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REGULAR ARTICLE

Placental microstructure and efficiency in cloned bovines: a design-based stereological approach

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Abstract Somatic nuclear transfer allows scientists to produce identical copies of individuals (clones) and offers the potential for research in various fields. However, the production of cloned bovines is inefficient, partly because of high rates of embryonic mortality associated with abnormal placental development, although the exact mechanisms are still unclear. In the present study, we have applied design-based stereological methods to quantify placental morphology in cloned and non-cloned Nelore bovines (280–297 days of gestation). We have found no differences for any of the following parameters: number and volume of placentomes, villous volume and surface area, volume and number of star volume units and placental efficiency. Therefore, placental deficiency is unlikely to be attributable to its villous component. Nevertheless, new studies should focus on other placental constituents, e.g. capillaries, trophoblast cells and feto-maternal exchange surface area.

Keywords Clones · Fetus · Placenta · Stereology · Bovine

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Introduction

Nuclear transfer biotechnology or embryonic reconstitution allows scientists to produce identical copies of individuals (clones) and this technology offers the potential to stimulate research in various fields (Gonçalves et al. 2002). However, the production of cloned bovines is relatively inefficient partly because of high rates of embryonic mortality (Hill et al. 2000).

Embryonic mortality has been observed at both the beginning and end of gestation and is normally accompanied by fetal malformation, birth mortality, abnormalities in placentome development, haemorrhagic cotyledons, hydroallantois and oedema (Hill et al. 2001). More recently, studies have demonstrated that these losses and alterations are associated with abnormal placental development, although the placental components affected have not always been identified (Hill et al. 2001; Schlafer et al. 2000).

As in other mammals, the fetal villi in human placentas are responsible for the transport and exchange of nutrients

from the mother to the fetus and their formation is an important factor in normal fetal development and wellbeing (Mayhew 2002). Thus, a quantitative approach for studying fetal villi is important in determining whether structural and functional alterations that might compromise placental efficiency occur in cloned animals. Although several morphometric studies have been carried out on villi in human placentas (Mayhew 1990, 2001; Mayhew and Wadrop 1994), little information is available for large mammals such as bovines.

In the present study, we have applied stereological sampling and estimation tools to quantify placental morphology in cloned and non-cloned bovines. In particular, estimates are made of the sizes and numbers of villi and these are used to provide surrogate estimates of placental efficiency expressed per kilogram of fetal weight.

Materials and methods

Four placentas of somatic nuclear transfer (SNT) cloned Nelore bovines (*Bos indicus*; between 280 and 297 days of gestation) and four placentas of non-cloned Nelore bovines (between 284 and 294 days of gestation) were investigated. Animals were obtained from the Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, Brazil. Non-cloned animals were from in-vivo-produced embryos after natural gestation. All placentas came from non-problematic pregnancies. The whole experimental procedure was approved by the Animal Care Commission of the College of Veterinary Medicine and Animal Science, University of São Paulo (application number: 04/04045–0)

SNT process

SNT was performed by Prof. Flávio Vieira Meirelles from the Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos in Pirassununga, São Paulo, Brazil. The full protocol is described elsewhere (Miglino et al. 2007). Briefly, cumulus oocyte complexes of cows were aspirated from ovaries collected in an abattoir and matured in vitro in fresh TCM 199 medium. After a maturation period of 18 h, cumulus cells were removed and first polar body oocytes 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 Eagle's medium and used as nucleus donors after synchronisation for 5 days by serum starvation. At 30 min before nucleus transfer, skin cells were individualised 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 ms. Reconstructed zygotes were kept in synthetic oviductal fluid (SOF) medium for 2 h before chemical activation with 5 mM ionomycin for 5 min and with 6-dimethylaminopurine (6-DMAP) for 3 h. Activated zygotes were co-cultured with a layer of granulosa cells in 100 ml drops of SOF medium supplemented with 10% fetal calf serum (Gibco, Invitrogen, Carlsbad, Calif., USA) overlaid with mineral oil for 7 days. On day 7 after fusion, embryo quality was evaluated and grade 1 and 2 blastocysts were transferred to recipients.

With respect to the origin of the cell lines, four donors were used. They comprised two female and two male Nelore bovines (mean body weight: 414 kg for females and 504 kg for males). There were no detectable differences amongst donors.

A total of eight animal recipients was used, i.e. for cloned (n=4) and non-cloned (n=4) fetuses. All were Nelore bovines aged 2 years and their mean body weight was 440 kg. All animals were housed under similar conditions.

Animal and placenta recoveries after birth

During birth, animals were assisted by an obstetrician who offered the animals first aid such as the mechanical stimulation of breathing, artificial heating and other relevant clinical treatments. All placentas were mechanically released from the uterus (see details below) with relatively ease after parturition and their placentomes were sampled as described below.

Collection of placentomes

After birth, fetal body weight was determined. Placentas were separated from the uterus by means of a placentome base incision and then weighed without separating the cotyledons from the caruncles. A multistage sampling scheme based on systematic uniform random (SUR) sampling was applied. SUR sampling was chosen because of its efficiency (Gundersen et al. 1999).

Initially, a quadratic plastic test lattice of squares (the total plastic lattice area was ~180 cm^2 and area per square ~1–3 cm^2) was positioned randomly over the whole placenta (including those placentomes closed to the umbilical insertion site) and a SUR sample of placentomes, selected independently of their size, was obtained. To be included, placentomes were required to lie entirely within a square or to touch its acceptance borders, but not the forbidden borders or their extensions. The plastic lattice used here was an application of the unbiased counting frame published by Gundersen (1977). In terms of placentome size, the lattice gave large placentomes a 50%

chance of being sampled, and similarly for small placentomes.

Approximately 15–48 placentomes were obtained from each cloned placenta and 15–56 placentomes from each non-cloned placenta. The dimensions (length, width and thickness or depth) of these selected placentomes were measured (Table 1). For each placenta, three to four randomly chosen placentomes were used to estimate tissue shrinkage by comparing their volumes before and after processing and embedding. The rest of the placentomes from each placenta was processed for the stereological analysis of key structural quantities.

Histology

Placentomes were perfused with modified Karnovsky solution: 5% glutaraldehyde plus 1% formaldehyde in sodium cacodylate buffer (pH 7.4, 0.125 M). After fixation, they were sectioned transversely for volume estimation (see details below). Placentome slabs were systematically and randomly selected to produce isotropic uniform random sections (IUR) by means of the orientator method (Mattfeldt et al. 1990).

Placentome samples were immersed for 72 h in the modified Karnovsky solution in sodium cacodylate buffer. They were post-fixed in 2% osmium tetroxide in cacodylate buffer (EM Sciences, Hatfield, Pa., USA), block-stained with a solution of uranyl acetate (EM Sciences), dehydrated in graded ethanol concentrations and propylene oxide (EM Sciences) and block-embedded in Araldite (502 Polyscience, EM Sciences).

Blocks were cured at 60–70°C for 3 days. For light microscopy, sections (1–2 μm thick) were cut with glass knives in an RMC ultramicrotome (TM RL), collected onto glass slides, dried on a hot plate, stained with toluidine blue and mounted under a coverslip with a drop of Araldite. SUR samples of microscopical fields of view were obtained by scanning sections and moving to new fields at fixed distances determined by the x- and y-axis controls on the microscope stage. Section images were acquired by using a Leica DMR Microscope coupled with a DFC 300 FX Leica digital camera. Magnifications were calibrated by using stage micrometer scales as external standards.

Table 1 Fetal weight, placental weight and placentome number, weight and dimensions in cloned and non-cloned bovines. Values are group means (CV)

Variable	Cloned[a]	Non-cloned[a]
Fetal weight (kg)	39.75 (0.19)	35.75 (0.09)
Placental weight (kg)	7.62 (0.89)	8.03 (0.48)
Number of placentomes	62.3 (0.44)	71 (0.47)
Placentome weight (total, kg)	7.61 (0.91)	8.04 (0.47)
Placentome weight (mean, g)	124 (0.47)	114 (0.37)
Placentome length (cm)	10.6 (0.16)	9.03 (0.28)
Placentome width (cm)	6.4 (0.14)	5.8 (0.18)
Placentome depth (cm)	3.43 (0.34)	2.95 (0.22)

[a] Group differences were not significant for all variables

Stereological estimations

Reference volume of placentomes

Placentome volumes were estimated by means of the Cavalieri Principle. Each placentome was cut transversely and serially sectioned to generate a certain number of slices. Then, the length of the placentome was divided by the number of slices to obtain a slab thickness. Subsequently, a test-point system was placed over each slab in order to estimate its area. The placentome volume was estimated by applying the following formula:

$$\mathrm{Vplac} = \mathrm{d} \times (\mathrm{a}/\mathrm{p}) \times \Sigma \mathrm{P}$$

where *d* was the average slice thickness, *(a/p)* was the area associated with each test point and ΣP was the total number of points hitting the cut surfaces of slabs.

In order to obtain the total volume, all placentome volumes in a given placenta were summed and this value, Vref, was multiplied by the inverse of the fraction (Fn^{-1}) that they represented in that placenta. The error variance of the Cavalieri estimate of volume was estimated according to Gundersen et al. (1999) and Nyengaard (1999) and found to be 0.06 for cloned bovines and 0.07 for non-cloned bovines.

Volume density of chorionic villi

The fraction of total placentome volume occupied by villi was also estimated by point counting. An SUR sample of fields was selected and test points randomly superimposed. We counted the total number of points falling within placentomes ($\sum$Pref) and the total falling on villi ($\sum$Pvilli). Volume density was then estimated simply as $V_v = \sum \mathrm{Pvilli} / \sum \mathrm{Pref}$.

The error variance for ratios (V_V) was estimated according to Gundersen et al. (1999) and Nyengaard (1999). The error variance for V_v estimates was 0.05 for cloned bovines and 0.07 for non-cloned bovines.

Total volume of villi

The total volume of villi (Vvilli) in total placentome volume was estimated indirectly by multiplying villous volume density by total placentome volume.

Surface density of villi

An SUR sample of fields was selected and villous surface density (Sv) was estimated by using a test system of lines superimposed on images projected onto a computer screen. Then, the total number of points hitting villi (ΣPv) and the total number of villous intersections (ΣI) were computed and used in the formula below, where ℓ represents the length of line associated with a test point and corrected for linear magnification (Mayhew1990):

$$S_v = 2 \times \Sigma I / \ell \times \Sigma Pv.$$

Surface area of villi

The villous surface area (Svilli) in the placentomes was estimated by multiplying the surface density (Sv) by the total placentome volume (Vref) as in Mayhew (1990):

$$\text{Svilli} = \text{Sv} \times \text{Vref}.$$

Mean star volume of villi (Vstar)

The star volume provides a local, direct and unbiased estimate of a particle volume that has a strict mathematical definition, i.e. it is the volume of a particle in a given compartment as seen from all points within this compartment. The star volume estimate is obtained by means of length measurements through point-sampled intercepts and mean star volume is averaged over all point-sampled intercepts (Lee and Mayhew 1995). The final estimation of the mean star volume of villi (Vstar) was made by applying the following formula: $\text{Vstar} = \pi/3\overline{1}\text{o}^3$ where $\overline{1}\text{o}^3$ was the mean of all cubed intercept lengths.

Number of star volume units

The number of star volume units (NVstar) was estimated by dividing the total volume occupied by the villi in all placentomes of a given placenta by the mean star volume of villi. Although providing the number of star volume units, rather than the number of true architectural units, this estimate offers an indirect comparative indicator of the total number of villous units in different placentas (Mayhew and Wadrop 1994): $\text{NVstar} = \text{Vvilli}/\text{Vstar}$.

Placental efficiency

Relative placental efficiencies (PE) for each organ were estimated by means of two formulae: PE1 = placental weight/fetal weight; PE2 = villous surface area/fetal weight.

Tissue shrinkage

The method for estimating tissue shrinkage effects was based on that described previously (Wulfsohn et al. 2004; Brüel and Nyengaard 2005). Pieces of each placentome (3–4 tissue blocks) taken at random locations from each placenta were weighed and their wet weights were converted into volumes assuming a tissue density of 1.06 $g.cm^{-3}$

All tissue blocks were exhaustively and serially cut at 1-μm thickness and stained with toluidine blue. The volume of each sample was then estimated by using the Cavalieri principle with a section sampling interval of 1 in 10. Sectional areas were estimated by point counting.

Statistical analysis

As regards descriptive statistics, the means and coefficients of variation were calculated for each variable in each group. Comparisons between cloned and non-cloned bovines were drawn by means of one-way analysis of variance when two conditions were simultaneously fulfilled, i.e. data distributions were normal and variances were homogeneous. Otherwise, comparisons were drawn by using one-tail and two-tail *t*-tests or a non-parametric test, i.e. the Mann-Whitney test. Null hypotheses of no differences between groups were accepted at a probability level of $P>0.05$.

Results

Tissue shrinkage estimate

By comparing the two volume estimates for each placentome tissue block, the estimated volumetric shrinkage of organs was obtained. Values amounted to 8%–11% (within-group CV values: 0.12–0.15) and there were no significant differences between groups. Consequently, final estimates were not corrected for shrinkage effects because these group estimates retained their comparative worth.

Gross morphology

Fetal weight (group mean: CV) was 39.75 kg (0.19) in cloned bovines and 35.75 kg (0.09) in non-cloned animals. Corresponding placental weights were 7.62 kg (0.89) and 8.03 kg (0.48) and total placentome weights were 7.61 kg (0.91) and 8.04 kg (0.47) in non-cloned bovines (Table 1). None of the apparent differences between groups were significant.

Placentomes were conical, distributed by placental surface and divided into principal and accessory types. Furthermore, some areas on the placentomal surface of cloned animals were devoid of placentomes, resulting in a non-significant 13.9%

increase in the interplacentomal part of fetal membranes. However, there were no detectable differences in either placentome form or the number and distribution of them between cloned and non-cloned animals.

The total number of placentomes per placenta was 62.3 (0.44) in cloned bovines and 71 (0.47) in non-cloned bovines, whereas mean placentome weights were 124 g (0.47) g and 114 g (0.37), respectively (Table 1). Length, width and depth of placentomes were 10.6 cm (0.16), 6.4 cm (0.14) and 3.43 cm (0.34), respectively, for cloned bovines and 9.03 cm (0.28), 5.8 cm (0.18) and 2.95 cm (0.22), respectively, for non-cloned animals (Table 1). Again, the differences between groups were not significant.

Microstructure

Tissue samples comprised uterine epithelium, endometrial stroma, trophoblast and mesenchymal cells. The difference in the microstructure of placentomes between non-cloned and cloned bovines was the lack of organisation of both villous trees and the cryptal uterine epithelium in the latter (Figs. 1, 2). In the intervillous spaces in cloned bovines, haemorrhagic areas were also visible, as were shed trophoblast cells that could also be found between villi and the uterine epithelium and in the uterine epithelium (Fig. 3).

Stereological estimates

Reference volume

Total placentome volume was 8,716 cm^3 (0.90) in cloned bovines and 6,074 cm^3 (0.41) in non-cloned animals. The mean volume of placentomes in cloned bovines was 123 cm^3 (0.48) in cloned bovines and 99 cm^3 (0.47) in non-cloned animals (Table 2). No differences between group means were detected.

Volume density and total volume of villi

We found no significant between-group differences in these variables. The volume density of villi in total placentome volume was 0.30 (0.32) in cloned bovines and 0.26 (0.38)

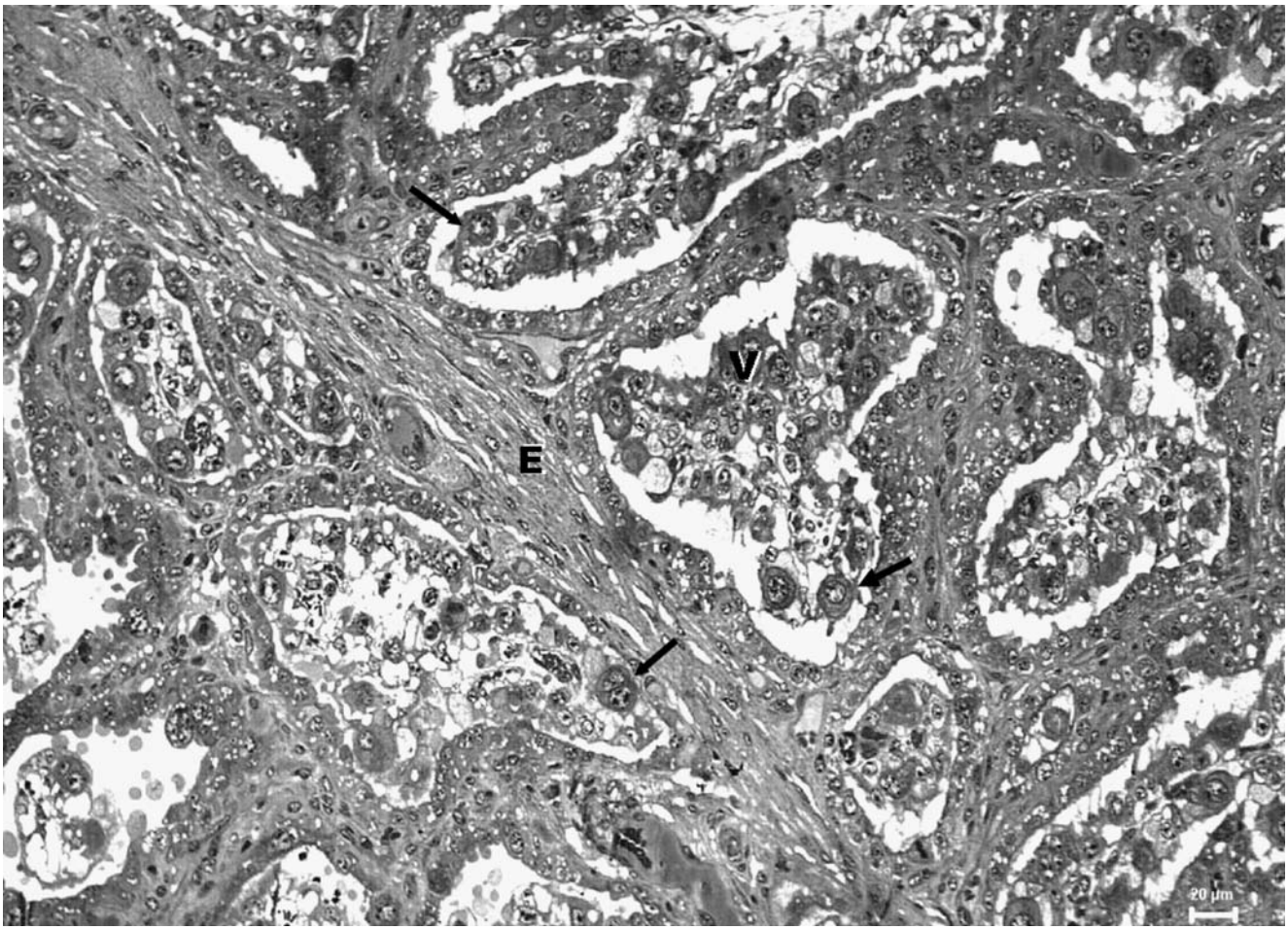

Fig. 1 Placentome microstructure of a non-cloned bovine showing the normal organisation of villi (*E* endometrium, *V* fetal chorionic villus, *arrows* trophoblast cells). Toluidine blue. *Bar* 20 µm

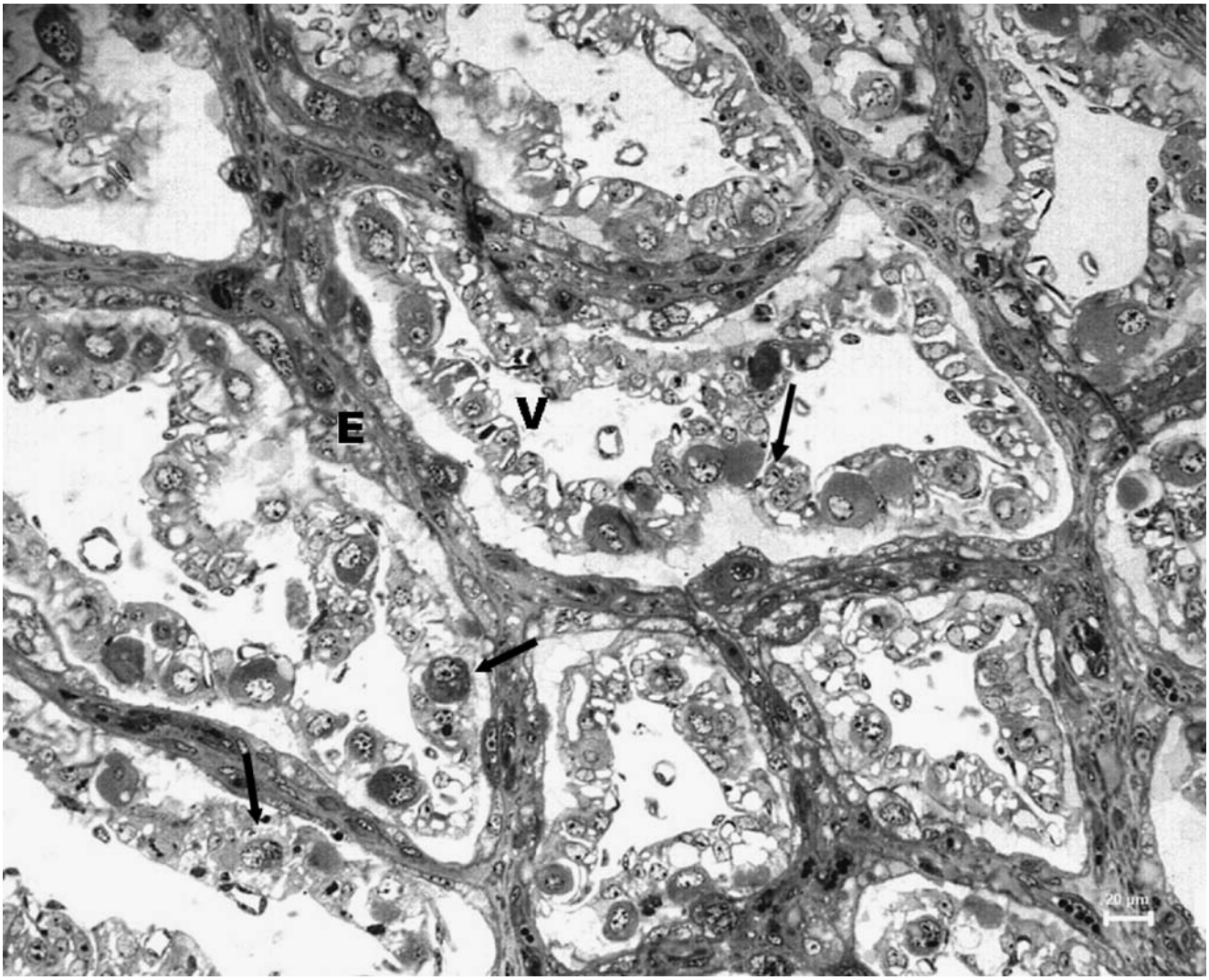

Fig. 2 Placentome microstructure in a cloned bovine showing a less-well-organised villous arrangement (*E* endometrium, *V* fetal chorionic villus, *arrows* trophoblast cells). Toluidine blue. *Bar* 20 µm

in non-cloned bovines. The total placentome volume occupied by villi was 3,090 cm^3 (1.22) in cloned animals and 1,610 cm^3 (0.54) in non-cloned bovines.

Surface density and total surface area of villi

Similarly to relative and absolute volumes of villi, no differences were seen in relative or absolute surfaces. The surface density of villi was 12,220 $m^2.m^{-3}$ (0.02) in placentomes of cloned bovines and 12,340 $m^2.m^{-3}$ (0.23) in non-cloned animals. In cloned bovines, the villous surface area was 107 m^2 (0.89) and, in non-cloned bovines, 69.8 m^2 (0.16; Table 2).

Star volumes and numbers

Villous star volume was 8.8×10^5 μm^3 (0.36) in cloned bovines and 11.8×10^5 μm^3 (0.56) in non-cloned animals. These group differences were not significant. In cloned bovines, the number of star volume units was 3.02×10^9 (0.89) and, in non-cloned bovines, 1.38×10^9 (0.09). Again, these group differences were not significant (Table 2).

Placental efficiency

PE1 in cloned bovines was 0.21 $kg.kg^{-1}$ (0.93) and 0.22 $kg.kg^{-1}$ (0.40) in non-cloned animals. Corresponding PE2 values were 2.85 $m^2.kg^{-1}$ (0.92) and 1.95 $m^2.kg^{-1}$ (0.09). Inter-group differences were not significant (Table 2).

Discussion

The present study has compared fetal weights, placentome morphometry and relative placental efficiency in cloned (280–297 days of gestation) and non-cloned (284–294 days

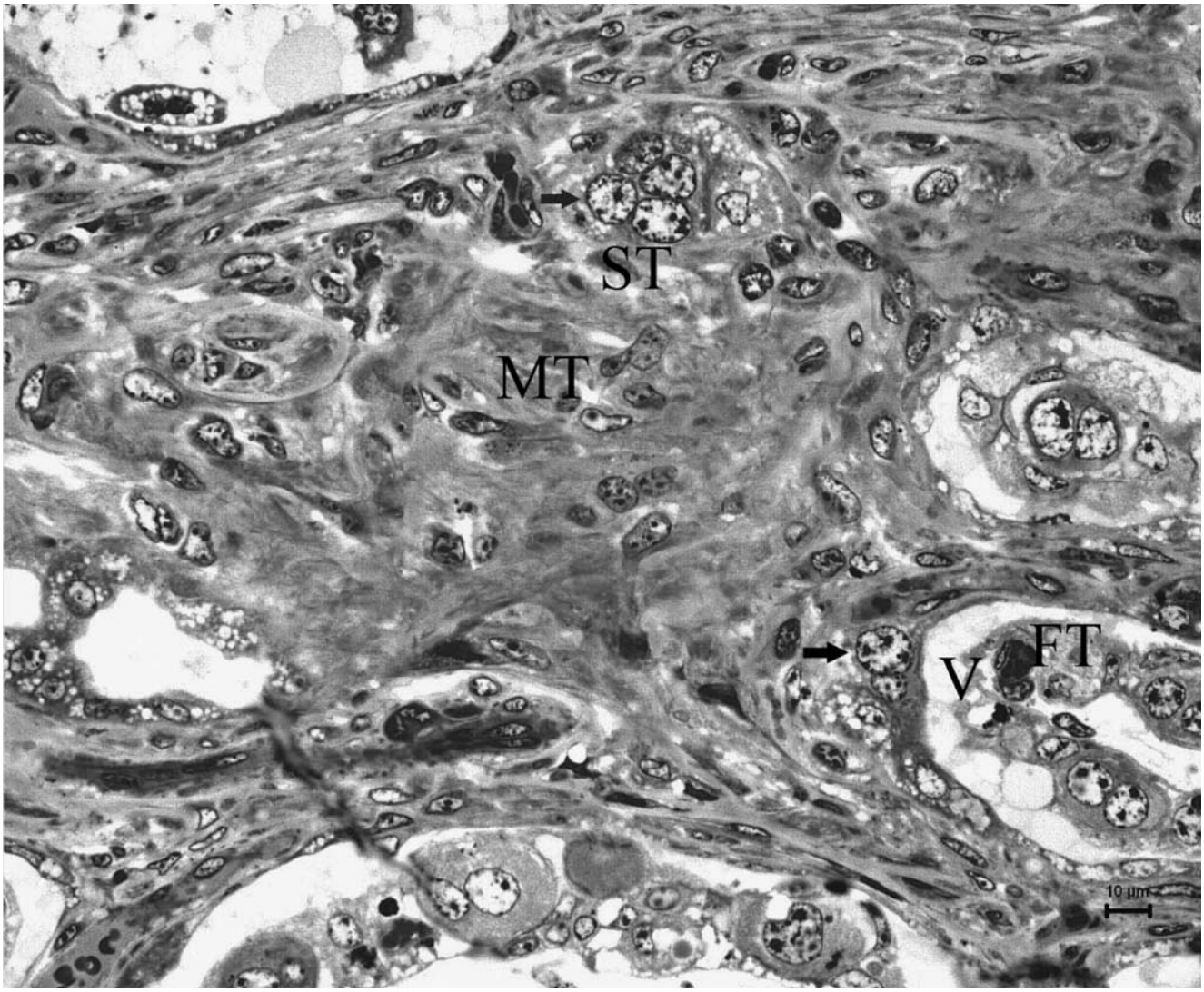

Fig. 3 Details of placentome microstructure in a cloned bovine. The following structures can be seen: maternal tissue (*MT*) possessing shed trophoblast cells (*ST*, *arrows*), fetal tissue (*FT*) containing chorionic villi (*V*). Toluidine blue. *Bar* 10 µm

of gestation) bovines. We have detected no significant differences between these groups for any of the estimated variables.

Stereological studies on placental microstructure have been applied mainly to human placentas (Jackson et al. 1987; Simpson et al. 1992; Mayhew and Burton 1997; Mayhew et al. 2003; Mayhew 2006, 2007; Mayhew and Barker 2001). Such analyses provide three-dimensional spatial information with the twin benefits of unbiased and efficient estimates of functionally relevant structural quantities. With the exception of mice (Coan et al. 2004) and horses (Allen et al. 2002; Wilsher and Allen 2003), few other species have been studied by using stereological techniques (Kannekens et al. 2006) rather than two-dimensional or model-based alternatives (Björkman 1954; Schlafer et al. 2000; Bertolini et al. 2002; Miles et al. 2004, 2005).

Cloned animals: gross changes

The development of bovine fetuses following the transfer of in-vitro-fertilised or cloned embryos may be associated with the occurrence of subtle abnormalities, including increased fetal birth weight, altered organ development, diminution of metabolism performance (Constant et al. 2006), increased perinatal mortality, hydrallantois, increased pregnancy loss and alterations in placentome number and placental structure. Collectively, these abnormalities are referred to as large offspring syndrome (Farin et al. 2004; Constant et al. 2006).

In earlier studies on various cloned animals, fetal weight was found to be lighter (Tanaka et al. 2001), larger (Constant et al. 2006; Miglino et al. 2007) or unchanged (Fletcher et al. 2007) compared with that of non-cloned controls. Our data regarding the weight of both cloned and

Table 2 Estimates of placentome size, composition and efficiency (PE1 and PE2) in cloned and non-cloned bovines. Values are group means (CV)

Variable	Cloned[a]	Non-cloned[a]
Mean placentome volume (cm^3)	123 (0.48)	99 (0.47)
Total placentome volume (cm^3)	8,716 (0.90)	6,074 (0.41)
Villous V_V ($cm^3.cm^{-3}$)	0.30 (0.32)	0.26 (0.38)
Villous volume (cm^3)	3,090 (1.22)	1,610 (0.54)
Villous S_V ($m^2.m^{-3}$)	12,220 (0.02)	12,340 (0.23)
Villous surface area (m^2)	107 (0.89)	69.8 (0.16)
Villous star volume (μm^3)	8.8×10^5 (0.36)	11.8×10^5 (0.56)
Number of Vstar units	3.02×10^9 (0.89)	1.38×10^9 (0.09)
PE1 ($kg.kg^{-1}$)	0.21 (0.93)	0.22 (0.40)
PE2 ($m^2.kg^{-1}$)	2.85 (0.92)	1.95 (0.09)

[a] Group differences were not significant for all variables

non-cloned fetuses, as determined between 280–297 or 284–294 days of gestation, respectively, showed no differences. Similarly, placental weight was increased in cloned animals such as mice (Tanaka et al. 2001) and bovines (Constant et al. 2006; before the increase in fetal weight), although it was unaltered in sheep (Fletcher et al. 2007). In mice, placentomegaly occurs as a result of an increase in size of the spongiotrophoblast layer (Tanaka et al. 2001). In nuclear-transfer-cloned bovines complicated by hydroallantois, placental weight was greater than in non-cloned bovines (Constant et al. 2003). Placental weights estimated in vivo and in vitro in pregnant Holstein cows at 222 days of gestation showed a significant increase in cloned subjects (Miles et al. 2004).

The volume of bovine placentome estimated by the Cavalieri principle has not previously been reported. Instead, volumes have been estimated directly by liquid displacement (Kannekens et al. 2006) or, indirectly, from placentome weight (Hill et al. 2001; Kannekens et al. 2006; Fletcher et al. 2007). In cloned sheep, total placentome weight is larger early in gestation (105–134 days) than towards the end of gestation (135–154 days) when there are no differences between cloned and non-cloned animals (Fletcher et al. 2007). An increase in placentome size has been reported in Nelore cloned bovines at the end of gestation (Miglino et al. 2007). In nuclear-transfer-cloned bovines complicated by hydroallantois, the means and total placentome weights are substantially heavier than in non-cloned bovines (Constant et al. 2003).

To the best of our knowledge, this is the first time that placentome volume has been estimated by using a design-based stereological method, i.e. the Cavalieri principle. Hence, the more accurate estimations have revelaed a non-significant 24% increase in the placentome volume of cloned bovines.

Since an early gestational stage has not been investigated in the present study, we cannot ascertain whether the placentomes would have been larger, as reported by Fletcher et al. (2007) for cloned sheep. On the other hand, the figures reported by Miglino et al. (2007) for placentome size in Nelore cloned bovines are questionable, since authors do not provide the reader with any statistical data.

In ruminants, placental weight is influenced by the size and number of placentomes. Moreover, the pattern reported in the literature is one of the total number of placentomes tending to decrease in cloned animals (Miles et al. 2004; Miglino et al. 2007), although, in some cases (as in the present experiments), this decrease does not attain statistical significance. A possible explanation is that the fusion of small placentomes that occurs in Nelore cloned bovines leads to an increase in placentome size (Miglino et al. 2007) and weight (Tanaka et al. 2001; Constant et al. 2003; Farin et al. 2004; Miles et al. 2004; Constant et al. 2006) and a reduction in the total number of placentomes (Miles et al. 2004).

Cloned animals: qualitative and quantitative microstructural changes

In the present study, a less well-organised endometrial structure was observed in the placentas of cloned bovines, i.e., the presence of an atypical villi tree when compared with non-cloned animals. We also noted a more distinct separation between villi and crypts in our Nelore cloned animals.

These findings are in marked contrast to previously published work on Nelore cloned bovines (Miglino et al. 2007) where cloned and non-cloned bovines were reported to have a similar placental histological structure at the materno-fetal interface. Paradoxically, the same authors (Miglino et al. 2007) stated that the caruncular surfaces were dilated and accommodated complexes of more than one primary villus in cloned bovines crypts. These two microstructural features seem to be contradictory.

The lack of endometrial organisation is consistent with other studies performed on the placenta of cloned sheep in which increases in the maternal epithelium and in maternal and fetal connective tissue have been reported (Fletcher et al. 2007) and with studies performed on cloned bovines noting a thinning of the maternal epithelium and an increase in trophoblast cell surface (Constant et al. 2006).

Of note tissue selection in our study was randomised with respect to orientation by using IUR sections and randomised for position by SUR sampling (Gundersen et al. 1999; Nyengaard 1999; Mayhew 2007). These sampling regimes gave all orientations and positions of sampling the same chance of occurrence. However, this seems not to have been the case in the other Nelore cloned bovine study (Miglino et al. 2007).

Another noteworthy structural feature is the presence of atypically positioned trophoblast cells in the intervillous spaces or between villi and the maternal crypts. The same trophoblast cell arrangement has also been reported in cloned sheep (Fletcher et al. 2007) and, following the same terminology, we have referred to these as "shed trophoblast cells" when they have detached from the fetal villi and as "intact trophoblasts" when they are present at the edge and tips of the fetal villi.

The occurrence of "shed trophoblast cells" is totally independent of tissue orientation, since the latter has been randomised by generating IUR in the tissue and giving all parts of this the same chance of being sampled (see Materials and methods for a full explanation of the orientator method).

An increased volume of chorionic villi has been reported in placentas from bovine embryos produced in vivo compared with in vitro (Farin et al. 2004). In the present study, no differences have been seen in either villi volume density or total villi volume between cloned and non-cloned bovines. In a recent investigation of placentomes from cloned bovines complicated by hydroallantois, the volume density of the fetal component, i.e. fetal connective tissue and trophoblast cells, was significantly higher in cloned bovines because of an increase in the volume density of fetal connective tissue (Constant et al. 2006).

In sheep placentomes, an increase has been observed in the volume density of shed trophoblast cells, whereas intact trophoblast cells in villi occupy a smaller fraction of placentome volume (Fletcher et al. 2007). However, the same authors have found no differences in the total fractional volume of trophoblast cells, i.e. intact and shed trophoblast cells in dead cloned fetuses.

We have noted no significant differences in the surface density of villi between cloned and non-cloned bovines. This finding is in agreement with data previously published for SNT cloned bovines complicated by hydroallantois (Constant et al. 2006). Because of dangers inherent in the "reference trap" (Mayhew et al. 2003), we have also monitored villous surface areas but, again, discerned no change in cloned animals. In SNT cloned bovines with hydroallantois, the total surface of trophoblast was not statistically different from that in controls before day 220 but was greater thereafter (Constant et al. 2006).

We have estimated placental efficiency in two main ways but neither have revealed any differences between groups. The first approach (the ratio between placental and fetal weights) expresses the placenta mass supporting each kilogram of fetal mass. Similar or inverse relationships have been used elsewhere (Miles et al. 2004; Constant et al. 2006). In cloned sheep, Fletcher et al. (2007) have shown that placental growth precedes fetal growth and the ratio of the fetal:placentome weight is lower than that in controls after gestation day 220. Our second estimator of placental efficiency refers villous surface area to fetal weight and has provided a useful index of the exchange surface area supporting each unit of fetal weight.

Conclusions and remarks for future studies

The present study has investigated whether placental microstructure and efficiency are quantitatively different in cloned bovines versus their normally produced counterparts. Although no statistical differences involving placentome-related structures have been observed, future investigations might profitably focus on placentome constituents such as capillaries, trophoblast cells, connective tissues and maternal epithelium. At least in Nelore cloned bovines, placental efficiency seems unlikely to be compromised by changes in placental villous components suggesting changes at physiological rather than anatomical levels.

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