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Large Offspring or Large Placenta Syndrome? Morphometric Analysis of Late Gestation Bovine Placentomes from Somatic Nuclear Transfer Pregnancies Complicated by Hydrallantois

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ABSTRACT

Somatic nuclear transfer (NT) in cattle is often complicated by fetal oversize (i.e., large offspring syndrome), hydrallantois, and placentomegaly in late gestation. The aims of this work were to obtain data on the placentome structure in NT-recipient cows with hydrallantois (NTH) and to relate these with fetal and placental weights to better understand the abnormalities observed in NTH pregnancies during the third trimester. Pregnant cows were slaughtered between Gestation Days 180 and 280. The fetuses were weighed, and the placentomes were numbered and weighed. Placentomes were examined by histologic and stereological techniques. Macroscopic data showed that placental overgrowth preceded fetal overgrowth, and the ratio of the fetal to the total placentome weight in the NTH group was lower than that in controls after Gestation Day 220. This suggests that placental overgrowth is due to placental default rather than due to fetal overgrowth, as shown also by stereological analysis showing primary deregulation of the growth of cotyledonary tissues. Observed alterations, such as thinning of the maternal epithelium within placentomes and increased trophoblastic surface, could be secondary adaptations. Thus, placental growth deregulations would be due to modifications of the expression of placental factors. Various examples of placental deficiency were observed, suggesting that some fetal abnormalities observed in NTH calves, such as enlarged heart, enlarged umbilical cord, and abdominal ascites, are consequences of placental dysfunction. Therefore, the condition described by the term "large offspring syndrome" might better be described by "large placenta syndrome," because this syndrome affects an average of 50% of late-gestation NT pregnancies. No conclusion can be drawn from this work on apparently normal pregnancies.

assisted reproductive technology, conceptus, developmental biology, placenta, pregnancy

INTRODUCTION

Cloning by nuclear transfer (NT) of somatic cells is associated with high rates of embryonic and fetal mortality in different species [1–5]. In cattle, a poorly developed placenta with very few placentomes and little vascularization has frequently been described in early pregnancy [2, 6]. Later in pregnancy, somatic cloning in cattle is associated with hydrallantois (i.e., excessive accumulation of allantoic fluid), which is one of the main causes of fetal mortality during the third trimester [7–9]; placentomegaly, a reduced number of placentomes [10]; and an increased birth weight, which is also referred to as large offspring syndrome [11–14].

In early pregnancy, it is easier to relate defects of placental development to the frequent early embryonic death associated with NT [2, 6]. Sometimes the placenta succeeded in developing but was associated with excessive weight. Although it can be suspected that placentomegaly is associated with modifications of placental efficiency, the nature of these modifications is not yet established, nor are their consequences on fetal growth.

The bovine placenta is composed of 60–120 placentomes, which are mushroom-shaped structures distributed along the uterine mucosa within which fetal villi (cotyledon) and maternal crypts (caruncle) are interdigitated and closely apposed. Placentomes appear during the second month of pregnancy and are all in place at the end of the first trimester. Within a placentome, the maternofetal interface consists of a microvillous interdigitation of uterine and trophoblastic epithelia, which starts to develop by Gestation Day 20, before placentome formation. To understand whether the fetal or maternal tissue allocation are disturbed in the placentomes of somatic clone pregnancies complicated by hydrallantois (NTH), we have studied the structural organization of placentomes by stereology at the time of apparition of clinical signs. Stereologic analysis is a useful morphometric technique that allows derivation of three-dimensional structures from two-dimensional sections of these structures, which improves knowledge about the placenta and its function [15].

The objectives of this work were to compare tissular composition of placentome in control and NT pregnancies complicated by hydrallantois and to relate these data with fetal weight and placental weight to establish relationships between fetal growth and placental growth.

MATERIALS AND METHODS

Ethical Approval

The experiment was performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Research work on cloned animals was approved by the INRA ethics committee (Ethical and Precaution Committee for Agronomical Research Application) in December 1999.

Embryo Production

Oocyte preparation. Bovine ovaries were collected at the abattoir, washed several times with fresh saline, and transported in sterile PBS at 33°C to the laboratory within 3 h of collection. Cumulus oocyte complexes (COCs) were

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aspirated and matured as described elsewhere [16]. In brief, COCs were aspirated from 2–7-mm follicles, washed, and selected morphologically for in vitro maturation. Groups of 30–40 COCs were incubated in TCM 199 (Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies, Paisley, Scotland), 10 μg/ml FSH (Stimufol; Merial, Lyon, France), and 1 μg/ml LH for 22 h at 39°C in a humidified atmosphere of 5% CO_2 in air. At the end of the maturation period, oocytes from the same batch were used either to provide a recipient cytoplast for cloning or for in vitro fertilization (IVF).

Somatic NT. Somatic cloning was performed as described elsewhere [17]. After maturation, cumulus cells were removed, and oocytes were enucleated (oocytes in metaphase II and polar body chromatin were removed). Donor cells from adult skin biopsies were cultured over several passages to obtain either a growing or a quiescent population of cells on the day of NT. Four genotypes (A, B, C, and D) from Holstein cows and one Holstein bull were used. The cells were mechanically scrapped, pelleted, and resuspended in fresh TCM 199. Each isolated cell was inserted under the zona pellucida of the recipient cytoplast and fused by electrofusion [18].

In vitro development of NT embryos. All of the reconstituted embryos were cultured under the same conditions in microdrops of 50 μl of B2 medium (CCD, Paris, France) with 2.5% FCS and were seeded with Vero cells. The droplets were overlaid with mineral oil (M8410; Sigma, Rhône-Mérieux, Lyon, France) and incubated for 7 days at 39°C under 5% CO_2. By Day 7, expanding or early hatching blastocysts (grades 1 and 2) were transferred into recipient heifers.

Control IVF embryo. IVF embryos were obtained from the same batches of in vitro matured oocytes. Embryos were made with the semen from the same Holstein bull and were cultured in B2 medium either as described above for NT (for 2 pregnancies) or in sequential medium without serum (for 4 pregnancies) [19].

Embryo Transfer

Animals. Recipient animals were normally cycling Charolais, Normande, or crossbred heifers raised in the same conditions and transported to the experimental farm by the age of 12–14 mo after thorough serologic tests to confirm the absence of infectious diseases. They were synchronized for embryo transfer by the age of 15–18 mo.

Estrous synchronization. Estrous cycles were synchronized in each group of recipients for 9 days by means of a progestagen implant (Crestar; Intervet, Angers, France) associated with a prostaglandin analogue injection (2 ml of Estrumate; Intervet, Angers, France) 2 days before implant removal. After estrous detection, heifers that had embryos of synchronous ages (i.e., ±24 h) and a palpable corpus luteum were selected for embryo transfer.

On Day 7, grade 1 and 2 blastocysts that were developed in vitro after NT or IVF were transferred nonsurgically (by a single transfer) into the uterine horn ipsilateral to the corpus luteum by use of the miniaturized embryo transfer syringe and sheath (IMV, L'Aigle, France) under epidural anesthesia.

Control Pregnancy Production

Holstein heifers and dairy cows from two experimental farms were artificially inseminated (AI) with frozen sperm from Holstein bulls to provide third-trimester controls (i.e., fetuses that were more than 180 gestation-days old).

NT Pregnancy Monitoring

Pregnancy diagnosis was performed at Gestation Day 35 ± 2 by transrectal ultrasonography with a 5.0-MHz probe (Ultrascan 9000; Alliance Medical, Russellville, MO). Pregnant recipients were then observed on Days 50, 64, and 90 of pregnancy. Beginning on Day 120, recipients underwent repeated transabdominal ultrasonography with a 3.5-MHz probe (Starvet 3; Hospimedi, St. Crépin Ibouvillers, France) every 2–3 wk until calving.

Hydrallantois was diagnosed by ultrasonography when an increase in the volume of fetal fluids associated with difficulty in locating the fetus within the uterine cavity was observed, together with visualization of placental edema and hyperechogenicity of placentomes. Clinical findings, such as rapid increase of abdominal circumference of the recipient and reluctance to walk or eat, were also used to confirm the diagnosis. During the third trimester of gestation, 18 recipients that received a diagnosis of hydrallantois were humanely slaughtered within 1–2 wk after diagnosis. These fetuses were considered to be nonviable, because in our experience, fetal death and abortion generally occur 1–4 wk after the onset of the disease. The uterus and the fetus were collected for examination. One NT recipient that received a diagnosis of hydrallantois was delivered by cesarean section (C-section) 24 h after induction of delivery with dexamethasone, because the fetus was considered to be viable due to the advanced gestational age (274 days) at the time of diagnosis. Treatment of hydrallantois by allantocentesis to mechanically remove the fluids was not performed, because a study involving noncloned cows demonstrated that the allantoic cavity might rapidly fill up again [20]. Moreover, we thought this treatment might affect tissue structure and, therefore, bias the study.

Collection of Placenta Samples

Control animals (ten in the AI group and six in the IVF group) were slaughtered under the same conditions as the NTH animals during the third trimester of pregnancy. Two cows in the AI group were delivered by C-section at term after induction with dexamethasone.

The fetuses were weighed, and all the placentomes were dissected away from the uterine mucosa, numbered, and weighed. For the recipients delivered by C-section (one in the NTH group and two in the AI group), only one placentome was collected during the surgery and weighed.

Histologic and Stereologic Analyses

Staining. One placentome from the same area of the pregnant horn (close to the fetus) from each cow was randomly selected for further analysis. From each of six cows, four placentomes were selected for intraplacental comparison. A 5-mm-thick slice was obtained from the width of each placentome and cut in two, three, or four pieces representing all areas, from the bottom of the crypts to the fetal side. Samples were fixed in 4% formalin, dehydrated, and embedded in paraffin wax with the same orientation. Sections of 7 μm were stained with hematoxylin-eosin-safran (HES) for histologic analysis or with a trichrome stain for morphometric analysis. For the trichrome stain, slides were successively stained with nuclear red (CI: 60760; Merck, Darmstadt, Germany) for 10 min, 1% (w/v) Orange G (CI: 16230; RAL, Bordeaux, France) in deionized water for 6 min, and 2% (w/v) aniline blue (CI: 42755; RAL) in deionized water (pH 6) for 10 min. Slides were rinsed with distilled water between each staining.

Stereological analysis. Placentomes from 14 control (AI and IVF) and 11 NTH pregnancies were used for stereological analysis. Sections were examined with a 25× objective lens and a 10× ocular lens on a Leitz DMRB microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Olympus DP50 digital camera (Tokyo, Japan). Images were captured and recorded with Viewfinder Lite 1.0 and Studio Lite 1.0 software (Pixera Corporation, Los Gatos, CA). One section from each of two to four different blocks from the same placentome was analyzed, and ten fields (360 points) were counted in each section. The first field location was chosen at random, and subsequent adjacent fields were systematically selected 1 mm apart with the aid of a stage micrometer. The proportions of maternal and fetal tissues in placentome were quantified using point counting with an isotropic L-36 Merz grid superimposed on captured images with Adobe Photoshop 4.0 LE software (San Jose, CA). The volume densities (V_V) of trophoblast, fetal connective tissue, maternal epithelium, and connective tissue were calculated using the formula $V_V = P_a/P_T$, where P_a is the total number of points falling on the given tissue, and P_T is the total number of points applied to the section [21]. The total weight of placental components was calculated by multiplying total placentome weight by volume density for each component (before the calculations were performed, we confirmed that the volume of 1 g of placentome is 1 cm^3).

The surface density (surface per gram of placenta) of the trophoblast (S_V) was measured by counting the line intercepts with the same grid in the same fields and was calculated using the formula $S_V = 2 \times I_a/L_T$, where I_a is the number of intercepts of the trophoblast with the line, and L_T is the total length of the lines applied [21]. The total surface of the trophoblast (S_T) was calculated by multiplying S_V by the total placentome weight.

The relative surface of the trophoblast (S_R) was obtained by dividing S_T by the sum of the weight of the fetus and the weight of the fetal components of the placentomes [22].

The arithmetic mean barrier thickness of maternal epithelium and trophoblast was calculated using the formula $B_T = V_V/S_V$, where V_V is the volume density of the maternal epithelium or trophoblast, and S_V is the surface density of the maternal epithelium or trophoblast [21].

Statistical Analysis

Data were analyzed using SAS software, version 8 (SAS Institute, Cary, NC). First, differences for gestational age, fetal weight, total placentome weight, mean placentome weight, and placentome number were compared between control and NTH pregnancies and between two gestational periods (before and after Day 220) using the Student *t*-test. Pearson correlation analyses were

TABLE 1. Mean ± SD for morphometric and stereological parameters in AI and IVF pregnancies.

Parameter	AI (n = 10)	IVF (n = 6)	*P* value
Morphometric			
No. of placentome	81 ± 30	103 ± 22	0.07
Mean placentome weight (g)	77 ± 50	56 ± 45	0.18
Fetal weight (FW) (kg)	28.4 ± 13.6	21.5 ± 9.5	0.30
Total placentome weight (TPW) (kg)	5.9 ± 1.7	5.4 ± 1.8	0.63
FW/TPW ratio	5.1 ± 1.4	3.9 ± 1.3	0.12
Gestational age (days)	233 ± 31	222 ± 26	0.48
Stereological			
Volume densities (V_v) (%)			
Fetal tissue	58 ± 3	57 ± 4	0.88
Trophoblast	42 ± 3	43 ± 4	0.88
Fetal connective tissue	22 ± 3	20 ± 2	0.13
Maternal tissue	35 ± 3	37 ± 5	0.23
Maternal epithelium	31 ± 2	30 ± 2	0.23
Maternal connective tissue	11 ± 3	13 ± 3	0.36
Surface density (S_v) (μm^{-1})	0.022 ± 0.002	0.020 ± 0.002	0.13
Maternal epithelium thickness (μm)	10.3 ± 1.3	10.0 ± 1.2	0.73
Trophoblast thickness (μm)	14.5 ± 2.0	15.0 ± 1.7	0.65

performed to relate these findings with each other in both groups separately. The fetal and total placentome weights were analyzed by general linear models. The effect of the animal group (controls or NTH) was introduced in all models.

Second, stereological differences between control and NTH groups were tested using the Student *t*-test. Pearson correlations were performed to relate stereological findings with each other and with gestational age, fetal weight, and total placentome weight in both groups separately. The MIXED procedure was used to test the variability of stereological findings for 4 placentomes from the same placenta.

Differences between groups and correlations were considered to be statistically significant if $P < 0.05$. Results are presented as means ± standard deviation.

RESULTS

After in vitro culture, blastocyst rates were 26.4%, 42.9%, 38.9%, 36.1%, and 20.5%; pregnancy rates at 35 days were 39%, 46%, 39%, 26%, and 38% of the transferred blastocysts; and calving rates were 6.4%, 19.8%, 8.2%, 2.3%, and 15% for genotypes A, B, C, D, and the bull, respectively, thus showing different developmental potential for different cell lines as shown previously. Although genotype C presented in more NTH pregnancies than other pregnancies, there was no predominant genotype in the cases analyzed.

Gross Placental and Fetal Measurements

Altogether, 10 AI pregnancies, 6 IVF normal pregnancies, and 18 NT pregnancies with NTH were used for gross examination. There was no difference between AI and IVF for all characteristics studied (Table 1), so data were pooled into a single control group. Mean gestational age was similar in controls and NTH (229 ± 29 days vs. 221 ± 27 days; $P = 0.43$). One NTH fetus was a male, whereas only three control fetuses were females. Therefore, the sex of fetuses could not be taken into account for statistical analyses.

General observations on the data: identification of two NTH groups. The mean placentome number was not significantly different between the control and NTH groups (90 ± 23 vs. 78 ± 24; $P = 0.17$). Table 2 presents correlation coefficients with gestational age for measurements involving fetuses and placentas from control and NTH pregnancies. Placentome number was constant during the third trimester of pregnancy in the control group ($r = -0.10$; $P = 0.72$) but tended to increase with gestational age in the NTH group ($r = 0.47$; $P = 0.05$) (Table 2).

The mean placentome weight, the total placentome weight, and the fetal weight were positively correlated with the stage of gestation in the control and NTH groups (Table 2). In the control group, mean placentome weight was negatively correlated with placentome number ($r = -0.70$; $P < 0.01$), but this was not the case in the NTH group ($r = -0.23$; $P = 0.36$). However, two populations of NTH fetuses could be distinguished: one with a mean placentome weight and placentome number within the normal range and one with a mean placentome weight heavier than expected for the respective placentome number (Fig. 1). The former population involved cows slaughtered before Day 220, and the latter population involved cows slaughtered after Day 220 (except

TABLE 2. Mean values (± SD) and correlation coefficients with gestational age for measurements on fetuses and placentas from control and NTH pregnancies, during the third trimester of gestation.

	Controls (n = 16)[a]			NTH (n = 18)		
Parameter	Mean ± SD	r[b]	*P* value[c]	Mean ± SD	r[b]	*P* value[c]
No. of placentome	90 ± 23	−0.10	0.72	78 ± 24	0.47	0.05
Mean placentome weight (g)	68 ± 28	0.54	<0.05	123 ± 46	0.51	<0.05
Total placentome weight (TPW) (kg)	5.7 ± 1.7	0.67	<0.01	9.3 ± 3.6	0.82	<0.01
Fetal weight (FW) (kg)	25.8 ± 12.4	0.92	<0.01	33.9 ± 13.8	0.95	<0.01
FW/TPW ratio	4.7 ± 1.5	0.76	<0.01	3.7 ± 1.1	0.28	0.27
Gestational age (days)	229 ± 29			221 ± 27		

[a] Missing data on one placenta.
[b] Pearson correlation coefficients (r) with gestational age.
[c] *P* values of correlation with gestational age.

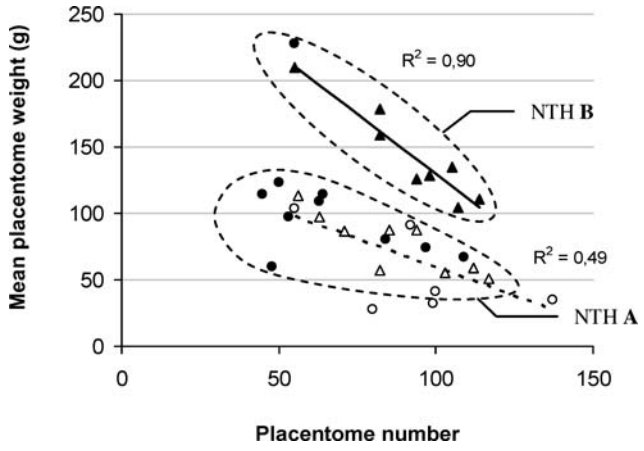

FIG. 1. Mean placentome weight in relation to placentome number during the third trimester of pregnancy in control (empty symbols) and hydrallantois (NTH) (filled symbols) pregnancies. The two groups A and B of NTH cows delimited on the figure corresponded to cows slaughtered before (group A) (circles) and after (group B) (triangles) 220 days of gestation, excepted one cow in group B. Lines are linear regression curves for the NTH group after Day 220 (solid line) and the control group (interrupted line).

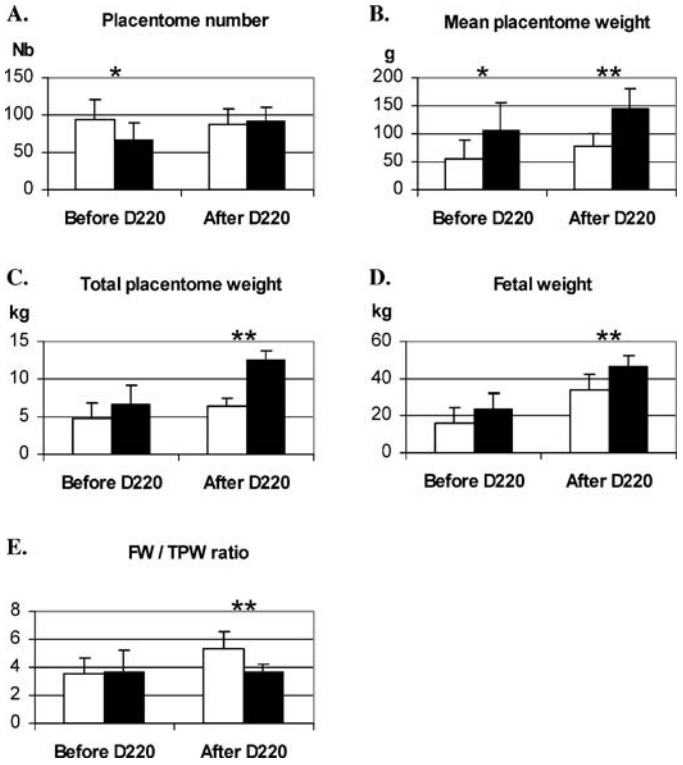

FIG. 2. Placentome number (**A**), mean placentome weight (**B**), total placentome weight (TPW) (**C**), fetal weight (FW) (**D**), and ratio of fetal weight to total placentome weight (**E**) in controls (white bars) and hydrallantois (NTH) (black bars) pregnancies within two periods of the third trimester of pregnancy (mean ± standard deviation). Statistical differences between the control and NTH groups within each period are indicated by $*P < 0.05$ and $**P < 0.01$.

for one cow slaughtered at Day 215). Subsequently, data from NTH and controls pregnancies were compared within two gestational periods, before Day 220 (cows [$n = 7$] in the control group were slaughtered on Day 201 ± 20 [range, Days 180–220], and cows in the NTH groups [$n = 10$] were slaughtered on Day 202 ± 16 [range, Days 172–220]) and after Day 220 (cows [$n = 9$] in the control group were slaughtered on Day 250 ± 9 [range, Days 235–260], and cows [$n = 8$] in the NTH group were slaughtered on Day 245 ± 15 [range, Days 221–263]).

Characterization of NTH groups. Before Day 220, placentome number was lower in the NTH group, compared with the control group (67 ± 22 vs. 94 ± 27 placentomes; $P < 0.05$). However, because the mean placentome weight was higher in the NTH group (106.7 ± 47.8 vs. 55.1 ± 33.3 g; $P < 0.05$), the total placentome weight was not different between the groups (6.7 ± 2.5 vs. 4.7 ± 2.2 kg; $P = 0.12$) (Fig. 2, A–C). Moreover, because fetal weight was not different between the NTH and control groups (23.8 ± 8.6 vs. 15.8 ± 9.1 kg; $P = 0.09$), the ratio of fetal weight to total placentome weight was similar between the groups (Fig. 2, D and E).

After Day 220, placentome number was not different between the NTH and control groups (92 ± 19 vs. 87 ± 21; $P = 0.61$), but the mean placentome weight (143.9 ± 35.9 vs. 77.2 ± 22.3 g; $P < 0.01$), the total placentome weight (12.6 ± 1.2 vs. 6.4 ± 1.0 kg; $P < 0.01$), and the fetal weight (46.5 ± 6.3 vs. 33.6 ± 8.5 kg; $P < 0.01$) were higher in the NTH group (Fig. 2, A–D). Moreover, the ratio of fetal weight to total placentome weight was smaller in the NTH group, compared with the control group (3.7 ± 0.5 vs. 5.3 ± 1.3 kg; $P < 0.01$) (Fig. 2E).

In the control group, because fetal weight increased between these two periods ($P < 0.01$) and the total increase in placentome weight was not statistically significant, the ratio of fetal weight to total placentome weight increased ($P < 0.05$). In the NTH group, both fetal weight and total placentome weight increased ($P < 0.01$), and the ratio of fetal weight to total placentome weight did not vary ($P = 0.99$) (Fig. 2).

Multivariate analysis. Fetal weight was not influenced by the treatment group (NTH vs. controls, $P = 0.51$) but depended on the gestational period ($P < 0.01$) and total placentome weight ($P < 0.05$) (Table 3), which explained 79% of the variability in fetal weight. Total placentome weight was influenced by the treatment group, gestational period, placentome number, and mean placentome weight ($P < 0.01$) (Table 3), which explained 94% of the variability in the total placentome weight. Total placentome weight was not influenced by fetal weight in any of the models studied. The ratio of fetal weight to total placentome weight was not influenced by the treatment group ($P = 0.71$) but depended on the gestational period ($P < 0.05$), the total placentome weight ($P < 0.01$), the interaction between gestational period and total placentome weight ($P < 0.01$), and the fetal weight ($P < 0.01$) (Table 3), all of which explained 94% of the variability in the ratio of the fetal weight to the total placentome weight.

Histologic Characteristics of the Placentomes

The typical organization of the synepitheliochorial placenta was maintained in NTH placentomes (Fig. 3). In some of them, however, the uterine epithelium was flattened with small nuclei. Fetal connective tissue enlargement without signs of edema, such as reduced cell density and vessels dilatation, was observed in other NTH placentomes. The edema observed around the placentomes in NTH fetuses was not observed within the placentomes. In all but one NTH placentome in which there was some degree of cytoplasmic enlargement, binucleate cells seemed to be of equal size.

Finally, relative differences in the different placental compartments could be evidenced by stereological findings in the NTH placentome, as shown below.

TABLE 3. Multivariate general linear models of fetal weight, total placentome weight and fetal weight/total placentome weight ratio.

Parameters & variables	LSM ± SEM	*P* value
Fetal weight		
Treatment group		
NTH	31.7 ± 2.1 kg	0.51
Controls	29.2 ± 2.5 kg	
Gestational period		
≤D220	24.0 ± 2.4 kg	<0.01
>D220	36.9 ± 2.1 kg	
Total placentome weight		<0.05
Total placentome weight		
Treatment group		
NTH	8.3 ± 2.4 kg	<0.01
Controls	6.9 ± 2.8 kg	
Gestational period		
≤D220	6.9 ± 2.5 kg	<0.01
>D220	8.3 ± 2.3 kg	
Placentome number		<0.01
Mean placentome weight		<0.01
Fetal weight/total placentome weight ratio		
Treatment group		
NTH	4.2 ± 0.13	0.71
Controls	4.3 ± 0.12	
Gestational period		
≤D220	4.4 ± 0.12	<0.05
>D220	4.2 ± 0.12	
Total placentome weight		<0.01
Gestational period × TPW		<0.01
Fetal weight		<0.01

Stereological Findings

Relative volumes of specific tissues and S_V. Ten AI pregnancies, 4 IVF normal pregnancies, and 11 NTH pregnancies were used for stereological analyses. There were no differences between the AI and IVF groups, so data were pooled into a single control group. None of the relative characteristics varied within the period of pregnancy studied in any of the two treatment groups (data not shown). Thus, these data were analyzed without taking gestational age into account.

Results of the stereological analysis of the placentomes are shown in Table 4. Specific tissue differences were observed between the control and NTH groups. The mean V_V of the fetal component was higher in the NTH group, compared with the control group (47% ± 6% vs. 42% ± 3%; $P < 0.05$). This was due to an increase in mean V_V of the fetal connective tissue (17% ± 6% vs. 12% ± 3%; $P < 0.05$) and a decrease in the mean V_V of maternal epithelium (18% ± 4% vs. 22% ± 3%; $P < 0.05$). The mean S_V was not different between the control and NTH groups ($P = 0.88$) (Table 4). The maternal epithelium was thinner in the NTH group, compared with the control group (8.5 ± 1.7 vs. 10.2 ± 1.2 μm; $P < 0.01$).

Total weights of specific tissues and S_T. In a preliminary study, 4 placentomes (which were obtained from the middle and tip of the pregnant and nonpregnant horn) from each of 2 NTH and 4 control pregnancies were examined by stereological techniques to check that a mean measurement for one placentome was representative of all placentomes within one placenta. No significant difference was observed for all characteristics within placentomes from the same placenta (Table 5). Thus, relative values obtained from only one placentome were used to calculate total weights and trophoblastic surface, using total placentome weight. Because of C-sections and missing total placentome weights for 2 control pregnancies, absolute values were calculated for only 10 controls and 10 NTH pregnancies.

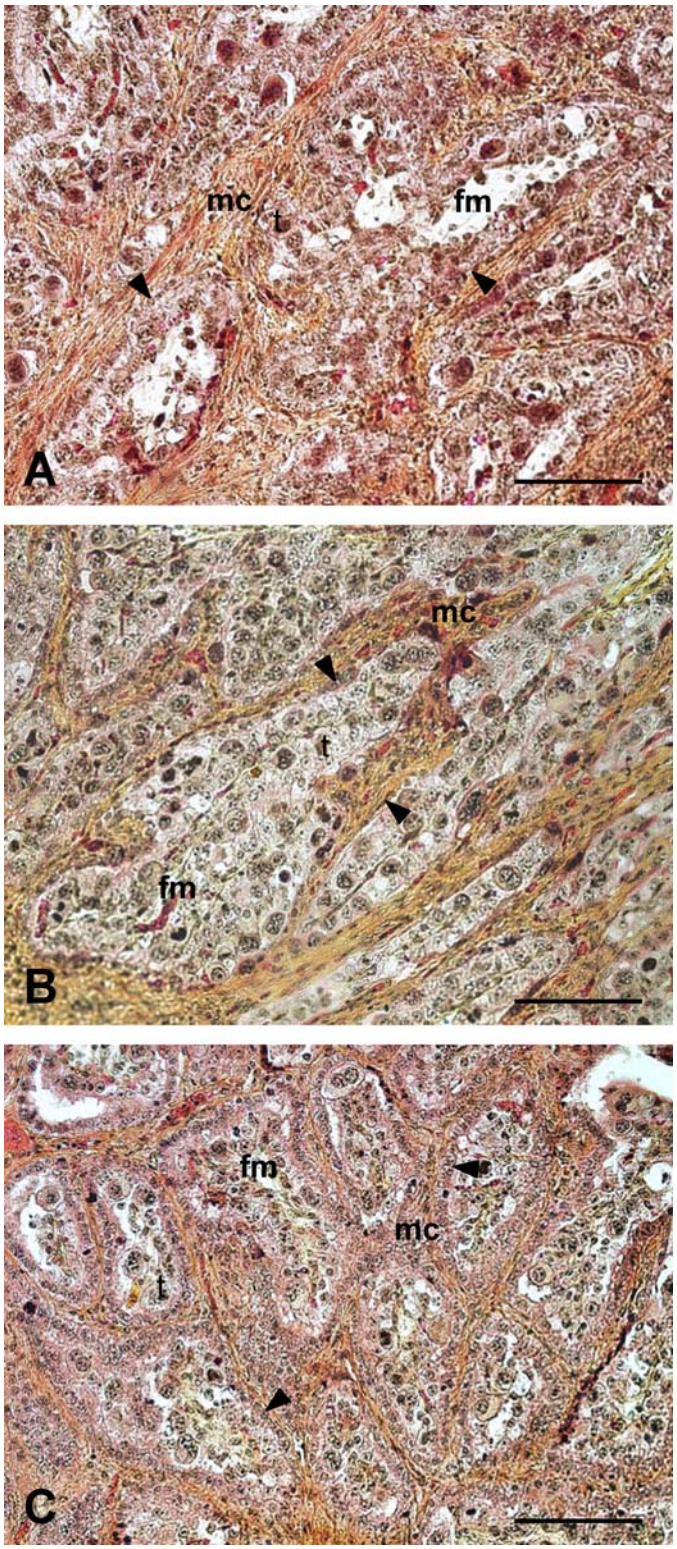

FIG. 3. Histological sections of placentomes from hydrallantois (**A**, **B**) and control (**C**) pregnancies at the third trimester. **A**) Enlargement of the fetal connective tissue without signs of edema. **B)** Smaller uterine epithelium (arrow heads). **C**) Control placentome. Hematoxylin-eosin-safran staining. fm, fetal mesenchyme; mc, maternal connective tissue; t, trophoblast; arrow heads, maternal epithelium. Bar = 100 μm.

Before D220, the total weight of the different placentome components was not different between the NTH and control groups. After Day 220, the total weight of all components were higher in the NTH group, compared with the control group (*P*

TABLE 4. Stereological parameters for placentomes from control and NTH pregnancies in the third trimester of gestation (mean ± SD).

Stereological parameters	Controls (n = 14)	NTH (n = 11)	*P* value
Volume densities (V_V) (%)			
Fetal tissue	42 ± 3	47 ± 6	<0.05
Trophoblast	31 ± 2	30 ± 3	0.58
Fetal connective tissue	12 ± 3	17 ± 6	<0.05
Maternal tissue	58 ± 3	53 ± 6	<0.05
Maternal epithelium	22 ± 3	18 ± 4	<0.05
Maternal connective tissue	36 ± 4	35 ± 6	0.66
Surface density (S_V) (μm^{-1})	0.021 ± 0.002	0.022 ± 0.003	0.88
Maternal epithelium thickness (μm)	10.2 ± 1.2	8.5 ± 1.7	<0.01
Trophoblast thickness (μm)	14.6 ± 1.9	14.2 ± 1.5	0.59

< 0.01) (Fig. 4). In the control group, total weights were not significantly different between the two gestational periods, but in the NTH group, they were higher after Day 220 (data not shown). The ratios of the total weight of each of the placentome components to the fetal weight were not different between the NTH and control groups before Day 220. The ratio of total fetal tissue weight to fetal weight after Day 220 was higher in the NTH group, compared with the control group ($P < 0.01$), whereas the ratio of the total maternal tissue weight to the fetal weight was not statistically different ($P = 0.19$) (Table 6).

Before Day 220, S_T was not statistically different between the NTH and control groups (155 ± 42 vs. 125 ± 38 m^2; $P = 0.38$), whereas it was more than 2-fold higher in the NTH group after Day 220 (276 ± 51 vs. 133 ± 26 m^2; $P < 0.01$) (Fig. 4G). S_T did not vary between the two gestational periods in the control group ($P = 0.71$) but was higher after Day 220 in the NTH group ($P < 0.01$).

S_R was not different between the NTH and control groups before Day 220 (5.3 ± 0.8 vs. 5.1 ± 0.7 m^2/kg; $P = 0.71$), and it tended to be higher in the NTH group, compared with the control group, after Day 220 (5.3 ± 0.9 vs. 4.2 ± 1.0 m^2/kg; $P = 0.06$).

DISCUSSION

As a preliminary remark, all abnormalities observed in the present study were observed when hydrallantois was diagnosed after Day 220, thus suggesting that these observations are linked to gestational age rather than directly to hydrallantois.

The macroscopic data show that placentomegaly associated with NT in cattle does not occur in response to the increase in fetal weight nor to compensate for the reduced placentome number. Indeed, the increase in mean placentome weight preceded the increase in fetal weight in NT pregnancies. Fetal weight was significantly higher in NT pregnancies than control pregnancies after Day 220, despite the facts that most NT fetuses were females, most control fetuses were males, and male fetuses are slightly heavier than females under normal conditions. The data are therefore reinforced by this skewed sex ratio. Moreover, in the multivariate analysis presented here, fetal weight was determined by total placentome weight, but the contrary was not true.

Fetal pathological data were not presented in this study, because they are still being analyzed. Preliminary data show that most fetuses had an enlarged umbilical cord, and several presented with abdominal ascites. A normal histologic structure was observed in all tissues studied (unpublished data). The relative weights of kidneys, heart, and liver were each heavier in NTH fetuses, as shown previously in a limited number of fetuses [16].

In terms of placental growth kinetics, several studies have shown that placental growth during pregnancy in cattle is initially exponential, reaching a plateau between Gestation Days 190 and 250 [23–26]. The 220-day threshold after which fetal and placental growth in the NTH group became markedly different from growth in the control group is therefore consistent with normal gestational data.

It has been suggested in previous studies that the presence of enlarged placentomes in NTH resulted from a compensatory mechanism [2, 10, 27, 28] that is similar to that reported after surgical removal of caruncles in ewe [29]. It may be the case that, before Day 220 but after Day 220, placentome number was similar in the NTH and control groups, whereas the mean placentome weight in the NTH group was heavier than that in the NTH group (Fig. 2), demonstrating that enlarged placentomes are not only due to a compensatory growth mechanism as a result of reduced placentome number.

TABLE 5. Intraplacental comparison: stereological parameters of four placentomes, by cow (mean ± SD).

Stereological parameters	Means of four placentomes: Control cow 1	Control cow 2	Control cow 3	Control cow 4	NTH cow 5	NTH cow 6	*P* value for placentome effect
Volume densities (V_V)							
Fetal tissue	0.49 ± 0.02	0.45 ± 0.05	0.42 ± 0.04	0.45 ± 0.06	0.48 ± 0.07	0.49 ± 0.04	0.76
Trophoblast	0.32 ± 0.02	0.33 ± 0.04	0.31 ± 0.02	0.35 ± 0.05	0.32 ± 0.05	0.31 ± 0.02	0.08
Fetal connective tissue	0.17 ± 0.03	0.12 ± 0.02	0.11 ± 0.03	0.10 ± 0.01	0.16 ± 0.04	0.18 ± 0.05	0.19
Maternal tissue	0.51 ± 0.02	0.55 ± 0.05	0.58 ± 0.04	0.55 ± 0.06	0.52 ± 0.07	0.51 ± 0.04	0.76
Maternal epithelium	0.20 ± 0.01	0.23 ± 0.03	0.22 ± 0.01	0.20 ± 0.02	0.21 ± 0.01	0.16 ± 0.02	0.65
Maternal connective tissue	0.31 ± 0.01	0.32 ± 0.08	0.36 ± 0.03	0.34 ± 0.04	0.31 ± 0.07	0.35 ± 0.05	0.69
Surface density (S_V) (μm^{-1})	0.018 ± 0.001	0.021 ± 0.001	0.022 ± 0.001	0.020 ± 0.002	0.023 ± 0.003	0.019 ± 0.002	0.34
Maternal epithelium thickness (μm)	11.3 ± 0.9	10.8 ± 1.0	10.0 ± 0.4	10.2 ± 1.4	9.1 ± 0.9	8.6 ± 0.7	0.41
Trophoblast thickness (μm)	17.9 ± 1.0	16.0 ± 1.3	14.0 ± 1.1	17.7 ± 2.7	14.2 ± 1.7	16.1 ± 0.5	0.22

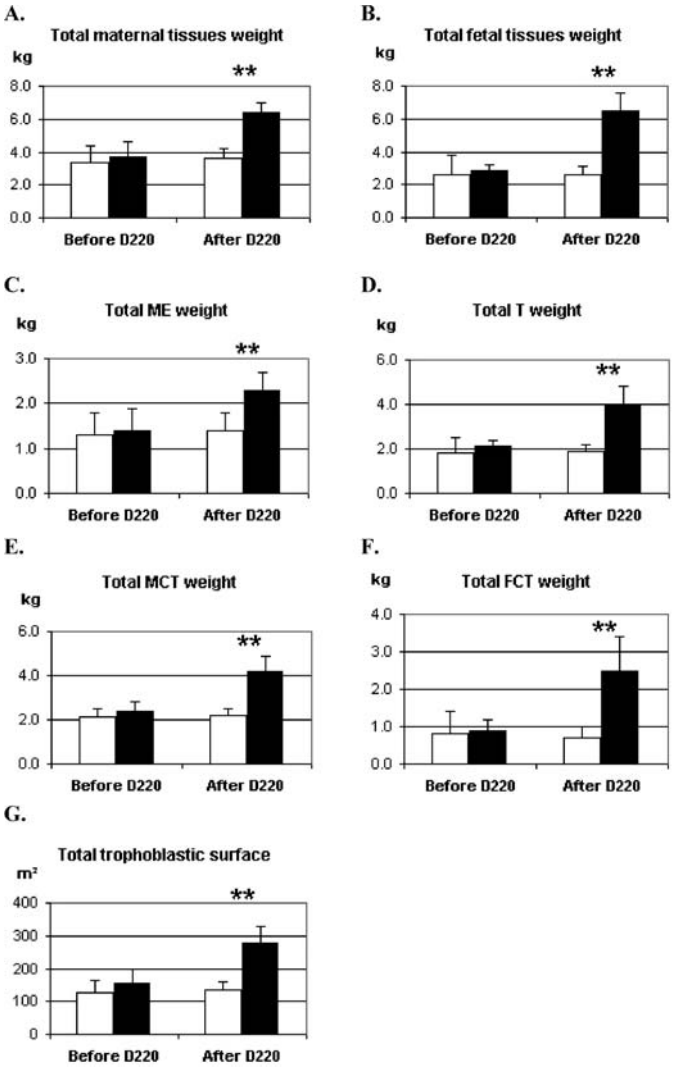

FIG. 4. Weight of the different components of placentome (**A**–**F**) and total surface of the trophoblast in control (white bars) and NTH (black bars) pregnancies within two periods of the third trimester of pregnancy (mean ± standard deviation). ME, maternal epithelium; T, trophoblast; MCT, maternal connective tissue; FCT, fetal connective tissue. Statistical differences between the control and NTH groups within each period are indicated by $*P < 0.05$ and $**P < 0.01$.

Furthermore, the edema around the placentomes is a relatively thin, jelly-like layer, and the increased weight is mainly due to enlarged placentome tissue.

Our macroscopic data are in agreement with data from Lee et al. (2004), who have shown significantly higher placental weight in NT pregnancies at Gestation Day 50 (in fetal membranes), Day 100 (in caruncles), and Day 150 (in both fetal membranes and caruncles), whereas fetal weight was not different between NT and control pregnancies at any of the three stages studied [30]. Cloning in mice is also associated with increased and disconnected fetal and placental weight in late gestation [31, 32]. In our laboratory, excess placental weight was observed in NT mouse fetuses before excess fetal weight and placental weight further increased when fetal overweight was noted. Extreme phenotypes were sometimes observed, with term development of a placenta in the absence of a fetus [33].

Other laboratories, however, have found significantly increased fetal weight as early as Day 80 in NT cattle [34]. It is therefore difficult to estimate an exact timing for the onset of increased fetal weight, because it may vary according to laboratories and both donor cell and oocyte phenotype [2, 34].

Thus, the first part of this study shows that placentomegaly was due to placental growth deregulation and suggests that it was a response and maybe an adaptation to placental dysfunction.

The stereological data are in agreement with the macroscopic data and clarify placental growth deregulations. No major histological abnormalities were observed in NTH placentomes in the third trimester of pregnancy, which accords with the report of Ravelich et al. (2004), who also observed that the cellular morphology of the placentomes in the control group (AI and IVF) was similar to that in the NT group at Days 50, 100, and 150 of gestation [35].

Despite the apparently normal histologic structure of NTH placentomes, objective measurements performed with stereologic techniques on placentome sections revealed that the growth of the maternal and fetal components of the placentomes were not parallel in NTH pregnancies during the third trimester. Indeed, the growth of fetal tissue, and especially fetal connective tissue, was favored. Fetal connective tissue was 3.6-fold heavier in NTH than in controls after Day 220, whereas total placentome weight was 2.1-fold heavier in NTH (data not shown). Moreover, the ratio of total cotyledon weight to fetal weight increased in the NTH group, whereas it decreased in the control group, suggesting that, although both the growth of placental tissues of fetal origin and the growth of fetal tissues per se were disturbed, they were differentially regulated. Finally, the ratio of the total caruncle weight to the fetal weight was not different between the NTH and control groups, suggesting that excessive growth of the uterine tissue was a response to overgrowth in fetal tissues (fetus and cotyledon) that was maybe due to growth factors of cotyledonary origin, such as those of the insulin-like growth factors system [35].

In mice, placental abnormalities observed after cloning are much more dramatic. The main faults are an expansion of the spongiotrophoblast, an increased number of glycogen cells, and enlargement of the spongiotrophoblast cells [31]. Nevertheless, placentas could support full development of the fetus. Ohgane et al. (2004) found a positive correlation between the extent of hypermethylation of the Sall3 locus in the placenta only and placental weight [36], suggesting that epigenetic modifications due to somatic NT are responsible for alterations of placental

TABLE 6. Placentome tissues weight/fetal weight (FW) ratios in controls and NTH within the two gestational periods (mean ± SD).

	Before D220			After D220		
Weight ratio	Control (n = 3)	Clones (n = 4)	*P* value	Control (n = 7)	Clones (n = 6)	*P* value
Maternal tissue weight/FW	0.16 ± 0.05	0.15 ± 0.04	0.58	0.12 ± 0.03	0.14 ± 0.02	0.19
Epithelium weight/FW	0.06 ± 0.02	0.05 ± 0.02	0.59	0.05 ± 0.02	0.05 ± 0.01	0.84
Maternal connective tissue weight/FW	0.10 ± 0.03	0.09 ± 0.02	0.64	0.07 ± 0.01	0.09 ± 0.02	0.08
Fetal tissue weight/FW	0.12 ± 0.06	0.12 ± 0.03	0.88	0.09 ± 0.02	0.15 ± 0.02	<0.01
Trophoblast weight/FW	0.08 ± 0.03	0.08 ± 0.01	1.00	0.07 ± 0.01	0.09 ± 0.01	<0.05
Fetal connective tissue weight/FW	0.04 ± 0.03	0.04 ± 0.02	0.78	0.02 ± 0.01	0.06 ± 0.02	<0.01

gene expression resulting in placental overgrowth, independently of fetal growth. Such a differential methylation level between the chorion and the fetus was observed for the X inactivation-specific transcript locus in cattle NT clones [37].

The structural alterations of NTH placentomes are probably evidences of functional alterations of placentas. Some observations in this study are in agreement with a reduced placental efficiency in NTH, such as the ratio of fetal weight to total placentome weight, which was reduced in the NTH group after Day 220.

In the present study, placentome number was very variable. There were significantly less placentomes in the NTH group before Day 220, compared with the control group, but not thereafter. Cloned fetuses have been reported to have less placentomes than control fetuses during the first trimester [2, 30, 38] but not after 5 mo of gestation [27, 39]. Therefore, it may be hypothesized that supplementary placentomes may have developed later in cloned pregnancies or that only cloned pregnancies with sufficient placentome number went on term. These placentomes were fully developed placentomes and did not appear as primitive placental structures (such as adventitious placentation) or accessory placentomes, as has been described in case of uterine disease or lack of placentomes [20]. Even if placentome number is normal at the end of NT pregnancies, the developmental defects observed earlier may induce placental adaptations to maintain pregnancy, which are responsible for perturbations in placental functions and fetal development. On the other hand, although recipients were killed on evidence of hydrallantois and not randomly, it could be suggested that more-severely affected placentas and fetuses were slaughtered earlier, therefore skewing data on the number of placentomes. Placental and fetal growth, however, appeared to be markedly different from that in controls only after Day 220, so earlier placentas may not be affected much more.

The thinning of the maternal epithelium and the increase in S_T, however, may be facilitating transplacental exchanges, but they could also have occurred in response to alterations in placental efficiency. More work on placentome vascularization, which is a predominant factor of placental exchanges capabilities and transporter systems (such as SLC2A1 and SLC2A3), is needed to draw conclusions about this subject. In the present study, however, it seems paradoxical that the placenta should be less efficient because the fetus is overgrown in late gestation and that, usually, placental deficiency is associated with reduced fetal weight [40, 41].

This study raises other minor points requiring comment. It must be noted that no differences between the IVF and AI groups were found in our study despite observations from the literature showing that fetuses produced by IVF and their placentas have a higher incidence of hydrallantois, compared with fetuses and placentas produced by AI [28, 42]. In our laboratory, however, IVF embryos cultured in B2 medium with 2.5% FCS did not produce calves that were heavier than those produced by AI [17]. Moreover, most of the IVF fetuses used in this study were obtained after culture of the embryos in sequential medium without serum [19], which produces normal calves (unpublished data).

The study of 4 different placentomes from different placental sites from the same pregnancy has shown that the histological structure of one placentome was representative of all placentomes in one pregnancy and could therefore be used to evaluate total weight of specific compartments and S_T. The findings obtained with this method were compatible with previously published data from control pregnancies. Thus, in control placentas, the S_V estimated by stereological analyses was in agreement with findings in a previous stereological study of the bovine placenta (~200 cm^2/cm^3 between 15 and 40 wk of pregnancy) [22], as was the proportion of maternal and fetal tissues in the placentome [24, 26]. If placental function can be related to stereological measurements, these results would suggest that the smaller placentomes are functionally comparable to the larger placentomes.

Finally, as shown in previous studies, there is a delay in placental development (e.g., few placentomes and hypovascularization) in early gestation associated with fetal demise in most extreme cases [2, 6]. Even earlier, a shift in the ratio of the inner cell mass to the trophoblast surface has been shown to be increased in NT blastocysts [43], probably resulting in alterations in the relationship between the embryo and the trophoblast. The late gestation effects of these defaults on placentome development remain unknown, and the observations made here may be related to very early events in embryonic development and altered genetic reprogramming, with particular emphasis on imprinted genes, as discussed by others [44].

In conclusion, this work demonstrates that, although the cellular structure of the placenta is very moderately altered in late-gestation somatic clone pregnancies complicated by hydrallantois, the ratio of the fetal tissue to the maternal tissue within the placentomes and the ratio of the fetal weight to the total placentome weight are altered, suggesting that placental growth factors and cellular metabolism, but not placental histological structure, are responsible for these abnormalities. Moreover, it shows that placental overgrowth was not due to fetal overgrowth only. The structural alterations of NTH placentas observed must, as signs of placental dysfunctions, have had consequences on fetal development. Thus, the term "large offspring syndrome" currently used to characterize all abnormal phenotypes of NT (and IVF) embryos should be replaced by "large placenta syndrome" or "abnormal placenta syndrome." Placental dysfunctions could not account for all abnormal fetal phenotypes, however, and thus the proposition by Farin et al. (2006) of a classification with four degrees of "abnormal offspring syndrome' might be better adapted [45]. Finally, it must be kept in mind that only pathological pregnancies with hydrallantois were studied. This syndrome only affected 25%–75% of the pregnancies over 90 days (depending on the cell line), which represents a subtype of all NT pregnancies. No conclusions can be drawn from this work on the placenta of apparently normal NT calves.

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