increasing eNOS and nNOS mRNA in penile tissue in healthy (Chapter 2) and pre-diabetic male rats (Chapter 3).

Chapter 2 revealed increased eNOS and nNOS mRNA one hour after injection but no effect of chronic treatment. In contrast, increased nNOS occurred in chronically treated pre-diabetic rats while eNOS was dependent on final injection, similar to the healthy rats (Chapter 3). The reasons for the differences in mRNA content increases between healthy and pre-diabetic rats can only be speculated since neither the active constituent nor the receptor activating gene transcription is known. Transcription deregulation has been shown in diabetic populations for genes of transcription factors

from overexposure to cytotoxic fatty acids (Johnstone *et al.*, 2011) and cellular messengers involved in gene transcription for inflammatory responses (Siqueira *et al.*, 2010). As such, the induction of mRNA transcription of nNOS in healthy rats may be quickly regulated to homeostatic levels within 24 hours whereas in pre-diabetic rats, the increase in nNOS is not as tightly regulated so chronic ethanol extract administration increases nNOS mRNA levels, which remain high over 24 hours.

The current study has limitations when explaining the ability of NA ginseng to enhance erectile function. Firstly, this study only measured levels of eNOS and nNOS mRNA content in penile tissue. These levels may not translate into increased protein content. Furthermore, the protein content may not correlate to NO activity and, as such, further studies are needed to determine NO activity in penile and brain tissue of NA ginseng-treated rats. In addition, a time course for NA ginseng's effects at improving erectile function and NO modulation are required; however, these objectives were outside of the scope of the current study. Finally, pathways through which NA ginseng produces effects on sexual performance need to be discovered to calculate appropriate dosing.

Although effects were detected on the NO/cGMP pathway in penile tissue and on nNOS expression in the PVN, a disconnect exists between these effects and the enhancement of erectile function. NA ginseng was able to increase eNOS and nNOS mRNA content in the penis and nNOS immunoreactivity in the PVN after one hour of dosing without any effects of chronic treatment. Conversely, NA ginseng was able to increase CE with chronic treatment where dosing did not occur for 24 hours before

testing. As such, although NO activity may contribute to some parameters of sexual behavior, other mechanisms must exist.

Alternate mechanisms through which NA ginseng may be producing its effects include activating other mediators of erection in supraspinal regions such as dopamine and influencing spinal cord modulation. Ginseng has already been shown to modulate dopamine release when administered with drugs of abuse (Shim *et al.*, 2000; Kim *et al.*, 2006; Lee *et al.*, 2008). Dopamine has a pro-erectile effect on brain regions including the PVN (Baskerville *et al.*, 2009). Furthermore, increased dopamine has been indirectly attributed to improvements of male sexual behavior in NA ginseng-treated rats (Murphy *et al.*, 1998) and effects in this current study may be occur due to influences on the dopamine system. In addition, although NA ginseng has not been shown to affect spinal control of erections, it cannot be ruled out as a possible site of action. The spinal cord directly controls erectile response in males (McKenna, 2000) so any influence on the erectile pathway may enhance erectile response.

Since increased eNOS or nNOS mRNA or protein occurred with NA ginseng injections one hour prior to measurements but did not translate to increased *in copula* performance in healthy rats, it is possible that small improvements of erectile mediators can affect *ex copula* reflexes but are insufficient to influence mating for which much more erectile improvement is required. Indeed, *in copula* erectile function is mediated by a complex interplay of neurochemical and hormonal inputs (Pfaus *et al.*, 2001). Future studies can investigate telemetry of the cavernosal bodies during copulation to determine

if penile rigidity is increased during mating even if measures of sexual behavior do not indicate increased erectile function. In addition, tests of telemetry of the corpus cavernosum in anesthetised rats would help determine if acute (and chronic) NA ginseng administration increases intracavernous pressure during electrostimulation. These experiments would help determine the contribution of the NO/cGMP pathway in NA ginseng's pro-erectile effects.

Ginsenosides, regardless of their very low oral bioavailability, are detected in the blood of canines after oral administration 10 minutes after injection (Song *et al.*, 2010). In addition, although ginsenosides are not NO donors, they cause an increase in corpus cavernosum relaxation and cGMP levels with 10 minutes of exposure (Chen and Lee, 1995; Murphy and Lee, 2002). As such, the rapid effects of ethanol extract on increased mRNA content after administration seem to be physiologically possible within one hour of injection.

The ginsenoside(s) responsible for the increase in erectile function and cellular mediators of erectile function with acute NA ginseng treatment must be rapidly absorbed and reach concentrations necessary to produce their effects. Ginsenosides are divided into two groups based on their structures: 20(*S*)-protopanaxadiol (ppd) ginsenosides have sugar moieties at either C-3, C-20, or both positions while 20(S)-protopanaxatriol (ppt) ginsenosides have a hydroxyl group at C-3 and sugar moieties at C-6, C-20, or both (Attele *et al.*, 1999). Ppd-ginsenosides include Rb1 and Rd while ppt-ginsenosides include Rg1 and Re (Gillis, 1997). Notably, the ethanol extract used in this study has

increased levels of Rb1 and Rd. Intestinal absorption of ginsenosides occurs largely via passive diffusion and, as such, ppd-ginsenosides have greater intestinal absorption and systemic exposure due to their smaller molecular size and lipid solubility (Liu *et al.*, 2009). Also, ppd-ginsenosides have a much greater half-life compared to ppt-ginsenosides (13-18 hours compared to <4 hours, respectively) and were detectible in plasma 48 hours after oral administration (Liu *et al.*, 2009). Even though the time to maximal plasma concentration for ppd-ginsenosides does not occur until 6-10 hours after oral injection, they provide the best targets for increasing erectile function after 30 minutes of dosing (with naïve pre-diabetic rats and *ex copula* tests) and cellular mediators after one hour of dosing. In addition, given their long half-life they also provide the best candidates for producing chronic effects of increased *in copula* erectile function in healthy and pre-diabetic rats (after sexual experience). Furthermore, since both ppd-ginsenosides Rb1 and Rd differ between ethanol and aqueous extracts and are part of the six ginsenosides that constitute over 70% of the ginsenoside content of NA ginseng (Lim *et al.*, 2005), they provide the best candidates for producing the pro-erectile effects seen in the ethanol extract.

The metabolite of ginsenoside Rb1, Compound K, which is the main form absorbed after intestinal bacteria metabolism, has been shown to phosphorylate and active eNOS *in vivo* (Tsutsumi *et al.*, 2011). In penile tissue, increased eNOS activity would lead to increased production of cGMP through increased NO levels, which would contribute to erection induction. In addition, ginsenoside Rb1 has anti-diabetic and insulin-sensitizing effects; Rb1 stimulated glucose transport by promoting the

translocation of GLUT1 and GLUT 4 (Shang *et al.*, 2007; Shang *et al.*, 2008). Furthermore, given the long half-life of Rb1, any improvement in diabetic symptoms could cause long-term improvements that reduce the intensity of secondary pathologies of diabetes and, alongside increased in eNOS phosphorylation, it may contribute to pro-erectile effects. In addition, ginsenoside Rd which is also a ppd-ginsenoside, increases endogenous oxidative defences in senescence mice by improving the glutathione/glutathione disulphide ratio and decreasing markers of lipid peroxidation (Yokozawa *et al.*, 2004). As such, with increased endogenous scavenging for ROS, more NO is free to act on penile smooth muscle cells to induce penile erections with ginsenoside Rd treatment (Hidalgo-Tamola and Chitaley, 2009).

In regards to ppt-ginsenosides, ginsenoside Rg1 may be able to further increase NO production which it has been shown in bovine aortic endothelial cells (Kim *et al.*, 1992) and in penile tissue alongside increased measures of sexual performance (Wang *et al.*, 2010). The increase in NO production would not be scavenged by ROS due to the antioxidant effects of ginsenosides Rd and Rb1. One final consideration for the differences between extracts, the ethanol extract may provide the minimum effective dose (MED) required for pro-erectile effects given that its ginsenoside concentration is greater (28%) than the aqueous extract (14%). If multiple ginsenosides are able to meet the MED required then synergistic, pro-erectile effects of NA ginseng can contribute to the improvements of erectile function *in copula* and during reflex tests seen in this study.

Ginseng extracts have been shown to improve self-reported measures of erectile function in humans and improve erection rigidity while men were exposed to audio and visual stimulation (Hong *et al.*, 2002; Jang *et al.*, 2008). However, NA ginseng has not been tested in human populations. The results of this study indicate a potential for ethanol extract of NA ginseng to improve erectile function in healthy and diabetic human populations with ED. Given that a greater proportion of diabetic men do not report positive responses to PDE5 inhibitors (Rendell *et al.*, 1999), NA ginseng may provide another pharmacological treatment option and is well tolerated with reports of a few mild side effects (Gillis, 1997). A combination therapy of NA ginseng with PDE5 inhibitors may provide addition benefits over PDE5 inhibitors alone. In summary, NA ginseng can increase endogenous production of NO and therefore cGMP content while PDE5 inhibitors prevent the degradation of cGMP. As such, cGMP can activate the cellular mediators needed to initiate an erection.

In conclusion, the results of this study demonstrated the effects of two NA ginseng extracts on erectile function and sexual behavior in healthy and pre-diabetic rats with ED. Moreover, ethanol extract of NA ginseng was successful at increasing erectile function *in copula* in healthy rats with both central (PVN) and peripheral (penile tissue) sites of action. In pre-diabetic rats, ethanol extract increased erectile function *in copula* as well as penile reflexes with a peripheral site of action (penile tissue), although central sites of actions were not investigated. In summary, ethanol extract of NA ginseng can be used for enhancing erectile function in healthy or pre-diabetic male rats and future tests may show a translation to increased erectile function in humans.

4.2. References

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Ethics Approval

April 8, 2010

This is the Original Approval for this protocol

Dear Dr. Coolen:

Your Animal Use Protocol form entitled:
Effects of Ginseng on Sexual Function
Funding Agency Ontario Research Fund -R3908A08

has been approved by the University Council on Animal Care. This approval is valid from **April 8, 2010 to April 30, 2011**. The protocol number for this project is **2010-217 which replaces 2006-049 which has expired.**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received. If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - L. Coolen, W. Lagerwerf