The Use of Stereology Method to Estimate the Volume of Feto-Maternal Exchange Area of the Bovine Placentome during Gestation

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ABSTRACT

The functional surface areas of the feto-maternal unit of the bovine placentomes were quantified from Day 100 to 260 of gestation by using stereology. This study was achieved using intact placentomes obtained from an abattoir. There was no change in volume and surface densities of binucleate cells, fetal trophoblast, fetal and maternal tissues, and maternal epithelium with gestation age, although the total volume of these components increased with gestation age from Day 126 to 260. The total surface area of the feto-maternal interface increased in a similar pattern as the placentomal components without a change in the fetal to maternal tissue ratio when estimated with stereology. This is one of the recent studies in cattle to quantify and describe the functional surface area of intact placentomes at different stages of gestation and it emphasised the differences between the yak and cattle placentomal development. Anat Rec, 299:1571–1577, 2016. © 2016 Wiley Periodicals, Inc.

Key words: placenta; stereology; bovine; volume density; surface area

In cattle (and other ruminants) the placentome is the specialized point of contact between the allantochorion and endometrium, where the vast majority of the feto-maternal exchange occurs (Liu et al., 2010). The placentome is made up of the caruncle (maternal tissue) and the cotyledon (fetal tissue). At the feto-maternal interface (FMI), where the exchange of nutrients and gases takes place, both the caruncle and the cotyledon consist of primary, secondary and tertiary branching villi. These develop throughout pregnancy and, indeed, their progressive development is essential for normal fetal growth. Development failure of the placentome can be associated with abnormal development of the fetus, whilst failure of the final stages of maturation of the placentome is associated with retention of the fetal membranes (Boos et al., 2003).

Placentome development is complex with many factors involved but its success is essential for normal fetal growth during gestation. Better data on the normal process of development, particularly the relationship between the fetal and maternal components, would aid significantly in assessing how factors such as nutrition, affect placentome development. Several methods have been used to assess the development of the bovine placentome, some requiring sophisticated equipment. For example, Pfarrer et al. (2001) followed the development

Abbreviations: FMI = feto-maternal interface; SEM = standard error of means.

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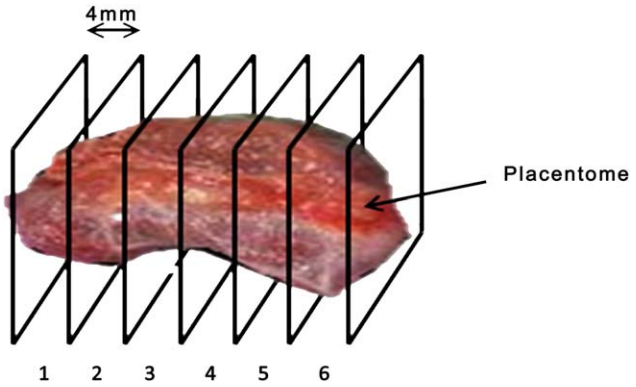

Fig. 1. Illustrates series of parallel vertical cutting planes of 4mm thick slices of placentome. This was randomised by rotation of the placentomes before cutting.

of the vascular structure of the placentome using a scanning electron microscopic, while Ferrell (1991) measured the RNA, DNA and protein concentrations in the cotyledons and caruncle. The latter study measured the weight of fetal membranes, cotyledons and caruncles after separation at two time points near term – this was taken further by Laven and Peters (2001) who evaluated placentome, caruncle and cotyledon weight and size across most of gestation.

One study (Laven and Peters, 2006) measured tissue area using an image analyser. This gave relative volumes of tissue but was a long, slow method that limited the amount of tissues which could be viewed and assessed. Stereology uses test grids to estimate surface area, volume density and absolute volumes of the placenta and has significant speed advantages over simple image analysis. However, only two studies have used this method to examine the ruminant placenta: Liu et al. (2010) used stereology to evaluate placentas from Day 60 to 211 of gestation from thirty-one yak, while Kannekens et al. (2006) evaluated the placenta from a single cow at Day 135 of gestation. The latter study needs expanding with more material from a wider spread of gestation.

The aim of the present study was to use stereology to estimate the functional surface area and volume density of the feto-maternal unit within the bovine placentome from Day 100 to 260 of gestation.

MATERIALS AND METHODS

Animals and Tissue Sampling

Uteri ($n = 25$) from early to near term pregnant Friesian, Jersey and crossbred cows (100 to 260 days of gestation) were obtained from a local abattoir. These uteri were categorized into five stages of gestation by measuring the crown rump length of the fetus (Winters et al., 1942). Stage 1: gestation age 100–125 days ($n = 5$); Stage 2: gestation age range 126–150 days ($n = 5$); Stage 3: gestation age range 151–175 days ($n = 5$); Stage 4: gestation age range 176–200 days ($n = 5$); Stage 5: gestation age range 201–260 days ($n = 5$).

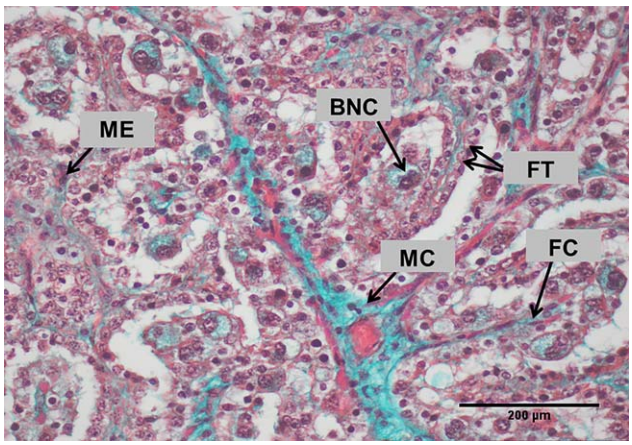

Fig. 2. Bovine placentome at Gestation Stage 2 (126–150 days) showing binucleate cells (BNC); fetal connective tissue (FC); fetal trophoblast (FT); maternal connective tissue (MC) and maternal epithelium (ME). Scale bar 200µm.

Tissue Preparation and Histological Techniques

All placentomes were carefully dissected from the uteri and arranged according to size from largest to smallest. Every eighth placentome was selected provided it was > 15 mm in diameter, otherwise the next correct size placentome was chosen to ensure unbiased sampling (Howard and Reed, 2010). The selected placentomes were then fixed by perfusion fixation followed by immersion in 10% formalin. Parallel cuts of 4 mm thick slices were made around the vertical axis of each selected placentome and one of these slices was randomly selected using systematic random sampling and cut into blocks, fixed in paraffin wax and embedded. Thereafter six 5 µm thick tissue sections from each block were cut using a manual microtome, mounted and stained with Masson's trichrome (Bancroft et al., 2008) (Fig. 1).

Stereology

One stained section was randomly selected using systematic random sampling per block per uterus and visualized using an Olympus light microscope (XC50, Japan) with an image magnification of 200X (Fig. 2). A 9 x 9 uniformly spaced point grid was generated by using Image J computer-assisted software (http://rsb.info.nih.gov/ij/index.html) and overlaid on the sections. Three fields of view (top, middle and lower part of the section) were selected from one of the six 5 µm section and analysed (Laven and Peters, 2006). In all, 243 points were assessed for each selected placentome (Fig. 3).

To estimate the surface density and total surface area of the FMI, a test grid comprising of eight uniformly spaced cycloids was generated and overlaid on three fields of view on each placentome section using image J cycloid arc plugging (Fig. 4). For each of these the minor vertical axes were parallel to the vertical cutting direction of the sectioning plane. The following tissues of interest were distinguished, counted and volume densities were estimated in each section: binucleate trophoblast cells, fetal connective tissue, maternal epithelium, and maternal connective tissue.

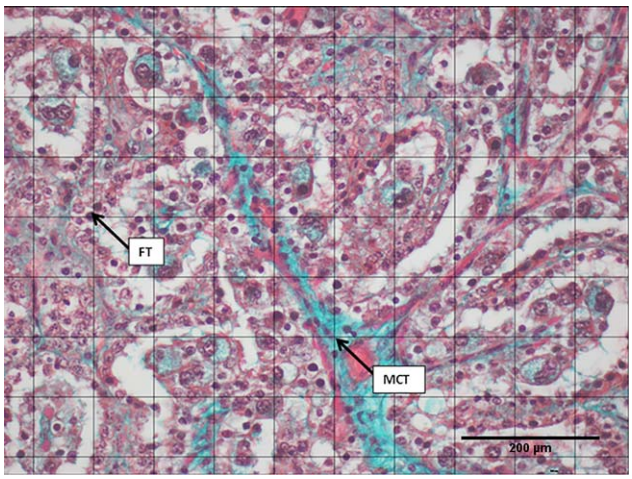

Fig. 3. Bovine placentome at Gestation Stage 2 (126-150 days) with a 9x9 point grid generated by image analysis (Image J software) overlaid on the image. Examples of points falling on a tissue of interest are represented by arrows: fetal trophoblast (FT); Maternal connective tissue (MCT). Scale bar 200µm.

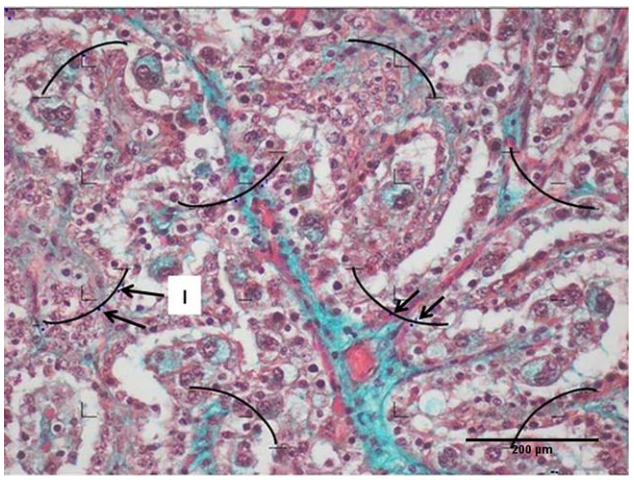

Fig. 4. Bovine placentome at Gestation Stage 2 (126-150 days) with cycloid grid generated from image analysis (Image J software) overlaid on the image. Examples of points of intersection (I) crossing the FMI are represented by arrows. Scale bar 200µm.

Volume Density

Volume density was defined as the "volume proportion of one phase within a reference volume" (Howard and Reed, 2010). The volume densities (V_v) of each tissue (binucleate trophoblast cells (BNC), fetal connective tissue, maternal epithelium, and maternal connective tissue) were estimated using the equation:

$$V_v = P(i)/P(t)$$

Where $P(i)$ is the total number of points falling on the tissue of interest and $P(t)$ is the total number of points falling within the placentomes.

Total Volume

Total placentome volume [$V(i)$]was obtained by measuring the volume of the total placentomes dissected from each uterus by the water displacement method using a graduated measuring cylinder. The total volume of each of the tissues of interest in the whole placentome was estimated by multiplying total placentome volume by volume densities for each tissue of interest.

$$V(i) = V_{(tot)} * V_v$$

Where $V(i)$ is the total volume of the tissue of interest and $V_{(tot)}$ was the volume of reference of all the placentomes

Surface density. Surface density (S_v) is the area of an interface within a unit reference volume (Howard and Reed, 2010). The number of intersections (I_i) crossing the feto-maternal junction was counted as described by Kannekens et al. (2006). The number of points falling within the field of interest (P_i) was then used to evaluate the total length of all cycloid used. The length of test line per point (l/p) was calculated by the computer-assisted software (Image J) and estimated using the following equation (Howard and Reed, 2010):

$$S_v = \frac{2\sum_{i=1}^{n} I_i}{l/p \bullet \sum_{i=1}^{n} P_i}$$

Total surface area. The total surface area of the feto-maternal interface S_{tot} (FMI) in the placentome was estimated by multiplying the surface density (S_v) by the total volume of the placentome as follows:

$$S_{tot} = S_v * V_{(tot)}$$

Statistical analysis. Data were analysed using general linear models (SAS 9.2, SAS Institute) to obtain the least square means and standard error of means (SEM) for volume densities, surface densities and total surface areas of bovine placentomes at different gestational ages. Analysis of variance was used to test for significant differences between different gestational stages. Tukey's range test was used to compare the means for individual stages where the ANOVA was significant.

To further analyse the association between techniques used and the relationship between gestation and relative volume of maternal and fetal tissue a mixed model was used with volume of placentome tissue as the outcome variable, technique used (stereology or water displacement), type of tissue (fetal or maternal) and stage of gestation as fixed effects and cow as random effect. This was followed by a second mixed model with ratio of fetal to maternal tissue as the outcome variable, technique used (stereology or water displacement) and stage of gestation as fixed effects and cow as random effect

RESULTS

Volume and Surface Densities of Placentome Components

The volume density of BNC, fetal trophoblast, fetal connective tissue, maternal connective tissue and epithelium did not change with gestational age. There was no

TABLE 1. Volume densities (%) of placentome components and surface density of FMI of bovine placentomes in each stage of gestation

	Volume density					
Gestation stage	Binucleate cells	Fetal trophoblast	Fetal connective tissue	Maternal connective tissue	Maternal epithelium	Surface density (FMI) mm^{-1}
100–125	5.0 (1.82)	42.4 (2.58)	17.4 (1.26)	25.3 (4.83)	9.9 (3.89)	1.5 (0.19)
126–150	6.6 (0.26)	46.1 (6.53)	12.0 (1.77)	26.67 (4.06)	8.6 (1.55)	1.9 (0.12)
151–175	8.0 (0.92)	43.6 (6.78)	12.2 (2.07)	11.7 (2.49)	24.5 (6.20)	1.5 (0.10)
176–200	6.5 (1.71)	37.7 (2.91)	16.3 (1.53)	24.6 (6.04)	14.8 (4.90)	1.6 (0.13)
201–260	4.4 (1.34)	40.9 (4.13)	15.2 (1.39)	26.5 (2.95)	13.0 (2.42)	1.4 (0.15)
P value	0.340	0.809	0.105	0.104	0.096	0.187

TABLE 2. Total volume densities (mL) and Total surface area of FMI (means (SE) of bovine placentomes in each stage of gestation

	Total volume (mL)						
Stage of gestation	Placentome volume	Binucleate cells	Fetal trophoblast	Fetal connective tissue	Maternal connective tissue	Maternal epithelium	Total surface area of FMI m^2
100–125	779 (118.3)^{a}	47 (21.6)^{a}	325 (46.6)^{a}	133 (16.7)^{a}	210 (62.4)^{a}	68 (25.4)^{a}	1.131 (0.21)^{a}
126–150	1134 (72.9)^{a}	75 (6.3)^{ab}	515 (74.2)^{ab}	140 (24.5)^{a}	311 (57.3)^{a}	99 (21.0)^{a}	2.131 (0.19)^{ab}
151–175	1940 (380.7)^{b}	169 (55.5)^{ab}	816 (166.5)^{bc}	250 (73.8)^{a}	211 (3.8)^{a}	496 (154.5)^{b}	2.841 (0.53)^{b}
176–200	2940 (188.4)^{c}	201 (57.1)^{c}	1120 (120.9)^{c}	479 (59.1)^{b}	690 (123.3)^{b}	454 (155.4)^{b}	4.751 (0.54)^{c}
201–260	4500 (154.3)^{d}	200 (60.5)^{c}	1861 (243.8)^{d}	687 (72.1)^{c}	1180 (112.2)^{c}	579 (105.1)^{b}	6.431 (0.75)^{d}

Different superscripts within columns indicates means that are significantly different ($P < 0.05$).

TABLE 3. Effect of stage of gestation on mean volume of fetal and maternal placentome tissue estimated using either stereology or water displacement

Stage of gestation	Fetal tissue stereology (mL)	Maternal tissue stereology (mL)	Fetal tissue volume (mL)	Maternal tissue volume (mL)
100–125	506 (79.9)^{a}	274 (49.1)^{a}	415 (62.98)^{a}	364 (55.3)^{a}
126–150	734 (73.3)^{ab}	404 (77.7)^{ab}	565 (36.3)^{ab}	569 (36.6)^{a}
151–175	1230 (266.2)^{bc}	706 (167.7)^{b}	837 (164.2)^{b}	1100 (216.5)^{b}
176–200	1800 (187.1)^{c}	1140 (57.5)^{c}	1260 (80.7)^{c}	1680 (107.7)^{c}
201–260	2930 (329.8)^{d}	1580 (142.9)^{d}	1630 (71.8)^{d}	2880 (127.1)^{d}
P value	<0.0001	<0.0001	<0.0001	<0.0001

Different superscripts within columns indicates means that are significantly different ($P < 0.001$).

significant effect of gestation stage on mean surface density with the highest surface density of 1.9 mm^{-1} from Day 126–150 of gestation (Table 1).

Total Volumes and Surface Areas of Placentome and Components

All measures increased between Stage 1 (100–125 days) and Stage 5 (201–260 days; Table 2). There was a significant increase in placentome volume, BNC and fetal trophoblast from Days 100 to 260 days of gestation (i.e., Stages 1–5; $P < 0.001$). However, most of the increases between stages were not statistically significant. For all tissues, volume at Stage 2 was not significantly greater than Stage 1, which was consistent with the change in total placentome volume. Volume at Stage 3 was only greater than at Stage 1 for fetal and maternal epithelial tissue, not for connective tissue or BNC. By Stage 4, all tissues were significantly larger by volume than at Stage 1. There was a significant increase in all tissues between Stages 4 and 5 (176–260 days) except for maternal epithelium and BNC. The changes seen in the total surface area of the FMI in Stage 1 was significantly less than that at Stage 3, which was significantly smaller than Stage 4 which in turn was smaller than Stage 5 (Table 2).

Volume of Fetal and Maternal Tissue within the Placentome

The total volume of both fetal and maternal components increased between Stage 1 and Stage 5 ($P<0.0001$; Table 3). There was a significant effect of Stage on placentome volume and significant interaction between technique and tissue type, and tissue type and Stage ($P < 0.001$). All other main effects and interactions were not significant ($P > 0.3$; Figs. 5 and 6).

For the ratio of fetal to maternal tissue as the outcome variable, there was no significant effect of Stage or interaction between Stage and technique on tissue ratio

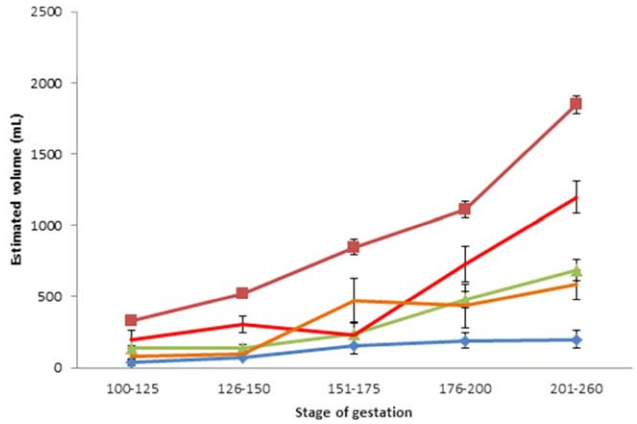

Fig. 5. Change in total volume of placentome components; binucleate cells (◆), fetal trophoblast (■), fetal connective tissue (▲), maternal connective tissue (●) and maternal epithelium (●) from 100 to 260 days of gestation. Error bars represent Standard Error of the Means.

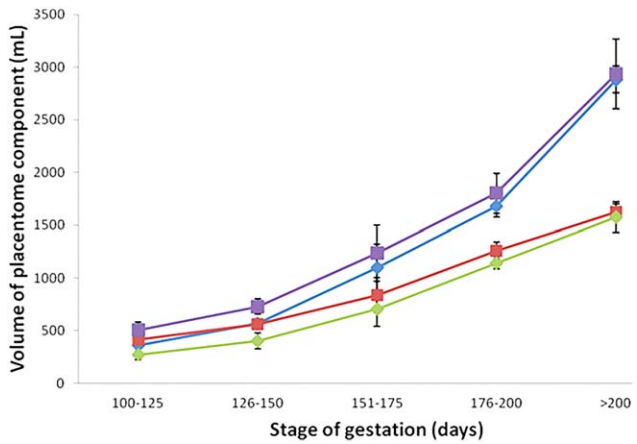

Fig. 6. Change in the volume of fetal and maternal tissue in the placentome during gestation as estimated using stereology (fetal [■]; maternal [■]) and water displacement (fetal [■]; maternal [◆]). Error bars represent Standard Error of the Means.

($P > 0.6$) but there was a significant difference between estimates using stereology and those using water displacement technique ($P < 0.001$). Overall mean ratio of fetal to maternal tissue was 0.844 using water displacement and 1.9 using stereology. Analysing the techniques separately, there was a significant association between gestational age and ratio of fetal to maternal tissue for water displacement ($P = 0.006$) but not for stereology ($P = 0.863$; Fig. 7).

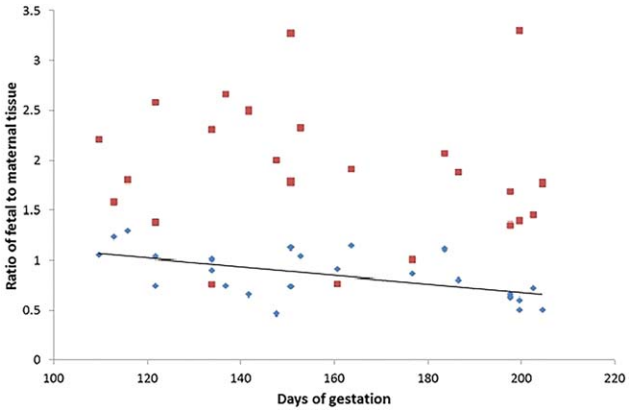

Fig. 7. Effect of gestational age on ratio of fetal to maternal tissue (by volume) in placentome estimated using water displacement (◆) or stereology (■). Solid line is line of best fit for water displacement ($R^2 = 0.333$). There was no line of best fit for stereology ($R^2 = 0.01$).

DISCUSSION

This study is the first study of cattle that has successfully used stereology to describe and quantify the changes in the fetal and maternal components of intact placentomes during gestation. The key findings of the study are, that, despite the marked change in volume of placentomes which occurs during gestation, there were no significant changes in the relative volume densities of any of the tissues measured (binucleate cells, fetal trophoblast, fetal connective tissue, maternal connective tissue and maternal epithelium) or in the surface density of the FMI.

The first study to use stereology in the bovine placentome was Kannekens et al., (2006). As only one cow at one gestational age (135 days) was used, the study was more of an evaluation of the technique. The relative volume densities reported by Kannekens et al., (2006) were 46% for fetal trophoblast, 6% for fetal connective tissue, 8% for maternal connective tissue and 40% for maternal epithelium (BNCs were not reported), whereas the equivalent figures for 126–150 days of gestation for this study were 46%, 12%, 26.7%, and 8.6%, respectively. The figures for fetal trophoblast are the same in the two studies, but the proportion of fetal connective tissue recorded in the current study is higher than that reported by Kannekens et al., (2006). This difference is even more pronounced for maternal connective tissue, while the relative volume density of maternal tissue is significantly less. The reasons for these differences are unclear but are likely to be related to differences in classification of tissue. However, this discrepancy is still present when fetal and maternal volume densities are compared. Kannekens et al. (2006) reported a relative volume density of maternal tissue of 48% compared to the 37% found in this study. That result is consistent with that of Laven and Peters (2006) who reported that at around 135 days the proportion of maternal tissue (by volume) in the placentome was approximately 48%. The relative proportion of maternal tissue is also much lower than the proportion reported by Liu et al. (2010) in the yak at the same stage (57%). Some of this difference between the yak and the results of the current study may be related to the measurement of BNC which would have been found in both maternal and fetal tissue and accounted for approximately 7% of relative volume density.

The results for surface density are more similar (converting cm^{-1} to mm^{-1}), with the average surface density around 1.9 mm^{-1} in the current study compared to the 15.5 mm^{-1} reported by Kannekens et al. (2006). Both of these results are markedly lower than the 33.8 mm^{-1} reported by Liu et al. (2010) and 225 mm^{-1} found in *Bos taurus* (Baur 1972). The difference in value between

the current study and previous studies could be attributed to the correction factor that was not used in this study.

Overall this study found no change in the relative volume densities of the measured tissues (and the sum of the fetal and maternal tissues) or in surface density of FMI between 100 and 260 days of gestation. In contrast, Liu et al. (2010) reported that in the yak the relative volume density of BNC and caruncular endometrium decreased while that of fetal villi increased. Those authors reported no change in surface density over gestation—consistent with this study. These differences between the yak and the current study in NZ dairy cattle may reflect species differences in placentome development.

As total placentome volume in the current study changed significantly with gestational age, all tissue types increased similarly such that there was not a significant difference between Stages. The pattern of increase was similar for both total volume and tissue volume except that the difference between stages tended to be less distinct for the tissue types than for total placentome volume. However for all measures there was a significant difference between Stage 4 (Day 176–200) and Stage 5 (201–260), which differs from the report by Liu et al. (2010) in yak, where there was no change between Day 181–210 and >210. The reason for this difference is likely the marked increase in total placentome volume in cattle (2940 vs. 4500 mL for Stage 4 and 5, respectively) which did not occur in yak (2255 vs. 2206 mL for Day 181–210 and >2010, respectively).

The estimated total surface area of the FMI increased similarly with gestational age. The estimated mean at Days 126–150 of gestation in the current study was 2.131 m^2 which is considerably smaller than the 18.5 m^2 reported by Kannekens et al. (2006). One reason for the difference could be that Kannekens et al. (2006) estimated and corrected for tissue shrinkage due to fixation of the tissue which was not done in this study. Also, the current study showed a large amount of variation (1.131–6.431 m^2), which meant that the result published for one cow by Kannekens et al. (2006) falls within this variation. The large variation found here presents a strong case for conducting such experiments with more than one animal, as done here.

The estimated surface area found in this analysis at all stages was smaller than the mean results reported by Liu et al. (2010); this was true even when the data from Stages 1–5 (100–260 days) in this study were compared to the results reported by Liu et al. (2010) from 91 to >211 days (1.1–6.43 vs. 9.07–65.13 m^2, respectively). This suggests that even though the total volume of placentomes in the yak is smaller, the surface area of the FMI is greater.

However it could be that the different method of estimating the volume of placentomes used by Liu et al. (2010) (Cavalieri principle) underestimated the placentome volume compared to water displacement, but this does not explain the large difference in surface density between the two species which is the key driver of the increased total surface area of the FMI. In contrast to a previous study using image analysis (Laven and Peters, 2006) which found that the ratio of fetal:maternal tissue by volume increased as gestation progressed, this study found no effect of gestational age on the relative proportions of fetal:maternal tissue by volume when volume was estimated by stereology. In contrast the ratio of fetal to maternal tissue by volume was estimated by water displacement of intact placentomes which decreased as gestation progressed (consistent with the results reported by both Reynolds et al. (1990) and Laven and Peters (2001)). Using stereology, Liu et al. (2010) reported results similar to Laven and Peters (2006). This implies that the proportion of fetal tissue by volume increased in yak placentomes as gestation progressed. The proportion of fetal tissue was much smaller in early gestation in the yak (36% compared to 50% in the cow), although proportions nearer term were similar (54 vs. 55%, respectively). The reason for the difference between this study and that reported by Laven and Peters (2006) is unclear.

Although there was no change with gestational age, the mean proportion of fetal tissue by volume estimated by stereology was always much greater than the proportion estimated by using water displacement of separated placentomes. As the density of placentome tissue is approximately 1 and consistent across gestation, this difference is consistent with the difference between the volume data from both Kannekens et al. (2006) and Laven and Peters (2006), who both used intact placentomes. It is also in agreement with the weight data reported by Reynolds et al. (1990), and Laven and Peters (2001), who both manually separated placentomes into caruncles and cotyledons. Kannekens et al. (2006) concluded that the difference was due to insufficient separation of maternal and fetal tissue, although both Reynolds et al. (1990) and Laven and Peters (2001) reported that there was only limited or no failure of separation (far below the level of separation required to produce the differences between stereology of intact placentomes and water displacement of separated placentomes seen in this analysis). The most likely reason for the difference is that the physical estimation of fetal and maternal proportion by volume using the water displacement method measures the whole placentome including both the tissue where exchange occurs and the non-exchange tissue such as the endometrial stalk and the non-villous areas of the cotyledon, whereas stereology focuses on the exchange area. This finding contradicts the assumptions of Kannekens et al. (2006), that both techniques were similar. The differences between the techniques are therefore due to the non-exchange tissue having a higher proportion of maternal tissue than the exchange tissue. As stereology focuses on the exchange tissue, it is likely that the results from stereology reflect placentome capacity better than the results from simple separation.

This study has confirmed that stereology is a useful technique to study placental development in the cow, and has highlighted differences between cattle and yak in placentome development and relative tissue proportions. As stereology focuses on the interface between fetal and maternal villi, its results are focussed on placental capacity which cannot be measured by simpler methods such as placentome separation and water displacement, as those measures include tissue which does not function as exchange tissue.

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