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RESEARCH ARTICLE

FSH and LH Secretion from *in-vitro* Cultured Buffalo Anterior Pituitary Cells Following Treatment with Diethyl-Stilbestrol and Medroxy-Progesterone and Their Effects on Ovarian Activity and Hematological Variables of Female Rabbits

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A B S T R A C T

Aims of this study were: to investigate whether FSH and LH secretion from *in-vitro* cultured buffalo adenohypophyseal cells can be increased by supplementing culture media with diethyl-stilbestrol and medroxy-progesterone, respectively; to monitor bioactivity of these *in-vitro* produced gonadotropins and to see if these gonadotropins have any adverse effects on hematology and internal body organs of female rabbits. Pituitary glands collected from 36 adult buffaloes slaughtered at a local abattoir were used. The anterior pituitary cells were cultured *in-vitro* using medium RPMI-1640 (code R6504-Sigma) enriched with 10% fetal calf serum and GnRH and treated with 0.5 or 1.0 mg/100 ml diethyl-stilbestrol, and 2.5 or 5.0 mg/ml medroxy-progesterone, or left as untreated control. The results showed that FSH and LH concentrations from cultures treated with low or high dose of respective steroids were higher ($P<0.05$) than those for controls. Treatment of pre-pubertal female rabbits with *in-vitro* extracted FSH increased serum FSH and LH concentrations, ovarian size and number of developing follicles (GFs) on the ovaries compared to controls ($P<0.01$). However, rabbits treated with *in-vitro* produced extract of LH showed increased serum FSH and LH, while there was no effect on ovarian size and number of GFs. Moreover, treatment of rabbits with both gonadotropins had no effects on body weight, hematological variables and internal body organs. In conclusion, diethyl-stilbestrol and medroxy-progesterone enhanced the secretion of FSH and LH, respectively, from cultured pituitary cells. Moreover, *in-vitro* produced FSH increased ovarian size, serum FSH and LH and stimulated ovarian activity, while *in-vitro* produced LH neither increased ovarian size nor stimulated ovarian activity.

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INTRODUCTION

Pituitary gonadotropins i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH) are required for normal reproductive functions in all mammals. In females, these hormones support folliculogenesis and ovulation (Kumar and Sait, 2011), while in males; they initiate and maintain spermatogenesis (Thackray *et al*., 2010). There is strong evidence that LH modulates leptin mRNA expression, which controls ovarian functions (Siawrys and Smolinska, 2013). These gonadotropins are extensively used worldwide for reproductive health management in human and animals; their use has proved effective for the treatment of true anestrus in mature females (Khalilzadeh *et al*., 2013) and stimulation of ovarian functions in young females to induce puberty (Rawlings *et al*., 2003; Daya, 2004; Lasota *et al*., 2014). This not only shortens the length of calving intervals and enhances calf crop and milk production in adult animals, but also brings early puberty in young females.

It is well established that successful *in-vitro* fertilization (IVF) and embryo transfer (ET) require stimulation of the ovary and suppression of the pituitary (Balasch *et al.*, 2001). Thus, exogenous gonadotropins are the key hormones required to maximize IVF success and induce super-ovulation in ET technology (Rahman *et al*., 2014). However, in Pakistan, these gonadotropins are

quite expensive, as they are mostly imported from abroad and their production locally in the country will serve the purpose of their inexpensive availability.

Previous research has shown that FSH and LH can be derived from pituitary extracts (Umer *et al*., 2009; Naveed *et al*., 2014). Various factors like GnRH (Ortmann *et al.*, 1999), ovarian steroids (Stormshak and Bishop, 2008) and insulin-like growth factor-1 (Baker *et al.*, 2000) can influence the secretion of gonadotropins from *in-vitro* culture of anterior pituitary.

Buffalo adenohypophysis has been successfully used to develop *in-vitro* cultures and secretions of FSH and LH have been obtained in the culture medium (Chand *et al.*, 2005). Recently, Naveed *et al*. (2014) extracted FSH from *in-vitro* cultured anterior pituitary cells of male buffalo calves; administration of this FSH to adult rabbits increased testis size, and serum levels of FSH and total cholesterol.

Aims of this study were: i) to investigate whether the production of FSH and LH from cultured buffalo anterior pituitary cells can be increased by supplementing culture medium RPMI-1640 (Rosswel Park Medical Institute-1640, code R6504-Sigma) with diethyl-stilbestrol and medroxy-progesterone, respectively, ii) to monitor bioactivity of these *in-vitro* produced gonadotropins in terms of their effects on ovarian activity of female rabbits, and iii) to see if these gonadotropins have any adverse effects on certain hematological variables and internal body organs of female rabbits.

MATERIALS AND METHODS

Collection and processing of pituitary glands: Pituitary glands from 36 adult healthy buffaloes slaughtered at a local abattoir were collected, as described elsewhere (Akhtar *et al*., 2012). Immediately after collection, each gland was shifted to the laboratory in Medium-199 (code M3769, Sigma). After removing the posterior part, the anterior part of each gland was sliced into <0.1 mm thick tissue fragments.

Preparation of cell suspension: The fragments of adenohypophysis were subjected to trypsinization using 0.05% Trypsin solution (Naveed *et al*., 2014). Cell suspension was subjected to repeated centrifugation and re-suspension of sediment in fresh trypsin solution till mono-dispersed cells were obtained. Cells were then re-suspended in Medium-199 and 5µL suspension was stained with Trypan Blue Stain (Sigma: 108K 2349). The cells stained as blue were considered dead. The concentration of live cells in suspension was recorded using Neubauer hemocytometer and sufficient amount of Medium-199 was added to achieve a concentration of $6x10^6$ cells per ml (Umer *et al*., 2009).

Culture of pituitary cells: *In-vitro* culture of pituitary cells was performed, as described previously (Umer *et al*., 2009). Briefly, 10 µL cell suspension was mixed with 25 ml of culture medium RPMI-1640, supplemented with 10% fetal calf serum, incubated under a mixture of 10% CO_2 and 90% filtered air at 38°C for 6h and stimulated with GnRH (Lecirelin, Fatro-Italy) at 1 ng/100 ml of medium. After further incubation, the medium was replaced with RPMI-1640 medium containing 0.5 or 1.0 mg/100 ml diethyl-stilbestrol to stimulate production of FSH and 2.5 or 5.0 mg/100 ml medroxy-progesterone for stimulation of LH secretion (Narasimhan and Anderson, 1981). After incubation and centrifugation, supernatant was passed through 0.22 µm syringe filter and preserved at -20°C. These preserved culture extracts were analyzed for FSH and LH levels through bovine ELISA kits (ERK B1007 and B1010, respectively, Endocrine Technologies Inc.).

Experimental rabbits and treatments: Twenty-four pre-pubertal female rabbits, aged 10-12 weeks, were randomly divided into six equal groups A, B, C, D, E and F. Rabbits of groups A and B were given extract having FSH activity ≈4.0 and 40.0 IU, respectively. Rabbits of group C (Control 1) received placebo treatment for FSH. Similarly, rabbits in groups D and E were given extract having LH activity ≈8.5 and 85 IU, respectively, while those of group F (Control 2) were given placebo treatment for LH. These extracts were given in equally divided doses twice daily for five days. Rabbits in control groups were injected culture free medium RPM1-1640 processed in the same way as for treatment groups.

Post treatment monitoring: Body weight of experimental rabbits was recorded at the start and end of treatments. About 2.0 ml blood was collected from each rabbit before treatment (Day 0) and one day after the last injection (Day 6). Each blood sample was divided into two parts; one part (1.5 ml) was used for determination of serum concentrations of FSH and LH, using commercially available kits (BioCheck Inc). The remaining 0.5 ml blood was heparinized and examined for RBC count, WBC counts, PCV and hemoglobin concentration, following standard procedures.

Examination of internal body organs: On Day 6 of the experiment, each rabbit was euthanized humanely. Number of developing GFs present on the ovaries was recorded. Length, width, weight and volume of each ovary were recorded (Uberoi and Meyer, 1967). For these parameters of ovarian size, values of the right and the left ovary of each animal were pooled to take their averages. Tissue samples were taken from liver, kidney, spleen and ovaries and processed for hematoxylin and eosin staining, following the standard procedure (Zahra *et al*., 2016).

Statistical analysis: Mean values (±SE) for various variables were computed. To study the magnitude of variation in these variables among different groups, the data were subjected to ANOVA, using General Linear Model. Least significant difference test was applied for multiple mean comparisons, where necessary.

RESULTS

Production of gonadotropins by cultured gonadotropic cells: Among the cell cultures, FSH concentrations were higher ($P<0.05$) in the culture treated with either 0.5 or 1.0 mg/100 ml of diethyl-stilbestrol compared to control (Table 1). Similarly, LH concentrations were higher ($P<0.05$) in the culture treated with 2.5 and 5.0 mg/100 ml

of medroxy-progesterone compared to control. However, non-significant differences in FSH and LH concentrations were observed between cultures treated with low and high doses of hormone (Table 1). This indicates that secretions of FSH and LH by the cultured buffalo pituitary cells were stimulated by supplementation of culture media with diethyl-stilbestrol and medroxy-progesterone, respectively.

Physical expression and body weight of rabbits: No hypersensitivity or toxic signs were expressed physically by any of the experimental rabbits up to 24 hours after the last injection. Moreover, there was no sign of swelling at the site of injection.

There were no differences in mean body weight of rabbits between Day 0 and Day 6 in any group, including controls (Table 2). Similarly, body weight of rabbits did not differ among 3 groups of each gonadotropin treatment. It indicates that *in-vitro* produced FSH and LH from buffalo adenohypophysis had no effect on body weight of pre-pubertal female rabbits.

Hematological variables: As shown in Table 2, differences in various hematological variables among rabbits of control and treatment groups before (Day 0) and after treatment (Day 6) for each gonadotropin treatment were non-significant. Similarly, differences in these parameters between two treatment groups for each gonadotropin (low and high dose) were also non-significant. This shows that *in-vitro* produced FSH and LH from buffalo adenohypophysis had no adverse effect on hematological variables of pre-pubertal female rabbits.

Serum FSH and LH concentrations: In rabbits treated with both low, as well as high, dose of *in-vitro* extracted FSH, the serum FSH and LH concentrations on Day 6 were significantly higher ($P<0.01$) compared to those at Day 0 (Table 2). However, the magnitude of increase in FSH concentration was much higher (about 100 fold) compared to that of LH (7-12 fold). A similar trend was seen for the serum concentrations of FSH and LH in rabbits given *in-vitro* extracted LH. However, there were no differences in serum concentrations of FSH and LH in rabbits of both control groups between Day 0 and Day 6 of the experiment. Moreover, low or high doses of *in-vitro* produced FSH or LH showed no difference in serum FSH or LH levels of rabbits.

Table 1: Effects of low and high doses of diethyl-stilbestrol and medroxy-progesterone on secretions of FSH and LH by *in-vitro* cultured buffalo adenohypophyseal cells

Treatments	Dose (mg/100 ml)	Gonadotropin production	
		FSH (ng/ml)	LH (ng/ml)
Diethyl-stilbestrol	0.5 (Low)	0.21±0.18[a] (0.009-0.870)	-
	1.0 (High)	2.33±0.54[a] (1.57-3.09)	-
	Control	0.09 ±0.07[b] (0.001-0.291)	-
Medroxy-progesterone	2.5 (Low)	-	75.58±36.45[a] (10.01-159.40)
	5.0 (High)	-	241.8±142.9[a] (45.70-438.00)
	Control	-	0.05±0.03[b] (0.012-0.171)

Values (mean±SE) with different letters within a column differ significantly from one another ($P<0.05$). Ranges are given within parentheses.

Ovarian morphometry: As shown in Table 3, rabbits given low, as well as high, dose of extracted FSH showed higher ovarian length compared to control ($P<0.01$). However, there was no difference in ovarian length of rabbits given either low or high FSH dose. Similar trend was seen for the width, weight and volume of the ovaries. Moreover, the number of GFs on the ovaries of rabbits treated with low and high dose of *in-vitro* extracted FSH showed a similar trend as that of various parameters of ovarian size. However, the effect of *in-vitro* extracted LH on ovarian length, width, weight, volume and the number of GFs was non-significant.

Histological observations: No gross or histopathological changes could be seen in any of the internal organs examined. Ovaries of the rabbits treated with *in-vitro* extracted FSH showed multiple numbers of developing or developed follicles. . However, rabbits of control groups showed occasional small developing follicles. No corpora lutea could be detected on ovary of any rabbit of treated or control groups.

DISCUSSION

Production of FSH and LH from cultured pituitary cells: One objective of the present investigation was to explore the possibility of stimulation of FSH and LH secretion from *in-vitro* cultured buffalo adenohypophyseal cells through supplementation of culture medium with diethyl-stilbestrol and medroxy-progesterone, respectively. The results revealed that supplementation of culture media with low, as well as high, dose of sex steroids significantly increased the production of FSH and LH by cultured buffalo adenohypophyseal cells. However, the effect of the low and high dose of each steroid hormone was similar. Increased secretion of LH by gonadotrophs in the presence of estradiol and dexamethasone was also reported by Ortmann *et al.* (1999) and Baker *et al*. (2000). According to Thackray *et al.* (2010), gonadal steroids and peptides modulate FSH and LH levels via feed back to the anterior pituitary and the hypothalamus. Lesoon and Mahesh (1992) observed that incubation of pituitary cell culture separately for different time periods following supplementation with progesterone enhanced the secretion of LH and FSH when incubated for 1-6 hr, whereas incubation for periods beyond 12 hrs inhibited FSH and LH release by cultured pituitary cells. In the present study, supplementation of culture medium with 1.0 mg/100 ml of diethyl-stilbestrol or 5.0 mg/100 ml medroxy-progesterone yielded 2.33±0.54 and 241.80±142.90 ng/ml of FSH and LH, respectively. Umer *et al*. (2009) reported FSH concentration of 2.42±0.11 mIU/ml (3.66ng/ml) in extract obtained from adult buffalo gonadotropic cells cultured *in-vitro*. Similarly, Naveed *et al*. (2014) observed FSH activity equivalent to 3.89±0.57 mIU/ml (5.89ng/ml) in the extract of cultured adenohypophyseal cells of male buffalo calves, following supplementation of culture medium with stilbestrol. These workers, however, did not monitor LH levels in these extracts. Inter-species DNA sequence differences may contribute to divergent hormonal regulation, resulting in observable differences in FSH secretion across species (Bernard *et al*., 2010).

Table 2: Values of body weight and various hematological variables in pre-pubertal female control rabbits and those treated with *in vitro* produced FSH and LH

Variables	Treatment days	FSH treated groups			LH treated groups		
		A (4.0 IU)	B (40.0 IU)	C (Control-1)	D (8.5 IU)	E (85.0 IU)	F Control-2)
Body weight (g)	0	722±12.25	698±15.81	695±10.44	711±12.25	708±08.09	721±08.09
	6	712±12.25	706±12.25	705±08.09	696±15.81	717±08.09	696±10.44
RBC count (10^6/µL)	0	5.56±0.19	6.26±0.19	5.93±0.24	5.52±0.19	5.72±0.19	5.97±0.24
	6	5.76±0.19	5.98±0.19	5.93±0.24	5.62±0.19	5.54±0.19	6.00±0.24
WBC count (10^3/µL)	0	7.76±0.30	7.64±0.30	7.83±0.39	7.78±0.30	7.44±0.30	7.53±0.39
	6	7.38±0.30	7.38±0.30	7.47±0.39	7.70±0.30	7.64±0.30	7.67±0.39
Hemoglobin concentration (g/dL)	0	11.32±0.18	11.12±0.18	10.60±0.23	10.60±0.18	10.72±0.18	10.73±0.23
	6	10.70±0.18	10.66±0.18	10.40±0.23	10.36±0.18	10.86±0.18	10.60±0.23
Packed cell volume (%)	0	34.80±0.81	34.00±0.81	31.67±1.05	34.60±0.81	34.80±0.81	34.67±1.05
	6	32.80±0.81	32.40±0.81	33.00±0.15	34.00±0.81	33.40±0.81	33.33±1.05
Serum FSH (mIU/ml)	0	4.38±1.94[a]	3.54±1.94[a]	3.61±2.45[a]	4.53±1.94[a]	3.13±1.94[a]	4.09±2.45[a]
	6	422.41±7.63[b]	462.70±7.63[b]	3.71±2.45[a]	308.40±7.63[b]	273.15±7.63[b]	9.18±2.45[a]
Serum LH (mIU/ml)	0	3.85±1.13[a]	4.12±1.13[a]	3.48±1.32[a]	4.27±1.13[a]	3.58±1.13[a]	3.67±1.32[a]
	6	22.85±3.13[b]	41.12±3.13[b]	4.48±2.52[a]	34.27±3.13[b]	43.58±3.13[b]	5.67±2.52[a]

Values (mean±SE) with different letters within the same row for each gonadotropin differ significantly (P<0.01).

Table 3: Biometrical values of ovaries and number of Graafian follicles of control rabbits and those treated with *in vitro* produced FSH and LH

Variables	FSH treated groups			LH treated groups		
	A (4.0 IU)	B (40.0 IU)	C (Control-1)	D (8.5 IU)	E (85.0 IU)	F (Control-2)
Ovarian length (cm)	0.73±0.01[a]	0.71±0.01[a]	0.60±0.01[b]	0.60±0.01[b]	0.57±0.01[b]	0.59±0.01[b]
Ovarian width (cm)	0.25±0.00[a]	0.24±0.00[a]	0.23±0.00[b]	0.22±0.00[b]	0.22±0.00[b]	0.23±0.00[b]
Ovarian weight (mg)	26.11±0.61[a]	26.11±0.61[a]	15.90±0.79[b]	16.17±0.61[b]	16.06±0.61[b]	15.67±0.79[b]
Ovarian volume (ml^3)	0.29±0.01[a]	0.30±0.01[a]	0.15±0.01[b]	0.17±0.01[b]	0.17±0.01[b]	0.15±0.01[b]
Graafian follicles (No)	9.20±0.31[a]	8.40±0.31[a]	0.67±0.39[b]	0.70±0.30[b]	0.40±0.43[b]	0.17±0.39[b]

Values (mean±SE) with different letters in a row for each gonadotropin differ significantly (P<0.01).

Bioactivity of *in-vitro* produced FSH and LH: As the other objective of the experiment, the bioactivity of *in-vitro* produced FSH and LH was monitored in terms of ovarian size and activity in female rabbits. Treatment of rabbits with FSH extracted from *in-vitro* cultured anterior pituitary cells from buffalo adenohypophysis resulted in the increase of ovarian size (length, width, weight and volume), which may be due to increased follicular activity in the ovaries of the treated rabbits, as seen histologically. This increased follicular activity following treatment with low or high dose of *in-vitro* extracted FSH appears to be associated with increased levels of serum FSH levels in treated rabbits compared to controls. Growth and development of antral follicles has been shown to be stimulated by FSH, however, they are highly dependent on gonadotropins to successfully achieve ovulatory size (Minj *et al*., 2007). Previously, Roth *et al.* (2002) also reported significant increase in width of ovaries in cows after FSH treatment.

In the present study, *in-vitro* extracted LH had no effect on ovarian length, width, weight, volume and the number of GFs, although it increased serum FSH levels in the treated female rabbits. It is well known that LH is involved in the final maturation and ovulation of developed ovarian follicles (Kumar and Sait, 2011). Moreover, the magnitude of the increase in serum FSH in rabbits treated with *in-vitro* produced LH was much lower compared to that seen following treatment with FSH. Furthermore, no active corpora lutea were seen on the ovaries of any rabbit, which is due to the fact that rabbit is an induced ovulatory animal (Rebollar *et al*., 2012) and corpus luteum would be formed only after mating with the male. However, the mechanism of increase in serum FSH following LH treatment and its physiological significance is not clear and needs further investigations.

In the present study, neither of the two *in-vitro* produced gonadotropins had any effect on the live body weight of female rabbits. According to Idris *et al*. (2012), treatment of rats with FSH or LH had no effect on their body weight, while thyroid hormones resulted in significant increase in the body weight of rats. Later, Naveed *et al*. (2014) were also unable to demonstrate any significant change is body weight of male rabbits following treatment with FSH produced from *in-vitro* cultured pituitary cells of male buffalo calves.

Hematological variables: Another objective of the present study was to see whether *in-vitro* produced FSH and LH have any adverse effects on certain hematological variables and internal body organs of female rabbits. Fortunately, neither of the *in-vitro* produced gonadotropin had any adverse effect on the internal body organs such as liver, kidneys and spleen. These gonadotropins also showed no undesirable effects on the hematological variables of the treated rabbits. Moreover, the values of various hematological variable recorded in this study were within the normal ranges reported for the female rabbits (Ozkan *et al*., 2012). In a previous study, Naveed *et al*. (2014) also reported that LH extracted from *in-vitro* cultured gonadotropic cells of male buffalo calves had no effects on most of the hematological variables of male rabbits. Similarly, Reza *et al.* (2001) did not observe any pathological changes in the liver following ovarian hyper-stimulation syndrome induced by administration of human exogenous gonadotropins in rabbits. However, they reported congestion in the uterus, with ovaries having multiple corpora lutea after ovulation, congestion on the lungs and hemorrhages present on the kidneys.

Results of the present study revealed that supplementation of culture media with diethyl-stilbestrol and medroxy-progesterone stimulated the secretion of FSH and LH from cultured pituitary cells of buffaloes. The results of bioactivity recorded in the female rabbits were also encouraging. However, number of rabbits

included in each experimental group was low and further studies with higher number of rabbits or females of other species are suggested.

Conclusions: i*n-vitro* cultured buffalo adenohyphyseal cells can be used for the production of FSH and LH. Supplementation of culture media with diethyl-stilbestrol and medroxy-progesterone enhanced the secretion of FSH and LH from these cells. Moreover, *in-vitro* produced FSH increased ovarian size, serum FSH and LH and stimulated ovarian activity in pre-pubertal rabbits, while *in-vitro* produced LH showed no such activity. These gonadotropins had no adverse effects on the hematology and internal body organs of female rabbits.

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