APOPTOSIS IN PREIMPLANTATION BOVINE EMBRYOS
AND METHODS USED FOR ITS DETECTION*

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Abstract
Apoptosis or programmed cell death acts to eliminate cells that are damaged, are no longer required or are developmentally incompetent. Incidence of apoptosis has been suggested as an additional criterion for assessing embryo quality and predicting embryo viability. The advances in knowledge of apoptosis in bovine preimplantation embryos are described. The investigations at molecular and cellular level, including biochemical and morphological features of apoptosis and methods used for its detection are discussed. Selected ingredients of media involved in the process of programmed cell death are also presented.

Key words: apoptosis, cattle, embryo, TUNEL

Apoptosis has received much attention because of its potential role in early embryonic loss and sub-optimal developmental conditions. In the normally developing embryo some cells spontaneously undergo apoptosis, which might be involved in the elimination of abnormal cells (Betts and Madan, 2008). It has been speculated that apoptosis also plays a function in eliminating defective embryos (Jurisicowa and Acton, 2004). During in vitro embryo culture apoptosis is probably caused by sub-optimal conditions and may therefore also be an indicator of embryo quality (Pomar et al., 2005). It has been shown that apoptosis represents a mechanism which allows the preimplantation embryo to overcome stress. For example, in response to heat shock only limited proportion of cells (27.6%) undergoes apoptosis, which suggests that the degree of apoptosis is an important determinant in embryonic fate (Paula-Lopes and Hansen, 2002). Developmental potential of a postimplantation embryo is affected by apoptotic incidence in preimplantation stages (Loureiro et al., 2007). The degree and pattern of programmed cell death in early embryos significantly impact implantation

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and pregnancy. In light of the reasons listed above, the knowledge of mechanisms regulating apoptosis is essential for improving the technology of in vitro embryo production.

**Molecular pathways of apoptosis**

The elimination of unnecessary cells is essential in development, but if not carefully regulated apoptosis can lead to the death of the organism. At the molecular level various stimuli can induce apoptosis e.g. withdrawal of growth factors, ionising irradiation, DNA-damaging agents. The tumour suppressor p53 is known to cause apoptosis in response to the action of the above factors. Protein p53 acts as a transcription factor for the Bax gene. Bax is a member of the family of Bcl-2 proteins. The various members of this family promote (bad, bak, bax, bcl-x<sub>S</sub>, bik, hrk) or inhibit (bcl-2, bcl-w, bcl-x<sub>L</sub>, bfl-1, brag-1, mcl-1, NR13) activity of execution caspases (Jurisicowa and Acton, 2004). In apoptosis cascade the activation of caspases is the “point of no return” and is regulated by the proteins of the Bcl-2 family. Activated caspases cleave proteins that turn cell survival pathways off, and death promoting activators on. Caspases are the executors of cell death. In some forms of apoptosis, the extrinsic apoptotic pathway is initiated by activation of the apical caspase-8 following death receptor ligation. In other forms, cellular stress leads to activation of the intrinsic apoptotic pathway initiated by the apical caspase-9 (Jousan et al., 2008 a; Loureiro et al., 2007). These pathways converge upon activation of the executioner caspase-3 and -7 (Vandaele et al., 2007). Both upstream and downstream caspases as well as other proteases (e.g. calpains) cooperate to regulate apoptosis in a cell-specific manner. The intrinsic pathway is triggered by cytochrome <i>c</i> release from mitochondria (Jousan et al., 2008 a; Loureiro et al., 2007; Vandaele et al., 2007). The mechanism by which cytochrome <i>c</i> crosses the mitochondrial outer membrane is critically regulated by the opposing actions of the Bcl-2 family proteins. The activated proapoptotic proteins such as Bad, Bid, Bax and Bak are involved in formation of specific channels or coformation of megachannels (mPTP) in the outer mitochondrial membrane. These channels are responsible for the release of apoptogenic proteins such as cytochrome <i>c</i>, Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI), AIF (apoptosis inducing factor), Omi/HtrA2, procaspase-9 and -3 and endonuclease G from mitochondrial intermembrane space. Smac/DIABLO is the second (after cytochrome <i>c</i>) activator of caspases, functioning through the release of caspases from the inhibitory effects of IAPs (inhibitors of apoptotic proteases).

Both members of Bcl-2 family and members of death effector cysteine protease family - the caspases, are expressed in preimplantation embryos (Jousan et al., 2008 a; Loureiro et al., 2007; Vandaele et al., 2007; Gjørret et al., 2004, 2007; Knijn et al., 2003). However, the value of their evaluation as a reliable apoptosis detection method is questionable. The latest research (Vandaele et al., 2008) investigated if the mRNA expression of a set of genes involved in apoptosis (Bax, Bcl-2, caspase-3 and -7) at an earlier point in the apoptotic cascade could be a good marker for apoptosis in in vitro produced bovine embryos. None of the analysed genes were differently expressed in staurosporine-treated in comparison with non-treated embryos, which means that
mRNA expression of Bax, Bcl-2, caspase-3 and-7 cannot be used as a reliable apoptosis detection method (Vandaele et al., 2008).

It is well known that Ca\textsuperscript{2+} is a key mediator of apoptosis in different cell lines. Research in the bovine has also proved that Ca\textsuperscript{2+} signalling mediates apoptotic cell death during early embryonic development via activation of Ca\textsuperscript{2+}-dependent proteases, micro-calpain and caspase-12 (Sergeev and Norman, 2003). The calpain family members can be classified as typical calpains, which are further divided into ubiquitous and tissue-specific calpains, and atypical calpains (reviewed in Suzuki et al., 2004). Ubiquitous calpains occur in two isoforms, \(\mu\)-calpain and m-calpain requiring micromolar or millimolar calcium concentrations respectively for their activation \textit{in vitro}. These heterodimeric enzymes consist of a small regulatory subunit (30 kDa) and a large catalytic subunit (80 kDa). Calpains have the potential to both positively and negatively modulate the caspase cascade during apoptosis. Calpain activity is, in turn, regulated by caspases through depletion of the endogenous calpain inhibitor, calpastatin. Calpains down-regulate the caspase cascade during the initiation or early execution phase of apoptosis. On the other hand, facilitated by caspase-mediated degradation of calpastatin, calpains eventually participate in the dying process by causing plasma membrane disruption. This delayed effect could be responsible for the phenomenon of “secondary necrosis” (Ben-Aharon et al., 2005; Suzuki et al., 2004).

**Morphological features of apoptosis at the cell level**

The apoptotic cell rounds up and separates from neighbouring cells. Condensed and misshapen nuclei with clumped chromatin can be observed, which are smaller than intact healthy nuclei. The DNA degradation into oligonucleosomal fragments is also observed. The membrane-bound fragments containing organelles, resembling apoptotic bodies seen in other types of cell are the example of cytoplasmic fragmentation. Although it has been proposed that cytoplasmic fragments are the equivalent to apoptotic bodies as they contain fragmented DNA and express active caspases, the failure to reduce fragmentation with caspase inhibitors (Xu et al., 2001) and observed active caspases in all fragments (Spanos et al., 2002) support the contention that apoptosis and cytoplasmic fragmentation are not always related. Fragments that have persisted for some time may undergo secondary necrosis, with membrane and DNA changes indistinguishable from those seen during apoptosis (Spanos et al., 2002).

It is not difficult to differentiate between apoptotic and necrotic cell. Cells undergo necrosis when exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia, injury, bacterial toxins) and lose their membrane integrity. This leads to an influx of water and extracellular ions. Moreover, as a result of plasma membrane breakout cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, \textit{in vivo}, necrotic cell death is often associated with intense inflammatory response. Intracellular organelles e.g. mitochondria, as well as the entire cell swell and rupture (cell lysis) (Jurisicowa and Acton, 2004).

**Apoptosis in embryos**

Cytoplasmic and nuclear fragmentation, extensive chromatin condensation and marginalization, DNA fragmentation and phagocytosis are observed in 70–80% of all...
in vitro produced blastocysts from mice, human, and practically in all bovine blastocysts (Gjørret et al., 2003). Many studies reported apoptosis in at least one apoptotic nucleus of almost every in vivo and in vitro blastocyst (Vandaele et al., 2007; Gjørret et al., 2003, 2007; Neuber et al., 2002). This data indicates that apoptosis is a universal process during normal bovine embryo development. However, in vitro produced blastocysts have features of a higher degree of apoptosis than their in vivo counterparts.

Although there is a general absence of morphological features of apoptosis before compaction or blastocyst formation of mammalian embryos, an earlier wave of apoptosis occurs, manifesting itself as blastomere fragmentation at the 1- and 2-cell stage in mice and 2–8 cell stage in humans (Kamjoo and Brison, 2002), which is the time when major genome activation occurs. It is speculated that the period of major genome activation induces competence for apoptosis and activates expression of genes that serve to suppress cell death in developing embryos (Jurisicowa and Acton, 2004). This turned out to be true also for bovine IVF embryos as apoptosis has been observed above 8-cell stage when embryonic genome is activated (Gjørret et al., 2003, 2007). Fahrudin et al. (2002) reported DNA fragmentation for the first time in 6- to 8-cell stage of the in vitro produced bovine embryos. Also Gjørret et al. (2003) observed nuclear condensation for the first time in the 6-cell stage in vitro produced embryos (IVP) and the 8-cell stage in vivo developed embryos. TdT-mediated dUTP nick-end labelling (TUNEL) reaction was first observed in IVP embryos at the 6-cell stage and in the 21-cell stage of the in vivo developing embryos. Interestingly, DNA fragmentation was detected even earlier in bovine embryos produced by somatic cell nuclear transfer (NT), i.e. at the 4-cell stage, and a marked increase in the extent of DNA-fragmented nuclei was apparent at the 6- to 8-cell stage (Fahrudin et al., 2002). However, recent studies of Vandaele et al. (2007) using fluorescence detection of active caspases showed that in bovine embryos apoptosis starts as early as in 2-cell stage. Since the transcripts of different apoptotic genes are present in all stages of preimplantation embryo development (Vandaele et al., 2007, 2008; Jurisicowa and Acton, 2004; Lonergan et al., 2003; Rizos et al., 2002) this suggests that apoptotic machinery can start from the very beginning of embryo development. On the basis of observations listed above, it may be concluded that the onset of apoptosis is probably regulated in a stage-specific manner, but certain features of apoptosis may be differentially regulated and independently modulated by the mode of the bovine embryo production (after Gjörret et al., 2003).

In bovine blastocysts apoptotic nuclei are mostly located in the inner cell mass – ICM (Fouladi-Nashta et al., 2005; Pomar et al., 2005; Fahrudin et al., 2002; Neuber et al., 2002), whereas in humans apoptotic cells are randomly distributed in an embryo. Since the ICM is the lineage that will form the foetus, it may be speculated that the regulation of ICM is more sensitive than regulation of trophectoderm cells – TE (Neuber et al., 2002). Apoptosis may also be involved in removing redundant ICM cells that have retained TE potential after blastocyst formation. It has been suggested that the aberrant TE : ICM ratio may be related to the large offspring syndrome of IVP embryos (Knijn et al., 2003).
Apoptosis as a result of in vitro culture

Appearance of apoptotic characteristics is accelerated by in vitro conditions but their expression is insignificant prior to compaction stage of the embryo (Gjørret et al., 2003). The dead cell index (DCI, total number of apoptotic nuclei/total number of nuclei) tends to increase as the in vitro culture time increases (Neuber et al., 2002). In vitro culture conditions are thought to increase apoptotic incidence, decrease embryonic cell number, decrease implantation rates and increase foetal resorption (Gjørret et al., 2003). Still, attempts are being made to find conditions optimising in vitro culture. There are many papers reporting supplementation of medium with growth factors, hormones and other ingredients that improve embryo development and decrease apoptosis.

Among growth factors the most important role is played by Insulin-like Growth Factor I (IGF-I). It has been shown that supplementation of culture medium with IGF-I has a beneficial effect on embryo development (Block et al., 2008; Jousan et al., 2008 a; Sirisathien and Brackett, 2003). Few previous studies stated that IGF-I lowers the apoptotic index by decreasing the number of apoptotic cells per embryo. Sirisathien and Brackett (2003) reported that only embryos reaching the blastocyst stage on day 8 showed significant effects of IGF-I treatment (lower percentage of TUNEL positive cells and higher blastocyst cell number) when compared to controls. However, recent research of Block et al. (2008) showed that the IGF-I has no significant effect on blastocyst cell number nor the number of apoptotic blastomeres. Their experiment showed that the positive effect of IGF-I is achieved by altered expression of transcripts important for embryo development and survival (Block et al., 2008). Additionally, the latest work checking the impact of short-term culture supplemented with IGF-I on in vitro produced bovine preimplantation embryos showed that this growth factor prevents heat shock-induced apoptosis through activation of the Phosphatidylinositol 3-Kinase/Akt pathway (Jousan et al., 2008 b).

Another factor with anti-apoptotic activity for bovine embryos is insulin. Bovine blastocysts derived from cultures supplemented with insulin showed a significant decrease in apoptosis determined by the TUNEL assay (Augustin et al., 2003). Beneficial effect of insulin on bovine embryo development was observed only when the culture medium was supplemented with glucose. Glucose is an essential energy substrate for development of bovine blastocyst, especially after activation of the embryonic genome. Impaired glucose transport can increase apoptosis in preimplantation embryos. However, high glucose level can be detrimental to embryo (Augustin et al., 2003; Jimenez et al., 2003). Other data showed that insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase/Akt also termed as protein kinase B or Rac kinase (PI3-kinase-Akt) pathway mediate insulin-specific signalling that results in anti-apoptotic activity (Jousan et al., 2008 b; Tseng et al., 2002). Apoptosis in embryo induced by high glucose can be reversed in part by insulin treatment.

The next factor regulating the process of apoptosis is growth hormone (GH). This hormone acts as survival factor during in vitro culture. GH reduces apoptosis by altering the Bax to Bcl-2 ratio during early embryogenesis and significantly increases the number of ICM and TE cells in bovine expanded blastocysts (Kölle et al., 2002).
Another factor that may be involved in the process of apoptosis in embryo is foetal calf serum (FCS). It contains “survival” peptide growth factors as well as “death” factors such as Tumour Necrosis Factor (TNF), which may induce cellular death. However, Fouladi-Nashta et al. (2005) who investigated the effect of serum supplementation on apoptosis index in IVP bovine embryos have found no differences in the number of apoptotic nuclei between embryos cultured in medium supplemented with FCS and without serum. However, in earlier investigations of Rizos et al. (2003) it has been shown that the presence of serum in culture medium resulted in a significant increase in the level of expression of proapoptotic Bax protein.

Epidermal growth factor (EGF) has also been checked for its beneficial effects on bovine embryo development; however, there was no effect on the level of apoptosis measured by TUNEL assay (Sirisathien and Brackett, 2003). As for action of tumour necrosis factor-alpha (TNF-α) on embryonic development it has been reported that only in the embryos above the 9-cell stage TNF-α increased the percentage of blastomeres undergoing apoptosis (Soto et al., 2003).

Methods for assessing apoptosis in bovine embryos

Basic methods

The most basic methods of observing apoptotic features include observation in fluorescence microscope after staining the embryo with specific fluorophores.

While the apoptotic cell rounds up and separates from neighbouring cells, the phospholipid phosphatidylserine translocates from the inner plasma membrane leaflet to the outer. This feature can be monitored by incubating the embryo in fluorescein isothiocyanate (FITC)-conjugated annexin V. Cells undergoing apoptosis show a marked increase in annexin V binding.

By labelling DNA with specific fluorochromes such as DAPI (4’,6-diamidino-2-phenylindole) or Hoechst, nuclei with clumped chromatin can be observed using fluorescence microscopy. Using the same approach the condensed and misshapen nuclei can be observed.

TUNEL method

Currently, the most popular technique for assessing apoptosis in embryos is TUNEL method (Vandaele et al., 2008; Gajda et al., 2008; Bryła et al., 2007; Gjørret et al., 2004, 2007). As previously mentioned, degradation of DNA into oligonucleosomal fragments is one of the features of apoptosis. Multimers of 180–200 bp fragments migrate as a ladder on an agarose gel. Due to the small number of cells in the embryos it is not possible to use electrophoresis to look for DNA laddering. Development of TUNEL – TdT-mediated dUTP nick-end labelling enables assessment of nuclear DNA fragmentation in situ. Deoxynucleotidyl transferase (TdT) catalyses the incorporation of biotinylated deoxyuridine at the sites of the DNA breaks (Figures 2 and 3). The signal may be amplified by avidin-peroxidase, enabling detection by fluorescent microscopy (Neuber et al., 2002). However, TUNEL reaction fails to determine how DNA degradation is generated, because nuclei of cells undergoing necrosis are also labelled (Gjørret et al., 2003). Also, there is a possibility of misinterpretation of TUNEL results (Vandaele et al., 2008). DNA strands may break during fixation.
Apoptosis in bovine embryos

Figure 1. Blots from analysis of the protein level of proapoptotic Bax and antiapoptotic Bcl-2 in immature (GV) bovine oocytes. The aim was to determine the degree of apoptotic changes in immature bovine oocytes based on expression of bax and bcl-2 genes and to determine the relationship between activity of glucose-6-phosphate dehydrogenase (G6PDH) in immature oocytes and expression of apoptotic genes. Biochemical analysis of G6PDH activity was performed using brilliant cresyl blue (BCB) staining. Oocytes with low G6PDH activity stained blue and were described as BCB+ in contrast to oocytes whose high G6PDH activity reduced the dye, making them colourless. Lanes 1-3 GV oocytes, Lane 1 – BCB+ oocytes; lane 2 – BCB- oocytes; lane 3 – control oocytes (K)

Figure 2. Bovine blastocysts after TUNEL assay – DAPI staining of blastomere DNA
Figure 3. Bovine blastocysts after TUNEL assay – FITC staining of apoptotic cells only (stained sperm heads (arrows) are also visible on lower blastocyst)

Figure 4. Caspase-3 activity in bovine embryos: expanding blastocyst (1) and middle blastocyst (2); A – visible light; B – red fluorescence corresponding to active caspase-3 (100× magnification)
Moreover, inadequate tissue handling may induce DNA damage and cause labelling of apparently normal nuclei. However, the morphological appearance of the TUNEL reaction in necrotic cells is different because the mode of nuclear disintegration is by karyolysis rather than by karyorhesis as seen in apoptosis (Gjørret et al., 2003). Thus more than one feature of apoptosis must be observed for correct identification of apoptosis. Still, apoptosis may not always be associated with DNA degradation, as a result of which not all TUNEL-positive nuclei are in the process of programmed cell death (Gjørret et al., 2003). TUNEL-positive nuclei lacking apoptotic morphology may represent blastomeres suffering the fate of secondary necrosis, which is a normal process in apoptotic cells that are not being adequately removed by phagocytosis (Gjørret et al., 2003). In some cases a cell with fragmented morphology can be TUNEL negative. It is advisable regarding apoptotic only nuclei displaying both apoptotic morphology and positive TUNEL reaction. Morphological evaluation is crucial because different cell death pathways share biochemical features.

**qPCR and RT-PCR method**

Quantitative assessment of apoptotic gene products is possible using RT-PCR or “real time” qPCR. The semiquantitative RT-PCR is the frequently used approach for analysing the relative transcript abundance in embryos. Adding a known amount of rabbit β-globin RNA prior to RNA extraction or utilizing endogenous transcript for housekeeping genes as standard helps to estimate the quantity of the RT-PCR product. The advantage of qPCR over RT-PCR is the possibility of analysing several genes in a very limited cDNA sample derived from single embryo, as well as accuracy.

By employing qualitative or quantitative RT-PCR assays, expression of different genes was studied including genes involved in apoptosis. The majority of the genes are expressed in a stage-specific manner, showing two major patterns of expression: starting after the onset of genomic activity or expression throughout the period before and after the onset of embryonic transcription, indicating maternal and embryonic activity. All of the observations indicate that conditions of post-fertilization culture influence mRNA expression in the resulting bovine embryos. The observed alterations in RNA expression may be directly linked with the quality of these embryos (Rizos et al., 2002). The results of almost all previous research showed that good quality embryos show a high expression level of antiapoptotic proteins (Bcl-2, Bcl-w, Bcl-xL), and low expression level of proapoptotic proteins, mainly Bax gene (Lonergan et al., 2003; Augustin et al., 2003; Spanos et al., 2002). The morphologically poor embryos are characterised by the reverse situation. That is why the statement that the ratio of pro- and antiapoptotic genes of Bcl-2 family can be used as a marker of embryo quality is considered as a rule. The earlier mentioned paper of Vandaele et al. (2008) questions the usefulness of analysis of mRNA expression of Bax, Bcl-2, caspase-3 and -7 as apoptosis detection method. The authors also suggest the superiority of protein analysis over mRNA analysis – “immunofluorescent staining of caspase-3 and -7” according to them, “is a better choice whereas for Bcl-2 no reliable and practicable alternative is available at the moment”. Our research (Opiela et al., 2008) also showed the discrepancy between results obtained at the transcript level compared to the protein level. Results obtained at the transcript level seem to indicate that oocytes
subjected to brilliant cresyl blue staining (BCB) show a tendency towards apoptosis. However, results obtained at the protein level did not confirm this conclusion (Opiela et al., 2008). In light of Vandaele et al. (2008) research, conclusions perhaps should not be drawn on the basis of RNA level gene expression only. Every analysis done on protein level gives a more accurate answer as we still do not know all the paths and mechanisms involved in transcribing the information from RNA to protein.

**Caspase activity**

Apoptosis in embryos can be assessed by measurement of caspase activity. It may be carried out using fluoroprobe which incorporates adequate caspase recognition sequence into bifluorophore-derivitised peptide that mimics the structure loop conformation present in native protease cleavage sites. Embryos are incubated in a given fluoroprobe for about an hour and then caspase activity is assessed using a fluorescence microscope. Fluorescence intensity is quantified by special software and the pixel intensity per unit area is determined (Paula-Lopes and Hansen, 2002). Another approach uses the fluorescein-labelled broad spectrum caspase inhibitor. The caspase inhibitor enters the cell and irreversibly binds to activated caspase in the order of decreasing activity, labelling them and allowing them to be visualised and localised (Spanos et al., 2002). It is also possible to apply a peptide substrate for certain caspase. The substrate has two fluorophores conjugated on each side of the target caspase cleavage site. The fluorescence is quenched in intact substrate due to the folded peptide structure. Upon cleavage by the active caspase present in every cell the fluorescence can be obtained (Opiela et al., 2007; Men et al., 2003) (Figure 4).

Active caspase-3 was detected on day 8 in *in vitro* produced bovine embryos using immunocytochemical visualization (Gjørret et al., 2004, 2007). Interestingly, this activation concurs with changes in nuclear morphology, but precedes DNA degradation detectable by TUNEL (Gjørret et al., 2004, 2007). However, the direct correlation needs further investigation.

**Analysis of proteins involved in apoptosis using immunofluorescence, immunohistochemistry and western blot**

Immunofluorescence and immunohistochemistry are used to detect death inducing transcripts, specific proteins regulating apoptosis and also downstream effectors like caspases. By immunofluorescence Vandaele et al. (2007) showed expression of active caspase-3 and -7 in 2-cell bovine embryos. According to these authors, immunofluorescence detection of apoptotic proteins is more precise and free from false positive labelling as in TUNEL due to detection of necrotic cells (Vandaele et al., 2007, 2008). Using immunohistochemistry Kölle et al. (2002) showed the pattern of bcl-2 and bax protein expression in bovine embryos. Both methods simultaneously give information on expression and localization of the target protein. Such knowledge about the different stages of certain protein expression enables apoptosis modulation in cells.

Western blot analysis is being also employed to investigate possible correlation between blastocyst quality and apoptosis by detecting expression of certain proteins. In western blot technique protein preparations are solubilised in Laemmli’s sample buffer and separated on a discontinuous SDS gel system consisting of polyacrylamide
stacking and separating components. Electrophoretic proteins are electrotransferred to membranes (PVDF or nitrocellulose), membranes are blocked to prevent non-specific binding and then are incubated first with the primary antibody directed against target protein in an appropriate dilution, and then in a labelled secondary antibody (e.g. biotinylated or conjugated with horseradish peroxidase). Immune complexes are most frequently detected by chemiluminescence and visualised after few minutes exposure to X-ray film (hyperfilm). Immunoreaction signals are quantified on the film by densitometry using an image analysis system (Figure 1).

Yang and Rajamahendran (2002) studied the pattern of Bcl-2 and Bax expression in immature bovine oocytes and in vitro produced bovine embryos of different qualities. The result of their research implies that the ratio of BCl-2 to Bax may be used to gauge the tendency of embryos and oocytes towards either survival or apoptosis.

**Comet assay**

DNA cleavage and damage of embryos can also be analysed by single cell microgel electrophoresis (comet assay). The target embryos are suspended in agarose gel layered onto a microscope slide. Under the fluorescent microscope the characteristic comet like appearance of fragmented DNA out of the nucleus of the blastomeres can be visualised (Takahashi et al., 2000). The comet assay is used to detect fragmented DNA in embryos subjected to DNA damaging agents, i.e. UV-light or oxidative stress. The comet assay can be used to follow both DNA damage and repair following exposure to toxic chemicals. According to Fabian et al. (2003) comet assay is an appropriate method for studying apoptosis in preimplantation embryos, and it appears to be more sensitive than the classically used morphological analyses.

**Conclusions**

It is generally assumed that apoptosis represents an embryo at risk. It is well accepted that the level of apoptosis is a marker of embryo quality, and that treatments which decrease apoptosis increase developmental competence of the embryo. However, cell loss itself does not lead to embryonic death. One must remember that apoptosis prevents a damaged cell from contributing abnormal daughter cells to the embryo. Additionally, apoptosis regulates the total ICM cell number preventing abnormal development of the foetus, such as an enlarged offspring. “While limited apoptosis is a mechanism for embryonic homeokinesis to remove damaged cells without disturbing the embryonic potential to further develop, more extensive apoptosis may leave the embryo with too few cells to sustain development. Given the importance of apoptosis in determining embryonic survival in response to stress, manipulation of apoptosis responses in embryos may lead to new methods for improving systems for in vitro production of embryos or for reducing embryonic mortality in female animals” (cited by Paula-Lopes and Hansen, 2002).

**References**


Apoptosis in bovine embryos


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Apoptoza w przedimplantacyjnych zarodkach bydlęcych oraz metody stosowane do jej oznaczenia

STRESZCZENIE

Apoptoza jest procesem, w wyniku którego następuje eliminacja komórek uszkodzonych, zbędnych oraz rozwojowo niekompetentnych. Ocena zaawansowania apoptozy służy jako jedno z kryteriów jakości zarodka oraz pozwala bardziej efektywnie prognozować jego zdolność rozwojową.

W pracy przeglądowej przedstawiono osiągnięcia w poznaniu przebiegu procesu apoptozy na poziomie molekularnym i komórkowym, z uwzględnieniem szlaków biochemicznych oraz cech morfologicznych w zarodkach bydlęcych. Pokrótce omówiono metody stosowane dla oznaczania apoptozy. Ponadto, przedstawiono wpływ niektórych związków dodawanych do pożywki stosowanej w hodowli in vitro przedimplantacyjnych zarodków na programowaną śmierć komórek.