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Cell cycle and apoptosis in normal and cloned bovine near-term placentae

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ABSTRACT

The bovine maternal epithelium is composed of cuboidal cells interspersed with low columnar cells having centrally located nuclei. Bovine trophoblast is composed of two cell types: mononuclear trophoblastic and giant trophoblastic cells that can have two or more nuclei. Number of apoptotic cells and proliferative cells are variable in both cell populations. This study compared tissue growth and apoptosis by flow cytometry in the cell population found at distinct placental regions (central region of placentomes, ≤1-cm microplacentomes and the interplacentomal region) between normal and cloned near-term bovine pregnancies. After a morphological comparison between regions and groups (controls vs. clones), a lesser proportion of diploid to tetraploid cells was observed in the central region of placentomes and in microplacentomes from cloned-derived pregnancies. In addition, cloned animals had a fewer apoptotic cells in the central region of the placentome and in interplacentomal region and a greater proliferative capacity in all regions (cells in G_2/M) near term as opposed to control animals. These results may reveal the existence of a relationship between such changes in the proportions of uterine and trophoblastic epithelial cells at the end of pregnancy and normal placental function. This could be related to faulty placentation in early pregnancy, placental insufficiency during pregnancy or lack of placental and/or fetal maturation in late pregnancy, which may contribute to some

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of the abnormalities after *in vitro* embryo manipulations, such as poor preparation and initiation of parturition, prolonged gestation and lesser post-natal survival in some cloned animals.

1. Introduction

The ruminant placenta is classified as synepitheliochorial and has a cotyledonary organization in which both fetal and maternal villi are discernible as discrete structures (placentome) on the uterine epithelium. Close contact between maternal and fetal tissues occurs only in the placentome, which is the most highly vascularized portion of the placenta (Leiser and Kaufmann, 1994; Cross et al., 1994; Wooding and Flint, 1994).

In the bovine placenta, approximately 15–20% of the fetal trophoblast is comprised of giant trophoblastic (GT) cells, producing hormones and bioactive products important for conceptus growth and development (Wooding, 1982; Green et al., 2000; Schlafer et al., 2000). Previous findings indicate that polyploidy is a normal feature in the development of most GT cells (Klisch et al., 1999). Thus, GT cells arise from a completion of karyokinesis (mitosis) not followed by cytokinesis, which characterizes proliferative inability (Winsatt, 1980). Such cells appear as early as day 17 of gestation in cattle, being, in their initial forms, deeply within the trophoblast, migrating later from the trophoblast cell layer to fuse with uterine epithelial cells, forming short-lived trinucleate cells (Wooding, 1992). This phenomenon occurs throughout pregnancy in cattle, with the number of GT cells slightly increasing throughout pregnancy, but the number and period of existence of trinucleate cells declining towards the end of gestation (Wooding, 1992; Green et al., 2000), as the placenta matures and parturition approaches.

Development of a fertilized egg into a multicellular complex organism involves cell replication and growth, as well as a progressive cell differentiation. Cell division and differentiation are proportional to cell apoptosis or cell death during the development and growth of both immature and adult organisms (Young and Heath, 2001). In development of the placenta, the balance between cell proliferation and cell death by apoptosis is important, having an important role in placental function, with both processes being inversely proportional throughout gestation (Boss et al., 2003). Flattened cells in the maternal epithelium are present at the end of gestation, which are committed to cell proliferation, suggesting that changes of cellular weight could be a consequence of alterations in metabolic needs (Stallmach et al., 2001). Likewise, apoptotic corpuscles can be found in maternal and fetal epithelium and at a greater percentage in the fetal epithelium, due to phagocytosis by the fetal tissue, being important for tissue remodeling (Miyoshi and Sawamukai, 2004). Previous reports implicated certain *in vitro* embryo manipulations, such as *in vitro* fertilization (IVF) and cloning by nuclear transfer (NT) procedures, as predisposing factors to abnormal placental development during pregnancy, affecting fetal growth and viability, including life *ex utero* (Behboodi et al., 1995; Bertolini et al., 2002b, 2004; Batchelder et al., 2005, 2007a, b; Cibelli et al., 1998; Hashizume et al., 2002; Hill and Chavatte-Palmer, 2002; Hill et al., 1999, 2000, 2001; Miglino et al., 2007).

The extent of tissue proliferation/apoptosis can be determined by flow cytometry, which enables the evaluation of a large number of cells in a short period of time. With the evaluation of DNA content, proportion of cells with abnormal amounts of DNA (aneuploidy) and at different phases of the cell cycle, DNA degradation, and amount of cell proliferation can be easily and readily determined (Cibas, 1995; Keren, 1994; O'Leary, 1998; Orfao et al., 1995; Shapiro, 1995). Such evaluation is important for the evaluation of both normal and abnormal conditions in different tissues. In this view, the use of flow cytometry analyses for the study of placental abnormalities after cloning by NT procedures may be valuable for the identification of cause-and-effect mechanisms responsible for variations in development and for the uncovering of novel, unknown physiological processes during pregnancy. Therefore, the aim of this study was to compare the patterns of cell cycle, cell proliferation, apoptosis and proportion of diploid to tetraploid cells in uterine and trophoblastic epithelium by flow cytometry in samples of tissue collected from distinct placental regions (central region of placentomes, $\leq$1-cm microplacentomes, and the interplacentomal region) between near-term control and cloned bovine pregnancies.

2. Materials and methods

2.1. Animals and tissue sampling

Near-term bovine placentae from normal pregnancies ($n = 5$) were obtained at a local slaughterhouse in São Paulo State. Upon collection, the uterus was opened, the fetus removed, and the crown-rump length used to estimate the stage of gestation. Placentomes of cloned cattle ($n = 5$) produced by nuclear transfer (NT) were obtained at CYAGRA Brasil Inc., delivered at term (290 days) by cesarean section. Placental tissues from controls and clones were excised, cut into various small fragments (0.5 cm) from different placentomal regions (central region of the placentome, interplacentomal region and $\leq$1.0 cm microplacentomes) and immediately snap-frozen in liquid nitrogen to be used for flow cytometric analysis. Tissues for histology from the same three areas were transferred to 10% formalin in 0.1 M phosphate buffer for 48 h, embedded in paraplast and sectioned at 5 μm using an automatic microtome (Leica RM2155, Germany). Sections were stained with haematoxylin and eosin (HE) and were examined with an Olympus BX40 microscope (Zeiss KS400 image analysis system 3.4; Fig. 1A–G), for placental methodology, complete description and morphology data see our previous publications (Miglino et al., 2007, 2008)

3. Flow cytometry (FACS)–DNA content and cell cycle analysis

Fragments stored in liquid nitrogen were placed into citrate buffer, pH 7.6, and processed according to Vindelov et al. (1983). In brief, samples were filtered through a 30-μm mesh (Spectra Mesh Nylon Filters, Sigma Chemical Co., St. Louis, MO, USA) to separate stroma and debris, to be subsequently incubated in 30 mg/mL trypsin (Sigma Chemical Co.) for 10 min, followed by 10 min in 5 mg/mL trypsin inhibitor (Sigma Chemical Co.) and 0.1 mg/mL ribonuclease A (Sigma Chemical Co.), all at RT. Then, 415 μg/mL propidium iodide (Sigma Chemical Co.) was added to the samples 15 min prior to flow cytometric analysis. At least 10,000 events were acquired using Cell Quest Software (Beckton Dickinson, San Jose, CA, USA). DNA content was measured using a FACSCalibur Flow Cytometer (Beckton Dickinson) equipped with an air-coupled 15-mW, 488-nm argon ion laser. The proportion of cells in apoptosis (sub-G_1 or DNA fragmentation) or in each phase of the cell cycle (G_0/G_1, S, G_2/M) was calculated using the ModFit Software analysis (Beckton Dickinson). Data were analyzed by the χ^2 test or Fisher's Exact test, for $P < 0.05$. Flow cytometry was used to quantify the DNA content identifying different cell populations (aneuploid, diploid, tetraploid and polyploid) and analyze cell proliferative activity related to cell distribution on cell cycle.

4. Results

The flow cytometric analysis of samples from control placentomes had two cell populations with distinct characteristics in size and granularity: diploid cells and tetraploid cells (Fig. 2). There were distinctions in cell cycle phases between placental regions classified as interplacentomal, central region and microplacentomes, and between groups (Fig. 3).

In control animals (Fig. 2), proportion of diploid to tetraploid cells was different ($P < 0.05$) in interplacentomal regions (89.1% ± 4.1 to 10.9% ± 4.1) in comparison with the central region of placentomes (19.7% ± 2.8 to 80.3% ± 2.8) and with microplacentomes (20.7% ± 1.4 to 79.3% ± 1.4). In addition, there was a significant increase in number of cells in apoptosis and a decrease in quiescent cells (G_0/G_1) in the interplacentomal region (26.2% ± 6.4 and 46.8% ± 7.0, respectively) when compared with the central placentomal regions (11.1% ± 5.3 and 61.2% ± 10.6, respectively) and microplacentomes (1.0% ± 0.1 and 56.1% ± 0.1, respectively), as depicted in Fig. 3. An increased proliferative capacity (G_2/M) was observed in the region of the microplacentomes (33.1% ± 0.1) in comparison with central placentomal (20.4% ± 5.4) or interplacentomal (24.0% ± 0.5) regions, while no differences between regions were observed for the proportion of cells at the S phase.

In cloned animals (Fig. 2), the proportion of diploid to tetraploid cells was different between interplacentomal regions (83.3% ± 4.8 to 16.0% ± 4.6) and both central region of placentomes (38.7% ± 4.2 to 59.0% ± 4.7) and the microplacentomes (57.4% ± 3.5 to 41.5% ± 3.4). Also, a greater percentage of

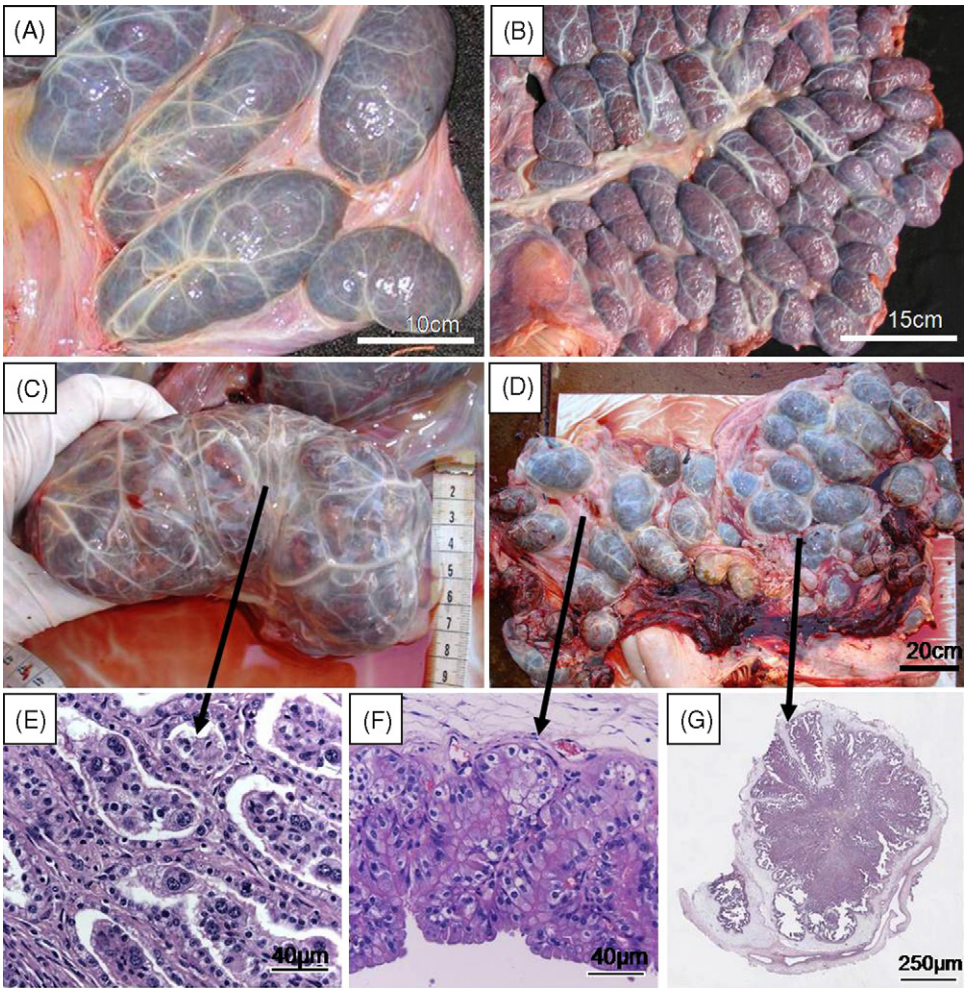

Fig. 1. Photography of normal and cloned placentae at 290 days of pregnancy. (A and B) Bovine control placentomes. (Cand D) Bovine cloned placentomes. Note the placentome size in C and D related to (A) and (B). Light microscopy of placentomes related to position and type. (E) Central region of the cloned cattle placentome with uterine cell presence, well organized, mononuclear trophoblastic cells and BNC, with few apoptotic cells in this region. (F) Interplacentomal region showing trophoblastic epithelium with folds and (G) microplacentomes in cloned cattle placenta showing all histological aspect of the tissue and relation with uterus. HE stains. Scale bars: E: 40 μm, F: 40 μm, G: 250 μm.

quiescent cells (G_0/G_1 phases) was observed in interplacentomal regions (40.1% ± 6.5) and in microplacentomes (38.5% ± 7.9), whereas an increased proliferative capacity (G2/M phases) was observed (Fig. 3) in the central region of placentomes (71.6% ± 7.2) compared with interplacentomal regions (53.6% ± 9.7) and microplacentomes (59.1% ± 6.2).

Differences ($P < 0.05$) in ploidy and cell cycle were also observed between the placentae of control and cloned animals in the regions that were assessed. The proportion of diploid compared with tetraploid cells was different in the central region of placentomes and in microplacentomes ($P < 0.001$), with cloned animals showing an increase in tetraploid cells, being similar in the interplacentomal regions (Fig. 2). Moreover, the number of apoptotic cells was less in the central region of placentomes (1.1% ± 0.5) and in interplacentomal regions (1.1% ± 0.8) from cloned than control animals (11.1% ± 5.3 and 26.2% ± 6.4, respectively). Moreover, the percentage of quiescent cells (G_0/G_1) was less ($P < 0.05$)

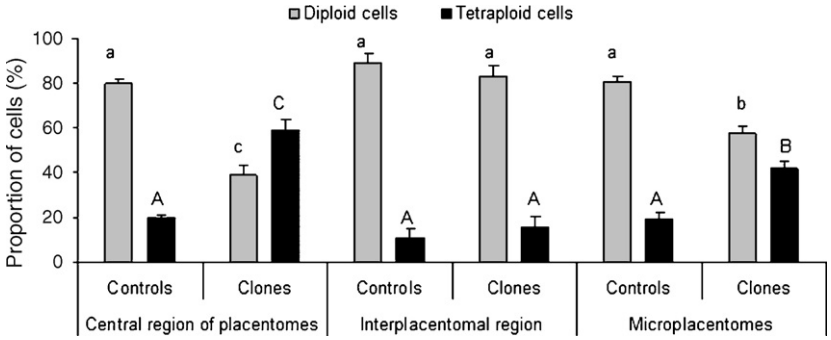

Fig. 2. Flow cytometric population profile from the central region of placentomes, interplacentomal region and microplacentomes obtained from normal and cloned bovine placentae, showing two populations of cells: diploid cells and tetraploid cells. a,b,c: columns for diploid cells; A,B,C: columns for tetraploid cells; Column bars without common superscripts differ; $P < 0.05$.

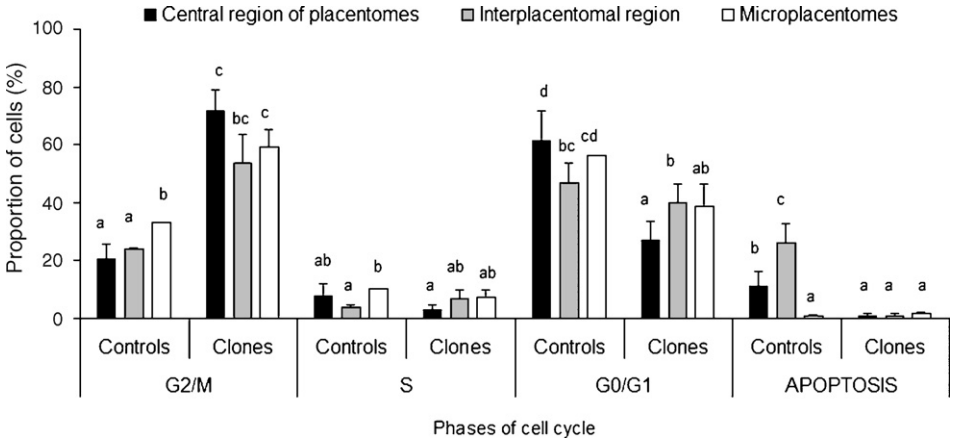

Fig. 3. Flow cytometric analysis of the distribution of cell cycle phases from the central region of placentomes, interplacentomal regions and microplacentomes obtained from normal and cloned bovine placentae. a,b,c,d: Column bars, within the same phase of the cell cycle, without common superscripts differ; $P < 0.05$.

in cloned animals than controls in the central region of placentomes (27.0% ± 6.5 compared with 61.2% ± 10.6, respectively) and in microplacentomes (38.5% ± 7.9 compared with 56.1% ± 0.1, respectively). In all regions where assessments were made, a greater number of cells (Fig. 3) at the G_2/M phase were detected in the cloned group (59.3% ± 12.2) compared with the control group (22.4% ± 6.3).

5. Discussion

Through the combination of beams of radiation, important information from a tissue can be obtained by flow cytometry, such as size, granularity/complexity and morphology of cells (Silva et al., 2004). In cattle, determination of ploidy by *in situ* hybridization indicated most nuclei of trophoblast cells are tetraploid and that both nuclei always lie together. These findings show that polyploidy is a normal feature of the development of most binucleated cells in normal placentae of cattle (Klisch et al., 1999).

Binucleate cells (BNC) usually take part of about 20% of the trophoblast cells, serving for at least two functions during pregnancy: to form the maternal-fetal junction and to produce hormones and bioactive products, which are essential to the success of implantation, subsequent placentomal development and regulation of fetal growth (Wooding, 1982, 1992; Green et al., 2000; Schlafer et al., 2000).

Ruminant BNC(s) are filled with granules that contain gestation-specific proteins and glycoproteins, such as placental lactogen hormone (PL) and other bioactive substances (Wooding and Wathes, 1980; Landim et al., 2007). Interestingly, proportion of binucleate cells in normal near-term placentas is in accordance with the flow cytometric analysis used in the present study, in which around 20% of tetraploid cells were found in the central region of placentomes and microplacentomes. Most likely, such tetraploid cells represent the binucleated cell population rather than dividing cells. Presence of tetraploid cells in interplacentomal regions was also found in the present study. These findings are in agreement with Wooding et al. (1996) where there were binucleated cells in cotyledonary and intercotyledonary regions. These studies indicate the presence of functionally specialized regions of the placenta, emphasizing the multifunctional activity of binucleated cells in ruminants.

Under physiological conditions, the placenta grows continuously during pregnancy, developing faster than fetus in early pregnancy, reaching a peak by the beginning of the third trimester, to subsequently reduce its pattern of tissue growth by late pregnancy (Eley et al., 1978; Prior and Laster, 1979; Ferrell, 1989; Reynolds et al., 1990). Flow cytometry in normal near-term bovine placenta in the present study confirmed that pattern, as the presence of a lesser percentage of cells in G_2/M and greater percentages in G_0/G_1 indicates cell proliferation is decreased in term placentae.

During placental development, cell proliferation and cell death by apoptosis are inversely proportional processes throughout gestation, being relevant for normal conceptus growth (Boss et al., 2003). In early gestation, the human placenta is characterized by a proliferative activity of cytotrophoblast cells (Mochizuki et al., 1998), with cells in proliferation having an important role in the nutrition of the concept (Björkman, 1969). However, in late gestation there is no physiological significance for maintaining or continuing the proliferation process. This is appears to have occurred in the control pregnancies because fewer cells at the G_2/M phases and greater numbers of cells in G_0/G_1 were observed in all placental regions.

As expected, a greater rate of apoptotic figures was detected in placentomal or interplacentomal regions in this study. Physiologically, apoptosis allows elimination of unnecessary cells to maintain tissues homeostasis (Barreto Filho and Marques Junior, 1993) and has been postulated to have a role on placenta maturation and detachment in late pregnancy (Martins et al., 2004). Results from the present study in normal term placentae of cattle are consistent with these previous reports (Smith et al., 1997; Leers et al., 1999; Pfarrer et al., 1999; Austgulen et al., 2002) with a large number of cells in apoptosis at term contributing to maternal-fetal disconnection. Also, apoptosis in organ remodeling may serve the function of prostagenic producing cell removal.

In the present study, the increase in tetraploid cells in placentomes or microplacentomes of cloned pregnancies may be more a reflection of compensatory tissue growth or angiogenic activity for a less efficient placenta than due to a greater number of binucleate cells. The relative population of binucleate cells changes in late gestation and at the time of parturition in normal cows, and its proportion is altered in placentae of cloned calves (Hashizume et al., 2002). This is consistent with findings by Miles et al. (2004), in which a reduction in fetal villi and binucleate cell volume densities in Day-222 IVF-derived placentomes appeared to compensate for decreased feto-maternal contact with an increased proportion of tissue volume of blood vessels in the maternal caruncles and the ratios of blood vessel volume density to-placentome surface area within the cotyledonary and caruncular tissues. The mRNA relative abundance and protein contents for angiogenic factors in cotyledonary and caruncular tissues from control versus IVF-derived pregnancies were not different between groups, except for IVF-derived caruncles, which had an increased in protein peroxisome proliferator-activated receptor gamma (PPARg), a factor that upregulates VEGF,gene expression being associated with angiogenesis and tissue remodeling in the mammalian placenta and other organs (Barak et al., 1999; Bamba et al., 2000). Interestingly, Bertolini et al. (2006) demonstrated that maternal concentrations of bovine PL in IVF-derived pregnancies were less than controls during the last 8 weeks of gestation, becoming similar as parturition approaches. When combined, those results indicated differential patterns of placental function and secretion exist between control and manipulated animals, supporting the concept that compensatory growth or functional mechanisms exist during late gestation of *in vitro*-derived pregnancies in cattle.

Greater rates of cells in G_2/M, less apoptosis and fewer cells in G_0/G_1 in cloned placental tissues may reflect a need for placental growth or activity due to placental gene deregulations, dysfunctions

or insufficiency. Disturbances in placentation and placental function after *in vitro* embryo manipulations by cloning or IVF have been associated with changes in pattern of fetal growth in early and late gestation, affecting pre- and post-natal survival (Behboodi et al., 1995; Stice et al., 1996; Cibelli et al., 1998; Hill et al., 1999, 2000, 2001; Bertolini et al., 2002a; Batchelder et al., 2005, 2007a,b). Placental underdevelopment in early pregnancy appears to precede growth restriction or fetal demise (Hill et al., 2000; Bertolini et al., 2002b). If early pregnancy is not terminated by faulty placentation, a biphasic conceptus growth pattern usually follows, with and initial period of growth restriction (Bertolini et al., 2002b; Chavatte-Palmer et al., 2006), followed by a period of compensatory growth, which is preceded by changes in placental development (Bertolini et al., 2002b, 2004; Batchelder et al., 2005). Such events culminate either with fetal death or with the delivery of larger IVF and SCNT calves with less postnatal survival and morphologically altered placentae (Bertolini and Anderson, 2002; Bertolini et al., 2002a, 2004, 2006; Batchelder et al., 2005; Miglino et al., 2007). Changes in placental micro-architecture have been postulated to cause faulty placentation and compensatory growth (Hill et al., 2000; Bertolini et al., 2002b, 2004). This placental compensatory mechanism and morphological changes are not surprising because placental tissue can have great plasticity under unfavorable conditions through mechanisms to adapt to adverse nutritional (McEvoy et al., 1997; Perry et al., 1999) and environmental (Ferrell, 1989; Krebs et al., 1997; Penninga and Longo, 1998) circumstances, which can promote morpho-histological changes in the placenta to compensate and modulate fetal and placental growth, in atypical pattern as seen after *in vitro* embryo manipulations by IVF and cloning by NT.

Binucleated cells in cattle synthesize progesterone (Reimers et al., 1985; Ullman and Reimers, 1989; Boss et al., 2003, 2006), being also implicated in estrogen synthesis (Ben David and Shemesh, 1990; Schuler et al., 2005). Progesterone has an important role in maintaining the homeostasis of the maternal-fetal interface regulating cell differentiation and growth and on placental development. In cloned animals, a proportion of 61.3% of tetraploid cells (putative binucleated cells) were present in placentomes in the present study. This value is greater than in control pregnancies (19.7%), suggesting placental regulation exerted by these cells may be altered in pregnancies of cloned animals. Moreover, results in the present study comparing pregnancies of cloned and control cattle inidicated a lesser frequency of apoptosis in all regions of the placenta from cloned animals. Boss et al. (2003) and Miyoshi and Sawamukai (2004) reported a lesser percentage of cells in apoptosis in pregnancies with placental retention and absence of placental maturity. This observation could be an indicator of inefficient maturation of the placenta of cloned pregnancies of cattle near term. Indeed, a large proportion of IVF- and NT-derived pregnancies fail to terminate normally, resulting in prolonged gestation of grossly over-sized fetuses with poor post-natal survival.

6. Conclusion

In summary, flow cytometric data obtained in the present study from the near-term placenta in control pregnancies of cattle revealed proliferation and apoptotic patterns and proportion of diploid to tetraploid cells at each distinct region that were in the expected normal physiological range. Conversely, placental tissues from cloned animals had more proliferation activity and a reduced rate of apoptosis in the central region of placentomes and interplacentomal areas when compared with control animals. Such phenomena may be associated with faulty placentation in early pregnancy, placental insufficiency or even a lack of placental and/or fetal maturation towards the end of pregnancy, events that are common after *in vitro* embryo manipulations such as cloning by NT.

Conflict of interest statement

There are no financial competing interests for all authors.

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