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Effect of some neurotransmitters on pituitary in bull-camel

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The present in vitro study is an attempt for profound exploration of the influence of some inhibitory (GABA and taurine) and excitatory neurotransmitters (glutamic acid and L-Argnine) on pituitary gland gonadotropic hormones in camels. For this purpose, neurotransmitters were added individually to pituitary cells in vitro. Results of current study revealed that addition of GABA alone has a potent inhibitory influence on gonadotropins release; an action which was dose-dependent. In addition, exaggerated high dose of GABA added to culture resulting in depression of GnRH activity. Inhibitory neurotransmitter taurine preserved gonadotropins release within their normal values and maintained the stimulatory potentials of GnRH. On the other side, nitric oxide had a stimulatory action at small dose rates and a depressive effect at higher dose rates. Glutamic acid as the major endogenous excitatory neurotransmitter had a potent dose-dependent excitatory effect upon FSH and LH as well as GnRH stimulatory influence.

Camels are the principal animals living in arid areas because they exhibit certain characteristics that enable them to survive the severe drought conditions. Camel meat, milk and other byproducts are indispensable for human life. This is beside the importance of camel as a race animal. Male camel is a seasonal breeder with a marked peak (the rut) during winter and spring as well as it is sexually quiescent for the remainder of the year. According to favorable environmental conditions, mature male undergoes a series of neuroendocrine changes that activate sexual activity. FSH, LH and testosterone (T) have the upper hand for regulating testicular performance and reproductive potency in the camel. Libido and sexual activity during rutting was attributed to the increased concentrations of FSH, LH and T; conversely decreased sexual activity and low fertility during the non-rutting season is linked to decreased levels of these hormones as well as increased prolactin (PRL) levels (Deen *et al.,* 2005).

Several studies have demonstrated that GnRH neurons do not possess steroid receptors, hence steroid control of GnRH secretion appeared to be mediated by other inhibitory or excitatory neurotransmitter neurons, which relay signals to the GnRH neurons (Donoso *et al.,* 1994; Tillbrook and Clarke, 2001). In this concern, previous reports revealed that inhibitory neurotransmitters as gamma-amino-butyric acid (GABA) play a physiological role in the control of anterior pituitary hormone secretion. On the other side, Bonavera *et al.,* (1993); Donoso *et al.,* (1994) reported involvement of glutamic acid, as stimulatory neurotransmitter, in gonadostate theory of puberty onset which comprises the hypothalamic pituitary gonadal system by stimulating LH release through FSH acting directly on LHRH neurons.

Therefore, the present in vitro study is an attempt for profound exploration of the influence of some inhibitory (GABA and taurine) and excitatory neurotransmitters (glutamic acid and L-Argnine) on pituitary gland gonadotropins in normal camel bulls.

Material and methods

Sixty pituitaries of apparently healthy bull camels during the breeding season were randomly collected from different slaughter houses (El-Warak, El-Basateen and El-Moneb). The pituitaries were placed immediately in phosphate buffer saline (PH 7.3) and transported in ice bag to the laboratory to be used for tissue culture technique.

In vitro determination of neurotransmitters and GnRH effect on pituitary gonadotropins. The procedure was performed under sterile conditions in a laminar flow cabinet (Scott *et al.,* 1980). All glasswares were heat - sterilized in autoclave at 121°C for 15 minutes. Liquids were sterilized by filtration using 0.2 µm membrane filter and 20 µl/ml antibiotic antimycotic solutions was added.

The procedure of Boguslawa *et al.,* (2003) was adopted to prepare pituitary culture in which camel pituitary glands were washed by 70 % ethyl alcohol for 30 seconds then by PBS. The capsule was removed and the glands were washed twice with minimal essential medium "MEM". The anterior lobes were separated and

chopped into l.0 mm cubes with scalpel. The fragments were washed five times with 5 ml MEM using sterile plastic pipette. Pieces were enzymatically dispersed during 10 minutes incubation in shaking water bath at 37° C in 0.1 % trypsin followed by mechanical dispersion by drawing the tissue fragments in and out of a pipette for 10 minutes. The trypsin solution was replaced by 10 ml PBS and the fragments were washed twice by centrifugation at 1500 rpm for 5 minutes.

The final precipitate was resuspended by adding 2 ml MEM containing fetal calf serum (50 ml / L). An aliquot was taken for counting the cells using hemocytometer. Viability was assessed by trypan blue to exclude dead cells which were stained blue. The cell suspension was diluted with MEM containing fetal calf serum (50 ml/L) then 2 ml portions containing 10^6 cells were placed in each culture well. The plates were incubated in CO_2 incubator at 37° C in water – saturated atmosphere and 5 % CO_2 for 48 hours to allow attachment of cells to the dishes.

The medium was collected by gentle aspiration from the 2 days old cells (Scott *et al.,* 1980) and considered as control. The wells were washed twice by MEM then 1.0 ml fresh MEM containing the different doses of the tested material (neurotransmitters and GnRH) was added to each well as follow:

- GnRH was added at doses of 5, 10 and 20 i.u./ml MEM.
- GABA as well as taurine was added at a rate of 0.1, 0.5 and 1.0 mM/ml MEM (Zhilin and Glenn, 2003).
- L-Arginine was added at concentrations of 10.89, 21.77 and 43.55mM (Carlos *et al.,* 1997).
- L-glutamic acid was added at 0.5, 1.0 and 2.0 mM (Eagle *et al.,* 1955).

In all excitatory and inhibitory neurotransmitters, the application time was one hour in the CO_2 incubator (Carlos *et al.,* 1997) after which the medium was aspirated using sterilized pipette and kept at -20ºC till gonadotropins assay. Moreover, one ml MEM containing the same concentration of the tested neurotransmitters plus 10 i.u.GnRH / ml medium were added to the appropriate wells. Six hours later, the medium was collected and kept at -20ºC as GnRH and/or neurotransmitter - treated samples (Boguslawa *et al.,* 2003).

In all experiments, samples were used at three replicates.

Immunological assay of gonadotropin. Levels of FSH and LH in the media samples were determined using the indirect enzyme - linked immunosorbent assay (ELISA) as outlined by Voller *et al.,* (1979). In this respect, PMSG (Folligon, Intervet Co., Holland), equivalent to camel FSH and hCG (Follutein, Zibo Pan Xin Chemicals Co., LTd) equivalent to camel LH, were used as antigens (Aboul-Ela *et al.,* 1986).

All data were subjected to statistical analysis according to Snedecor and Cochran, (1994).

Results

Effect of GnRH on FSH and LH release from male camel pituitary cell cultures during breeding season. Table 1 showed that GnRH had a potent stimulatory effect upon the release of gonadotropins from pituitary cells. It was also reported that GnRH at concentration 10 i.u. / ml showed its agonistic effect on the release of gonadotropins from pituitary cell culture; an effect which did not differ significantly than that upon applying 20 i.u. GnRH / ml.

Effect of GABA on FSH and LH release from camel pituitary cell culture before and after GnRH. From table 2 it appeared that GnRH supplementation alone resulted in significant release of both FSH and LH. GABA neurotransmitter at dose rate of 0.1 and 0.5 mMol/ml before GnRH led to significant inhibition of FSH and LH release from camel pituitaries. Addition of GABA (0.1 and 0.5 mM / ml) together with GnRH did not affect either the stimulatory effect of GnRH or the neurotransmitter inhibitory effect on gonadotropins release. The highest dose of GABA blocked the effect of GnRH on gonadotropins.

Effect of taurine on FSH and LH release from camel pituitary cell culture before and after GnRH. In healthy pituitary cell cultures (table 3), GnRH addition led to a significant release of both FSH and LH levels. Meanwhile, addition of taurine at different concentrations did not affect the release of both gonadotropins from the pituitary cell cultures either before or after GnRH supplementation.

Effect of L-Arginine on FSH and LH release from camel pituitary cell culture before and after GnRH. Table 4 disclosed that the effect of L-Arginine on the release of FSH and LH in healthy male camel pituitary cell cultures was dose-dependent. At dose rate of 10.89 mM, the neurotransmitter stimulated gonadotropin release as well as augmented GnRH effect. Other doses

did not show a significant effect on gonadotropin release.

Effect of L-glutamine on FSH and LH release from male camel pituitary cell culture before and after GnRH. Table 5 displayed that addition of L-glutamine to the media resulted in stimulation of both gonadotropins release, particularly LH, and augmented GnRH induced action. This effect was dose-dependent.

Table (1): Effect of GnRH on FSH and LH release from male camel pituitary cell cultured during breeding season (Mean ± Standard error).

Gonadotropin		Gonadotropin level (i.u. / ml)		
		5	10	20
FSH	Before GnRH	14.19±2.03	12.96±1.87[a]	13.69±0.11[a]
	After GnRH	16.17±2.42	18.65±1.03[b]	20.09±1.19[b]
LH	Before GnRH	10.06±1.05[a]	11.19±0.81[a]	12.07±1.12[a]
	After GnRH	16.13±0.71b	15.94±1.08[b]	17.01±1.14[b]

In the same row, means with different superscript differ significantly from before GnRH treatment values at P ≤ 0.01, while in the same column means with different capital letters differ significantly at P ≤ 0.05.

Table (2): Effect of GABA on FSH and LH release from healthy male camel pituitary cell culture during breeding season (Mean ± Standard error).

Concentration of GABA	FSH		LH	
	Before GnRH	After GnRH	Before GnRH	After GnRH
Control	13.22 ± 0.37aA	18.17 ± 0.76bA	11.29 ± 0.95aA	16.05 ± 0.57bA
0.1 mM/ml	9.38 ± 1.02aB	14.33 ± 0.32bB	7.76 ± 0.25aB	12.17 ± 1.31bB
0.5 mM/ml	6.16 ± 0.69aC	16.24 ± 2.67bAB	4.55 ± 0.46aC	7.70 ± 0.77bC
1.0 mM/ml	12.19 ± 2.09AB	12.78 ± 2.56AB	11.01 ± 1.23A	12.12 ± 1.68AB

In the same row, means with different small letters differ significantly from before GnRH treatment values at P ≤ 0.01, while in the same column means with different capital letters differ significantly at P ≤ 0.05.

Table (3): Effect of taurine on FSH and LH release from camel pituitary cell culture during breeding season (Mean ± Standard error).

Dose of taurine	FSH		LH	
	Before GnRH	After GnRH	Before GnRH	After GnRH
Control	13.56±1.09a	17.89±0.69b	10.96±0.82a	14.76±1.03b
0.1 mM/ml	12.22±0.91a	16.32±0.82b	11.22±1.03a	15.28±1.11b
0.5 mM/ml	13.10±0.84a	17.21±1.09b	11.43±1.09a	14.90±0.67b
1.0 mM/ml	13.17±0.72a	18.87±1.06b	10.98±1.13a	15.10±0.88b

In the same row or columns, means with different small letters differ significantly from before GnRH treatment values at P ≤ 0.05.

Table 4: Effect of L-Arginine on FSH and LH release from male camel pituitary cell culture during breeding season (Mean ± Standard error).

Dose of L-Arginine	FSH		LH	
	Before GnRH	After GnRH	Before GnRH	After GnRH
Control	13.02 ± 0.58aA	16.15 ± 0.76bA	11.36 ± 0.49aA	13.72 ± 0.47bA
10.89mM/ml	15.99 ± 0.46aB	19.66 ± 1.11bB	14.35 ± 0.13aB	17.17 ± 0.76bB
21.77mM/ml	10.00 ± 0.45C	10.23 ± 1.51C	9.01 ± 0.31C	11.22 ± 1.87AC
43.55mM/ml	7.16 ± 0.39D	6.96 ± 1.69D	8.55 ± 0.61CD	10.36 ± 0.41CD

In the same row, means with different small letters differ significantly from before GnRH treatment values at P ≤ 0.05, while in the same column means with different capital letters differ significantly at P ≤ 0.01.

Table (5): Effect of L-glutamine on FSH and LH release from male camel pituitary cell culture during breeding season (Mean ± Standard error).

Dose of L-glutamine	FSH		LH	
	Before GnRH	After GnRH	Before GnRH	After GnRH
Control	12.85 ± 0.66aA	15.98 ± 0.52bA	11.07 ± 0.61aA	14.67 ± 0.74bA
0.5 mM/ml	14.95 ± 0.45aB	18.26 ± 0.22bB	14.15 ± 0.71aB	19.02 ± 0.63bB
1.0 mM/ml	17.83 ± 0.46aC	21.43 ± 0.36bC	17.70 ± 0.44aC	21.87 ± 0.39bC
2.0 mM/ml	17.42 ± 0.56aCD	21.01 ± 0.61bCD	17.99 ± 0.56aCD	21.92 ± 0.45bCD

In the same row, means with different small letters differ significantly from before GnRH treatment values at P ≤ 0.05, while in the same column means with different capital letters differ significantly at P ≤ 0.001.

Discussion

In the current study, table 1 showed the effect of different doses of GnRH (5, 10 and 20 i.u. / ml medium) on the release of FSH and LH from male camel pituitary cell cultures during breeding season. In this concern, GnRH had a potent local stimulatory effect upon the release of both gonadotropins from pituitary cells. Also, there was a positive correlation between GnRH concentration and level of gonadotropins. These results are similar to those of Scott *et al.,* (1980); Boudjemaa *et al.,* (2000); Hinojosa *et al.,* (2004) in rats and Clarke *et al.,* (1983); Henderson *et al.,* (1989) in sheep who showed that exposure of monolayer cell culture of pituitaries to GnRH induced a significant increase in the amount of released gonadotropins into the media. Also, it was suggested by Boudjemaa *et al.,* (2000) that this effect of GnRH was through cAMP.

Therefore, the present study in camel emphasizes the direct stimulatory effect of hypothalamic GnRH on pituitary cells to release gonadotropins. Moreover, it could be suggested that the influence of GnRH was dose-dependent as gonadotropins level was unchanged by the increase of GnRH to higher concentrations.

GABA is the main inhibitory neurotransmitter of the central nervous system secreted by Purkinje cells of the cerebellum, spinal cord, retina and granule cells of olfactory bulb (Brenner, 2000). It is also present in several non-neural tissues including the endocrine organs as pituitary gland and testis (Geigerseder *et al.,* 2003). In the present study, data in table 2 showed that GABA at dose rate of 0.1 and 0.5 mM / ml before GnRH led to significant inhibition of FSH and LH release from camel pituitaries. Addition of GABA (0.1 and 0.5 mM / ml) together with GnRH did not affect either the stimulatory effect of GnRH or the neurotransmitter inhibitory effect on gonadotropins release. Concerning the inhibitory action of GABA on gonadotropins and male reproduction, Kawano and Daikoku (1981) recorded this effect on gonadotropins release particularly LH. Moreover, implantation of a potent GABA agonist into the medial preoptic-anterior hypothalamic area was able to reduce LH secretion (Lamberts *et al.,* 1983). Masugl and Fukuko (1987) reported that GABA can reduce pulsatile LH secretion by acting on hypothalamic ARC. In vitro study of Lux-Lantos *et al.,* (2001) showed that activation of pituitary GABA receptors by the specific agonist baclofen inhibited pituitary hormone secretion. The characteristic inhibition was suggested to be resemblance of these receptors to the typical presynaptic GABA sites described in the central nervous system.

From the present results, 2 dose rates of GABA exhibited the inhibitory effect on gonadotropins release; a finding which coincided with the study of Zemkova *et al.,* (2008). In addition, in the present study, the 2 inhibitory doses of GABA did not prevent the stimulatory influence of GnRH upon the release of gonadotropins; a result which came in agreement with that of Virmani *et al.,* (1990) who showed that the effect of GABA was independent on GnRH action and occurred via binding to receptors and mediated by the activation of voltage-sensitive calcium channels. The highest concentration of GABA (103 µg / ml) led to abolish its direct inhibitory effect on gonadotropins and tended to induce an inhibitory mechanism on GnRH stimulatory effect. Therefore, Nakayama *et al.,* (2006) stated that it was controversial whether GABA was stimulatory or inhibitory for the secretory output of anterior pituitary cells while in cultured pituitary cells, Ryo and Yoshitaka (2010) demonstrated that $GABA_A$ receptor activation induced excitation in GnRH neurons, which might facilitate their neuromodulatory functions by increasing their spontaneous firing frequencies.

Thus, it could be concluded that GABA alone has a potent inhibitory influence on gonadotropins release; an action which was dose-dependent. GABA action does not interfere with the stimulatory effect of GnRH unless exaggerated high dose of GABA was supplemented to the culture resulting in depression of GnRH activity.

Taurine is an inhibitory neurotransmitter related to inhibitory sulfur-containing amino acids and can cross the blood-brain barrier (Salimäki *et al.,* 2003). In pituitary cell cultures, GnRH addition led to a significant release of both FSH and LH levels. Meanwhile, addition of taurine at different concentrations did not affect the release of both gonadotropins from the pituitary cell cultures either before or after GnRH supplementation. These results came in accordance with studies of Maria *et al.,* (2000); Yang *et al.,* (2010 a) who showed that the effect of taurine on male reproduction was poorly understood and the proposed inhibitory role of taurine, and its metabolic precursor, hypotaurine, did not affect the secretion of LH or FSH

following infusion. Moreover, El-Saied (2010) found that prolonged S.C injection of 1 / 20 LD_{50} of taurine preserved reproduction in mature male Albino rats as it maintained pituitary and serum FSH and LH as well as serum testosterone levels within their normal values. In addition, it preserved semen quality; a finding which was concomitant with higher total antioxidant activity and minimal histopathological changes. In vivo and in vitro studies of Yang *et al.,* (2010 b) showed that taurine had biphasic effect on basal testosterone secretion as low concentrations could stimulate testosterone secretion, whereas high concentration of taurine could inhibit testosterone secretion.

Therefore, it could be concluded that taurine preserved gonadotropins release within their normal values and maintained the stimulatory potentials of GnRH. Furthermore, the effect of neurotransmitters upon gonadotropins was multifactorial depending upon site of neurotransmitter application, type of specific receptor activated, dose rate and period of application.

Nitric oxide (NO) is a reactive gaseous molecule, originally described as the endothelial-derived relaxing factor (Wink and Mitchell, 1998). Nitric oxide molecule is generated as a byproduct from the conversion of L-arginine to L-citrulline (Forstermann *et al.,* 1991). Data in table 4 revealed that L-Arginine at dose rate of 10.89 mM stimulated gonadotropin release as well as augmented GnRH effect. This stimulatory effect of NO came in accordance with the findings of Ceccatelli *et al.,* (1993) who showed that NO had an autocrine mediation of pulsatile secretion of gonadotropins and a paracrine effect on growth hormone secretion. Furthermore, the study of Leonor *et al.,* (1998) concluded that NO, acting at the pituitary level, stimulated gonadotropins secretion through a calcium-dependent cGMP-independent mechanism. In this respect, Bachir *et al.,* (2001) provided an evidence for the presence of several elements controlling NOS I expression in gonadotrops and demonstrated that GnRH, the prime regulator of gonadotrops function and cAMP mighty induce the transcription of NOS I in these cells. Dixit and Parvizi (2001); Jaroszewski *et al.,* (2003) implicated NO in the control of gonadotropin secretion at both hypothalamic and hypophyseal levels, LH surge mechanism and sexual behavior. Pampillo *et al.,* (2002) reported the role of NO in male reproduction and involved it in many aspects related to expression of normal libido and fertility as well as stated that NO mediated the stimulatory effect of D-aspartate on LHRH in hypothalamus; an action which was blunted when NOS was inhibited. Nicole *et al.,* (2011) described the role of NO to regulate the reproductive function. The authors proposed that NO controls the bursting activity of GnRH neurons, coordinate GnRH release from neuroendocrine terminals and provides a regulatory mechanism that may be required for appropriate delivery of GnRH to the pituitary gland.

Regarding the effect of higher doses, the current results revealed a depression in gonadotropins release and GnRH activity. These findings coincided with the results of Félix *et al.,* (2007) who described that in the pituitary gland, NO exerted inhibitory action on PRL and growth hormone as well as GnRH-induced secretion on gonadotropins. Moreover, the in vivo study of El-Saied (2010) in adult male Albino rats clarified that successive L-arginine administeration for 8 successive weeks was associated with depressed pituitary and serum FSH and LH as well as serum testosterone levels.

Thus, from the present study it could be concluded that nitric oxide had a dual mechanism on gonadotropins release and GnRH; a stimulatory action at small dose rates and a depressive effect at higher dose rates.

Glutamic acid, the major endogenous excitatory neurotransmitter, is a non-essential amino acid. The carboxylate anions and salts of glutamic acid are known as glutamates (Shigeri *et al.,* 2004). Table 5 displayed that addition of L-glutamine to the media resulted in stimulation of both gonadotropins release, particularly LH, and augmented GnRH induced action. This effect was dose-dependent at dose rates of 0.5 and 1.0 mM. These results came in accordance with a series of previous studies. In this concern, results of in vitro studies revealed direct hypophysial actions of glutamate on luteinising hormone secretion from the adenohypophysis (Zanisi and Messi, 1991; Brann, 1995). Moreover, Attademo *et al.,* (2006) showed that neuropeptide glutamic acid-isoleucine might induce LH secretion by a direct mechanism through release into the median eminence or modulation of GnRH neurons located in the preoptic area or by modulation of the GnRH terminals located in the median eminence or by an additive effect involving other

neurotransmitters or neurohormones. Hrabovszky and Liposits, (2008) added that populations of secretory cells in various endocrine organs, including the adenohypophysis, also expressed the glutamatergic markers.

Therefore, the present study concluded that glutamic acid as the major endogenous excitatory neurotransmitter had a potent dose-dependent excitatory effect upon FSH and LH as well as GnRH stimulatory influence.

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