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Cellular Remodelling by Apoptosis During Porcine Placentation

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Contents
The mechanisms that regulate the apoptosis are essential to the normal development and maintenance of homoeostasis and play an important role in placental development in mammals. During porcine pregnancy, there must be a proper cellular remodelling to achieve a normal gestational development. Knowledge of pig physiology during pregnancy will explore options to increase the productivity of this species of high economical value. The purpose of this work was to study the cell morphology and apoptosis of porcine placentas from early, mid and late pregnancy. For that purpose, high-resolution light microscopy and transmission electron microscopy were performed to the study of cell morphology. TUNEL, the apoptosis index (IAp) and the expression of c-FLIP through immunohistochemistry technique were used to the study of apoptosis. High-resolution light microscopy and transmission electron microscopy confirmed the presence of placental cells with ultrastructural apoptotic features. Apoptotic nuclei were detected by TUNEL in different placental structures and phagocytes containing apoptotic bodies. The IAp in villi was 9.34% at early, 0.82% at mid and 23.85% at late pregnancy. Statistically significant differences were found between periods ($p < 0.05$). In previous studies, we determined a differential induction of the apoptotic routes in the placental villi in agreement with the gestational period. A co-expression of receptors and mitochondrial proteins in placental connective tissue was detected, but the immunolocalization of c-FLIP would indicate an endogenous inhibition of the extrinsic pathway. In conclusion, in swine there exists differential activation of inducing apoptotic pathways in different placental structures according to the gestational period.

Introduction

The early development in mammals is characterized by the contribution of nutrients from the maternal tissues through the placenta, which is in apposition or fusion with foetal membranes and the endometrium, allowing the physiological interchange between the embryos/fetuses and the mother (Hafez and Hafez 2002; Wooding and Burton 2008).

Early gestation in pigs is characterized by a rapid development of the uterus and the embryos (Bazer et al. 2009). The adhesion to the uterine endometrium takes place through the interdigitations between the trophectoderm microvilli and the surface uterine epithelium that are interconnected through an adherent glucocalix covering completely the maternal/foetal interface. All the chorion is in intimate contact with the uterine mucosa, without invading it nor destroying it, resulting in a diffuse, folded, adeciduate, non-invasive and true epitheliochorial placenta (Ender and Carter 2004; Merkis et al. 2005, 2006; Sanchis et al. 2011).

In the porcine placenta, between the adjacent cells of both epithelia, there are thigh junctions and desmosomes, allowing the maintenance of the epitheliochorial structure and cellular communication during the porcine gestational development (Miglino et al. 2001; Koncurat et al. 2002; Cristofolini 2010).

The apoptosis is an essential, permanent, dynamic and interactive biological process by which an organism eliminates the undesirable cells without causing an inflammatory response (Angosto 2003; Zinkel et al. 2005). The mechanisms that regulate this type of programmed cellular death are essential to the normal development and maintenance of tissue homoeostasis, specially during placentation (Barrio et al. 2003; Huppertz and Kingdom 2004; Martins et al. 2004; Straszewski-Chavez et al. 2005; Huppertz et al. 2006; Rote et al. 2010).

Apoptosis is induced through two major signalling pathways: the intrinsic mitochondrial and the extrinsic membrane receptor-mediated pathway (Angosto 2003). In the intrinsic induction, proapoptotic and antiapoptotic mitochondrial proteins, members of the family of Bcl-2, are involved. The apoptotic extrinsic route is characterized by the activation of cellular death receptors that bind to its specific ligand. The best characterized transmembrane receptors are FAS, DR4 and TNF-R1 (Angosto 2003; Hua et al. 2003; Zhang et al. 2004).

Within the triggered molecular events that follow the activation of the receptors, the recruitment and activation of procaspase-8 take place, which activates caspasas responsible for cellular dismantling (Hua et al. 2003; García Fuster 2005).

A type of apoptosis inhibitor molecule, named cellular FLICE-inhibitory proteins (c-FLIP), structurally similar to procaspase-8, has been described (Scaffidi et al. 1999; Yeh et al. 2000). The c-FLIP proteins partial or completely block the processing of procaspase-8, inhibiting the apoptotic signal (Krueger et al. 2001; Mathas et al. 2004; Mezzanzanica et al. 2004; Solé Serra 2006). c-FLIP is an important regulator of death receptor-mediated apoptosis in bovine corpus luteum and in murine embryos (Yeh et al. 2000; Hojo et al. 2010); whereas in human placentas, the presence of c-FLIP at the early and late pregnancy has been determined playing a critical role in the survival of placental cells (Ka and Hunt 2006).

In pigs, one of the mechanisms of tissue remodelling regulation during placental development and growth is the apoptosis (Merkis et al. 2007). Okano et al. (2007) have studied the porcine placental remodelling by apoptosis during the peri-implantation window and in the post-partum endometrium. Nevertheless, the involvement of apoptosis in the remodelling of porcine placentas from gestational periods after implantation is still unclear. In recent studies, we have determined different molecules involved in the

extrinsic and intrinsic apoptotic induction pathways in placentas from cross-bred swines of different gestational periods (Cristofolini et al. 2008, 2009a,b; Merkis et al. 2010). Nevertheless, in some placental structures, an overlapping of both routes has been observed (Cristofolini 2010).

To recognize some of the basic mechanisms that regulate the maintenance of porcine placental homoeostasis through the identification of molecules that take part in the cellular remodelling, will allow to deepen the knowledge about the mechanisms of the physiological apoptotic process in normal porcine pregnancy and evaluate in future, the role of this process in placental pathological mechanisms involved in failures during gestation, such as desynchronization between *concepti* and uterine microambience. These failures may produce embryonic/foetal death without apparent specific cause, reducing production in this species of high productive value in our region.

The aim of this work was to study placental cell remodelling by apoptosis in porcine placental tissues from early (28 days), mid (60 days) and late gestation (114 days). The apoptotic cell morphology will be analysed by high-resolution light microscopy and transmission electron microscopy. Detection and quantification of apoptotic cells and the rate of apoptosis (IAp) will be performed by TUNEL technique, and the expression of c-FLIP protein will be determined by immunohistochemistry. This knowledge may illuminate the functions of the apoptosis in cell remodelling of maternal–foetal interface during pregnancy in pigs.

Materials and Methods

Animals and tissue collection

Cross-bred swines from different slaughterhouses located in Río Cuarto city, Argentine (33.1° S; 64.3°O) were used. According to the *antemortem* and *post-mortem* examinations, the animals were considered free of disease. From these bristles, a total of 15 animals were processed: 28 days of gestation (n = 5), 60 days (n = 5) and upon maturity (114 days of pregnancy) (n = 5). In every case, the reproductive tract was removed immediately after slaughter, washed with saline solution of Hank′s (SSH) containing sodic penicillin G, streptomycin sulphate and fungizone (Gibco, Grand Island, NY USA) and maintained at 4°C until processing in the laboratory. Palpation was made to detect the location of the embryos or fetuses. The uterine horns were opened carefully and longitudinally with an incision on the anti-mesometrial edge to observe the implantation site. Embryos or fetuses were removed from each gestating bristle and used to the determination of the gestational age of the placentas according to their crown-rump length (Marrable 1971).

Tissue samples were taken from five placentas of every gestational period (one placenta was randomly chosen from each animal). The samples were gathered from mesometrial-endometrial and foetal placental tissues and used for light microscopy, electron microscopy, high-resolution light microscopy, TUNEL technique and immunohistochemical analysis.

Conventional histological technique

Portions of approximately 6 mm of placental tissue were fixed in 4% (v/v) buffered-saline formaldehyde pH 7.2–7.4 at 4°C, embedded in paraffin and cut in ± 4 μm histological sections.

Electron microscopy

Portions of approximately 1 mm^3 of placental tissue were fixed in 2.5% glutaraldehyde in 0.2 M S-collidine pH 7.4, post-fixed in 1% osmium tetroxide in 0.2 M S-collidine pH 7.4, dehydrated in increasing concentration acetone, embedded in EMbed 812 resin and sectioned with an ultramicrotome. The ultra-thin sections (± 60 nm) were cut and placed on cupper grids, counterstained with saturated uranyl acetate and aqueous lead citrate. The sections were examined in transmission electron microscope Elmiskop 101 (Siemens, Germany).

High-resolution light microscopy

For high-resolution light microscopy (HRLM), the placental samples processed by transmission electron microscopy technique were used. An ultramicrotome was used to obtain the semi-thin sections (± 0.25 μm). These sections were counterstained with toluidina′s blue and were cover-slipped in DPX (Merk, Alemania) embedding agent. They were then observed in a light microscope Axiophot (Carl Zeiss, Thornwood, NY, USA) fitted with a high-resolution digital camera Powershot G6 7.1 megapixels (Canon INC, Tokyo, Kanto, Japan). Digital images were captured with Axiovision 4.6.3 software (Carl Zeiss, Göttingen, Germany).

TUNEL technique, quantification of apoptotic index and statistical analysis

Paraffin-embedded tissues were used for TUNEL technique. Nuclei DNA fragmentation was detected *in situ* using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method (ApopTag® Plus Peroxidase In Situ Apoptosis; Chemicon International, Temecula, CA, USA) (Schmitz et al. 1991). Analysis was conducted as described in kit protocol, following the recommendation of a pretreatment of slides with tritium. The results were expressed as quantitative. From the relation between the number of cells with fragmentation of DNA and the number of total cells, the apoptotic index (IAp) for the different periods gestation was determined, using the following formula:

$$\mathrm{IAp} = \frac{\mathrm{TUNEL-positive\ cells}}{\mathrm{Total\ cells}} \times 100$$

The quantification was carried out by a single operator on two slides per animal/placenta, two sections per slide and five fields per section. Values were expressed in means and standard deviations. An ANOVA test was conducted to assess the effect of day of gestation on the IAp index in placental villi. A *post hoc* LSD Fisher test was performed when a

statistically significant difference was found. The software InfoStat version 2009 was used.

Immunohistochemical analysis

Immunohistochemical techniques were performed in paraffin-embedded tissues using mouse monoclonal antibody anti-c-FLIP$_{s/l}$, working dilution 1/50 (G-11: sc-5276, Santa Cruz, Biotechnology, Inc. USA); biotinylated secondary antibodies pool, streptavidin conjugated to horseradish peroxidase (LSAB®+Systems HRP; Dako Cytomation, Glostrup, Region Hovedstaden, Denmark) and 3,3′-diaminobenzidine chromogen solution (Liquid DAB+Substrate Chromogen System, Dako Cytomation). The sections were counterstained with Mayer's haematoxylin and cover-slipped in Entellan (Merk) embedding agent. We confirmed in subsequent experiments that omission of the primary antibody served as a sufficient control. Therefore, a negative control consisted of omission of the primary antibody. The staining intensity was evaluated by a semi-quantitative scoring system as follows: (−): negative, (+): weak, (++): moderate and (+++): strong immunostaining. Simultaneously, immunohistochemical negative controls were carried out. Photomicrographs of representative fields of immunohistochemistry were evaluated with an Axiophot microscope (Carl Zeiss) fitted with a high-resolution digital camera Powershot G6 7.1 megapixels (Canon INC). Digital images were captured with Axiovision 4.6.3 software.

Results

The presence of uterine and trophectoderm epithelial cells with structural and morphologic characteristics associated with the apoptotic phenomenon was determined in the different gestational periods analysed by high-resolution light microscopy. At Day 28 of pregnancy in the apex of placental villi, uterine epithelial cells containing a big central nucleus, abundant euchromatin and one or two compact nucleoli were observed. Between these epithelial cells, some cells with evident apoptotic characteristic were found, with decreased cellular size, pycnotic nuclei and heterochromatin arranged on the internal face of the nuclear membrane (Fig. 1).

In placental villi of 60 days of gestation, apoptotic bodies phagocyted by neighbouring maternal epithelial cells were observed. These cells would be acting as non-professional phagocytes, eliminating the genetic fragmented material and cell remains packed into small, plasmatic membrane-bound, sealed vesicles, as result of the process of cellular death by apoptosis (Fig. 2).

By transmission electron microscopy (TEM), placental cellular ultrastructure was analysed. In the trophoblast epithelium from placental samples at late gestation, multiple nuclear fragments were observed, which were forming dense bodies attached to the internal face of the nuclear membrane, morphological changes characteristic of an early apoptotic stage (Fig. 3).

At Day 28 of pregnancy analyses by TEM showed chorionic cells with signs of an advanced apoptotic process, with decreased cellular size, condensation of cytoplasm, increase in intracellular density, chromatin condensation and arranged of chromatin in finely granular masses of uniform texture that adhere to the inner surface of the nuclear membrane (Fig. 4).

Nuclei at very initial phases of chromatinic marginalization can be also identified with TUNEL technique. In Figure 5a–f, apoptotic nuclei are observed in different placental structures during porcine pregnancy. TUNEL-positive nuclei were observed in villi of 28, 60 and 114 days of pregnancy, respectively (Fig. 5a,b,c). We have highlighted the presence of apoptotic cells in glands of different stages of functionality (Fig. 5d). We have shown TUNEL-positive cells in blood vessels at the early pregnancy (Fig. 5e) and the presence of phagocytes cells containing apoptotic bodies TUNEL-positive in the uterine stroma at Day 60 of gestation (Fig. 5f).

Figure 6 expresses the quantification of apoptotic cells determined through the apoptotic index (IAp). In placental villi of early pregnancy, a IAp of 9.34% was detected; at mid-gestation, the apoptotic index decreased to 0.82% and increased considerably (23.85%) towards the end of pregnancy. Statistically significant differences were detected in the IAp of

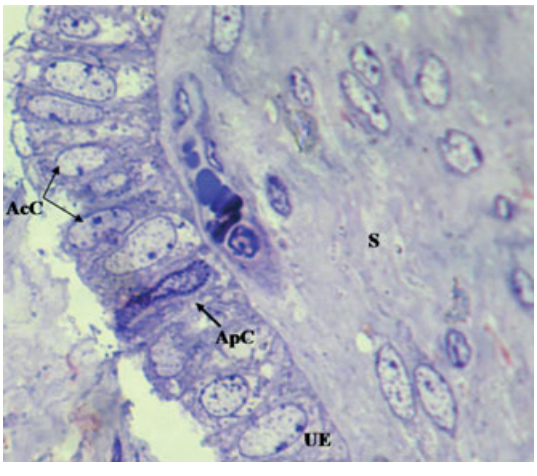

Fig. 1. Photograph of HRLM at Day 28 of pregnancy. Uterine epithelial cells with apoptotic characteristic were observed. AcA, active cells; ApC, apoptotic cells; S, strome; UE, uterine epithelium (1000×)

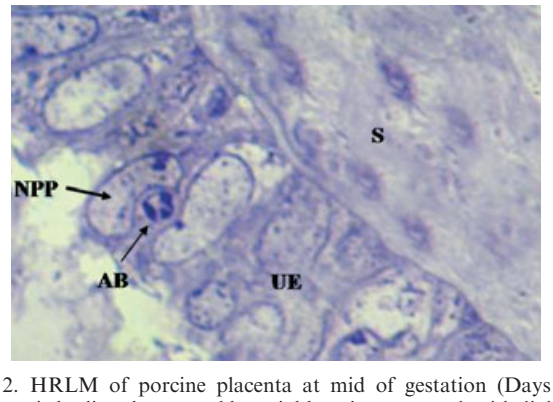

Fig. 2. HRLM of porcine placenta at mid of gestation (Days 60). Apoptotic bodies phagocyted by neighbouring maternal epithelial cells were observed. AB, apoptotic bodies; NPP, non-professional phagocyted; S, strome; UE, uterine epithelium (1000×)

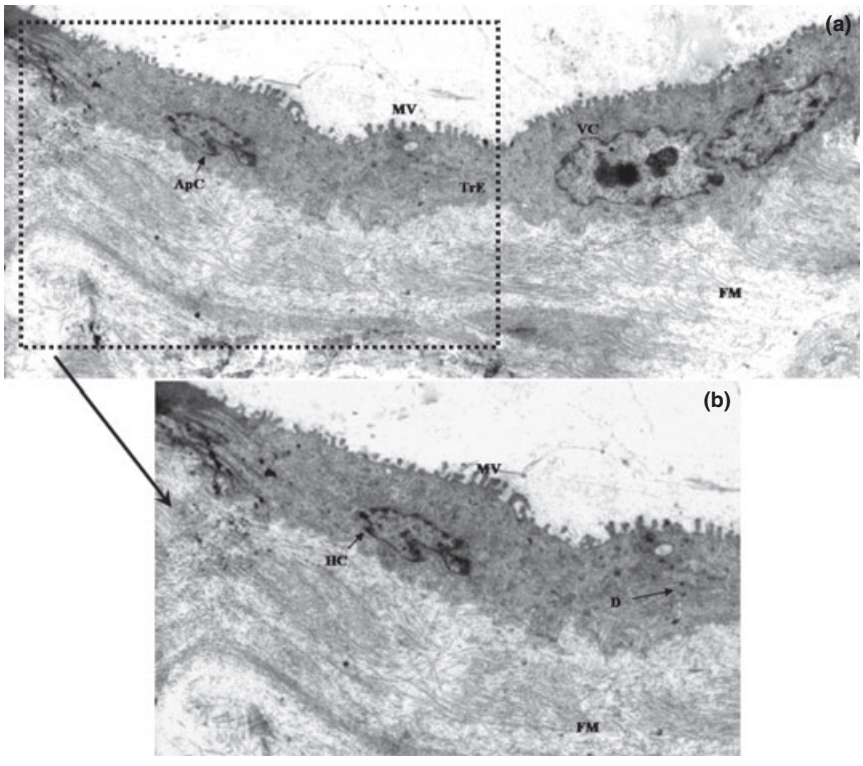

Fig. 3. Microphotograph of TEM from porcine placenta at late gestation. Trophoblastic epithelial cells with apoptotic characteristic were observed. ApC, apoptotic cell; D, desmosome; FM, foetal mesenchyme; HC, heterochromatin condensed; MV, placental microvilli; TrE, trophoblastic epithelium; VC, viable cell (a: 2784×; b: 3500×)

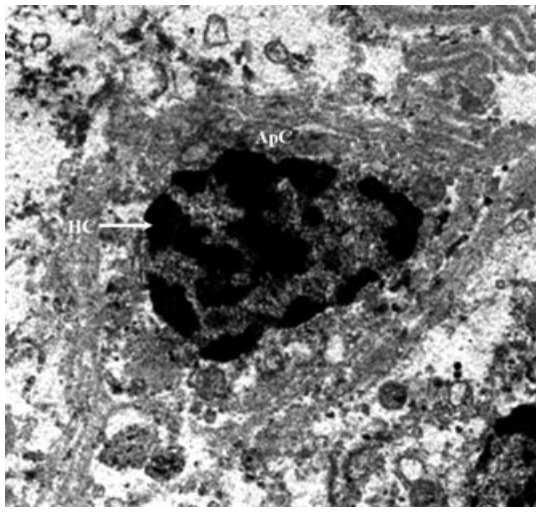

Fig. 4. Microphotograph of TEM at early gestation were showed chorionic apoptotic cell. ApC, apoptotic cell; HC, heterochromatin condensed (3600×)

placental villi with regard to the gestational period ($p < 0.05$).

We have determined the immunolabeling intensity of the c-FLIP protein in villi, strome and mesenchymal connective tissues, blood vessels and glandular tissue of placental samples at early, mid and late porcine pregnancy (Table 1). At Days 28, 60 and 114 negative immunostaining of c-FLIP was found in the uterine luminal and trophoectoderm epithelia, in the glandular epithelium and in blood vessels. The c-FLIP immunoreactivity observed in stroma and foetal mesenchyme was moderate in placentas of Days 28 and 60 and weak at late pregnancy (114 days) (Fig. 7a,b,c). No inespecific c-FLIP binding was detected in negative control slide (Fig. 7d).

Discussion

In previous studies, we have documented that as porcine gestation advances and the nutritional demand grows due to the continuous growth and development of the *concepti*, the placenta undergoes a physiological process of remodelling necessary for the regulation of the materno/foetal exchange, allowing a suitable balance that assures a successful pregnancy (Merkis et al. 2005, 2007). These physiological changes observed in the placental porcine structure, such as progressive increase in density of blood vessel of small calibre at Day 55 of gestation and increase in the epithelial area due to the development of tertiary placental villi at late pregnancy are indispensable to the exchange of substances and nutrients between the mother and the *concepti*, allowing the individual development of every embryo in the uterine horns (Merkis et al. 2005, 2006).

The relation between the programmed cellular death by apoptosis and the physiological maintenance of tissue homoeostasis, especially in pregnant uteri, has been studied in several species (Huppertz and Kingdom 2004; Kokawa et al. 1996; Martins et al. 2004; Straszewski-Chavez et al. 2005).

In pigs, we have demonstrated the expression of Bax protein in cells of maternal/foetal interface at early and late pregnancy, demonstrating that the apoptotic phenomenon would be triggered through the signalling of the intrinsic pathway (Cristofolini et al. 2009a,b), although at mid-gestation, we have determined the presence of cellular death transmembrane receptors FAS and other members of super-

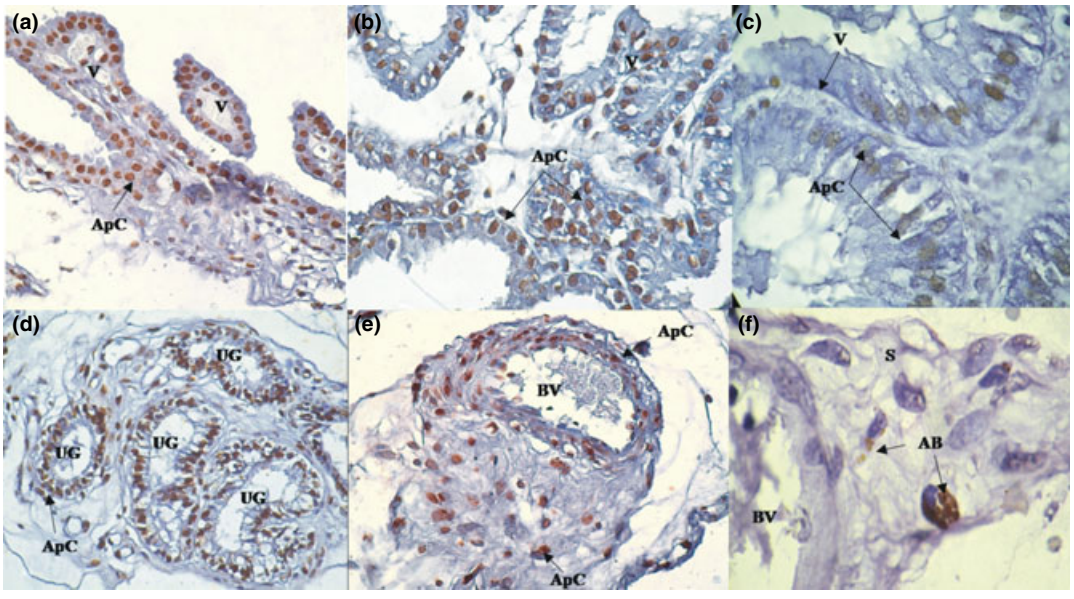

Fig. 5. Photograph the TUNEL technique in different placental structures during porcine pregnancy. (a, b, c) Apoptotic nuclei in villi of 28, 60 and 114, days of pregnancy, respectively. (d) Apoptotic cells in gland. (e) TUNEL-positive cells in blood vessels of Day 28. (f) Phagocytes cells containing apoptotic bodies in the uterine stroma at Day 60. AB, apoptotic bodies; ApC, apoptotic cell; BV, blood vessels; UG, uterine glands; V, villi (*a*: 200×; *b, d and e*: 400×; *c* and *f*: 1000×)

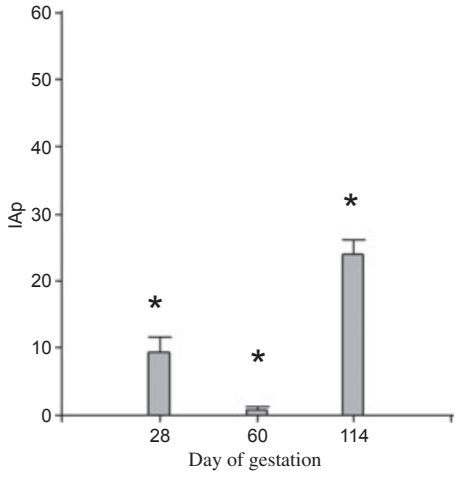

Fig. 6. Quantitative analysis of apoptotic cells (IAp) in placental villi at 28, 60 and 114 days of pregnancy. Values are expressed as means ± SD. *p < 0.05

Table 1. Semi-quantitative determination of immunolocalization of c-FLIP in uterine epithelium, trophoblastic epithelium, stroma, foetal mesenchyme, maternal blood vessels, foetal blood vessels and uterine glands in porcine placenta of 28, 60 and 114 days of pregnancy [(−): negative immunostaining, (+): weak immunostaining, (++): moderate immunostaining and (+++): strong immunostaining]

Gestacional period (days)	Villi c-FLIP	Connective tissue c-FLIP	Blood vessels c-FLIP	Gland c-FLIP
±28	UE (−)	S (++)	MBV: (−)	UG(−)
	TrE (−)	S (++)	FBV: (−)	
±60	UE (−)	S (++)	MBV: (−)	UG(−)
	TrE (−)	FM (++)	FBV: (−)	
±114	TrE (−)	FM (+)	FBV: (−)	

UE, uterine epithelium; TrE, trophoblastic epithelium; S, strome; FM, fetal mesenchyme; MBV, maternal blood vessels; FBV, fetal blood vessels, U, uterine glands.

family TNF-R1, demonstrating the induction of the extrinsic pathway in placental villi (Cristofolini et al. 2008; Merkis et al. 2010). The co-expression of membrane receptors and mitochondrial proteins observed in stroma and foetal mesenchyme demonstrates a very complex interconnection of specific signals of cellular death throughout the placentation in the pig (Cristofolini 2010).

In the present report, of immunoexpression of protein c-FLIP in stroma and foetal mesenchyme throughout the placentation in swine, we have established the action of the endogenous inhibitor c-FLIP, blocking the extrinsic signalling of membrane receptors. This demonstrates that the apoptotic process observed in stroma and foetal mesenchyme throughout the porcine gestation is mediated by the intrinsic pathway induction. This is in agreement with other authors, who have informed that the overlapping of both pathways in the majority of conditions is minimal (Zinkel et al. 2005; Hojo et al. 2010).

TUNEL technique allowed us to identify apoptotic cells in every placental structure of the gestational periods analysed. Moreover, the nuclei at very initial phases of chromatinic marginalization could also be identified. We highlight the finding of professional and non-professional phagocytic cells, containing TUNEL-positive apoptotic bodies inside, in the endometrium of placentas of 60 day of pregnancy, demonstrating the apoptotic process (Cristofolini 2010).

In the present study in the maternal/foetal interface statistically significant difference of the apoptotic index (IAp) was detected in accordance with the gestational period. At early pregnancy, a high rate of apoptotic cells was detected, allowing the maintenance of tissue homoeostasis through a suitable cellular remodelling

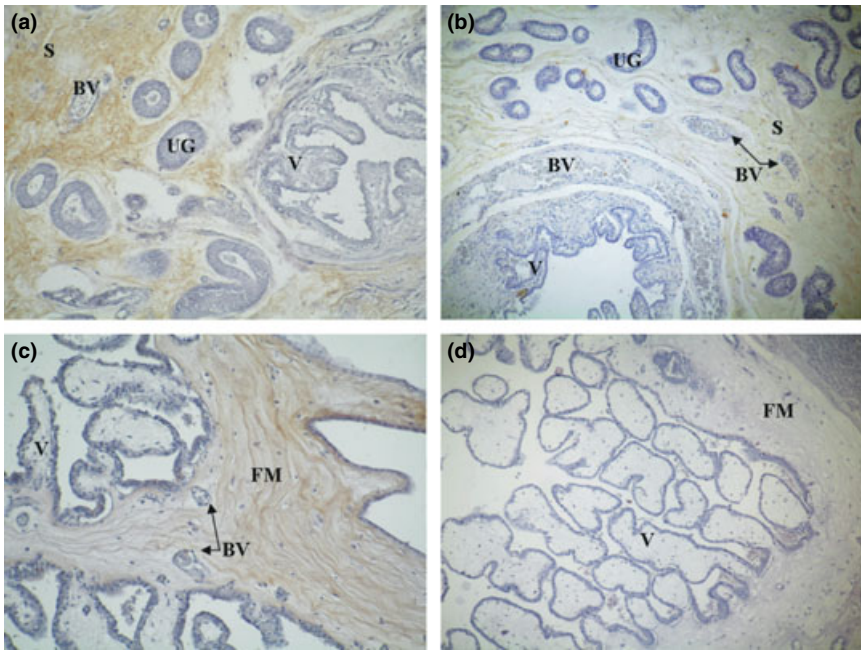

Fig. 7. Immunoexpression of c-FLIP in different structures of porcine placenta at 28 (*a*), 60 (b) and 114 (c) days of gestation. (d), negative control**.** BV, blood vessels; FM, foetal mesenchymal; (S), strome; UG, uterine glands; V, villi. (a, c and d: 200×; b: 100×)

in the interface, allowing the materno/foetal contact in this period of pregnancy. On the other hand, towards the mid of pregnancy, a significant decrease of IAp was observed, which coincides with the period of maximum placental growth, approximately Day 60 of pregnancy, when fetuses mostly increase in size with respect to placental development (Merkis et al. 2005). However, the highest IAp was observed in placental villi at term, indicating the fundamental role of programmed cell death in epitheliochorial and non-invasive placenta. The increase in apoptosis expression results necessary for the induction of farrowing. These findings are consistent with the previously established by Bertoja et al. (2005), Huppertz et al. (2006) and Martins et al. (2004) in murine, human and bovine placentation, respectively, and with our finding in goat placenta (unpublished data). In this way and given the type of placentation in swines, the apoptosis in term pregnancies would be responsible for the loss of contact between trophoblastic and maternal epithelial cells, allowing the elimination of foetal membranes during the farrowing, without loss of maternal tissues. According to Okano et al. (2007), the apoptosis would be involved in tissue remodelling, allowing the maintenance of a proper tissue balance necessary for uterine involution in sows during post-farrowing.

The implementation of the high-resolution light microscopy and transmission electron microscopy allowed us to determine morphological changes associated with the apoptotic phenomenon in placental cells, during porcine pregnancy. Especially, high-resolution light microscopy provided a structural study of porcine placenta with higher definition and resolution than using a conventional light microscopy, allowing the localization of phagocytic epithelial cells with apoptotic bodies. Moreover, we emphasize the use of high-resolution light microscopy and transmission electron microscopy as excellent complementary tools for the detection of apoptotic process in the placenta of pigs (Lynch et al. 2005; Cristofolini 2010).

In conclusion, we have shown the apoptotic phenomenon in different gestational periods of swines, which is involved in the maintenance of internal physiological balance of the placenta, mainly in the maternal/foetal interface, ensuring the nutrition of the *concepti* and facilitating the mechanisms of delivery. Through this work, we describe an interconnection and regulation of cell death signals in different placental structures during pregnancy in cross-bred gilts. The knowledge of the mechanisms that influence the apoptosis pathways during pig placentation may help to generate some strategies in the future to increase porcine production. The relevance of our findings is based on the deepening of knowledge about swine placental physiology, especially in placental remodelling in species of high productive value, such as the pig, characteristic of our geographical livestock activity.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Cristofolini A and Sanchis G contributed in acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and final approval of the version to be submitted; Moliva M, Alonso L, Chanique A and Koncurat M contributed in the acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be submitted; Merkis contributed in the conception and design of the study, acquisition of data, analysis and interpretation of data, revising the article critically for important intellectual content and final approval of the version to be submitted.

References

Angosto M, 2003: Bases moleculares de la apoptosis. Anal Real Acad Nal Farm **69**, 36–63.

Barrio E, Calvo M, Romo A, Alvarez R, Gutierrez J, Fernández Longás A, 2003: Crecimiento intrauterino restringido: estudio de la apoptosis en la placenta. An Pediatr **58**, 51–54.

Bazer F, Spencer T, Johnson G, Burghardt R, Wu G, 2009: Comparative aspects of implantation. Reproduction **138**, 195–209.

Bertoja A, Zenclussen M, Wollenberg I, Paeschke S, Sollwedel A, Gerlof K, Woiciechowsky C, Volk H, Zenclussen A, 2005: Upregulation of Bcl-2 at the foetal-maternal interface from mice undergoing abortion. Scand J Immunol **61**, 492–502.

Cristofolini A, 2010: Estudio de la remodelación celular durante la placentación porcina. Tesis Doctoral. Biblioteca Universidad Nacional de Río Cuarto.

Cristofolini A, Merkis C, Barroso F, Vaquer V, Lloret M, Moschetti E, Koncurat M, 2008: Detección de fibrinógeno, FAS B-10, FAS ZB4 y FAS C-20 durante la placentación porcina**.** REDVET. Available: http://www.veterinaria.org/revistas/redvet/n070708.html. (accessed 1 July 2008).

Cristofolini A, Merkis C, Barroso F, Vaquer Balmaceda V, Allende F, Chanique A, Koncurat M, 2009a: Inmunolocalización de proteínas mitocondriales reguladoras durante la placentación porcina. REDVET. Available: http://redalyc.uaemex.mx/src/inicio/ArtPdfRed.jsp?iCve=63617144001. (accessed 15 September 2009).

Cristofolini A, Merkis C, Barroso F, Vaquer Balmaceda V, Allende F, Chanique A, Koncurat M, 2009b: Inmunoreactividad de las proteínas Bcl-2 y Bax durante la placentación porcina. RAPA **30**, 1–7.

Ender A, Carter A, 2004: What can comparative studies of placental structure tell us?-A review. Placenta **25**, S3–S9.

García Fuster M, 2005: La vía de señalización del receptor apoptótico FAS en la adicción a opiáceos. Tesis Doctoral. Universitat de Barcelona. Available: http://hdl.handle.net/10803/984. (accessed 1 July 2005).

Hafez E, Hafez B 2002: Reproducción e inseminación artificial en animales. Ed McGraw-Hill, DF, Mexico.

Hojo T, Al-Zi'Abi M, Komiyama J, Manabe N, Acosta T, Okuda K, 2010: Expression and localization of cFLIP, and antiapoptotic factor, in the bovine corpus luteum. J Reprod Dev **56**, 230–235.

Hua Z, Sohn S, Kang Ch, Cado D, Winoto A, 2003: A Function of Fas-associated death domain protein in cell cycle progression localized to a single amino acid at its C-terminal region. Immunity **18**, 513–521.

Huppertz B, Kingdom J, 2004: Apoptosis in the trophoblast-role of apoptosis in placental morphogenesis. J Soc Gynecol Investig **11**, 353–362.

Huppertz B, Kadyrov M, Kingdom J, 2006: Apoptosis and its role in the trophoblast. Am J Obstet Gynecol **195**, 29–39.

Ka H, Hunt J, 2006: FLICE-inhibitory Protein: expression in early and late gestation human placentas. Placenta **27**, 626–634.

Kokawa K, Shikone T, Nakano R, 1996: Apoptosis in the human uterine endometrium during the menstrual cycle. J Clin Endocrinol Metab **81**, 4144–4147.

Koncurat M, Maglione C, de Vargas V, 2002: Ultraestructura placentaria porcina en diferentes períodos gestacionales. Rev Cs Vet **3**, 1–6.

Krueger A, Baumann S, Krammer P, Kirchhoff S, 2001: FLICE inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol **21**, 8247–8254.

Lynch C, Johnson J, Vaccaro C, Thurberg B, 2005: High-resolution Light Microscopy (HRLM) and digital analysis of Pompe disease pathology. J Histochem Cytochem **53**, 63–73.

Marrable AW, 1971: The Embryonic Pig: A Chronological Account. Pitman Medical, London, England.

Martins V, Marques Junior A, Vasconcelos A, Martins E, Santos R, Lima F, 2004: Placental maturation and expulsion in Holstein and Nelore cows. Arq Bras Med Vet Zoote **56**, 157–167.

Mathas S, Lietz A, Anagnostopoulos I, Hummel F, Wiesner B, Janz M, Jundt F, Hirsch B, Jöhrens-Leder K, Vornlocher H, Bommert K, Stein H, Dörken B, 2004: c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor–induced apoptosis. J Exp Med **199**, 1041–1052.

Merkis C, Cristofolini A, Franchino M, Moschetti E, Koncurat M, 2005: Relación entre Area Total y Area Epitelial de vellosidades placentarias porcinas en diferentes estadíos gestacionales. InVet **7**, 47–54.

Merkis C, Cristofolini A, Franchino M, Sanchis E, Moschetti E, Koncurat M, 2006: Angiogénesis placentaria durante la gestación porcina. REDVET. Available: http://www.veterinaria.org/revistas/redvet/n040406.html. (accesssed 1 April 2006).

Merkis C, Cristofolini A, Koncurat M, 2007: Apoptotic phenomena during porcine placentation. REDVET.Available: www.veterinaria.org/revistas/redvet/n010107.html. (accesssed 1 January 2007).

Merkis C, Cristofolini A, Sanchis E, Koncurat M, 2010: Expression of death cellular receptors FAS/CD95 and DR4 during porcine placentation. En revisión por consultores. Int J Morph **28**, 829–834.

Mezzanzanica D, Balladore E, Turatti F, Luison E, Alberti P, Bagnoli M, Figini M, Mazzoni A, Raspagliesi F, Oggionni M, Pilotti S, Canevari S, 2004: CD95-Mediated Apoptosis is impaired at receptor level by cellular FLICE-inhibitory protein (long form) in wild-type p53 human ovarian carcinoma. Clin Cancer Res **10**, 5202–5214.

Miglino M, Pereira F, Santos T, Carvalho F, 2001: A morfologia placentaria dos suinos domesticos. Revisao Arq Cien Vet Zool **4**, 71–76.

Okano A, Ogawa H, Takahashi H, Geshi M, 2007: Apoptosis in the porcine uterine endometrium during the estrous cycle, early pregnancy and post partum. J Reprod Dev **53**, 923–930.

Rote N, Wei B, Xu C, Luo L, 2010: Caspase 8 and human villous cytotrophoblast differentiation. Placenta **31**, 89–96.

Sanchis G, Taglialegna A, Cristofolini A, Merkis C (2011) Porcine uterine and placental extracellular matrix molecules throughout pregnancy. Int J Morph **29**, 1438–1443.

Scaffidi C, Schmitz I, Krammer P, Peter M, 1999: The role of c-FLIP in modulation of CD95-induced apoptosis. J Biol Chem **274**, 1541–1548.

Schmitz G, Walter T, Seibl R, Kessler C, 1991: Nonradioactive labelling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. Anal Biochem **192**, 222–231.

Solé Serra C, 2006: Estudio de FAIM y FLIP: dos moléculas antagonistas del receptor FAS, reguladas por NGF y con efectos promotores del crecimiento neurítico. Tesis Doctoral. UdL CMB Ciències Mèdiques Bàsiques.

Straszewski-Chavez S, Abrahams V, Mor G, 2005: The role of a Apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. Endocr Rev **26**, 877–897.

Wooding P, Burton G, 2008: Comparative Placentation. Structures, Functions and Evolution. Springer-Verlag, Berlín Heidelberg.

Yeh W, Itie A, Elia A, Ng M, Shu H, Wakeham A, Mirtsos C, Suzuki N, Bonnard M, Goeddel D, Mak T, 2000: Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity **12**, 633–642.

Zhang J, Zhang D, Hua Z, 2004: FADD and its phosphorylation. IUBMB Life **56**, 395–401.

Zinkel S, Hurov K, Ong C, Abtahi F, Gross A, Korsmeyer S, 2005: A role for proapoptotic BID in the DNA-damage response. Cell **122**, 579–591.

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