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Placentation in cloned cattle: Structure and microvascular architecture

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Abstract

To elucidate the morphological differences between placentas from normal and cloned cattle pregnancies reaching term, the umbilical cord, placentomes and interplacentomal region of the fetal membranes were examined macroscopically as well as by light and scanning electron microscopy. In pregnancies established by somatic nucleus transfer (NT), the umbilical cord and fetal membranes were edematous. Placentomal fusion was common, resulting in increased size and a decreased number of placentomes. Extensive areas of the chorioallantoic membrane were devoid of placentomes. An increased number of functional or accessory microcotyledons (<1 cm) were present at the maternally oriented surface of fetal membranes. Extensive areas of extravasated maternal blood were present within the placentomes and in the interplacentomal region. The crypts on the caruncular surface were dilated and accommodated complexes of more than one primary villus, as opposed to a single villus in non-cloned placentae. Scanning electron microscopy of blood vessel casts revealed that there was also more than one stem artery per villous tree and that the ramification of the vessels failed to form dense complexes of capillary loops and sinusoidal dilations as in normal pregnancies. At the materno-fetal interface, however, the trophoblast and uterine epithelium had normal histology. In conclusion, the NT placentas had a range of pathomorphological changes; this was likely associated with the poor clinical outcome of NT pregnancies.

Keywords: Cloning; Fetal membranes; Microvasculature; Placentation; Nucleus transfer

1. Introduction

Animal cloning is one of the most important biotechnical advances of recent years, and the results of somatic nucleus transfer (NT) in ruminants have served to clarify the genetic and epigenetic aspects of cloning [1]. However, NT can be an inefficient process, with a limited number of pregnancies reaching term [2],

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compared to pregnancies established by IVF [3]. In cattle studies, only 6% [4] or 8% [5] of the embryos transferred to recipient cows resulted in healthy, long-term viable calves. Large offspring syndrome is another feature of NT in cattle and sheep [6]. Moreover fetal loss sometimes involves death of the surrogate mother [7]. Finally, cloning by NT is a bio-medical tool with great potential for agricultural, economical and social impact but lacks regulatory and consumer acceptance [8,9].

As the main organ of materno-fetal interaction, the placenta plays a critical role in maintaining pregnancy [10–12]. Indications of placental failure in NT pregnancies include anomalies such as large offspring syndrome, altered placental and fetal membrane proteins, increased placental weight, and placentome enlargement and edema in cattle [3,6,13–18]; placentomegaly in the mouse [19]; and placental insufficiency in the sheep [20].

The objectives of the present study were to elucidate the macroscopic and microscopic morphology of the placenta in NT bovine pregnancies that went to full term. The study includes the umbilical cord, the number and structure of the placentomes and the interplacentomal areas of the fetal membranes. Within the placentomes, fetal villi interdigitate with the maternal caruncular crypts; their ramifications were described from vascular casts studied by scanning electron microscopy.

2. Materials and methods

Authorizations for this research were provided by the Veterinary Bioethics Committee of the School of Veterinary Medicine, University of Sao Paulo; UNESP, Jaboticabal; FZEA, Pirassununga; and EMPRAPA, Brasília.

2.1. Enucleation, nucleus transfer and culture

Cumulus oocyte complexes (COC) of cows (*Bos taurus* and *Bos indicus*) were aspirated from ovaries collected at an abatoir and matured *in vitro* in TCM 199 medium. After a maturation period of 18 h, cumulus cells were totally removed and oocytes with the first polar body were selected for enucleation. This procedure was conducted after aspirating the first polar body and surrounding cytoplasm with a pipette needle. Fibroblasts from adult male and female animals were collected from ear skin, cultured *in vitro* in Dulbecco's modified Eagles's medium (DMEM), and used as nucleus donors after synchronization for 5 days by serum starvation. Thirty minutes before nucleus transfer, skin cells were individualized by treatment with trypsin. A single fibroblast nucleus was then introduced into the perivitelline space of the enucleated oocyte followed by a single DC pulse of 1.5 kV/cm for 65 μs. Reconstructed zygotes were kept in synthetic oviductal fluid (SOF) medium [21] for 2 h before chemical activation with 5 mM ionomycin for 5 min and 6-dimethylaminopurine (6-DMAP) for 3 h. Activated zygotes were co-cultured with a layer of granulosa cells in 100 μL drops of SOF medium supplemented with 10% fetal calf serum (Gibco^{TM}, Invitrogen, Carlsbad, CA, USA) overlaid with mineral oil for 7 days. On day 7 after fusion, embryo quality was evaluated and grades 1 and 2 blastocysts were transferred to recipients. For further details of the protocols, see Mello et al. [22] and Yamazaki et al. [23].

2.2. Collection of material

Material was collected from 19 pregnancies (*Bos taurus* three, *Bos indicus* 12 and two cross breed) established by NT. Details are given in Table 1. In 15 cases, the calf was delivered by Cesarian section. During this procedure, one to four placentomes with adjacing interplacentomal tissue were removed from the uterus (eight cases) using clamps and a knife for histological examination (see below). In a subset of seven pregnancies, the cow was slaughtered following delivery of the calf by cesarian section and then the uterine horns were opened, injected with fixative, and the fetal membranes inspected. Nude or cotyledon-free areas of chorioallantoic membrane were recorded. Then the uterus was totally everted to register the size, shape and distribution of the placentomes. As controls, pregnant uteri were obtained from 12 animals at the slaughterhouse. Only pregnancies judged to be near term (>210 days, according to crown-rump-length) were included as controls [24].

2.3. Light and scanning electron microscopy

For light microscopy, placentomes were perfused through chorioallantoic blood vessels with 4% buffered paraformaldehyde, processed by standard procedures and embedded in Paraplast. Sections were cut at 5 μm and stained with hematoxylin and eosin, Van Gieson, and picrosirius [25]. The PAS reaction was applied as a histochemical method for detection of glycosaminoglycans. For the study of semithin sections prepared by routine methods, fragments of placentomes were fixed in 2.5% glutaraldehyde, 0.1 M PBS, pH 7.4 [26]. Selected tissue samples were washed in 0.1 M PBS

Table 1
Pregnancy outcome and placental material examination at term in 19 bovine pregnancies established by somatic nucleus transfer

Cloned calf (institutes)	Sex	Delivery	Parturition (day/month/year)	Birth weight (kg)	Length of gestation (d)	Outcome	Material collected	Observations
1. Penta (UNESP)	Female[a]	Caesarian section	11/07/2002	42	294	Survived 30 days	Four placentomes and intercotyledonary area	Increased amount of Wharton's jelly and vessel enlargement due to edema
2. Marcolino (USP)	Male[b]	Caesarian section	29/04/2003	34	290	Survived	One placentome	Enlargement of umbilical cord
3. Mortolino I (USP)	Male[b]	Mother died with intrauterine fetus	28/12/2003	32	229	Intrauterine fetal death at 7 months (concurrent with maternal death)	Uterus and placenta	Umbilical cord pathology and hydroallantois
4. Mortolino II (USP)	Male[b]	Mother died with intrauterine fetus	20/01/2004	30	252	Intrauterine fetal death at 8 months (concurrent with maternal death)	No material collected	Edema of umbilical cord but with normal vessel lumina
5. Bela Viva (USP)	Female[a]	Caesarian section	15/04/2003	41	290	Survived	Four placentomes	Umbilical cord pathology and edema of amnion
6. Bela Morta (USP)	Female[a]	Caesarian section	16/04/2003	40	260	Stillborn	Two placentomes and delivered placenta	Umbilical cord pathology
7. Lenda (EMBRAPA)	Female[c]	Normal parturition	04/11/2003	45	287	Survived	Samples of placentomes	Normal cord
8. Vitoria III (EMBRAPA)	Female[d]	Dystocia	03/02/2004	66	305	Survived 1 day	Samples of placentomes	Umbilical cord pathology
9. Vitoriosa (EMBRAPA)	Female[d]	Caesarian section	05/02/2004	62	294	Survived 45 days	Samples of placentomes	Umbilical cord pathology
10. Independência (FZEA)	Female[a]	Caesarian section	07/09/2004	37	283	Survived 15 days	Uterus and placenta	Umbilical cord pathology (edema and enlargement of vessel lumina)
11. Clone 3.1 (FZEA)	Male[a]	Caesarian section	04/03/2005	51	290	Survived 3 days	Two placentomes and interplacentomal region	Umbilical cord pathology
12. Clone 3.3 (FZEA)	Male[a]	Caesarian section	08/03/2005	37	290	Survived	Uterus and placenta	Umbilical cord pathology
13. Clone 3.4 (FZEA)	Male[a]	Caesarian section	12/03/2005	14	292	Survived 7 hours	Three placentomes and nude area	Normal umbilical cord but increased amount of Wharton's jelly at the junction between cord and placenta
14. Clone 3.5 (FZEA)	Male[a]	Caesarian section	19/03/2005	57	289	Stillborn	Four placentomes (three large and one very small)	had abdominal dilatation and calf had a normal cord
15. Clone 3.6 (FZEA)	Male[a]	Caesarian section	20/03/2005	35	293	Survived	Samples of placentomes and interplacentomal region	Umbilical cord pathology
16. Clone 4.1 (FZEA)	Male[a]	Caesarian section	12/04/2005	55	290	Survived	Samples of placentomes and interplacentomal region	Umbilical cord pathology
17. Clone 4.2 (FZEA)	Male[a]	Caesarian section	13/04/2005	51	290	Survived	Uterus and placenta	Umbilical cord pathology
18. Clone 2.1 (FZEA)	Female[a]	Caesarian section	28/07/2005	37	289	Survived 2 days	Uterus and placenta	Umbilical cord pathology
19. Clone 8.1 (FZEA)	Female[a]	Caesarian section	20/02/2006	40	290	Survived	Samples of placentomes and interplacentomal region	Umbilical cord pathology

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[a] Nellore.

[b] Cross breed (*Bos indicus* × *Bos taurus*).

[c] Holstein Friesian.

[d] Simmental.

(pH 7.4) and postfixed in 1% osmium tetroxide solution (Polysciences Inc., Warrington, Pennsylvania, USA) for 1 h. After serial washes, the samples were dehydrated in ethanol (70–100%) and propylene oxide (EM Grade, Polysciences Inc.). They were kept under gentle agitation in a solution of 1:1 propylene oxide and Spurr's resin for 12–16 h, before being embedded in pure Spurr's resin for 4–5 h, and dried at 60 °C for 72 h. Semithin sections (1 μm) were made on an ultramicrotome (Leica Ultracut UCT) and stained with toluidine blue.

For analysis of the microvasculature by scanning electron microscopy, placentomes and interplacentomal areas were injected through chorioallantoic vessels with Mercox under controlled manual pressure (Mercox® CL-2R, Vilene, Tokyo, Japan). Tissue samples were immersed in 20% sodium hydroxide solution at 50 °C, washed for 24 h, dried at 36 °C, sputter-coated with gold (EmiTech K550), and observed with the scanning electron microscope (Leo 435 VP). For further details of vessel corrosion casting, see Leiser and Pfarrer [27].

3. Results

3.1. Umbilical cord

The umbilical cords of 17 of the 19 NT calves had a greatly enlarged allantoic duct, in comparison to control calves, and an increased amount of Wharton's jelly, resulting from gross edema (Table 1). As exemplified in *B. indicus* (Fig. 1A), these phenomena were shown in a section of umbilicus located 10 cm apart from the transition of the umbilicus to the skin (Figs. 1B and 2B, D and F) with corresponding controls (Fig. 2A, C and E). In the allantoic duct, this edema infiltrated the wall, enlarging particularly the mucosa and adventitia (Fig. 2F). In the two arteries and two veins, the longitudinal folds of vessel wall, which normally project into the lumen, were smoothed out in the NT cord and the edema penetrated the outer or adventitial layer (Fig. 2A–D). The muscular layer of all umbilical blood vessels was thicker in NT animals (Fig. 2A–D).

3.2. Fetal membranes

Macroscopically, the fetal membranes were edematous in all 19 cloned placentas, giving them a gelatinous appearance (Fig. 3A and B). In places, the edema was so pronounced that it obscured the placentomes from view on the fetal side of the placenta.

Seepage of maternal blood into spaces between the chorion and septal tips of caruncles near the base of the

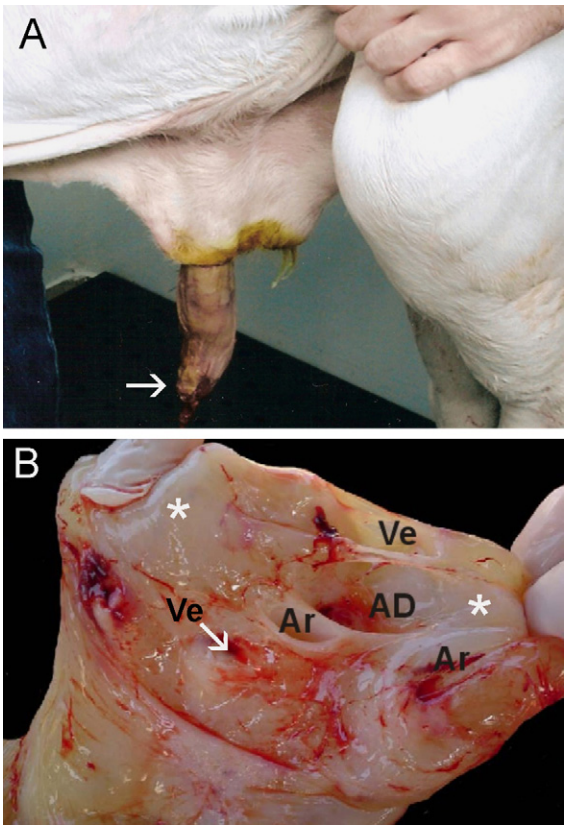

Fig. 1. Umbilical cord of just born NT calf (Bos indicus: Independência). (A) The umbilicus was cut approximately 10 cm from the transition of umbilicus to the skin (arrow). (B) The corresponding umbilical cross-section with enlarged arteries (Ar) and veins (Ve) in pairs as well as the allantoic duct (AD) being distinctly stretched (*) by the fingers of the assistant. The Wharton's jelly around these vessels was swollen due to edema.

villi was a normal feature during the second half of bovine control pregnancy (Fig. 4) [28]. In these hemophagous regions or hematomes, which, on the fetal side of placentome, macroscopically appeared as a distinctly undulated rim (Fig. 4A) or in cross-section as punctuated areas (Fig. 4B), some maternal erythrocytes were hemolysed (Fig. 4C) and/or phagocytosed by columnar trophoblast cells (Fig. 4D). In NT placentas, however, there was much more extensive extravasation of maternal blood into these areas (Fig. 5A–D). Moreover, at section, extravasation was deeper in the placentome (Fig. 5B and C). Histological section confirmed that large areas of blood extravasation occurred under the edematous allantochorion and also around the stem villi (Fig. 5C and D). Extravasation could also generally been seen from the chorionic side in intercotyledonary areas (Fig. 5E).

The size of placental cotyledons was very variable in NT placentas. Data for 12 control placentas and a subset

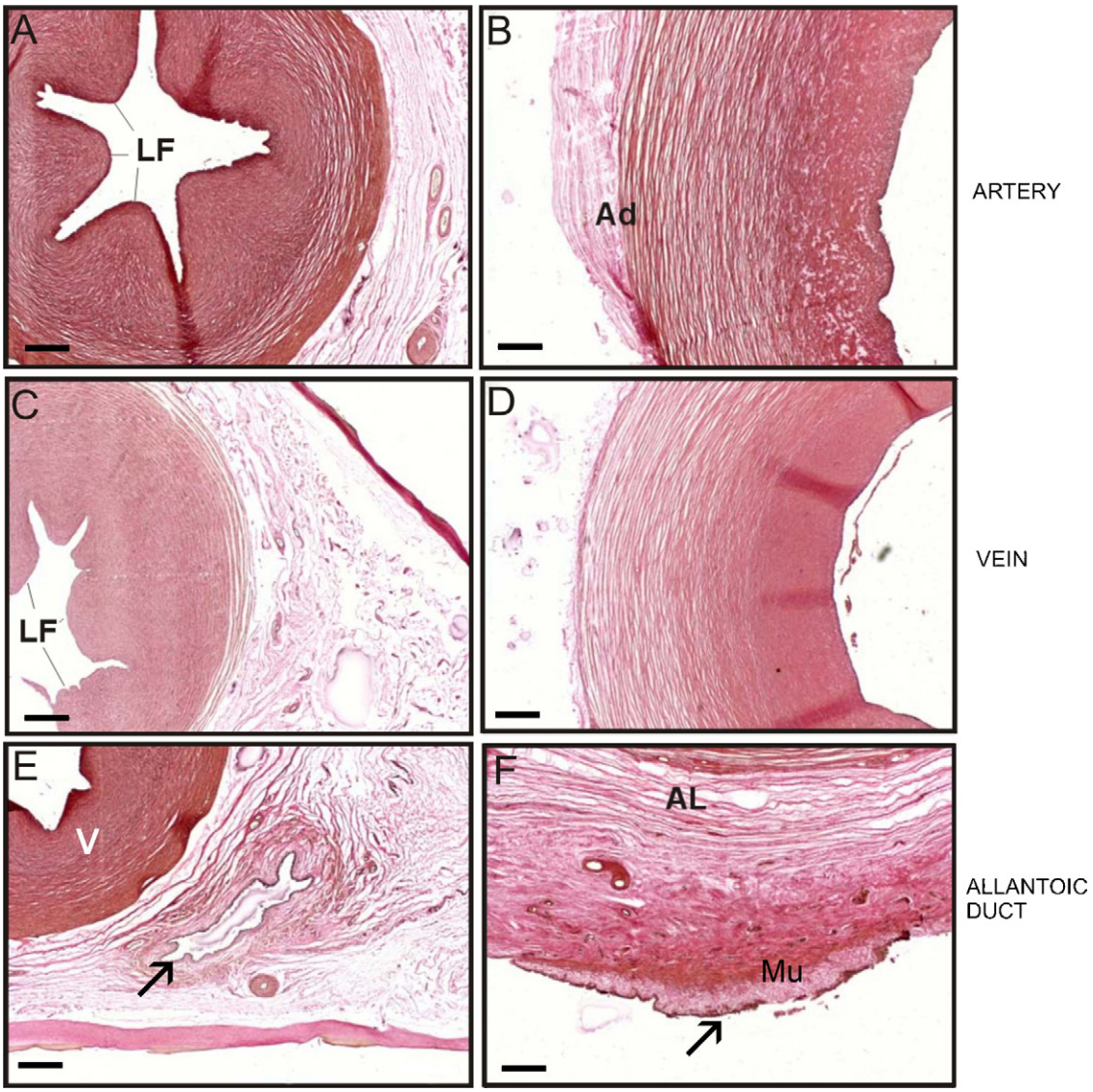

Fig. 2. Comparison of umbilical cord histology in control (A, C and E; left) and term NT pregnancy (B, D and F; right). The luminal diameter of the umbilical artery (A and B) and vein (C and D) was greatly increased, resulting in smoothing of the luminal folds (LF) of the walls. Edema penetrated the adventitial vessel layer (Ad), particularly in the artery. The allantoic duct (E and F) was dilated and the mucosal (Mu) and adventitial layers (AL) were edematous. Arrows: allantoic epithelium; V: vein. Embedded in Paraplast, Van Gieson stain. In all panels, scale bar = 500 μm.

of eight NT placentas are shown (Table 2). The cotyledons were arbitrarily divided according to diameter into very large (>11 cm), large (6–11 cm), medium (3–6 cm), small (1–3 cm) and very small (<1 cm); the latter were regarded as accessory placentomes (see below). There were two primary differences between NT and control placentas. Firstly, one or more very large cotyledons or placentomes occurred in all eight NT placentas (Fig. 6A and Table 2) but in only one control placenta. Close inspection of the form and arrangement of the placentomes revealed that these cotyledons probably had formed by amalgamation of several smaller ones; this probably resulted from extreme growth of the placentome margins, leading to fusion of several caruncles (Fig. 6B). Furthermore, extensive areas were devoid of placentomes, resulting in an increase in the interplacentomal part of the fetal membranes in NT cattle (Fig. 6C). This did not occur in control cattle. Secondly, very small cotyledons (<1 cm) were found in all eight NT fetal membranes. When accessory placentomes occurred in control animals, they normally were few in number and formed a cluster

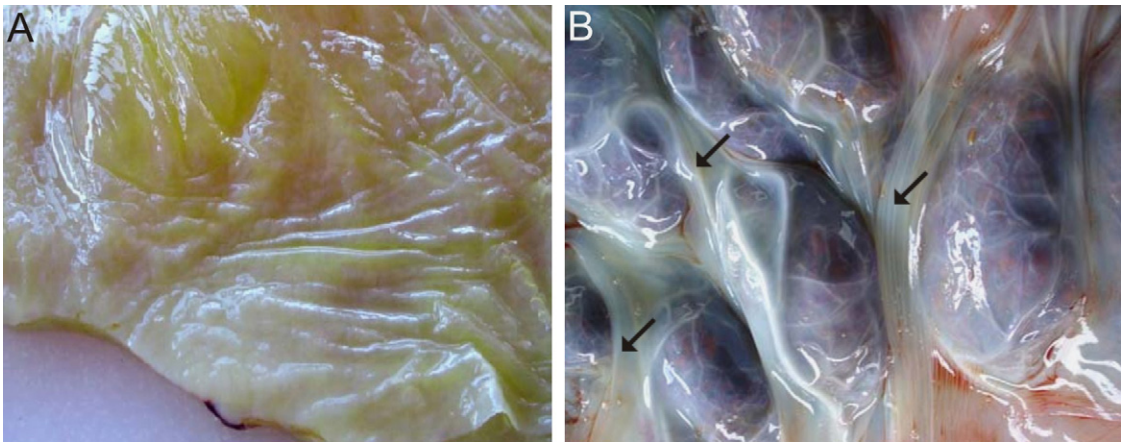

Fig. 3. Fetal membranes in NT pregnancies of cattle at term seen from the allantoic side. (A) The membranes were highly edematous, giving them a gelatinous appearance. (B) Chorioallantoic membrane with edema (arrows) that was particularly pronounced between the placentomes.

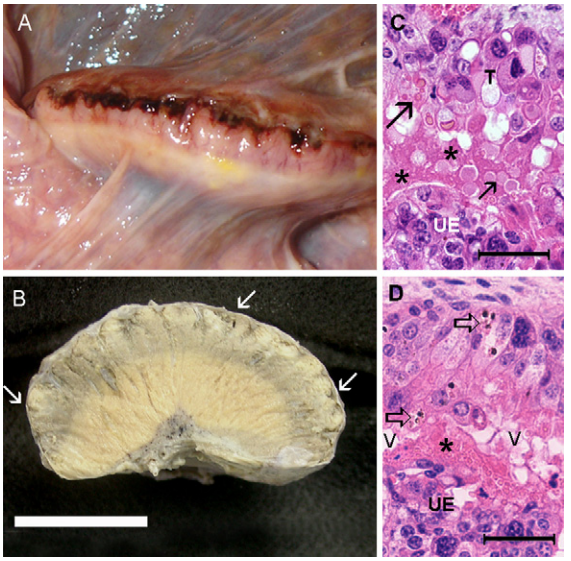

Fig. 4. Extravasation of maternal blood in term placentas of control cattle. (A) Overview of unfixed placentome with a thin and slightly undulating rim of extravasation of blood on the fetal side. B: Transverse section of paraformaldehyde fixed placentome showing punctuate extravasation of blood beneath the fetal surface (arrows). (C) Small hematomal area of control at early extravasation stage of maternal blood located between septal uterine epithelium (UE) and trophoblast (T). The erythrocytes extravasated from the uterine epithelium in part are normally sized (*) in part were increased by beginning hemolysis (small arrow). Irregular cuboidal trophoblast cells contain a few erythrophagocytosis (large arrow). (D) In comparison to C, a temporally advanced development stage of hematoma is shown. Totally hemolysed erythrocytes, appearing as vacuoles (V), intermingled with blood (*) which has recently moved (by diapedesis) from the uterine epithelium (UE). Some columnar trophoblast cells contained hemosiderin as residues (arrows) from earlier erythrophagocytosis (scale bars = 40 μm).

in association with a single caruncle. In cloned placentas, however, they were isolated and each was associated with a single small caruncle (Fig. 6D). The total number of cotyledons (Table 2) was smaller in NT placentas than in control placentas.

The materno-fetal interface was revealed by manual separation of the cotyledon from the caruncle (Fig. 7A and B). In comparison with controls, the caruncular crypts of NT cattle were greatly extended and contained remnants of fetal tissue (Fig. 7B). In control placentas, usually one primary villus of the cotyledon occupied each primary crypt of the caruncle and the two separated without rupture of the villus. In NT placentas, however, several primary villi occupied each crypt and there was no longer clean separation of the fetal tissue. This interpretation was supported by corrosion casts of the blood vessels, in which the subepithelial capillary network mirrored the three-dimensional form of the villus (Fig. 8). The villi of controls were slim and long-tipped (Fig. 8A and B). In contrast, in NT placentas, the base of the villous complex was broad and several irregularly shaped primary villi descended from it, well separated from each other and ending in distinct tips (Fig. 8C and D).

Light microscopy of the materno-fetal interface (Fig. 9A and B) revealed no perceptible difference between control and NT placentas. On the fetal side, the cotyledonary trophoblast was typically of uninuclear, cuboidal to flat cells in contact with the basal membrane of the epithelium, whereas the globular giant cells, usually with two nuclei, were detached from the basal membrane. On the maternal side of the interface, trinuclear cells were found within the uterine epithelium. These cells are formed following migration of the trophoblast giant cells and their fusion and hybridization

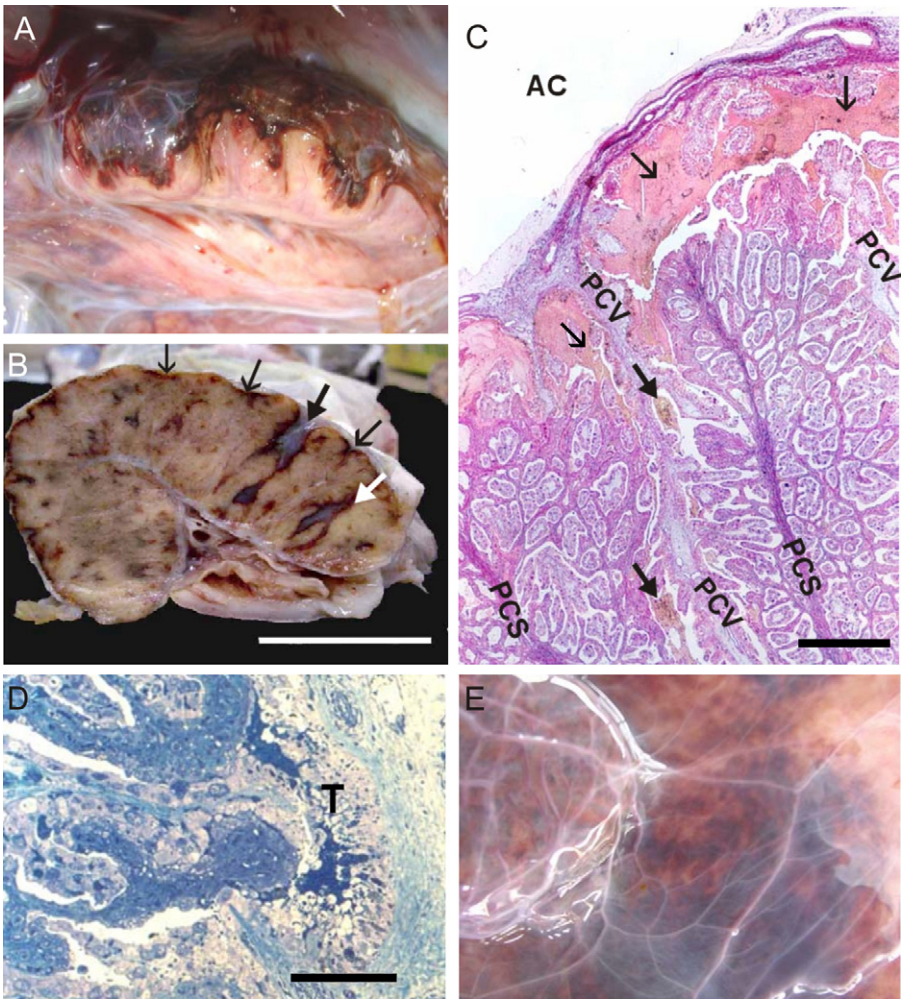

Fig. 5. Extravasation of maternal blood in placentas of NT pregnancies of cattle at term (as compared to normal placentas shown in Fig. 4). (A) Overview of unfixed placentome with extensive extravasation of blood on the lobulated fetal side. (B) Transverse section of mushroom-shaped placentome with extravasation of blood, not only along the rim of the chorionic plate (small arrows; as in controls), but also in the middle parts of the placentome (large arrows). Scale bar: 3 cm. (C) Histological section taken from B. Besides very distinct inclusions of extravasated blood situated along the placentomal chorionic plate (small arrows), hematomal areas occurred in the middle part of the placentome (large arrows). AC: allantoic cavity, PCV: primary cotyledonary villi, PCS: primary caruncular septa. Picrosirius, scale bar = 500 μm. (D) Semithin section of placentome showing increased extravasation of maternal blood, which was located between the dark septal epithelium of the caruncle (left) and the clear columnar trophoblast of the cotyledon (right). The trophoblast (T) was hemophagous, as indicated by its content of erythrocytes. Toluidine blue (scale bar = 160 μm). (E) Extravasation of brownish blood into the interplacentomal area, seen from the allantoic cavity through the allantochorion.

with uterine epithelial cells [29]. Trophoblastic giant cells were best visualized with the PAS reaction for glycoproteins (Fig. 9C); the villous connective tissue was clear with relatively large, thin-walled blood vessels, but the caruncular septal connective tissue was dark and rich in the collagen fibres that provided the structural integrity of the placentome.

The accessory placentomes formed in NT placentas contained all the above components, suggesting, at least from a histological standpoint, that they were functional (Fig. 9C).

4. Discussion

The high rate of pregnancy loss following somatic nucleus transfer in cattle is associated with placental anomalies [4,7]. In the present study we examined placentas from 19 NT pregnancies from which two were aborted, two cows died with a fetus, and 15 were carried to term by comparing them with normal term placentas. Features have been associated with pregnancy loss or limited survival time of the calves after birth by deficiencies such as a reduction in placentome number,

Table 2
Size and number of placental cotyledons per fetal membrane of bovine NT pregnancies

	Very large (>11 cm)	Large (6–11 cm)	Medium (3–6 cm)	Small (1–3 cm)	Subtotal	Accessory placentome (<1 cm)	Total
Control pregnancies							
1	0	33	22	2	57	6	63
2	0	22	55	8	85	4	89
3	0	54	35	12	101	4	105
4	0	40	12	8	60	3	63
5	0	37	48	13	98	10	108
6	0	15	74	20	109	0	109
7	0	19	44	6	69	17	86
8	0	40	60	29	129	1	130
9	0	21	65	46	132	36	168
10	0	22	65	29	116	13	129
11	0	22	14	3	39	4	43
12	4	14	10	19	47	8	55
Mean	0.3	28.3	42.0	16.3	86.8	8.8	95.7
S.D.	1.2	12.3	22.9	13.1	32.0	9.9	36.5
NT pregnancies							
Mortolino I	2	26	13	5	46	38	84
Vitoriosa	5	30	30	16	81	56	137
Vitoria III	3	37	23	5	68	47	115
Independencia	5	39	47	5	96	24	120
Clone 3.3	2	42	22	2	68	1	69
Clone 4.2	11	13	6	1	31	51	82
Clone 2.1	1	28	17	8	54	5	59
Clone 8.1	15	5	0	0	20	0	20
Mean	5.5	27.5	19.8	5.3	58.0	27.8	85.8
S.D.	5.0	12.9	14.6	5.1	25.4	23.4	37.8

increased placentome size and edema of the fetal membranes. Together with cord anomalies, they likely contributed to the high perinatal and postnatal morbidity associated with NT pregnancies. However we are unable to speculate what kind of deficiencies of the placenta or the uterus ultimately contributed to calve's limited viability.

The analysis of the bovine cloned placenta proteins showed an over expression of TIMP-2, which may cause abnormalities such as enlarged and dysfunctional placentas [30]. Also, the gene expression in trophoblast of cloned placentas have alterations as inappropriate major histocompatibility complex (MHC) expression [31,32] which when aberrant, cause immune-mediated abortion [33]. Hitherto scant attention has been paid to the structural correlates of the reduction in placentome number and increase in placentome size [11,12]. This was a focus of the present study. We observed disruptions in maternal–fetal interactions ranging from apparent loss or amalgamation of uterine caruncles to inappropriate relations between the maternal and fetal components of the placentome that negatively impacted on the fetal vasculature. These findings can likely be extrapolated to the large number of pregnancies that end between the second trimester and term [7]. In contrast, the histology of the exchange areas, including the binucleate trophoblast cells, was similar in NT and control pregnancies, because the tissue samples taken for the NT could not exclude injured areas like extended hematomic formations and materno-fetal detachment. Therefore, this might explain why this subset of pregnancies was able to continue to term.

4.1. Placentome number and size

Failure to form a placenta or a reduction in placentome number may be a factor in early pregnancy loss in cattle [34] and sheep [20,35]. In cattle, placentas from second trimester abortions often have a reduced number of placentomes. However, in NT pregnancies that survived until 150 days of gestation, placentome number was similar to that in controls [34]. Moreover these authors found an increase in the total weight of the caruncles, which form the maternal component of the placentome [34]. In our material from term pregnancies, the number of placentomes with a diameter >1 cm was

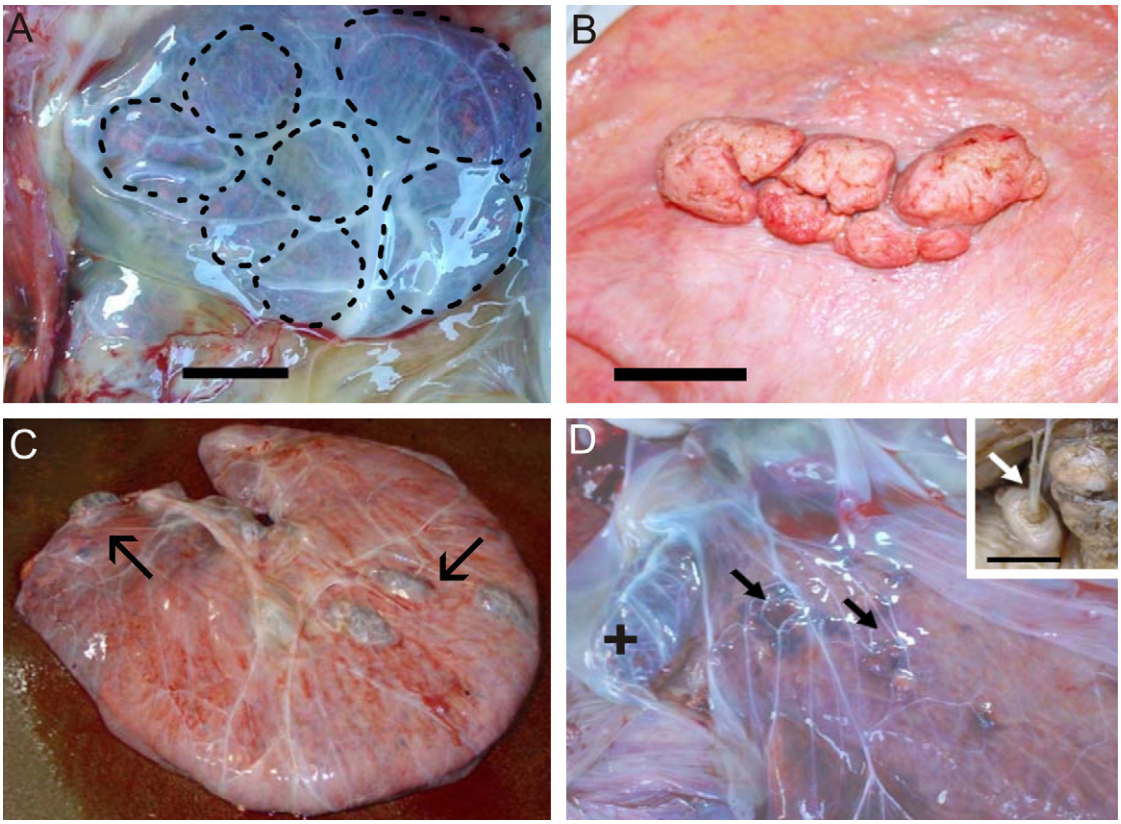

Fig. 6. Abnormal placentation in NT pregnancies of cattle at term. (A) This very large placentome may have formed by the amalgamation of several smaller ones, as indicated by the broken lines. Scale bar: 4 cm. (B) Aggregation of several small uterine caruncles which in part are separated by grooves. Scale bar: 2 cm. (C) Only very few cotyledons can be seen grouped (arrows) on the extended interplacentomal area of the manually everted sac of fetal membranes. (D) Very small or accessory placentomes (arrows) besides normal placentomes (+) are common in NT pregnancies. Inset shows an accessory placentome supplied by a fetal vessel (arrow). Detail figure scale bar = 1 cm.

significantly different between NT pregnancies and controls. However there were significantly more very large placentomes with a diameter >11 cm. Compensatory overgrowth of individual placentomes has been reported in early [2,14] and late [34] NT pregnancies where the number of placentomes was reduced. Phenomena which were also observed near term in our research. On closer examination, these placentomes were apparently formed by aggregation of several normal sized placentomes, in a process that appeared to involve amalgamation of the caruncles in part yet visible by grooves. This interpretation was supported by the occurrence of areas that were completely devoid of caruncles or placentomes. This was very different from the orderly arrangement of placentomes that is seen in normal pregnancy [36,37].

A second difference between NT and control pregnancies was the frequent occurrence of accessory placentomes with a diameter <1 cm. Those have been reported before, but were regarded as non-functional [15]. In contrast, based on our histological analysis, these tiny placentomes were functional. When they were included in the total, there was a significant 86% increase in placentome number in NT pregnancies at term compared to controls. We suggest that these accessory placentomes reflect an adaptation that contributed to the continuation of these pregnancies to term.

4.2. Edema of membranes and cord

Edematous and gelatinous membranes have been reported previously in NT pregnancies [13,34] and this was a consistent finding in our study. We also saw gross edema of the umbilical cord with enlargement of the allantoic duct and umbilical vessels. These findings reflected a disturbance in fluid homeostasis and were likely related to the fetal hydrops and hydroallantois that are the main cause of NT pregnancy loss in the third trimester [2,7,34]. Normally there is no difficulty in clamping the cord of newborn calves [38]. However the

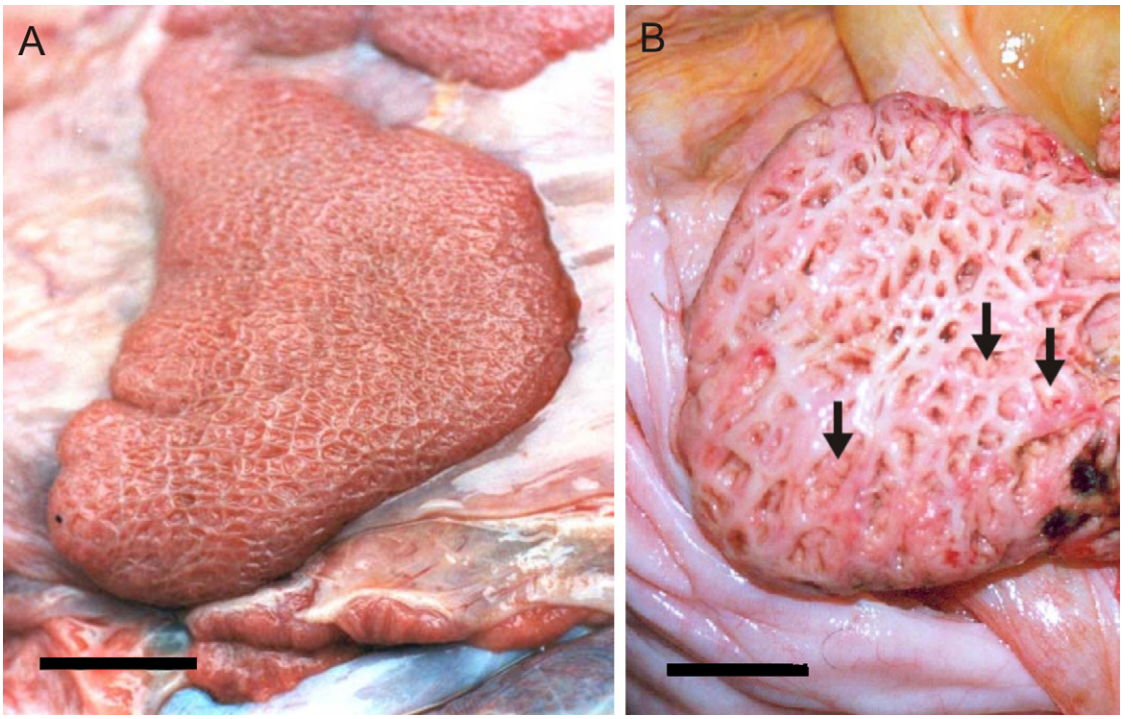

Fig. 7. Caruncular portion of the placentome in cattle at term. (A) Control pregnancy showing a caruncle with crypts of normal size. (B) Caruncle from an NT pregnancy with extensively enlarged crypts which, following separation of the fetal membrane from the uterus, were partly filled by ruptured chorionic villi (arrows). Scale bar A and B = 2 cm.

swollen cords of NT calves were difficult to close and represented a potential route of infection that may contribute to postnatal morbidity [4,7]. In addition to edema, we also noted blood extravasation in the fetal membranes and cotyledons. This is mentioned in only a few previous reports [14].

4.3. Vascular morphology

Not only were the caruncles larger in NT pregnancies [34], we found that the crypts were wider than normal. Perhaps as a result, there was disruption of the normal pattern of placentome formation, where each crypt was occupied by a single branching villus. It was apparent from vascular casts that a single crypt could be occupied by two or more fetal villi; this overcrowding resulted in reduced branching of the villi. To our knowledge, this has not been described earlier. It is important in that it suggests that placental function may be severely affected, even when the number of placentomes is normal.

4.4. Histology, including binucleate cells

In NT placentas in the present study, the histology of the maternal–fetal interface was deemed as normal, consistent with pregnancies carried to term. There apparently was a sufficiently large exchange area to enable adequate transfer of respiratory gases and nutrients. The recent histomorphostereological study of Constant et al. [39] clearly showed that the very moderate alterations of NT placentomes in relation to control, such as thinning of maternal epithelium, increased trophoblast surface and enlargement of fetal connective tissue, can not be the primary defaults of third-trimester NT placentas.

In contrast to cattle, major causes of placental abnormality and dysfunction sheep placentas were distinct reduction of villous vascularization and hypoplasia of trophoblast with inclusion of the giant cells [40,41]. Histological normality in NT placenta, as seen in the bovine here, does not exclude that there is aberrant molecular biology, as shown with an abnormal profile in proteomics [30].

There also appeared to be normal numbers of binucleate trophoblast cells (BNC). Pregnancy serum protein 60 (PSP60) is secreted by these cells and used as a routine pregnancy test. It was noteworthy that significantly higher concentrations of PSP60 were measured in the blood of recipients of NT embryos that subsequently lost their pregnancy [2].

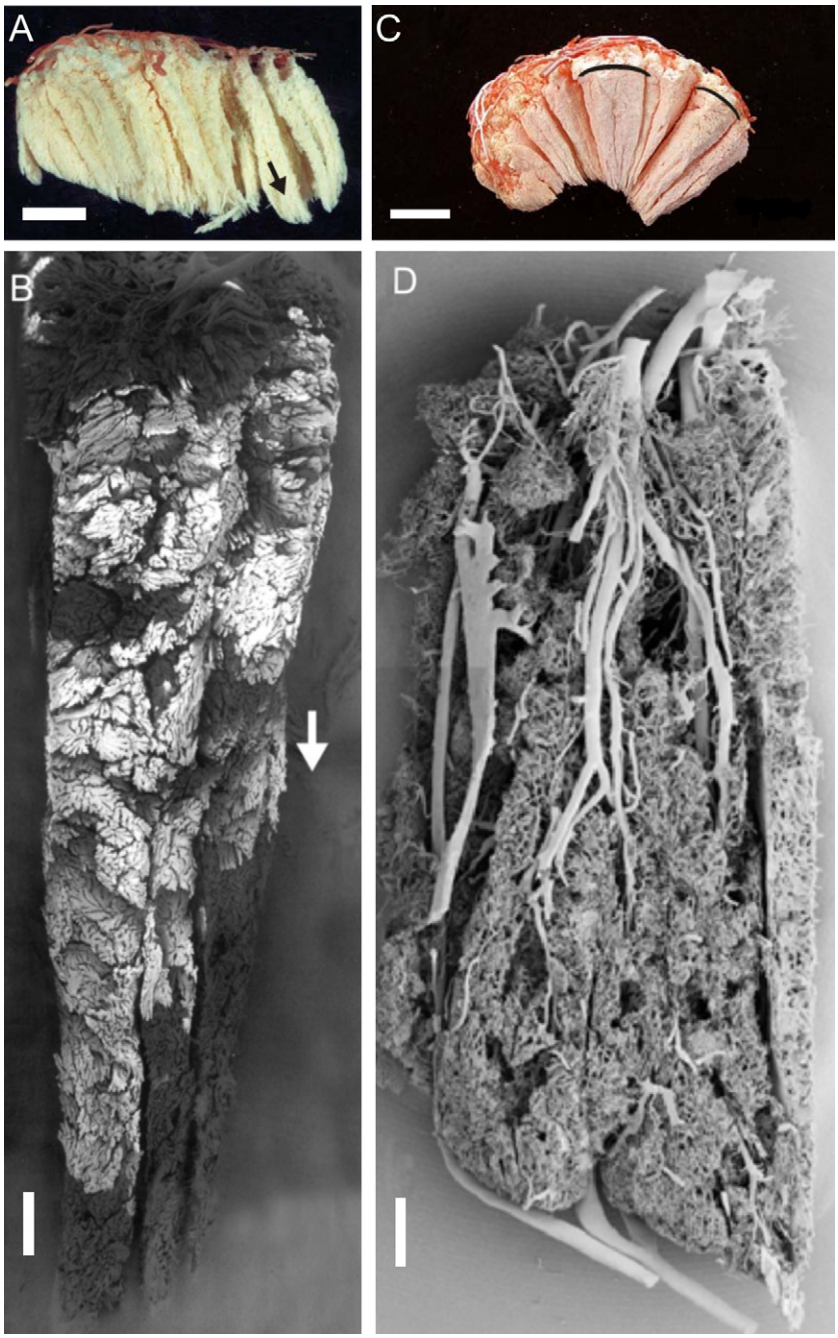

Fig. 8. Fetal microvasculature of bovine placenta at term, shown by corrosion casting of blood vessels. (A and B) Control: low magnification (A) shows long and slim villous trees or primary villi. When magnified by scanning electron microscopy (B), these formed cone-like entities strictly oriented in a feto-maternal direction (arrow). (C and D) NT pregnancy: low magnification (C) shows broadly based primary villi complexes (brackets) which were rather clumpy and short. When magnified by scanning electron microscopy (D), broad vascular complexes formed a base from which villous trees of differing length arose to pass in a feto-maternal direction, well separated from each other. Scale bars B and D = 1 mm; A and C = 8 mm.

4.5. Findings in relation to pregnancy loss

The low success rate of NT was attributed to five periods of loss [7]; those that concern us here are losses in the third trimester and postnatal deaths. The main cause of third trimester losses was hydroallantois and fetal hydrops, usually attributed to inadequate placentation [7]. We studied pregnancies that survived to term,

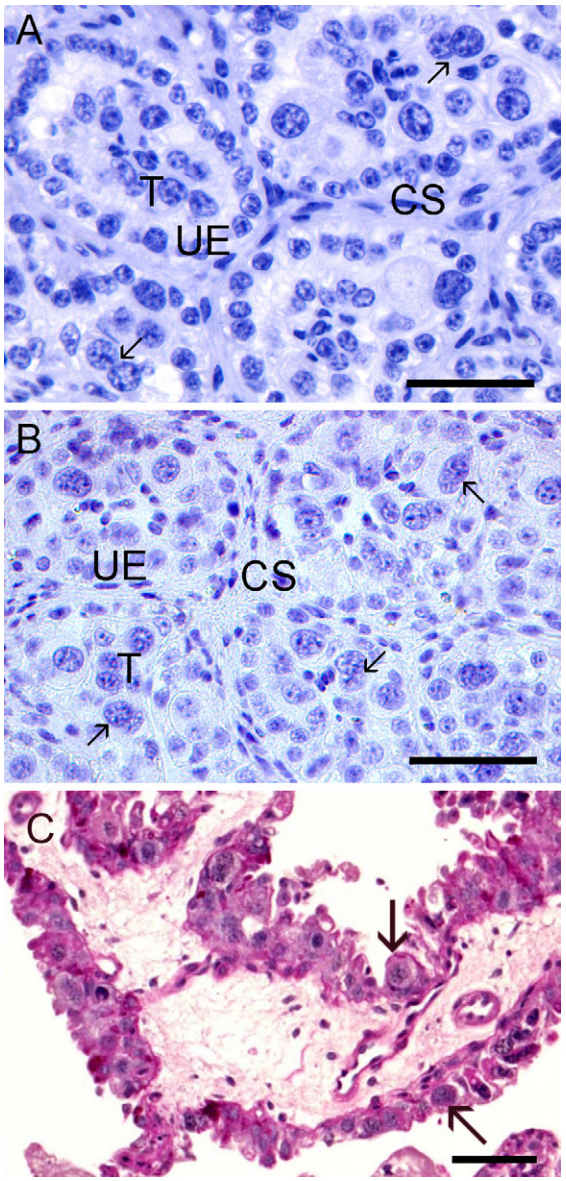

Fig. 9. Light microscopy of materno-fetal interface of cattle near term. Paraformaldehyde fixed sections of placentomes from control (A) and NT (B) pregnancies show similar histology. Tertiary fetal villi of the cotyledon form islands inside the coarse network of caruncular septa (CS). The villi have a clear stroma bordered by trophoblast (T) which besides uninucleate clear cells, shows darker binucleate or giant cells (arrows), whereas the septa generally are rather dark with a cuboidal uterine epithelium (UE). Toluidine blue staining. Scale bar 100 μm. (C) Histological PAS reaction of trophoblast in an accessory cotyledon from an NT pregnancy. Global binucleate cells, interspersed between uninucleate cells, can be seen in the trophoblast layer (arrows); an observation which suggests normal function of this epithelium. Embedded in Paraplast. Scale bar = 50 μm.

yet they displayed similar anomalies including edematous fetal membranes and marked enlargement of some cotyledons. Therefore this subset of pregnancies seems to form part of a continuum. It is important to note that there was no reduction in placentome number. On the contrary, placentome number was often supplemented by many small cotyledons <1 cm in diameter. Histological analysis indicated that they were functional (as previously discussed). The large and normally sized cotyledons had deranged vascular structure, but normal histology at the materno-fetal interface.

There are several causes of perinatal and postnatal demise, but enlarged umbilical veins and arteries, as observed here, have been implicated in loss of calves caused by sepsis in umbilical structures [4,7].

The underlying causes of these defects in placentation are thought to be epigenetic. The clearest evidence for this is that placental abnormalities do not occur in the offspring of clones [4]. Some may arise from the in vitro procedures alone. Differences in placentome size and number were associated with in vitro produced embryos (without nucleus transfer) and may contribute to large calf syndrome [3,16]. However, defective imprinting of genes following transfer of a somatic cell nucleus to the oocyte is thought to be the main explanation for the subsequent failures in placentation.

In summary, although some macroscopic features observed in tissues from clone-derived term pregnancies in our study were in agreement with findings from other reports, important novel pieces of information regarding the placental morphology and tissue microarchitecture were revealed. The majority of the cloned calves had enlarged umbilical cords (a common occurrence after cloning by NT); this was characterized by an increase in allantoic duct size and in thickness of both the duct and the associated blood vessel walls, which, in turn, appeared to be caused by both excessive tissue growth and edema. The presence of very small and very large functional placentomes in clones was associated with broad areas lacking in placentation, likely formed by the amalgamation of smaller placentomes and by the fusion of nearby caruncles. Interestingly, caruncular crypts in clones were distended, harboring more than one irregularly shaped primary villous, with more than one stem artery per villous tree and decreased capillary loops and sinusoidal dilations. In addition, postnatal survival was substantially reduced in cloned calves. Collectively, the range in placental abnormalities and related tissues from cloned-derived term pregnancies here the first time are shown in the context of clinical, macroscopical and histological aspects, providing support to the concept

that such anomalies have a direct effect on placental function, which may be an impact on fetal growth, development, and postnatal survival.

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