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Effect of Neuromedin S (NMS) on Growth Hormone and its Relationship with NMS Induced Testosterone Secretion in Male Rhesus Monkeys (*Macaca mulatta*)

Shakeel Ahmed* and Sarwat Jahan

Reproductive Physiology Laboratory, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan

ABSTRACT

Neuromedin S (NMS), an anorexigenic neuropeptide was first discovered in rat brain. It is a ligand for receptor FM4/TGR-1 which is also called as NMU receptor type II (NMU2R). Mainly it is expressed in SCN and involved in regulation of food intake and dark light circadian rhythms. In rodents and higher primates its stimulatory role in HPG axis is reported. Growth hormone (GH) is released from anterior pituitary and directly or indirectly play very important role in regulation of HPG axis. In the present study the pathway of stimulatory role of NMS was investigated in the regulation of HPG axis. For this purpose, after NMS administration plasma testosterone (T) and growth hormone (GH) levels were determined in four normally fed and 48 hours fasted adult male rhesus monkeys. Fifty nmol (50 nmol) of NMS was injected through a cannula affixed in saphenous vein. Blood samples were collected individually 60 minutes before and 120 minutes after NMS administration at 15 minutes intervals. Plasma T and GH concentrations were determined by using specific Enzyme Immunoassay (EIA) kits. 48 h fasting significantly ($P<0.001$) decreased plasma T levels but it did not cause any significant ($P>0.05$) change in plasma GH levels compared to normal fed monkeys. NMS injection induced a significant increase ($P<0.05$) in T and GH concentrations compared to saline treated animals suggesting the possible involvement of GH in NMS induced secretion of testosterone. In summary our results suggest that NMS is a positive modulator of HPG axis and pituitary hormones like GH might be playing an intermediate role.

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SA performed the experiments and wrote the article. SJ supervised the research work and helped in preparation of manuscript.

Key words
Neuromedin S, Tesosterone, Growth hormone, Reproduction, Monkeys

INTRODUCTION

Neuromedin S (NMS) is a 36-amino acid peptide which binds with the G protein-coupled receptor FM4/TGR-1 also called neuromedin U receptor type-2 (NMU2R) and is highly expressed in the suprachiasmatic nucleus (SCN) of the hypothalamus (Mori *et al*., 2005). The expression of NMS receptor is restricted almost only to the central nervous system having abundant expression in SCN and paraventricular nucleus (PVN) (Guan *et al*., 2001; Nakahara *et al*., 2004). The presence of receptor within SCN, suggests its ligand role in regulation of circadian rhythms and hypothalamic hormones like corticotropic releasing hormone (CRH) and gonadotropic releasing hormone (GnRH) secretion (Mori *et al*., 2005) while its PVN presence implies its role in feeding and the regulation of hypothalamus pituitary adrenal (HPA) axis. It has been demonstrated that NMS has higher expression in the hypothalamus (Rucinski *et al*., 2007), which suggests the predominance of NMS in central regulatory processes. NMS mRNA has higher expression in the hypothalamus, testes and spleen (Mori *et al*., 2005). The presence of NMS mRNA in testes (Fujii *et al*., 2000), suggests its possible role in reproduction. Central administration of NMS in female rats stimulate luteinizing hormone (LH) secretion (Vigo *et al*., 2007) and peripheral administration of NMS induces T secretion in rhesus monkeys in a dose dependent manner which indicates that it may have a very important role in regulation of reproductive functions.

Reproductive functions are vitally controlled by hypothalamus pituitary gonadal (HPG) axis. This axis regulates secretion of pituitary gonadotropins, follicle stimulating hormone (FSH) and LH by pulsatile release of hypothalamic decapeptide GnRH. All these hormones play a major role in gonadal maturation and functions (Plant, 2008). Many internal and external factors may affect the proper functioning of HPG axis. The most important factor is the nutritional status of an individual (Bronson, 1985; Cameron, 1996; Wade *et al*., 1996; Wade and Jones, 2004). The observations of Pirke and colleagues suggested the possible role of specific nutrients on reproductive function in the human (Pirke *et al*., 1986). Many studies

* Corresponding author: shakeelzoologist@gmail.com
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on rats, cattle and hamsters, have also shown that the metabolic deficiencies affect the testicular size and sperm production, as caused by other environmental factors such as photoperiod and social cues (Lincoln and Short, 1980; Walkden-Brown *et al*., 1994). The exact mechanism that how metabolic fuel deficiencies arrest the neural networks which regulate the intermittent GnRH discharge is not completely understood. Metabolic deficiency suppressed GnRH secretion is associated with increased levels of gamma-aminobutyric acid (GABA) due to over expression of GABA synthesizing enzymes (Leonhardt *et al*., 1999). The study of Mahesh and Brann (2005) showed that excitatory amino acids (EAA) stimulate LH secretion.

In mammals, hormone secretion and needs of the organism are precisely balanced in a particular state. Mainly from the different hypothalamic nuclei, releasing or inhibitory factors define the final concentrations of many pituitary hormones in circulatory system (Schibler and Sassone-Corsi, 2002). Higher brain sites with an integrative system control these nuclei. The afferent inputs to these areas of brain may be of hormonal or neural origin. The neural networks, controlling hormone release include feedback loops in which the released signaling molecule directly or indirectly modifies its pattern of secretion (Schibler and Sassone-Corsi, 2002).

Hypothalamus controls a variety of homeostatic processes such as metabolic control, reproduction, thermoregulation, lactation, cardiovascular function, feeding, drinking, sleep-wake cycle and hormone secretion. Hypothalamus delivers its secretions through the hypophyseal portal system to the anterior pituitary gland which in turn regulate the secretions of other endocrine glands (Everitt and Hokfelt, 1990; Bernardis and Bellinger, 1993, 1998).

A complex network of hormonal system is required for spermatogenesis and steroidogenesis, which are normal testicular functions. Like other glands testes are also controlled by secretion of certain hormones. These hormones are the primary regulators while the local paracrine and autocrine chemicals produced by the cellular parts of testes work to establish the important microenvironment for sperm development. Steroidogenesis, spermatogenesis and testicular functions are controlled by the complex interaction of autocrine, paracrine and endocrine signals (Heindel and Treinen, 1989; Spiteri-Grech and Nieschlag, 1993; Gnessi *et al*., 1997; Abney, 1999; Hull and Harvey, 2000; Roser, 2001; Welt *et al*., 2002; Huleihel and Lunenfeld, 2004; Petersen and Soder, 2006).

GH belongs to protein family (Niall *et al*., 1971). It is required for pubertal maturation and sexual differentiation. It is also involved in gametogenesis, gonadal steroidogenesis, and ovulation. During pregnancy GH is also needed for fetal nutrition, growth, development of mammary gland and lactation. These roles reflect the effect of GH on the secretion and action of FSH and LH (Chandrashekar and Bartke, 1998), directly and indirectly through insulin-like growth factor I production. Moreover, production of GH in mammary and gonadal tissues reflects paracrine or autocrine actions of extra pituitary GH. Experimental studies showed that GH affects gonadal differentiation, steroidogenesis, gonadotrophin secretions and gametogenesis (Zachmann, 1992; Franks, 1998).

Compelling evidences suggest that GH plays an important role in the reproductive process. The presence of GH receptors has been documented in the ovary (Mathews *et al*., 1989; Lobie *et al*., 1990). In male reproductive system, GH receptors are found ubiquitously including sertoli and leydig cells, vas deferens, seminal vesicles and prostate gland (Lobie *et al*., 1990). GH also plays very important role in testicular development and growth. GH deficiency in human is associated with abnormally small testes. Similarly, pituitary and testicular GH may affect testicular function including gametogenesis and steroidogenesis (Spiteri-Grech and Nieschlag, 1992).

In the present study it was hypothesized that NMS is possibly involved in the regulation of HPG axis by affecting the secretion of GH. For this purpose, the effect of NMS on GH secretion and its relationship with T secretion was observed in normal, fed and 48-h fasted male monkeys.

MATERIALS AND METHODS

Animals and catheterization

Four adult normal male monkeys (*Macaca mulatta*) of age and weight ranging from 6-8 years and 7-10 kg, respectively ept in standard colony environment of primate facility at Department of Animal Sciences, Quaid-i-Azam University Islamabad. During experiment normally fed animals were given daily with fresh fruits, boiled potatoes, eggs and bread at specific times according to their body weights while water was available ad libitum to both fed and 48 h fasted monkeys. The normal feeding was carefully observed for one month in both fed and fasted groups prior to the start of experiment. A cathy cannula (Silver Surgical Complex, Karachi, Pakistan; 0.8 mm O.D/22 G×25mm) was affixed in the sephnous vein after anesthesizing the animals with Ketamine HCl (10 mg/kg BW, *im*), to bring about all the chemical administration and sequential blood sampling. A butterfly tubing (24 G×3/4″ diameter and 300 mm length; JMS Singapore) was attached with free end of the cannula. A single intravenous injection of NMS/saline was given after 60 min start of experiment. Five samples

were collected in both fed and fasted groups before NMS/saline injection and eight samples were collected after injection. All the sampling was performed after full recovery of animals from sedation. All experiments were approved by the Departmental Committee for Care and Use of animals at Quaid-i-Azam University Islamabad, Pakistan.

Pharmacological reagents

Pharmacological reagents used in the study are Heparin (Sinochem Ningbo, China), Ketamine HCl (Rotexmedica, Trittau, Germany), Human Neuromedin S (Anaspec, USA). All the working solutions were prepared in saline solution (0.9% NaCl).

Blood sampling

Blood sampling (2-3 ml) was done at regular intervals of 15 min in both fed and 48 h fasted animals using heparinized syringes. An equivalent quantity of heparinized (5 IU/ml) saline was injected after each sample withdrawal. Samples were collected 60 min before and 120 min after NMS administration. The time of NMS (50 nmol) administration was considered as 0 min. All blood samples were obtained between 1100-1500 h. All experiments were performed in a couple of weeks in order to reduce the alterations in hormonal levels associated with seasonal changes. Samples were centrifuged for 10 min at 3000 rpm, and then plasma was pipetted out and stored at -20˚C until analyzed.

Hormonal analysis

T and GH concentrations were quantitatively determined by using EIA kits (Amgenix Inc. USA). The minimum limit of detectable T levels was upto 0.05 ng/ml; intra-assay and inter-assay coefficients of variation were 6.4% and 4.4%, respectively and the minimum detectable limit for both GH levels was 0.05 ng/ml. Intra-assay and inter-assay coefficients of variation were <8%. All the procedures of EIA were followed as provided with the kits.

Statistical analysis

All the data were presented as mean±SEM. T and GH concentrations after NMS and saline administration were compared by one-way ANOVA followed by post hoc Dunnett's multiple comparisons test. Student's t test was employed to compare mean pre- and post-treatment T and GH concentrations, under 48-h fasting and normal fed conditions.

Statistical significance was set at $P \leq 0.05$. All the data were analyzed by using statistical software GraphPad Prism version 5.

RESULTS

Basal plasma T and GH concentrations

Basal plasma concentrations of T (ng/ml) and GH (ng/ml) during 1-h before saline/NMS administration in fed and 48-h fasting monkeys are shown in Figure 1. Plasma T concentrations significantly ($P < 0.001$) decreased in 48-h fasting monkeys compared to normal fed monkeys but it did not cause any significant change ($P > 0.05$) in basal plasma GH levels.

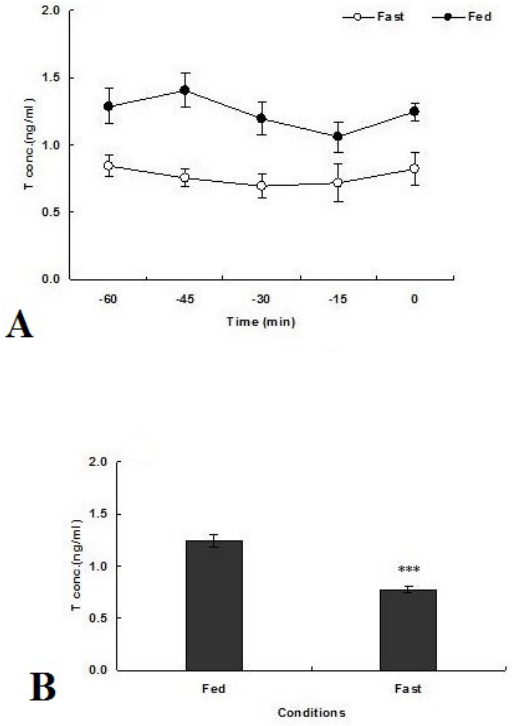

Fig. 1. (A) Changes in mean (±SEM) basal plasma T concentrations (ng/ml) during 1-h period before NMS/saline administration in fed and 48-h fasting adult male monkeys (B) Overall mean (±SEM) basal plasma T concentrations (ng/ml) during 1-h period before NMS/saline administration in normal fed, and 48-h fasting adult male monkeys. ***P<0.001 vs fed (Student's t test).

Effect of NMS on plasma T and GH secretion

The plasma T and GH concentrations (ng/ml) before and after saline/NMS administration in normal fed monkeys are given in Figure 2A. At 30 min after NMS administration significant ($P<0.05$) increase in T secretion was observed. Maximum levels of T concentrations were observed at 60 min of NMS treatment compared to 0 min sample (Fig. 2B). GH secretions significantly ($P<0.05$) increased after 45 min of NMS injection compared to 0 min sample. Maximum levels of GH concentrations ($P<0.001$)

were observed at 90 min of NMS injection compared to 0 min sample (Fig. 2B). Comparison between pre- and post-treatment also showed a significant increase in T ($P<0.05$) and GH ($P<0.01$) secretion after NMS administration (Fig. 3).

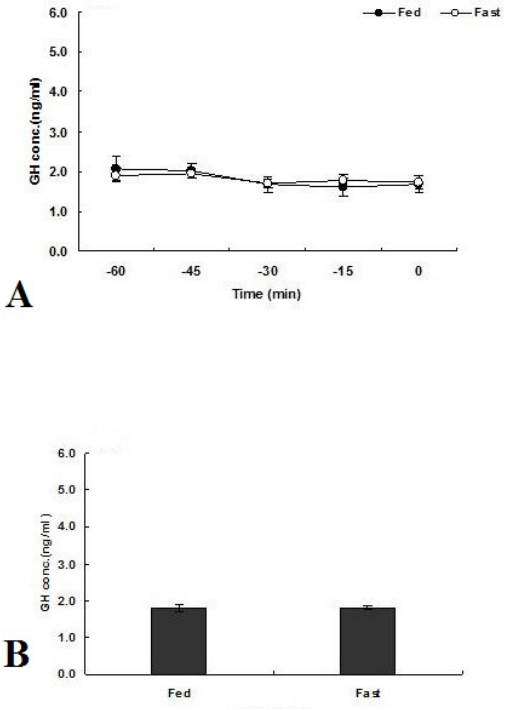

Fig. 2. (A) Changes in mean (±SEM) basal plasma GH concentrations (ng/ml) during 1-h period before NMS/ saline administration in fed and 48-h fasting adult male monkeys (B) Overall mean (±SEM) basal plasma GH concentrations (ng/ml) during 1-h period before NMS/ saline administration in normal fed, and 48-h fasting adult male monkeys. P>0.05 vs fed (Student's t test).

Effect of NMS on plasma T and GH secretion

The plasma T and GH concentrations (ng/ml) before and after saline/NMS administration in 48-h fasting monkeys are given in Fig. 4A. At 60 min after NMS administration significant ($P<0.05$) increase in T secretion was observed. Maximum levels of T concentrations were observed at 75 min of NMS treatment compared to 0 min sample (Fig. 4B). NMS treatment in 48-h fasted monkeys significantly ($P<0.01$) increased GH concentrations after 60 min of injection. Maximum levels of GH concentrations ($P<0.001$) were observed at 90 min of NMS injection compared to 0 min sample (Fig. 4B). Comparison between pre- and post-treatment also showed a significant increase in both T ($P<0.05$) and GH ($P<0.01$) levels after NMS administration (Fig. 5).

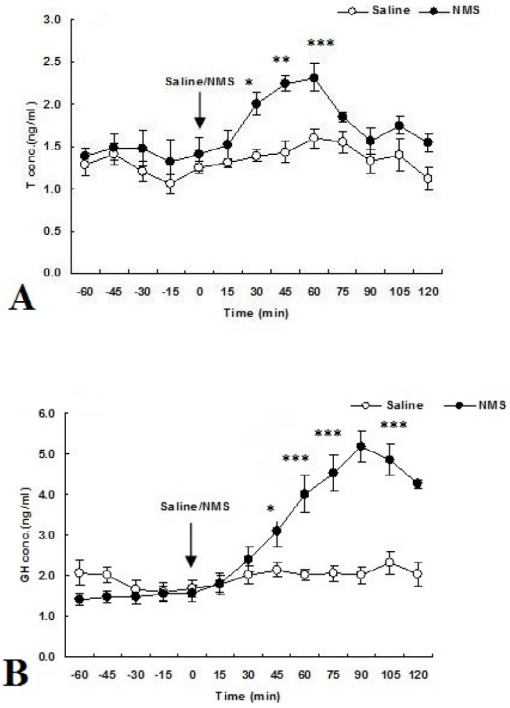

Fig. 3. (A) Mean (±SEM) changes in plasma T levels (ng/ml) before and after saline/NMS administration (at 0 min) in normal fed adult male monkeys. (B) Mean (±SEM) changes in plasma GH levels (ng/ml) before and after saline/NMS administration (at 0 min) in normal fed adult male monkeys. *P<0.05, **P<0.01, ***P<0.001 vs 0 min (ANOVA followed by post hoc Dunnett's test).

DISCUSSION

GH plays very important role in autocrine/paracrine and endocrine regulation of reproduction. It is involved in the control of growth, differentiation, proliferation, apoptosis and the secretory activities of reproductive organs. It also regulates the response of reproductive structures to GnRH and gonadotropins (Sirotkin, 2005). GH and its receptors are present in large number of tissues and cells including pituitary, uterus, mammary gland, placenta, leydig cells, granulosa cells, theca cells, cumulus cells of oocyte and many other reproductive and non-reproductive tissues (Hull and Harvey, 2000, 2001; Kaiser *et al*., 2001; Marchal *et al*., 2003). Previously it was observed that NMS causes its effects on reproductive axis through metabolic hormones like adipokines. It was hypothesized that NMS might be playing its stimulatory role in HPG axis through stimulation of the GH hormone. To find out this relationship the effect of peripheral administration of NMS on GH and T secretion was investigated in normal fed and 48-h fasting monkeys.

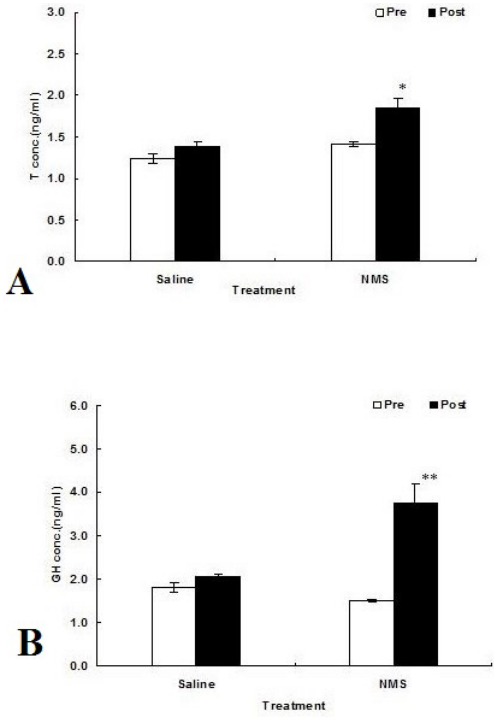

Fig. 4. (A) Comparison of mean (±SEM) plasma T concentrations (ng/ml) in 60 min pre- and 120 min post saline/NMS in fed adult male monkeys. (B) Comparison of mean (±SEM) plasma GH levels (ng/ml) in 60 min pre- and 120 min post saline/NMS in fed adult male monkeys. *P<0.05 vs pre-treatment ,**P<0.01 vs pre-treatment (Student's t test).

Fasting suppressed basal plasma T levels (P<0.001) suggesting that short term fasting has inhibitory effect on HPG axis in monkeys but no significant change was observed in case of GH concentrations. The inhibition of T was possibly due to suppressed GnRH secretion, as it was evident in previous findings that inhibitory effect of short term fasting on HPG axis in monkeys is due to inhibition of GnRH secretion (Wahab *et al*., 2008) and not by changes in pituitary response to GnRH or changes in testicular response to LH (Cameron and Nosbisch, 1991). GH plays an important role in regulation of metabolic activities during fasting conditions (Norrelund, 2005; Moller and Jorgensen, 2009) but there are discrepancies in GH release in fasting periods in different animals. Among two groups of healthy human adult males, 24-h fasting induced a significant rise in GH levels in one group while in second group GH levels remained same to the initial pre fasting values (Alkén *et al*., 2008). Similar results were also observed in young healthy human females (Beer *et al*., 1989). Several other studies also showed that up to 2.5 days fasting did not cause significant change and the GH levels remained same in adult human females (Bergendahl *et al*., 1999; Norrelund *et al*., 2001; Darzy *et al*., 2006; Sakharova *et al*., 2008). Thissen and colleagues found negative effect of fasting on GH secretion in men (Thissen *et al*., 1994). In rats 24-h fasting did not effect GH levels but five days fasting caused significant decrease in GH secretion (Ohashi *et al*., 1995). In our study 48-h fasting caused no effect (P>0.05) on GH secretion in rhesus monkeys. On the basis of these findings it is very difficult to understand this differential role of fasting on GH secretion but it is more logical to say that species differences and periods of fasting employed might have contributed in these different responses.

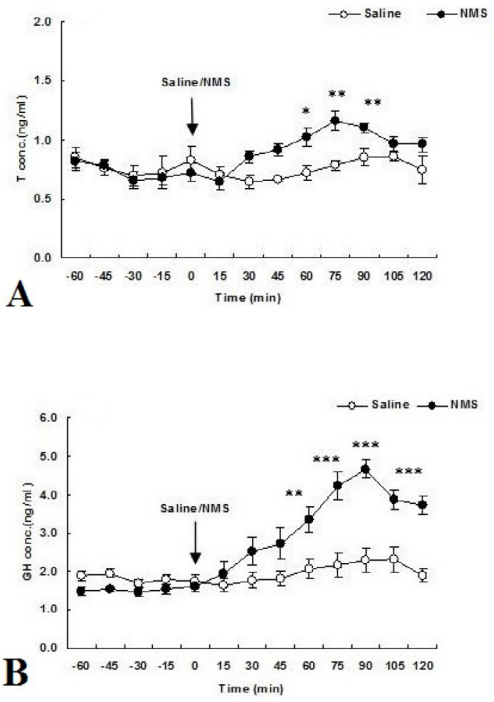

Fig. 5. (A) Mean (±SEM) changes in plasma T concentrations (ng/ml) before and after saline/NMS administration (at 0 min) in 48-h fasting adult male monkeys. (B) Mean (±SEM) changes in plasma GH concentrations (ng/ml) before and after saline/NMS administration (at 0 min) in 48-h fasting adult male monkeys. *P<0.05, **P<0.01, ***P<0.001 vs 0 min (ANOVA followed by post hoc Dunnett's test).

In our study single peripheral injection of NMS (50 nmol) significantly increased (P<0.05) T secretion in both normally fed and 48-h fasting animals. On the basis of these results it may be suggested that NMS has ability to overcome the fasting suppressed inactivity of HPG axis. Our results are in accordance with the findings of a previous study where *iv* administration of NMS

significantly induced T secretion in dose dependent manner in rhesus monkeys. This increase in T secretion is more likely due to increase in LH from pituitary and GnRH from hypothalamus. The positive role of NMS on gonadotropin release was not unpredicted as NMU, which acts through the same receptor, influenced LH secretion in OVX female rats when centrally injected (Quan *et al*., 2003, 2004). Vigo and his colleagues also found stimulatory role of NMS on LH secretion in female rats (Vigo *et al*., 2007). In present study increase in T secretion after NMS administration might also be due its stimulatory effect on LH release. These results suggested that more likely NMS is also a potent regulator of male gonadal axis in monkeys. The exact mechanism of this stimulatory response of NMS on LH secretion is yet not clear. However possibly NMS modulates expression of neuropeptides in ARC (Ida *et al*., 2005). ARC is the main site with abundant expression of NMU2R (Mori *et al*., 2005), involved in control of reproduction and energy balance. So it may be concluded that this stimulatory role of NMS in HPG axis is due to activation of ARC pathways.

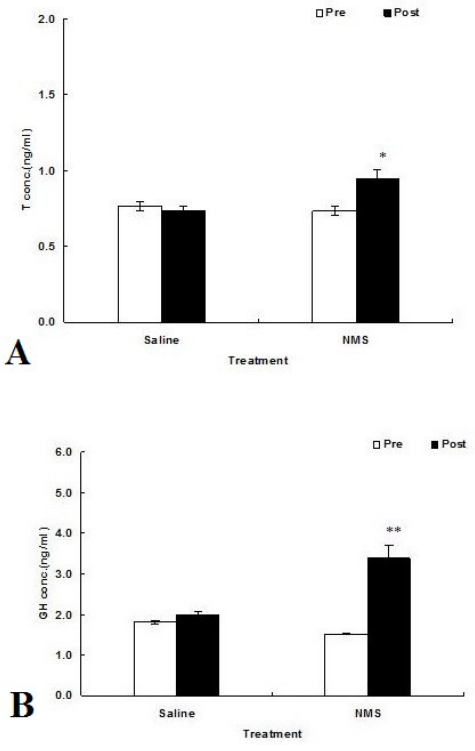

Fig. 6. (A) Comparison of mean (±SEM) plasma T levels (ng/ml) in 60 min pre- and 120 min post saline/NMS in 48-h fasting adult male monkeys. (B) Comparison of mean (±SEM) plasma GH levels (ng/ml) in 60 min pre- and 120 min post saline/NMS in 48-h fasting adult male monkeys. *P<0.05, **P<0.01 vs pre-treatment (Student's t test).

Kisspeptin and galanin like peptides, which have abundant expression in ARC, are most suitable candidates for this intermediatory action (Gottsch *et al*., 2004; Tena-Sempere, 2006). NMS also induced LH secretion in fasting female rats at diestrus. Similar response was noticed in underfed animals with different stimuli e.g. kisspeptin and galanin like peptide (Castellano *et al*., 2005, 2006). These observations are clear evidence that NMS has ability to counteract the inhibitory effect of metabolic stress on the gonadotropic axis and potentiate its role in regulation of energy balance and reproduction. The most important findings of our study were that in fasting conditions, the T response to NMS administration was delayed compared to normal fed monkeys. It is suggested that the suppression of GnRH release by metabolic fuel deficiency might be the result of decrease in NMS receptor signaling to GnRH neurons or the neurons afferent to GnRH neurons. Further studies are required to understand the exact reason for this delayed response.

A significant increase (P<0.01) in GH concentrations after NMS administration in both fed and 48-h fasting adult male monkeys suggesting that irrespective of the metabolic status of animals NMS stimulated GH secretion. The possible mechanism involved in the regulation of GH by NMS, is through the alpha-melanocyte stimulaying hormone (α-MSH) and beta-endorphin (β-END) from Pro-opiomelanocortin (POMC) in ARC. Both α-MSH and β-END are the products of the POMC gene (Smith and Funder, 1988). These POMC products stimulate the release of GHRH from hypothalamus. It was shown by Dupont and colleagues that 2 µg and higher dose of β-END resulted in a significant stimulation of plasma GH release from 6 to 10 and 20 to 30-fold respectively (Dupont *et al*., 1977). Another study (Bricaire *et al*., 1973) showed that α-MSH induced GH release in 18 among 23 normal males. Similarly, a significant rise in GH secretion by α-MSH administration in children suffering from hypopituitarism was observed (Bernasconi *et al*., 1975). NMS expression at the SCN, PVN within the brain (Mori *et al*., 2005; Ida *et al*., 2005) may regulate the POMC mRNA expression at ARC. NMS *icv* administration led to the augmentation of POMC mRNA levels in the ARC and elevated expression of c-Foss in ARC POMC neurons (Mori *et al*., 2005). These outcomes propose the involvement of α-MSH in NMS regulated feeding behavior and pituitary hormones regulation.

CONCLUSION

In summary, our results suggested that NMS is a presumptive regulator of pituitary hormones like GH and PRL. So, it is plausible that NMS might play its positive

role in HPG regulation through the stimulation of pituitary hormones like GH and PRL. Various pathways may be considered as suitable candidates for this regulation but it is very difficult to confirm the exact pathway of NMS action in this regard. Further studies are required to confirm the exact mechanism of this regulation. Our results suggest that NMS is a modulator of metabolic and reproductive axis. It induces T secretion GH secretion in both fed and fasting conditions but its effect was delayed in fasting monkeys compared to NMS treated normal fed monkeys. In fasting conditions, the effect of NMS administration showed similar response suggesting the possible role of GH in T modulation but due to unknown reasons, in normal fed monkeys the rise in GH and T levels were quite different after NMS injection. In future further studies will confirm the exact role of NMS on GH induced reproductive functions.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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