Teratogenic action of the thyroid stimulating hormone and its interaction with trypan blue

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The role that the thyroid gland may play in embryonic development has been under investigation for many years. A direct relationship between thyroid gland function and development is best illustrated in the amphibia (see Kollros, 1961, for pertinent literature). Attempts to demonstrate a similar influence of thyroxin on development in mammals has led to controversial results, in part due to the difficulty of separating the metabolic from the possible developmental effects of the hormone. In order for thyroxin to exert a direct effect upon the mammalian embryo it must cross the placenta. The bulk of the evidence seems to favor the viewpoint that the placenta is rather impermeable to thyroxin during the early stages of gestation and that this permeability increases near term. This is apparently true of the rabbit and man (Osorio & Myant, 1960) and the rat (Hamburgh, Sobel, Koblin & Rinestone, 1962; Roy & Kobayashi, 1962).

The thyroids of offspring from thyroidectomized female guinea-pigs treated with thyrotropin (TSH) during pregnancy did not differ in weight or histological appearance from those of fetuses from the normal control mothers (Peterson & Young, 1952). Injection of TSH into pregnant rats resulted in stimulation of maternal thyroids but no stimulation was observed in embryonic thyroids (Tobin, 1941). Hypophysectomy of pregnant rats neither altered the histological appearance or weight of the fetal thyroid at term from those observed in thyroids of fetuses from normal mothers (Hamburgh et al. 1962) nor influenced iodine uptake by the fetal thyroid (Nikitovitch & Knobil, 1955). These reported results suggest that maternal thyrotropin and injected thyrotropin do not affect the maturation of the fetal thyroid gland, presumably because thyrotropin does not cross the placenta from mother to fetus.

Thyroid dysfunctions in pregnancy have been repeatedly investigated, but the exact role that the thyroid hormone plays in embryonic and fetal development is not known. Evidence has been reported that women who are hypothyroid have a higher incidence of reproductive failure than women with normal thyroid function or those who receive supplemental hormone therapy (Moore, 1950; Mann, Shaver & Cooke, 1958; Hoet, Gommers & Hoet, 1960; Osorio & Myant, 1960).
In addition to frank malformations, there is a high incidence of mental retardation in living offspring of untreated hypothyroid mothers. Maternal hyperthyroid states are apparently without effect on the human embryo. Thyroid-less animals have been shown to be able to conceive and produce essentially normal young (Davenport & Swingle, 1927; Chu, 1944; Stempak, 1962). Hamburgh, Lynn & Weiss (1964) concluded that the development of the rat fetus was not markedly influenced by the presence or absence of the thyroid hormone. However, Langman & van Faassen (1955) reported a high percentage of eye defects following partial thyroidectomy in rats.

Trypan blue has been reported to decrease the thyroxin-binding capacity of plasma (Crispell, Coleman & Hyer, 1957). Yamada (1960) demonstrated that after repeated injections of trypan blue into male rats the thyroid weight and serum protein-bound iodine had decreased and the thyroidal uptake of radio-iodine was suppressed. Exogenous TSH given to these rats restored hormone secretion. Shimoda, Tomizawa, Yamada & Shichijo (1962) and Yamada et al. (1965) suggest that trypan blue may compete with thyroxin for serum protein binding sites and the free thyroxin thus produced inhibits TSH secretion by the pituitary, causing hypofunction of the thyroid gland.

The present experiment was designed to test for interaction between the thyroid gland and its hormone, the thyroid stimulating hormone of the pituitary gland, and the teratogenic activity of trypan blue. The working hypothesis was that if exogenous TSH would return the hypofunctioning thyroid gland to near normal, as reported in the literature, this might offset the reported inhibitory action of trypan blue on the thyroid gland and in so doing perhaps affect the teratogenic outcome of trypan blue treatment.

**MATERIALS AND METHODS**

Virgin females of Wistar Albino rats (Albino Farms, Red Bank, New Jersey) were used in this study. The animals were maintained on a Rockland Complete Rat Diet, *ad lib.* Day 0 of pregnancy was considered to begin on the morning sperm was found in the vaginal smear. Thyrotropin (Nutritional Biochemicals, Cleveland, Ohio) was administered intraperitoneally at a dose of either 5, 10, or 20 USP units per animal. The thyrotropin was prepared in sterile saline and administered in one-quarter doses at 24 h intervals during the 7th, 8th, 9th and 10th days of gestation. Trypan blue was prepared as a 2% aqueous solution and injected intraperitoneally during the 8th day of pregnancy either as a teratogenic dose of 14 mg/100 g maternal body weight or as a subteratogenic dose of 0-6 mg/100 g maternal body weight. Pregnancy was terminated on the 20th day, uterine resorption sites counted, and the fetuses recovered, weighed, and fixed in Bouin’s fluid or 95% alcohol, for subsequent examination for malformations.

The fetuses fixed in 95% alcohol were prepared for staining with alizarin red
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for visualization of the skeleton. The internal thoracic and abdominal organs of Bouin's-fixed fetuses were removed and examined for abnormalities. The head of each Bouin's-fixed fetus was sectioned freehand with a razor blade into slices of approximately 2 mm in thickness. This procedure allowed easy visualization of defects in the head, face, eyes and brain, which were the predominant locations of abnormalities in this experiment.

RESULTS

Table 1 presents the results of treating pregnant rats with varying concentrations of exogenous TSH and a teratogenic concentration of trypan blue. The injection of 5 units of TSH in conjunction with a teratogenic dose of trypan blue had no effect on the teratogenicity of trypan blue. When 10 or 20 units of TSH were injected in conjunction with the dye, there was an apparent increase in both resorptions and malformed survivors. More striking was the observation that 20 units of thyrotrophin injected during the 7th–10th days of gestation caused

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mothers</th>
<th>No. of implantation sites</th>
<th>No. and % resorbed or dead</th>
<th>No. and % survivors malformed</th>
<th>% implantation sites affected by treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue on day 8 (14 mg/100 g)*</td>
<td>18</td>
<td>187</td>
<td>82 (43.8)</td>
<td>82 (78.2)</td>
<td>87.8</td>
</tr>
<tr>
<td>5 units TSH, 1/2 dose on days 7, 8, 9, 10</td>
<td>10</td>
<td>97</td>
<td>8 (8.2)</td>
<td>1 (1.1)</td>
<td>9.3</td>
</tr>
<tr>
<td>5 units TSH plus trypan blue</td>
<td>17</td>
<td>182</td>
<td>81 (44.5)</td>
<td>84 (83.2)</td>
<td>90.7</td>
</tr>
<tr>
<td>10 units TSH, 1/2 dose on days 7, 8, 9, 10</td>
<td>10</td>
<td>109</td>
<td>4 (3.7)</td>
<td>1 (0.95)</td>
<td>4.6</td>
</tr>
<tr>
<td>10 units TSH plus trypan blue</td>
<td>8</td>
<td>82</td>
<td>58 (70.1)</td>
<td>19 (79.3)</td>
<td>94.0</td>
</tr>
<tr>
<td>20 units TSH, 1/2 dose on days 7, 8, 9, 10</td>
<td>20</td>
<td>224</td>
<td>74 (33.0)</td>
<td>37 (24.6)</td>
<td>49.6</td>
</tr>
<tr>
<td>20 units TSH plus trypan blue</td>
<td>8</td>
<td>80</td>
<td>49 (61.8)</td>
<td>30 (96.8)</td>
<td>98.8</td>
</tr>
</tbody>
</table>


one-third of the implantation sites to be resorbed and one-quarter of the survivors to be malformed. Because of the high incidence of malformations following treatment with a teratogenic dose of trypan blue, this experiment did not reveal whether or not any interaction existed between the dye and the hormone. It was decided that the use of a subteratogenic dose of trypan blue might yield the sought-after information.
Table 2 gives the results of this experiment. Ten units of thyrotropin injected over a 4-day period early in pregnancy (days 7, 8, 9 and 10) caused only four resorptions and one malformed fetus in 109 implantation sites. A subteratogenic dose of trypan blue administered as a single intraperitoneal injection during the 8th day of gestation caused one resorption and no malformations in 122 implantation sites. When the subteratogenic dose of trypan blue was given in conjunction with 10 units of TSH, there was only a slight increase in resorptions and malformations. After administration of 20 units of thyrotropin together with the dye, there was found to be no change in the resorption rate (33 % with TSH alone and 32.5 % with TSH and trypan blue combined); however, there was a marked rise in the incidence of malformations, from 24.6 % with TSH alone to 59.7 %. Almost three-fourths of the implantation sites were affected by the combined treatment whereas only one-half were affected with thyrotropin alone.

The types of malformations seen following treatment with trypan blue or thyrotropin show many similarities and a few marked dissimilarities. Table 3 lists the major defects seen after treatment with dye and hormone. Eye malformations and hydrocephalus were the most frequent abnormalities present in fetuses from mothers treated with either teratogen. Two malformations, taillessness and megalophthalmia, were found only following treatment with trypan blue. Agnathia was observed exclusively in fetuses from TSH treated mothers. When the two teratogens were used in conjunction with one another, it was found that a teratogenic dose of trypan blue used with any concentration of TSH predisposed to malformations found when trypan blue was used alone. When TSH was used in teratogenic concentration together with a subterato-

Table 2. Effects of intraperitoneal injections of thyrotropin and a subteratogenic dose of trypan blue

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mothers</th>
<th>No. of implantation sites</th>
<th>No. and % resorbed or dead</th>
<th>No. and % survivors malformed</th>
<th>% implantation sites affected by treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue on day 8 (0-6 mg/100 g)*</td>
<td>9</td>
<td>122</td>
<td>1 (0.82)</td>
<td>0</td>
<td>0.82</td>
</tr>
<tr>
<td>10 units TSH, 1/2 dose on days 7, 8, 9, 10</td>
<td>10</td>
<td>109</td>
<td>4 (3.7)</td>
<td>1 (0.95)</td>
<td>4.6</td>
</tr>
<tr>
<td>10 units TSH plus trypan blue</td>
<td>15</td>
<td>187</td>
<td>11 (5.9)</td>
<td>7 (4.0)</td>
<td>9.6</td>
</tr>
<tr>
<td>20 units TSH, 1/2 dose on days 7, 8, 9, 10</td>
<td>20</td>
<td>224</td>
<td>74 (33.0)</td>
<td>37 (24.6)</td>
<td>49.6</td>
</tr>
<tr>
<td>20 units TSH plus trypan blue</td>
<td>15</td>
<td>169</td>
<td>55 (32.5)</td>
<td>68 (59.7)</td>
<td>72.8</td>
</tr>
</tbody>
</table>

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genic dose to trypan blue, the incidence of the major malformations was the same as if TSH had been used alone.

Table 3. Incidence (%) of major malformations observed after treatment with tyrotropin and trypan blue

<table>
<thead>
<tr>
<th>Malformation</th>
<th>Trypan blue (teratogenic dose)</th>
<th>Trypan blue (teratogenic dose plus any dose of TSH)</th>
<th>Trypan blue (subteratogenic dose plus 20 units TSH)</th>
<th>TSH (20 units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anophthalmia</td>
<td>29.9</td>
<td>34.8</td>
<td>29.8</td>
<td>32.8</td>
</tr>
<tr>
<td>Hydrocephalus</td>
<td>15.4</td>
<td>12.4</td>
<td>35.4</td>
<td>41.0</td>
</tr>
<tr>
<td>Exencephaly</td>
<td>15.1</td>
<td>11.4</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Microphthalmia</td>
<td>5.7</td>
<td>9.1</td>
<td>12.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Vertebral</td>
<td>5.7</td>
<td>5.3</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Encephalomeningocele</td>
<td>5.4</td>
<td>9.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Megalophthalmia</td>
<td>3.9</td>
<td>1.9</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Tailless</td>
<td>3.6</td>
<td>2.4</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Agnathia</td>
<td>1.4</td>
<td>5.7</td>
<td>6.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Gastroschisis</td>
<td>14.0</td>
<td>7.4</td>
<td>10.4</td>
<td>8.1</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of this experiment offer additional evidence that agents may interact to produce congenital malformations. The relationship between teratogenic agents and the genetic constitution, physiological and nutritional state of the mother and fetus is not clearly understood. It appears safe to assume a complex interplay among these factors. An interaction between genetic and nutritional factors has been demonstrated in mice. Runner (1959) has shown that a 24 h fast during the 9th day of pregnancy can increase the incidence of spontaneously occurring skeletal defects. Woollam & Millen (1960) reported the teratogenic activity of hypervitaminosis A in rats to be enhanced by cortisone and hypothyroidism. Two or more chemical agents may each be present in concentrations too low to cause a teratogenic effect yet interact to produce a significant teratogenic effect (Wilson, 1964). Not all interactions necessarily result in potentiation. Runner & Dagg (1960) have postulated that interaction between different teratogenic agents may result in (1) an interference effect, (2) a non-additive effect, (3) an additive effect, or (4) a reinforcing effect (the total effect is greater than the effect of each teratogen added together). The effect of hormones on teratogenic action has been extensively investigated by Woollam & Millen (1960). They found cortisone and methylthiouracil increased the numbers of malformations in offspring of hypervitaminosis A rats. Thyroxin, at certain doses, gave complete protection from the teratogenic effects of the hyper-
vitaminosis condition while insulin merely reduced these effects and estradiol
had no effect. Woollam & Millen also reported that the teratogenic effects of
X-irradiation could be enhanced by the hormones, thyroxin, cortisone, and
insulin.

The results of the present experiment raise questions as to the nature of the
teratogenic action of the thyrotrropic hormone as well as to the relation of the
hypophysis and thyroid glands to normal and abnormal development. The early
stages of embryonic development presumably do not require the thyroid hor-
mone since placental transfer is low and the fetal gland is not functioning.
The accumulation of radioactive iodine by human fetal thyroids during the
4th month of pregnancy has been interpreted as indicative of the onset of the
ability of the fetal thyroid gland to function (Hodges, Evans, Bradbury & Keettel,
1955). Hall & Kaan (1942) demonstrated that the thyroid gland of the 18-day-old
rat fetus had hormone activity (birth occurred at 20½ or 21 days in these rats).
At the time of administration of thyrotropin, in the present study, neither the
embryonic anterior hypophyseal lobe nor the thyroid gland consisted of more
than a few cells in the process of invaginating from the stomadeal depression or
evaginating from the foregut. In addition, all available evidence suggests that
TSH does not cross the rat placenta. It would appear, then, that the site of
primary action of exogenously supplied thyrotropin must be within the mother
or placenta. The principal effect of TSH administration is a stimulation of the
thyroid gland causing cellular hypertrophy, release of stored colloid, and a
stimulation of hormone synthesis. It is difficult to postulate teratogenic action
from this knowledge of TSH function. The possibility exists that certain un-
known contaminants were present in the samples of thyrotropin used in these
experiments although the sample specifications furnished by Nutritional Bio-
chemicals report only a trace of organic iodide and ash. The major effect of
release of thyroxin by the thyroid gland is described as a generalized increase in
metabolism with a fall in liver glycogen, increased excretion of nitrogen in the
urine, and a slight lowering of the respiratory quotient indicative of increased
fat and protein utilization. Such a hyperthyroid state has not been associated
with an abnormal outcome of pregnancy. On the other hand, hypothyroidism
is thought to be related to various difficulties during the gestational period and
thought, by some at least, to be responsible for frank malformations, especially
of the nervous system (Mann et al. 1958; Greenman et al. 1962). The experi-
mental production of hypothyroidism by partial thyroidectomy has been re-
ported to cause malformations in rats by Langman & van Faassen (1955).
Stempak (1962) repeated the experiments of Langman & van Faassen, using a
different strain of rats, and was unable to confirm their findings.

Our experiment demonstrates that with the proper dosage and at a particular
time in rat gestation the thyrotrropic hormone can cause death and resorption of
developing embryos and induce malformations in the surviving fetuses. The
nature and site of action of this hormone remains to be determined. If the
placenta is impermeable to TSH, as current literature suggests, then the site of action must be sought for within the placenta or maternal organism.

SUMMARY

1. Pregnant Wistar Albino rats were injected intraperitoneally with trypan blue and thyroid stimulating hormone (TSH). Trypan blue was administered as a single injection during the 8th day of pregnancy. TSH was administered during the 7th–10th days, one-quarter of the total dose each day. Autopsy was on the 20th day.

2. The injection of 10 or 20 USP units of TSH in conjunction with a teratogenic dose of trypan blue (14 mg dye per 100 g maternal body weight) caused a slight increase in resorptions and malformations over the numbers observed following trypan-blue treatment alone.

3. The injection of 20 units of TSH alone caused one-third of the implantation sites to be resorbed and one-fourth of the survivors to be malformed.

4. A subteratogenic concentration of trypan blue (0.6 mg/100 g) did not cause malformations in fetuses of treated rats. A subteratogenic dose of trypan blue injected in conjunction with 20 units of TSH more than doubled the incidence of malformations (60%) over that observed following TSH injection alone (25%). The incidence of resorption was not changed.

5. These results demonstrate that, under the conditions of this experiment, the thyroid-stimulating hormone is teratogenic to the developing fetus of the Wistar Albino rat and that some interaction occurs between exogenously supplied TSH and trypan blue.

RÉSUMÉ

Action tératogène de l'hormone stimulant la thyroïde et son interaction avec le bleu trypan

1. Des rattes Wistar Albinos, gestantes, reçoivent une injection intrapéritonéale de bleu trypan et d'hormone stimulant la thyroïde (TSH). Le bleu trypan est administré en une seule dose, le 8ème jour de la gestation. TSH est administrée en plusieurs doses (un quart de la dose totale chaque jour) du 7ème au 10ème jour de la gestation. Les rattes sont autopsiées le 20ème jour.

2. Après injection simultanée de 10 ou 20 unités USP de TSH et d'une dose tératogène de bleu trypan (14 mg. de colorant pour 100 g. de poids du corps maternel), le nombre de résorptions et de malformations est un peu plus élevé qu'après traitement au bleu trypan seul.

3. Après injection de 20 unités de TSH, un tiers des sites d'implantation sont résorbés et un quart des survivants sont malformés.

4. Une concentration de bleu trypan (0.6 mg./100g.) inférieure à la concentration tératogène ne provoque pas de malformations chez les foetus des rattes traitées. Après injection simultanée d'une dose de bleu trypan inférieure à la
dose tératogène et de 20 unités de TSH, le nombre de malformations (60 %) est plus du double de celui qu'on observe après une injection unique de TSH (25 %). Le taux des résorptions n'est pas modifié.

5. Ces résultatsmontrent que dans les conditions expérimentales, l'hormone stimulant la thyroïde est tératogène pour le foetus de ratte Wistar Albino en voie de développement et il semble qu'une certaine interaction existe entre le TSH administré et le trypan bleu.

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REFERENCES

Teratogenicity of TSH and trypan blue


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