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A STUDY OF THE TERATOGENIC POTENTIAL
OF ORAL HORMONAL PREGNANCY TESTS
IN THE WHITE RAT

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Introduction

Hormonal pregnancy tests have been used for the early diagnosis of pregnancy or amenorrhea of short duration since the late 1940's. They are considered simple, inexpensive, accurate and, until recently, safe diagnostic agents that could be given up to several weeks into pregnancy. They contain naturally occurring or synthetic progesterones with estrogens. Their mechanism of action is based on progesterone induced withdrawal bleeding in the estrogen primed (proliferative phase) endometrium. That is, orally or parentally administered progesterone with estrogen is given over a two to three day period then abruptly discontinued. If the woman is not pregnant the progesterone stimulated endometrium will be sloughed and bleeding induced. However, if she is pregnant the endometrium will be maintained by progesterone output from her own corpus luteum and/or the developing placenta.

Hormonal pregnancy tests were initially developed from progesterone and estrogen compounds used in the treatment of primary amenorrhea. Their composition and usage was refined by numerous physicians into the oral and injectable pregnancy tests of today. Most hormonal pregnancy tests are composed of from 2.5mg to 10mg norethisterone acetate or 50mg ethisterone (progesterones) with from .02 to .05mg ethinyl estradiol (estrogens). Numerous companies around the world have produced and marketed them under various names: U.S.A. -- Squibb "Gestest" and Roussel "Pro-Duosterone"; Canada -- Will "Duphaston", Barlowe and Cote "Estro-Prodial" and "Ethisterone"; West Germany -- Schering A.G. and Knoll A.G. "Primodos oral" and "Duogynon simplex"; Jordan and U.A.R. -- Schering A.G. "Duogynon" and "Primodos forte". 
The teratogenic potential of any drug administered during pregnancy has generally been thoroughly investigated by the manufacturer and the F.D.A. Because of the use of hormonal pregnancy tests since the 1940's, there had been much clinical evidence of their efficacy with no reports of associated congenital malformation. Thus, hormonal pregnancy tests were considered safe to the embryo with only the adverse effects on the user being investigated. In 1961, the F.D.A. examined the clinical literature and passed hormonal pregnancy tests as safe and effective for their intended purpose. However, since 1962 there has been mounting controversy regarding the teratogenic potential of hormonal pregnancy tests. Some researchers have found no teratogenic connection while others have implicated them in masculinization of the female fetus, congenital neural tube defects (meningo(myelo)cele and hydrocephalus), congenital heart and VACTEL malformations, spontaneous abortions, still births, and congenital limb bud defects.

Review of the Literature

In 1962 Dubowitz reported masculinization (non-adrenal female pseudohermaphordism) of a female infant whose mother had used hormonal pregnancy tests. Masculinization of the female fetus had long been known as an adverse side effect of progesterone therapy for spontaneous abortion. However, this was the first time a hormonal pregnancy test had been mentioned as a possible cause. Then in 1967, Gal et al did a retrospective study of the mothers of 100 babies born with spina bifida and hydrocephalus and compared them to 100 matched controls who had delivered healthy babies. There was a significant difference in the number of hormonal pregnancy tests used by the affected group as compared to the controls. Thus, there was a possible association between congenital neural tube defects and hormonal pregnancy tests. Mitchell et al in a study of heart disease in 56,109
birth suggested a possible correlation with hormonal therapy during pregnancy and congenital transposition of great vessels (T.G.V.) of the heart. Nora and Nora in a study of 10 patients with the congenital anomaly known by the acronym VACTERL (vertebral, anal, cardiac, tracheal, esophageal, limb defects) found 8 had been exposed to hormonal pregnancy tests. Also, a retrospective study of 224 children born with congenital heart disease found that a significant number had received progesterone with estrogen during the critical period of cardiogenesis, resulting in heart malformations, primarily transposition of the great vessels. In addition Levey et al. did a retrospective study of 76 cases born with T.G.V. and concluded that hormonal pregnancy tests or sex hormones during pregnancy could be a predisposing factor in congenital T.G.V. Most recently, Janerick et al. found that women who had used oral contraceptives during pregnancy, hormonal supportive therapy, or hormonal pregnancy tests had a higher number of offspring with congenital limb bud defects when compared to matched control mothers. Lastly, exogenous hormones have been implicated in increased spontaneous abortion rate and increased still birth numbers. Brotherton and Craft found that out of 91 spontaneous abortions 7.6% had taken hormonal pregnancy tests. The Royal College of General Practitioners’ survey on the outcome of pregnancy found a 10% abortion rate after "primodos" administration. Crombie et al. found a significant excess of hormone prescriptions in a survey of still births. Thus, retrospective studies have implicated hormonal pregnancy tests as possible teratogens.

In contrast several researchers have found conflicting evidence to the previous works. Dubowitz and Smithells studied 189 mothers who had
used hormonal pregnancy tests and found that none of the female offspring suffered from masculinization. Laurence et al. 28 in a study similar to Gal's, compared 271 mothers of spina bifida children to 323 matched controls and found no significant association between hormonal pregnancy tests and children with neural tube defects. In 1973 Oakley and Flynt 29 did a retrospective study of Atlanta women who gave birth to malformed children. Out of 433 women, 46 had taken hormonal pregnancy tests during the first trimester. The proportion of women with positive histories of hormonal pregnancy tests in each malformed group did not differ significantly from the proportion observed in the total. However, he concluded that there was an unusually large number with esophageal atresia and that studies other than retrospective ones could give more convincing evidence. Lastly, in response to the findings of Nora and Nora, David and O'Callaghan 30 tested the hypothesis that hormonal pregnancy tests may cause esophageal atresia as part of VACTEL syndrome. They compared the sale of hormonal pregnancy tests and occurrence of esophageal atresia over a 30 year period in South West England; but they found no linear trend to indicate an association. Thus, there has been conflicting research on hormonal pregnancy tests as teratogens.

Statement of Purpose

In 1973 the F.D.A. reviewed the clinical evidence since 1961 and the National Academy of Science - National Research Council Drug Efficacy's report and made the recommendation to withdraw approval of hormonal pregnancy tests. 31 Thus, the F.D.A. reported in the Federal Register (1973) that "Gestest" made by Squibb and other similar preparations could be potentially dangerous during pregnancy, and that proof of the drug's safety was lacking.
In response to the recommendation, Squibb removed "Gestest" from their products even though "Gestest" was listed in the 1974 P.D.R. However, the final ruling had not been issued by the F.D.A. and legally hormonal pregnancy tests could be produced and marketed in the U.S. In 1975 Roussel, makers of "Pro-Duosterone", were still selling their product; and did not plan on removing it from the market. Then in March 1975, the F.D.A. made their final ruling and all hormonal pregnancy tests were discontinued in the U.S.

Of particular interest is that the initial F.D.A. approval in 1961 and the recommendation to withdrawal approval were based entirely on clinical evidence supplied by the manufacturers and found in the literature quoted. As far as could be determined no animal studies had been used in the evaluation of the product. For example, previous to March 1975, the medical director and vice-president of Roussel felt that the clinical evidence was sufficient proof of safety, and that animal studies were unnecessary. Squibb reported that "Gestest" had been evaluated on only clinical evidence. Also, the F.D.A. stated that they had neither conducted nor authorized any animal experimentation on the teratogenic potential of this recently controversial drug.

The purpose of this study was to investigate the teratogenic potential of oral hormonal pregnancy tests on pregnant Sprague Dawley rats. The drug was administered in both physiological and potentially teratogenic dosages to the animals. The mothers were sacrificed and the fetuses were examined for various congenital anomalies.
Material and Methods

Animals: Thirty-eight female Sprague-Dawley albino rats of 200-250 gram weights were divided into two groups. The control group consisted of fifteen rats and the experimental group consisted of twenty-three rats. The control group was further divided into three subgroups of five animals each and the experimental group into four subgroups of five animals each and one subgroup of three animals. The females were purchased as timed pregnant rats from Charles Rivers Laboratories, Massachusetts. The rats had been paired with male animals for fertilization from 5 P.M. to 8 A.M. Pregnancy was indicated by a vaginal smear the following morning, and a positive smear was recorded as day zero of pregnancy. On the seventh day of pregnancy the animals were shipped by truck to the University of Connecticut Health Center. Throughout the experimental period water and a commercial diet were allowed ad libitum to all the animals.

Medication: Pro-Duosterone with the formula (ethisterone - 50.00mg and ethinyl estradiol - 0.03mg) was used as the model hormonal pregnancy test in this experiment. The ethisterone and ethinyl estradiol were obtained as pure hormones from the Sigma Chemical Company. Using pure hormones instead of prepared Pro-Duosterone eliminated any possibility of not being able to obtain the drug should it be removed from the market. The basic animal dosages of ethisterone (204 µg) and ethinyl estradiol (0.122 µg) were calculated for a 225 gram rat at a level proportional by weight to that consumed by a reference woman at 55 Kg. The appropriate dosages were weighed on a Sartorius electronic balance type 2472. The drug (ethisterone and ethinyl estradiol) was suspended in a solution of
normal saline with 0.4% polysorbate 80. The polysorbate 80 was added to assure a uniform suspension, as ethisterone and ethinyl estradiol were not water soluble and could possibly precipitate out before the appropriate dosage was drawn off. In addition, the hormones were passed through a forty-four micron pharmaceutical sieve prior to weighing and suspension. Reduction in particle size greatly reduced precipitation time once in solution. The hormone and saline mixture was prepared fresh at the beginning of each three day administration period and kept refrigerated at 38 F. to prevent any loss of potency or degradation of the drug.

The 5 experimental groups and 3 control groups received the following dosages:

- **Group I, controls** - nothing
- **Group II, controls** - 0.025 ml normal saline
- **Group III, controls** - 0.025 ml normal saline, 0.4% polysorbate 80 solution
- **Group IV** - basic dosage (204 μg ethisterone and 122 μg ethinyl estradiol) in 0.025 ml normal saline 0.4% polysorbate 80 solution.
- **Group V** - one-half the basic dosage (102 μg ethisterone and 0.061 μg ethinyl estradiol) in 0.025 ml normal saline 0.4% polysorbate 80 solution.
- **Group VI** - two times the basic dosage (408 μg ethisterone and 0.244 μg ethinyl estradiol) in 0.025 ml normal saline 0.4% polysorbate 80 solution.
Group VII, five times the basic dosage (1020 μg ethisterone and 0.610 μg ethinyl estradiol) in 0.025 ml normal saline 0.4% polysorbate 30 solution.

Group VIII, twenty times the basic dosage (4080 μg ethisterone and 1.240 μg ethinyl estradiol) in 0.025 ml normal saline 0.4% polysorbate 80 solution.

Timing of Administration: Each animal received 0.025 ml of suspension by stomach tube three times per day at four hour intervals (9 A.M. - 1 P.M. - 5 P.M.) on the 11th, 12th, and 13th day of pregnancy. The first two doses (9 A.M. and 1 P.M.) were at the standard dosages, however, the third dose (5 P.M.) was at double the concentration of hormones in 0.025 ml. The double concentration was necessary to duplicate the human administration pattern of four times per day. The three day regimen also duplicated the human treatment pattern of three consecutive days. 41 The 11th, 12th, and 13th days were selected because they covered the organogenic period in the rat that would include most of the human anomalies reported in the literature following hormonal pregnancy tests. Thus, the drug was given during the rat's critical period for the anomalies being investigated in this study. 42-45 The 13th day in the rat is the critical period in development of the appendages (digits and limbs), vertebra, skull, the ribs and the secondary palate. The 12th and 13th days are when final sexual differentiation occurs and anal and genital anomalies can occur. The three day period would also be critical for development of the sense organs, fusion of facial processes, and spinal anomalies (spina bifida, etc.) if they are to occur in the rat. The earlier 11th day is important for development of the tail and esophagus.
Data Collected: On the 19th day of pregnancy the animals were sacrificed by ether anesthesia, the fetuses removed, and the following data collected:

1. Number of alive, dead, and resorbed fetuses in each group
2. Weight and crown-rump length of each live fetus
3. Sex of each live fetus.

The incidence of the following anomalies was recorded:

1. Cleft lip
2. Cleft palate
3. Cleft lip and palate
4. Facial clefts
5. Anencephaly
6. Exencephalus
7. Hydrocephalus
8. Spina-bifida (meningocele)
9. Spina-bifida (myelomeningocele)
10. Spina-bifida (myeloschisis)
11. Anophthalmia
12. Microphthalmia
13. External ear position and anomalies
14. Anal and genital anomalies
15. Hypoplastic tail
16. Joined twins
17. Limb and paw defects

In addition, fetuses were stained with alizarin red according to the Humason method to allow examination of the skeletal system for anomalies of:

1. Ribs
2. Vertebra
3. Limbs
4. Digits
5. Skull

Weight, Crown-rump length, and Sex determination: Before weighing, fetuses were blotted dry to remove excess fluids and blood. They were then weighted on a Sartorius electronic scale type 1106. Crown-rump length
was measured along the dorsal surface with a flexible millimeter ruler from the tip of the nose to the base of the tail. Sex was determined by the method described by Rugh. Crown-rump measurements and sex were determined by one investigator.

Skeletal examination: All skeletal elements were examined under a dissecting microscope at approximately 7X by a single observer. The number of vertebrae and ribs was recorded; and the skeleton examined for rudimentary and malformed ribs, limbs, digits, skull and facial bones.

RESULTS

For each treatment group the following variables were determined:

1. Mean number of implantation sites
2. Mean number of live fetuses
3. Percent of resorbed fetuses
4. Percent of fetuses that were male
5. Mean total fetal weight of each group (fig. 1)
6. Mean individual fetal weight
7. Mean individual fetal length.

One-way analysis of variance was performed for variables one to five described above. Statistical analysis found no significant difference at the .05 level for these variables (fig. 2). No discernible difference between groups for variables six and seven were observed (fig. 3).

All fetuses were examined for the external malformations listed in the material and methods. Infrequent minor anomalies were detected in both
control and experimental groups; however, there was no tendency for an increased frequency in the groups receiving the hormones. No major external anomalies such as: cleft palate, spina-bifida, hydrocephalus, etc. were found in any fetuses (fig. 13, 14, 15). Only one fetus in the group receiving 2X of the basic dose had a deformed leg (fig. 4).

Minor anomalies found included: extended tail tips, dark spots on the medial dorsal surface, and one to two enlarged digits. Extended tail tips were defined as an extra soft tissue tip greater than 1 mm on the end of the tail (fig. 5). Numerous fetuses in all groups had extended tail tips less than 1 mm; however, only two fetuses in the 5X group had extended tail tips. Four fetuses with medial dorsal dark spots were found in the saline and polysorbate groups (fig. 6). Dissection showed no spinal defects in any of these specimens. Enlarged digits were found on three specimens from the saline, 2X, and 5X groups (fig. 7). In general, none of these anomalies were related to experimental treatments, but instead represented random developmental events.

Skeletal examination of the fetuses revealed vertebral variations, supernumerary ribs, and malformed ribs. No major deformations of the limbs, paws, or skull were detected.

Variations in the vertebra occurred in the center portion and were classified as bilobed or split centra (fig. 8). They were found in several control and experimental groups. A chi square test for significance was performed between the three control groups and the five experimental groups. The three control groups were also compared to the 20X plus 5X groups, and the 20X group. The controls had a significantly lower incidence (.05 level)
of vertebral variations as compared to the experimental groups. (fig. 9)

Supernumerary or 14th rudimentary ribs were also found in several controls and experimental groups. The rudimentary ribs varied from small ossification centers to partially formed ribs (fig. 10). In addition, they were found as unilateral and bilateral structures. Statistical analysis revealed a significant increase at the .01 level for rudimentary ribs in the three experimental groups (1/2X to 20X, 5X + 20X, and 20X). Deformed ribs appeared as wavy ribs (fig. 11) and appeared in groups: 1X, 2X, and 5X. However, the incidence was too small for statistical analysis (fig. 12).
figure 1
# One Way Analysis of Variance

## For Variables One to Five

<table>
<thead>
<tr>
<th></th>
<th>normal saline polysorbate</th>
<th>1/2X</th>
<th>1X</th>
<th>2X</th>
<th>5X</th>
<th>20X</th>
<th>F ratio</th>
<th>F prob.</th>
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<tr>
<td># of sites</td>
<td>11.4</td>
<td>11.4</td>
<td>11.0</td>
<td>11.2</td>
<td>12.2</td>
<td>11.6</td>
<td>13.6</td>
<td>12.0</td>
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<td>% resorbed</td>
<td>12.3</td>
<td>3.5</td>
<td>9.1</td>
<td>5.4</td>
<td>19.7</td>
<td>0</td>
<td>11.8</td>
<td>33.3</td>
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<tr>
<td>% Male</td>
<td>48</td>
<td>41.8</td>
<td>46</td>
<td>45.3</td>
<td>55.1</td>
<td>46.6</td>
<td>43.3</td>
<td>33.3</td>
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<tr>
<td># of live fetuses</td>
<td>10</td>
<td>11</td>
<td>10</td>
<td>10.6</td>
<td>9.8</td>
<td>11.6</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Total fetal weight</td>
<td>26.8</td>
<td>25.8</td>
<td>26.7</td>
<td>28.9</td>
<td>26.5</td>
<td>33.7</td>
<td>32.8</td>
<td>21.7</td>
</tr>
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</table>

**Figure 2**
figure 3
Deformed rear leg
2X Group

figure 4
No tail tip Polysorbate group

figure 5
Tail tip less than 1mm - Normal group

figure 5
Tail tip greater than 1mm - 5X group

figure 5
Dark spot on dorsal surface of fetus from polysorbate group

figure 6
Enlarged digits on fetus from group 2X

figure 7
Chi Square test of Control and Treatment Groups

Bilobular and Split Centra

<table>
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<th>Groups</th>
<th>Probability</th>
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<tr>
<td>I  Controls - Groups ( \frac{1}{2}X ) to 20X</td>
<td>P = .0129</td>
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<tr>
<td>II Controls - Groups 5X + 20X</td>
<td>P = .01</td>
</tr>
<tr>
<td>III Controls - Group 20X</td>
<td>P = .02</td>
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Rudimentary Ribs

<table>
<thead>
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<th>Groups</th>
<th>Probability</th>
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</thead>
<tbody>
<tr>
<td>I  Controls = Groups ( \frac{1}{2}X ) to 20X</td>
<td>P = .00005</td>
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<tr>
<td>II Controls - Groups 5X to 20X</td>
<td>P = .000002</td>
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<tr>
<td>III Controls - Group 20X</td>
<td>P = .00001</td>
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[figure 9]
figure 8  Split and bilobular centra.

figure 10  Bilateral rudimentary 14th rib.
Deformed ribs

figure 11
<table>
<thead>
<tr>
<th>Group</th>
<th>% Rudimentary or Extra Ribs</th>
<th>% Bilobular or Split Centra</th>
<th>% Malformed Ribs</th>
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<tr>
<td></td>
<td>Unilateral - Bilateral</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.27</td>
<td>2.27</td>
<td>6.82</td>
</tr>
<tr>
<td>Saline</td>
<td>5.45</td>
<td>9.09</td>
<td>20.</td>
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<tr>
<td>Polysorbate</td>
<td>3.57</td>
<td>10.71</td>
<td>25.</td>
</tr>
<tr>
<td>1/2X</td>
<td>28.27</td>
<td>58.70</td>
<td>19.57</td>
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<tr>
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<td>2X</td>
<td>18.52</td>
<td>22.22</td>
<td>38.89</td>
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<tr>
<td>5X</td>
<td>24.44</td>
<td>26.67</td>
<td>24.44</td>
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<tr>
<td>20X</td>
<td>33.33</td>
<td>41.67</td>
<td>20.83</td>
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figure 12
Fetuses from groups: Normal, Saline, Polysorbate, 1X, 2X, 5X (upper left to right)

figure 13
Fetuses from groups: Normal, Saline, Polysorbate, 1X, 2X, 5X, (upper left to right)

figure 13
Fetuses from groups: Normal, Saline, Polysorbate and $\frac{1}{2}X$

(upper left to right)

figure 14
Fetuses from groups: 1X, 2X, 5X and 20X (upper left to right)

figure 15
DISCUSSION

Recently hormonal pregnancy tests have been implicated as potentially teratogenic agents. Retrospective studies have associated them with masculinization of the female fetus, spinal defects, VACTEL syndrome, and increased spontaneous abortion rate. In response, the F.D.A. in March 1975 withdrew approval of hormonal pregnancy tests because of their reported teratogenic potential. This study examined the effect of hormonal pregnancy tests on the fetuses of albino rats. The study stayed within physiologic dose limits (1/2X to 20X) with no attempt to study the effect of experimental teratogenic dosages.

In general, the results of the experiment did not support the contention that hormonal pregnancy tests are potentially dangerous to rat fetal development. This study found no increase in the per cent of resorbed fetuses in treatment groups. This was in contrast to Brotherton and Craft\textsuperscript{25} and Crombie et al\textsuperscript{26} who had reported increased spontaneous abortions and still births in humans with the use of pregnancy test agents.

In this experiment, no genital anomalies were found and no increase in percentage of male fetuses in treatment groups. Masculinization could not be clearly identified as no dissection of fetuses was performed in the present study. The present study did not examine for esophageal atresia, anal anomalies, T.G.V., and tracheal anomalies, as reported by Nora\textsuperscript{21}, Levy\textsuperscript{22}, and Mitchell\textsuperscript{23}.

Skeletally there were significant increases in bilobular and split vertebra centra and extra rudimentary ribs in the experimental group. However, the centra and ribs may not be anomalies but variations of normal skeletal structures. It is difficult to speculate how these variations would have expressed themselves in fully developed rats. Kuhns and Hormell\textsuperscript{47} reported that scoliosis and orthopedic problems in children are associated with variations and numerical alterations in the vertebra. However, Kimmel and Wilson\textsuperscript{48} indicated that extra
rudimentary ribs and bilobular centra should not be classified as abnormal and they may only be a sign of embriotoxicity. Also, Bertilli and Donati stated that the incidence of extra ribs is too high in normal rabbits to be classified as an abnormality; however, it may be an indicator of drug activity.

In conclusion, this study did not find conclusive evidence that oral hormonal pregnancy tests at physiological doses are a teratogenic agent in white rats.
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