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# MODULATION OF GONADOTROPHIN RELEASE FROM PRETREATED MALE CAMEL PITUITARY CELL CULTURE WITH INHIBIN AND BOTH CALCIUM AGONIST AND ANTAGONIST

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## ABSTRACT

**The present study was conducted to identify the modification carried out by both calcium agonist (Cacl2) and antagonist (VHcl) and inhibin like material (OTLP4) on the GnRH stimulated release of FSH and LH from male camel pituitary cell culture collected during breeding and non breeding seasons. The data obtained showed that 100 and 200 ul/ ml of GnRH was able to release both FSH and LH from pituitaries collected during the year. OTLP4 as an inhibin-like preparation was able to block FSH release from the pituitary especially during non breeding season. Calcium chloride pretreatment has a pronounced effect on LH release without any effect on FSH. The higher dose of calcium chloride unable to release LH from pituitaries collected during breeding season and it may be due to the calcium desensitizing effect. VHcl has a potent effect in blocking LH release to the media by all doses used. The blocking effect on FSH was obtained only when VHcl was used in a higher dose. The use of VHcl in the field for treatment of cardiovascular diseases and its effect on male reproduction need further *in vivo* investigation**.

## INTRODUCTION

It is well known that the principle site of action of gonadotrophin releasing hormone (GnRH) is on the pituitary gonadotrophs. The pituitary action of GnRH can be divided conveniently into an immediate release of FSH and LH within minutes **(Henderson et al., 1989)** in sheep and **(Lorena et al., 2004)** in rats. Intermediate action in the form of synthesis of gonadotrophins **(Senovilla et al., 2005)** and long term morphological changes lasting several days **(Xie et al., 2008)**. Most if not all of these actions are initiated following interaction with high affinity steroreceptors on gonadotrophs (**Lewis,2007)**.

It is documented that pituitary gonadotrophs were regulated by both steroid testosterone and non steroid inhibin as reported early by **Wang et al., (1988)** and recently by **Fafioffe et al.,( 2004).** They documented that both follicular and testicular inhibin origins act on the pituitary and modulate the stimulation exerted by GnRH. Since inhibin and GnRH do not compete for pituitary binding in vitro **(Boudjemaa et al., 2000 )** , it is appears that they may interact at the same post –binding sites.

Calcium can serve not only as an intracellular messenger, but also as an extracellular controlling the gating properties of plasma membrane

channels and acting as agonist for G protein-coupled calcium sensing receptors **(Zivadinovic et al.,2002)**. They also reported a cell type-specific role of calcium in the control of calcium signaling and secretion.

**Hirsh (2003)** reported that ion channel located in the plasma membrane provided one mean to mediate cellular adaptation to local environmental change.

**Van-Goor et al.,(2001)** and **Fiordelisio et al., (2007)** reported that calcium influx through the voltage-gated calcium channel (VGCC) was required for hormonal secretion in anterior pituitary cells and this channel expression was regulated by steroids in rodents. Also, operated calcium channel (VOCCs) plays a significant role in the regulation of intracellular calcium in cardiovascular, neuronal, pituitary and skeletal tissues as reported by **Tammela and Vuorela (2004)** who added that rat pituitary cell line possessing L-type VOCCs and can be blocked by verapamil.

Verapamil hydrochloride (VHcl) a phenyl alkylamine (PAA) is a calcium antagonist which is widely used in the therapy of cardiovascular disorders **(Lopatin and Nichols,2001 and Mason et al., 2003)**. It induced hyperprolactinemia and proopiomelanocortin (POMC) gene expression in calf **(Kile and Amoss,1988)** and mouse pituitary cell line **(Ikeda et al., 2007)**. **Hirsh (2003)** reported that calcium channel blockers have an adverse effect on male fertility.

Therefore, the present study was conducted to clarify the modification carried out by calcium agonist (Cacl2), calcium antagonist (verapamil hydrochloride) and inhibin (OTLP4) on the release of FSH and LH from male camel pituitary cell culture stimulated by GnRH during breeding and non breeding seasons.

## MATERIAL AND METHODS

Collection of ovine testicular lymph (OTL) was carried out according to the method of **Cower et al., (1964)** while the separation of protein fraction 4 was done according to **Eddie et al., (1979)** with some modification from **Abou-Aziza (2007).**

Male camel pituitaries during breeding (December to April) and non breeding (remaining of the year) were collected from slaughter houses and transported in ice bag to the lab. The glands with their enveloping capsules were washed by 70% ethyl alcohol for 30 sec. then by Ca-Mg free P.B.S 7.3. The capsules were removed and the glands were washed twice with Modified Earlie's Media (MEM, M4767, Sigma Ltd.Co.USA) as described by **Boguslawa et al., (2003)**. The anterior lobes were separated and shopped into 1 mm cubes with scalpel. The fragments were washed five times with 5 ml MEM 7.3. The pieces were enzymatically dispersed during 10 minutes incubation in shaking water bath at 37C in o.1% trypsin in Ca-Mg free P.B.S 7.3 and the fragments were washed five times by centrifugation at 1500 r.p.m 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 cells using hemocytometer. Viability of cells assessed by trypan blue exclusion as dead cells were detected by staining blue **(Boguslawa et al., 2003)** and the mean viable cells were calculated according to **Sigma catalogue (1993)** Fig (1 and 2) using the following equation:

$$\text{Viable cells / ml} = \frac{\text{Number of viable cells in 5 squares} \times 2 \times 10^6}{5}$$

Two ml portion containing million cells were placed in each culture well for 48 hrs. Media was collected as a control sample (without GnRH). The wells were washed twice then 1 ml fresh serum free MEM was added together with three different doses of GnRH (Receptal-Intervet Co. Holland ) 50µl, 100µl and 200µl (**Abou-Aziza, 2007)**to appropriate the optimum dose for FSH and LH release to the media (table, 1). Every dose used and hormone measured in the current study has specific control. Another wells were washed twice then 1 ml fresh serum free MEM was added together with three different concentration of OTLP4 ( 0.1, 0.3 and 0.6) µl/ ml( **Abou-Aziza, 2007)**; verapamil hydrochloride ( Isoptin,Abbott,Kahira harm.&Chem.Ind.Co.) with a concentration of (1.0,10.0 and 100.0)µl / ml **(Kile and Amoss 1988)** and calcium chloride in a concentration of (1.0,10.0 and 100.0)µg/ ml **(Kile and Amoss 1988).** The culture medium was collected after three days and kept at – 20Cº as GnRH free samples (Fig.3). The cells were washed once with 2 ml MEM. 1 ml MEM containing the same concentration of the tested samples plus 200µl GnRH was added to the appropriate dishes. Six hours later, the media were collected and kept at – 20 Cº as GnRH treated samples. All samples were used at three replicates.

Two types of antisera for both FSH (PMSG, Folligon, Intervet Co. Holland) and LH (hCG, Chorulon, Intervet Co. Holland) were prepared in rabbits and mice according to the method of **Taduez (1974).** FSH and LH antisera checkerboard titration with the standard and the slope of log doses response curves were done according to **Spiegel (1981).** Both hormones were estimated in the media using indirect enzyme linked immunosorbent assay (ELISA) as outlined by **Voller et al., (1979).** The potency of prepared FSH and LH antisera against 150 ng Folligon and 300 ng Chorulon in rabbits were (b=0.152 and negative control Y=0.08) and (b=0.158 and negative control Y=0.06) respectively. In mice, the potency was (b=0.263 and negative control Y= 0.03) for FSH and (b=0.311 and negative control Y=0.04) for LH. The b values of the log dose response curves of FSH and LH were 0.384 and 0.371 respectively. Significant differences were determined by analysis of variance according to **Snedecor (1971)**.

## RESULTS

Data presented in table (1) showed that the levels of both FSH and LH in the media without GnRH were not changed except during breeding, LH level was changed significantly. Addition of GnRH to the incubation media with pituitaries collected during breeding season, LH was significantly higher at all doses used in the experiments, while a significant release of FSH was observed only at higher doses

( 100 and 200µl/ml). During non breeding collection, the sensitivity of cells to GnRH treated media was decreased specially for FSH, it was released only at a very high dose (200 µl/ml). For LH, the release also was decreased at limited two higher doses (100 and 200 µl/ml).

Table (2) revealed that pretreatment with OTLP4 was resulted in blocking FSH release by GnRH during breeding collection when OTLP4 at a dose of 0.3 and 0.6 µl/ ml. During non breeding collection, the release of FSH was completely blocked by all doses used. The blocking effect of OTLP4 was very mild on the release of LH, it takes place only at a very high dose (0.6 µl/ ml) either in breeding or non breeding pituitary collections.

Table (3) showed that Cacl2 pretreated cells had no significant effect on the release of FSH by GnRH neither in breeding nor non breeding. On the other hand, a pronounced release of LH to the media by GnRH was observed especially during non breeding collection at all doses used for Cacl2. During breeding collection, a higher dose of Cacl2 (100µg/ ml) not affected LH release. Concerning VHcl pretreated pituitary cells, a small(1.0µl/ ml) and medium (10 µl/ ml) doses unable to block the release of FSH by GnRH either breeding or non breeding. Verapamil had a potent effect in blocking LH release to the media by all doses used, only unable to block this release during non breeding collection when it was used in small dose (1.0 µl/ ml).

**Table (1): The effect of GnRH on FSH and LH secretion from male camel pituitary cells.**

| Dose of GnRH (µl/ml) | Hormone conc. (ng/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FSH | | | | LH | | | |
| | Breeding | | Non Breeding | | Breeding | | Non Breeding | |
| | Without GnRH | With GnRH | Without GnRh | With GnRH | Without GnRh | With GnRH | Without GnRh | With GnRH |
| 50 | 14.09 ± 1.13 | 12.83 ±1.78 | 14.17 ± 1.11 | 16.23 ± 3.12 | 9.63 [a] ± 0.99 | 14.29* ± 1.80 | 10.13 ± 1.90 | 14.10 ± 2.23 |
| 100 | 14.37 ± 1.60 | 24.43* ± 3.73 | 12.50 ± 1.20 | 15.96 ± 3.30 | 12.57 ± 1.01 | 19.94* ± 2.39 | 11.57 ± 0.91 | 25.52** ± 3.80 |
| 200 | 14.70 ± 2.30 | 33.23** ± 4.91 | 14.20 ± 1.60 | 21.86** ± 1.72 | 14.21 [a] ± 1.11 | 25.73* ± 2.16 | 13.30 ± 1.93 | 23.38* ± 3.22 |

In the same raw, within the same season and hormone, values having the mark* and ** are significantly different at P<0.05 and at P<0.01 respectively.
In the same column, values have the mark(a) are significantly different at P<0.05.

**Table (2): The effect of OTLP4 on FSH and LH secretion by GnRH (200µl/ ml) from male camel pituitary cells.**

| Dose of OTLP4 (µl/ ml) | Hormone conc. ( ng/ ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FSH | | | | LH | | | |
| | Breeding | | Non Breeding | | Breeding | | Non Breeding | |
| | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH |
| 0.1 | 9.63 ± 1.37 | 17.73** ± 1.12 | 10.33 ± 2.12 | 11.15 ± 1.11 | 16.87 ± 1.63 | 27.65* ± 3.61 | 11.62 ± 1.11 | 25.62** ± 3.12 |
| 0.3 | 12.85 ± 1.25 | 15.55 ± 2.20 | 12.90 ±2.73 | 14.53 ± 1.66 | 14.30 ± 1.30 | 23.47* ± 3.31 | 1o.53 ± 2.12 | 23.33* ± 4.10 |
| 0.6 | 14.56 ± 1.36 | 15.57 ± 1.65 | 13.92 ± 1.13 | 13.35 ± 1.71 | 11.00 ± 1.23 | 14.55 ± 1.15 | 11.06 ± 1.23 | 14.55 ± 1.15 |

In the same raw, within the same season and hormone, values having the mark* and ** are significantly different at P<0.05 and at P<0.01 respectively.

**Table (3): The effect of Cacl2 on FSH and LH secretion by GnRH (200µl/ ml) from male camel pituitary cells.**

| Dose of Cacl2 (µg/ ml) | Hormone conc. ( ng/ ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FSH | | | | LH | | | |
| | Breeding | | Non Breeding | | Breeding | | Non Breeding | |
| | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH |
| 1.0 | 15.35 ± 2.36 | 13.85 ± 2.15 | 14.15 ± 2.42 | 17.65 ± 1.33 | 11.10 ± 2.11 | 19.00* ± 1.12 | 10.75 ± 2.39 | 19.73* ± 2.13 |
| 10.0 | 11.38 ± 1.44 | 13.40 ± 1.53 | 10.85 ± 2.11 | 16.65 ± 1.29 | 14.08 ± 2.22 | 22.85* ± 2.61 | 12.10 ± 3.21 | 27.50** ± 3.11 |
| 100.0 | 13.05 ± 2.34 | 13.50 ± 2.80 | 13.55 ± 1.73 | 13.16 ± 3.22 | 10.50 ± 2.11 | 16.25 ± 2.93 | 11.20 ± 2.02 | 20.74* ± 2.32 |

In the same raw, within the same season and hormone, values having the mark*and ** are significantly different at P<0.05 and at P<0.01 respectively.

**Table (4): The effect of VHcl on FSH and LH secretion by GnRH (200µl/ml) from male camel pituitary cells.**

| Dose of VHcl ( µl/ ml) | Hormone conc. (ng/ ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FSH | | | | LH | | | |
| | Breeding | | Non Breeding | | Breeding | | Non Breeding | |
| | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH | Without GnRH | With GnRH |
| 1.0 | 11.65 ± 2.61 | 20.60* ± 2.23 | 10.40 ± 1.47 | 19.45* ± 2.11 | 12.35 ± 1.52 | 9.50 ± 1.32 | 11.40 ± 2.13 | 19.52* ± 1.63 |
| 10.0 | 10.60 ± 1.90 | 19.75* ± 2.85 | 10.10 ± 2.31 | 20.21* ± 2.93 | 11.85 ± 1.43 | 14.55 ± 1.63 | 13.95 ± 1.55 | 14.65 ± 1.56 |
| 100.0 | 16.53 ± 1.23 | 16.65 ± 2.49 | 16.33 ± 2.12 | 17.50 ± 2.35 | 16.93 ± 1.36 | 17.85 ± 1.31 | 15.40 ± 1.20 | 15.45 ± 2.50 |

In the same raw, within the same season and hormone, values having the mark* is significantly different at P<0.05.

## DISCUSSION

The hypothalamic GnRH is the key hormone of reproduction, it causes the release of trophic hormones FSH and LH from anterior pituitary that affect gonadal function. Gonadal steroids and non steroid inhibin in turn feedback to alter hypothalamo-hypophyseal function through an indirect mechanism. In this respect, several studies have demonstrated that gonadotrophs and GnRH neurons do not possess steroid receptors **(Tilbrook and Clarke ,2001).** Therefore, recent studies were focused on other mechanisms controlling the release of gonadotrophins as by transmitters on different pituitary ion channels.

The data obtained showed that FSH and LH in the media without GnRH were mainly not significant except at breeding collection for LH. The higher doses of GnRH used (100 and 200 µl /ml) were able to stimulate the release of FSH and LH during breeding and non breeding collections, a data previously reported by **Lorena et al., (2004).**

The effect of OTLP4 as an inhibin-like preparation pretreated male camel pituitary cells showed that, medium (0.3 µl/ml) and large (0.6 µl/ml) doses were blocked the release of FSH by GnRH during breeding but it completely blocked during non breeding at any dose used which indicated higher sensitivity during non breading season, a data in agreement with the work of **Bleach et al.,(2001)** and **Abou-Aziza (2007).** The effect of OTLP4 on LH release by GnRH was mild as only obtained at higher dose (0.6 µl/ml). The specificity on FSH was previously reported by **Mann et al.,(1992)** and **Fafioffe et al.,(2004)** who reported that if inhibin whether follicular or testicular origin, it acts on pituitary, it should modulate the stimulation of FSH exerted by GnRH. **Hertal et al.,(1999)** identified inhibin binding sites on gonadotrophs and **Boudjemaa et al., (2000)** showed that both inhibin and GnRH were compete for cAMP production.

Concerning the effect of Cacl2 as calcium agonist on the release of gonadotrophins by GnRH, data showed no significant effect on the release of FSH neither breeding nor non breeding pituitaries that may indicated a mechanism of FSH release was away from the calcium channels. On the other hand, Cacl2 has a pronounced effect on the release of LH to the media by GnRH specially during non breeding collection.This data is in agreement with **Kasahara et al., (1994)** and **Zemkova et al.,(2006)** who reported that GnRH modulated the release of LH through calcium influx and it so called calcium mobilizing agent. They added that, calcium influx was played a critical role in GnRH regulation of LH subunit gene transcript and it may be acting through calcium to activate calcium calmodulin dependent protein kinase type II. The higher dose of calcium chloride ( 100 µg/ml) unable to release LH to the media by GnRH during breeding collection and it may be due to its desensitizing effect as previously reported by **Zivadinovic et al., (2002)** who

mentioned that elevation of calcium above physiological level decreased calcium in many pituitary cell types. The desensitizing effect of hormone as GnRH was also previously reported by **Winters (2004).**

Pretreatment of pituitary cells by calcium blocking agent as verapamil hydrochloride on the release of gonadotrophins by GnRH was observed. Small (1.0µl/ml) and medium (10.0µl/ml) doses unable to block the release of FSH either breeding or non breeding but it have a potent effect in blocking LH release. The blocking effect by verapamil in GnRH induction of calcium influx in neonatal rat gonadotrophs was observed by **Slanar et al.,(1997).** Also, **Tammela and Vourela (2004)** reported that rat pituitary cell line possessing L-type calcium channel and calcium can be blocked by verapamil. **Hirsh (2003)** reported that calcium channel blockers have an adverse effect on male fertility.Therefore, the use of verapamil hydrochloride in the treatment of cardiovascular diseases and its effect on reproduction need further investigation *in vivo* studies.

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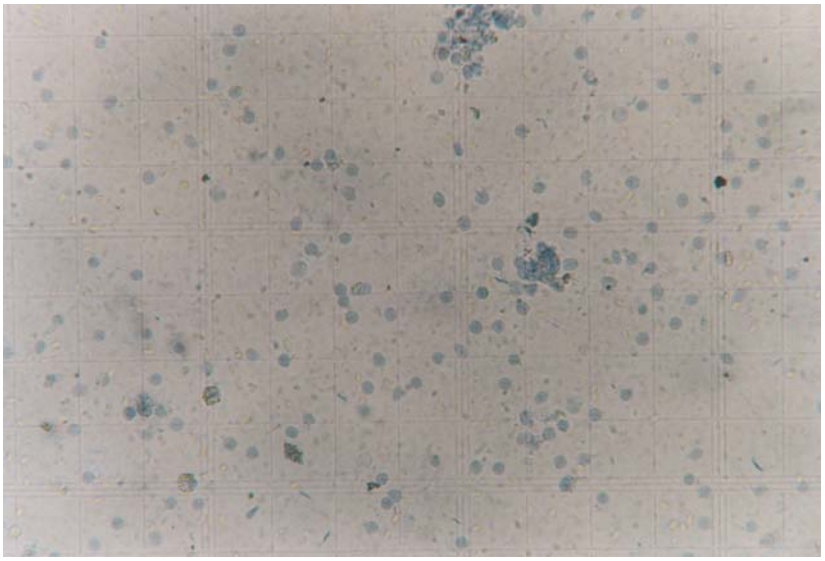


**Fig.(1):** Trypan blue exclusion for dead pituitary cells by staining blue.

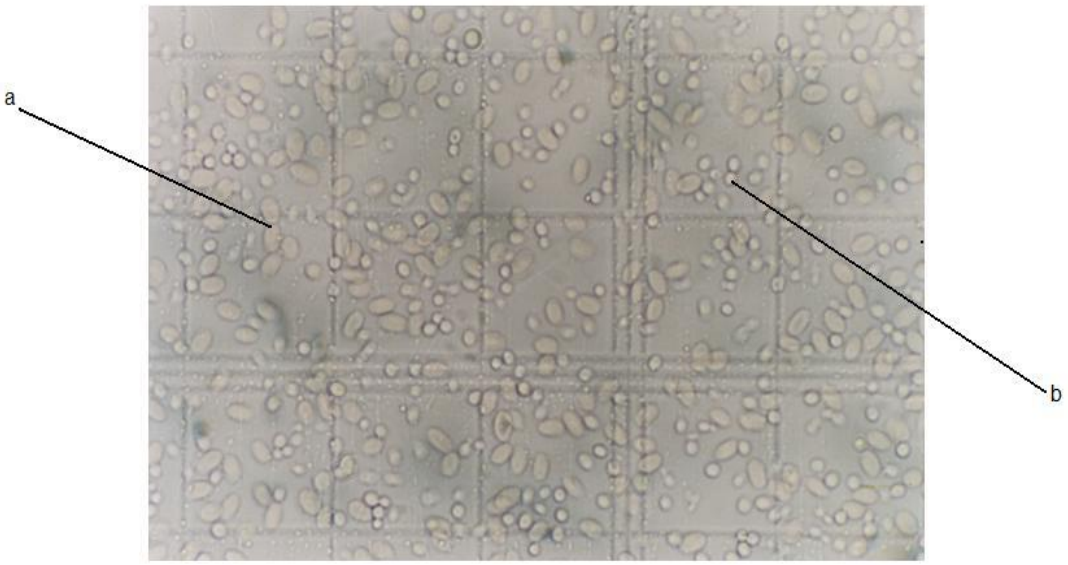
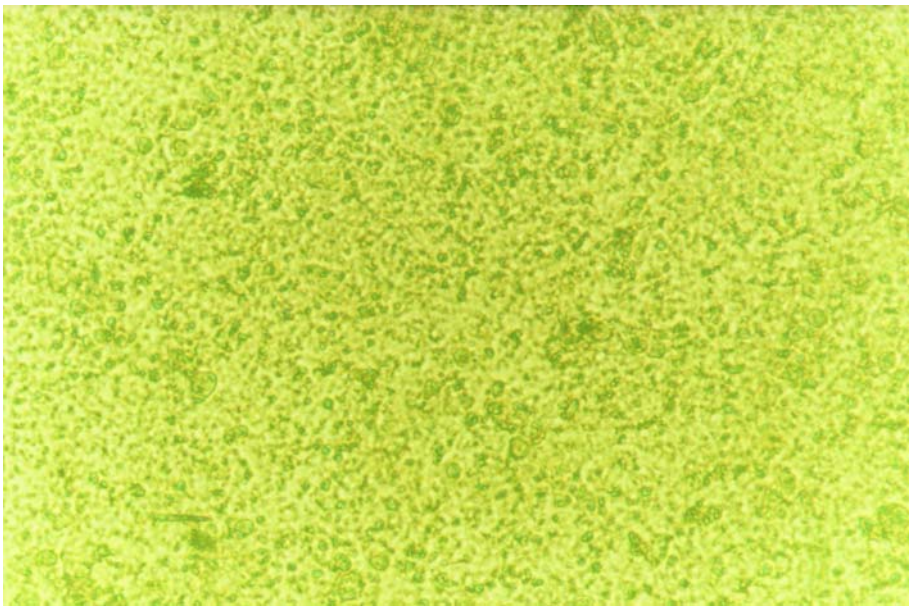



**Fig.(2):**Suspended pituitary cells on hemocytometer showing elliptical red cells(a) and alive round pituitary cells (b).



**Fig(3):** Plate showing 3-days old pituitary cell culture under inverted microscope.