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Chapter 6

Apoptosis as the Major Cause of Embryonic Mortality in Cattle

Helena Moreira da Silva, Loide Isabel Valadão and Fernando Moreira da Silva

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Abstract

Besides several mechanisms such as autophagy, necroptosis, and pyroptosis, programmed cell death (PCD) also includes apoptosis which is characterized by membrane blebbing, chromatin condensation, and DNA fragmentation which involves a number of membrane receptors and a cascade of signal transduction steps resulting in the activation of an ample number of proteases known as caspases. Even though this mechanism plays a significant role in the progressions of gamete maturation and embryo development, contributing to the formation of different organs and structures, they also lead to the death of blastomeres and, consequently, the occurrence of structural abnormalities, increasing embryo and fetal mortality. Therefore, understanding the mechanisms involved in apoptosis dysregulation may contribute to the development of new therapeutic methods to prevent various developmental abnormalities. The purpose of this chapter is to review recent research into the mechanisms of programmed cell death, focusing on apoptosis during embryo development leading to embryo mortality. The final section includes a discussion of the implication of the findings for future research on reducing embryo mortality in the bovine.

Keywords: apoptosis, bovine, embryo mortality

1. Introduction

During apoptosis, cell suicide is controlled by the genes involved in the induction or prevention of apoptosis. These genes play crucial roles in the activation/inactivation mechanisms of apoptosis, upstream/downstream of effector molecules, and in the signaling pathways, which will be reviewed below.

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In fact, apoptosis was primarily observed by Karl Vogt in Neuchâtel, Switzerland, in the year of 1842, although he did not use this term. He noticed, in the midwife toad (*Alytes obstetricans*) embryos, that cells in the notochord, a cartilaginous skeletal structure, would disappear during development, being replaced by cells of the vertebrae. Despite having documented that some cells disappeared during development, Vogt did not focus his research on this phenomenon. This problem was addressed again only when Walther Flemming, using more advanced staining techniques on the cell nucleus, observed what he called chromatolysis, the diminishing of nuclear material in dying cells. He provided then a more precise description of the whole process in 1885. However, only in 1965, this topic was addressed once again [1].

Although PCD is a term often used as a synonym to the designation of apoptosis, which indicates an endogenous cell suicide program used to eliminate useless or damaged cells, in fact, other forms of regulated cell death, e.g., the autophagy, necroptosis, and pyroptosis, included in the non-apoptotic pathways of PCD exist [1–3]. During embryogenesis, the elimination of the cell by apoptosis is an important way of molding the tissues and shaping the body. But apoptosis occurs not only during embryo development. It occurs also after birth and during cell turnover and tissue homeostasis [4]. For example, it occurs in brain cells, which undergo apoptosis either prior to or after birth, to eliminate excess brain cells and streamline nerve impulses. Apoptosis also occurs in some cancers, where activation of the apoptotic pathways may prevent the spread of neoplastic cells and contain the cancer. However, dysregulation of apoptosis is also associated with aging decline, multiple diseases (e.g., Alzheimer's disease and amyotrophic lateral sclerosis), and malignant cancer, as well as abnormalities in development [5–7].

Embryo mortality is generally defined as the loss of the conceptus before day 42 of pregnancy, i.e., before the complete development of the organs. It is well documented that about 30% of all embryos are not able to survive. Of all lost embryos, approximately 80% of the embryos are lost before day 17, while 10–15% are lost between days 17 and 42. Only 5% of pregnancies are lost after the 42nd day [8].

Although diverse causes for early mortality exist, it is well documented that short-term exposure to heat stress before and after insemination results in low conception rates or embryonic death, due to an elevated uterine temperature, affecting cattle reproductive performance mainly in spring and summer. Other than the temperature, nutritional factors, infectious agents, and animal's welfare in general, contribute to low conception rates resulting from embryo mortality. Whether this is due to an abnormal hormonal environment, such as reduced progesterone secretion, or the ovulation of a defective oocyte has not been determined [8].

Previous *in vitro* and *in vivo* studies by our group [2] demonstrated that situations causing discomfort to cattle were associated to the activation of the genes responsible for embryo apoptosis, including Cx43, CDH1, DNMT1, and HSPA14, resulting in an acceleration of apoptosis in embryonic cells.

The apoptosis mechanism acts on three levels [9]: at the membrane level, where specific receptors mediating death signals have been identified; at the nuclear level, as the genome itself contains genes that are transcribed in response to molecules triggering the apoptotic process

(e.g. p53); and at the cytoplasm level, where the signal transduction pathways actuate in response to diverse stimuli. These signal transduction pathways are different for each initial receptor and generally integrate cysteine proteases—the so-called "caspases" (CASP)—which are the central regulators of apoptosis. The initiator caspases (including, CASP-2, -8, -9, and -10) are closely coupled to pro-apoptotic signals. Once these caspases are activated, they cleave and activate downstream effector caspases (including CASP-3, -6, and -7), which in turn execute apoptosis by cleaving cellular proteins.

2. Mechanisms of apoptosis

Apoptosis has been described as a stepwise process [3], which includes cell's shrinking and loss of intercellular connections, condensation of chromatin, blebs appearing in the membrane cells, collapse of nucleus, and finally the collapse of the cells in different fragments—the so-called apoptotic bodies—further engulfed by the phagocytes or the neighboring cells [9].

The first step respects the onset of apoptosis, which can be achieved by different pathways: the intrinsic pathway (also named mitochondrial pathway) and the extrinsic pathway (also called dead receptor pathway) are the two best understood pathways. But some authors [9–11] refer to an additional pathway involving T-cell-mediated cytotoxicity and the perforin-granzyme-dependent killing of the cell via either granzyme A or granzyme B. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution, pathway, which is initiated by the cleavage of CASP-3 [9] (**Figure 1**), starting step two of the process. Apoptosis is considered an irreversible process from this point on; once the effector caspases are activated, the death of the cell always occurs [12].

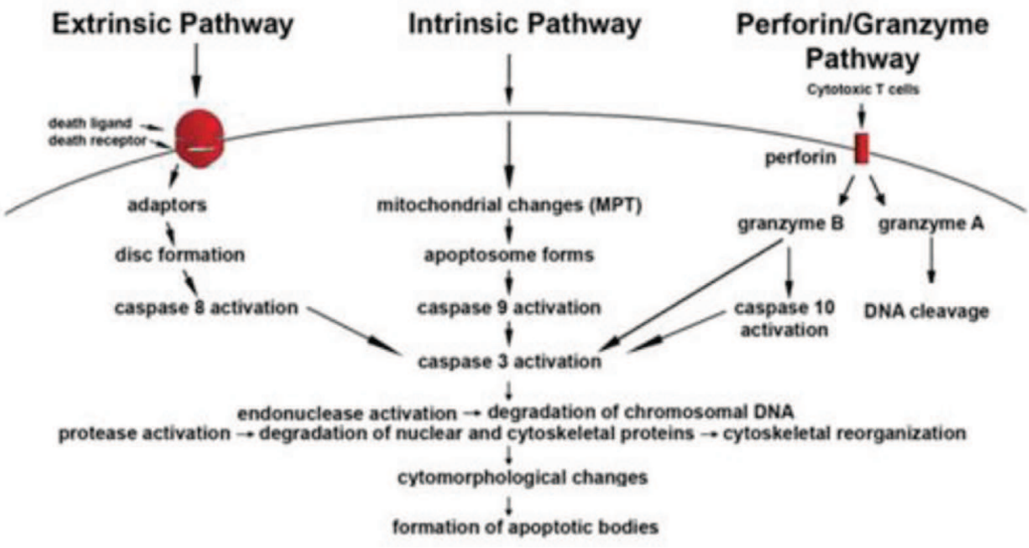

Figure 1. Schematic representation of apoptotic events (adapted from [9]).

Resulting from the action of activated effector caspases, nuclear chromatin marginates and condenses, while the DNA undergoes fragmentation. Taking a morphological approach, the chromatin condensation that occurs in an interphase nucleus upon incubation in a mitotic extract is very similar to chromatin condensation occurring in an apoptotic nucleus as DNA ends up being broken up into smaller pieces by enzymes that are activated as a part of the apoptotic program [13]. This fragmentation can be visualized by gel electrophoresis since it produces such a characteristic pattern. Terminal deoxynucleotidyl transferase dUTP nick-end labeling is a commonly used assay that can detect DNA fragmentation. This method is usually referred to as TUNEL assay. This assay labels 3′-OH DNA ends that are produced during apoptotic DNA cleavage. The characteristic nuclear feature of apoptosis can also be histologically seen as shrunken or fragmented nuclei when the cells are labeled with DNA-binding dyes, such as the Hoechst [14].

Nowadays, there are several fluorescent probes that can be used to monitor caspase activity in living cells.

After DNA fragmentation, the cytoplasmic membrane bulges irregularly, due to the decoupling of the cytoskeleton from the plasma membrane. The bulge sooner or later will bleb off from the plasma membrane, taking part of the cytoplasm with it. The irregularly shaped cell membrane loses the ability to maintain the intercellular connections with the neighboring cells, and also the integrity required to maintain transmembrane gradients [15]. The blebbing of the apoptotic cellular membrane can be easily observed on electron micrographs. It is usually described as an early feature distinguishing apoptotic and necrotic cell. Membrane blebbing has been associated to caspase-mediated activation of the Rho-associated Kinase ROCK I [16]. The membrane blebbing is followed by cell fragmentation into membrane-bound apoptotic bodies (**Figure 2**), containing cytoplasm and highly packed organelles. The recruitment of local phagocytes ensures the clearance of dying cellular debris, which is why apoptosis does not incite an inflammatory reaction [40]. Besides, they also have a protective role for surrounding cells and may serve as a signal for the recruitment of progenitor cells, for tissue regeneration. Recently, it has also been hypothesized that some microblebs could be released from the apoptotic cell that could be used as a biomarker for disease [17].

Externalization of phosphatidylserine residues at the outer plasma membrane of apoptotic cells may be detected via Annexin V, a dye that signals the existence of disrupted cell membranes [19]. Bounding with FITC-labeled Annexin V (for fluorescence) allows the identification of apoptotic cells in fluorescent microscopy. A strength in this technique is the sensitivity (they can detect a single apoptotic cell at an early stage of the process). However, it has the disadvantage to also label the membranes of necrotic cells, since they also present loss of membrane integrity. Annexin is often used in conjunction with a vital stain, such as propidium iodide, to distinguish between cells in early or late apoptosis, or the propidium iodine and trypan blue to differentiate cells in apoptosis and necrosis [9].

2.1. The extrinsic pathway

The extrinsic pathway is activated by extracellular ligands that bind to the cell-surface death receptors (e.g., FAS ligand or other TNF superfamily receptors), a transmembrane protein, which leads to the activation of inducer caspases 8 and 10 [20]. Different ligands have their specific cell death-receptors, and thereby, different stimuli may use different receptors.

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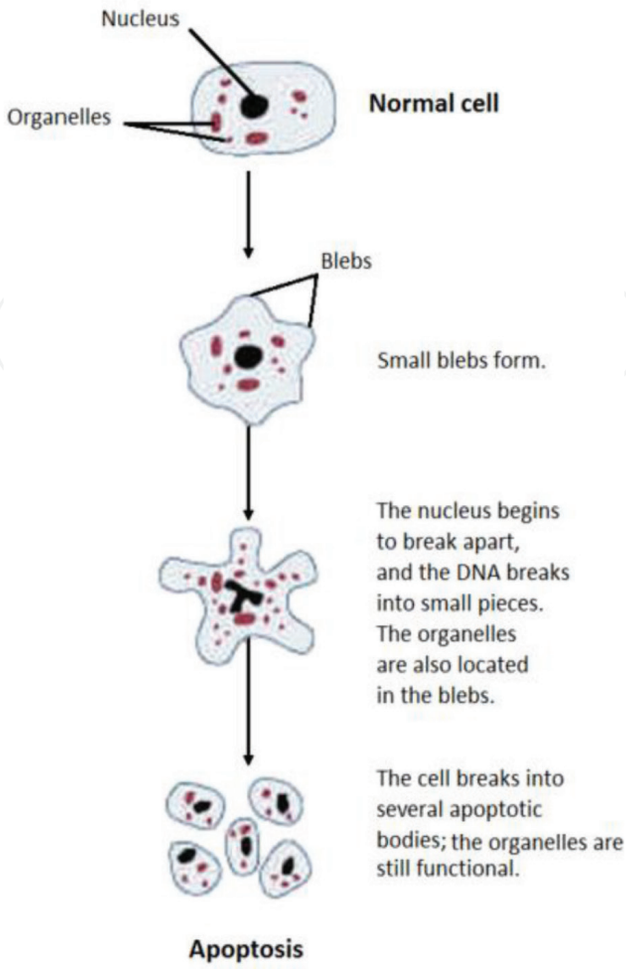

Figure 2. Schematic representation of apoptosis (adapted from Wikipedia [18]).

Activation of the cell death receptors at the cell surface induces the formation of a death-inducing signaling complex (DISC) that activates CASP-8, which is released into the cytosol to cleave the effector CASP-3, -6 and -7 (**Figure 3**). These caspases are associated with the breakdown of the cell cytoskeleton and the activation of an endonuclease, which will induce the DNA fragmentation [9].

The intrinsic pathway can also link to the extrinsic pathway, as a way to amplify the apoptotic phenomenon. This link is established through the interaction of CASP-8 with one of the pro-apoptotic molecules of the Bcl-2 family, that will stimulate the intrinsic cascade. Besides the DISC activation of CASP-8, this caspase can also be activated by a feedback loop derived from the intrinsic apoptotic pathway mediated by the release of cytochrome C from the cell mitochondria [21].

2.2. The intrinsic pathway

The intrinsic pathway is activated by intracellular endogenous or exogenous stimuli, such as ischemia, oxidative stress, or in response to chemical stressors or other stimuli originating chromatin fragmentation. Disruption of the intracellular homeostasis is at the origin of the release of signals for cell suicide, like the release of cathepsins from the lysosomal lumen [22].

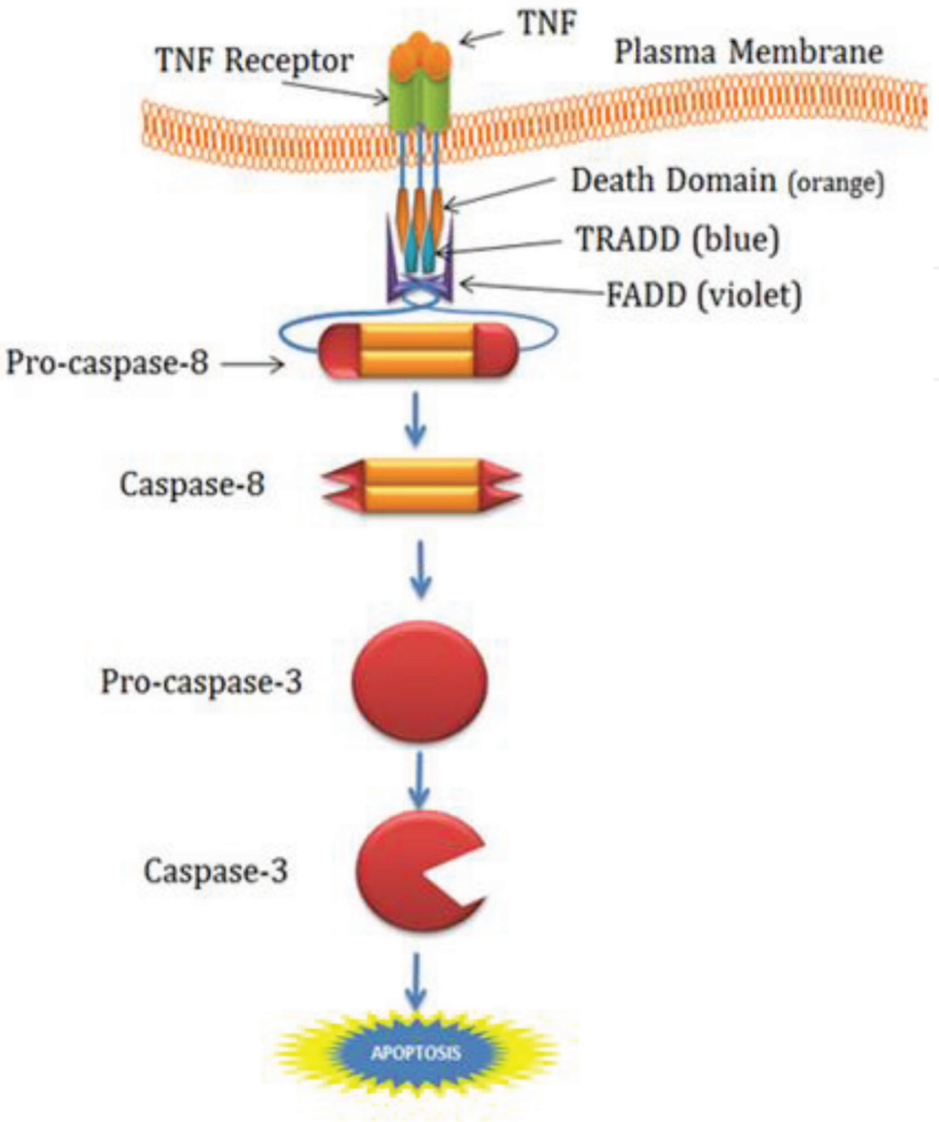

Figure 3. Extrinsic pathway of apoptosis (adapted from [9]).

The mitochondria are key players in this pathway [23], although the endoplasmic reticulum has also been implicated. After an apoptotic insult, the permeability of the mitochondrial membranes is disrupted, allowing the influx of ions and the efflux of some molecules localized within the mitochondria (e.g., cytochrome C) and the activation of Bax/Bak channels and APAF-1 [22]. These events lead to the activation of CASP-9, which in turn cleaves and activates the executioner CASP-3, -6, and -7 [24]. This pathway represents the caspase-dependent cascade, but the intrinsic apoptotic pathway also presents a caspase-independent cascade of events [23].

Although the caspase activation may originate from the death of the cell, in mammals, in particular circumstances, cells can undergo caspase-independent apoptosis. This form of apoptosis is mediated by the mitochondrial dysfunction, with loss of the mitochondrial membrane potential, which originates the translocation of an apoptosis-inducing factor (AIF) and a mitochondrial-derived protease—the endonuclease G—into the nucleus. In there, they

induce large-scale DNA fragmentation with subsequent peripheral condensation of chromatin that results in cell death [25]. Although the caspase-dependent and caspase-independent apoptotic pathways are separate, there is evidence of crosstalk between the two [26].

In the extrinsic pathway, the cell death-inducing signal for the programmed cell death is triggered by an external stimulus. For receiving such an external death-inducing signal, cells possess plasma membrane receptors specific to each stimulus and thus the extrinsic signaling of apoptosis, which in most cases is a cytokine, is also known as the receptor-mediated programmed cell death pathway. The most studied cytokine to induce extrinsic pathway of apoptosis is an extracellular messenger protein called tumor necrosis factor (TNF), produced by the cells of the immune system in response toward adverse conditions (**Figure 3**).

3. Apoptosis in female gametogenesis

In females, oogenesis is the process by which the female gametes, the oocytes or ova, are created. It begins with the allocation of the embryonic precursors of adult gametes, known as primordial germ cells (PGCs), into the gonadal ridges and forms the primordial sex cords, which will ultimately originate primordial follicles. Besides ovarian cell death that has been studied for over a century, recently in March 2018 [27], Regan and collaborators postulated that apoptosis is an integral part of normal ovarian cell's development and has limited predictive capability regarding oocyte quality or the ensuing pregnancy rate. In the ovary, the mechanisms underlying decisions of life and death involve cross dialog between pro-apoptotic and pro-survival molecules [28]. Even though apoptosis in the post-pubertal ovary is frequently observed in granulosa cells at all stages of follicle development, in fetal life, it only occurs in the oocyte [28]. In consequence, a large population of ovarian follicles in mammal ovaries is lost, limiting the number of ovulations and restricting the full reproductive potential of a species [29, 30].

Atresia of ovarian follicles has been divided into three phenotypes, each one presenting a different mechanism of initiation and regulation [27]. In the "antral atresia," the middle prolific layers of granulosa cells are affected by the apoptosis, which progresses to the antrum. The "basal atresia," occurs in the granulosa cells closest to the basal lamina, in very small antral follicles; these cells prematurely luteinize and begin to produce progesterone, but they do not complete luteinization and become apoptotic. The third form of apoptosis, often named as "terminal differentiation apoptosis," occurs in the preovulatory follicles and resembles that of the epidermal skin cells sloughing off, with the granulosa cells being shed into the antral fluid [27, 31]. In the male, apoptosis also has a significant relation with fertility.

4. Apoptosis and the gamete's quality

Fewer than 10% of oocytes collected in IVF programs become live births [32], leading to research on the relationship between apoptosis and oocyte quality. As in mice, the level of granulosa cell apoptosis increases in older IVF animals, in association to a decrease in the

oocyte quality, which is reflected in lower fertilization, pregnancy, and live birth rates [33]. In 1996, Sugino and collaborators [34] reported the relationship between the frequency of apoptotic granulosa cells and the size of the follicles in IVF programs. These findings were supported by several authors [35–37] leading to the hypothesis that the amount of cell-free DNA level in the follicular fluid samples obtained during follicle aspiration would correlate with the level of apoptosis in granulosa cells and thereby could be used as a predictor of oocyte quality and IVF-embryo transfer outcome.

5. Apoptosis after fertilization

In 2010, our team [14] studied the relationship between the arrest of bovine embryos in different stages of development and the level of apoptosis in embryonic cells, as measured by the TUNEL assay. It has been demonstrated that among embryos 7 days after IVF, those of nine cells to morula presented a higher incidence of apoptotic cell ratio (ACR) when compared with blastocysts. It was further demonstrated that embryos with higher ACR also showed higher cytoplasmic fragmentation and that higher ACR was associated with embryonic arrest. These findings lead the authors to hypothesize that an apoptosis level above a given threshold could be harmful to embryo development [14]. Notwithstanding, further research is foreseen to determine the pathways of the apoptotic process and especially to establish the ACR threshold detrimental to embryo development since nowadays it is well accepted that early cleaving embryos will result in a significantly higher proportion of good quality embryos compared with late cleavage (62.5 versus 33.4%, $P < 0.0001$) [38].

Besides, a lower apoptotic cell ratio in early cleaved embryos may anticipate higher embryo qualities. During compaction and blastulation of bovine embryos, it is often observed excluded cells between the developing embryo and the zona pellucida, which have poor gap junction communication with the embryo. Arrested and developing embryos contain different proportions of cells with the classic features of apoptosis, [39] including cytoplasmic, nuclear, and DNA fragmentation. In the embryo, the larger the number of apoptotic cells in an embryo, the lower is the ability of that embryo to survive in culture [40].

6. Apoptosis in the implantation process

Endometrial receptivity depends on a complex interplay of many factors that integrates the diverse mechanisms regulating tissue homeostasis, including apoptosis [41]. Endometrial function and in particular the endometrial receptivity are critical limiting factors for pregnancy success because, for implantation, pregnancy, and subsequent birth of the offspring, the endometrium should be ready to accept and interact with the embryo. Embryo implantation depends on some morphologic and biochemical modifications of the endometrium during the estrous cycle, orchestrated by the action of ovarian steroids on its receptors. These changes are exerted by cytokines, growth factors, adhesion molecules, transcriptional factors, and many others [42].

As it happens in other hormone-responsive cells, the endometrium presents a cyclic pattern of apoptosis, both in the epithelial and stromal cells, that allows the constant regeneration of the tissue and ensures fertility [43]. In women, it has been shown that the equilibrium between cell proliferation and apoptosis controls the endometrial cells, dictating its fate toward destruction and shedding at menstruation or toward survival, and prepare the mucosal layer of endometrium for the implantation of the embryo [44]. Endometrial receptivity represents a very short, self-limited period in which the endometrium does not reject the embryo [45].

Von Rango and collaborators [46] detected signs of apoptosis in the deep glandular epithelium and stroma at the beginning of the implantation window, in the human endometrium, that extended to other endometrial epithelia later on the luteal phase. The authors hypothesized that this pattern of apoptosis might have implications for the decidualization processes formation in endometrium during the late secretory phase. Supporting this hypothesis, Joswig and collaborators [47] demonstrated that apoptosis occurs in the uterine epithelium of the implantation chamber, as detected by the TUNEL assay and the immunolocalization of active CASP-3. It was also sporadically observed in decidual cells adjacent to the implantation chamber [47]. Apoptosis may, therefore, contribute to tissue remodeling during implantation and establishment of the placenta. Zhang et al. [48] demonstrated that the embryo controls the transcription of the apoptosis-inducing factor (AIF—a participant in the intrinsic, caspase-independent pathway) in the maternal endometrium around the time for implantation, both temporally and spatially. AIF transcription is maintained in basal levels in the surface and glandular superficial epithelia, those epithelia that interact with the trophoblast in mice. During the invasion phase, in implantation, its transcription increases in the sub-luminal stroma at the implantation sites, but not in the interplacentary sites. The authors concluded that apoptosis is vital for embryo implantation, decreasing the apoptosis in the apposition phase and contributing to the success of the blastocyst invasion. Any disturbance in the apoptosis pattern could lead to infertility and recurrent pregnancy lost.

However, most studies available were developed in species with a decidual placenta. So, it is possible that species-specific differences exist related to the physiology of implantation, such as the moment for implantation and the type of implantation, that are still to be elucidated. The detection of apoptosis in the endometrium during embryo implantation surely gives important information about the endometrial receptivity. Besides, the exact mechanisms and factors mediating the apoptotic process in the endometrium are not fully understood in many species, additional research of this problem would possibly let us to comprehend the mechanisms of endometrial receptivity better and to improve new predictors of IVF results.

Apoptosis was also detected in the trophoblast, under physiological conditions [49], which was mediated by the Bcl-2 gene and the Fas receptor [50, 51].

During placentation, ultrastructural studies showed that apoptosis contributes actively to the shaping and reorganization of fetal membranes [52], in a mechanism associated with the Fas-mediated signaling pathways. In post-implantation embryos, apoptosis is involved in organogenesis and in processes such as removing anomalous, inappropriate, nonfunctional, or damaging cells, and adjusting cell numbers. Although being a crucial phenomenon for embryo viability and pregnancy survival, this topic falls out of scope in this review.

7. Conclusions

Apoptosis is a process that tackles diverse specific intracellular signaling cascades that culminate in the enzymatic activation leading to programmed cell death. The enzymes most widely linked to apoptotic cell death are caspase enzymes. Caspase enzymes are cysteine proteases that are present in cells as zymogens until activated. Even though the mechanisms of apoptosis are present through the entire life, they start at the gametic formation as well as during all stages of embryo development. It first appears in the 32- to 64-cell embryo and can be demonstrated during the whole embryogenesis, when it plays an essential role in virtually all of the stages of development necessary to produce a normally developed newborn. It is also crucial for embryo-maternal interaction and seems to regulate the implantation and placenta formation. Evidences have accumulated that the formation of inborn anomalies or intrauterine death, induced by different developmental toxicants, result from distortions of the normal pattern of PCD in the embryo. Various chemical agents and physical factors have been shown to exert their effect by disturbing the apoptotic process occurring during gametogenesis. For that same reason, in response to environmental stressors, apoptosis may be an important event in embryo losses in early pregnancy. The mechanisms of apoptosis as well as its regulation in the early embryonic period has been studied in women or in rodent species, as models for human fertility, but limited information exists regarding farm animal species. This dearth of evidence opens new avenues for research on the apoptosis role in early pregnancy and fertility of livestock.

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Conflict of interest

The authors declare, for all legal purposes, the absence of any conflict of interest related to this paper.

Author details

Helena Moreira da Silva, Loide Isabel Valadão and Fernando Moreira da Silva*

*Address all correspondence to: joaquim.fm.silva@uac.pt

University of the Azores, Faculty of Agrarian Sciences and Environment Group of Animal Reproduction, IITAA, Portugal

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