Effect of Neuromedin S (NMS) on ghrelin suppressed testosterone secretion in adult male rhesus monkey

QUAID-I-AZAM UNIVERSITY
ISLAMABAD

By

Hussain Ahmed

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2011

Effect of Neuromedin S (NMS) on ghrelin suppressed testosterone secretion in adult male rhesus monkey

A thesis submitted in partial fulfillment of the requirements

for the degree of

MASTER OF PHILOSOPHY

IN

REPRODUCTIVE PHYSIOLOGY

By

Hussain Ahmed

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2011

Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is own composition. No part of this thesis has been previously presented for any other degree.

Hussain Ahmed

بسم الله الرحمن الرحيم

IN THE NAME OF ALLAH

THE MOST MERCIFUL

THE MOST BENEFICENT

AND

THE MOST COMPASSIONATE

DEDICATED TO :

My Loving caring family

Teachers and Friends

CERTIFICATE

This thesis submitted by Hussain Ahmed is accepted in its present form by the Department of Animal Sciences as satisfying the thesis requirement for the degree of Master of Philosophy in Reproductive Physiology.

Supervisor
Ass. Prof. Dr. Sarwat Jahan

External Examiners:
Dr. Syed Murtaza Hassan Andrabi

Chairperson
Prof. Dr. Muhammad Shahab

Date: 27-09-2011

Table of Contents

ABBREVIATIONS

ACTH	ADRENO CORTICOTROPIC HORMONE
AP	ANTERIOR PITUITARY
ARC	ARCUATE NUCLEI
CNS	CENTRAL NERVOUS SYSTEM
CRH	CORTICOTROPIN RELEASING HORMONE
GABA	GAMMA AMINOBUTYRIC ACID
GnRH	GONADOTROPIN RELEASING HORMONE
GPCRs	G PROTEIN COUPLED RECEPTORS
GHS-R	GROWTH HORMONE SECRETOGOGUE RECEPTOR
HPA	HYPOTHALAMIC PITUITARY ADRENAL AXIS
HPG	HYPOTHALAMIC PITUITARY GONADAL AXIS
im	INTRAMUSCULAR
iv	INTRAVENOUS
icv	INTRA CEREBROVENTRICULAR
LH	LUTEINIZING HORMONE
NMS	NEUROMEDIN S
NMU	NEUROMEDIN U
NMU1R	NEROMEDIN U RECEPTOR -1

List of Tables

List of Figures

Acknowledgements

Praise is to Allah, Lord of the Worlds. The Most Beneficent, the Most Merciful, Who is the entire source of knowledge and wisdom endowed to mankind; Who gave me courage and potential to pursue this goal Whom I believe that He never spoils any effort of good deeds. Blessings of Allah be upon His Prophet Muhammad (PBUH)", the city of knowledge and blessing for entire creature, who has guided his Ummah to seek knowledge from Cradle to Grave, and enabled me to win honor of life.

It is a matter of great pleasure to express my sincere regards to my honorable supervisor Assistant Prof. Dr. Sarwat Jahan, Department of Animal Sciences for affectionate supervision, inspiring attitude, masterly advice and encouragement. Without her useful intellectual suggestions, it would have been impossible for me to complete this tedious work.

I would like to extend my thanks to Dr. Muhammad Shahab Chairman Department of Animal Sciences, for providing me all the privileges during my research work.

I wish to extend my greatest appreciation gratitude and thanks to Mr. Shakeel Ahmed Senoir lab fellow, for his affectionate efforts, guidance, support, encouragement and patience. He has been my continuous support and sourse of inspiration through out my research work.

I must acknowledge my debt to my lab fellows, Sana Baba, Satwat Malik, Beenish Ishtiaq, Sanjay Kumar, Huma irah, Asi a, Mehreen Khan, Ambreen Taj, Shumaila Gul, Sadaf Khan, Col.ZAB, Mahwish, Adila, Saadia for their kind help and cooperation during my research work. I am also thankful to them for their nice company and time they provided me with beautiful memories that I will treasure through out my life.

I am realy thankful to my senior lab fellows Ayesha Ambreen, Hizbullah, Kamran, Riffat Gilani Mam Naheed and Dr.Latafat Amin Khan for their valueable help motivation and suggestion.

I am obliged to Mr.Naeem Masih, M. Saeed, Lab Assistant and SamiUllah for their help and support and motivation during my research.

I express my special thanks to my friends Wasim Akram, Naqeeb Ullah, Ali Raza Shah, Dr.Fazal Wahab (KMU),Dr.Naseer Khattak, Zahid Ali Shah, Javaid Khan, Nadim Khan, *Habib Khan, Sher Ali Wazir, Sher Ali Kakar, Naseeb, Farhad, Rauf Lakhan, Qaiser Khan, Haris, Captain Irfaan, M. Farooq, Wasim Akhter, ACP, Asif Khan, Safdar Khan, Liaq Khan (NDU), Adeel Butt,Naumaan Ali khan, Azam jaan Afridi and Pir Rafiq, whom I will ever remember for their continued encouragement, moral support and cooperation during the critical moments.*

I cant forget fabulous company and robust attitude of my friends especially Mr.Shakeel Ahmed (Sheky), Wasim Akram, Naqeeb kakar, Ali Raza and Dr.Latafat Amin Khan.

Special thanks to my M.Sc Class fellows Faiza Younus, Sumera Awais, Shabana Nazeer Sadaf Khan, Saadia Qayyum and Rabia Anjum for their best wishes and regards.

Very special thanks to dearest sincere loving and bosom friend Ubaid-ur-Rahman for his love, support, care, patience, guidance and who blessed me with unforgetable memories and contributed a lot to the completion of my thesis.

No words can express my thanks to my loving parents and my brothers Hamad Ahmad, Talal Ahmad Bashir Ahmad (LRH) and Engineer Shabir Ahmad and Sisters whom love, affection, prayers, care and support helped me not only during my studies but throughout my life and other family members especially my cousins Wahab (ACCA),Razzaq and A.Q Khan whom words are meaningless for what they have given to me.

A non-payable debt to my loving parents, their wish motivated me in striving for higher education; they prayed for me, shared the burden and made sure that I sailed through smoothly.

Last but not the least I pay gratitude to my very cute brothers Hamad, Talal and sister Sarwat sher, I really miss their company and they mine company during my study for their nice company while at home and through messaging while at hostel for their best wishes and best regards.

In the end I am thankful to all those who helped me.

Hussain Ahmed

Abstract

Neuromedin S (NMS), a 36 amino acid peptide, identified in rat brain as ligand for the G protein-coupled receptor FM4/TGR-1, also termed neuromedin U receptor type-2 (NMU2R). Its central expression is restricted to the suprachiasmatic nucleus and involved in the regulation of dark light rhythms and suppression of food intake. Ghrelin on the other hand is a 28 amino acid peptide produced in the stomach and has a stimulatory role in food intake and energy homeostasis. Stimulatory role of NMS and inhibitory role of ghrelin on hypothalamic pituitary gonadal axis (HPG) is reported in rodents. The potential contribution of these two peptides in the control of reproductive axis in higher primates remains unexplored. In the present study the stimulatory role of NMS was investigated on ghrelin suppressed testosterone secretion in adult male rhesus monkeys. Four adult male rhesus monkeys were used in this study. Fifty nmol of NMS and 2μg/Kg ghrelin were injected through a teflon cannula implanted in saphenous vein. Blood samples were collected individually for NMS and ghrelin 60 min before and 120 min after NMS and ghrelin administration at 15 min intervals. To study the effect of NMS on ghrelin suppressed plasma testosterone secretion samples were collected 45 min before the administration of ghrelin, then NMS was administered after 60 min of ghrelin injection and samples were collected for 120 min after NMS injection. The plasma testosterone concentrations were determined by using specific Enzyme Immunoassay (EIA). Ghrelin significantly ($P<0.05$) decreased plasma testosterone secretion after 45 min and levels remained low till 60 min. NMS blocked this decline caused by ghrelin and further stimulated ($P<0.001$) plasma testosterone secretion from 30 to 60 min after its administration. In conclusion the present study suggests that NMS has an ability to restore the inhibitory effect of ghrelin on testosterone secretion and further stimulated testosterone secretion in adult male rhesus monkey. This response might be regulated through HPG axis however further study is recommended to understand the exact mechanism of action of these two peptides in regulation of reproductive behaviour in primates.

INTRODUCTION

In Mammals, gonadal functions critically rely on a complex regulatory network of systemic (endocrine) and locally-produced (paracrine and autocrine) signals. Although it has been known that conditions of negative energy balance are frequently linked to lack of puberty onset and reproductive failure, only recently the mechanisms involved in the coupling of reproductive function and body energy stores have been partially elucidated (Fernandez-Fernandez *et al.*, 2004). Central and peripheral endocrine signals primarily involved in the control of energy balance and metabolism, control reproductive function by acting at different levels of hypothalamic pituitary–gonadal axis, thus providing the basis for the link between energy homeostasis and fertility (Tena-Sempere *et al.*, 2002; Fernandez-Fernandez *et al.*, *2005).*

Neuromedins

The neuromedins are an extremely versatile group of neuropeptides, belong to the tachykinin family. Tachykinin peptides are one of the largest family of neuropeptides, found from amphibians to mammals (Helke *et al.*, 1990). The first members of neuromedins were described in the porcine central nervous system (CNS) (Minamino *et al.*, 1983) and were mainly designated on the basis of their receptor preference (K: kassinine-like, B: bombesin-like, N: neurotensin- like, etc.). These neuropeptides are abundantly expressed in those CNS structures which regulate endocrine, behavioral and autonomic processes (Minamino *et al.*, 1985). Neuromedin B and C appear to be especially important in the regulation of behavioral endocrine and autonomic processes (Ohki-Hamazaki, 2000). Neuromedin N has a well-established effect on the hypothalamic– pituitary–adrenal (HPA) axis (Malendowicz *et al.*, 1993) and displays marked action on thermoregulation (Dubuc *et al.*, 1988).

Neuromedin U (NMU) was first described as a potent smooth muscle stimulating peptide (Minamino *et al.*, 1985). Neuromedin U originally isolated from porcine spinal cord, is a brain–gut peptide that has potent contractile activity on uterine smooth muscle (Minamino *et al.*, 1985). The peripheral activities of NMU include smooth muscle contraction, blood pressure elevation and modification of intestinal ion transport, whereas centrally, NMU suppresses feeding and induces the release of stress-mediating molecules such as adrenocorticotropic hormone and corticosterone (Minamino *et al.*, 1985; Hanada *et al.*, 2001). However, its profound neural expression (Honzawa *et al.*, 1987) raised the possibility that, like other neuromedins, it may play a significant role in central regulation. Neuromedin U has proved to exert considerable effect on thermoregulation, feeding (Nakazato *et al.*, 2000) and circulation (Chu *et al.*, 2002) and to activate the HPA axis after either peripheral (Malendowicz *et al.*, 1994) or central administration (Wren *et al.*, 2002). Further studies have reinforced the involvement of the corticotrophin releasing hormone (CRH) neurons in the central processing of neuromedin U-evoked phenomena. HPA activation, behavioral activation and the inhibition of gastric acid secretion all appear to be CRH-dependent (Wren *et al.*, 2002; Hanada *et al.*, 2003; Mondal *et al.*, 2003).

Neuromedin S

Neuromedin S is a member of this peptide family (Mori *et al.*, 2005) which has closely resemblance with Neuromedin U (Minamino *et al.*, 1985). This nomenclature stems from the fact that this neuropeptide is highly expressed in the suprachiasmatic nucleus of the hypothalamus. Although neuromedin S shares C-terminal structures with neuromedin U, the N-terminal portion has no sequence homology to other known peptides, and these two neuromedins are coded by two different genes (Mori *et al.*, 2005). Furthermore, neuromedin U and S have been demonstrated to share their receptors, FM-3/GPR66 and FM-4/TGR-1. On the other hand, while they exhibit similar affinity to FM-3/GPR66, which may be responsible for the similarities between their effects, neuromedin S binds with higher affinity to FM-4/TGR-1 (Mori *et al.*, 2005), the receptor which may mediate the genuine physiological actions of this neuropeptide. The FM-4/TGR-1 receptor is confined almost only to the CNS and its expression is highest in the hypothalamus, especially in the pararaventricular (Guan *et al.*, 2001) and suprachiasmatic nuclei (Nakahara *et al.*, 2004). The paraventricular expression argues for a putative role of the receptor in the regulation of the HPA axis and feeding, while the suprachiasmatic receptors may govern the sleep/wake cycle and the circadian rhythm of temperature, motor phenomena and hypothalamic hormone (e.g. gonadotropin hormone releasing hormone and CRH) secretion. Outside the hypothalamus, the receptor is found in highest abundance in the hippocampus, the amygdala, the thalamus and the cerebellum, which suggests its putative role in thermoregulation of behavior, emotions and motor phenomena (Raddatz *et al.*, 2000). Moreover, the distribution (abundant hypothalamic expression, mainly in the suprachiasmatic, paraventricular and arcuate nuclei) of neuromedin S itself raises the possibility that, similarly to other neuromedins (and especially neuromedin U), it may play a role in the regulation of hypothalamic functions (Mori *et al.*, 2005). It has been demonstrated that neuromedin S expression is markedly higher than that of neuromedin U in the hypothalamus (Rucinski *et al.*, 2007), which suggests that Neuromedin S is predominant in central regulatory processes. Neuromedin S has been demonstrated to influence the circadian rhythm (Mori *et al.*, 2005), feeding (Ida *et al.*, 2005; Shousha *et al.*, 2006) and pituitary Luteinizing hormone secretion (Vigo *et al.*, 2006). The activation of paraventricular CRH secretion and pro-opiomelanocortin (POMC) release from the arcuate nucleus appear to play crucial roles (Ida *et al.*, 2005). The CRH-related endocrine (HPA activation), autonomic (temperature) and behavioral (anxiety-related motor phenomena) processes may also be influenced by neuromedin S. It has been determine that CRH (Monnikes *et al.*, 1992; Menzaghi *et al.*, 1994) and dopamine (Majovski *et al.*, 1981) play especially important roles in the mediation of behavior. The CRH is the central regulator of HPA activation (Vale *et al.*, 1981), anxiety (Skutella *et al.*, 1994), stress-related motor paradigms (Monnikes *et al.*, 1992; Menzaghi *et al.*, 1994) and neuromedin S evoked hypophagia (Ida *et al.*, 2005).

The SCN is the site of the master circadian pacemaker in mammals and important for the regulation of energy balance (Reppert *et al.*, 2001). NMS was suggested to be involved in circadian oscillation systems and feeding regulation (Ida *et al.*, 2005). The tissue distribution of NMS mRNA in rats was investigated using quantitative RT-PCR. The expression of NMS mRNA was mainly found in the central nervous system, spleen and testis. In the brain, NMS mRNA was expressed predominantly in the SCN, with only very slight expression in other regions (Fujii *et al.*, 2000). In situ hybridization histochemistry, as well as RT-PCR analysis, showed that the NMS mRNA expression was restricted to the SCN in rat brain (Mori *et al.*, 2005). The SCN is divided into the ventrolateral portion, where the neuropeptide vasoactive intestinal polypeptide (VIP) is expressed, and the dorsomedial portion, where the neuropeptide arginine vasopressin is expressed (Moore *et al.*, 2002). NMS mRNA was expressed in the ventrolateral SCN, in a similar manner to VIP mRNA (Mori *et al.*, 2005). The SCN is involved in the organization of daily metabolic activity and the regulation of energy balance (Kreier *et al.*, 2003). Because NMU is an anorexigenic neuropeptide involved in the central regulation of feeding behavior, NMS also play a significant role in feeding regulation (Wren *et al.*, 2002). Icv (intracerebroventricular) injection of NMS reduced 12- hr food intake during the dark period in a dose-dependent manner. Icv injection of 3 nmol NMS and NMU into rats resulted in a significant decrease in 12-h food intake. On the other hand, at doses of 0.5 nmol and 1 nmol, only NMS injection suppressed food intake (Miyazato., 2008). Icv administration of NMS augmented the levels of POMC mRNA in the Arcuate nucleus (ARC) and CRH mRNA in the paraventricular nucleus (PVN), and induced c-Fos expression in POMC neurons in the ARC (Miyazato *et al.*, 2008). Pretreatment with both SHU9119 (an antagonist for α-melanocyte stimulating hormone (α-MSH)) and α-hCRF (an antagonist for CRH) attenuated NMS-induced suppression of food intake in a dose-dependent manner in fasted rats (Ida *et al.*, 2005). These results suggest that α-MSH in the ARC and CRH in the PVN are involved in NMS action on feeding.

Ghrelin

Ghrelin is a 28 amino-acid peptide (Yang *et al.*, 2008), characterized as the endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) (Howard *et al.*, 1996). Ghrelin got its name from the word 'ghre' from the proto-indo-European language, meaning to grow and 'relin' as it had GH-releasing activities. It is predominantly produced in the stomach by the X/Alike cells with in the oxyntic glands of the gastric fundus mucosa (Sakata *et al.*, 2002). It is an orexigenic peptide and a long-term regulator of energy homeostasis (Bluet-Pajot *et al.*, 2005). Ghrelin homologs have been identified in a number of different species, including human, rhesus monkey, rat, mouse, pig, gerbil, chicken, bullfrog, eel, sheep, goldfish and tilapia (Parhar *et al.*, 2003).

The biological actions of ghrelin are conducted through interaction with its specific cell surface receptor, namely the GHS-R. The cognate ghrelin receptor belongs to the large family of G protein-coupled, seven-transmembrane domain receptors (McKee *et al.*, 1997). Ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken and pufferfish. This strict conservation suggests that ghrelin and its receptor serve important physiological functions (Palyha *et al.*, 2000). Ghrelin acts on the GHS-R and activates phospholipase C to generate inositol triphosphate (IP3) and diacylglycerol (DAG), resulting in an increase of intracellular Ca2+, indicating that the ghrelin receptor is coupled to a Gq subunit (Malagon *et al.*, 2003).

Ghrelin receptor has a wide spread distribution in various tissues, suggesting multiple paracrine, autocrine and endocrine roles for ghrelin. GHS-R1a is highly expressed in the hypothalamus and pituitary, this is consistent with ghrelin's observed action on the pituitary and its role in the control of appetite, food intake and energy balances (Howard *et al.,* 1996). Within the hypothalamus, immunostaining studies detect ghrelin's expression in the internuclear spaces between the lateral hypothalamus, the arcuate nucleus (ARC), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN), and the ependymal layer of the third ventricle (Cowley *et al.*, 2003). Interestingly, GHS-R1a expression has also been reported in areas of the CNS that affect biological rhythms, mood, cognition, memory and learning, such as the hippocampus, pars compacta of the substancia nigra, ventral tegmental area, dorsal and medial raphe nuclei (Guan *et al.*, 1997).

Ghrelin and GHSR-1a expression were also detected in reproductive tissues including placenta, ovary and testis (Tena-sempere *et al.*, 2002). Within testis expression of ghrelin gene and protein has been reported in interstitial rat Leydig cells (Barreiro *et al.*, 2002). However, a specific feature of testicular expression of ghrelin in the human is the presence of this peptide, albeit at low levels, in Sertoli cells (Gaytan *et al.*, 2004). Ghrelin and GHSR-1a expression were also detected in reproductive tissues including placenta, ovary and testis (Tena-sempere *et al.*, 2002).

Peripherally produced ghrelin has been shown to cross the blood-brain barrier. Banks *et al.*, (2002) identified a saturable system transporting human ghrelin from brain-to-blood and from blood-to-brain. ghrelin possesses a strong and dose-dependent GH-releasing effect, both in vitro and in vivo in humans and rats. Intravenrticular (iv) and Intracerebroventricular (icv) injection of ghrelin induces potent GH release. This effect seems to result from ghrelin binding to GHSR-1a on somatotrophic cells at the pituitary as peripherally produced ghrelin has been shown to cross the blood-brain barrier (Kojima *et al.*, 1999; Malagon *et al.*, 2003). Ghrelin is expressed in hypothalamic neurons that are adjacent to the third ventricle between the dorsal, ventral, PVN and ARC hypothalamic nuclei. In the ARC nucleus these ghrelin-containing neurons send efferent fibers onto NPY- and AgRP-expressing neurons to stimulate the release of these orexigenic peptides and onto POMC neurons to suppress the release of this anorexigenic peptide. In this way ghrelin stimulate food intake (Shintani *et al.*, 2001; Cowley *et al.*, 2003). Ghrelin has been reported to either inhibit or stimulate insulin secretion in animals and humans (Wang *et al.*, 2008).

Expression of ghrelin and its receptor in various reproductive organs, such as placenta, testis, Leydig cells, rat ovary, mouse embryo and endometrium suggests that ghrelin may play role in the regulation of reproductive function at least partially, in a paracrine or autocrine manner (Caminos *et al.*, 2003). In the rat testis, ghrelin expression was selectively detected in Leydig cells at advanced stages of maturation, regardless of their fetal or adult origin and is under the hormonal control of pituitary LH, similarly in humans its expression is evident in interstitial mature Leydig cells of testis. However, in the human this peptide, albeit at low levels, is present also in Sertoli cells (Gaytan *et al.*, 2004). Ghrelin expression in the testis is, partially, under the control of pituitary LH. This is in good agreement with the fact that testicular LH/hCG receptors are expressed in Leydig cells (Tena-Sempere *et al.*, 2002). In the ovary, expression of ghrelin was demonstrated in steroidogenically active luteal cells and interstitial hilus cells. Likewise, expression of GHS-R type 1a was demonstrated in Sertoli and Leydig cells of the testis and follicular, Luteal and interstitial hilus cells in the ovary (Gaytan *et al.*, 2003).

Ghrelin was initially identified (and named) by virtue of its ability to elicit GH secretion (Kojima *et al.*, 1999), compelling evidence has demonstrated that the biological actions of ghrelin are much more diverse than those originally described, and include endocrine and non-endocrine effects. Detailed reviews of the potential biological roles of ghrelin have been recently published elsewhere (Korbonits *et al.*, 2004; van der Lely *et al.*, 2004). Interestingly, ghrelin was shown to be a potent orexigenic signal, acting at the hypothalamus (Korbonits *et al.*, 2004; van der Lely *et al.*, 2004).

Ghrelin has been recently postulated to be a peripheral signal for energy insufficiency, which may play a major role in the long-term control of body weight (Zigman and Elmquist, 2003). However, the effects of ghrelin on reproduction are not restricted to its expression and/or actions in the gonads and placenta. Ghrelin has also direct actions on the brain and the pituitary. Thus, central administration of ghrelin suppressed pulsatile LH secretion in ovariectomized female rats (Furuta *et al.*, 2001). A similar finding has been reported recently in the rhesus monkey (Vulliemoz *et al.*, 2004) and sheep (Iqbal *et al.*, 2006).

It has been shown that ghrelin inhibits LH secretion in vivo in prepubertal male rats, adult males and cyclic females, as well as in gonadectomized animals (Fernandez-Fernandez *et al.*, 2004).The inhibitory effects of ghrelin upon LH secretion are mimicked by the un-acylated form of the molecule (Martini *et al.*, 2006), previously regarded as biologically inert. In addition, ghrelin decreases hypothalamic GnRH release and LH responsiveness to GnRH in vitro (Fernandez-Fernandez *et al.*, 2006).

Central injections of ghrelin resulted in a significant reduction in LH levels in rats, while FSH levels remain unaltered in prepubertal male rats and gonadectomized male and female rats (Furuta *et al.*, 2001; Fernandez-Fernandez *et al.*, 2004). Whereas acute administration of ghrelin inhibits FSH and LH secretion in humans, while chronic infusion was found effective in reducing LH pulsatility (Lanfranco *et al.*, 2008; Kluge *et al.*, 2007). In contrast, a recent study by Messini and colleagues found that intravenous injections of ghrelin had no effects on the basal and GnRH stimulated LH and FSH release in women. These studies indicate sexual dimorphism in the actions of ghrelin in regulating gonadotropin secretion in humans (Messini *et al.*, 2009). Numerous other effects have been attributed to ghrelin. These include stimulation of prolactin, ACTH, AVP, promotion of slow-wave sleep, memory retention and anxiety-like behavior and stimulation of milk secretion (Wren *et al.*, 2001; Mozid *et al.*, 2003; Nakahara *et al.*, 2006).

The hypothalamic-pituitary-adrenocortical axis is stimulated following acute administration of ghrelin in vitro. Ghrelin stimulate the release of significant amounts of GHRH, CRH and AVP from hypothalamic explants (Mozid *et al.*, 2003). In rats, Intracerebroventricular administration of ghrelin has been shown to increase the plasma AVP concentration suppress pulsatile LH secretion (Furuta *et al.*, 2001). Intravenous ghrelin infusion also cause an increase in cortisol and aldosterone levels, while others have shown inhibition of the release of thyroid-stimulating hormone (Arvat *et al.*, 2001). However, Stimulatory effect of ghrelin and its analogs on PRL secretion in humans is far less age and gender dependent than the effect on GH secretion. Ghrelin also stimulates lactotroph and corticotroph secretion in an age- and gender-independent manner, both in human and in animal models (Broglio *et al.*, 2003). Ghrelin was also able to inhibit testosterone secretion in vivo and in vitro and partially prevented the normal timing of balanopreputial separation,an external index of puberty onset, in rats (Martini *et al.*, 2006; Fernandez-Fernandez *et al.*, 2005; Tena-Sempere *et al.*, 2002). Ghrelin induces a dose-dependent inhibition of hCG and cAMP-stimulated testosterone secretion in vitro. This effect is associated with a significant decrease in hCG stimulated expression of several mRNAs encoding key factors in the steroidogenic route (Tena-sempere *et al.*, 2002). Three different doses of NMS have been used by Jahan *et al.*, (2011) to determine its effect on testosterone secretion in adult male rhesus monkeys. According to their results NMS caused dose dependent increase in testosterone secretion but has inhibitory effect on cortisol secretion in adult male rhesus monkeys. In this study the effect of NMS on testosterone secretion was assessed after inhibition by ghrelin in adult male rhesus monkeys (*Macaca mulatta*).

MATERIALS AND METHODS

Animals

Four adult male rhesus monkeys (*Macaca mulatta*), 5-8 years of age, were utilized in this study. The animals were given numbers as 0201, 0202, 0203, and 0204. The ages of the animals were calculated using a dental formula described by Haigh and Scot (1965). The body weight of the animal at the time of the experiment ranged from 5-8 kg. The animals were housed in individual cages and were maintained under standard colony conditions at the Primate Facility of the Quaid-i-Azam University, Islamabad. They were provided with standard monkey food supplemented with fresh fruits and vegetables. Water was available *ad-libitum*. The appetite of the animals was monitored for 1 month prior to the beginning of the experiments. All animals used in the study were able to finish their food within 5-10 min. Daily feeding protocol consisted of fruits (6:00am), boiled potatoes (9:00am), eggs (11:00 am) and bread (1:00pm). The animals were given diet according to their body weights.

Chair restraining

The monkeys were trained for chair-restraint prior to initiation of the experiment in order to minimize the stress and sedation factor. Under ketamine sedation (Ketamax, Rotexmedica, Trittau, Germany 5 mg/kg BW, im) animals were affixed to a primate chair. After recovery from sedation the animals were allowed to sit on the chair for gradually increasing periods. The animals were habituated to chair restraint over a period of couple of months.

Catheterization

To permit sequential withdrawal of the blood samples and iv administration of drugs, the

animals were anesthetized with Ketamine hydrochloride (5 mg/kg BW, im), and a cathy cannula (Silver surgical complex, Karachi, Pakistan; 0.8 mm O.D/22 G×25mm) was inserted in the saphenous vein, 30 min before initiation of sampling and the animals were restrained to the chair. The free end of the cannula was attached to a syringe via butterfly tubing (24 G×3/4" diameter and 300 mm length; JMS Singapore PTE LTD, Singapore). Blood sampling and infusion of drugs were carried out when the animals had fully regained consciousness.

Pharmacological reagents

The following drugs were used in this study.

Heparin (Rotexmedica, Trittau, Germany)

Ketamine HCl (Ketavet; Park-Davis, Berlin, FRG)

Human Neuromedin S (AnaSpec, USA).

Human ghrelin (Sigma Aldrich, Israel)

Working solutions of NMS and ghrelin were made in normal saline. (0.9% NaCl).

Experimental protocol

Sequential blood samples (2.0 ml each after every 15 min) were obtained at 45 min before ghrelin was injected. After 60 min of ghrelin treatmernt (2μg/Kg), NMS (50 nmol) was injected intravenously and after that blood samples were collected for 120 min, in hepranized syringes. Following withdrawal of each sample, an equal volume of hepranized (5 IU/ml) saline was injected into the tubing. Similarly for NMS and ghrelin individual sampling blood samples were obtained after 15 min interval each. Blood samples were collected 60 min before NMS/ghrelin treatment (50 nmol and 2μg/Kg respectively) and 120 min afterwards. All blood sampling were carried out between 11:00 am 03:00 pm. Blood samples were immediately centrifuged at 3000 rpm for 10 min and plasma was separated and stored at -20°C until analyzed.

Hormonal analysis:

Testosterone concentrations were quantitatively determined by using EIA kits (Amgenix Inc, USA). Principle and procedure of the assay is as follow.

Testosterone Enzyme Immunoassay (EIA) test:

Testosterone EIA test kits were used to determine testosterone concentrations. The assay was carried out as described in the protocol provided with the kit.

Principle of the test:

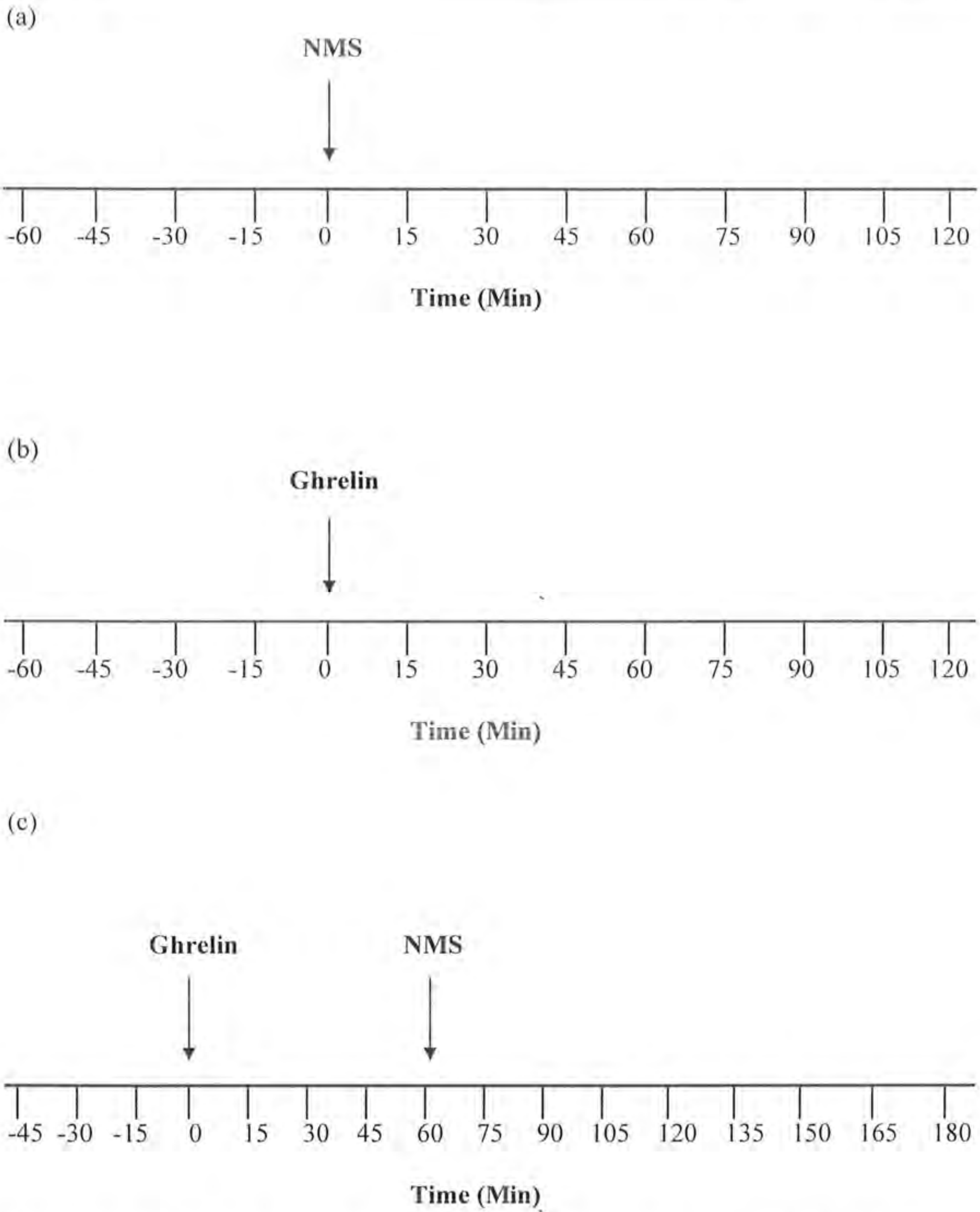
The Testosterone EIA is based on the principle of competitive binding between testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti-Testosterone. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 10μl of Testosterone standards, controls, patient samples, 100μl Testosterone-HRP conjugate reagent and 50μl rabbit anti-Testosterone reagent at 37°C for 90 minutes. During the incubation, a fixed amount of HRP-labeled Testosterone competes with the endogenous Testosterone in the standard, sample, or quality specific Testosterone antibody. Unbound Testosterone peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled Testosterone in the sample. A standard curve is obtained by plotting the concentration the standard versus the absorbance. The Testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve..

Assay Procedure

1. Desired number of coated wells was secured in the holder.
2. Standards, specimen and controls (10µl) were dispensed into the appropriate wells.
3. Testosterone-HRP Conjugate Reagent (100µl) was dispensed into each well.
4. Rabbit anti-Testosterone reagent (50µl) was dispensed to each well.
5. The microwell plate was incubated at 37°C for 90 minutes.
6. The microwells were rinsed and flicked 5 times with the distilled or deionized water.
7. TMB Reagent (100µl) was dispensed into each well and was mixed gently for 10 seconds.
8. The microwell plate was incubated at room temperature (18-25°C) for 20 minutes.
9. The reaction was stopped by adding 100µl of Stop Solution to each well.
10. The content of microwells was mixed gently for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
11. The absorbance read at 450nm with a microtiter well reader within 15 minutes.
12. The results were expressed in ng/ml.

Statistical Analysis

All data were presented as mean ± SEM. Student's t test was employed to determine differences between pre and post treatment testosterone levels. ANOVA followed by post hoc test (Tuckey's test) was used to determine testosterone concentration difference between control, NMS and ghrelin treatments. Statistical significance was set at $P<0.05$. Data were analyzed by using Graphpad prism version 5.



Protocol for NMS and ghrelin administration and blood sampling (a) NMS, (b) Ghrelin and (c) Ghrelin and NMS.

RESULTS

Effect of single intravenous injection of NMS (50nmol) on plasma testosterone concentration

The individual and mean ±SEM testosterone profiles before and after a single 50nmol i.v injection of NMS in four conscious chair restrained habituated adult male rhesus monkeys are presented in Table 1 and Fig. 1A, 1B and Fig. 2. Slight increase in plasma testosterone concentrations were noticed after NMS administration but significant increase was observed from 30 min to 60 min as compared to 0 min sample. At 30 min the concentration was 2.01±0.13 ($P<0.05$) and 45 min and 60 min hormonal level increased to 2.24±0.10 ($P<0.01$) and 2.31±0.16 ($P<0.01$) respectively as compared to control at 0 min (1.41±0.20). A gradual decrease in plasma testosterone concentrations were observed from 75 minutes to 120 minutes.

Pre treatment and post treatment mean values after NMS administration were also compared and a significant increase in mean plasma testosterone concentrations (pre 1.41±0.03 ng/ml; post 1.85±0.11) was observed (Table 2 and Fig.3). This increase in plasma testosterone level was significant ($P<0.05$).

Effect of single intravenous injection of ghrelin (2µg/kg) on plasma testosterone concentration

After 30 minutes of ghrelin administration (2µg/kg) there was a significant decrease ($P<0.05$) in plasma testosterone levels from 1.60±0.16 at 0 min to 0.81±0.16 at 30 min. Ghrelin administration caused a highly significant decrease ($P<0.001$) in plasma testosterone concentration was observed. The values were 0.47±0.15 at 45 min, 0.23 ±0.06 at 60 min and 0.21 ±0.07 at 75 min respectively as compared to 0 min sample i.e 1.60±0.16. The individual ad mean plasma testosterone profiles before and after a single i.v injection of ghrelin (2µg/kg body weight) in four conscious chair restrained habituated adult male rhesus monkey are presented (Table 3 and Fig. 4A, 4B and Fig. 5).

The mean values of post treatment also showed a significant decrease in testosterone concentrations as compared to pre treatment samples as describe in Table 4 and Fig. 6.

Effect of single intravenous injection of NMS (50nmol) and ghrelin (2μg/kg) on plasma testosterone concentration

The individual and mean ±SEM plasma testosterone profile before and after a single i.v. injection of ghrelin (2μg/kg body weight) and NMS (50nmol) of the four adult male rhesus monkeys are presented in Table 5, Fig. 7A, 7B and Fig. 8. After ghrelin administration upto 30 min plasma testosterone levels decreased to 0.69±0.10 but this decrease in plasma testosterone level was non-significantly (P>0.05) different as compared to control at 0 min i.e 1.07±0.13. Then a significant decrease was observed from 45 min to 60 min of ghrelin administration as compared to control at 0 min sample. These values were 0.51±0.08 (P<0.05) at 45 min, 0.38±0.07 (P<0.01) at 60 min as compared to control at 0 min sample. After 60 min of ghrelin, 50nmol of NMS was administered slight increase in plasma testosterone concentrations was observed after 15 min but this increase was non significant (P>0.05) as compared to 60 min sample of ghrelin administration. But from 30 min to 60 min of NMS treatment plasma testosterone level increased significantly (P<0.001) as compared to 60 min sample of ghrelin treatment. This increase was almost similar as was noted when NMS was given individually to these animals. These values were 1.21±0.15 at 30 min, 1.57±0.10 at 45 min and 1.81±0.05 at 60 min respectively after NMS injection.

Mean values of pre treatment, ghrelin treatment and NMS treatment were also compared as shown in Table 6 and Fig. 9. There was significant decrease (P<0.01) in mean plasma testosterone concentrations in post ghrelin treatment as compared to pre ghrelin treatment (pre ghrelin treatment 1.23± 0.09 to post ghrelin 0.60±0.10 ng/ml). Post treatment of NMS caused a non significant (P>0.05)) increase in plasma testosterone concentrations as compared to mean post ghrelin treatment (ghrelin 0.60±0.10 ng/ml to 1.09±0.16 Post NMS).

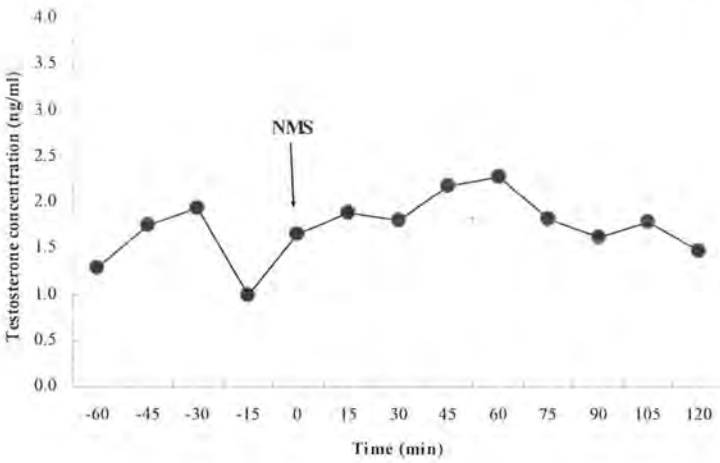
Intravenous administration of 50nmol of NMS significantly increased plasma testosterone level from 0.60±0.10 to 1.09±0.16 after it was suppressed by ghrelin (P<0.01).

Table: 1. Individual and mean ±SEM plasma testosterone concentrations in adult male rhesus monkey before and after NMS administration (n=4)

Time	Animal # plasma testosterone concentrations (ng/ml)				Mean±SEM
	201	202	203	204	
-60	1.29	1.62	1.16	1.48	1.39±0.10
-45	1.75	1.68	1.07	1.46	1.49±0.15
-30	1.94	1.70	0.97	1.27	1.47±0.22
-15	1.00	1.97	1.49	0.79	1.31±0.26
0	1.66	1.82	1.11	1.04	1.41±0.20
15	1.89	2.15	1.69	1.38	1.77±0.16
30	1.81	2.39	1.86	1.97	2.01±0.13*
45	2.19	2.05	2.51	2.21	2.24±0.10**
60	2.28	2.01	2.17	2.77	2.31±0.16**
75	1.82	2.00	1.80	1.75	1.84±0.05
90	1.63	1.96	1.21	1.47	1.57±0.16
105	1.79	2.01	1.69	1.49	1.75±0.11
120	1.47	1.78	1.31	1.61	1.54±0.10

*** =P< 0.05, ** =P<0.01 vs control at 0 min**

(a) # 201



(b) # 202

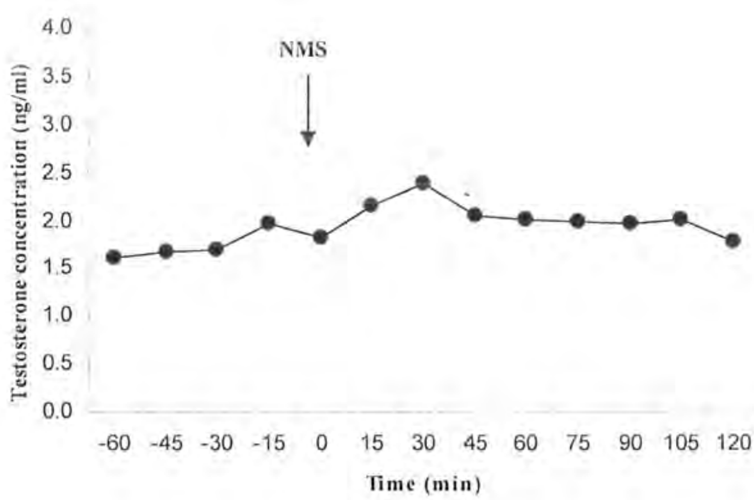
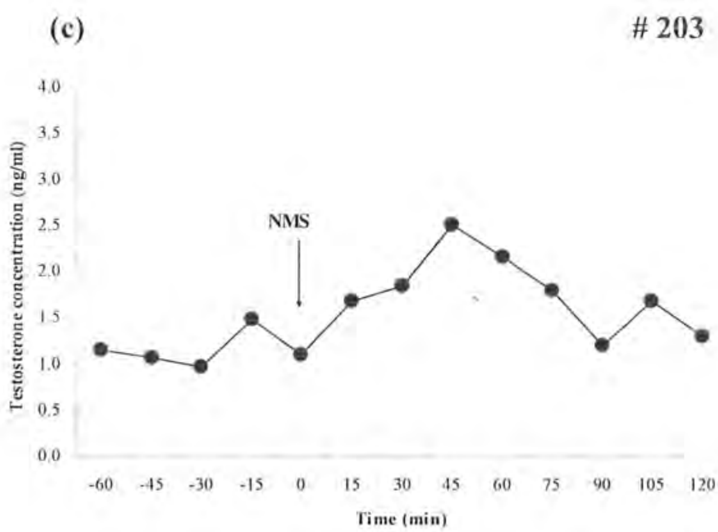


Fig: 1A. Changes in individual plasma testosterone concentrations (ng/ml) in adult male rhesus monkey before and after NMS administration (arrow).



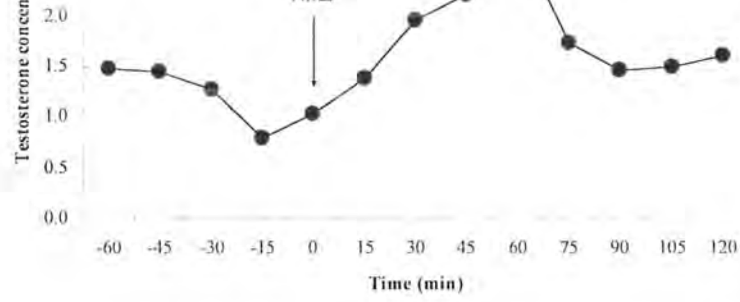


Fig: 1B. Changes in individual plasma testosterone concentrations (ng/ml) in adult male rhesus monkey before and after NMS administration (arrow).

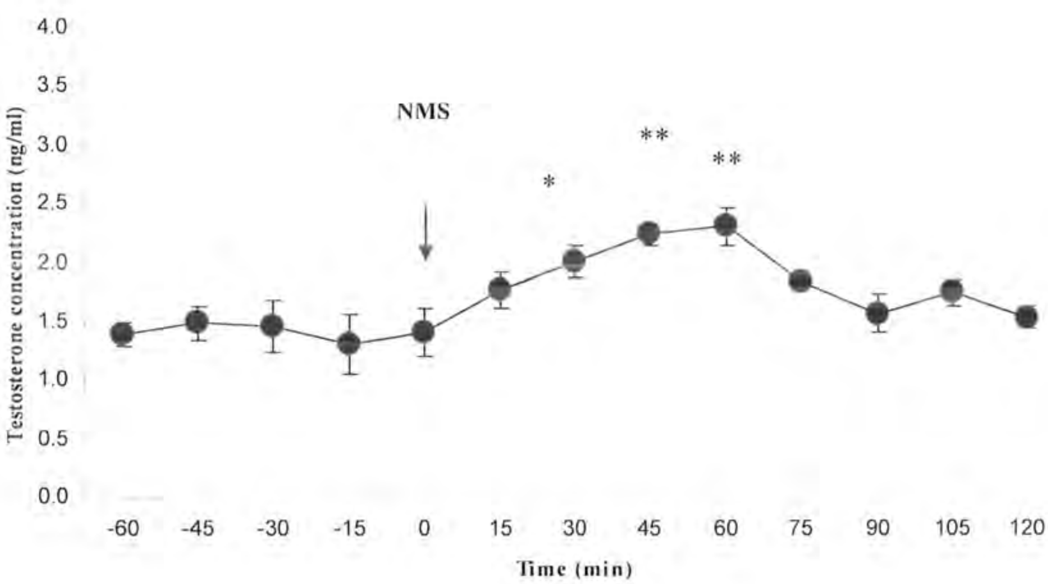


Fig: 2. Mean (±SEM) plasma testosterone concentrations before and after NMS administration as iv injection (arrow) in conscious chair restrained adult male rhesus monkey (n=4).

***=P< 0.05, ** =P<0.01 vs control at 0 min**

Table: 2. Mean (±SEM) plasma testosterone concentration (ng/ml) at various segments before and after NMS treatment in adult male rhesus monkey (n=4).

	Pre Treatment	NMS Treatment
Time(Min)	-60 to 0	15 to 120
Mean ± SEM	1.41±0.03	1.85±0.11*

*** = P < 0.05**

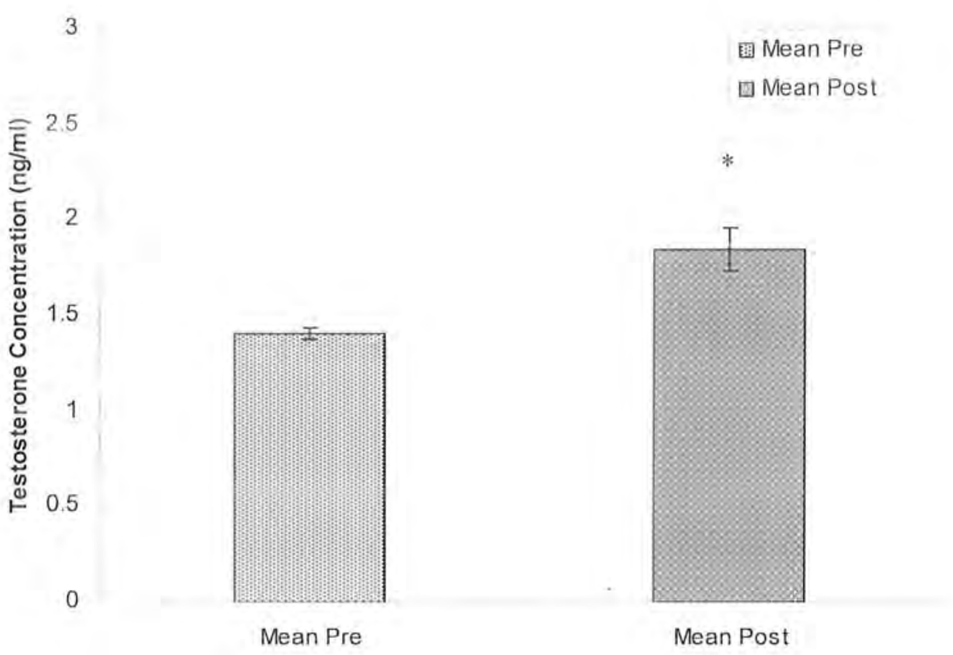


Fig: 3. Comparison of mean ± SEM plasma testosterone concentrations observed in pre and post administration of NMS in adult male rhesus monkey (n=4).
***= P < 0.05 vs Pre treatment**

Table: 3. Individual and mean ±SEM plasma testosterone concentrations in adult male rhesus monkey before and after ghrelin administration (n=4).

Time	Animal # Plasma testosterone concentration (ng/ml)				Mean±SEM
	201	202	203	204	
-60	1.04	0.92	0.89	1.25	1.02±0.08
-45	1.63	1.65	1.00	1.69	1.49±0.16
-30	1.99	1.85	1.21	1.81	1.71±0.17
-15	1.56	1.79	1.06	1.04	1.36±0.19
0	1.80	1.93	1.44	1.24	1.60±0.16
15	1.79	1.09	0.91	0.82	1.15±0.22
30	1.27	0.75	0.55	0.68	0.81±0.16*
45	0.90	0.24	0.44	0.28	0.47±0.15***
60	0.39	0.14	0.18	0.20	0.23±0.06***
75	0.43	0.11	0.15	0.17	0.21±0.07***
90	0.88	0.44	0.53	0.29	0.53±0.13
105	1.25	0.45	0.98	0.87	0.89±0.17
120	0.93	0.84	1.09	0.99	0.96±0.05

- **= P< 0.05, ***= P< 0.001 vs control at 0 min**

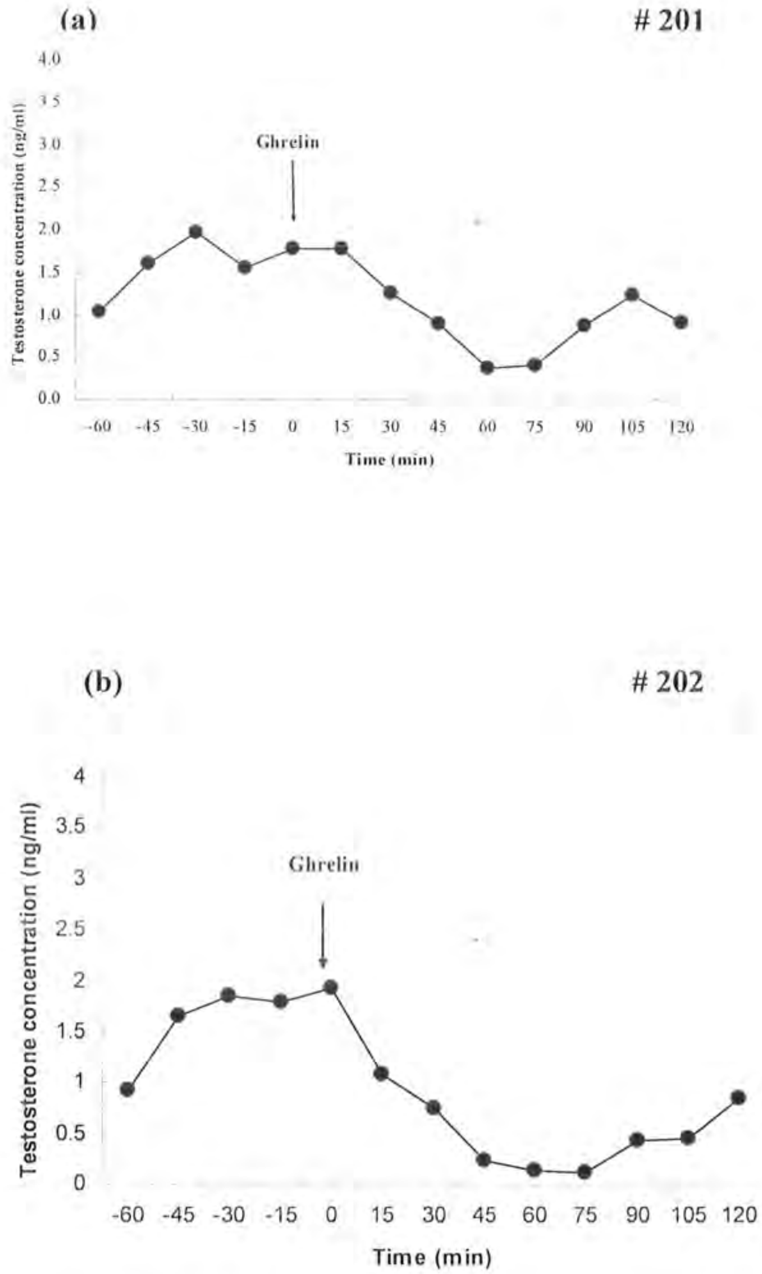
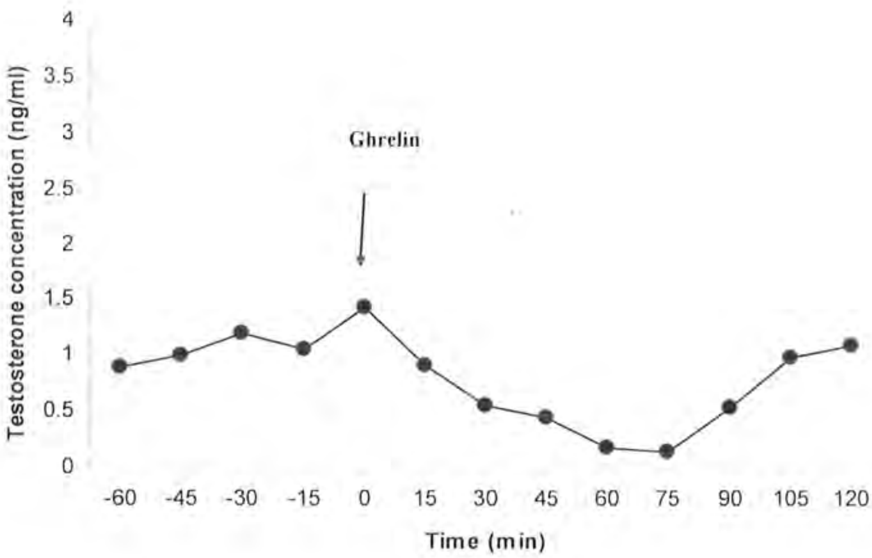


Figure: 4A. Changes in individual plasma testosterone concentrations (ng/ml) in adult male rhesus monkey before and after ghrelin administration (arrow).

(c) # 203



(d) # 204

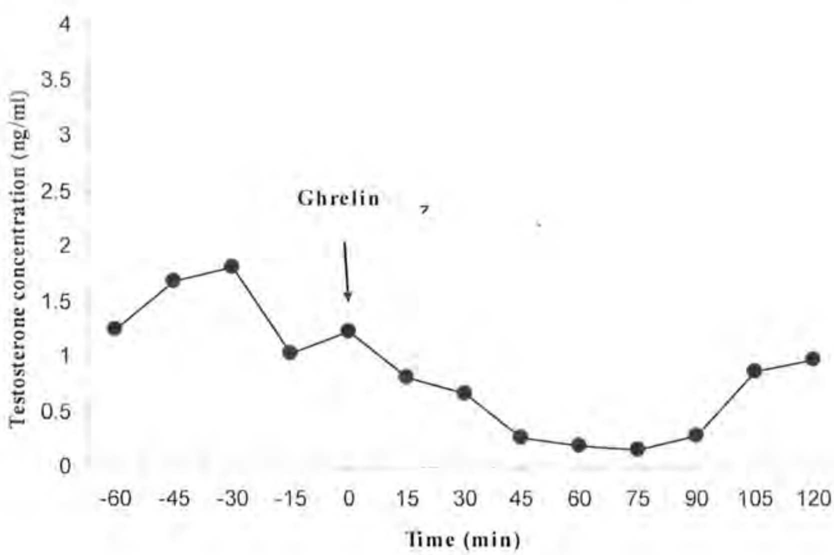


Figure: 4B. Changes in individual plasma testosterone concentration (ng/ml) in adult male rhesus monkey before and after ghrelin administration (arrow).

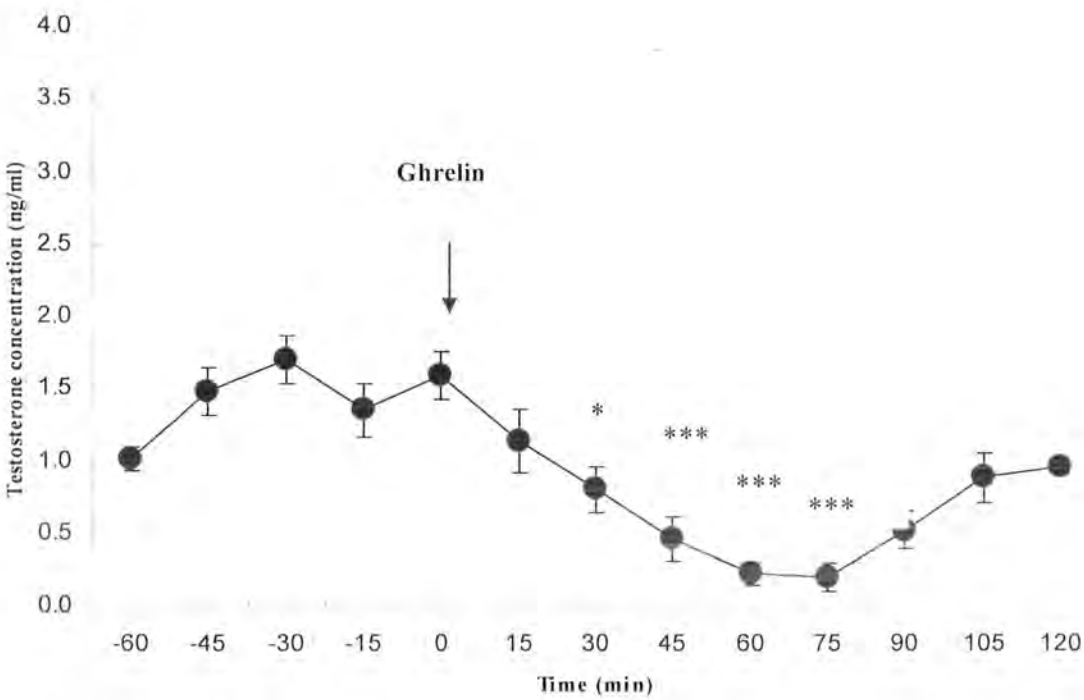


Fig: 5. Mean (±SEM) plasma testost erone concentrations (ng/ml) before and after ghrelin administration as iv injection (arrow) in conscious chair restrained adult male rhesus monkey (n=4).

***= P< 0.05, ***= P< 0.001 vs control at 0 min**

Table: 4. Mean (±SEM) plasma testosterone concentrations (ng/ml) at various segments before and after ghrelin treatment in adult male rhesus monkey (n=4).

	Pre Treatment	Ghrelin Treatment
Time(Min)	-60 to 0	15 to 120
Mean ± SEM	1.46±0.10	0.64±0.13**

**** =P < 0.01 vs Pre ghrelin pre treatment**

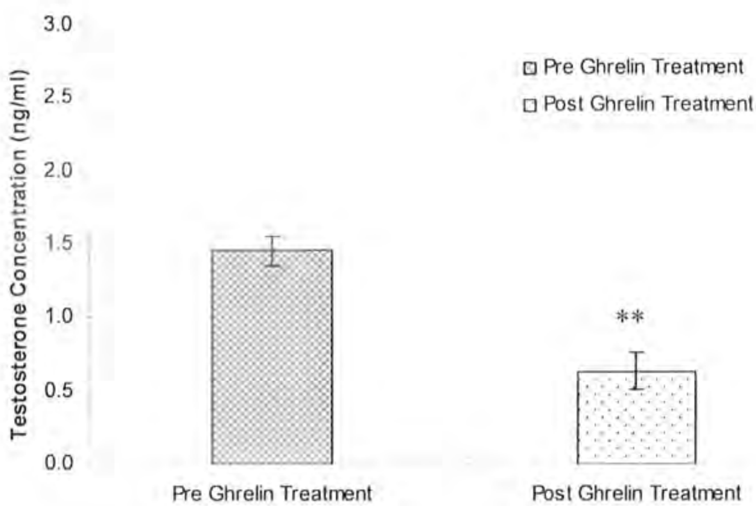


Fig: 6. Comparison of mean ± SEM plasma testosterone concentrations observed in pre and post ghrelin administration in adult male rhesus monkey (n=4).
**** =P < 0.01 vs Pre ghrelin Treatment**

Table: 5. Individual and Mean (±SEM) plasma testosterone concentrations in adult male rhesus monkey before and after ghrelin and NMS administration (n=4).

Time	Animal # Plasma testosterone concentrations (ng/ml)				Mean±SEM
	201	202	203	204	
-45	1.37	1.01	1.24	0.85	1.12±0.12
-30	1.40	1.05	1.45	1.20	1.28±0.09
-15	1.69	1.26	1.25	1.68	1.47±0.12
0	1.02	0.73	1.20	1.33	1.07±0.13
15	0.87	0.57	0.84	1.07	0.84±0.10
30	0.67	0.50	0.61	0.97	0.69±0.10
45	0.54	0.35	0.41	0.72	0.51±0.08**a**
60	0.32	0.27	0.33	0.59	0.38±0.07**ab**
75	0.35	0.39	0.57	0.93	0.56±0.13
90	1.01	0.92	1.34	1.57	1.21±0.15***
105	1.48	1.37	1.65	1.80	1.57±0.10***
120	1.85	1.66	1.83	1.91	1.81±0.05*****c**
135	1.62	1.05	1.01	1.16	1.21±0.14
150	0.88	0.75	0.86	1.11	0.90±0.08
165	0.67	0.53	0.62	0.84	0.66±0.06
180	0.76	0.62	0.73	1.06	0.79±0.09

a= P < 0.05 vs 0 min, ab= P< 0.01 vs 0 min, ***= P <0.001 vs 60 min, c= P < 0.01 vs 0 min

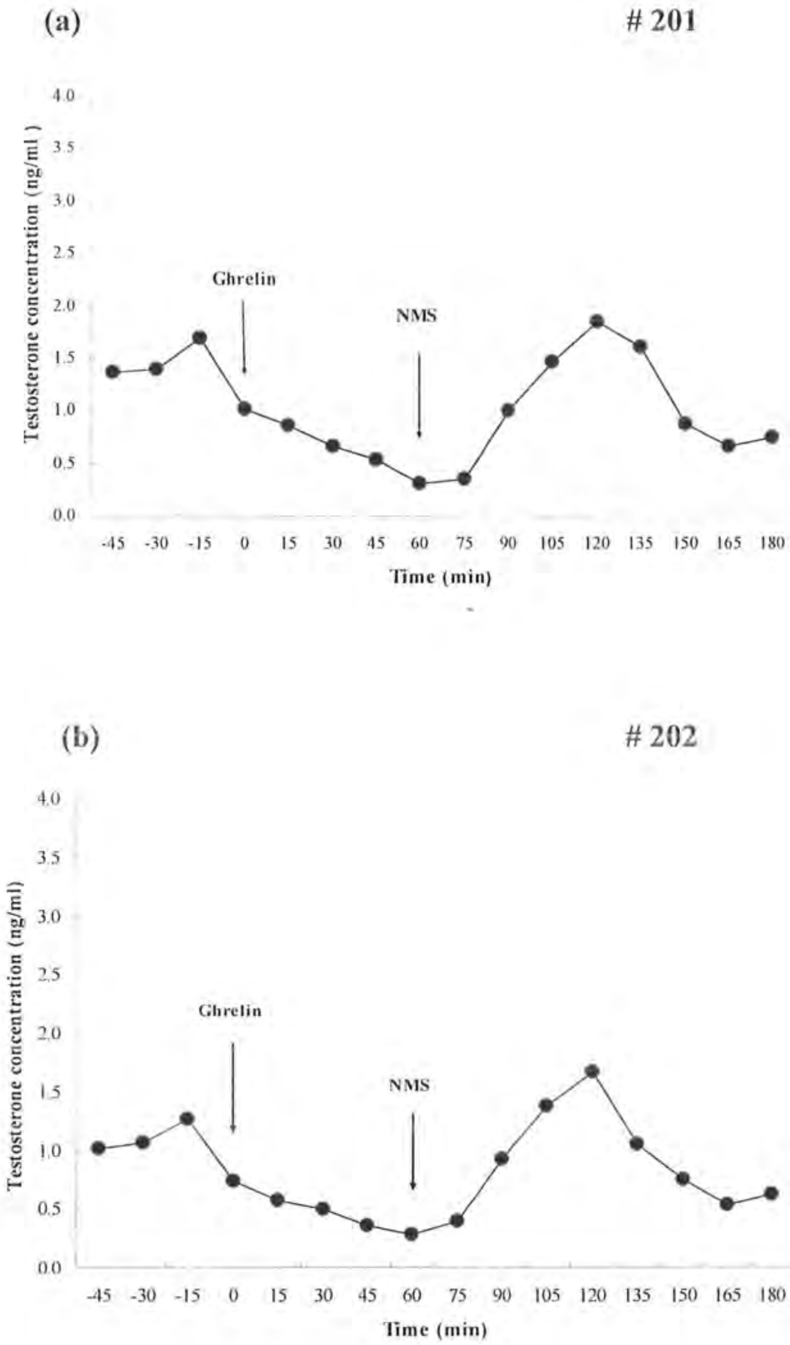
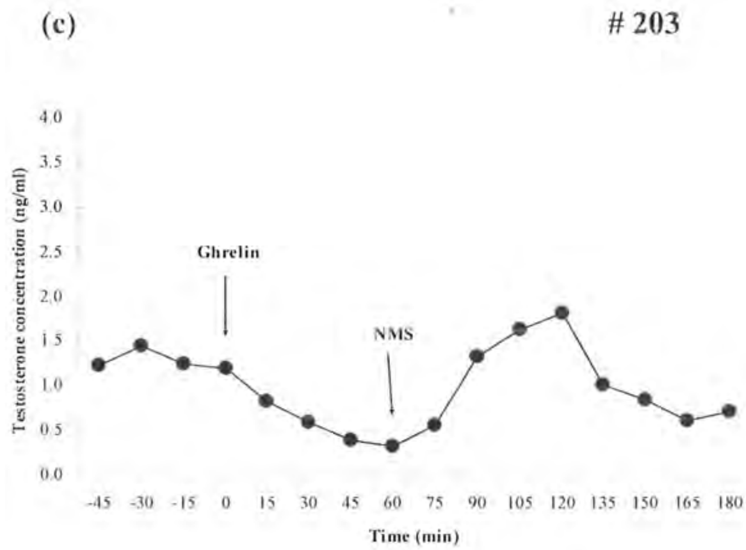


Fig: 7A. Changes in individual plasma testosterone concentration (ng/ml) in adult male rhesus monkey before and after ghrelin and NMS administration (arrow).



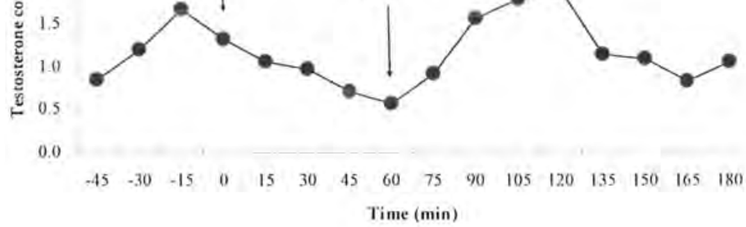


Fig: 7B. Changes in individual plasma testosterone concentrations (ng/ml) in adult male rhesus monkey before and after ghrelin and NMS administration (arrow).

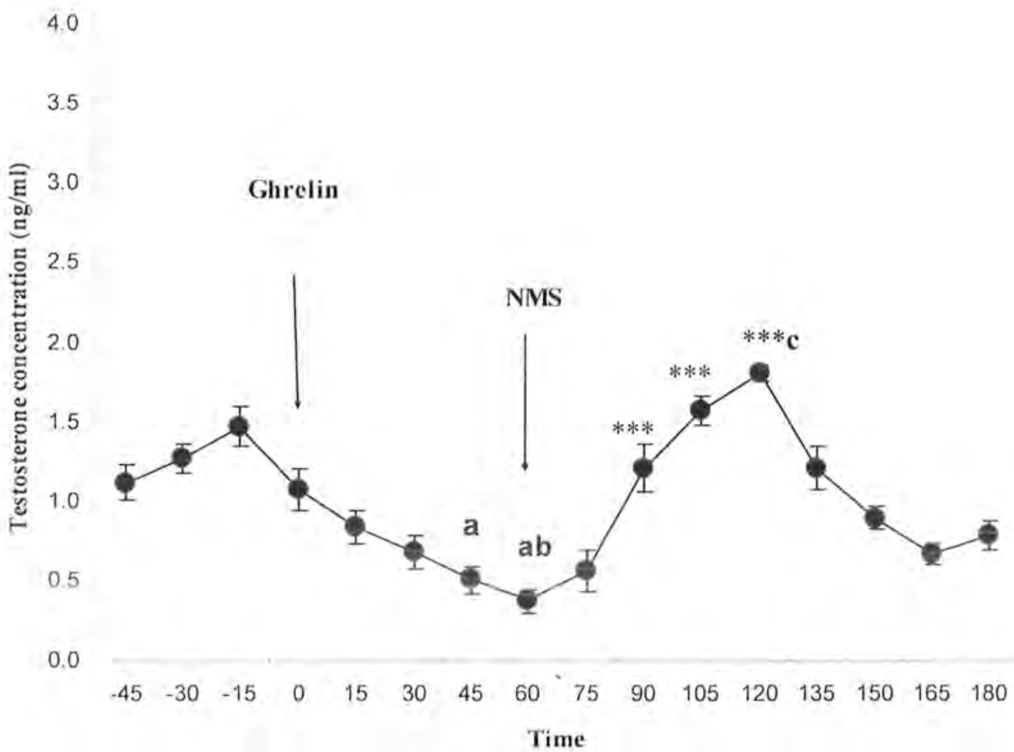


Fig: 8. Mean (±SEM) plasma testosterone concentrations before and after ghrelin and NMS administration as iv bolus (arrow) in conscious chair restrained adult male rhesus monkey (n=4).

a= P < 0.05 vs 0 min, ab= P< 0.01 vs 0 min, ***= P <0.001 vs 60 min, c= P < 0.01 vs 0 min

Table: 6. Mean (±SEM) plasma testosterone concentrations (ng/ml) at various segments before and after ghrelin and NMS treatment in adult male rhesus monkey (n=4).

	Pre Treatment	Ghrelin Treatment	NMS Treatment
Time(Min)	-45 to 0	15 to 60	75 to 180
Mean ± SEM	1.23±0.09	0.60±0.10**	1.09±0.16

****= P < 0.01 vs Pre treatment**

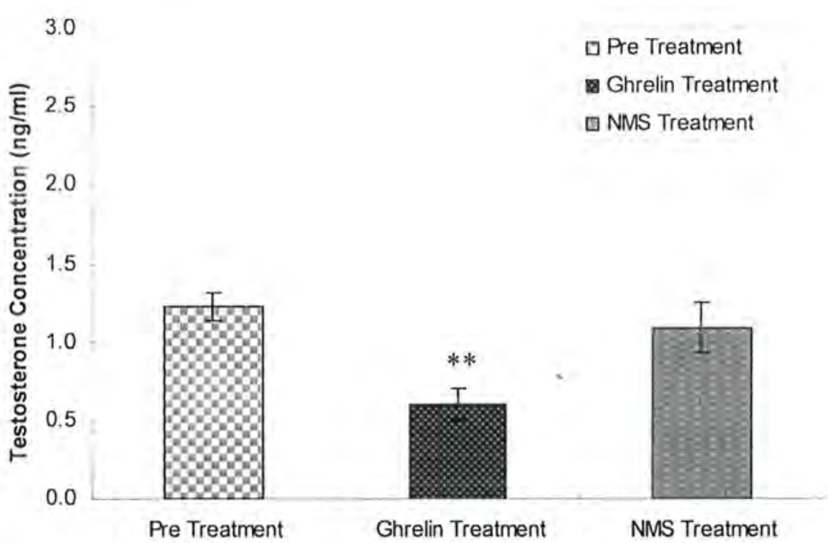


Fig: 9. Comparison of mean ± SEM plasma testosterone concentrations observed in pre and post ghrelin administration and post administration of NMS in adult male rhesus monkey (n=4).

****= P < 0.01 vs Pre treatment**

DISCUSSION

NMS is an anorexigenic neuropeptide, expressed at the SCN within the brain, while ghrelin is an orexigenic gut peptide both of which play an important role in the regulation of food intake, circadian rhythms and anorexigenic activity (Kojima *et al.*, 1999; Mori *et al.*, 2005; Ida *et al.*, 2005). In rodents the endocrine actions of NMS has been reported but not much data is available in higher primates. On the basis of our previous findings that NMS induces testosterone secretion in a dose dependant manner in adult male rhesus monkey (Jahan *et al.*, 2011). The present study was designed to investigate the stimulatory effect of NMS on ghrelin suppressed testosterone secretions in adult male rhesus monkeys as according to Tena Sampere *et al.*,(2002) suggested that ghrelin has inhibitory effect on testosterone secretion in rodents. In the present study 50nmol NMS concentration were used and had shown a stimulatory effect on testosterone secretion. These results are in accordance with our previous findings (Jahan *et al.*, 2011). Vigo *et al.*, (2006) observed in rodents that NMS has stimulatory effect on LH secretion suggesting that NMS may play a pivotal role in the regulation of HPG axis. The ability of NMS to influence testosterone secretion was not totally unpredicted as Vigo *et al.*, (2006) has reported stimulatory effect on HPG axis in rodents and similarly the actions of NMU, which operates through the same NMU2R centrally, having opposite role to NMS on LH release had inhibitory effect in ovariectomized female rats (Quan *et al.*, 2003, 2004). This induced secretion of testosterone might be following the same pathway e.g HPG axis.

Expression of mRNA of NMS in the testes makes a possible action of this neuropeptide at gonadal level (Mori *et al.*, 2005). It has been well established that the circadian rhythm is generated by an endogenous pacemaker generated in the SCN, and that precise rhythmic oscillation of this pacemaker is coordinated by various neurochemical substances (Lowrey and Takahashi, 2000; Reppert and Weaver, 2001). The data obtained in this study regarding the role of NMS in testosterone secretion suggest that NMS function as a regulator of the testosterone secretion in adult male rhesus monkeys. Present data and previous studies (Mori *et al.*, 2005) suggest that NMS shifted phase of the testosterone concentration, and increase the amplitude and the period of pulses of testosterone in a dose dependent manner.

The exact neuroendocrine circuitry responsible for such a positive effect of NMS on testosterone secretion remains to be confirmed yet. However, considering that NMS is able to modulate neuropeptide expression at the ARC (Ida *et al.*, 2005) which is a major center for the integrated control of energy balance and reproduction with abundant expression of NMU2R; it is plausible that the central mechanism, whereby NMS stimulating testosterone secretion, involves the activation of ARC pathways. Potential candidates for such an intermediary action, such as kisspeptin and galanin-like peptide, which are prominently expressed at the ARC (Gottsch *et al.*, 2004; Tena-Sempere, 2006) may have role in HPG axis stimulation. Vigo *et al.*, (2006) also reported that stimulatory effects of NMS on LH secretion were detected after suppression of gonadotropin secretion by short-term fasting, with exaggerated responses *vs.* control females at diestrus. Enhanced LH secretory responses to different stimuli (such as kisspeptin and galanin like peptide) have been detected in underfed animals (Castellano *et al.*, 2005). In any event, this observation evidences that NMS is able to counteract the inhibitory effect of energy insufficiency on the gonadotropic axis, thus reinforcing the potential role of this neuropeptide in the joint regulation of reproductive function and energy balance. The central effects of NMS on gonadotropin secretion, reported in study by (Vigo *et al.*, 2006) do not exclude additional actions of this neuropeptide at other levels of the HPG axis. In this regard, expression of NMU1R and NMU2R has been very recently observed in mouse pituitary, which suggest its possible action at the pituitary level (Vigo *et al.*, 2006). The present study demonstrate for the first time the role of ghrelin in testosterone secretion in adult male rhesus monkey.

The hypothalamus has been identified as the main source of ghrelin in the central nervous system, the GHS-R1a receptor mRNA has been found in many areas of the brain. In rats, systemic administration of ghrelin reduces the GnRH pulse frequency in vivo. The involvement of NPY in the mediation of the effects of ghrelin on pulsatile GnRH secretion is indicated by the complete abolition of the effects of ghrelin by the NPY-Y5 receptor antagonist (Lebrethon *et al.*, 2007). GnRH secretion by hypothalamic fragments from ovariectomized females is also significantly inhibited by ghrelin (Fernandez-Fernandez *et al.*, 2005). In mammalian and nonmammalian species, ghrelin affects gonadotropin release acting at the level of the hypothalamus as well as directly on the pituitary gland (Fernandez-Fernandez *et al.*, 2005). This is the very first experiment on adult male rhesus monkeys regarding the role of ghrelin in reproduction and results showed that ghrelin not only suppressed testosterone secretion in rodents and humans but it also suppressed testosterone secretion in adult male rhesus monkeys. In pituitary, ghrelin suppresses LH pulse frequency in rats, sheep, monkeys (Fernandez-Fernandez *et al*., 2004) and humans (Lanfranco *et al*., 2008). Ghrelin delays pubertal onset in male rats (Fernandez-Fernandez *et al*., 2005). In rats, ghrelin is able to downregulate *Kiss1* expression in the hypothalamic medial preoptic area and this could be a contributing factor in ghrelin-related suppression of pulsatile LH secretion (Forbes *et al*., 2009). In women during the menstrual cycle, administration of ghrelin does not affect basal and GnRH-induced LH and FSH secretion (Messini *et al*., 2009). Ghrelin has been shown to stimulate LH release in goldfishes (Unniappan *et al*., 2004) and in carps (Sokolowska-Mikolajczyk *et al*., 2009). Expression of ghrelin has been demonstrated in rodents and sheep by immunostaining in Leydig cells (Tena-Sempere *et al*., 2002). Ghrelin is also present in the human testis and particularly in Leydig and Sertoli cells but not in germ cells (Gaytan *et al*., 2004). In human testis, the expression of ghrelin by Leydig cells is apparently linked to the degree of cell differentiation (Gaytan *et al*., 2004). The present study was designed to elucidate the stimulatory role of NMS, inhibitory role of ghrelin on testosterone secretion and stimulatory role of NMS on ghrelin suppressed plasma testosterone secretions in adult male rhesus monkeys. The present study was designed to explore effect of these two peptides on testosterone secretion in adult male rhesus monkeys which are secreted from different sites in the body but seem to be antagonistic to each other regarding food intake and plasma testosterone secretions.

Ghrelin is inversely correlated with the serum testosterone levels in patients with normozoospermia, obstructive azoospermia, or varicocele suggesting that ghrelin has an indirect effect on spermatogenesis (Ishikawa *et al*., 2007). Our results are also in accordance to previous findings by Tena-sempere *et al*., (2002) suggested that ghrelin suppresses testosterone secretion in rat testicular slices in vitro. So it can be suggested that ghrelin has inhibitory effects on testosterone secretion both in rodents and primates. The inhibitory role of ghrelin on testosterone concentration was observed just after 30 minutes and maintained for 75 minutes. On the basis of above discussion it may be concluded that both these peptides have opposite effect on testosterone secretions in adult male rhesus monkeys.

The present experiment was designed to investigate the combined effect of orexigenic and an anorexigenic peptide. It was noticed that after the administration of NMS, the inhibitory effect of ghrelin was diminished and with in 30 to 60 min interval it almost blocked decrease in testosterone secretion and further stimulated testosterone secretion in adult male rhesus monkeys, as it was before ghrelin administration. These results are in accordance with the individual effects of these peptides on testosterone secretions. It may be concluded from these results that both these peptides play antagonistic role on testosterone secretions in adult male rhesus monkeys. HPG axis and HPA axis both may be involved in controlling such behavior but the exact mechanism is still unknown, so a detailed study is recommended to confirm the exact mechanism of action of these two peptides.

REFERENCES

Arvat E., Maccario M., Di vito L., Broglio F., Benso A., Gottero C., Papotti M., Muccioli G., Dieguez C., Casanueva FF., Deghenghi R., Camanni F and Ghigo E. 2001. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a non natural peptidyl GHS and GH releasing hormone. *J Clin Endocrinol Metab.* 86: 1169–1174.

Banks WA., Tschop M., Robinson SM and Heiman ML. 2002. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther.* 302: 822-827.

Barreiro ML., Gaytan F., Caminos JE., Pinilla L., Casanueva FF., Aguilar E., Dieguez C and Tena-Sempere M. 2002. Cellular location and hormonal regulation of ghrelin expression in rat testis. *Biol Reprod.* 67: 1768–1776.

Bluet-Pajot MT., Tolle V., Zizzari P., Tomasetto C., Grouselle D and Epelbaum J. 2005. Ghrelin, a striking example of neuroendocrine peptide pleiotropy. *Med Sci.* 218: 715-721.

Broglio F., Benso A., Castiglioni C., Gottero C., Prodam F., Destefanis S., Gauna C., Van der-Lely AJ., Deghenghi R., Bo M., Arvat E and Ghigo E. 2003. The endocrine response to ghrelin as a function of gender in humans in young and elderly subjects. *J Clin Endocrinol Metab.* 88: 1537–1542.

Caminos JE., Tena-Sempere M., Gaytan F., Sanchez-Criado JE., Barreiro ML., Nogueiras R., Casanueva FF., Aguilar E and Dieguez C. 2003. Expression of ghrelin in the cyclic and pregnant rat ovary. *Endocrinology.* 144: 1594–1602.

Castellano JM., Navarro VM., Fernandez-Fernandez R., Nogueiras R., Tovar S., Roa J., Vazquez MJ., Vigo E., Casanueva FF., Aguilar E., Pinilla L., Dieguez C and Tena-Sempere M. 2005. Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology.* 146: 3917-25.

Chu C., Jin Q., Kunitake T., Kato K., Nabekura T., Nakazato M., Kangawa K and Kannan H. 2002. Cardiovascular actions of central neuromedin U in conscious rats. *Regul Pept.* 105: 29–34.

Cowley MA., Smith RG., Diano S., Tschöp M., Pronchuk N., Grove KL., Strasburger CJ., Bidlingmaier M., Esterman M., Heiman ML., Garcia-Segura LM., Nillni EA., Mendez P., Low MJ., Sotonyi P., Friedman JM., Liu H., Pinto S., Colmers WF., Cone RD and Horvath TL. 2003. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron.* 7: 649–661.

Dubuc I., Nouel D., Coquerel A., Menard JF., Kitabgi P and Costentin J. 1988. Hypothermic effect of neuromedin N in mice and its potentiation by peptidase inhibitors. *Eur J Pharmacol.* 151: 117–121.

Fernandez-Fernandez R., Tena-Sempere M., Aguilar E and Pinilla L. 2004. Ghrelin effects on gonadotropin secretion in male and female rats. *Neurosci Letters.* 362: 103– 107.

Fernández-Fernández R., Tena-Sempere M., Navarro VM., Barreiro ML., Castellano JM., Aguilar E and Pinilla L. 2005. Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies. *Neuroendocrinology.* 82: 245– 255.

Forbes S., Li XF., Kinsey-Jones J and O'Byrne K. 2009. Effects of ghrelin on Kisspeptin mRNA expression in the hypothalamic medial preoptic area and pulsatile luteinising hormone secretion in the female rat. *Neurosci Letters.* 460: 143–147.

Fujii R., Hosoya M., Fukusumi S., Kawamata Y., Habata Y., Hinuma S., Onda H., Nishimura O and Fujino M. 2000. Identification of neuromedin U as the cognate ligand of the orphan G protein-coupled receptor FM-3. *J Biol Chem.* 275:21068–21074.

Furuta M., Funabashi T and Kimura F. 2001. Intracerebroventricular administration of ghrelin rapidly suppresses pulsatile luteinizing hormone secretion in ovariectomized rats. *Biochem Biophys Res Commun.* 288: 780–785.

Gaytan F., Barreiro ML., Caminos JE., Chopin LK., Herington AC., Morales C., Pinilla L., Paniagua R., Nistal FF., Casanueva E., Aguilar E., Dieguez C and Tena-Sempere M. 2003. Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. *J Clin Endocrinol Metab.* 88: 879–887.

Gaytan F., Barreiro ML., Caminos JE., Chopin LK., Herington AC., Morales C., Pinilla L., Paniagua R., Nistal FF., Casanueva E., Aguilar E., Dieguez C and Tena- Sempere M. 2004. Expression of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in normal human testis and testicular tumors. *J Clin Endocrinol Metab.* 89: 400–409.

Gottsch ML., Clifton DK and Steiner RA. 2004. Galanin-like peptide as a link in the integration of metabolism and reproduction. *Trends Endocrinol Metab.* 15: 215-221.

Guan XM., Yu H., Palyha OC., McKee KK., Feighner SD., Sirinathsinghji DJ., Smith RG., Van der Ploeg LH and Howard AD. 1997. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res.* 48: 23–29.

Guan XM., Yu H., Jiang Q., Van Der Ploeg LH and Liu Q. 2001. Distribution of neuromedin U receptor subtype 2 mRNA in the rat brain. *Brain Res Gene Expr Patterns.* 1: 1–4.

Hanada T., Date Y., Shimbara T., Sakihara S., Murakami N., Hayashi Y., Kanai Y., Suda T., Kangawa K and Nakazato M. 2003. Central actions of neuromedin U via corticotropin-releasing hormone. *Biochem Biophys Res Commun.* 311: 954–958.

Helke CJ., Krause JE., Mantyh PW., Couture R and Bannon MJ. 1990. Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. *FASEB J.* 4: 1606–15.

Honzawa M., Sudoh T., Minamino N., Tohyama M and Matsuo H. 1987. Topographic localization of neuromedin U-like structures in the rat brain: an immunohistochemical study. *Neuroscience.* 23: 1103–1122.

Howard AD., Feighner SC., Cully DF., Arena JP., Liberator PA., Rosenblum CI., Hamelin M., Hreniuk DL., Palyha OC., Anderson J., Paress PS., Diaz C., Chou M., Liu KK., McKee KK., Pong SS., Chaung LY., Elbrecht A., Dashkevicz M., Heavens R., Rigby M., Sirinathsinghji DJ., Dean DC., Melillo DG., Patchett A., Nargund RP., Griffin PR., DeMartino JA., Gupta SK., Schaeffer JM., Smith RG and van-der Ploeg LHT. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science.* 273: 974–977.

Ida T., Mori K., Miyazato M., Egi Y., Abe S., Nakahara K., Nishihara M., Kangawa K and Murakami N. 2005. Neuromedin S is a novel anorexigenic hormone. *Endocrinology.*146: 4217–4223.

Iqbal J., Kurose Y., Canny B and Clarke IJ. 2006. Effects of central infusion of ghrelin on food intake and plasma levels of growth hormone, luteinizing hormone, prolactin, and cortisol secretion in sheep. *Endocrinology* 147 : 510–519.

Ishikawa T., Fujioka H., Ishimura T., Takenaka A and Fujisawa M. 2007. Ghrelin expression in human testis and serum testosterone level. *J Androl.* 28: 320–324.

Jahan S., Ahmed S., Shah NA and Hizbullah. 2011. Role of Neuromedin S in Plasma Testosterone and Cortisol Concentrations in Male Adult Rhesus Monkey (*Macaca mulatta*). *Pak J Zoo.* 43: 147-153.

Kluge M., Uhr M., Bleninger P., Yassouridis A and Steiger A. 2007. Ghrelin suppresses secretion of Luteinizing hormone in humans. *J Clin Endocrinol Metab.* 70: 920–923.

Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H and Kangawa K.1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature.* 402: 656–660.

Kojima M., Haruno R., Nakazato M., Date Y., Murakami N., Hanada R., Matsuo H and Kangawa K. 2000. Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3). *Biochem Biophys Res Commun.* 276: 435–438.

Korbonits M., Goldstone AP., Gueorguiev M and Grossman AB. 2004. Ghrelin – a hormone with multiple functions. *Front. Neuroendocrinol.* 25: 27–68.

Kreier F., Yilmaz A., Kalsbeek A., Romijn JA., Sauerwein HP., Fliers E and Buijs RM. 2003. Hypothesis: shifting the equilibrium from activity to food leads to autonomic unbalance and themetabolic syndrome. *Diabetes.* 52: 2652–2656.

Lanfranco F., Bonelli L., Baldi M., Me E., Broglio F and Ghigo E. 2008. Acylated ghrelin inhibits spontaneous luteinizing hormone pulsatility and responsiveness to naloxone but not that to gonadotropin-releasing hormone in young men: evidence for a central inhibitory action of ghrelin on the gonadal axis. *J Clin Endocrinol Metab.* 93: 3633–3639.

Lebrethon MC., Aganina A., Fournier M., G´erard A., Parent AS and Bourguignon JP. 2007. Effects of in vivo and in vitro administration of ghrelin, leptin and neuropeptide mediators on pulsatile gonadotrophin-releasing hormone secretion from male rat hypothalamus before and after puberty. *J Neuroendocrinol.* 19: 181–188.

Lowrey PL and Takahashi JS. 2000. Genetics of the mammalian circadian system: photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. *Annu Rev Genet.* 34: 533-562.

Malagon MM., Luque RM., Ruiz-Guerrero E., Rodrıguez- Pacheco F., Garcıa-Navarro S., Casanueva FF., Gracia-Navarro F and Castaño JP. 2003. Intracellular signalling mechanisms mediating ghrelin stimulated growth hormone release in somatotropes. *Endocrinology.* 144: 5372–5380.

Malendowicz LK., Nussdorfer GG., Markowska A., Nowak KW and Torlinski L. 1993. Effects of neuromedin-N on the pituitary– adrenocortical axis of dexamethasone-suppressed rats. *Neuropeptides.* 24: 1–4.

Malendowicz LK., Nussdorfer GG., Markowska A., Tortorella C., Nowak M and Warchol JB. 1994. Effects of neuromedin U (NMU)-8 on the rat hypothalamo–pituitary–adrenal axis. Evidence of a direct effect of NMU- 8 on the adrenal gland. *Neuropeptides.* 26: 47–53.

Martini AC., Fernandez-Fernandez R., Tovar S., Navarro VM., Vigo E., Vazquez MJ., Davies JS., Thompson NM., Aguilar E., Pinilla L., Wells T., Dieguez C and Tena-Sempere M. 2006. Comparative analysis of the effects of ghrelin and un-acylated ghrelin upon luteinizing hormone secretion in male rats. *J Endocrinol.* 147: 2374–2382.

McKee KK., Palyha OC., Feighner SD., Hreniuk DL., Tan CP., Phillips MS., Smith RG., van-der Ploeg LH and Howard AD. 1997. Molecular analysis of rat pituitary and hypothalamic growth hormone secretagogue receptors. *Mol Endocrinol.* 11: 415–423.

Menzaghi F., Heinrichs SC., Merlo-Pich E., Tilders FJ and Koob GF. 1994. Involvement of hypothalamic corticotropin-releasing factor neurons in behavioral responses to novelty in rats. *Neurosci Lett.* 168: 139–142.

Messini C., Dafopoulos K., Chalvatzas N., Georgoulias P and Messinis IE. 2009. Effect of ghrelin on gonadotrophin secretion in women during the menstrual cycle. *Hum Reprod.* 24: 976–981.

Minamino N., Kangawa K and Matsuo H. 1983. Neuromedin B: a novel bombesin-like peptide identified in porcine spinal cord. *Biochem Biophys Res Commun.* 114: 541 548.

Minamino N., Sudoh T., Kangawa K and Matsuo H. 1985. Neuromedins: novel smooth-muscle stimulating peptides identified in porcine spinal cord. *Peptides*. 6: 245–248.

Miyazato M., Mori K., Ida T., Kojima M., Murakami N and Kangawa K. 2008. Identification and functional analysis of a novel ligand for G protein-coupled receptor, Neuromedin S. *Regul Pept.* 145 : 37–41

Mondal MS., Date Y., Murakami N., Toshinai K., Shimbara T., Kangawa K and Nakazato M. 2003. Neuromedin U acts in the central nervous system to inhibit gastric acid secretion via CRH system. *Am J Physiol Gasterointest Liver Physiol*. 284: 963–969.

Monnikes H., Heymann-Monnikes I and Tache Y. 1992. CRF in the paraventricular nucleus of the hypothalamus induces dose-related behavioral profile in rats. *Brain Res.* 574: 70–76.

Moore RY., Speh JC and Leak RK. 2002. Suprachiasmatic nucleus organization. *Cell Tissue Res*. 309: 89–98.

Mori K., Miyazato M., Ida T., Murakami N., Serino R., Ueta Y., Kojima M and Kangawa K. 2005. Identification of neuromedin S and its possible role in the mammalian circadian oscillator system. *EMBO J*. 24: 325–335.

Mozid AM., Tringali ML., Forsling MS., Hendricks S., Ajodha R., Edwards P., Navarra AB., Grossman M and Korbonits. 2003. Ghrelin is released from rat hypothalamic explants and stimulates corticotrophin-releasing hormone and Arginine-vasopressin. *Horm Metab Res.* 35: 455–459.

Nakahara K., Hanada R., Murakami N., Teranishi H.,Ohgusu H., Fukushima N., Moriyama M., Ida T., Kangawa K and Kojima M. 2004. The gut–brain peptide neuromedin U is involved in the mammalian circadian oscillator system. *Biochem Biophys Res Commun*. 318: 156–161.

Nakahara K., Nakagawa M., Baba Y., Sato M., Toshinai K and Date Y. 2006. Maternal ghrelin plays an important role in rat fetal development during pregnancy. *Endocrinology*. 147: 1333–1342.

Ohki-Hamazaki H. 2000. Neuromedin B. *Prog Neurobiol*. 62: 297–312.

Palyha OC., Feighner SD., Tan CP., McKee KK., Hreniuk DL., Gao YD., Schleim KD., Yang L., Morriello GJ., Nargund R., Patchett AA., Howard AD and Smith RG. 2000. Ligand activation domain of human orphan growth hormone

(GH) secretagogue receptor (GHS-R) conserved from Pufferfish to humans. *Mol Endocrinol.* 14: 160–169.

Parhar IS., Sato H and Sakuma Y. 2003. Ghrelin gene in cichlid fish is modulated by sex and development. *Biochem Biophys Res Commun.* 305: 169–175.

Quan H., Funabashi T., Furuta M and Kimura F. 2003. Effects of neuromedin U on the pulsatile LH secretion in ovariectomized rats in association with feeding conditions. *Biochem biophys Res Commun.* 311: 721-727.

Quan H., Funabashi T and Kimura F. 2004. Intracerebroventricular injection of corticotrophin-releasing hormone receptor antagonist blocks the suppression of pulsatile luteinizing hormone secretion induced by neuromedin U in ovariectomized rats after 48 hours fasting. *Neurosci Lett.* 369: 33–38.

Raddatz R., Wilson AE., Artymyshyn R., Bonini JA., Borowsky B., Boteju LW., Zhou S., Kouranova EV., Nagorny R., Guevarra MS., Dai M., Lerman GS., Vaysse PJ., Branchek TA., Gerald C., Forray C and Adham N. 2000. Identification and characterization of two neuromedin U receptors differentially expressed in peripheral tissues and the central nervous system. *J Biol Chem.* 275: 32452–32459.

Reppert SM and Weaver DR. 2001. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol.* 63: 647–676.

Rucinski M., Ziolkowska A., Neri G., Trejter M., Zemleduch T., Tyczewska M., Nussdorfer GG and Malendowicz LK. 2007. Expression of neuromedins S and U and their receptors in the hypothalamus and endocrine glands of the rat. *Int J Mol Med.* 20: 255–259.

Sakata I., Nakamura K., Yamazaki M., Matsubara M., Hayashi Y., Kangawa K and Sakai T. 2002. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides.* 23: 531–536.

Shintani M., Ogawa Y., Ebihara K., Aizawa-Abe M., Miyanaga F., Takaya K., Hayashi T., Inoue G., Hosoda K., Kojima M., Kangawa K and Nakao K. 2001. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes.* 50: 227–232.

Shousha S., Nakahara K., Sato M., Mori K., Miyazato M., Kangawa K and Murakami N. 2006. Effect of neuromedin S on feeding regulation in the Japanese quail. *Neurosci. Lett.* 391: 87–90.

Sokolowska-Mikolajczyk M., Socha P., Szczerbik and Epler P. 2009. The effects of ghrelin on the in vitro spontaneous and GnRH-A stimulated luteinizing hormone (LH) release from the pituitary cells of common carp (Cyprinus carpio L.). *Comp Biochem Physiol A.* 153: 386–390.

Tena-Sempere M and Barreiro ML. 2002. Leptin in male reproduction: the testis paradigm. *Mol Cell J Endocrinol.* 188: 9–13.

Tena-Sempere M., Barreiro ML., Gonzalez LC., Gaytan F., Zhang FP., Caminos JE., Pinilla L., Casanueva FF., Dieguez C and Aguilar E. 2002. Novel expression and functional role of ghrelin in rat testis. *J Endocrinol.* 143: 717–725.

Unniappan S., Canosa LF and Peter RE. 2004. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. *Neuroendocrinology.* 79: 100–108.

Van der Lely AJ., Tschop M., Heiman ML and Ghigo E. 2004. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocrinol Rev.* 25: 426–457.

Vigo E., Roa J., Lopez M., Castellano JM., Fernandez-Fernandez R., Navarro VM., Pineda R., Aguilar E., Dieguez C., Pinilla L and Tena- Sempere M. 2006. Neuromedin S as novel putative regulator of luteinizing hormone secretion. *Endocrinology.* 148: 813-823

Vulliemoz NR., Xiao E., Xia-Zhang L., Germond M., Rivier J and Ferin M. 2004. Decrease in luteinizing hormone pulse frequency during a five-hour peripheral ghrelin infusion in the ovariectomized rhesus monkey. *J Clin Endocrinol Metab.* 89 : 5718–5723.

Wang WG., Chen X., Jiang H and Jiang ZY. 2008. Effects of ghrelin on glucose-sensing and gastric distension sensitive neurons in rat dorsal vagal complex. *Regul Pept.* 146: 169–175.

Wren AM., Seal L., Cohen MA., Brynes AE., Frost GS., Murphy KG., Dhillo WS., Ghatei MA and Bloom SR. 2001.Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab.* 86: 5992-5995.

Yang J., Brown MS., Liang G., Grishin NV and Goldstein JL. 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell.* 132: 387–396.

Zigman JM and Elmquist JK. 2003. From anorexia to obesity—the Yin and Yang of body weight control. *Endocrinology* 144: 3749–3756.