

Research article

PLACENTAL IODOETHYRONINE DEIODINASES EXPRESSION IN PREGNANT COWS EXPOSED TO PROPYLTHIOURACIL (PTU) AND THYROID AXIS ACTIVITY OF THEIR CALVES

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The aim of our study was to investigate if the thyroid axis of newborn calves is affected by prenatal application of propylthiouracil (PTU). The study included 20 late pregnant Holstein cows. One group (n=10) was treated with PTU (4 mg/kg of BW daily) from day 20 before expected calving until the day of calving. The other group (n=10) was non-treated. Placental samples of dams were obtained for measuring mRNA expression of iodothyronine deiodinases type I (D1), type II (D2) and type III (D3). After parturition calves were separated from the dams and included in the study. Blood samples were taken daily from each calf starting on the day of birth until day 7 of age. Blood T₃, T₄ and TSH concentrations were measured. PCR analysis of the placental tissue revealed an abundance of all three types of placental deiodinases in non-treated cows, and a significant elevation of mRNA levels for all three types of deiodinases after PTU treatment. Calves that originated from dams treated with PTU had significantly lower T₃ and T₄ and significantly higher TSH concentrations compared to non-treated calves during the first 2 days of life. Starting from day 4 until day 6 of life the opposite effect was observed meaning that calves prenatally exposed to PTU had significantly higher T₃ and T₄ and slightly lower TSH. Our study, for the first time, provides information related to iodothyronine deiodinases mRNA expression in bovine placenta, and confirm that PTU treatment of pregnant dams provokes depression of thyroid function in newborns during the first days of life.

Key words: cows, placental deiodinases, neonate, thyroid axis, PTU

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INTRODUCTION

Thyroid hormones (THs), that include thyroxine (T_4) and triiodothyronine (T_3), are crucial regulators of growth and development of fetal and neonatal calves [1]. Their secretion is under regulation of the hypothalamic-pituitary-thyroid axis that is active during both prenatal and postnatal life [2]. Fetal thyroid status is under a strong influence of the maternal thyroid axis due to the fact that the placenta is permeable for iodine, thus improving fetal TH synthesis [3,4]. There is evidence that maternal T_4 can cross the placenta in humans [5] and may modulate fetal development before, as well as after, the onset of the fetus's own TH production [6,7]. Not much T_3 is transferred from the mother to the fetus in humans [6]. Up to our knowledge, there is no evidence about TH transfer through the placenta in ruminants. The placenta contains three types of iodothyronine deiodinase that may modulate fetal TH metabolism and activity due to the fact that the placenta receives a relatively large proportion of fetal cardiac output [8]. Iodothyronine deiodinase type III (D3) is most dominant in the placenta and it metabolites T_4 to reverse T_3 (rT_3) during pregnancy. Iodothyronine deiodinase types I and II (D1 and D2) primarily convert inactive T_4 to active T_3 , although research has also shown that D1 might inactivate T_4 [9]. D3 is more active in the early pregnancy period, protecting the fetus from excessive TH activation, while D2 increases its activity with gestational age leading to a prepartal surge in plasma T_3 .

The high level of fetal TH that maintain in the blood after birth provoke high levels of T_4 and T_3 in 1 day old neonatal calves [10]. During the next several days, TH concentrations significantly decrease [11]. Although, hypothalamic-pituitary-thyroid axis is functional at birth there is a strong evidence that it is immature. Thus, Davicco and coworkers (1982) [12] showed that THs secretion stimulated by TSH results in significantly higher plasma THs concentrations in 21 days old than in 3 hours old calves, indicating on lower receptor abundance for TSH during the early postnatal period. Consequently, authors concluded that fetal thyroid axis is essential in providing sufficient amounts of THs for early postnatal development [12]. It is well known that the degree of maturation of the thyroid axis at birth is essential for an adequate response of the newborn to environmental conditions immediately after birth [1,13].

Hypothyroidism is the most frequent type of thyroid disorder in neonatal calves [11,14]. Offspring hypothyroidism in the first days of neonatal life is usually caused by maternal hypothyroidism that is combined with fetal hypothyroidism.

Hypothyroidism in cattle may be induced by 6-propylthiouracil (PTU), which is a well-known chemical inhibitor of the thyroid function on both central (thyroid gland) and peripheral level [15]. On the central level, it inhibits thyroid peroxidase enzymes activity responsible for TH formation, while on the peripheral level it suppresses the conversion of T_4 to T_3 by blocking D1 function [16]. PTU effect is dose-dependent and reversible [17]. It was earlier proved that PTU in doses of 4 mg/kg body weight (BW), by acting on central and peripheral level, significantly decreases both T_4 and T_3 in dams treated in daily fashion [18-20]. Doses of 1-2 mg/kg BW per day only inhibit

the peripheral conversion of T_4 in T_3 [21,22]. Unlike D1, D2 and D3 are insensitive to PTU. PTU may readily cross human placenta and suppress fetal thyroid hormone secretion [23-25], in the same manner as in older ruminants [26].

Up to our knowledge, no studies have been published to date to assess the exact rate of neonatal hypothyroidism in calves after prenatal exposure to PTU. The objectives of the present study were to evaluate whether *in utero* exposure to PTU induces neonatal hypothyroidism in calves, and to explore the underlying mechanism of its action.

MATERIALS AND METHODS

Animals and treatment

Twenty Holstein cows averaging 600 kg of body weight (BW) were selected and placed in the study on day 20 before expected calving. Thereafter, cows were divided into two groups of equal size ($n = 10$). One group was treated with PTU and the other was non-treated. PTU (Sigma Chemical Company) was fed 4 mg/kg BW daily from day 20 before expected calving until the day of calving. Daily amounts were mixed with 25 mL of maltose syrup as a carrier and applied orally 4 hours after the morning feeding. Non-treated cows received only 25 mL of maltose syrup once a day. Cows were weighted on the beginning of the study. About 5 days before parturition, cows were moved to maternity stalls. After parturition calves were immediately separated from the dams and placed in individual boxes in a byre where the temperature ranged from 15 to 20 °C. Calves were divided in two groups of equal size. First group ($n = 10$) that originated from dams treated with PTU was marked as PTU-treated and the other group that originated from non-treated dams was marked as non-treated group.

The animal-related component of the study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade in accordance with the National Regulation on Animal Welfare.

Placental collection and analysis

After parturition, placental samples were obtained. Placentas were washed with cold (4 °C) tris-buffered saline (TBS) solution to remove maternal blood contamination. For analysis caruncles (maternal placenta) and cotyledons (fetal placenta) were manually separated, vascular and connective tissues were removed and divided into small pieces (< 75 g). One set of samples was snap-frozen in liquid nitrogen before storage at - 80 °C for measurement and RNA assessment.

RNA extraction and reverse transcription

Total RNA from placental tissue was extracted using TrIZol® Reagent (Invitrogen, Life Technologies USA). Briefly, tissue was weighed and homogenized in 1ml TrIZol® Reagent per 100 mg of tissue using Potter-Elvehjem teflon-glass homogenizer. Homogenates were then incubated at 30°C for 5 min, 0.2 mL of chloroform was

added and the homogenate was shaken vigorously 15 s and incubated for 3 min at 30 °C. Samples were centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase, containing RNA, was mixed with 0.5 mL of isopropanol, incubated at 30°C for 10 min and centrifuged at 12,000 g for 10 min at 4°C. The resulting RNA pellet was re-suspended in 75% ethanol, centrifuged (7,500 g, 5 min, 4°C), dried on air, and dissolved in 100 µl 0.1% diethylpyrocarbonate (DEPC)-treated water. For the synthesis of cDNAs, a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. Namely, 2 µg of total RNA was reverse transcribed using MultiScribe™ Reverse Transcriptase (50 U/µl) in the presence of 2 µL Random Primers, 0.8 µL 100 mM dNTP Mix, 1 µL of RNase Inhibitor and 10xRT Buffer in a final volume of 20 µL. The cDNAs were stored at -20 °C.

Semi-quantitative PCR

Specific primers were designed to selectively amplify D1 F 5'TGGTGGTAGACACAATGACGAA3', R 5'GGCCAGATTTACCCTTGTAGGA3', D2 F 5'CCACCTTCTGGACTTTGCCA3', R 5'GGAAGTCAGCCACGGATGAG3', D3 F 5'TCACTCCCTGAGGCTCTG3', R 5'CCCAGTAAATGCCTACGGATG3' and ATP synthase (ATP5B) F 5'CATCGTGGCGGTCATTGG3', R 5'AATGGTCCCTTACTGTGCTCTC3'. For each set of primers control experiments were performed, to define the linear range for PCR amplification. As additional controls, PCR reactions lacking only the template were run for each set of reactions. For polymerase chain reaction (PCR), appropriate dilutions of cDNA samples were mixed with PCR buffer containing 0.2 mM dNTPs, 2 mM MgCl₂, 0.25 µM primers for D1, D2, D3 and ATP5B, and 2U Taq polymerase in a total volume of 25 µl. cDNAs were amplified in Eppendorf Mastercycler® for 30 cycles using the following conditions: denaturation 94 °C/45 s; annealing 60 °C/1 min (D1), 59 °C/1 min (D2), 55 °C/1 min (D3) or 59 °C/1 min (ATP5B); extension 72 °C/1 min; final extension 72 °C/8 min. PCR products were electrophoresed on 2% agarose gels together with a MassRuler™ Low Range DNA Ladder, 50-1500 bp (Fermentas), and visualized under UV light using ethidium bromide. The intensity of PCR products were measured with an image analysis system GelDoc 1000 (BioRad, Hercules, CA).

Blood collection and analysis

Blood samples were taken daily by jugular venipuncture from each calf starting the day of birth until 7 days after calving. Samples were obtained with a sterile needle, collected into tubes and allowed approximately 30 min to clot spontaneously. Samples were subsequently centrifuged at 1,000 g for 20 minutes, and the serum was collected and stored at -18 °C until analyzed. To compare hormone concentrations without influence of daily rhythms, samples were taken 4 to 6 hours after morning feeding. TSH concentrations in the blood serum were measured by a quantitative enzyme immunoassay technique using ELISA kit (Endocrine, USA). The T₃ and T₄ concentrations in the blood serum were measured by radioimmunoassay kit (RIA; INEP-Zemun, Serbia) validated for use with bovine serum. The mean intra assay coefficients of variation (CV) for duplicate samples were 4.1% and 3.5% for T₄ and T₃, respectively. Inter assay CVs were below 10%

Statistical analysis

Results were subjected to statistical analysis using the Statistica package (Version 6.0). Data were subjected to repeated-measure ANOVA with 3 variables (calf, treatment, and time as a split-plot on the random treatment) to analyze the difference in each group according to time sampling. When “F” for treatment, time, or interaction showed statistical significance ($P < 0.05$), differences between mean values of different groups at the same time sampling were evaluated using the least significant difference (LSD) test.

RESULTS

mRNA level of D1, D2 and D3 in placental samples of dams

PCR analysis of the placental tissue revealed significant elevation of mRNA levels for all three types of examined deiodinases after PTU treatment. Namely, both activating deiodinases (D1 and D2) had significantly increased mRNA level detected in PTU-treated dams compared to non-treated dams (Figures 1 and 2, $*p < 0.05$ and $***p < 0.001$, respectively). Interestingly, mRNA level of D3, which is an enzyme involved in the inactivation of thyroid hormones, was also significantly elevated in placental samples of dams treated with PTU (Figure 3, $**p < 0.01$).

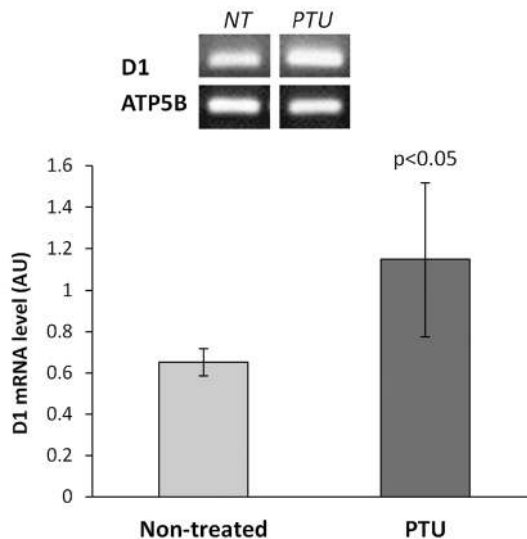


Figure 1. Effect of propylthiouracil (PTU) treatment on placental iodothyronine deiodinase type I (D1) mRNA levels. Representative agarose gel electrophoresis for the RT-PCR products for deiodinase type I (D1) and for ATP synthase (ATP5B) after ethidium bromide staining in placental RNA samples from non-treated (NT) and propylthiouracil (PTU)-treated cows. The graph is showing relative densitometric quantification of RT-PCR products of the mRNA of D1 expressed as D1 mRNA/ATP5BmRNA ratios in placental samples obtained from non-treated (NT) dams and from dams after PTU treatment (PTU). Data are presented as mean \pm SE ($n = 10$ animals per group) of the triplicate analysis of the RNA samples.

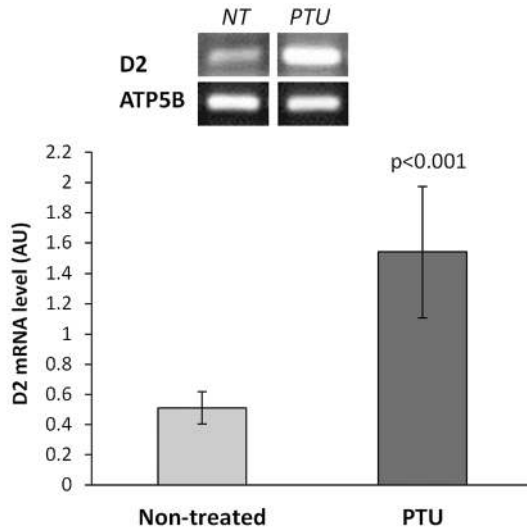


Figure 2. Effect of propylthiouracil (PTU) treatment on placental iodothyronine deiodinase type II (D2) mRNA levels. Representative agarose gel electrophoresis for the RT-PCR products for deiodinase type II (D2) and for ATP synthase (ATP5B) after ethidium bromide staining in placental RNA samples from non-treated (NT) and propylthiouracil (PTU)-treated cows. The graph is showing relative densitometric quantification of RT-PCR products of the mRNA of D2 expressed as D2 mRNA/ATP5BmRNA ratios in placental samples obtained from non-treated (NT) dams and from dams after PTU treatment (PTU). Data are presented as mean \pm SE ($n = 10$ animals per group) of the triplicate analysis of the RNA samples.

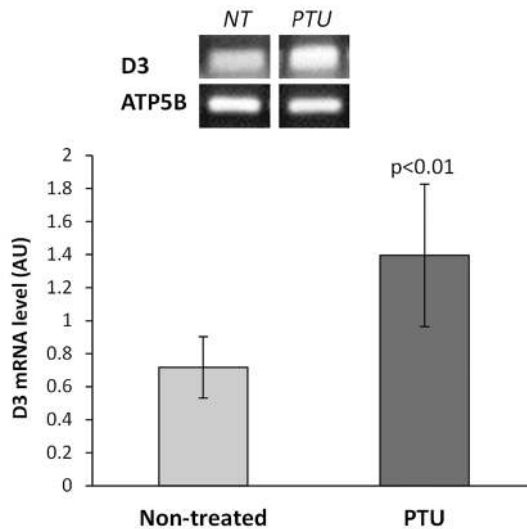


Figure 3. Effect of propylthiouracil (PTU) treatment on placental iodothyronine deiodinase type III (D3) mRNA levels. Representative agarose gel electrophoresis for the RT-PCR products for deiodinase type III (D3) and for ATP synthase (ATP5B) after ethidium bromide staining in placental RNA samples from non-treated (NT) and propylthiouracil (PTU)-treated cows. The graph is showing relative densitometric quantification of RT-PCR products of the mRNA of D3 expressed as D3 mRNA/ATP5BmRNA ratios in placental samples obtained from non-treated (NT) dams and from dams after PTU treatment (PTU). Data are presented as mean \pm SE ($n = 10$ animals per group) of the triplicate analysis of the RNA samples.

T₃, T₄ and TSH concentrations in calves

Treatment of dams with PTU has a significant impact on T₃ and TSH values in their calves. No significant effect of treatment on T₄ concentrations was observed. Concentrations of T₃, T₄ and TSH significantly changed with the age of calves. The significant treatment time interactions for all three measured hormones indicate on different directions of hormonal changes within groups (Table 1).

T₃ concentrations in non-treated calves were highest on the day of birth and then significantly decreased (Table 2). On the contrary, in the treated group, initial T₃ value at the day of birth was lowest and then increased. T₃ concentrations were significantly lower in the treated compared to the non-treated group on the day of birth, but significantly higher in treated compared to non-treated groups from days 4 to 6. In one week old calves, no significant difference in T₃ concentrations was observed between groups, although the values were higher in treated compared to non-treated calves. Similar trends of hormonal changes, as for T₃, were observed for T₄ (Table 3). As expected, an opposite effect of treatment was established for TSH (Table 4). Treated calves, compared to non-treated calves, had significantly higher TSH concentrations until day 3 of age. Thereafter, TSH concentrations were slightly lower in treated compared to non-treated calves.

Table 1. Effects of treatment, time and treatment x time interaction on statistical significance of difference

Variable units	Pooled “s”	P treatment effect	P time effect	P treatment × time
T ₃	1.743	<0.0001	<0.0001	<0.0001
T ₄	1892	0.425	<0.0001	<0.0001
TSH	3.814	<0.0001	<0.0001	<0.0001

“s” – pooled estimate of variance

Table 2. Mean (\pm SE) serum T₃ concentrations (mmol/L) in non-treated and PTU treated calves

Day related to birth	Non-treated	PTU treated
0	6.04 \pm 0.41 ^{a, A}	2.31 \pm 0.22 ^{a, B}
1	4.74 \pm 0.48 ^{b, A}	2.82 \pm 0.20 ^{ad, B}
2	4.30 \pm 0.39 ^{b, A}	3.45 \pm 0.30 ^{ac, A}
3	3.97 \pm 0.32 ^{b, A}	4.24 \pm 0.29 ^{bc, A}
4	3.62 \pm 0.23 ^{bd, A}	9.69 \pm 0.92 ^{e, B}
5	2.79 \pm 0.20 ^{cd, A}	7.26 \pm 0.42 ^{e, B}
6	2.80 \pm 0.24 ^{cd, A}	7.50 \pm 0.76 ^{e, B}
7	2.40 \pm 0.23 ^{c, A}	3.34 \pm 0.25 ^{bde, A}

a, b, c, d – means with different subscripts within a column differ (P < 0.05)

A, B - means with different subscripts within a row differ (P < 0.05)

Table 3. Mean (\pm SE) serum T_4 concentrations (mmol/L) in non-treated and PTU treated calves

Day related to birth	Non-treated	PTU treated
0	280.82 \pm 17.47 ^{a, A}	105.67 \pm 9.71 ^{a, B}
1	265.61 \pm 17.58 ^{a, A}	119.95 \pm 11.15 ^{a, B}
2	237.74 \pm 10.06 ^{a, A}	163.26 \pm 15.49 ^{b, B}
3	222.32 \pm 8.68 ^{b, A}	233.81 \pm 16.28 ^{c, A}
4	180.88 \pm 8.89 ^{c, A}	259.94 \pm 18.14 ^{c, B}
5	139.74 \pm 10.34 ^{d, A}	250.42 \pm 18.75 ^{c, B}
6	109.14 \pm 11.46 ^{d, A}	244.63 \pm 21.42 ^{c, B}
7	107.96 \pm 1.75 ^{d, A}	133.17 \pm 12.74 ^{ab, A}

a, b, c, d – means with different subscripts within a column differ ($P < 0.05$)

A, B – means with different subscripts within a row differ ($P < 0.05$)

Table 4. Mean ($M \pm SE$) serum TSH concentrations (mmol/L) in non-treated calves and PTU treated calves

Day related to birth	Non-treated	PTU treated
0	1.47 \pm 0.25 ^{a, A}	15.23 \pm 0.99 ^{a, B}
1	2.49 \pm 0.53 ^{ac, A}	13.36 \pm 0.84 ^{b, B}
2	3.68 \pm 0.36 ^{bcd, A}	8.38 \pm 1.44 ^{c, B}
3	1.80 \pm 0.44 ^{a, A}	8.51 \pm 0.55 ^{c, B}
4	2.75 \pm 0.64 ^{ad, A}	3.63 \pm 0.80 ^{d, A}
5	2.65 \pm 0.24 ^{ad, A}	1.79 \pm 0.23 ^{c, A}
6	2.65 \pm 0.30 ^{ad, A}	1.63 \pm 0.15 ^{c, A}
7	1.91 \pm 0.22 ^{a, A}	1.51 \pm 0.16 ^{c, A}

a, b, c, d – means with different subscripts within a column differ ($P < 0.05$)

A, B – means with different subscripts within a row differ ($P < 0.05$)

DISCUSSION

According to our results, PTU treatment during late pregnancy had a significant impact on the thyroid axis in newborn calves.

The thyroid status of neonatal calves, presented by blood TH concentrations, was studied by many authors [27-30]. It was established that serum T_4 and T_3 in calves aged up to 7 days, are significantly higher than in adult cows [11]. It may indicate on high neonatal storage of THs that are readily available after birth, meaning during the critical time for survival [31]. Similar results were observed in our study for the non-treated group of calves.

On the contrary, the newborn calves from dams treated with PTU, had significantly lower TH concentrations until the second day of neonatal life, compared to non-treated calves. Many authors proved that PTU readily crosses the human placental barrier [23-25] and higher concentrations of PTU were detected in the umbilical cord than in the mother's blood [36]. It may explain the higher risk of hypothyroidism in human fetuses from mothers exposed to PTU treatment [37]. Dose and frequency of application are the predominant factors that influence PTU concentration in the fetus [38]. Some studies showed that PTU could be highly accumulated in the human fetal thyroid tissues [39]. Since Horger and coworkers (1976) [26] suppressed TH secretion in pregnant sheep that orally received PTU, and induced fetal goiter, it may be considered that there is transplacental passage of PTU in ruminants. Additionally, Rumsey and coworkers (1985) [21] concluded that young animals, compared to adults, are more susceptible to PTU treatment. Considering the fact that the thyroid responses to PTU are dose related and that higher doses decrease thyroid gland secretion of T_4 and T_3 , our results obtained in the treated group of neonatal calves indicate that the transplacental passage of PTU during the last 20 days of pregnancy was in concentrations that were sufficient to induce suppression of fetal thyroid secretion of both hormones.

Due to the fact that there are no data in literature related to PTU induced hypothyroidism in neonatal calves, our results for thyroid hormone levels in PTU treated calves may be compared with those obtained on calves with goiter. Thus, Takahashi at coworkers (2000) [11] showed that newborn calves with congenital goiter had lower plasma T_4 concentrations from day of birth until 2 weeks of life, and lower T_4/T_3 ratios up to 4 weeks of neonatal life. Lower concentrations of THs in calves with congenital goiter, during the first 24 hours of neonatal life, were observed also by Guyot and coworkers (2007) [33]. In both studies the thyroid depression was explained by iodine deficiency observed during the prenatal period. Depressed TH concentrations activate feedback mechanisms and consequently induce a marked increase of blood TSH concentration [32], which was also proved in our study. It is well known that primary hypothyroidism is consistently combined with increased TSH levels [34]. Guyot and coworkers (2007) [33] detected higher serum TSH concentrations in calves with congenital goiter. Hypothyroidism induced by PTU in rats is associated with high TSH mRNA expression [35]. Higher concentrations of THs in treated calves, starting from day 4 until day 6, may be a result of compensatory effects to previous thyroid depression.

The other question important for thyroid status in neonatal calves that originates from PTU-treated dams, besides passage of PTU through the placenta, is related to the effect of PTU on placental deiodination. To the best of our knowledge, there are no data related to the expression and activity of bovine placenta iodothyronine deiodinases. Nevertheless, literature data for iodothyronine deiodinase expression and activity are available for other species including ovine [40]. In this study, we have examined the mRNA level of all three types of placental iodothyronine deiodinases (D1, D2 and D3). It was previously shown by other authors that D3 is predominantly

expressed form of placental iodothyronine deiodinase with a potentially beneficial function for the fetus, since it protects the fetus from the active form of TH [41].

The present study demonstrates, for the first time, that all three types of iodothyronine deiodinases are expressed in term bovine placenta. Our results related to D2 mRNA expression in non-treated cows are proved by other authors who confirmed an abundant expression of D2 in the placental samples [40]. It is supposed that the increase of placental D2 activity with gestational age is related to generation of intra-placental T_3 required for increased metabolic demands of tissues [42]. This explanation can be supported by our results obtained for PTU-treated animals. Namely, although D2 is not PTU-sensitive, treated cows showed a significantly higher expression of placental D2 mRNA, due to possibly increased metabolic demands of the placental tissue in cows with hypothyroidism [43]. The same was observed for brain tissue in domestic animals (chicken and sheep) with hypothyroidism [44]. As shown by Dentice and coworkers (2013) [45] D2 performs outer ring deiodination and its expression may be regulated by the concentration of THs. Martinez and coworkers (2013) [46] reported an increased expression of D2 in adipose and muscle tissues in food deprived mammals with decreased thyroid hormones concentrations.

In our study, it was observed that expression of placental D3 mRNA in non-treated cows was very similar to the level of D2 mRNA expression. Research done on other species showed lesser expression of D3, compared to D2, in the near term placenta [47]. This was explained by activation of mechanisms that promote production and reduce the clearance of TH in the near term fetuses. Since there are no data for D3 expression in the bovine placenta there are no comparable results in the literature. Possible explanation of abundant expression of placental D3 in high-yielding cows, as our dams were, might be the fact that these cows are exposed to markedly elevated estrogen levels before parturition which may interfere with the thyroid axis [48]. On the other hand, PTU-treated cows presented a significant elevation of placental D3 expression compared to non-treated cows. These results may be explained by an assumed higher concentration of placental T_3 induced by increased D2 activity on those cows. It is known that increased tissue T_3 concentration induce increased expression of D3, as was proven for brain tissue [49].

D1 is predominantly expressed in the liver, kidney and thyroid [50], but also in rat placenta as confirmed by Bates and coworkers (1999) [48]. Chan and coworkers (2003) [51] reported that expression of D1 in the human placenta was on the limit of detection in early pregnancy, and was not detectable in the late second trimester and term placenta. Our results showed that D1 was most abundant type of iodothyronine deiodinase in the placenta of non-treated cows. The high expression of placental D1 may be explained by increased level of blood T_3 in near term animals, since D1 expression is positively regulated by TH concentrations [52]. Forhead and coworkers (2006) [40] showed that cortisol surge during late pregnancy is an important factor that provokes physiological up regulation of D1 activity in the fetus and placenta toward term. This is, according to these authors, accompanied with a significant increase of

blood T_3 concentrations. PTU treated cows showed a significantly higher expression of placental D1 mRNA compared to non-treated cows. Increased placental D1 mRNA expression in these cows may be considered as a compensatory mechanism, since D1 activity is known to be blocked by PTU [53-57]. Additionally, decreased blood T_3 concentration in PTU treated animals, provoke increased D1 expression [58].

In conclusion, our study for the first time provides information related to all three deiodinases expressions in bovine placenta, and confirms that PTU treatment of pregnant dam provokes depression of thyroid function in newborns during first two days of neonatal life.

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Authors' contributions

DK organized and designed experiment. PD participated in experimental part related to animal study, carried out immunoassays and drafted the manuscript. ĐS participated in pilot study related to PTU dose for application. IV participated in experimental part related to animal study and in its design and coordination. RP participated in experimental part related to animal study and helped to draft the manuscript. MM performed the statistical analysis. ŽS participated in experimental part related to animal study, carried out the placental collection. AĐ carried out the molecular genetic analyses, participated in the PCR products electrophoresis and visualisation, and drafted the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Hillman NH, Kallapur SG, Jobe AH: Physiology of transition from intrauterine to extrauterine life. *Clin Perinatol* 2012, 39(4):769-83.
2. Feingold SB, Brown RS: Neonatal thyroid function. *NeoReviews*. 2010, 11(11):640-646.
3. Hidioglou M: Trace Elements in the Fetal and Neonate Ruminant: A Review. *Can Vet J* 1980, 21(12): 328–335.
4. Yarrington C, Pearce: Iodine and Pregnancy. *J Thyroid Res* 2011, 2011:934104.

5. James SR, Franklyn JA, Kilby MD: Placental transport of thyroid hormone. *Best Pract Res Clin Endocrinol Metab.* 2007, 21(2):253-64
6. Calvo RM, Obregón MJ, Ruiz de Ona C, Escobar del Rey F, Morreale de Escobar G: Congenital hypothyroidism, as studied in rats: Crucial role of maternal thyroxine but not of 3, 5, 3-triiodothyronine in the protection of the fetal brain. *J Clin Invest* 1990, 86:889–899, 32.
7. Morreale de Escobar G, Calvo RM, Obregón MJ, Escobar del Rey F Contribution of maternal thyroxine to fetal thyroxine pools in normal rats near term. *Endocrinology* 1990, 126:2765–2767
8. Emerson CH, Braverman LE: Peripheral deiodination of thyroid hormones in placenta and fetal membranes. *Horm Metab Res Suppl* 1984,14:56
9. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR: Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 2002, 23(1):38-89.
10. Kirovski D, Lazarević M, Baričević-Jones I, Nedić O, Masnikosa R, Nikolić JA: Effect of peroral insulin and glucose on circulating insulin-like growth factor-1, its binding proteins and thyroid hormones in neonatal calves. *Can J Vet Res* 2008, 72(3):253-8.
11. Takahashi K, Takahashi E, Ducusin RJ, Tanabe S, Uzuka Y, Sarashina T: Changes in serum thyroid hormone levels in newborn calves as a diagnostic index of endemic goiter. *J Vet Med Sci* 2001, 63(2):175-8.
12. Davicco MJ, Vigouroux E, Dardillat C, Barlet JP: Thyroxine, triiodothyronine and iodide in different breeds of newborn calves. *Reprod Nutr Dev* 1982, 22(2):355-62.
13. Kirovski D: Endocrine and metabolic adaptation of calves to extrauterine life. *Acta Veterinaria Beograd* 2015, 65(3):297-318.
14. Anderson PD, Dalir-Naghadeh B, Parkinson TJ: Iodine deficiency in dairy cattle, *Proceedings of the New Zealand Society of Animal Production* 2007, 67 248-254
15. Cooper DS: Antithyroid Drugs. *N Engl J Med* 2005, 352(9):905-17.
16. Taurog A: Hormone synthesis: thyroid hormone metabolism in: *Warner and Ingbar's The thyroid 8th ed.* New York: Lippincot Williams&Wilkins 2000, 61-84.
17. Villar D, Rhind SM, Arthur JR, Goddard PJ: Manipulation of thyroid hormones in ruminants – a tool to understand their physiological role and identify their potential for increasing production efficiency. *Aust J Agric Res* 2002, 53:259-270.
18. DeMoraes GV, Vera-Avila HR, Lewis AW, Koch JW, Neuendorff DA, Hallfor DM, Reeves JJ, Randel RD: Influence of hypo- and hyperthyroidism on ovarian function in Brahman cows. *J Anim Sci* 1998, 76(3):871-879.
19. Thrift TA, Bernal A, Lewis AW, Neuendorff DA, Willard CC, Randel RD: Effects of induced hypothyroidism or hyperthyroidism on growth and reproductive performance of Brahman heifers. *J Anim Sci* 1999, 77(7): 1833-1843.
20. Bernal A, DeMoraes GV, Thrift TA, Willard CC, Randel RD: Effects of induced hypothyroidism on ovarian response to superovulation in Brahman (*Bos indicus*) cows. *J Anim Sci* 1999, 77(10):2749-2756.
21. Rumsey TS, Bitman J, Tao H, Kozak AS: Changes in plasma concentrations of thyroxine and triiodothyronine in beef steers feed different level of propylthiouracil. *J Anim Sci* 1985, 60(6):1454-1462.
22. Elsasser TH, Rumsey TS, Norton S: Relationship between thyroid and somatotrophic axes in steers I: effect of propylthiouracil-induced hypothyroidism on growth hormone, thyroid

- stimulating hormone and insulin –like growth factor I. *Dom Anim Endocrinol* 1992, 9(4):261-171.
23. Rosenfeld H, Ornoy A, Shechtman S, Diav-Citrin O: Pregnancy outcome, thyroid dysfunction and fetal goitre after in utero exposure to propylthiouracil: a controlled cohort study. *Br J Clin Pharmacol* 2009, 68(4):609-17.
 24. Azizi F, Amouzegar A: Management of hyperthyroidism during pregnancy and lactation. *Eur J Endocrinol* 2011, 164(6):871-6.
 25. Mortimer RH, Cannell GR, Addison RS, Johnson LR, Roberts MS, Bernus I. Methimazole and propylthiouracil equally cross the perfused human term placental lobule. *J Clin Endocrinol Metab* 1997,82- 3099-102.
 26. Horger EO, Kenimer JG, Azukizawa M, Hershman JM, Kansal PC, Leaming AB: Failure of triiodothyronine to prevent propylthiouracil-induced hypothyroidism and goiter in fetal sheep. *Obstet Gynecol* 1976, 47(1):46-49.
 27. Hernandez MV, Etta KM, Reineke EP, Oxender WD, Hafs HD: Thyroid function in the prenatal and neonatal bovine. *J Anim Sci* 1972, 34 780-785.
 28. Davicco MJ, Vigouroux E, Dardillat C, Barlet JP: Thyroxine, triiodothyronine and iodide in different breeds of newborn calves. *Reprod Nutr Dev* 1982, 22, 355-62.
 29. Stojić V, Nikolić JA, Huszenica G, Šamanc H, Gvozdić D, Kirovski D: The plasma level of triiodothyronine, thyroxine and cortisol in newborn calves, *Acta Veterinaria Beograd* 2002,25, 85-96
 30. Kirovski D, Lazarević M, Stojić V, Šamanc H, Vujanac I, Nedić O, Masnikosa R: Hormonal status regulation of glycemia in neonatal calves during the first hours of postnatal life. *Acta Veterinaria Beograd* 2011, 61(4):349-361.
 31. Stojić V, Gvozdić D, Šamanc H, Jovanović I, Frantrić N: Thyroid gland hormones in newborn calves treated with clinoptilolite receiving different amounts of colostrum. *Acta Veterinaria Beograd* 2005,55, 3-10.
 32. Dietrich JW, Landgrafe G, Fotiadou EH: TSH and Thyrotropic Agonists: Key Actors in Thyroid Homeostasis. *J Thyroid Res.* 2012, 2012:351 864.
 33. Guyot H, Lebreton P, Alves de Oliveira L, Sulon J, Beckers J, Rollin F: Thyrotropin in newborn calves as a tool for diagnosing hypothyroidism. *British Cattle Veterinary Assoc* 2007,15(3): 271-275.
 34. Ladenson PW, Singer PA, Ain KB, Bagchi N, Bigos ST, Levy EG, Smith SA, Daniels G H, Cohen, HD: American Thyroid Association guidelines for detection of thyroid dysfunction. *Archives of internal medicine* 2000,160 (11):1573-1575.
 35. Taylor T, Wondisford FE, Blaine T, Weintraub BD: The paraventricular nucleus of the hypothalamus has a major role in thyroid hormone feedback regulation of thyrotropin synthesis and secretion. *Endocrinology* 1990, 126 1, pp. 317-324.
 36. Gardner DF, Cruikshank DP, Hays PM, Cooper DS: Pharmacology of propylthiouracil (PTU) in pregnant hyperthyroid women: correlation of maternal PTU concentrations with cord serum thyroid function tests. *J Clin Endocrinol Metab* 1986, 62 217-20.
 37. Momotani N, Noh JY, Ishikawa N, Ito K: Effects of propylthiouracil and methamizole on fetal thyroid status in mothers with Grave's hyperthyroidism. *J Clin Endocrinol Metab* 1997, 82 3633-6.
 38. Kampmann JP, Mølholm Hansen JE: Serum protein binding of propylthiouracil, *Br J Clin Pharmacol* 1983, 16 549–552.
 39. Feng Q, Gao Y, Li H: The effects of propylthiouracil on the fetal thyroid and serum concentration in pregnant women. *Zhong Nei Ke Za Zhi* 1996, 35 295-8.

40. Forhead AJ, Curtis K, Kaptein E, Visser TJ, Fowden AL: Developmental control of iodothyronine deiodinases by cortisol in the ovine fetus and placenta near term. *Endocrinology* 2006, 147(12):5988-94.
41. Kuiper GG, Kester MH, Peeters RP, Visser, TJ: Biochemical mechanisms of thyroid hormone deiodination. *Thyroid* 2005, 15(8):787-798.
42. Koopdonk-Kool JM, de Vijlder JJ, Veenboer GJ, Ris-Stalpers C, Kok JH, Vulsma T, Boer K, Visser TJ: Type II and type III deiodinase activity in human placenta as a function of gestational age. *Clin Endocrinol Metab.* 1996, 81(6):2154-8.
43. Galton VA, Martinez E, Hernandez A, St Germain EA, Bates JM, St Germain DL: The type 2 iodothyronine deiodinase is expressed in the rat uterus and induced during pregnancy. *Endocrinology* 2001,142(5):2123-8.
44. Rudas P, Rónai Z, Bartha T: Thyroid hormone metabolism in the brain of domestic animals. *Domest Anim Endocrinol.* 2005, 29(1):88-96.
45. Dentice M, Marsili A, Zavacki A, Larsen PR, Salvatore D: The deiodinases and the control of intracellular thyroid hormone signaling during cellular differentiation. *Biochim Biophys Acta* 2013, 1830(7):3937-45.
46. Martinez B, Soñanez-Organis JG, Vázquez-Medina JP, Viscarra JA, MacKenzie DS, Crocker DE, Ortiz RM: Prolonged food deprivation increases mRNA expression of deiodinase 1 and 2, and thyroid hormone receptor β -1 in a fasting-adapted mammal. *J Exp Biol* 2013, 216(24):4647-54.
47. Stulp MR, de Vijlder JJ, Ris-Stalpers C: Placental iodothyronine deiodinase III and II ratios, mRNA expression compared to enzyme activity. *Mol Cell Endocrinol* 1998, 25;142(1-2):67-73.
48. Bates JM, St Germain DL, Galton VA: Expression profiles of the three iodothyronine deiodinases, D1, D2, and D3, in the developing rat. *Endocrinology* 1999, 140(2):844-51
49. Pallud S, Ramaugé M, Gavaret JM, Lennon AM, Munsch N, St Germain DL, Pierre M, Courtin F: Regulation of type 3 iodothyronine deiodinase expression in cultured rat astrocytes: role of the Erk cascade. *Endocrinology.* 1999, 140(6):2917-23.
50. Maia AL, Goemann IM, Meyer EL, Wajner SM: Deiodinases: the balance of thyroid hormone: type 1 iodothyronine deiodinase in human physiology and disease. *J Endocrinol* 2011, 209(3):283-97.
51. Chan S, Kachilele S, Hobbs E, Bulmer JN, Boelaert K, McCabe CJ, Driver PM, Bradwell AR, Kester M, Visser TJ, Franklyn JA, Kilby MD: Placental iodothyronine deiodinase expression in normal and growth-restricted human pregnancies. *J Clin Endocrinol Metab* 2003, 88(9):4488-95.
52. Kim SW, Harney JW, Larsen PR: Studies of the hormonal regulation of type 2 5'-iodothyronine deiodinase messenger ribonucleic acid in pituitary tumor cells using semiquantitative reverse transcription-polymerase chain reaction. *Endocrinology* 1998, 139(12):4895-905.
53. Berry MJ, Kieffer JD, Harney JW, Larsen PR: Selenocysteine confers the biochemical properties characteristic of the type I iodothyronine deiodinase. *J Biol Chem* 1991, 266(22):14155-8.
54. Sanders JP, Van der Geyten S, Kaptein E, Darras VM, Kühn ER, Leonard JL, Visser TJ: Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. *Endocrinology* 1997, 138(12):5153-60
55. Manna D, Roy G, Mughesh G: Antithyroid drugs and their analogues: synthesis, structure, and mechanism of action. *Acc Chem Res.* 2013, 19;46(11):2706-15

56. Taurog A, Dorris ML, Hu WX, Guziec FS Jr: The selenium analog of 6-propylthiouracil. Measurement of its inhibitory effect on type I iodothyronine deiodinase and of its antithyroid activity. *Biochem Pharmacol* 1995, 49(5):701-9.
57. Koenig RJ: Regulation of Type 1 Iodothyronine Deiodinase in Health and Disease, *Thyroid*. 2005 15(8):835-40.
58. Köhrle J: The deiodinase family: selenoenzymes regulating thyroid hormone availability and action. *Cell Mol Life Sci*. 2000, 57(13-14):1853-63.

EKSPRESIJA DEJODINAZA U PLACENTI KRAVA TRETIRANIH SA PROPILTIOURACILOM (PTU) I AKTIVNOST TIREOIDNE OSOVINE NJIHOVE TELADI

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Cilj ovog rada bio je da se ispita da li je tireoidna osovina novorođene teladi promenjena nakon prenatalne aplikacije propiltiouracila (PTU). U ogled je uključeno 20 krava holštajn rase u kasnom graviditetu. Prva grupa (n=10) je tretirana PTU (4 mg/kg telesne mase, dnevna doza) počevši od 20 dana pre očekivanog teljenja do dana teljenja. Druga grupa (n=10) nije tretirana. Uzorci placente krava uzimani su u cilju određivanja ekspresije iRNK dejodinaza tipa I (D1), tipa II (D2) i tipa III (D3). Nakon teljenja, telad su odvojena od majki i uključena u ispitivanja. Uzorci krvi su uzimani dnevno od svakog teleta počevši od dana rođenja do 7. dana života. U krvi su merene koncentracije T_3 , T_4 i TSH. PCR analiza tkiva placente je pokazala zastupljenost sva tri tipa dejodinaza kod netretiranih krava, i značajno povećanje nivoa iRNK sva tri tipa dejodinaza nakon tretmana PTU. Telad koja su poticala od majki tretiranih PTU imala su značajno niže koncentracije T_3 i T_4 i značajno više koncentracije TSH u odnosu na telad poreklom od netretiranih majki, tokom prva dva dana života. U periodu od 4. do 6. dana života, utvrđen je suprotan efekat, što znači da su telad prenatalno izložena delovanju PTU imala značajno više koncentracije T_3 i T_4 i značajno niže koncentracije TSH u odnosu na netretiranu telad. Naša studija, po prvi put, daje informacije vezane za ekspresiju dejodinaza u bovinoj placenti i potvrđuje da tretman gravidnih majki sa PTU izaziva depresiju funkcije tireoidne osovine kod novorođenčadi tokom prva dva dana neonatalnog života.