Department of Pharmacology

GRANT MEDICAL COLLEGE, MUMBAI - 400 008, INDIA.

Report on In vivo Investigations into Estrogenic & Progestational Activity of P-28 an Ayurvedic Menstrual Corrective

INTRODUCTION

The Discovery of Gonadotropin Releasing Hormone (GnRH) as a single decapeptide hormone, synthesized and stored in the medial basal hypothalamus, and released in varying pulsatile frequencies at different times in the female menstrual cycle, providing the vital link between the neural and the endocrine system has become pivotal in our understanding of the functioning of the female reproductive cycle and the disorders associated with it. Thus it is today evident that pulsatile releases of GnRH into the hypophyseal portal system and then conducted to the anterior pituitary, where it stimulates the release of the gonadotropins -- FSH and LH, which in turn stimulate the ovarian secretion of estrogens and progesterones leading to endometrial proliferation, ovulation & secretory changes in a proliferated endometrium. This elucidation of the functioning and control of the hypothalamic-anterior pituitary-ovarian-uterine axis has formed the endocrinological & gynecological basis of diagnosis and treatment of the menstrual disorders in modern medicine. Thus depending on the specifics of individual cases, specific treatment is initiated with calibrated dosing and frequency of estrogen agonists / antagonists, progesterone agonists / antagonists, FSH and LH congeners, ovulation inducing drugs, ovulation suppressing regimes, &/or GnRH congeners.

On the other hand classical treatises of ayurveda like the Charak Samhita also detail various menstrual disorders and disorders of the

uterus and advocate a number of drugs of botanical origin including Saraca indica, Abroma augusta, Asparagus racemosus, Symplocos racemosa amongst others and their formulations in these very conditions (1, 2, 3, 4). We have previous demonstrated specific uterotropic antispasmodic activity of a compound ayurvedic formulation P-28 containing such botanical drugs against various different uterine spasmogens in in-vitro studies on isolated uterine smooth muscle preparations. This study was therefore undertaken to assess the estrogenic &/or progestorgenic effects of the compound ayurvedic formulation P-28 using conventional bio-assay protocols for assessing estrogenic or progestorgenic activities.

METHODS

Preparation of test drug material

Test drug P-28 was supplied by the manufacturers M/s Pharmaveda as a 20 mesh coarse powder blend. Each 850 grams of the test drug P-28 blend contained Ashoka (Saraca indica) 300 gms, Ulatkambal (Abroma augusta) 100 gms, Dashmooli (Ten roots) 100 gms, Lodhra (Symplocos racemosa)100 gms, Motha (Cypreus rotundus) 100 gms, Satavari (Asparagus racemosus) 100 gms and Ashwagandha (Withania somnifera) 25 gms. The dried powdered plant material (1kg) was extracted with 8 liters distilled water till the volume of the macerate went to one liter. This extract was used as test drug in the experiments

Study for estrogenic activity in intact immature female rats

Modified test protocol of that described by Wakeling and Valcaccia (5) was followed. Thirty immature albino female rats Haffkine strain (25 day old) from inbred colony were divided into 3 groups of 10

each. The animals were given free access to diet and water. All experiments were conducted using oral gavage of the test drug P-28 at a dose of 1 ml/100 gms twice daily for 8 days, accompanied by a vehicle control group (water, oral administration) and a positive control group estradiol (0.4 mg/kg/day) by oral gavage for 3 days from the sixth day. Vaginal smears were examined microscopically daily to detect vaginal cornification. The rats were killed by cervical dislocation 24 h following the last treatment. Vaginal opening or otherwise was recorded at the time of death. Uteri were excised, trimmed free of fat, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The uterus was then weighed. Uteri from 7 animals were used for glycogen estimation by anthrone method (6) while four excised uteri from each group were fixed in Bouin's fluid and processed for histological preparations. Haematoxylin and eosin stained slides were examined under microscope for changes in cellular organization.

Study for estrogenic activity in ovariectomised immature female rats

Immature albino female rats Haffkine strain (20 day old) from inbred colony were bilaterally ovariectomised under methoxy-urane anesthesia, randomly distributed into treatment groups and treatments were initiated 5 days after ovariectomy. Similar procedure, grouping and treatment durations as in intact immature rats were followed. However, here the positive control group received estradiol (0.04 mg/kg/day) by subcutaneous injection.

Study for estrogenic activity in hypophysectomised immature female rats

Immature albino female rats Haffkine strain (20 day old) from inbred colony were hypophysectomised under methoxy-urane anesthesia, randomly distributed into treatment groups and treatments were initiated 5 days after ovariectomy. Similar procedure, grouping and treatment durations as in intact immature rats were followed. The positive control group received estradiol (0.04 mg/kg/day) by subcutaneous injection similar to the ovariectomized group.

Study for progestational activity in immature female rabbits

The progestational activity of P-28 was determined by modified Clauberg test (7). Twenty immature female rabbits (700 - 800 g body weight) were procured from Haffkine Institute, Bombay. After a week of acclimatization, rabbits were weighed and randomly assigned to one of the 4 groups. Animals from group I to III were then primed with estradiol (5 mg/day in 0.2 ml arachis oil) for six consecutive days, and then received with vehicle injected subcutaneously (group I, control), progesterone injected subcutaneously (group II, positive control), test drug P-28 gavage orally at a dose of 1 ml/100 gms twice daily (group III, estrogen primed test group) or for five days. Animals in group IV received only the test drug P-28 orally at a dose of 1 ml/100 gms twice daily for all eleven days. 24 hours following the last treatment, rabbits were killed and the body weights and the uterine weights recorded. One half of the uterus was rinsed with cold saline. The endometrium was dissected and homogenized with cold distilled water. The homogenate was centrifuged and the supernant was used the determination of endometrial carbonic anhydrase activity.

The enzyme activity was estimated colorimetrically by modifying the Philpot method, one enzyme unit being defined as (TO - T)/T, where 'TO' is the reaction time without enzyme and T is the same with enzyme (8). The other part of the uterus and vagina were fixed in Bouin's solution and processed for microscopic examination. The uterine sections were graded for progestational activity according to the McPhail index (9). The McPhail index scoring used a grade of 1 through 4 in measuring the degree of progestational activity with 1 being the least and 4 being the highest level of stimulation. Uterine changes were assessed as (i) myometrial hypertrophy, the degree of smooth muscle myofiber enlargement associated with estrogen priming further stimulated by the progestational drug; (ii) endometrial stromal proliferation, the degree of stromal cell hypertrophy, edema and neovascularization; (iii) branching and tortuosity of the endometrial glands and (iv) cross-sectional diameter of the uterine horns measured in millimeters. This evaluation correlates uterine weights associated with treatment and dosage levels.

RESULTS

The results of the studies for estrogenic activity in intact immature female rats, ovariectomised immature female rats and hypophysectomised immature female rats are shown in tables 1, 2 & 3 respectively. Administration of P-28 in the intact immature female rats significantly increased the uterine weights as well as the uterine glycogen content as compared to the control group indicating that P-28 has estrogenic effects. However the increase in the uterine weights and uterine glycogen content was lower as compared to the group receiving estradiol. Proliferative changes induced in the

uterine endometrium on administration of P-28 was evidenced by increased height of luminal epithelium, with loose stroma and increased number of glands (Fig. 1), compared to control. The control animals presented a typical infantile condition (Fig. 2). Estradiol administration also induced similar proliferative changes (Fig. 3). Vaginal opening signifying initiation of puberty was seen both in the groups receiving estradiol as well as in the group receiving P-28. However premature initiation of menses, which is estrus in the immature female rats, indicated by cornification of vaginal cells was seen only in the group receiving estradiol.

In both the ovariectomised and hypophysectomised immature female rat population however P-28 failed to exhibit any estrogenic effects, the results being similar to the controls in all the parameters assessed. Estrogenic effects in these ovariectomised and hypophysectomised models being seen only with the administration of estradiol (Fig.4, 5).

The results of the studies for progestational activity of P-28 in the immature rabbit model are shown in table 4. As can be seen from the table administration of P-28, with or without estrogen priming, indicated significant progestational activity. However the progestational activity was lower than that seen with the administration of progesterones. Thus whereas the mean uterine weights in the control group was 1.4 grams, on administration of P-28 without estrogen priming significantly increased and almost doubled to 2.7 gms, which further increased to 3.3 gms when P-28 was administered after estrogen priming. The Progestational Activity

McPhail Index also showed a similar trend with values of 2.6, 4.2 & 4.9 respectively. The endometrial carbonic anhydrase levels, a enzyme marker of progestational activity also followed a similar pattern.

REMARKS

The discovery of GnRH as a single decapeptide hormone has finally crystallized the functioning of the anatomically dispersed and yet highly integrated and coordinated functional operations of the hypothalamic-pituitary-ovarian-uterine axis. This enhanced insight into the operation of the axis has formed the therapeutic basis for the use of specific agonists, antagonists and congeners in the clinical management of functional disorders in the axis.

In the present study, results indicate that the test polyherbal medication P-28 exhibits both estrogenic and progestational activity, but only when the hypothalamic-pituitary-ovarian-uterine axis is intact as is the case in the immature intact female rat and rabbit model. The study medication shows no such activity when either the ovaries or pituitary is removed. This implies that the test medication P-28 does not possess any inherent estrogenic &/or progestational activity per se nor any gonadotropic activity per se. However the estrogenic & progestational activity seen only when the hypothalamic-pituitary-ovarian-uterine axis is intact seems to indicate stimulation of secretion of endogenous gonadotropins which in turn would stimulate ovarian secretion of gonadal hormones. The enhanced progestational activity seen in estrogen primed population

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receiving P-28 as opposed to the population receiving P-28 not primed with estrogens seems to support such an interpretation.

The vaginal opening in immature female rats normally occurs in 35 days old animals and signals the initiation of puberty and culminates in the initiation of estrus indicated by appearance of cornified epithelial cells in the vaginal smears shortly thereafter. Whereas the vaginal opening and induction of estrous with exogenous estrogens in the present study are a direct squeal of the inherent estrogenic activity, the marginal preponement of pubertal initiation seen with the study medication which does not have any inherent gonadotropic activity per se nor any estrogenic &/or progestational activity per se implies hypothalamic activation of the axis which could be due to expression or up-regulation of the hypothalamic gonadal hormone receptors.

Dr. A.P.Saraf M.D.
Professor & Head,
Department of Pharmacology
Grant Medical College
Bombay - 400 008

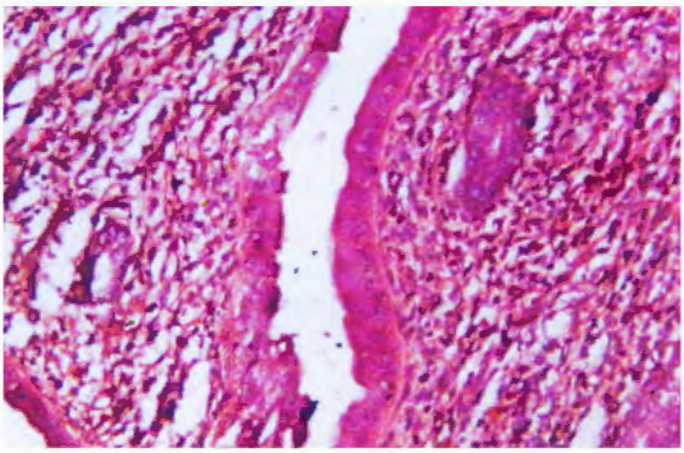

Fig. 1. Photomicrograph of haematoxylin and eosin stained transverse section of uterus of P-28 treated intact rat, showing proliferative stage (i.e., stimulated endometrium with loose stroma).

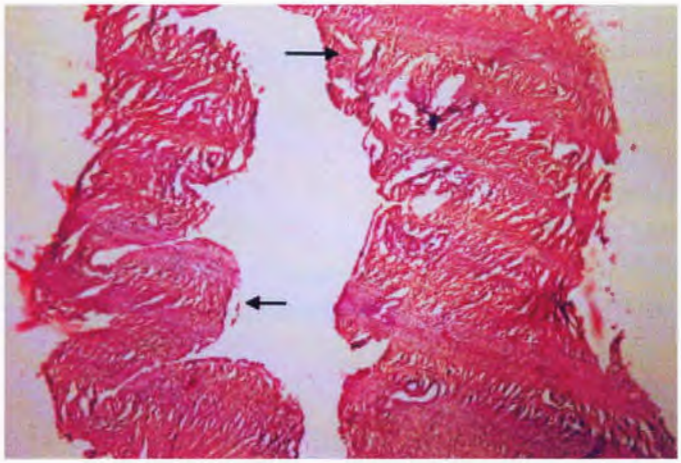

Fig. 2. Photomicrograph of haematoxylin and eosin stained transverse section of uterus of control intact rat, showing disintegrated endometrium (arrow mark).

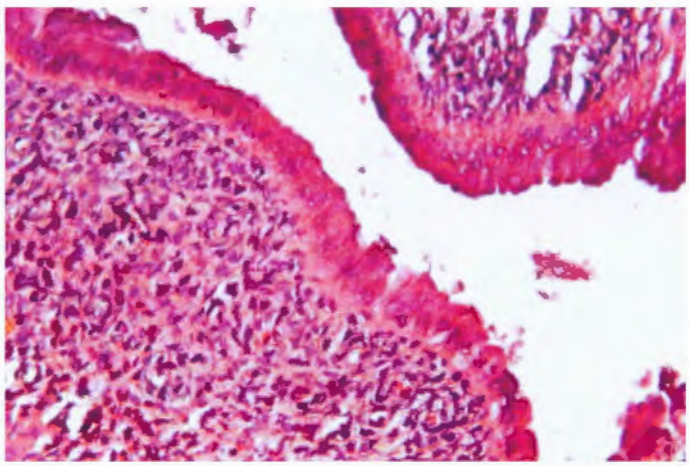

Fig. 3. Photomicrograph of haematoxylin and eosin stained transverse section of uterus of estradiol treated intact rat, showing proliferative stage (i.e., stimulated endometrium with loose stroma and glands).

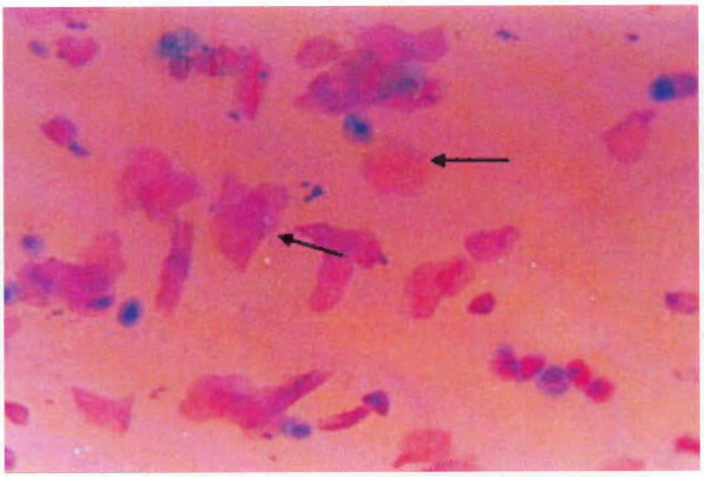

Fig. 4. Photomicrograph of methylene blue and eosin stained vaginal smear (in estrous) of estradiol treated overactomised rat, showing only cornified epithelial cells (arrow mark).

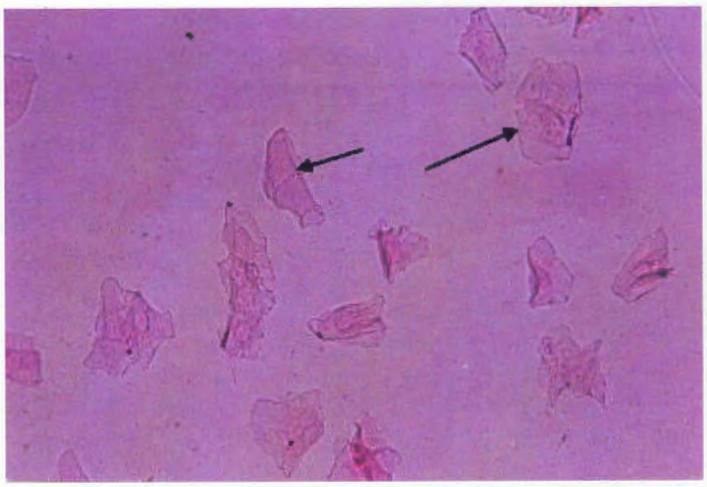

Fig. 5. Photomicrograph of methylene blue and eosin stained vaginal smear (in estrous) of estradiol treated hypophysectomised rat, showing only cornified epithelial cells (arrow mark).

Table 1
Effect of P-28 on the estrogenic endpoints in intact immature rats

Treatment	Uterine weight (mg, mean ± SE) (n = 10)	Vaginal cornification * (n = 10)	Vaginal Opening ** (n = 10)	Uterine glycogen content (microgram/mg of uterine tissue ± SE) (n = 7)
Intact control	142.2 ± 20.7	-	-	0.788 ± 0.059
Estradiol (0.4 mg/kg)	364.2 ± 5.5	+	+	1.835 ± 0.015***
P-28 (1 ml/100 gms)	281.8 ± 10.8	-	+	1.184 ± 0.105****

*Vaginal smears were taken on the last 2 days of treatment. (+) or (-) indicates the presence or absence of cornified cells in the smears, respectively.
**Vaginal opening or otherwise was recorded at the time of sacrificing at the end of treatment. (+) or (-) indicates the presence of vaginal opening & absence of vaginal opening, respectively.
*** $p < 0.01$, **** $p < 0.05$ as compared to control Students t test

Table 2

Effect of P-28 on the estrogenic endpoints in ovariectomised immature rats

Treatment	Uterine weight (mg, mean ± SE) (n = 10)	Vaginal cornification * (n = 10)	Vaginal Opening ** (n = 10)	Uterine glycogen content (microgram/mg of uterine tissue ± SE) (n = 7)
Ovariectomised control	49.2 ± 8.7	-	-	0.488 ± 0.059
Estradiol (0.4 mg/kg)	298.2 ± 19.3	+	+	1.425 ± 0.024***
P-28 (1 ml/100 gms)	48.8 ± 5.7	-	-	0.456 ± 0.046

*Vaginal smears were taken on the last 2 days of treatment. (+) or (-) indicates the presence or absence of cornified cells in the smears, respectively.

**Vaginal opening or otherwise was recorded at the time of sacrificing at the end of treatment. (+) or (-) indicates the presence of vaginal opening & absence of vaginal opening, respectively.

*** $p < 0.01$, as compared to control Students t test

Table 3

Effect of P-28 on the estrogenic endpoints in hypophysectomised immature rats

Treatment	Uterine weight (mg, mean ± SE) (n = 10)	Vaginal cornification * (n = 10)	Vaginal Opening ** (n = 10)	Uterine glycogen content (microgram/mg of uterine tissue ± SE) (n = 7)
Hypophysectomised control	53.2 ± 10.5	-	-	0.531 ± 0.032
Estradiol (0.4 mg/kg)	270.8 ± 15.3	+	+	1.553 ± 0.033***
P-28 (1 ml/100 gms)	48.7 ± 10.8	-	-	0.495 ± 0.059

*Vaginal smears were taken on the last 2 days of treatment. (+) or (-) indicates the presence or absence of cornified cells in the smears, respectively.

**Vaginal opening or otherwise was recorded at the time of sacrificing at the end of treatment. (+) or (-) indicates the presence of vaginal opening & absence of vaginal opening, respectively.

*** $p < 0.01$, as compared to control Students t test

Table 4
Progestational activity of P-28 in immature rabbits

Treatment, n = 5	Uterine weight (gms) (mean ± SE)	Progestational activity average modified McPhail score*	Uterine cross sectional diameter** (mm) mean (n = 5)	Endometrial Carbonic anhydrase content (enzyme units/gm wet tissue)
Control	1.4 ± 0.08	2.8	2.8	23 ± 3
Progesterone(0.5mg/kg)	4.2 ± 0.35***	6.0	4.5	228 ± 24***
Estrogen Primed +P-28	3.3 ± 0.23***	4.9	3.8	198 ± 19***
P-28	2.7 ± 0.31***	4.2	3.4	135 ± 42***

*Progestational activity was measured by modified MPhail index.
**Cross-sectional diameter of the uterine horns was measured in millimeters from mesometrial border to anti-mesometrial border.
*** $p < 0.05$ (Student's t test as compared to control).

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