Extraction and purification of Buffalo pituitary FSH ($_{bu}$FSH) with emphasis on its Biological Activity and histological changes in ovaries of Mice

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Abstract: The current study aimed to extract and purify Follicle stimulating hormone ($_{bu}$FSH) from anterior pituitaries of buffaloes and detection of the biological activity of the isolated hormone ($_{bu}$FSH). The pituitary glands were collected from slaughtering houses, quickly just after slaughter and stored in deep freezer at -20 ^{o}C. Extraction of $_{bu}$FSH was performed by ammonium sulphate precipitation method and purified by gel filtration chromatography and its purity was detected using Bio-Rad USA mini gel electrophoresis. Two experiments were performed. Experiment 1 was to design a standard log-dose response relationship of PMSG (Folligon®) and hCG (Pregnyl®) using 37 immature female albino mice. In the Second experiment, 20 immature female albino mice were divided into four groups, injected intraperitoneally (IP) with four doses (3.13, 6.25 12.5, 25 µg/ml) of purified $_{bu}$FSH, respectively, twice daily at 6 hours interval. 48-72hrs post injection; all mice were decapitated to dissect ovaries and genital tract from the surrounding tissues. Oocytes were collected from the ovaries using slicing method under stereomicroscope in Petri dishes containing M-PBS .and and the other ones preserved in 10%formalin for histological examination. The results revealed that crude $_{bu}$FSH was obtained as final product of extraction with total protein 3140 mg / kg. A maximum recovery rate (48 oocytes per mouse) was obtained with a maximum stimulation dose of 25 µg/ml $_{bu}$FSH. Histological examination of mice ovaries decapitated 72 hrs after treatment with gonadotropins revealed that multiple corpora lutea and follicles appeared in mice injected with different doses of PMSG plus 5IU hCG. The result also showed that injection of different doses of purified $_{bu}$FSH increased the number of follicles compared to the control and the best result was achieved by injection of 25µg /ml $_{bu}$FSH.
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1. Introduction:

Water buffalo contributes currently by 70% of milk and 40% of meat production in Egypt **(1).** These animals mostly show low productive and reproductive potentials such as late sexual maturity, silent heat, seasonal anoestrus, long periods of postpartum ovarian inactivity and consequently extended calving intervals which cause great economic losses in milk and meat production. Such reproductive problems in buffaloes could be due to genetic as well as environmental factors such as malnutrition, heat stress, bad hygiene, parasitic infection and pollution **(2, 3).** On the other hand, buffaloes have inherent lower fertility and poor superovulatory response than cows as buffalo's ovary has a smaller population of recruitable follicles at any given time than cow's ovary **(4).** Fertility and reproductive efficiency of buffaloes could be improved by superovulation using gonadotropic hormones preparations, such as pituitary gonadotropin (FSH-P), porcine follicle stimulating hormone (pFSH), equine chorionic gonadotropin (eCG) or pregnant mare serum gonadotropin (PMSG) **(5, 6).** But the ovulatory response to gonadotropins injection varied due to many factors such as source, batch and dose of gonadotropins, age and stage of estrous cycle of donor animal **(7).** recent study, improved that a single injection of a preparation of long-acting rbFSH (recombinant bovine FSH) produced similar superovulatory responses resulting in the production of good-quality embryos when compared with a pituitary-derived FSH preparation administered twice daily for 4 days **(8)**. Extraction and purification of gonadotropins were scarcely attempted from buffalo pituitary glands **(9)** compared with bovine **(10- 13)**, ovine **(14),** equine **(15)** and fish pituitary glands **(16).** Therefore, the present study aimed to extract and purify pituitary follicle stimulating hormone ($_{bu}$FSH) from buffalo pituitary glands. Furthermore, the purity and potency of the isolated $_{bu}$FSH will be determined in mice as a trial to improve the reproductive efficiency of buffaloes *in vivo* in the future

2. Material and Methods

Fifty seven female immature albino were kept under standard conditions of 12-hrs light/12-hrs dark at temperature 22±1 °C and fed on commercial rodent chow pellets containing 5% fiber and were supplied with clean water *ad libitum*. Mice were divided into nine groups; five groups (5 mice for each) and other four groups (8 mice for each). The latter ones were divided into 2 subgroups, subgroup (1) has 5 mice for each and subgroup (2) has 3 mice for each.

2.1. Collection, Extraction and purification of $_{bu}$FSH:

2.1.1. Collection of Buffalo pituitary glands: Pituitary glands were collected from buffaloes quickly just after slaughter from GERCO abattoir (El basattin, Cairo, Egypt), transported in thermos with ice bags to the laboratory and stored in deep freezer at -20 ^{o} C until used for hormone extraction.

2.1.2. Extraction of $_{bu}$FSH: According to **Wu *et al.* (10),** One kilogram of frozen buffalo pituitary glands thawed in 6 liters of distilled H_2O was added to protease inhibitors; phenylmethylsulphonyl fluoride (1mmol/l), pepstatin A (1.2µmol/l), leupeptin (1.7 µmol/l) and ethylene diaminetetraacetic acid (2mmol/l) at pH 5.0 and finely homogenized with a commercial blender. The mixture was then continuously stirred for 16 hrs and centrifuged at 3000 g for 1 hr at 5 ^{o} C. The supernatant was sequentially fractionated with1.8 mol/l and 3 mol/l of $(NH_4)_2SO_4$, respectively at pH 4.0. The precipitate from $(NH_4)_2SO_4$ was resuspended in 600 ml distilled H_2O and dialyzed against 50 mmol/l of ammonium bicarbonate for 16 hrs with frequent change of the buffer. The dialysate was then centrifuged at 3000 g for 1 hr. The resulting supernatant (crude $_{bu}$FSH) was further dialyzed against the equilibration buffer to be ready for purification step using gel filtration chromatography.

2.1.3. $_{bu}$FSH Purification by Gel Filtration Chromatography: 100mg of crude $_{bu}$FSH sample was prepared by dissolving in 2ml of ammonium bicarbonate (50mmol/l) at pH 8.0, at 5 C ^{o}. The prepared sample was applied to run down on the side of the column in one complete stream. Then, 1ml of running buffer was gently added. The remainder of the column was filled with running buffer. Flow rate was adjusted at 11.6ml/hr. The sample was followed along the column. Fractions were collected in tubes (5ml /tube). Three runs (R1-R2- R3) were performed along the column chromatography, from each run 19- 20 tubes or fractions were obtained. In each run, the protein concentration of all fractions obtained was determined, then the fractions with related protein concentration were pooled together and finally three peaks were obtained from each run. The protein concentrations of different fractions were estimated by Biuret reagent **(17).**

2.1.4. Detection of isolated $_{bu}$FSH purity by (SDS-PAGE): The purity of $_{bu}$FSH was detected using Bio-Rad USA mini gel electrophoresis according to **Laemmli (18)**. 20µl of diluted samples (10µl isolated $_{bu}$FSH and 10µl buffer) applied to each well. Standard pure human FSH (Siemens, Los angeles) is applied to the first and second wells with different concentrations. The run was terminated when the bromophenol blue 0.05% had reached the bottom of the gel. Then, the gel was soaked in 150ml staining solution for one hour to visualize protein in SDS-PAGE. The gel was washed several times with destaining solution until the background become completely clear.

2.2. Determination of Biological potency of purified $_{bu}$FSH activity:

To assess the biological potency of the purified $_{bu}$FSH, two experiments were performed.

2.2.1. Experiment No. 1 was designed to make a standard log-dose response relationship of PMSG (Folligon®) using 37 immature female albino mice which were divided into control group A (injected s/c with NaCl 0.9%) and 8 experimental subgroups; (B1, B2) , (C1, C2) ,(D1,D2), (E1, E2) which were injected with 1 , 2.5, 5, and 10 IU Folligon®; respectively and 48 hrs later , subgroups; B2, C2, D2 and E2 were injected with 5 IU hCG (Pregnyl®). Mice injected only with PMSG were decapitated 48hrs post injection while mice later on injected with hCG were decapitated 72 hrs to dissect ovaries and genital tract from the surrounding tissues and fats. Ovaries used for oocyte count placed in Petri dishes containing M-PBS and sliced under stereomicroscope to determine the number of oocytes and the other ones preserved in 10%formalin for histopathological studies.

2.2.2. In the Second experiment designed to determine the biological potency of the purified $_{bu}$FSH based on standard curve of PMSG ,20 immature female albino mice were divided into four groups; A, B, C, D which were injected s/c, respectively with 3.13, 6.25 12.5, 25 µg/ml of purified $_{bu}$FSH twice at 6 hours interval. 48hrs post injection; all mice were decapitated to dissect ovaries and genital tract from the surrounding tissues. Ovaries used for oocyte count placed in Petri dishes containing M-PBS and sliced under stereomicroscope to determine the number of oocytes and the other ones preserved in 10%formalin for histopathological studies.

2.3. Histopathological examination:

Mice ovarian tissue samples were stained with Haematoxylin and Eosin (H and E) for detection of different stages of follicular growth and presence or

absence of Corpora Lutea (CL) according to **Bancroft *et al.* (19).**

2.4. Statistical Analysis

All data were subjected to statistical analysis including the calculation of the mean (M), standard error of the mean (SE) and one way ANOVA at a confidence limit of 95% (P<0.01). Statistical analyses were conducted according to the method of **Armitage (18)** using practicing statistical analysis program (SPSS, version 16).Duncan's multiple range tests was used for testing pairs of means for comparison at a probability of 5**% (20-22).**

3. Results

3.1. Extraction and purification of $_{bu}$FSH.

3.1.1 Determination of protein concentration of crude $_{bu}$FSH

The protein concentrations of all crude $_{bu}$FSH fractions were shown in table (1) and three peaks were obtained from three runs (R1-R2- R3) performed along the column chromatograph in figure (1).

3.1.2 Detection of purity of isolated $_{bu}$FSH using (SDS-PAGE):

Purified buffalo pituitary extract ($_{bu}$FSH) samples obtained after gel filtration chromatography steps were analyzed under reducing conditions on Sodium dodecyl sulphatepolyacrylamide gel Electrophoresis (SDS-PAGE)compared to standard human FSH (Siemens, Los angeles, USA) as shown in figure (2).Lane 1, standard human FSH (15 µg/ml) , lane 2, standard FSH (30 µg/ml), lane 3, purified buffalo pituitary extract (25 µg/ml $_{bu}$FSH),lane 4 with purified buffalo pituitary extract (12.5 µg/ml $_{bu}$FSH), lane 5 with PMSG (10 IU), lane 6 with PMSG (5 IU) .Figure (2) showed that Major diffuse band opposite to standard FSH bands appeared in lane 3, while there was very faint band in lane 4 and another faint band in lane 5.

3.1. 3. Recovery of $_{bu}$FSH from 1kg frozen buffalo pituitary glands

The results of recovery percentage of total proteins from 1kg of frozen buffalo pituitary glands were shown in table (2) .The percentage of total protein recovered in each fraction was calculated with reference to the original preliminary extract as 100%

3.2. Determination of the biological potency of the purified $_{bu}$FSH in mice.

The pharmacological effect of PMSG and purified $_{bu}$FSH injections on folliculogenesis and oocyte development in the mice ovaries are shown in table (4). The mean number of oocytes resulted from injection of 1, 2.5,5 and 10 IU PMSG were 11 ±1.30, 13.40 ±1.50 , 18.00 ±0.70 , 22.40 ±1.36 , 33.00 ±1.30 oocytes/mouse in groups A, B, C, D and E ,respectively. The total number of oocytes increased from 13.40 ±1.50 to 33.00 ±1.30 oocytes/ mouse as a result of increase in the dose of PMSG from 1 IU to 10 IU. The mean number of oocytes resulted from injection of 6.25,12.5,25,50 µg/ml of $_{bu}$FSH in groups A, B, C, D and E were 20.20 ±1.15, 28.80 ±1.39, 47.80 ±2.63 and 31.75 ±1.65 oocytes/mouse; respectively. Also, total number of oocytes obtained from mice ovaries were increased (P< 0.001) in groups injected with different doses of $_{bu}$FSH compared to control as shown in figure (2). There were significant difference between different doses of purified $_{bu}$FSH (6.25, 12.5, 25 and 50µg /ml), respectively. Increasing stimulation dose resulted in an increase in number of recovered oocytes, the best result achieved by the dose of (25µg /ml $_{bu}$FSH) which gives recovery rate of 47.80 ±2.63 oocytes/mouse. No additional effect was found when using higher stimulation dose, and the number of recovered oocytes decreased significantly at dose (50 µg/ml) of purified $_{bu}$FSH.

3.3. Histopathological findings:

Figures (16 to24) showed that control group injected with normal saline(0.9%Nacl) revealed of growing scanty number of primordial and primary follicles, however injection of standard doses of PMSG plus 5IU hCG resulted multiple tertiary follicles, corpora haemorrhagica and multiple corpora lutea. The result also showed that injection of different doses of purified buffalo pituitary extract ($_{bu}$FSH), (6.25, 12.5, 25 and 50µg /ml) respectively, increased the number of follicles compared to the control and the best result was achieved by injection of 25µg /ml) of $_{bu}$FSH.

Table 1. Protein concentrations of $_{bu}$FSH fractions (g/dl) resulted from Sephadex G-100

The numbers of runs of $_{bu}$FSH extract through Sephadex G-100/protein conc. (g/dl)				
Fraction no.		R1	R2	R3
Fraction Tubes	1	0.012	0.016	0.019
	2	0.041	0.025	0.032
	3	0.043	0.045	0.046
	4	0.050	0.067	0.097
	5	0.058	0.081	0.058
	6	0.087	0.093	0.087
	7	0.062	0.069	0.111
	8	0.060	0.142	0.152
	9	0.095	0.167	0.134
	10	0.113	0.087	0.167
	11	0.074	0.098	0.254
	12	0.093	0.133	0.283
	13	0.124	0.178	0.311
	14	0.164	0.211	0.290
	15	0.264	0.255	0.267
	16	0.287	0.149	0.184
	17	0.153	0.076	0.113
	18	0.057	0.042	0.064
	19	0.032	0.021	0.043
	20	0.016		

Table 2. Recovery of $_{bu}$FSH from 1kg of frozen bovine pituitary gland

Fraction	Protein yield(mg)	Recovery (%)
Initial extract	8722.2	100
1.8 mol/L $(NH_4)_2SO4$ precipitate (fraction I)	6044.5	69.3
3 mol/L $(NH_4)_2SO_4$ precipitate (fraction II)	3698.2	42.4
Crude $_{bu}$FSH (fraction III)	3140	36.2
Final $_{bu}$FSH preparation	11.4	11.9

Table 3. The pharmacological effect of standard doses of Folligon® (PMSG) and purified FSH on folliculogenesis and oocyte development in mice ovaries.

Treatment	Oocyte Mean	±S.D.	±SE	Min	Max
Control	11.00[a]	2.915	1.304	8	15
PMSG 1IU	13.40[a]	3.362	1.503	9	17
PMSG 2.5 IU	18.00[b]	1.581	0.707	16	20
PMSG 5 IU	22.40[b]	3.050	1.364	19	26
PMSG 10 IU	33.00[c]	2.915	1.304	29	37
$_{bu}$FSH 6.25µg/ml)	20.20[b]	2.588	1.158	17	23
$_{bu}$FSH 12.5µg/ml	28.80[c]	3.114	1.393	25	32
$_{bu}$FSH 25µg /ml	47.80[d**]	5.891	2.634	40	55
$_{bu}$FSH 50µg /ml	31.75[c]	3.304	1.652	28	36
Total	25.00	11.430	1.723	8	55

**Superscripts of the same letter denote a non significant variation and with the different letters denote a significant variation. *(P< 0.001)*

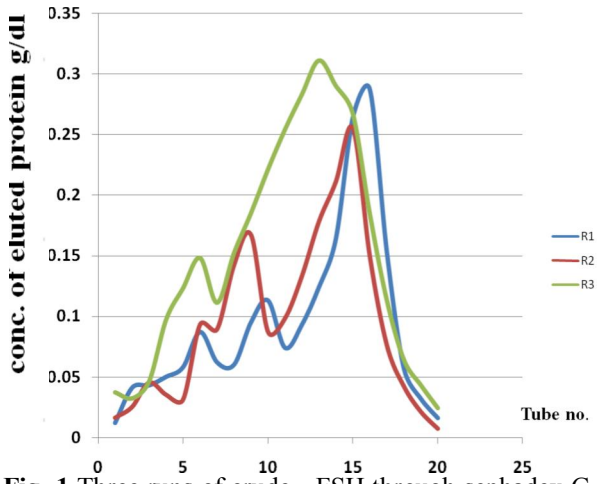

Fig. 1 Three runs of crude $_{bu}$FSH through sephadex G-100 showing concentrations of elution protiens.

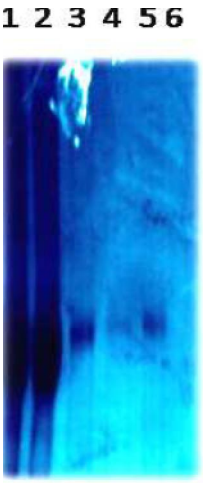

Fig. 2 SDS-PAG stained with commassi brilliant blue showing one band of the purifed buffalo pituitary extract ($_{bu}$FSH).

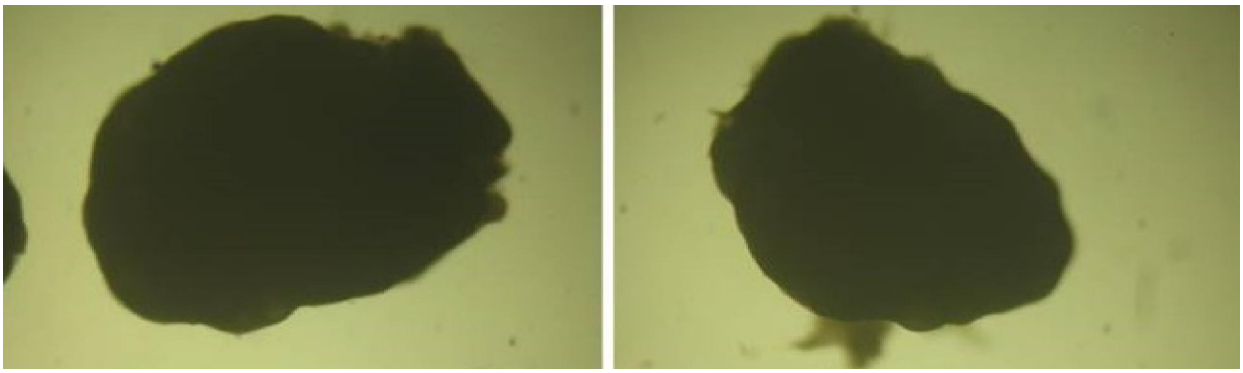

Fig. 3 Ovary of mouse (control group) injected with normal saline showing scanty number of follicles.

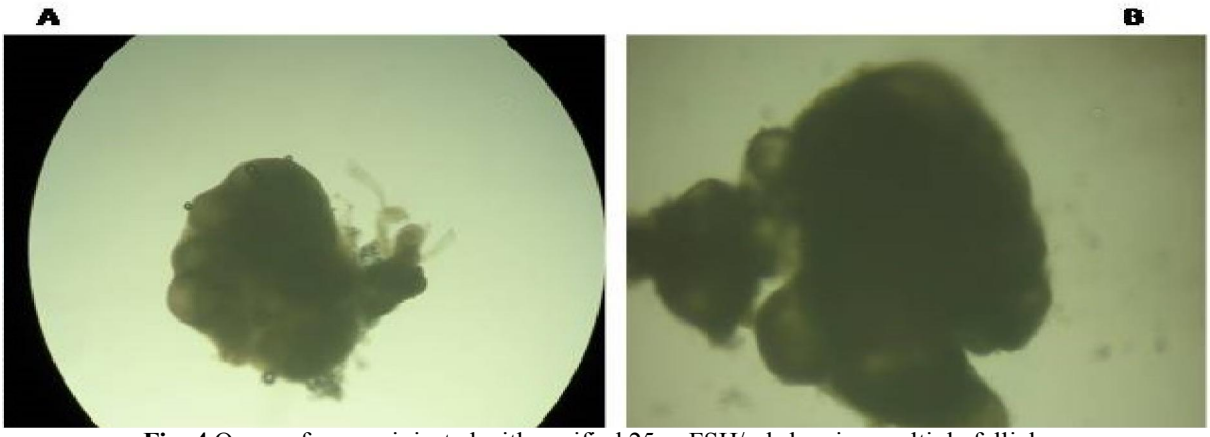

Fig. 4 Ovary of mouse injected with purified 25ug FSH/ml showing multiple follicles.

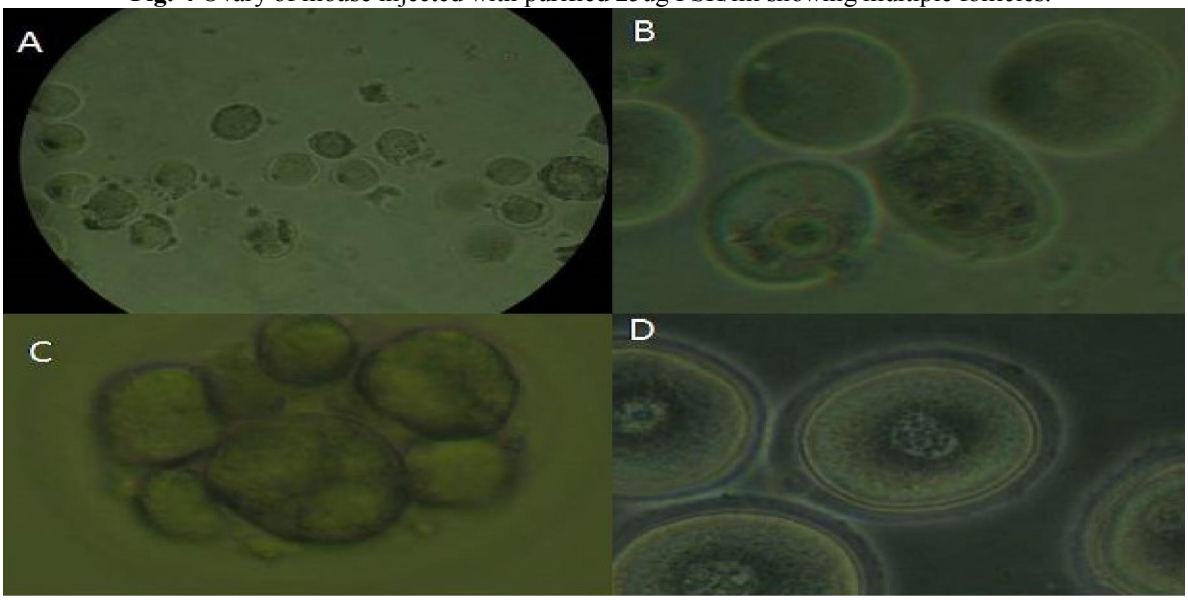

Fig. 5a-d: Showing freshly collected mice oocytes (A: oocytes 40x, B&C 100x, D 100x).

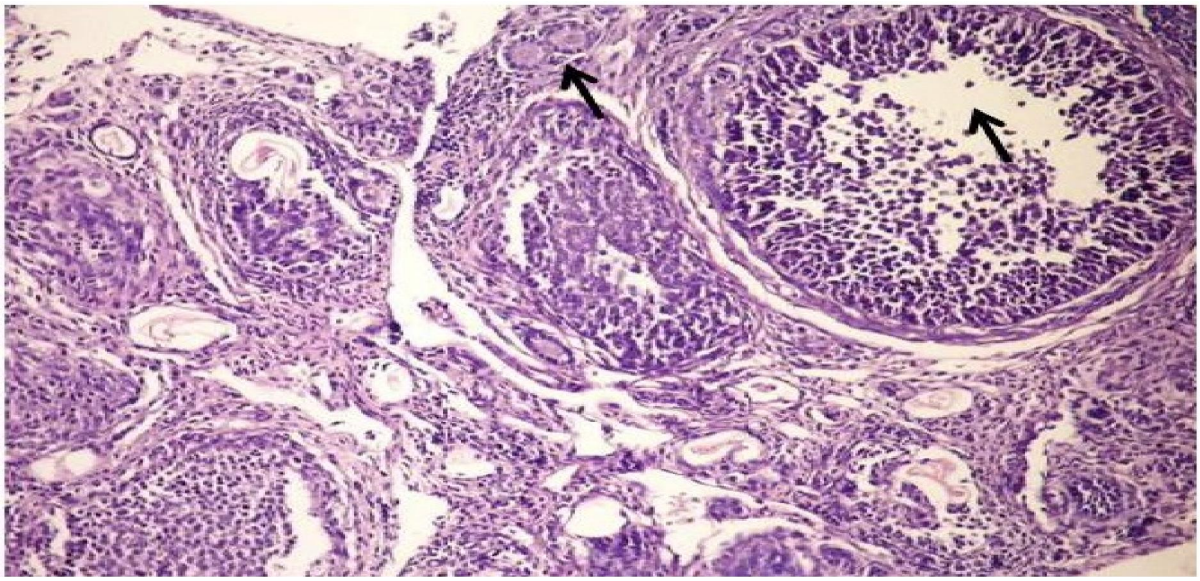

Fig. 6 Ovary of mouse from control group (H&E stain) showing primordial and primary follicles (200x).

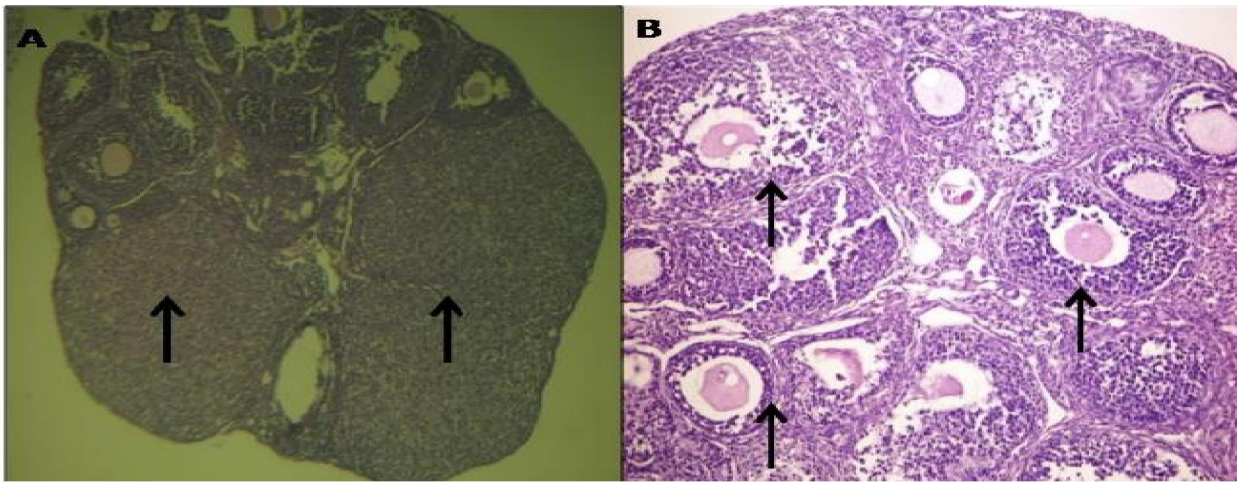

Fig. 7a,b: Ovary of mouse injected with 1i.u.PMSG, **A.** showing multiple corpora lutea (100x) from control group (H&E stain) showing primordial and primary follicles (200x),**B**. follicles of various developmental stages (200x) (H&E stain).

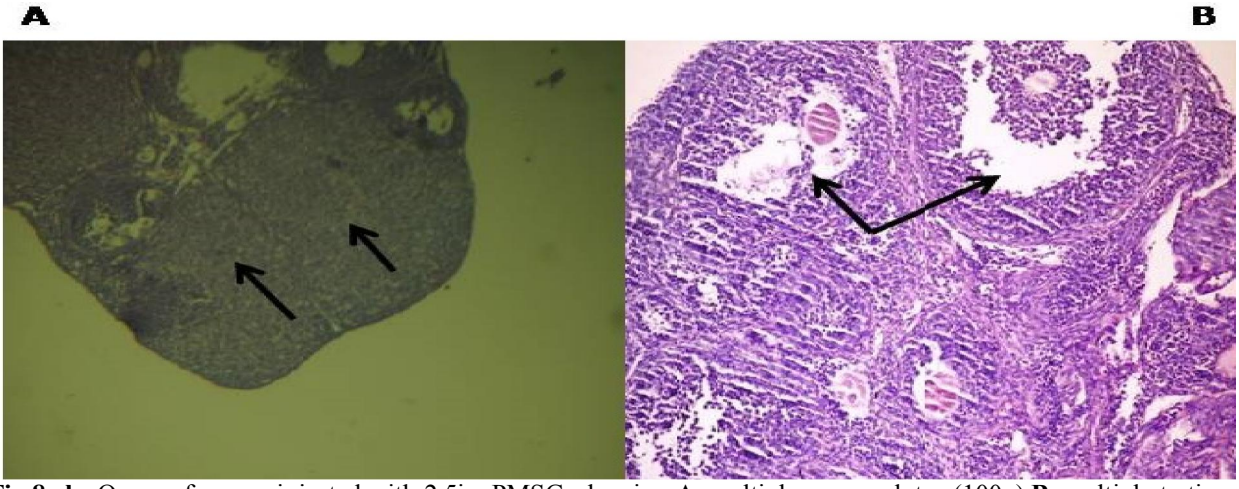

Fig.8a,b: Ovary of mouse injected with 2.5i.u.PMSG, showing **A.** multiple corpora lutea (100x) **B.** multiple tertiary follicles (200x) (H&E stain).

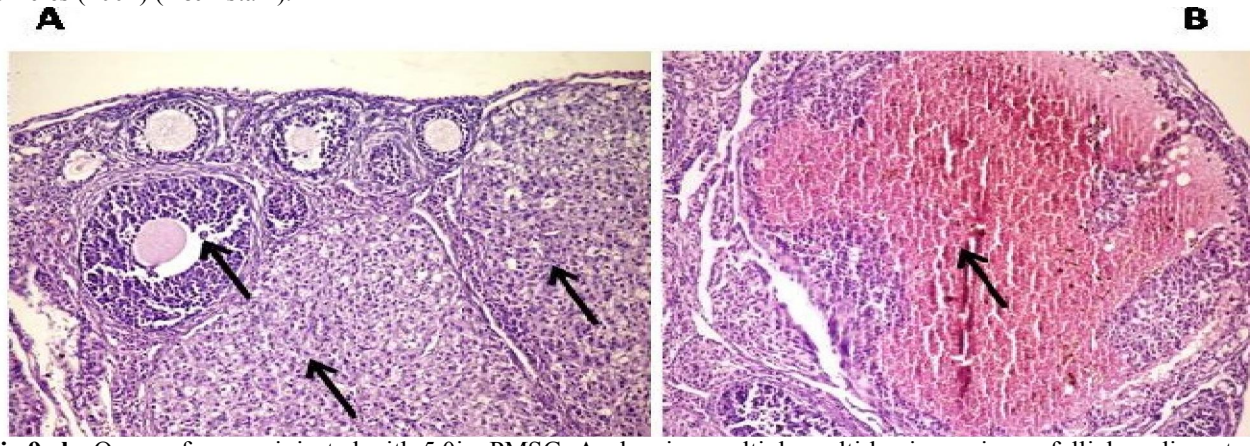

Fig.9a,b: Ovary of mouse injected with 5.0i.u.PMSG, **A.** showing multiple multi laminar primary follicles adjacent to two C.L., **B**. showing corpus haemorrhagicum (200x) (H&E stain).

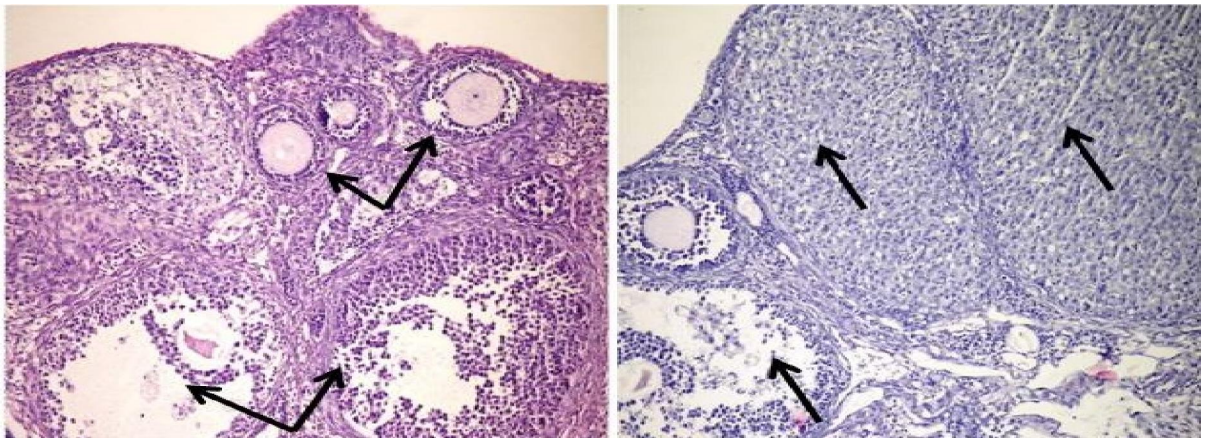

Fig. 10a,b: Ovary of mouse injected with 10 i.u.PMSG, showing two corpora lutea adjacent to primary follicles and tertiary follicles (200x) (H&E stain).

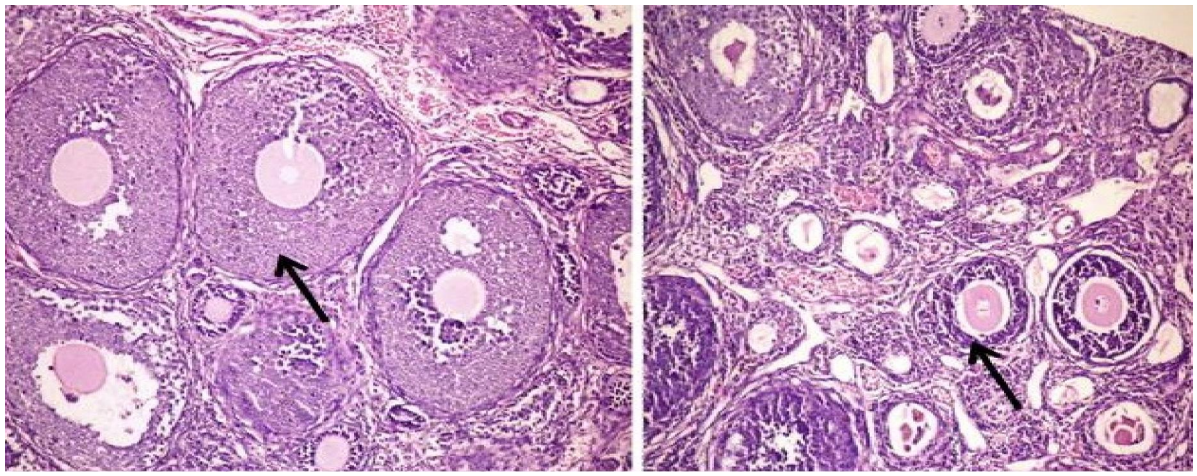

Fig.11a,b: Ovary of mouse injected with 6.25 ug/ml purified buFSH, showing multiple multi laminar primary and secondary follicles (200x) (H&E stain).

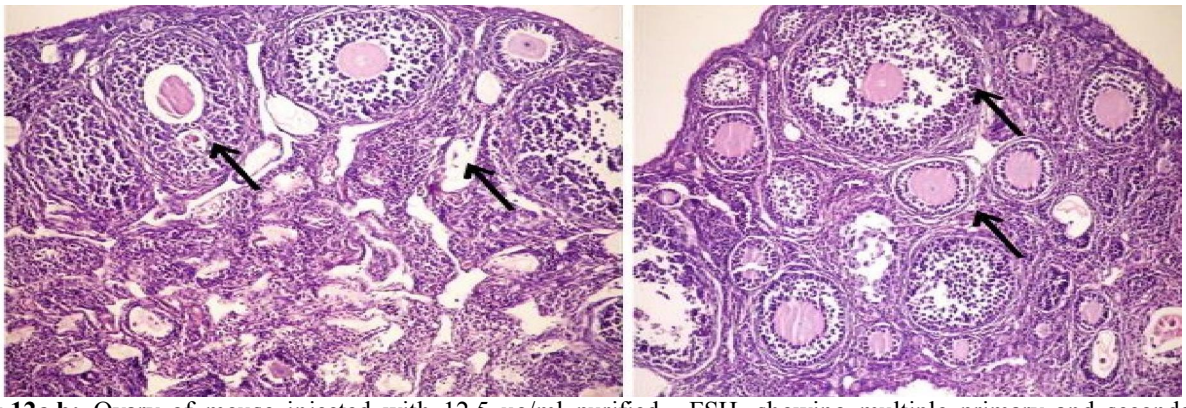

Fig.12a,b: Ovary of mouse injected with 12.5 ug/ml purified $_{bu}$FSH, showing multiple primary and secondary follicles (200x) (H&E stain).

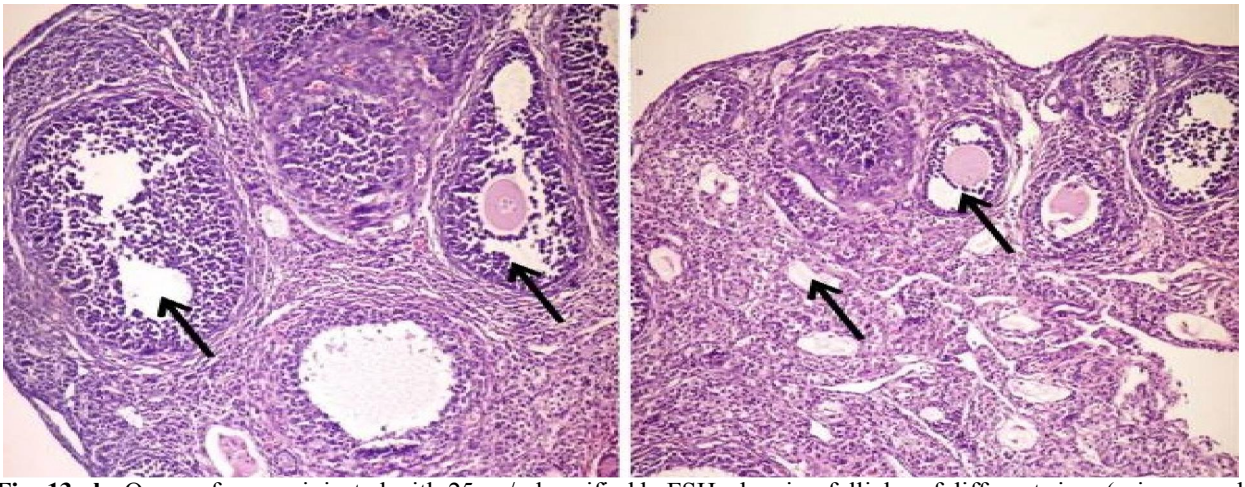

Fig. 13a,b: Ovary of mouse injected with 25 ug/ml purified buFSH, showing follicles of different sizes (primary and secondary follicles) (200x, H&E stain).

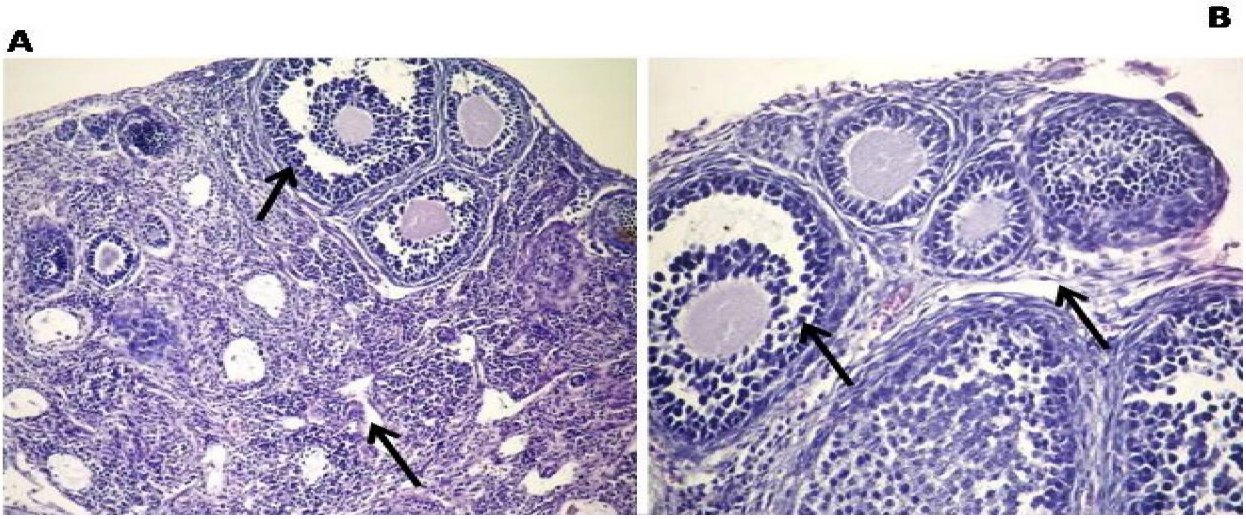

Fig. 14a,b: Ovary of mouse injected with 50 ug/ml purified $_{bu}$FSH, showing follicles of different sizes (primary and secondary and tertiary follicles, A: 200x, B:400x, H&E stain).

4. Discussion

The pituitary gonadotropins had attained a great importance in studying the reproductive pattern of domestic animals. So, many studies had been focused on the isolation and purification of FSH as well as its effect on reproduction in many animal species. $_{bu}$FSH can be used in clinical and practical field to improve reproductive efficiency of buffaloes and produce healthy oocytes capable for maturation and fertilization by natural manner.

In the present study, extraction and purification of $_{bu}$FSH was carried out through two phases, ammonium sulphate precipitation and gel filtration chromatography on sephadex G-100. In the first phase, crude $_{bu}$FSH was obtained as final product of extraction with total protein 3140 mg / kg. This result was consistent with that of **Hammam (8)** in buffaloes **; Reichert (23) and Wu *et al.* (10**) who obtained crude FSH from bovine pituitary glands after initial ammonium sulphate precipitation step with total proteins 3190 and 2880 mg / kg, respectively. While **Cheng *et al.* (9)** obtained crude FSH from bovine pituitary glands after precipitation by ammonium sulphate and acetone with yield of protein 5200 mg / kg. In the second phase, three fractions (F-A, F-B, F-C) were obtained through the application of the crude $_{bu}$FSH to sephadex G-100 within range of 4000-150,000 Da. The total protein of fraction (F-C) was 91.12µg/dl while the other two fractions were smaller and could not be detected after lyophilization. The number of fractions obtained appears to be consistent with those of **Wu *et al.* (10)** who obtained three fractions (F-A, F-B, F-C) from bovine pituitary gland after gel filtration step on sephadex G-100.

In the present study, a high degree of superovulation in mice was attained by twice-daily injections of mice with purified buffalo pituitary $_{bu}$FSH and not a single injection. It seems likely that the

ineffectiveness of a single injection of FSH to induce superovulation is a reflection of the short half-life of exogenous as well as endogenous FSH **(24, 25).**

In this work, the results showed that the injection of mice with a low dose of purified buffalo pituitary $_{bu}$FSH (6.25µg/ml) resulted in low ovulation rate (20 oocytes per mouse). While a maximum recovery rate (48 oocytes per mouse) was obtained when stimulating mice with amaximum stimulation dose of of $_{bu}$FSH (25 µg/ml), which was also significantly greater than the number ovulated in response to 10 IU PMSG (33 oocytes per mouse) which was the optimum dose of PMSG used .The number of recovered oocytes decreased significantly without any additional effect when using higher stimulation dose of purified $_{bu}$FSH (50 µg / ml). These results were consistent with **Ozgunen *et al.* (26)** who observed that increase of stimulation doses of FSH in immature female mice resulted in an increased number of recovered ova until maximum stimulation dose. They also reported that significant decrease in the number of recovered ova was observed using higher stimulation dose. The poor ovulatory response of high stimulation dose (50 µg /ml) of purified $_{bu}$FSH may be attributed to contamination of FSH with traces of LH. These results agreed with that of **Armstrong and Opavsky, (27)** who obtained high rates of ovulation, embryo development and recovery following infusion with a partially purified FSH preparation low in LH activity. This assured the importance of LH: FSH ratio vs half-life of gonadotropin preparations and the detrimental effect of high LH activity of gonadotropin used for superovulation of immature rats.

In vivo, FSH triggers granulosa cell proliferation in the early preantral phase, prevents atresia, induces the synthesis of luteinizing hormone receptors (LHR) and steroid hormones expression such as cytochrome P450 cholesterol side-chain cleavage (P450scc) mRNA in bovine, murine, porcine and human species **(29,30). Danilovich *et al.* (31**) Suggested that FSH is essential for the growth of the oocyte and follicular cells during the early antral stages. Also**, Adriaens *et al.* (29)** reported that FSH is the main driver of antral follicle growth in vivo and is essential to obtain developmentally competent oocytes during in vitro culture of follicles.Histological examination of mice ovaries performed 72 hrs after treatment with gonadotropins revealed that multiple corpora lutae and tertiary follicles appeared in mice injected with different doses of PMSG plus 5IU hCG. However, treatment of mice with PMSG or $_{bu}$FSH alone did not elicit formation of any corpora lutea (CL). It is known that both FSH and LH are required for follicle development and ovulation. The FSH stimulates granulosa cell growth and estradiol synthesis in certain primary follicles. Later on, in folliculogenesis, by the increase of LH receptors, LH stimulates theca cell growth and production of androgen which is then converted to estradiol in the granulosa cells. As a result, the dominant follicle emerges as a very efficient secretor of estradiol **(32).** Injection of hCG after PMSG lead acute rise of LH, an LH surge, triggers ovulation and development of the corpus luteum. **Barkan *et al.* (33**) reported that the preovulatory surge of LH induces ovulation by several mechanisms, including activation of proteases that weaken the follicular wall leading to rupture and extrusion of follicular content. These enzymes include a disintegrin and metalloproteinase with a thrombospondin-like motif (ADAMTS-1) and cathepsin L. In conlution, the crude $_{bu}$FSH was obtained as final product of extraction with total protein 3140 mg / kg. A maximum recovery rate of 48 oocytes per mouse was obtained by injecting mice with a maximum stimulation dose of 25 µg/ml $_{bu}$FSH. Histological examination of mice ovaries performed 72 hrs after treatment with gonadotropins revealed that multiple corpora lutea and tertiary follicles appeared in mice injected with different doses of PMSG plus 5IU hCG.

5. Conclusion

It can be concluded that Buffalo pituitary follicle stimulating hormone can be isolated from buffalo pituitary glands with high yield in pure form based on ammonium sulphate precipitation and purification on column chromatography. Our study suggested that the use of pure form of $_{bu}$FSH represent a significant improvement over previously used methods of superovulation, enabling large numbers of mice oocytes to be obtained that are capable of normal fertilization and development during the preimplantation stages.

Conflict of Interests

The authors have declared that there is no conflict of interest and there is no any fund for our research.

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