Suzuki, M., Takahashi, T. and Abe, T. The Effect of Chemical Ovulation-inducing Substances on Gonadotropin Secretion in Incubation Experiments of the Anterior Pituitary. Tohoku J. exp. Med., 1972, 108 (2), 123-131 — In order to clarify the in vitro action of clomiphene, F6066 and Lychium chinense Miller (LCM) on the pituitary, the anterior pituitary of the rat was incubated with these materials and the gonadotropin in the medium was assayed. 1) Clomiphene showed no increase of LH secretion upon its addition to the incubation medium containing the pituitary alone or in the presence of the hypothalamus. The addition of a large amount of clomiphene induced a slight increase in secretion of FSH but it was not statistically significant, while prolactin secretion was significantly stimulated by a small amount of clomiphene. Secretion of prolactin was stimulated even in the presence of the hypothalamus. 2) F6066 acted directly on the pituitary, markedly stimulating LH secretion. No significant changes of FSH and prolactin values were observed. 3) Extracts from LCM acted directly on the pituitary to stimulate LH secretion. 4) The results described above suggest that both clomiphene and F6066 act on the diencephalon-pituitary system. Clomiphene gives a more marked effect on prolactin, while F6066 acts on LH secretion more strongly. The action of LCM to induce ovulation is presumably consisted of a direct action on the pituitary to induce LH secretion. Further studies are necessary to elucidate the mechanism of action of these substances. — ovulation-inducing substances; clomiphene citrate; pituitary incubation; plant inducing ovulation

While clomiphene (Clomid) and F6066 give an excellent ovulation-inducing effect in women, a paradoxical response of inhibiting ovulation is noted in experimental animals. Therefore, concerning the mechanism of action of induction of ovulation, only hypotheses are available at present. As for the Lychium chinense Miller (LCM) which induces ovulation in rabbits at a considerably high rate, its mechanism of action has not been studied yet. We have carried out a study on these three drugs, using incubation method of the anterior pituitary of rat, to determine the in vitro influence of these substances on the pituitary and to clarify the mechanism of action of these drugs.

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MATERIALS AND METHODS

A. Experimental materials

The anterior pituitaries obtained from adult male Wistar rats of 150–200 g body weight were incubated and the hypothalamus removed from the same animal was added to the incubation medium.

Clomid (Shionogi Pharmaceutical Co. Ltd., Osaka), F6066 (Teikoku-zóki Pharmaceutical Co. Ltd., Tokyo), or extracts from Folium Lycii of LCM obtained by the method reported previously (Suzuki et al., 1972 b) were used as ovulation-inducing substances.

Immature female Wistar-Imamichi rats were used for bioassay of LH, immature female Wistar rats for FSH, and native Japanese pigeons for prolactin assay. NIH-LH-S12, FSH-S1, and NIH-prolactin-B2 were used as standards.

B. Experimental methods

Incubation method: The method described in detail in the previous paper (Suzuki et al. 1972 a) was used. The ovulation-inducing substances were used in two doses, 10 µg and 1 mg for Clomid and F6066, 1 and 10 mg for each of G–25F1 and IR–4B of LCM (Suzuki et al. 1972 b) per one of the anterior pituitary, respectively. Clomid, F6066 and LCM in a powder form were dissolved slowly in the aqueous solution at 37°C.

Method of preparation of hypothalamic extracts (subsequently abbreviated as HE): The same method as described in the previous paper (Suzuki et al. 1972 a) was used for the preparation of hypothalamic extract.

Method of gonadotropin assay: LH was determined by the ascorbic acid depletion method using the immature rat ovary according to Parlow (1958). FSH was assayed by the Parlow’s modification (1964) of the Steelman-Pohley’s method (1953) measuring the increase in rat ovarian weight. Prolactin was assayed by the measurement of the dry weight of pigeon crop sac mucosa according to Nicoll (1967). Details of these methods were described in the previous paper (Suzuki et al. 1972 a).

RESULTS

A. Effect of Clomid on gonadotropin activity in the incubation medium

1. Effect on LH activity

When 10 µg Clomid/one anterior pituitary was added to the incubation medium of the anterior pituitary, as in the case of the addition of 1 mg Clomid, LH activity showed slight decrease as compared with the group without such addition, but it was not statistically significant. When 1 mg Clomid was added to the incubation medium containing the anterior pituitary and the hypothalamus, LH activity fell significantly to 1.29 µg as compared with 2.81 µg in the control group (Table 1).

2. Effect on FSH activity

In the group with the addition of Clomid alone to the anterior lobe of the pituitary, the addition of 1 mg Clomid increased FSH activity to 108.2 µg, as compared with 73.6 µg in the controls. When Clomid was added to the incubation medium containing anterior pituitary and hypothalamus, the addition of 1 mg Clomid caused a decrease of FSH activity from 121.6 µg in the controls to 102.4 µg. However, no significant difference between the Clomid and the control groups was found in either case (Table 2).
3. Effect on prolactin activity

When anterior pituitary was incubated with 10 μg Clomid alone prolactin activity significantly increased from 24.5±3.5 mU in the controls to 234.9±65.6 mU. However, the addition of 1 mg Clomid caused no change. In the presence of hypothalamus, the addition of 10 μg and 1 mg Clomid caused significant increases in prolactin activity up to 207.3±34.5 and 242.6±69.9 mU respectively, as compared with 13.5±2.7 mU in the control group (Table 3).
B. Effect of F6066 on gonadotropin activity

1. Effect on LH activity

When anterior pituitaries were incubated with 10 µg and 1 mg of F6066, LH activity was increased to 6.2 µg and 3.65 µg, respectively, from the control level (0.82 µg). In the case of the presence of hypothalamus in the incubation medium, LH activity was also increased to 4.15 µg and 5.13 µg from control level (2.33 µg) when incubated with 10 µg and 1 mg of F6066, respectively (Table 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>OAAD±SE (%)</th>
<th>LH conc.±SE (µg)</th>
<th>P.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>22.8±2.4</td>
<td>0.82±0.17</td>
<td></td>
</tr>
<tr>
<td>P+F6066 10 µg</td>
<td>5</td>
<td>57.1±1.8</td>
<td>6.20±0.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P+F6066 1 mg</td>
<td>5</td>
<td>48.9±2.9</td>
<td>3.65±0.23</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>49.3±1.4</td>
<td>2.33±0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P+HE+F6066 10 µg</td>
<td>5</td>
<td>59.0±2.1</td>
<td>4.15±0.16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P+HE+F6066 1 mg</td>
<td>5</td>
<td>53.5±2.1</td>
<td>5.13±0.17</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

2. Effect on FSH activity

When the incubation of anterior pituitary was carried out with F6066 alone, in a dose of 10 µg or 1 mg, FSH activity was increased but no significant difference was found statistically between the F6066 and the control groups. When 10 µg and 1 mg of F6066 were added to the incubation medium of the anterior pituitary and the hypothalamus, FSH activity tended to decrease, although it was not significant (Table 5).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>Ovarian weight±SE (mg)</th>
<th>FSH conc.±SE (µg)</th>
<th>P.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>82.4±2.5</td>
<td>106.2±3.8</td>
<td>-N S</td>
</tr>
<tr>
<td>P+F6066 10 µg</td>
<td>5</td>
<td>91.8±4.3</td>
<td>130.4±6.3</td>
<td>N S</td>
</tr>
<tr>
<td>P+F6066 1 mg</td>
<td>5</td>
<td>103.6±7.4</td>
<td>176.4±10.9</td>
<td>N S</td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>104.5±2.8</td>
<td>182.5±4.4</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+F6066 10 µg</td>
<td>5</td>
<td>85.0±3.1</td>
<td>115.6±5.7</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+F6066 1 mg</td>
<td>5</td>
<td>84.6±5.2</td>
<td>114.1±8.1</td>
<td>-N S</td>
</tr>
</tbody>
</table>

3. Effect on prolactin activity

When anterior pituitary was incubated with 10 µg F6066 alone, prolactin activity was increased from the control level of 22.6±2.5 mU to 79.2±20.6 mU, but difference of the prolactin activity between the F6066 and the control groups was not statistically significant (Table 6).
TABLE 6. Effects of F6066 on prolactin activities in incubated medium of the anterior pituitary

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay pigeons</th>
<th>Increase of crop sac weight ± SE (mg)</th>
<th>Prolactin conc. ± SE (mU)</th>
<th>P.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>8.1±0.8</td>
<td>22.6±2.5</td>
<td>-N S</td>
</tr>
<tr>
<td>P+F6066 10 µg</td>
<td>5</td>
<td>14.3±4.2</td>
<td>73.2±20.6</td>
<td>-N S</td>
</tr>
<tr>
<td>P+F6066 1 mg</td>
<td>5</td>
<td>8.9±1.2</td>
<td>28.0±5.5</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>6.2±1.8</td>
<td>16.4±3.8</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+F6066 10 µg</td>
<td>5</td>
<td>5.8±2.4</td>
<td>13.2±5.8</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+F6066 1 mg</td>
<td>5</td>
<td>7.1±1.4</td>
<td>19.1±4.6</td>
<td>-N S</td>
</tr>
</tbody>
</table>

C. Effect of LCM on LH and FSH activities

1. Effect of fraction 1 (G-25F1) subjected to gel filtration using Sephadex G-25 medium on LH and FSH activity

   a. Effect on LH activity: The addition of 1 mg G-25F1 (Suzuki et al. 1972 b) to one anterior pituitary brought about a significant increase of LH activity. When the control level was 0.72 µg, upon the use of 1 mg and 10 mg of G-25F1, 2.15 µg and 2.30 µg of LH activity was recorded, respectively. In the presence of the hypothalamus, LH activity was increased upon the use of either 1 mg or 10 mg, but no significant increase was found statistically (Table 7).

   b. Effect on FSH activity: The addition of G-25F1 alone caused an elevation of FSH activity from the control level of 68.2 µg to 103.4 µg upon the use of 1 mg, but a depression to 41.4 µg was observed upon the addition of 10 mg, but these results are not significant statistically. Addition of the hypothalamus and G-25F1 also gave similar results. An increase of FSH activity was noted upon the addition of 1 mg, but a decrease was seen by the addition of 10 mg (Table 8). These changes are, however, not significant.

TABLE 7. Effects of plant extract separated by Sephadex G-25 (G-25F1) on LH activities in incubated medium of the anterior pituitary

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>OAAD ± SE (%)</th>
<th>LH conc. ± SE (µg)</th>
<th>P.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>24.5±2.1</td>
<td>0.72±0.09</td>
<td>-&lt;0.01</td>
</tr>
<tr>
<td>P+G-25F1 1 mg</td>
<td>5</td>
<td>39.9±3.3</td>
<td>2.15±0.25</td>
<td>-&lt;0.01</td>
</tr>
<tr>
<td>P+G-25F1 10 mg</td>
<td>5</td>
<td>40.8±2.9</td>
<td>2.30±0.23</td>
<td>-&lt;0.01</td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>38.6±1.8</td>
<td>2.05±0.10</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+G-25F1 1 mg</td>
<td>5</td>
<td>45.3±2.7</td>
<td>3.12±0.22</td>
<td>-N S</td>
</tr>
<tr>
<td>P+HE+G-25F1 10 mg</td>
<td>5</td>
<td>43.0±3.1</td>
<td>2.70±0.26</td>
<td>-N S</td>
</tr>
</tbody>
</table>
TABLE 8. Effects of plant extract separated by Sephadex G-25 (G-25F1) on FSH activities in incubated medium of the anterior pituitary

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>Ovarian weight ±SE (mg)</th>
<th>FSH conc. ±SE (µg)</th>
<th>P.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>88.2±5.2</td>
<td>68.2±4.6</td>
<td></td>
</tr>
<tr>
<td>P+G-25F1 1 mg</td>
<td>5</td>
<td>106.5±10.2</td>
<td>103.4±10.1</td>
<td></td>
</tr>
<tr>
<td>P+G-25F1 10 mg</td>
<td>5</td>
<td>66.2±5.9</td>
<td>41.4±4.7</td>
<td></td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>119.0±10.2</td>
<td>137.5±13.4</td>
<td></td>
</tr>
<tr>
<td>P+HE+G-25F1 1 mg</td>
<td>5</td>
<td>141.4±6.5</td>
<td>223.4±9.6</td>
<td></td>
</tr>
<tr>
<td>P+HE+G-25F1 10 mg</td>
<td>5</td>
<td>100.2±9.3</td>
<td>89.6±8.8</td>
<td></td>
</tr>
</tbody>
</table>

2. Effect of an extract from LCM (IR-4B) obtained by ion exchange chromatography of Amberlite IR-4B on LH and FSH activities

a. Effect on LH activity: When 1 mg of IR-4B (Suzuki et al. 1972 b) per one incubated anterior pituitary was added, LH activity significantly increased to 3.29 µg from 0.82 µg in the control. The addition of 10 mg also caused a tendency towards an increase. The addition of the hypothalamus and IR-4B caused no change whatsoever as compared with the controls (Table 9).

TABLE 9. Effects of plant extract separated by Amberlite IR-4B ion exchange chromatography on LH activities in incubated medium of the anterior pituitary

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>OAAAD ± SE (%)</th>
<th>LH conc. ±SE (µg)</th>
<th>P.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>26.8±4.6</td>
<td>0.82±0.29</td>
<td></td>
</tr>
<tr>
<td>P+IR-4B 1 mg</td>
<td>5</td>
<td>47.6±1.8</td>
<td>3.92±0.15</td>
<td></td>
</tr>
<tr>
<td>P+IR-4B 10 mg</td>
<td>5</td>
<td>33.5±3.6</td>
<td>1.35±0.17</td>
<td></td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>40.5±3.1</td>
<td>2.12±0.21</td>
<td></td>
</tr>
<tr>
<td>P+HE+IR-4B 1 mg</td>
<td>5</td>
<td>40.2±2.8</td>
<td>2.08±0.19</td>
<td></td>
</tr>
<tr>
<td>P+HE+IR-4B 10 mg</td>
<td>5</td>
<td>40.9±5.4</td>
<td>2.18±0.36</td>
<td></td>
</tr>
</tbody>
</table>

b. Effect on FSH activity: When IR-4B was added to the anterior pituitary, FSH activity did not change at all as compared with the controls regardless of the amount added. The addition of the hypothalamus and IR-4B also gave the same results (Table 10).

TABLE 10. Effects of plant extract separated by Amberlite IR-4B ion exchange chromatography on FSH activities in incubated medium of the anterior pituitary

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of assay rats</th>
<th>Ovarian weight ±SE (mg)</th>
<th>FSH conc. ±SE (µg)</th>
<th>P.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>5</td>
<td>92.8±4.2</td>
<td>94.2±4.4</td>
<td></td>
</tr>
<tr>
<td>P+IR-4B 1 mg</td>
<td>5</td>
<td>90.6±3.6</td>
<td>88.4±3.9</td>
<td></td>
</tr>
<tr>
<td>P+IR-4B 10 mg</td>
<td>5</td>
<td>90.8±5.5</td>
<td>90.2±5.3</td>
<td></td>
</tr>
<tr>
<td>P+HE</td>
<td>5</td>
<td>123.3±4.4</td>
<td>205.2±9.6</td>
<td></td>
</tr>
<tr>
<td>P+HE+IR-4B 1 mg</td>
<td>5</td>
<td>91.4±2.8</td>
<td>92.6±2.7</td>
<td></td>
</tr>
<tr>
<td>P+HE+IR-4B 10 mg</td>
<td>5</td>
<td>98.0±3.6</td>
<td>104.2±4.2</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Clomiphene citrate (Clomid), a derivative of triparanol (MER-29) and ethamoxytriphetol (MER-25), obtained through partial modification of the chemical structure of non-steroidal estrogenic substances such as diethylstilbestrol and chlorotriansene (TACE) was discovered by the investigators of Merrell Co., U.S.A. Since the discovery of its action of inducing ovulation in anovulatory women by Greenblatt et al. in 1961, attention was focussed on this drug and many studies have been carried out to evaluate the mechanism of its action. In the initial experiment, Greenblatt (1962) reported the inhibition of pituitary gonadotropin secretion by Clomid. In subsequent experiments, stimulation of gonadotropin secretion was demonstrated (Greenblatt 1962). Roy et al. (1962), Riley et al. (1964) and Thompson et al. (1965) reported the stimulation of gonadotropin secretion in the human by Clomid. Charles et al. (1963), on the other hand, failed to observe changes in urinary total gonadotropin and pointed out the possibility of a direct action on the ovary without being mediated by gonadotropin. Pildes (1965) suggested that Clomid would stimulate estrogen secretion by its direct action on the ovary and would cause an increase of gonadotropin secretion secondarily, based on their finding that urinary estrogen was increased in all cases in spite of an inconsistent increase in urinary total gonadotropin. At present, the majority of workers support the central theory, but whether Clomid acts directly on the pituitary or acts on the hypothalamus primarily and stimulate the pituitary secondarily has not been decided yet. As to experiments in which effect of Clomid on gonadotropin was studied, Holtkamp et al. (1960) administered Clomid on female rats but found no change in total gonadotropin content of the pituitary, and Nelson et al. (1962) administered 0.1–25.0 mg/kg/day of Clomid for 30 days, demonstrating a decrease in total gonadotropin content.

As described above, most of the reports are concerned with in vivo experiments and no studies were conducted on prolactin. In order to clarify the effect of Clomid on the pituitary in vitro without neurohumoral transmission, a short term incubation method was used to quantify LH, FSH and prolactin. To the incubation medium were added 10 μg and 1 mg of Clomid to see the direct effect of Clomid on the pituitary.

F6066 which appeared after discovery of Clomid is bis (p-acetoxyphenyl)-cyclohexylidenemethane with a position between stilbene and triphenylethylene type estrogen. Since it was used by Persson (1965 a, b) successfully to induce ovulation in anovulatory women its effectiveness has been recognized.

While the estrogenic activity of this compound is as low as approximately 1/1,000 of that of estradiol, this substance is antiestrogenic and antigestagenic at times and seems to participate in release and production of gonadotropin, changing the FSH/LH ratio, according to the report of Persson (1965 a, b). This was also confirmed by Hellinga et al. (1967). F6066 gives a similar effect to that of Clomid clinically but gives a reaction similar to Clomid or an opposite one at times in
animal experiments, so that nothing definite has been established on the site and mechanism of action of F6066. Although many investigators support a theory of a central action of Clomid, Hänngren (1964) adopted the theory of ovarian stimulation since the uptake of \(^{14}\)C-F6066 into the corpus luteum was rather pronounced, and Arnold (1967) also maintained the same theory since urinary estrogen increased after F6066 administration. According to the experiments of the authors, F6066 acts directly on the hypothalamo-pituitary to cause a marked secretion of LH, while no remarkable effect was noted in FSH and prolactin within the condition of methods of this paper.

Since Friedman et al. (1934) reported on a substance in alfalfa which induces ovulation in adult female rabbits, scattered reports have been found on the presence of substances which induced ovulation in adult female rabbits and of those with estrogenic activity or antiestrogenic activity in plant components. Two components in the course of analysis of Folium Lycii, which induced ovulation, were used for incubation of rat anterior pituitary to study the effect on LH and FSH secretion. Few such reports are yet available. In the experiment of the authors, G-25F1 and IR-4B directly acted on the pituitary to stimulate LH secretion significantly, but scarcely affected FSH.

In summary, the dynamic changes of LH, FSH and prolactin at the time when Clomid, F6066 and LCM were added singly or with hypothalamus to anterior pituitary incubated in vitro were studied. As each substance produced a characteristic pattern of gonadotropic secretion, a different mechanism of their action may be suggested. Species specificity of these substances should also be considered. It may also be considered that results from experiments in vitro are not the same as those in vivo and that difference of experimental procedures in vitro come up to difference of results. Accordingly, more detailed experiments may be necessary to make clear the mechanism of these ovulatory substances.

References


