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ORIGINAL ARTICLE

EFFECTS OF AN INDIGENOUS CONTRACEPTIVE HERBAL FORMULATION ON GONADOTROPHS OF THE PITUITARY GLAND OF THE RAT

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An indigenous contraceptive herbal formulation consisting of a mixture of *Lepidagathis longifolia, Palaquium sp* and *Phyllagathis rotundifolia* is being used by the Temuan Aborigins of Malaysia. Although the previous studies demonstrated that this contraceptive herbal formulation causes anovulatory estrous cycle, altered circulating hormone levels and fetal resorption in rats, but the effects of this formulation on the gonadotrphs of the pituitary gland are yet to be evaluated. The present study was designed to observe the morphometric changes of the gonadotrophs and the plasma concentrations of follicle stimulating hormone and leutinizing hormone. Thirty five Sprague-Dawley adult female rats were randomly divided into 5 groups. Experimental animals were given a combined herbal extract or individual herbal extract at a dose of 540 mg/kg/day subcutaneously for 7 days. Immunostained gonadotrophs were studied by using image analyzer. FSH and LH serum concentrations were determined using RIA. The FSH and LH concentrations were low in animals that received combined herbal extract ($p<0.01$). FSH concentration was noted to be significantly low in animals that received *P. rotundifolia* ($p<0.05$). The mean cell area and cell density of gonadotrophs of animals that received combined herbal extract were significantly low compared to control group ($p<0.05$). It was concluded that the herbal extracts do suppress the production of gonaotrophins along with the demonstrable suppresive effect on the FSH cells.

Key words *: Pitutary gland; Gonadotrophins; Gonadotrophs; Herbal contraceptive*

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Introduction

The use of traditional herbal contraceptives by women in Malaysia is well documented (1, 2, 3). Interest in evaluating the efficacy and safety of this herbs by reseachers in Malaysia was scattered (4, 5, 6, 7, 8).

Studies on the effect of an indigenous contraceptive herbal formulation consists of a mixture of three plant species (*Lepidagathis longifolia, Palaquium sp*. and *Phyllagathis rotundifolia*) and the individual contraceptive herbs on pregnant and nonpregnant rats have previously been conducted (7,9). Traditionally, the water concoction of the combined herbs was consumed as contraceptive every morning, about three to five days before the expected menses (10). On the other hand, the individual herb, *Lepidagathis longifolia* or *Phyllagathis rotundifolia* in particular could be consumed daily to prevent conception (8). The water-based extract of these combined herbs was known to impair blastocyst implantation, severely affect intrauterine fetal growth, fetal survival and parturation process in pregnant rats, and also inhibit the formation of the endometrial decidualisation and reduced estradiol and progesterone concentrations in pseudopregnant rats (7). The herbs cause anovulatory estrous cycle in rats (7). The acyclic animals treated with combined extract become ovulated upon eCG and hCG treatments (9). The

Table 1 : Effect of combined herbs, L. longifolia, Palaquium sp and P. rotundifolia on FSH and LH concentrations in rats. Data was presented in median(interquartiles) and analysed using nonparametric statistic Kruskal Wallis (KW) followed by Mann Whitney U (MW-U) for comparing between two groups.

	Group 1 Control (n=7)	Group 1 Combined herb (n=7)	Group 1 *L. longifolia* (n=7)	Group 1 *Palaquim sp* (n=7)	Group 1 *P. rofindifolio* (n=7)	'p' value K/W
FSH (ng/ml)	6.43(1.54)	2.83(0.74)**	4.08(3.10)	4.29(2.32)	3.79(2.57)*	p<0.01
LH(ng/ml)	0.41(0.36)	0.18(0.04)**	0.51(0.66)	0.33(0.52)	0.64(0.83)+	p<0.01

'n' is the number of animals
* p<0.05 compared with control group (Group1)
**p<0.01 compared with control group (Group 1)
* p<0.05 compared with combined herbs (Group 2)

present study was designed to demostrate the mechanism of action of these herbs by evaluating the pituitary gonadotrophs and circulatory gonadotrophins in nonpregnant rats.

Materials and Methods

Collection, Extraction and standardisation of plant material

The herbal plants were collected from the tropical rain forest in the area of Jempul in the state of Negeri Sembilan, Malaysia with the help of the Orang Asli (aborigines) who actually formulated the contraceptive herbs. The plant was identified by botanists of the Forest Research of Institute of Malaysia (FRIM) and voucher speciments were deposited in the Herbarium of the Institute (FRI 45579, FRI 45580).

A mixture of the dried ground roots and stems of *Lepidagathis longifolia, Palaquium sp*., *Phyllagathis rotundifolia* at a ratio of 1.7:6:4 (by weight) and of the individual plants were extracted with distilled water by using Sochlex apparatus. The extracts were then rotavaporised and freezed-dried. The extracts were reconstituted with distilled water at a concentration of 270 mg/ml and kept in small aliquots at -20^0 C until used. A dose of 540 mg/kg/day of the combined or individual herbal extracts were found effective to produce anovulatory estrous cycle.

Animals

Two month old female virgin albino rats weighing 180-200 gm with regular 4-day estrous cycle (at least two consegutive estrous cycle) were used in this study. Animals were obtained from the Animal House Unit of the Health Campus, USM and allowed one week for acclamatization. They were housed in polypropylline cages at 22 ± 1^0C, relative humidity of 35-60% with a 12:12 h light and dark cycle. Light was on from 6:00 to 18:00. All animals had free access to laboratory chow Gold coin* (M Sdn Bhd) and tap water.

Study Design

Thirty-five animals were randomly divided into 5 groups. Animals of group-2 were given combined herbal extract 540 mg/kg/day (0.2 ml) subcutaneously, whereas animals of group 3, 4 and 5 were given extracts of *Lepidagathis longifilia*, *Palaquium sp*. and *Phyllagathis rotundifolia* respectively at a dose of 540 mg/kg/day (0.2 ml) subcutaneously between 9:00 to 10:00. Animals of group-1 were used as control and were given same volume of vehicle. Treatment was started on the second day of the estrous cycle and continued daily for a period of one week. The body weight was recorded before treatment and on day 8 of the study. Vaginal smears were examined at 8:30 every day following the method described by Marcodes et al., 2002 (11) and evaluation of the phases of the estrous cycle were determined throughout the study period according to the criteria described by Marcodes et al,. 2002(11) and Freeman,1994(12).

Twenty four hours after the last dose, the animals of similar physiological state (vaginal smear showed diestrus) were placed under light ether anaesthesia. Laparotomy was performed and 5 ml of trunk blood was collected. Animals were sacrificed under anaesthesia by decapitation. Blood was centrifuged at 3000 rpm for 10 min. Serum was collected and stored at-20^0C until assayed. The pituitary glands were removed, weighed and preserved with 10% formaldehyde in normal saline. The study protocol was approved by the Animals Ethical Committee of the Health Campus of

Table 2 : Effect of combined herbs, L. longifolia, Palaquium sp and P. rotundifolia on the gonadotrophs of the pituitary gland of rats. Data was presented in median(interquartiles) and analysed using nonparametric statistic Kruskal Wallis (KW) followed by Mann Whitney U (MW-U) for comparing between two groups.

	Group 1 Control (n=7)	Group 2 Combined herb (n=7)	Group 3 *L.longifolio* (n=7)	Group 4 *Palaquium sp.* (n=7)	Group 5 *P.rotundifolio* (n=7)	'p' value K/W
Cell density FSH	177.83(63.43)	96.92(25.58)*	118.67(68.17)	153.58(75.10)	100.66(23.67)*	<0.01
Mean cell area (μm^2) FSH	27.71(9.26)	9.91(3.88)*	22.56(20.89)	26.87(11.41)	11.26(2.07)	<0.01
Volume density FSH	0.06(0.01)	0.04(0.02)	0.06(0.01)	0.06(0.05)	0.05(0.01)	n.s
Cell density LH	183.17(84.71)	174.61(34.66)	194.83(28.04)	183.75(67.79)	210.25(34.58)	n.s
Mean cell area (μm^2) LH	51.31(10.53)	48.02(11.51)	58.31(4.74)	52.67(13.74)	57.88(8.84)	n.s
Volume dendity LH	0.12(0.04)	0.11(0.01)	0.14(0.02)	0.13(0.02)	0.15(0.04)	n.s

"n" is the number animals
"n.s" no significant difference among all groups
*p<0.05 compared with control group (Group1)

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Hormonal assays

FSH and LH serum concentrations were determined using RIA kits supplied by BIOCODE, Belgium. All samples were analysed in duplicate and performed in one assay to avoid interdays variation. Results were expressed as ng/ml. The intraassays co-efficient of variations were found to be 6.23 for FSH and 3.46 for LH.

Immunohistochemistry and morphometry

The pituitary glands of all animals were processed and embedded in paraffin block. Serial sections of 4 μm thickness were performed from ventral to dorsal and immunostained using Avidin-Biotin-Complex method. Six sections from each pituitary gland were taken to immunostain with FSH and LH antibodies. The sections were incubated with peroxidase blocking agents (3%H_2O_2 in methanol) for 15 minutes and washed. Unmasking of the antigens were performed by treating the tissues with 0.01 citrate buffer in the microwave oven for 7 minutes (twice). The tissues were then incubated overnight with the primary antibody (FSH or LH, Dako, Denmark) at a concentration of 1:50. Then the tissues were incubated with the biotinylated secondary antibody for 30 minutes at room temperature and with streptavidin biotin complex for 30 minutes and later with HRP chromogenic substrate (DAB) and finally counterstained with haematoxyline.

Measurements of cell parameters (200X magnification) were made by using an image analysis system (Image-Pro Plus, Media Cybernatic Inc., Silver Spring, USA). The immunostained cells of each reference area (75,461.30 sq μm) were labeled and analysed. Eighteen reference areas (equal number from high, intermediate and low density areas) from each pituitary gland were analysed. Three main parameters i.e mean cell areas, volume density (Vv=∑cell area/reference area) and cell density (CD=number of cells/referance area) for each reference area were calculated (13).

Statistical analysis

All data were analysed using SPSS version 11.0. Data were expressed as median (interquartiles) and analysed using Kruskal Wallis and followed by Mann-Whitney U test for comparisons between two groups whenever appropriate. A value of $p<0.05$ was considered as significant.

Results

All experimental groups of animal lost their body weights between 2% to 7% whereas the control group of animal gained 5% body weight. There was no significant change in weight of the pituitary gland of the experimental animals compared to the control group.

Circulating gonadotrophins

Serum FSH and LH concentrations were significantly low in animals treated with the combined herbal extract (group 2) when compared

to the control animals ($p<0.01$). Although the FSH levels were low in animals that received the individual extracts, a significant difference when compared to control group was noted in animals that received *P. rotundifolia* (group 5). There was a significant increase in concentrations of LH in group 3 (*L. longifolia*) and group 5 (*P. rotundifolia*) when compared to the group of animals that received combined herbal extract. *(Table 1)*

Morphometry of the gonadotrophs

Mean cell area and cell density of the folliculotroph of animals that received combined herbal extract (group 2) were significantly low compared to the control animals ($p<0.05$). Significantly low level of cell density of folliculotroph ($p<0.05$) was noted in animals that received *P.rotundifolia* (group 5) compared to control group. Volume density of the folliculotroph was not significantly different from each other ($p>0.05$). The mean cell area, cell density and volume density of luteotroph of the treated and control animals were not significantly different ($p> 0.05$; Table 2).

Discussion

The present study showed that combined and individual herbal extracts affect the gonadotroph and circulating gonadotrophins in rats. The effect of the combined herbs was more towards suppressing the area and number of follicullotrophs and at the same time reducing the FSH and LH productions. These findings were in line with the previous findings where similar herbal formulation causes reduction in estradiol and progesterone concentrations in nonpregnant (9) and pseudopregnant rats (7). This study suggested that the combined herbs prevent the negative feedback induced by the low estradiol and progesterone concentration on the pituitary.

The effects of the individual herbs on gonadotrophs were slightly different compared to the combined herbs. Decreased levels of FSH and increased LH concentration were noted especially in animals that received *Phyllagathis rotundifolia*. The results support previous findings where hypoestrogenemia and normal progesterone levels were observed in animals that received the individual plant extracts (9) Although the individual herb did not show their affect on the luteotroph and LH concentration but they however showed the suppressing effect on the folliculotroph and inhibiting effect on FSH production which could be the main mechanism of action of the individual herb in inhibition of ovulation.

The overall findings of the study support the traditional use of the herbs as contraceptive. The combined herbs might directly affect the gonadotrophs or via hypothalamus, a much higher centre of the hypothalamic-pituitary-ovarian axis.

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