

# Maternal 3,3'-Diiodothyronine Sulfate Formation from Guinea Pig Placenta Perfused with 3,3',5-Triiodothyronine

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## Abstract

**Objective:** Serum 3, 3',5-triiodothyronine (T<sub>3</sub>) remains low in near-term fetus to prevent the growing fetus from undue exposure to its active catabolic effect in mammals. The present study was undertaken to gain insight in the role of placenta in T<sub>3</sub> metabolism, fetal to maternal transfer of T<sub>3</sub>, and its metabolites by in situ placenta perfusion with outer-ring labeled [<sup>125</sup>I]-T<sub>3</sub> in pregnant guinea pig, a species showing increased sulfated 3, 3'-diiodothyronine (T<sub>2</sub>S) levels in maternal serum in late pregnancy (term = 65 days), similarly to humans in pregnancy.

**Materials and Methods:** One-pass placenta perfusions performed on pregnant guinea pigs were studied between 58 - 65 days of gestation. In two separate experiments, the umbilical artery of the guinea pig placenta was perfused in situ at 37°C with outer-ring labeled [<sup>125</sup>I]-T<sub>3</sub>. Maternal sera and umbilical effluents were obtained for analysis at the end of a 60-minute perfusion, when the steady-state levels of radioactivity were reached in the placenta effluent after 30-minute.

**Results:** Sulfated [<sup>125</sup>I]-T<sub>2</sub>S was readily detected in the maternal serum as the major metabolite of T<sub>3</sub> following the perfusion of placenta with [<sup>125</sup>I]-T<sub>3</sub>, suggesting that placental inner-ring deiodinase and sulfotransferase may play an important role in fetal T<sub>3</sub> homeostasis and in the fetal to maternal transfer of sulfated iodothyronine metabolites.

**Conclusions:** The expression of type 3 deiodinase (D3) and thyroid hormone sulfotransferase activity in placenta may play an important role to protect developing organs against undue exposure to active thyroid hormone in late gestation in the fetus. The combined activities of D3 and sulfotransferase promoted a placental transfer of T<sub>2</sub>S into maternal circulation. The maternal circulation of T<sub>2</sub>S is fetal T<sub>3</sub> in origin and its role as a fetal thyroid function biomarker deserves further evaluations and studies.

**Key Words:** placental function; placental transfer; thyroid hormones; sulfation of thyroid hormone

## Introduction

Optimal level of thyroid hormone (TH) is essential for normal neurological development in developing mammals, including humans. A critical amount of TH is especially important in the central nervous system (CNS) maturation. Deficiency or excess of TH in the CNS during fetal and neonatal periods can lead to morphological and functional abnormalities [2, 3]. The most severe form of TH deficiency in human fetus and neonate is the syndrome of cretinism. A milder form of fetal TH deficiency is observed in children of women with high serum TSH during pregnancy who performed less well in IQ and other

neuropsychological tests [4, 5]. These findings underline the importance of TH in human brain development.

Thus, the study of fetal TH metabolism has been the subject of considerable interest. Iodothyronines detected in the fetus before the onset of fetal thyroid function must be maternal in origin [6]. The maternal-fetal transfer of TH and their metabolites is apparently a two-way street [1]. The high gradient between fetal and maternal serum concentrations of iodothyronine sulfates raises the possibility of significant fetal to maternal transfer of iodothyronine sulfoconjugates [1, 2]. Sack et al. [7] showed that umbilical cord cutting, thus removing the lamb from

placental type 3 deiodinase (D3) and fetal-to-maternal transfer, triggers hypertriiodothyroninemia in the newborn lamb and that the postnatal T3 peak can be delayed until well after the TSH peak by delaying umbilical cord cutting. Santini et al. [8] found that the placenta plays an important role in maintaining the low serum T3 in fetuses late in gestation. These findings suggest the importance of the placenta in fetal T3 metabolism, and it is possible that fetal-to-maternal transfer of the sulfated iodothyronines via placenta is one mechanism responsible for optimization of serum T3 concentrations in the fetus. Increasing fetal-to-maternal transfer of iodothyronines may occur in late gestation [1].

Sulfoconjugation is the dominant pathway in thyroid hormone metabolism during intrauterine development, particularly in the last trimester in the precocial species, including humans, guinea pigs, and sheep. It regulates the supply of T<sub>3</sub> via sulfation and deiodination, and facilitates fetal to maternal transfer of sulfated iodothyronines, e.g., 3,3'-diiodothyronine sulfate (T<sub>2</sub>S) [1, 9].

Sulfation, catalyzed by sulfotransferase enzymes (SULTs), is an important pathway of thyroid hormone metabolism, irreversibly converting T<sub>4</sub> to inactive reverse T<sub>3</sub> (rT<sub>3</sub>) rather than active T<sub>3</sub>. The human fetus and neonate have high levels of circulating sulfated iodothyronines (e.g., T<sub>4</sub>S, T<sub>3</sub>S, rT<sub>3</sub>S, and 3,3'-T<sub>2</sub>S); the placenta forms the link between the fetus and its mother and is involved in transfer of thyroid hormones early in pregnancy. We and others have examined the expression of the SULTs involved in iodothyronine metabolism during mammalian placental/uterus development [10, 11]. The placenta at late gestation may facilitate inactivation of T<sub>3</sub> and fetal to maternal transfer of 3,3'-T<sub>2</sub>S. Thus, it is possible that T<sub>2</sub>S or its derivatives, transferred from the fetus and appearing in maternal serum or urine, can serve as a biomarker of fetal thyroid function; and in humans we have previously reported W-Compound to fill this role [1].

The aim of present study was undertaken to gain insight in the role of placenta in the fetal to maternal transfer of T<sub>2</sub>S by in situ placenta perfusion with outer-ring labeled [<sup>125</sup>I]-T<sub>3</sub> in the pregnant guinea pig, a species showing increased T<sub>2</sub>S levels in maternal serum in late pregnancy (term = 65 days), similarly to humans [1]. We found that [<sup>125</sup>I]-3, 3'-T<sub>2</sub>S was readily detected in the maternal serum following the perfusion of placenta with [<sup>125</sup>I]-T<sub>3</sub>. This suggests that placental inner-ring deiodinase and sulfotransferase may play an important role in fetal T<sub>3</sub> homeostasis and in the fetal to maternal transfer of sulfated iodothyronine metabolites. It is consistent with the hypothesis that transferred sulfate iodothyronine (3,3'-T<sub>2</sub>S) in maternal compartment may serve as a biomarker for fetal thyroid function.

## Materials & Methods

### T<sub>2</sub>S radioimmunoassay

3,3'-T<sub>2</sub>S and [<sup>125</sup>I]-T<sub>2</sub>S were prepared by the method of Eelkman-Rooda et al., Mol and Visser [12, 13]. T<sub>2</sub>S was further purified and quantitatively recovered by reverse-phase high-pressure liquid chromatography (HPLC) with a preparative column, as described previously [14, 15].

The T<sub>2</sub>S radioimmunoassay (RIA) procedure was performed as reported previously [14, 15], with modifications as below. Serum samples (0.2 to 1.0 mL) were extracted with 2 vol 95% ethanol (final concentration, 63%) before assay. Since the extraction efficiency of T<sub>2</sub>S in serum exceeded 96% in preliminary experiments, final T<sub>2</sub>S concentrations were not corrected for recovery rate. The lower limit of detection of the assay was 3.3 fmol (2 pg), or 33.1 pmol/L in a 300 µL ethanol extract of serum [14]. Intra-assay variations were 1.9% to 9.1% and inter-assay variations were 6.0% to 19.5%, depending on the measured concentrations [15].

With respect to dose-responsiveness, we found close parallelism to the T<sub>2</sub>S standard curve in the range between 10-250 pg/tube [15]. In the measurement of serum T<sub>2</sub>S, 100 µL of ethanol extract was used, which

usually contains 5 to 100 pg per tube, corresponding to a T<sub>2</sub>S of 15 to 300 ng/dL (or 0.248 to 4.96 nmol/L) in the serum sample [15].

### In Situ Perfusion of Guinea Pig Placenta

Normal male and female guinea pigs (500 grams) were purchased from Elm Hill Breeding Labs (Chelmsford, MA), and mated in our laboratory (at Worcester). Plug dated pregnancies were studied between 58 -65 days of gestation. The animal use protocol was approved by Institutional Animal Care and Use Committee at the Medical School, University of Massachusetts, Worcester.

One-pass placenta perfusions were performed using a technique modified from Kihlstrom and Kihlstrom [16, 17]. Anesthesia was induced with an intramuscular injection (i.m.) of Ketamine/Rompun (70 mg Ketamine and 6 mg Rompun/100 grams body weight). A catheter of PE50 tubing was tied into the maternal carotid for sampling of maternal serum. Following maternal cannulation, a laparotomy was performed to expose a single fetus through a small uterine incision. Both uterus and placenta were left within the maternal abdominal cavity to minimize hemodynamic changes in the maternal circulation to the placenta. To facilitate cannulation, we induced the dilation of vessels by the topical application of 4% papaverine. The umbilical vein and one fetal artery were cannulated with PE60 polyethylene tubing. The remaining vessels leading to the fetus were tied off and the fetus was removed [17].

In two separate experiments, the umbilical artery of the guinea pig placenta was perfused in situ at 37°C with outer-ring labeled [<sup>125</sup>I]-T<sub>3</sub>. The constituents of the perfusion media were 0.14 nM [<sup>125</sup>I]-T<sub>3</sub>, 3% bovine serum albumin (BSA) in Krebs-Henseleit (KH) buffer (0.110 M NaCl, 2.4 mM CaCl<sub>2</sub>, 4.4 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 1.1 mM MgSO<sub>4</sub> 7H<sub>2</sub>O, and 25.0 mM NaHCO<sub>3</sub>, pH 7.4). Placental pressure was monitored with a string-gauge transducer and catheter positions were adjusted to maintain a placental pressure between 20-40 mmHg. Maternal sera and umbilical effluents were obtained for analysis at the end of a 60-minute perfusion, when the steady-state levels of radioactivity were reached in the placenta effluent after 30-minute [17].

### High-pressure Liquid Chromatography (HPLC): Identification of Iodothyronines

Iodothyronines were identified by HPLC as below [14, 15]. Radioactive metabolites were identified in maternal serum extracts of guinea pigs which were perfused in situ via the fetal placental artery with [<sup>125</sup>I]-T<sub>3</sub>. Six to ten milliliters of maternal samples, obtained at the end of the 60-minute perfusion were extracted with two volumes of 95% ethanol and subsequently lyophilized. The dried extracts were dissolved in one milliliter of H<sub>2</sub>O and purified with a LH-20 column as previously described [14]. The infusate and effluent samples were diluted appropriately with 0.025 N NaOH. After application to the HPLC µBondapak C18 column, the serum extract was eluted isocratically with a mixture (22:78 vol/vol) of acetonitrile and 0.02 M ammonium acetate, pH 4.0, at a flow rate of 2 ml/min. Radioactive peaks were identified by comparing their retention times to those of known synthetic iodothyronines eluted on HPLC under the same conditions. Radioactivity in serum extracts was expressed as percent of total activity of each sample (100%).

### Sources of Materials

[<sup>125</sup>I]3,3'-T<sub>2</sub> was prepared by radioiodination using the method described previously [18]. BSA, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, iodomethane, and dimethyl-formamide were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorosulfonic acid (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). The Biochrom 1010 ODS preparative column was obtained from Regis (Morton Grove, IL). Papaverine hydrochloride was purchased from Eli Lilly Co. (Indianapolis, IN) PE50 and PE60 poly-ethylene tubings were obtained from Fisher

Scientific Co. (Medford, MA). [<sup>125</sup>I]-T<sub>3</sub> was purchased from New England Nuclear (PerkinElmer Inc., Boston, MA) with its specific activity ranged from 1,133 to 2,000 μCi/ug.

### Statistical Analysis

In the current study, the serum sample sizes are small (10 and 4 in the pregnant and virgin group, respectively), we use “testing for difference between two means” [19]. The two-tailed hypotheses, can be proposed to ask whether, in the small population sampled, the mean (X<sub>1</sub>) of the first group is different from the mean (X<sub>2</sub>) of the second group. These hypotheses are commonly expressed in their equivalent forms: The t value for testing the hypotheses concerning the difference between two means is:

$$t = \frac{X_1 - X_2}{S_{x_1-x_2}}$$

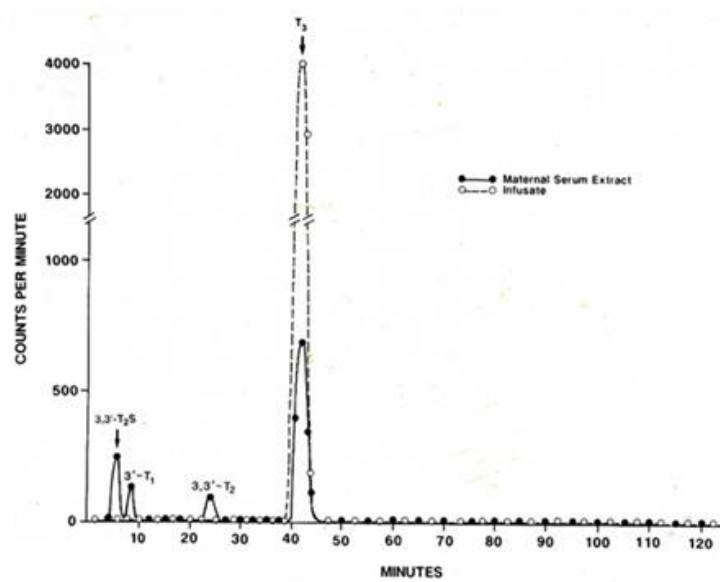
(The difference between two means divided by the standard error of the difference between the means.)

Significance was defined as  $p < 0.05$ . Results are reported as the mean  $\pm$  SD.

### Results

3, 3'-T<sub>2</sub>S concentrations were significantly elevated in third-trimester pregnant guinea pig sera [ $0.64 \pm 0.19$  nmol/L (n=10) vs.  $0.15 \pm 0.04$  nmol/L (n=4) in virgin female guinea pigs,  $p < 0.001$ ] [19].

After perfusion of the near-term guinea pig placenta with [<sup>125</sup>I]-T<sub>3</sub> for 60 minutes using a reservoir system we demonstrated a distinct [<sup>125</sup>I]-T<sub>2</sub>S peak by HPLC in maternal serum extracts (Figure. 1). The T<sub>2</sub>S peak accounted for a mean of 12.0% of the total radioactivity in maternal serum extracts (Table 1). The remaining radioactivity consisted of 8.9% for 3,3'-T<sub>2</sub>, 8.4% for 3'-T<sub>1</sub>, 26.7% for T<sub>3</sub> and 42.6% for free iodide suggesting a rapid inner-ring deiodination (Table 1). The sulfoconjugation appears to be a step to promote the placenta to maternal transfer of T<sub>3</sub> metabolites.



**Figure 1:** Identification of radioactive metabolites in maternal serum extract from one guinea pig whose placenta was perfused in situ with [<sup>125</sup>I]-T<sub>3</sub> via umbilical placental artery. The serum extract was applied and eluted from an HPLC column isocratically with a mixture (22:78 vol/vol) of acetonitriles and 0.02 M ammonium acetate, pH 4.0 at a flow rate of 2 ml/min.

A comparison of [<sup>125</sup>I]-T<sub>3</sub> infusion via the fetal placental artery in guinea pig (maternal serum) with via the fetal femoral vein in intact fetal lamb (maternal serum and urine in sheep) (20). Both guinea pig (58 – 65 d of gestation, term=65 d) and fetal lamb (139 – 143 d, term=150 d) are in near

term. Table 2 shows much higher percent of T<sub>2</sub>S radioactivity is found the ovine serum, 43.7%, in the first two hours of infusion than the guinea pig, 12.0% in the first hour of maternal serum. The amount of free iodide is similar in the Sheep (42.6%) and guinea pigs (44.6%).

Exp.	% Radioactivity	3,3'-T <sub>2</sub> S	3,3'-T <sub>2</sub>	3'-T <sub>1</sub>	I	T <sub>3</sub>
I.	Maternal Serum	14.2%	3.8%	9.8%	44.7%	24.3%
	Infusate	0.1%	0.9%	0.3%	4.8%	93.9%
	Effluent	0.1%	24.2%	4.4%	4.6%	66.8%
II.	Maternal Serum	9.7%	13.9%	7.0%	4.4%	29.0%
	Infusate	0.0%	1.0%	0.0%	4.4%	94.5%
	Effluent	0.0%	7.6%	1.0%	4.2%	86.7%
<b>Mean</b>	Maternal Serum	12.0%	8.9%	8.4%	42.6%	26.7%

**Table 1:** Identification of radioactive metabolites in maternal serum extract from guinea pigs whose placenta was perfused in situ with [<sup>125</sup>I]-T<sub>3</sub> via the fetal placental artery.

Exp.	% Radioactivity	T3S	3,3'-T2S	3'3'-T2	3'-T1	I-	T3	Serum of unconjugated
<b>Guinea pig:</b> Mean at 1 h	[ <sup>125</sup> I]-T3 via Maternal serum	0	12.0%	8.9%	8.4%	42.6%	26.7%	44%
<b>Sheep:</b> At 2h serum	[ <sup>125</sup> I]-T3 via fetal femoral vein (maternal serum)	0	43.7%	3.9%	0	44.6%	4.1%	8.0%
<b>0-1 h ± S.E. Urine</b>	[ <sup>125</sup> I]-T3 via fetal femoral vein (maternal urine in sheep)	2.0 ± 1.0%	46.4 ± 4.8%	-	-	42.4 ± 4.0%	-	6.2 ± 0.9%

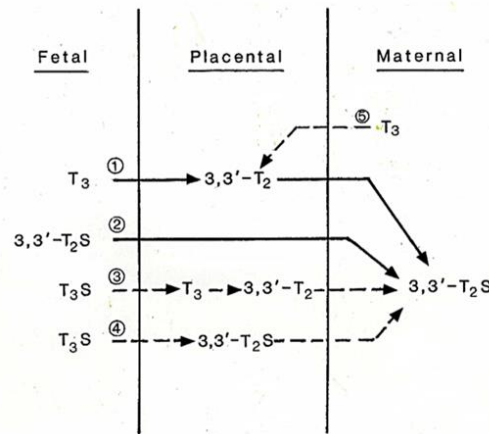
\* Data were from Reference 20.

**Table 2:** A comparison of [<sup>125</sup>I]-T3 infusion via the placental artery (Mat. Serum in Guinea pig) with via fetal femoral vein (Mat. Serum/Urine in sheep)\*.

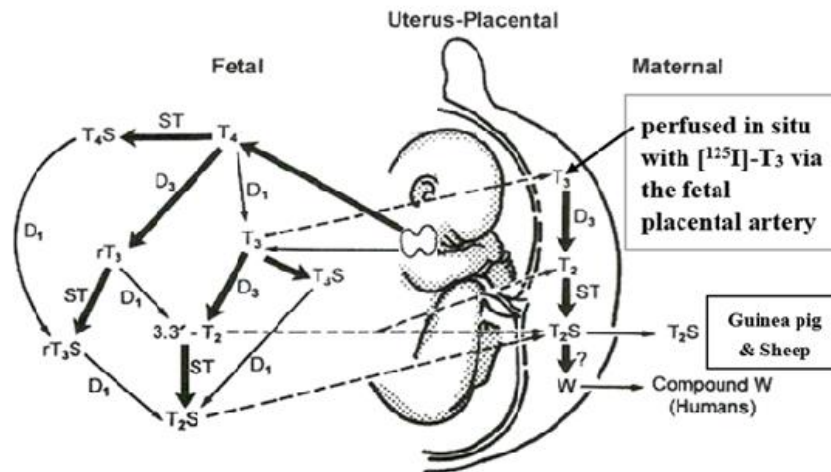
**Discussion**

The expression of type 3 deiodinase (D3) in placenta/uterus, embryonic and fetal tissues may protect developing organs against undue exposure to active thyroid hormone (Figures. 2 and 3). Also in adult subjects, D3

appears to be an important site for clearance of plasma T3 and production of plasma rT3 and 3, 3'- T2 [1, 2, 14]. To remove placental D3 and fetal-to-maternal transfer, Sack et al. [7] showed that umbilical cord cutting triggers hypertriiodothyroninemia in the newborn lamb.



**Figure 2:** Possible precursor caused the marked elevation of maternal serum 3, 3'-T2S level near term.



**Figure 3:** Postulation of fetal to maternal transfer of 3, 3'-T2S in near-term developing mammals, including humans. The placental type 3



*deiodinase (D3) and sulfotransferase (ST) are involved. Heavy solid lines indicate pathways that are more active in fetuses than in adults; thin solid lines, pathways that are less active in fetuses. Other broken lines represent unconfirmed pathways. [D1, D2, and D3: type I, type II, and type III iodothyronine deiodinases; ST: iodothyronine sulfotransferases (SULT)].*

In rats, as in humans, sulfation of iodothyronines is catalyzed by multiple sulfotransferases (SULT) isozymes in different tissue [1, 9]. We have identified rSULT1A1 and 1B1 mRNA in rat uterus but not 1C1 [1]. In rats, we found significant activities in uterus with an apparent Km of 0.62  $\mu$ M for T<sub>2</sub>, and activity varied during gestation [10]. In various substrates SULTs of iodothyronine, 3,3'-T<sub>2</sub> is by far the best of substrate as compared to other thyroid hormone analogs: 3,3'-T<sub>2</sub> > rT<sub>3</sub> >> T<sub>3</sub> > T<sub>4</sub> [10]. From Figure. 1 and Table 1, we find 3, 3'-T<sub>2</sub>S is the major metabolites in both intact fetal infusion in near-term fetal lamb [20] and in situ isolated fetal placental artery perfusion suggesting that T<sub>3</sub> is rapidly degraded by D3 [21] to 3, 3'-T<sub>2</sub> first and then sulfated to 3, 3'-T<sub>2</sub>S. SULT1A1 activity showed significant correlation with sulfation of 3,3'-T<sub>2</sub>, suggesting that this enzyme is primarily responsible for placental T<sub>2</sub> sulfation. It is likely that SULT isozyme in guinea pig and sheep in placenta/uterus that resembles rSULT1A1 that has high affinity to 3,3'-T<sub>2</sub>.

We and others have reported high concentrations of sulfated iodothyronines, including thyroxine sulfate (T<sub>4</sub>S), T<sub>3</sub> Sulfate (T<sub>3</sub>S), and reverse T<sub>3</sub> sulfate (rT<sub>3</sub>S), in sera of human and sheep fetuses [22 – 25]. Levels of these metabolites in maternal sera are low. We found Increased Levels of T<sub>2</sub>S– immune-cross-reactive material was found in both fetal and maternal sera during human pregnancy [25, 26].

In sheep study, we have shown T<sub>2</sub>S derived from T<sub>3</sub> of fetal origin is transferred to the maternal circulation and contributes significantly to the maternal urinary pool [1, 27 – 29]. We also have reported that T<sub>2</sub>S was the major metabolite in maternal compartment while labeled T<sub>4</sub> or T<sub>3</sub> was infused intravenously to fetuses in utero at late gestation [20, 28]. These findings suggest the importance of the placenta in fetal T<sub>3</sub> metabolism, and it is possible that placental fetal-to-maternal transfer of the sulfated iodothyronines is one mechanism responsible for optimization of serum T<sub>3</sub> concentrations in the fetus. Thus, fetal-to-maternal transfer of iodothyronines may increase in late gestation.

In the present study, we have observed that when the umbilical artery of the guinea pig placenta was perfused in situ with outer-ring labeled T<sub>3</sub> and 3, 3'-T<sub>2</sub>S was found to be the major product in maternal compartment. Again, this finding is consistent with the maternal circulation of 3,3'-T<sub>2</sub>S, in mammalian species, or its immuno-crossreactive material (W-Compound) is fetal T<sub>3</sub> in origin and could be used a fetal thyroid function biomarker [1, 26, 30, 31].

The limitations of the present study include that it is a small study with only two perfusions, no statistical analysis could be performed. In addition, no specific manipulations of D3 or SULTs with inhibitors are performed. SULT activity may be inhibited by xenobiotics [32]; D3 can be inhibited by propylthiouracil (PTU) [33]. Further studies with specific inhibitors may differentiate the role of D3 and SULTs in placental metabolism and transfer of iodothyronines. Finally, future study with in vitro perfused human placental lobule, that is collected and prepared immediately after delivery from normal uncomplicated pregnancy, may be more relevant to humans [34].

Recently, brominated flame retardants (BFRs) have been shown to disrupt thyroid hormone (TH) homeostasis through multiple mechanisms, including inhibition of enzymes that regulate intracellular levels of THs, such as sulfotransferases (SULTs). As shown in the present study, the placenta plays a critical role in expressing D3 and SULTs to prevent the developing fetuses from exposure to high level of active thyroid hormone T<sub>3</sub>, which is needed immediately after birth. This is concerning given that disruption of TH regulation within the placenta could potentially harm the

developing fetus [35]. The possibility of the maternal serum or urine levels of W-Compound also be used as a marker for BFR toxicity may be explored.

## Conclusions

The expression of type 3 deiodinase (D3) and thyroid hormone sulfotransferase activity in placenta may play an important role to protect developing organs against undue exposure to active thyroid hormone in late gestation in the fetus. The combined activities of D3 and sulfotransferase promoted a placental transfer of T<sub>2</sub>S into maternal circulation. This finding is consistent with our previous studies in human and sheep that the maternal circulation of 3, 3'-T<sub>2</sub>S, in mammalian species, or its immuno-cross-reactive material (W-Compound) is fetal T<sub>3</sub> in origin and its role as a fetal thyroid function marker deserves further evaluations and studies.

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