The effect of thyroxine, 3,5-dimethyl-3'-isopropyl-L-thyronine and iodized oil on fetal brain development in the iodine-deficient sheep

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Abstract. Studies have been carried out to investigate the role of maternal and fetal thyroid function in the effects of iodine deficiency on fetal brain development in sheep. Iodine deficiency was established with an especially prepared low-iodine diet of maize and pea pollard. The iodine-deficient sheep were mated and at the end of the second trimester of pregnancy (100 days gestation) were divided into groups which received either a sc injection of T\(_4\) or 3,5-dimethyl-3'-isopropyl-L-thyronine or an im injection of iodized oil. At 140 days gestation (10 days prior to parturition) comparison of the fetuses delivered by hysterotomy revealed that the retarded fetal brain development observed in iodine deficiency was greatly improved by T\(_4\) and by iodized oil. However, T\(_4\) and iodized oil failed to correct the reduction in the number and the increase in the length of synaptic appositions which were observed in the fetal cerebral cortex after iodine deficiency. In addition, the histological appearance of the fetal thyroid gland and the levels of plasma thyroid hormones were restored to normal. The administration of 3,5-dimethyl-3'-isopropyl-L-thyronine had no effect on the retarded fetal brain and body development of the iodine-deficient fetuses. The lack of response may be due to the inability of 3,5-dimethyl-3'-isopropyl-L-thyronine to cross the ovine placenta as no reduction in the abnormally elevated fetal plasma TSH was observed in spite of a fall in maternal plasma TSH and apparent restoration of maternal thyroid function. It is concluded that the retarded fetal brain development observed during iodine deficiency in sheep can be substantially improved by iodized oil or to a lesser extent by T\(_4\) administration at 100 days gestation and that this is dependent on the restoration of both maternal and fetal thyroid function which supports previous observations from this laboratory following fetal and maternal thyroidectomy. The persistence of some effects of iodine deficiency on the fetal brain suggests that irreversible damage may have occurred.

Iodine deficiency in the pregnant sheep has been shown to cause retardation of fetal brain development (1). A subsequent study demonstrated substantial though not complete reversal of this retardation by the injection of iodized oil at 100 days (2).

In order to investigate the role of the maternal and fetal thyroid function further studies have been carried out by comparison of the effects of iodized oil with those of T\(_4\) and the non-halogenated thyroid hormone analogue 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT). Thyroxine has been shown to be ineffective in crossing the placenta in late pregnancy (3) although there is more recent evidence of its passage to the fetus early in pregnancy in the rat (4,5). DIMIT has been shown to possess thyromimetic properties in a wide range of species (6–10). Pregnant ewes have been shown to respond to DIMIT, but it failed to prevent hypothyroidism in the thyroidectomised fetal lamb (11). The thyromimetic activity of DIMIT in the ewe was employed therefore to normalize maternal pituitary function and those tissues dependent on plasma T\(_3\) and allow fetal hypothyroidism to be studied in the absence of iodine.
Materials and Methods

Thirty-two Merino ewes aged three years were individually housed in pens and fed daily a specially selected low-iodine diet of crushed maize and pea pollard providing only 5–8 μg iodine per day (12). Ten of the ewes (controls) were supplemented at commencement with a single intramuscular injection of iodized oil containing 400 mg iodine (‘Lipiodol’ Viscous, May and Baker, Ltd, England). All the sheep received de-ionized water for drinking purposes. When severe iodine deficiency had been established by plasma T₄ levels of less than 20 nmol/l in the depleted animals, all 32 of the ewes were successfully mated with iodine-replete fertile rams and the dates of conception noted. At 100 days gestation, 4 ewes received T₄ as a sc injection of 0.5 mg of the free acid dissolved in 1 ml of 0.1 mol/l NaOH/saline on alternate days and 3 received 2 mg DIMIT sc in 2 ml of 0.1 mol/l NaOH/saline on alternate days. DIMIT was kindly supplied by Dr P. Block, River Research, 4059 River Road, Toledo, OH. Fifteen iodine-deficient ewes remained untreated. At 140 days gestation (i.e. 10 days before parturition), pregnancies were terminated because of the uncertainty of maintaining viable fetuses thereafter in the iodine-deficient group.

The fetuses were delivered by hysterotomy from the ewes under sedation and epidural anesthesia (1) and immediately bled, killed by an overdose of barbiturate, weighed and dissected. T₄ and TSH were determined on blood plasma by radioimmunoassay (12). Thyroids were removed, one for iodine analysis (12) and the other fixed with formalin and stained with hematoxylin and eosin.

Fetal brains were carefully dissected from the cranium (13) and divided into cerebral hemispheres and cerebellum, each of which was further subdivided sagittally along its midline and one half stored at −20°C until DNA (14) protein (15), and cholesterol (16) could be measured. The values for DNA, protein: DNA ratio and cholesterol: weight ratio were used to determine cell number, cell size and myelination, respectively (16). Water was determined by the weight change after drying the tissue samples in an oven at 105°C for 24 h.

The other half of each segment of brain was preserved in aldehyde fixative (4% formaldehyde with 1% glutaraldehyde in 0.1 mol/l sodium phosphate buffer at pH 7.4) for histological examination. The cerebellum was sectioned sagittally at 30 μm near the midline on a freezing microtome, the cut medial surface of the cerebellum being placed on a flat absorbent pad in the container of fixative, and held in contact by a small weight thus giving a flat medial surface that yielded complete sections of cerebellar cortex. The sections were immersed overnight in 0.1 mol/l sodium phosphate buffer containing 0.001% toluidine blue and 0.001% thionin and then mounted and dried on to the slides, briefly dipped in xylol and covered with DPX. The areas of the molecular, granular and medullary (white matter) layers were measured for the whole cerebellum by projections from a Leitz Micro- promar microscope at a magnification of × 28 on to a MOP electronic measuring tablet. The average thickness of the external germinal layer, where the granule cells are produced, was obtained by measuring the length and area of 10 segments of this layer, each about 0.15 mm long, and dividing the area by the length for each segment, and taking the mean.

The synapses were counted in the visual cerebral cortex because it is a highly defined area to sample efficiently the synapses made by pyramidal cells at all depths. A small block of occipital cortex was reduced to 0.5 mm thickness before being fixed in 1% osmium tetroxide for 24 h. After staining with uranyl acetate and embedding in Araldite®, sections of silver interference colour were stained with lead citrate and viewed in a Jeol 100S electron microscope. The lower part of layer 1 immediately above the cell bodies of layer 2 was selected and 6–10 photographs obtained from each brain at random positions at a magnification of 10 000. Prints of these photographs were examined under a dissecting microscope and all synapses (defined by a thickened membranous apposition and the presence of one or more vesicles) were counted. The lengths of the appositions were also measured with an ocular scale in the dissecting microscope.

Statistical significance was determined by the t-test using the pooled variance estimated by the analysis of variance.

All results for iodized oil treatment at 100 days gestation have been included from a previous report by Potter et al. (2) for comparison in the figures and tables.

Results

Physical appearance of the fetuses

Thyroxine administered to pregnant ewe at 100 days gestation restored the fetal wool coat which was sparse or absent in iodine-deficient fetuses and prevented the abnormal characteristics (dome-like cranium, subluxation of foot joints and retrognathia) frequently observed in iodine deficiency (1). Body weight however was not restored by T₄ even to the extent seen with iodine replacement at 100 days gestation (Fig. 1). By contrast DIMIT had no corrective effect.

Changes in the thyroid

Thyroid glands form iodine-deficient fetuses were enlarged and on histological examination showed a characteristic absence of colloid in the follicles (Fig. 2). The fetal thyroid glands were reduced considerably in weight after T₄ treatment (Fig. 1) but
values showed that those with iodine deficiency had significantly higher fetal body weight. Administration of T4 increased fetal body weight, whereas DIMIT produced no effect. The weight change reflected a significant increase in the number of cells (mg DNA) and a lesser extent the size of the cells (protein:DNA ratio) in the fetal cerebellum which after T4 treatment was not significantly different to those of controls but considerably greater than that observed for fetuses of iodine-deficient and DIMIT-treated ewes (Fig. 3).

The cerebral hemispheres of the control fetal brains weighed significantly more than those subjected to iodine deficiency, T4 or DIMIT treatment. However, the significant reduction in the number of cells in the cerebral hemispheres observed with iodine deficiency was not responsive to T4 or DIMIT treatment. Cell size in the cerebral hemispheres was normal for all treatments (Fig. 3).

Cholesterol and water content for the cerebellum and cerebral hemispheres are shown in Table 2. The significant reduction in cholesterol content observed in the cerebellum due to iodine deficiency was corrected by T4 but not DIMIT and a similar trend, which did not reach significance, was observed for the cholesterol:weight ratio (an index of the degree of myelination). For the cerebral hemispheres the cholesterol content was significantly below controls for all treatments, however, the cholesterol:weight ratio was restored to normal by T4 but not DIMIT. The ratio for DIMIT in fact was significantly below that for iodine deficiency.

When compared with the controls a small but significant increase in water content of the cerebellum and cerebral hemispheres was evident for iodine deficiency and DIMIT treatments.

Histology of the fetal brain

Cerebellum. The molecular and medullary areas, as proportions of the total area of cerebellar cortex, and the ratio of molecular to granular area (Table 3) all showed significant differences between the control and the iodine-deficient, thyroxine or

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**Fig. 1.** Thyroid, body and brain weights of fetuses aged 140 days after the administration of iodized oil (I, N = 8), T4 (N = 4), and 3,5-dimethyl-3′-isopropyl-L-thyronine (DIMIT, N = 3) to iodine deficient ewes at 100 days gestation. The means are compared with those of control (C, N = 10) and iodine deficient (ID, N = 15) fetuses (mean ± SEM). a: P < 0.05 C vs I, T4, T4, DIMIT. b: P < 0.05 ID vs I, T4, DIMIT.

were still significantly heavier than those of controls. DIMIT, however, produced no reduction in fetal thyroid weight when compared with the iodine-deficient group. Histological examination showed that iodine and T4 treatment at 100 days gestation restored the thyroid structure to resemble that of controls. In fact fewer but larger follicles filled with colloid were present (Fig. 2). Once again DIMIT had no restorative effect on size or structure of the iodine-deficient gland. These observations are supported by fetal thyroid iodine values which together with maternal and fetal plasma T4, T3 and TSH concentrations are shown in Table 1.

**Fetal development: biochemical observations**

A marked increase in fetal brain growth was produced by T4 administration over a 40-day period and this occurred particularly in the cerebellum. In the cerebellum the weight, which was significantly less for iodine deficiency, was increased by T4 to be not significantly different to that of the controls, whereas DIMIT produced no effect. The weight change reflected a significant increase in the number of cells (mg DNA) and to a lesser extent the size of the cells (protein: DNA ratio) in the fetal cerebellum which after T4 treatment was not significantly different to those of controls but considerably greater than that observed for fetuses of iodine-deficient and DIMIT-treated ewes (Fig. 3).
DIMIT treatments. The external germinal layer was significantly thicker in the iodine deficient and DIMIT groups when compared with the controls (Table 3).

Cerebral cortex. The visual cortex can be regarded as having six layers. Layer 1 stretches from the pial surface down to the underlying cell bodies of layer 2 and consists mainly of neuropil containing the terminal dendrites of neurons in lower layers.

Scanning in the electron microscope (17) failed to reveal qualitative differences in the structure of the synapses of the different experimental groups and so counts of synapses in the lower half of layer 1 were made and these are compared in Table 3. The mean areal density of synaptic appositions was less than controls for all treatment groups. For the iodine deficient groups the length of the synaptic apposition was 15% greater than the controls and was not corrected by T4 or DIMIT.
Table 1.
Effects of iodized oil (I), T₄ and 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT) on maternal and fetal thyroid function (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Plasma T₄ (nmol/l)</th>
<th>Plasma T₃ (nmol/l)</th>
<th>Plasma TSH (µg/l)</th>
<th>Thyroid iodine (µg)</th>
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<tbody>
<tr>
<td></td>
<td>Maternal</td>
<td>Fetal</td>
<td>Maternal</td>
<td>Fetal</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>126 ± 8b</td>
<td>203 ± 13b</td>
<td>2.77 ± 0.24</td>
<td>0.38 ± 0.05b</td>
<td>12.6 ± 4.7b</td>
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<tr>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(7)</td>
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<tr>
<td>Iodine deficient</td>
<td></td>
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<tr>
<td>17.6 ± 3.5a</td>
<td>15.5 ± 5.2a</td>
<td>3.00 ± 0.65</td>
<td>0.21 ± 0.04a</td>
<td>107 ± 15a</td>
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<tr>
<td>(15)</td>
<td>(15)</td>
<td>(12)</td>
<td>(12)</td>
<td>(13)</td>
</tr>
<tr>
<td>+ I at 100 days</td>
<td></td>
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<tr>
<td>155 ± 14b</td>
<td>208 ± 30b</td>
<td>2.95 ± 0.16</td>
<td>0.46 ± 0.08b</td>
<td>3.6 ± 0.4ab</td>
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<tr>
<td>(8)</td>
<td>(8)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
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<tr>
<td>+ T₄ at 100 days</td>
<td></td>
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<tr>
<td>149 ± 13b</td>
<td>174 ± 8b</td>
<td>2.46 ± 0.69</td>
<td>0.27 ± 0.07</td>
<td>4.0 ± 1.0ab</td>
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<tr>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(3)</td>
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<tr>
<td>+ DIMIT at 100 days</td>
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<tr>
<td>4 ± 2b</td>
<td>10 ± 3a</td>
<td>0.34 ± 0.16b</td>
<td>0.12 ± 0.06a</td>
<td>25 ± 7b</td>
</tr>
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<td>(3)</td>
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<td>(3)</td>
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</table>

Number of observations shown in parentheses. Statistical significance has been estimated from a log transformation of data. a: P<0.05 for control vs all other groups. b: P<0.05 for iodine-deficient vs + I, + T₄, and + DIMIT.

Discussion
The present investigations using the sheep as an experimental model, have confirmed the delayed fetal brain development observed at 140 days gestation (10 days before parturition) due to severe iodine deficiency (1). The substantial restorative effect of iodized oil, given as a single intramuscular injection at 100 days gestation, on fetal brain development in iodine deficiency has been reported previously (2). Such a dramatic restorative effect of iodized oil on fetal development could not be produced, however, by the administration of T₄, or its iodine-free analogue 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT).

Fig 3.
The weights, cell numbers and cell sizes of the cerebella and cerebral hemispheres of 140 day fetuses of control (C, N = 10), iodine-deficient (ID, N = 15), and iodine deficient ewes which received iodized oil (I, N = 8), T₄ (N = 4) or 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT, N = 3) at 100 days gestation. The means are compared with those of the control and iodine deficient fetuses (mean ± SEM). a: P<0.05 C vs ID, I, T₄, DIMIT. b: P<0.05 ID vs I, T₄, DIMIT.
Table 2.
Effect of iodized oil (I), T₄ and 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT) on water, cholesterol (CHOL) and the ratio of cholesterol and tissue weight (CHOL:WT) of the cerebellum and cerebral hemispheres from fetuses which were iodine-deficient for the first 100 days of gestation (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Cerebellum</th>
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<th>Cerebral hemispheres</th>
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<tbody>
<tr>
<td></td>
<td>Water (%)</td>
<td>CHOL (mg)</td>
<td>CHOL:WT (× 10⁵)</td>
</tr>
<tr>
<td>Control (10)</td>
<td>83.4 ± 0.3b</td>
<td>58.8 ± 2.2b</td>
<td>12.8 ± 0.4b</td>
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<tr>
<td>Iodine deficient (15)</td>
<td>84.8 ± 0.2b</td>
<td>44.5 ± 1.7b</td>
<td>11.6 ± 0.3a</td>
</tr>
<tr>
<td>+ I at 100 days (8)</td>
<td>83.2 ± 0.2b</td>
<td>54.7 ± 1.7b</td>
<td>12.3 ± 0.4</td>
</tr>
<tr>
<td>+ T₄ at 100 days (4)</td>
<td>84.2 ± 0.2</td>
<td>57.2 ± 2.2b</td>
<td>12.4 ± 0.1</td>
</tr>
<tr>
<td>+ DIMIT at 100 days (3)</td>
<td>85.1 ± 0.4a</td>
<td>42.1 ± 2.5a</td>
<td>11.1 ± 0.3a</td>
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<td></td>
<td></td>
<td>85.7 ± 0.2b</td>
<td>403 ± 9b</td>
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<tr>
<td></td>
<td></td>
<td>87.7 ± 0.1 b</td>
<td>307 ± 9a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86.1 ± 0.3b</td>
<td>356 ± 8b</td>
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<tr>
<td></td>
<td></td>
<td>86.6 ± 0.2b</td>
<td>355 ± 14b</td>
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<tr>
<td></td>
<td></td>
<td>87.9 ± 0.1b</td>
<td>264 ± 15b</td>
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<td></td>
<td>7.34 ± 0.45b</td>
<td>9.75 ± 0.16b</td>
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Number of observations shown in parentheses. a: P < 0.05 for C vs all other groups. b: P < 0.05 for ID vs I, + T₄, + DIMIT.

Thyroline, injected subcutaneously, produced a similar response to that of iodized oil in maternal and fetal thyroid histology and in plasma T₃ and T₄ concentrations, which were increased, and in TSH concentrations, which were depressed to normal levels. Brain growth improved also after T₄ administration, but body weight remained comparable or even less than that of iodine deficiency. This may be related to the fact that fetal plasma T₃, T₄ and thyroid iodine content after T₄ treatment were 86, 71 and 47%, respectively, compared to control values. In contrast, iodized oil treatment, which did restore body growth, increased fetal plasma T₃, T₄ and thyroid iodine content to 102, 121 and 593%, compared to control values. This suggests that with T₄ treatment, suboptimal amounts of iodine or thyroid hormones were available to the fetus thus limiting body growth in favour of the brain. This is consistent with the observations of Geel & Timiras (18) that body weight is more severely affected than the brain by lack of thyroid hormones. It is possible that brain ‘catch-up’ occurs more readily due to its relatively high metabolic rate and high priority for available nutrients for growth.

The administration of DIMIT produced no change in T₃ and T₄ concentrations in maternal and fetal plasma and despite TSH values in maternal plasma being lowered to normal, the levels of TSH in the fetus remained high. The histological appearance of the fetal thyroids was unchanged from that seen in the iodine-deficient fetus and similar retardation in brain and body growth was evident.

The lack of response to DIMIT was apparent also in the total cholesterol content and the cholesterol to tissue weight ratio of the cerebellum and cerebral hemispheres which remained similar to those of iodine deficiency. In contrast, T₄ or iodized oil restored to control values the total cholesterol content in the cerebellum and partly in the cerebral hemispheres. The cholesterol to tissue weight ratio in the cerebellum and cerebral hemispheres of iodized oil and T₄-treated groups were not significantly different from controls.

The responses produced by iodized oil and T₄ on fetal brain growth were the result of an increase in the number and size of cells, both of which occurred in the cerebellum and cerebral hemispheres. Despite the apparent ‘catch-up’ in cerebellar growth after iodized oil and T₄ administration, restoration of cellular structure was not achieved with T₄. This was indicated by the significantly reduced ratios of the molecular layer area relative to both the total area and granular layer area, which suggests impaired dendritic arborization of the Purkinje cells. The thick external germinal layer, which is so characteristic of iodine deficiency, was restored to normal after T₄ or iodized oil administration. Furthermore, in the cerebral cortex, both T₄ and iodized oil failed to correct the reduction in the number and the increase in the length of synaptic appositions which were observed for iodine deficiency (Table 3). This suggests irreversible damage due to maternal and fetal iodine deficiency during the first 100 days of pregnancy.
DIMIT had no restorative effects on the cellular structure of the brain.

The effects on the sheep fetus of thyroxine administered to the iodine-deficient ewe do not agree with those reported for 6 day-old hypothyroid rats in which injected thyroxine has been shown to restore brain growth and enzyme activity to normal (18,19) and in low doses to correct anomalies in the development of the cerebellar cortex in propylthiouracil-induced hypothyroidism (20). Moreover Dainat & Rebiere (21) reported that a single injection of thyroxine to hypothyroid rats augmented cerebellar protein content, from which it was concluded that the $T_4$ produced more rapid cerebellar protein synthesis and cellular division in the hypothyroid than in the normal state.

It is probable that the full recovery observed in the rat but not in the sheep fetus is related to the route of injection, which was direct to the rat and indirect via the mother in the sheep. The relatively impermeable nature of the ovine placenta to thyroxine would then modify any effect of the injected thyroxine by reduced transfer of $T_4$ or $T_3$ through the placental barrier (5). The possibility of some transfer of small amounts of $T_4$ cannot be excluded. However, transfer of free iodide from active deiodination of $T_4$ and $T_3$ in the placenta must have occurred.

The lack of response after DIMIT administration substantiates the observations of Bachrach et al. (11) who reported that DIMIT administration to the ewe failed to prevent hypothyroidism in the thyroidectomized lamb even when given at a dose that suppressed maternal $T_4$ concentrations. They concluded that failure of DIMIT to prevent hypothyroidism in the thyroidectomized fetal lamb could be attributed to its instability in ovine serum, the insensitivity of ovine fetal tissues to DIMIT or the inability of DIMIT readily to traverse the ovine placenta.

In this study, the fact that DIMIT reduced maternal plasma TSH to normal levels in the iodine deficient sheep suggests that maternal pituitary function and those tissues dependent on plasma $T_3$ have been normalized. This reduces the possibility that maternal hypothyroidism may limit the supply of nutrients (other than iodine) from the mother to the fetus. The high fetal plasma TSH levels and low thyroid iodine content indicate that fetal hypothyroidism and iodine deficiency still persist. The sustained effects of iodine deficiency
on the fetus after DIMIT administration therefore suggest that restoration of both maternal and fetal thyroid function is necessary. The importance of normal fetal and maternal thyroid function is supported by the previously described similar effects of fetal thyroidectomy at 60 and 98 days gestation (22,23) and, after maternal thyroidectomy, the retarded brain development attributed to the lack of maternal thyroid hormones early in pregnancy before the fetal thyroid begins to develop (24). Furthermore in rats it has been shown that substantial amounts of maternal thyroid hormones enter the rat fetus early in pregnancy (4,5).

The ability of iodized oil or T₄ injection at 100 days gestation to substantially reverse the effects of iodine deficiency on the development of the ovine fetal brain would seem to depend on the restoration of both maternal and fetal thyroid function. The inability of T₄ and iodized oil to reverse completely the effects of iodine deficiency on the ovine fetal brain emphasises the importance of normal maternal and fetal thyroid function early in pregnancy. The significance of these effects will be disclosed by studies in the postnatal period using T₄ and iodized oil earlier in pregnancy and postnatally.

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References


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