Preparation of Biologically Active Recombinant Buffalo Follicle Stimulating Hormone (rbuFSH) from Buffalo pituitaries

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DUE to the immunogenic differences in the FSH-β subunits among animal species and the need for preparing specific gonadotropic hormones specific for buffaloes, the present work was aimed to prepare recombinant buffalo follicle stimulating hormone for using in improving the fertility of Egyptian buffaloes. Production of recombinant buffalo follicle stimulating hormone (rbuFSH) in *E.coli* by using recent biotechnological tools .Buffalo anterior pituitaries were collected from slaughtered buffaloes ,the buffalo common α-subunit and FSH β-subunit cDNAs was cloned and expressed to produce recombinant FSH from buffalo species *in vitro*. The RNAs extracted from buffalo pituitary glands will be reverse-transcribed and amplified by PCR and RT-PCR. The cDNAs corresponding to both subunits of buffalo hormones was cloned into the expression vector *E.coli* cells (DH5α) and pGEMT-Easy vector and transfected into pET 28a, pET 15b cells or pCR2.1-TOPO vector. Subcloning of the different subunits was carried out in pET vectors. Expression of genes was determined in the transfected cells by Northern and Western blot analysis. Recombinant buffalo FSH secreted in culture media was characterized by an *in vitro* bioassay. The recombinant products were injected to immature female rats compared to PMSG(standard) to stimulate ovarian growth and inducing of multiple follicles. The results revealed that the possibility of producing recombinant buff. FSH in vitro which are possessing efficient biological activity,stimulated the ovarian activity of immature female rats compared with standard PMSG hormone , which can be applied in buffalo-cows for improvement of reproductive efficiency .

Keywords: Recombinant FSH, Buffalo, Pituitary glands- cDNA, Cloning, RT-PCR.

Introduction

Buffaloes are one of the main sources of meat and milk in Egypt and contributes 70% of milk and 40% of meat production [1]. Globally it counted about 188.31 million buffalo according to FAO, 2010[2], 97% in Asia, 2% in Africa , and 0.2% in Europe . Buffaloes are raring under harsh environment and suffering from different reproductive problems either due to genetic and/ or environmental factors (malnutrition, heat stress, bad hygiene, infection and pollution) .The reproductive process in buffaloes is very slow, represented in prolonged postpartum acyclicity and anoestrus which are considered major sources of economic losses to buffalo breeders [3,4]. Delayed onset of post-partum ovarian activity have been recorded in lactating buffaloes [5], which may be due to suppressed pituitary

responsiveness to exogenous GnRH challenge in post-partum buffaloes [6]. Also long anestrous periods due to cessation of cyclic activity for 3 weeks or more and prolonged luteal activity for 28 days or more [7]. On the other hand buffalo's ovary has a smaller population of recruitable follicles at any given time than cow's ovary [8].

Induction of multiple ovulations (superovulation) by hormonal treatment was one of the tools previously used to improve fertility and reproductive efficiency of buffaloes through injection of gonadotropic hormones preparations, such as pituitary gonadotropin (FSH-P), porcine follicle stimulating hormone (PFSH), equine chorionic gonadotropin (eCG) or pregnant mare serum gonadotropin (PMSG) [9], in order to trigger the development of more than one dominant follicle . FSH has conventionally been

derived from pituitary tissue, despite concerns that include contamination with other pituitary hormones such as LH [10, 11], batch-to-batch variation [10,12], and potential to spread disease-causing agents [10,11]. Initially, recombinant gonadotropins such as FSH were produced as two separate subunits oligomerized after synthesis [13,]. Later efforts included studies aimed at the expression of constructs that yielded both protein subunits fused together, which quickly became the standard production method because it required fewer steps during synthesis and purification [14].

The next focus of recombinant gonadotropin research included efforts to modify the subunits to yield increased *in vivo* biological half-life (and therefore decrease the number of doses required for adequate superovulation). By engineering up to four extra N-linked glycosylation consensus sites into the α - subunit, retention time was enhanced [15,16]. Increased retention time has also been achieved by adding a section of DNA that codes for a highly glycosylated protein to one terminus of the protein [17,18]. Acidic residues tend to have reduced glomerular filtration in the kidney and a protective effect within the liver [14, 18].

Some researchers have developed fused α and β constructs that have the ability to bind to multiple gonadotropin receptors [19]. In sheep, an increase in estradiol production as a result of treatment with a dual active FSH/LH gonadotropin was similar to a combination of individual FSH and hCO hormones, suggesting the ability of the dual gonadotropin was sufficient to activate both receptors and elicit multiple biological responses [11]. Further, the technology can be tweaked to include other gene domains such as angiogenic or steroidogenic factors [11].

Recombinant gonadotropin production from modified CHO cells has been similar to hFSH, bovine (bFSH) was initially produced in CHO cells and found to have biological activity [20], but is no longer available in sufficient quantities for commercial purposes. Other production methods have been explored for both FSH and LH and have resulted in a biologically active protein. These methods employ the use of bacteria [21,22], viruses [23], insects [24,25], tobacco plants [26], yeast [27,28], and transgenic milk [29,30]. The use of hosts such as bacteria and yeast may provide a safer alternative to CHO production due to the lack of FBS utilization. Conversely, these systems do not harbor the glycosylation capabilities employed by CHO cells, and in some cases hyperglycosylate proteins, which may or may not have positive effects on biological activity [31].

rFSH was considered valuable for induction of superovulation in cattle and horses as an effort to harvest multiple embryos used for embryo transfer [32] .Revelli et al. [33]demonstrated that the overall FSH dose needed to achieve ovulation was significantly lower with r-FSH, whereas all the other studied variables did not significantly differ with either treatment. Therefore, the aim of the present work was to prepare specific recombinant gonadotropic hormones specially FSH and LH from native breeds of buffalo-pituitaries to be used in improvement of buffalo fertility.

Material and Methods

Chemicals and biological materials:

The buffalo pituitaries were procured from a local abattoir (Al warrak, Giza). RNA extraction kit from the pituitary gland using Qiagen. TRI-reagent was purchased from Sigma Co., St. Louis, Missouri, USA. Oligo-dT primers were purchased from Biosearch technologies Co., USA. The cloning vector pGEMT-Easy was purchased from Promega Life Sciences and the bacterial expression vectors pET 28α, pET 15β were purchased from Novagen.

The *E.coli* strain DH5α and BL21(DE3)pLysS were purchased from Gibco BRL and Novagen respectively. All restriction enzymes and other enzymes were purchased from New England Biolabs (NEB), Beverly, MA, USA or MBI Fermentas. PMSG was purchased fromIntervet International BV., Boxmeer-Holland.

All the other reagents were obtained from Sigma Chemical Company, USA

Total RNA extraction from Buffalo Pituitary Glands

All the glassware and plastic ware treated with 0.1%DEPC (Diethyl PyroCarbonate) (Sigma) for 8-12 h, autoclaved and dried. Fresh buffalo pituitaries were removed from the skull of buffaloes within 30 min of slaughter. The pituitaries were cut into pieces of about 10 mg by weighting and rinsed twice in RNase free DEPC treated water and quickly frozen in liquid nitrogen.

RNA was extracted from the pituitary gland using Qiagen RN easy kit according

to the manufacturer protocol. Briefly, to each 0.2 grams tissue sample, 600 µl of RLT and 6 µl mercptoethanol was added and then homogenized using Qiagen tissue ruptor at 33,000 rpm for 40 seconds and centrifuged for 3 minutes at full speed. Then, 1 volume of 70% ethanol was added to the supernatant with pipetting and then transferred to the spin column and centrifuged at maximum speed. After washing with RW1 and PRE, the columns were transferred into new collection tubes to elute the RNA using 60 µl of RNase free water. RNA was estimated by measuring the absorbance of the eluted samples at 260 nm using NanoDrop2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA).The purity of purified samples was determined by calculation of OD_{260}/OD_{280} nm ratio and the ratio was recorded.

Samples showing A260/A280 in a range of 1.8-2.0 were used for first strand cDNAsynthesis [34]

Amplification of full length using RT-PCR

Full genes of LH and FSH were amplified by RT-PCR using newly designed primers.

Reverse transcription (RT) of the extracted RNA was performed for synthesis of cDNA followed by polymerase chain reaction (PCR) of full length as one step reaction using thermal cycler.

The following reagents as showed in Table 2 were added to a micro-centrifuge tubes and mixed gently by pipetting up and down several times. For control reaction, 5 µl of distilled water was added instead of sample as a negative control. The thermal profile of 2 genes was illustrated in Table 3.

TABLE 1. Primers for gonadotrophic hormones (FSH, LH, gonadotropins and TSH) forward and backword

Name	Primer design	Ref.no.
FSH-F-33	5`d ATGAAGTCCGTCCAGTTCTGC 3`	SS302219-15
FSH-R-33	5`d TTATTCTCTGCTTTCACTGAAGGAG 3`	SS302219-16
Gonadotropin F-33	5`d TCGCTCTGGCCATTTTGTCT 3`	SS302219-17
Gonadotropin R-33	5`d TTGGAGCATCTGGCTTGGAG 3`	SS302219-18
LH-F-33	5`d ATGGAGATGTTCCAGGGACT 3`	SS302219-21
LH-R-33	5`d TTAGAGGAAGAGGATGTCTGGG 3`	SS302219-21
TSH-F-33	5`d ATGACTGCTACCTTCCTGATGTC 3`	SS302219-19
TSH-R-33	5`d TTAGATAGAAAATCCCACCATATAGGA 3`	SS302219-20

TABLE 2. RT-PCR reaction contents

Component	Volume/reaction (µl)
RNase-free water	24
5X Qiagen RT-PCR buffer	10
dNTPs(10 mM of each dNTP)	2
Forward primer (10 µmol/µl)	3
Reverse primer (10 µmol/µl)	3
QiagenOneStep RT-PCR enzyme mix	2
RNase inhibitor (20U/µl)	1
Extracted RNA	5
Final reaction volume	50

TABLE 3. Thermal cycling conditions used for amplification of full LH and FSH genes

Step	Temperature (°C)	Time (min:sec)
Reverse transcription	50	60:00
Initial PCR activation step. Taq DNA Polymerase is activated by this heating step. Reverse transcriptase is inactivated and the cDNA template is denatured.	95	15:00
Three step cycling (35 cycles)		
Denaturation	94	0:30
Annealing	50	0:30
Extension	72	2:00
Final extension step	72	15:00

According to Nolan et al.,[35]

TA cloning of LH and FSH genes

pCR2.1-TOPO vector (TOPO TA Cloning system, Invitrogen, USA, Cat. No. K4520-01) was used as a transfer vector in the cloning process of two genes. The plasmid (pCR2.1-TOPO vector) is supplied linearized with single 3′-thymidine (T) overhangs for TA cloning. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector has single, overhanging 3′ deoxythymidine (T) residues. This allows LH and FSH amplicon to ligate efficiently with the vector. The TOPO cloning reaction was simply consist of 3 μl fresh PCR product, 1 μl salt solution (200 mMNaCl and 10 mM MgCl$_2$ to increase the number of transformants), 1 μl TOPO vector (10 ng/ μl) and 1 μl sterile water to a final volume of 6 μl. The reaction was gently mixed and incubated for 5 min. at room temperature.

Purification of the Recombinant His-tagged Subunits

Induced bacterial cells were harvested by centrifugation at 5,000g for 2 min at room temperature. 10 mL of lysisbuffer (20 mM Phosphate buffer (pH 7.8), 500 mM NaCl2 and 1 mM PMSF) was added. Cells were suspended by vigorous vor texing. Lysozyme was added to a final concentration of 1 mg/mL, mixed by gentle vortexingandkept on ice for 1 h with intermittent shaking. 1% Triton X-100 was added and the mixture was further incubated for 30 min. It was then centrifuged for 20 min at 14,000g at 4°C. Supernatant was collected (in case of thesoluble protein) and loaded on Ni- NTA matrix pre-equilibratedwith loading buffer (Buffer A, 20 mMPhosphate buffer (pH 7.8), 500 mMNaCl). Matrix was washed with 10 column volumes of loading buffer. Elution was carried out sequentially with 20 mMPhosphate buffer (pH 6.0), 500 mMNaCl, 50 mMimidazole (Buffer B) and 20 mM Phosphate buffer (pH6.0), 500 mMNaCl, 250 mM imidazole (Buffer C). All thefractions were analyzed by resolving equal volumes ofeach on 13% SDS-PAGE. Also the absorbance at 280 nm was measured for all the fractions followed by measuring the immunoreactivity. Protein was estimated according toprotocol given by Bradford [36]. Proteins were essentially resolved by high resolution SDS-Polyacrylamide slab gel electrophoresis system [37].

Biological estimation of the activity of the prepared recombinant buffalo FSH in vivo:

Estimation of both FSH *in vivo* was carried out according to the method of Steelman and Pohly[38], briefly, 3 μg recombinant buffalo FSH and different doses of PMSG (1-20 i.u.) were injected intraperitoneally for immature female rats (Ages less than 21 days)

All animals will be kept to a cage with *ad libitum* food and water, at a constant temperature (22°C) and light cycle (12 hr light) during the experimental period. The animals were

classified into the following groups:Group 1(n=5): Treated with normal saline. Groups 2-5(n=5/group): Treated with 1,5,10 and 20 i.u. PMSG(FSH), respectively as a single dose (IP) and considered standard for different doses of pregnant mare serum gonadotropin (FSH).Group 6(n=5): treated with 3μg of the prepared recombinant buffalo FSH for 3 days.All animals were decapitated 24 hrs post injection of injecting hormones (PMSG or rec. buff.FSH).

The genitalia and ovaries were weighted, the numbers of follicles, corpora hemorrhagica and corpora lutea were counted under stereomicroscope.

The ovaries and uteri were fixed in 10%formalin for histological examination.
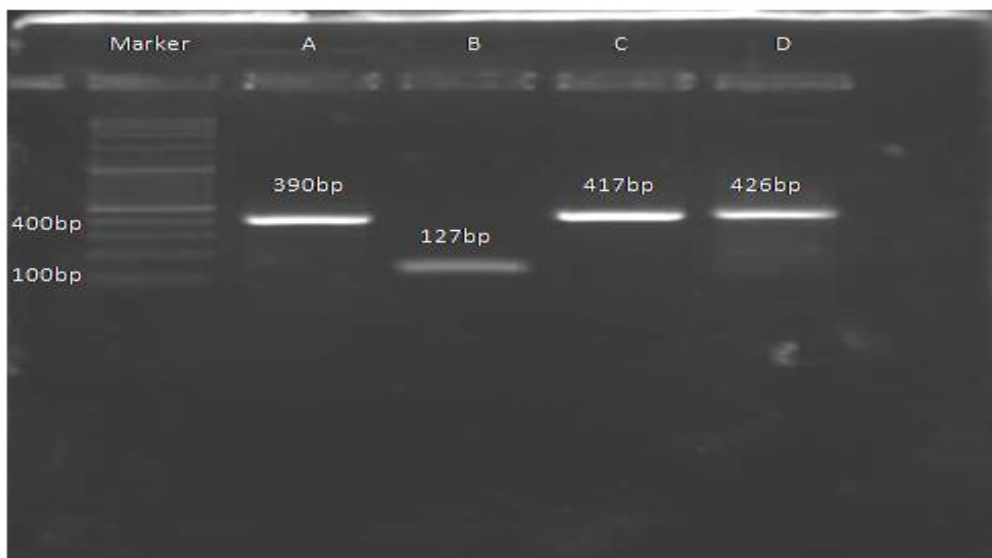
Results and Discussion

Pituitary glands obtained within half an hour of slaughter were immediately subjected to RNA isolation following the classical protocol [34] . The yield was in range of 2.2-3 pg/mg of the pituitary. The quality of the RNA as assessed by the A260:A280 ratio was clear bands and of good quality.

A total of about 15 pg of first strand cDNA was synthesized from a total of 3 pg RNA. Further the different pair of primers (Fig 2) used gave a gene specific amplification. The PCR product when analyzed on a 1% agarose gel yielded different amplicons sizes as follows FSH β 390bp, Gonadotropin α 127bp , TSH β 417 bp and LH β 426bp (Fig.1). Further PCR products were sequenced commercially using the specific primer to each gene. The sequenced genes were aligned to the other available sequences in the NCBI database of GenBank and there are very few differences in the coding sequences

Results are presented in Fig. 2 where agarose gel electrophoresis of PCR product demonstrated the amplification of LH and FSH genes to the expected molecular weight of each gene. The PCR product was successfully purified from gel using Qiaquick Gel Extraction Kit to be cloned later.

In Fig.3,where the white transformed bacterial colonies were observed within 24 hrs indicating the transformation of the competent *E. coli* cells.The four genes obtained from PCR reaction (a,b,c,d) were analyzed using sequencer (Table 3) and the results showed that the matching identity of these genes were with (FSHβ, FSHα, TSHβ, LHβ) genes, respectively.

Fig. 1. This figure shows PCR amplified products of different genes from buffalo pituitary first strand c DNA of FSH beta 390 bp (A), Gonadotropin alpha subunit (B), TSH beta 417 bp (C) And LH beta (D) . A 100 bp DNA Marker is loaded along with to ascertain the exact size.
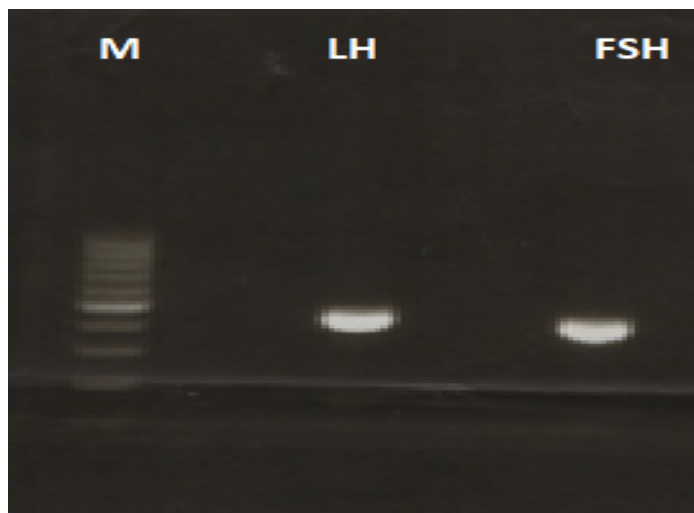
Fig. 2. Agarose gel electrophoresis showing the amplification the target genes by RT-PCR. Lane (M): 100 bp DNA Ladder.

Fig. 3&4. Transformed bacterial colonies on LB agar plate with ampicillin and X-gal for selection. White colonies represent the transformed E. coli cells by the pCR2.1-TOPO/LH and pCR2.1-TOPO/FSH constructs while the blue colonies represent the transformation by vector molecule without insert.

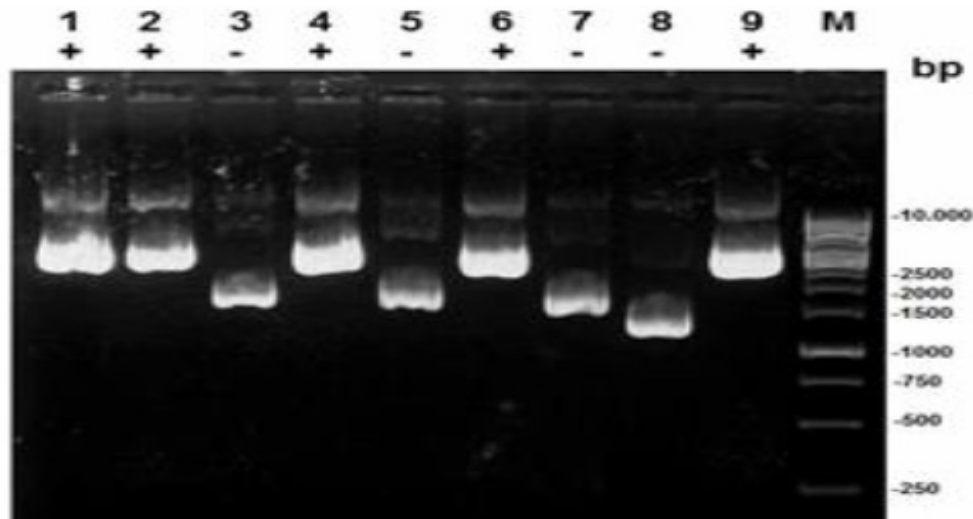**TABLE 4. Showing matching identity of four gene sequences (a,b,c,d) with genes from NCBI database**

Name	Length	Gene	Identity matching
a_1	371	Bubalusbubalisfollitropin subunit beta-like (FSH), mRNA	98%
a_2	370	Bubalusbubalisfollitropin subunit beta-like (FSH), mRNA	99%
b_3	103	Bubalusbubalisfollitropin subunit alph-like (FSH), mRNA	97%
b_4	109	Bubalusbubalisfollitropin subunit alph-like (FSH), mRNA	99%
c_5	390	Bubalusbubalis thyroid stimulating hormone, beta (TSHB), mRNA	99%
c_6	399	Bubalusbubalis thyroid stimulating hormone, beta (TSHB), mRNA	99%
d_7	407	Bubalusbubalis luteinizing hormone beta polypeptide (LHB), mRNA	100%
d_8	405	Bubalusbubalis luteinizing hormone beta polypeptide (LHB), mRNA	99%

Concentration of the purified fragment was determined using NanoDrop (Thermo Fisher Scientific, USA) .
The results showed the 65 and 85 ng/µl of LH and FSH, respectively.

TA cloning of LH and FSH genes

The different PCR products were cloned in pGEMT Easy vector .The *E.coli* component cells were efficiently transformed and about 85% of white colonies were obtained .The transformation was observed as shown in Fig. 3 in which the white transformed bacterial colonies were observed within 24 hrs indicating the transformation of the competent *E. coli* cells.

The recombinant plasmids were purified from nine white colonies of E. coli using miniprep protocol as described in methods [38]. Isolated plasmids DNA from these transformed bacteria were then detected by 1% agarose gel electrophoresis as shown in Fig 5. Samples naming 1, 2, 4, 6 and 9 were recorded as positive samples having higher molecular weights) than the negative samples.

Fig. 5. Electrophoretic analysis of the constructed plasmids after isolation by miniprep protocol. Lanes (1:9): the isolated plasmids from nine white colonies. Lane (M): 1kbp DNA Ladder. As appear, lanes 1, 2, 4, 6 and 9 represent the positive samples having higher molecular weights, of plasmid plus the inserted fragment, than the negative samples.
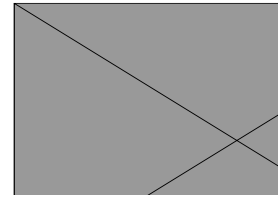
In the present work the obtained cDNAs from buffalo pituitaries was corresponding to the buffalo pituitary glycoprotein hormonal subunits have been cloned and expressed, and sequence was submitted to GenBank. The cDNA and the expressed protein for different subunits of bubaline glycoprotein hormones (FSH,LH, TSH and glycoprotein) into *E.coli* had the expected composition of the nucleotides and amino acids . The different beta subunits also have a varied similarity at the level of cDNA sequences and the deduced amino acid sequences. At the cDNAlevel the general similarity is ranged from 97 to 100 % for the various alpha and beta subunits. That the glycoprotein hormonal subunits were well conserved sequences across the species is well accepted [39,40]. The beta subunits of the glycoprotein hormones have been reported to be expressed at a very lower level in E. coli because of a self-attenuating region in the 5` sequence of the mRNA. The expression of the N-terminal gelation constructs of the bFSHβhave been shown to increase in this case [41].

Biological activity of recBuff.FSH.

Estimation of biological activity of recombinant buffalo FSH as compared with standard doses of pregnant mare serum gonadotropin(PMSG, 1-20 i.u.).

-Estimation of FSH-like activity of the prepared rec.buff FSH in immature female rats

As shown in the Figures (9a-c), the recombinant buffalo FSH possess high biological activity in immature female rats as compared with negative control (Fig.6a,b) and positive control (given different doses of PMSG as shown in Figs.7a,b & 8a,b). The activity of recBuffalo FSH in experimental rats was an increase in the volume and weight of genitalia(not shown here), enlargement and thinning of uterine horns and filled with large quantities of uterine secretion . The ovaries are large in size containing a high numbers of growing follicles at different developmental stages particularly Graffianfollicles .The present work proved that the prepared recombinant follicle stimulating hormone from pituitaries of buffaloes posse a high biological activity as estimated in experimental immature female rats in vivo.

Fig.(6a,b). Immature female rats treated IP with saline (-ve Control group), the ovaries contain scanty numbers of follicles, the uterine horns filled with scanty uterine secretions and the wall of uterus and uterine horns are tick and short in length.

Fig.(7a,b).Immature female rats treated with 1 i.u. PMSG IP, single injection, the uterine horns are thin, filled with huge amounts of uterine fluids,and the ovaries containing large numbers of follicles at different developmental stages.

Fig.(8a,b). Immature female rats treated with 20 i.u. PMSG IP, the ovaries are enlarged containing huge numbers of large and growing follicles, the uterine horns are filled with large amounts of uterine fluids. The uterine horns are short and the uterine wall is thin .

Fig.(9a-c). Immature female rats treated with 5ug recombinant buffalo FSH injected IP, the ovaries are enlarged containing huge sever numbers of large and growing follicles, the uterine horns are filled with medium amounts of uterine fluids. The uterine horns are short and the uterine wall is thin .

Conflict of interest
No conflict of interests

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تحضير هرمون تحفيز الجريبات المركب للجاموس(rbuFSH) والفعال حيويا من الغدد النخامية للجاموس

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بسبب الاختلافات المناعية في الوحدات الفرعية لهرمون تحفيز الجريبات-بيتا بين الحيوانات المختلفة والحاجة إلى تحضير هرمونات الجونادوتروبينات الخاصة بالجاموس. لذا كان الهدف من هذا العمل هو تحضير هرمون محفز للجريبات مركب لاستخدامه في تحسين خصوبة الجاموس المصري. إنتاج هرمون تحفيز الجريبات المركب (rbuFSH) في بكتيريا الاي كولاي عن طريق استخدام تقنيات البيولوجيا الحيوية الحديثة . فى هذه الدراسة تم تجميع الغدد النخامية الامامية من الجازر على وجه السرعة بعد الذبح مباشرة ثم عزل أر-أن-ايه وتحويلها الى cDNA واستنساخ كل من وحدات الفا وبيتا لهرمون تحفيز الجريبات والتعرف على التسلسل النيوكليتيدى لهرمونات الجونادوتروبين المتشابه ،وتضخيم حجمها باستخدام جهاز البلمرة المتسلسل. تم نسخ من RNAs المستخرجة من الغدد النخامية الجاموسية وتضخيمها بواسطة PCR و RT-PCR. تم استنساخ cDNAs المقابلة لكلتا الجزئين من هرمونات الجاموس في ناقلات الخلايا البكتيرية (E.coli) و (DH5α) وناقلات pGEMT-Easy واحداث عدوى إلى pET 15b ، pET 28a أو PIP2.1-TOPO. تم تحديد التعبير الجينى فى في الخلايا البكتيرية المصابة بالعدوى عن طريق التحليل نقاط الشمالية والغربية . تم استخلاص هرمون الجريبات المؤتلف (المركب) من المزرعة البكتيرية واختبار كفاءته البيولوجية فى اناث الجرزان غير البالغة والتعرف على التغيرات فى الجهازالتناسلى وعدد الجريبات المتكونه فى المبايض بالاضافة الى التغيير فى احجام واوزان الرحم والمبايض لحيوانات التجارب مقارنه بالمجموعات الضابطة السالبة والموجبة (اخذت هرمون تحفيز الجريبات المستخلص من الافراس الحوامل- PMSG)

. وقد اشارت النتائج الى ان الهرمون المحضر من جريبات الجاموس باستخدام التقنيات الحيوية يحمل كفاءة بيولوجية حيوية عالية ادت الى زيادة فى احجام واوزان الجهاز التناسلى لاناث الجزان الغير بالغة وزيادة كبيرة فى عدد الجريبات فى المبايض والتى يمكن فيما بعد من استخدامها فى تحسين الخصوبة فى الجاموس .

الكلمات الدالة: FSH المؤتلف ، الجاموس ، الغدد النخامية ، cDNA ، الاستنساخ ، RT-PCR.