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Minireview

APOPTOSIS DETECTION AS A TOOL FOR THE DETERMINATION OF ANIMAL EMBRYO QUALITY

A.V. MAKAREVICH[1], P. CHRENEK[1,2], P. MASSÁNYI[2], N. LUKÁČ[2], J. PIVKO[1]

[1]Slovak Agricultural Research Centre, Research Institute of Animal Production, Nitra, Slovak Republic;
[2]Slovak Agricultural University, Nitra, Slovak Republic

ABSTRACT

We present here a brief review of available world literature, as well as our own studies concerning process of programmed cell death – apoptosis in relation to quality and viability of mammalian embryos at preimplantation stages. A mini-review describes specific signs of apoptosis on the level of the cell membrane, cell organelles and cytoskeleton, as well as several techniques for the detection of apoptosis. Particular role of growth factors in suppression of apoptosis is mentioned. Moreover, quality of embryos following the gene microinjection or the exposure to elevated temperatures in association with apoptosis occurrence is discussed. In conclusion, detection and quantification of apoptosis occurrence may help to determine consequences of stress influences and to find out factors which inhibit apoptosis. Exact knowledge of processes involved in regulation of cell death may find out approaches for the improvement of embryo quality and survivability.

Key words: embryo, apoptosis, stress, micromanipulation, hyperthermia

IINTRODUCTION

Success of reproduction, fertility and health of offspring in farm animals depends on quality and viability of gametes and developmental potential of developed embryos. In this complex reproductive process a final outcome is influenced by several factors including genetic predisposition, health state and physical conditions. A basis for successful run of reproductive processes is hidden on the level of gametes (oocytes, sperm) and subsequent embryos. In particular, such micromanipulations as the gene microinjection for transgenesis or culture procedures, may destroy normal reproductive process, negatively influence gamete quality and decrease reproduction efficacy.

Biotechnological approaches, such as a cryoconservation, which serve to increase the usage of reproductive potential of genetically valuable animals, may negatively affect survival of gametes and embryos. Various environmental influences or manipulations may lead to damages and even death of cells, i.e. apoptosis or necrosis. Determination of these influences may have a great importance for the explanation of failures in reproductive processes. Research in this area is progressed and enables us to follow in details processes running on the cell level as well as factors regulating these processes.

Correspondence: E-mail: makarevic@scpv.sk
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APOPTOSIS AND APPROACHES FOR ITS DETECTION

Whilst cell **necrosis** is associated with the release of enzymes causing cell damages, inflammation of neighbouring tissue and finally cell lysis, **apoptosis** is a genetically programmed cell death (Wyllie et al., 1980). It is an active physiological process resulted in the elimination of damaged or harmful cells, whereas neighbouring cells are not subjected by this process (Schwartzman and Cidlowski, 1993). In normally developing morulas and blastocysts several cells spontaneously undergo programmed cell death – apoptosis, which might be involved in the elimination of abnormal cells (Hardy, 1999). However, at a higher occurrence, apoptosis is a detrimental for the blastocyst formation leading to the death of preimplantation embryos (Van Soom et al., 2000). Apoptosis in different cell types can be induced by external stimuli; quantification of apoptosis occurrence may serve for the evaluation of influence of culture conditions or experimental procedures. If a higher rate of apoptotic cells, than is physiologically normal, is revealed, it may indicate a decreased ability of embryos to develop into health offspring (Van Soom et al., 2000). This evaluation of apoptosis presence has a growing importance by the fact that the cells undergoing apoptosis may, at initial stages, seem to be quite normal.

Apoptosis in cells is manifested on the level of the membrane, nucleus (DNA) or cellular organelles (mitochondria, vesicles of endoplasmic reticulum and Golgi complex). At early stages several properties of the membrane are altered, for example redistribution of **membrane phospholipid – phosphatidylserine** occurs (Martin et al., 1995). Reorganization of the cytoskeleton in apoptotic cells is based on the disruption of microtubules and loss of microtubular structure (Martin and Cotter, 1990; Cotter et al., 1992) with consequent changes in cellular size and shape. During apoptosis, destruction of the **nuclear lamina**, which underlies the nuclear membrane and is involved in chromatin organization, has also been observed. The lamina is formed by polymers of intermediate filaments called lamins. During apoptosis, lamins are cleaved by caspases, causing the lamina to collapse and contributing to nuclear chromatin condensation and cell shrinkage (Thornberry and Lazebnik, 1998). At final stages apoptotic cells undergo fragmentation and formation of apoptotic bodies, which are quickly removed by phagocytosis (Hardy, 1997; Hardy, 1999). During apoptosis an extensive DNA degradation occurs. DNA strand is digested by cellular enzymes into single fragments of equal size (180-200 bp) (Arends et al., 1990). DNA fragmentation results into double-strand, low molecular weight fragments as well as single-strand breaks (nicks) of high molecular DNA (Martin et al., 1994). In relation to these manifestations different methods of apoptosis detection exist.

A simplest method is a morphological evaluation, when on the basis of nuclear staining it is possible to recognize certain signs characteristic for apoptosis like chromatin condensation, migration of chromatin clumps on the nuclear periphery (marginalization) and disintegration of cell nucleus into apoptotic bodies. **TUNEL technique** (terminal dUTP-transferase-mediated DNA nick end-labelling) is commonly used for the detection of apoptotic cells by means of digested DNA fragment labelling (Fig. 1 A,B) (Gavrieli et al., 1992). However, for years there has been a debate about its accuracy, due to problems in the original assay which caused necrotic cells to be inappropriately labelled as apoptotic (Grasl-Kraupp et al., 1995). Despite extensive using, a disadvantage of this technique is a possibility of false-positive labelling of necrotic cells. Furthermore, this assay enables to detect only later stages of apoptosis (Negoescu et al., 1996; 1998).

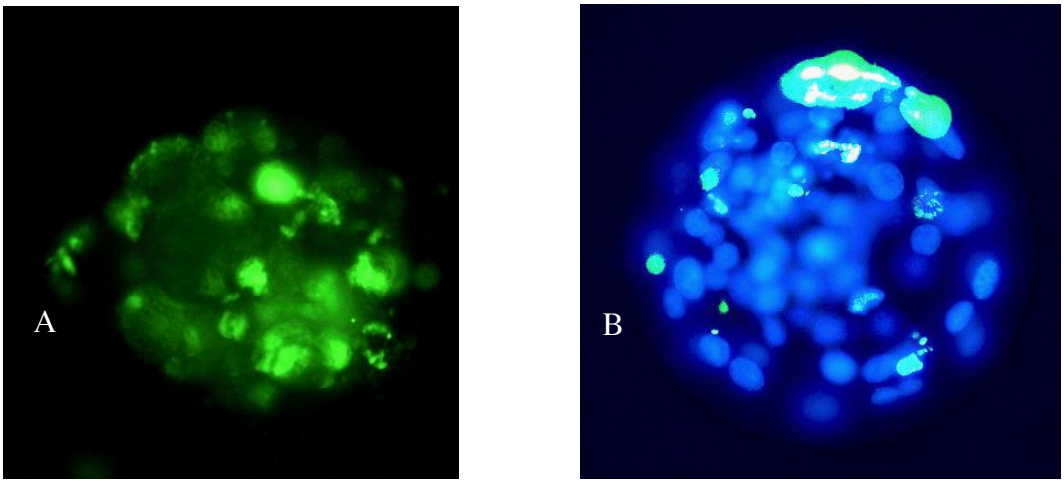

Fig. 1 A,B: Detection of apoptotic nuclei of rabbit embryos using TUNEL assay (A) with visualization of all nuclei (DAPI, B)

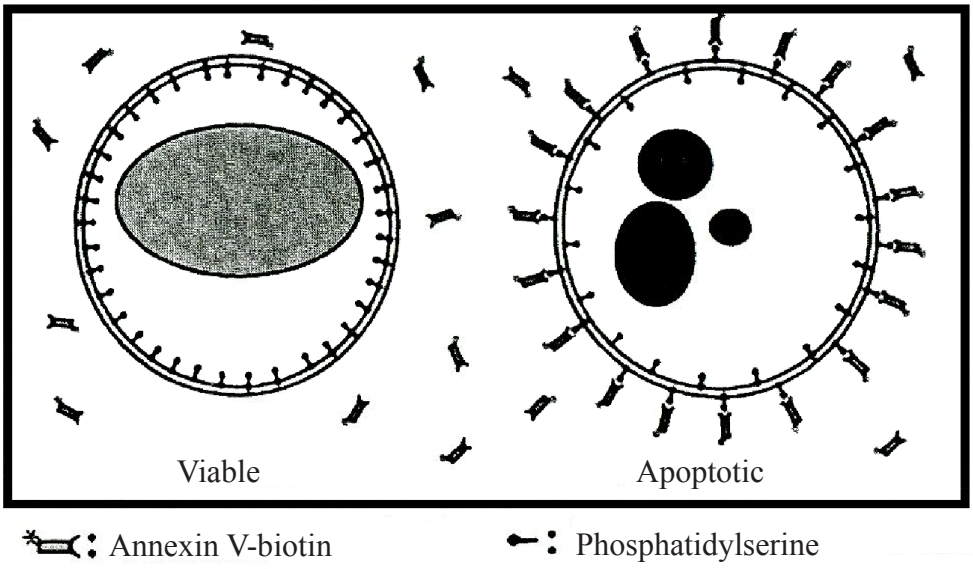

Fig. 2: Principle of Annexin V –binding assay (Van den Eijnde a kol., 1997)

Annexin V–binding is a further known method for the apoptotic cell detection (Vermes et al., 1995; van Engeland et al., 1998). This assay is based on the observation that after initiation of apoptosis phosphatidylserine - obligatory element of the inner part of the cell cytoplasmic membrane migrates from the inner site of the membrane on the cell surface (Fig. 2). This is a process of so-called phosphatidylserine externalization, when the membrane remains intact and phosphatidylserine is easy detectable by the binding with labelled annexin V (Fig. 3), which is a binding protein for phosphatidylserine (Martin et al., 1995). These alterations precede further morphological changes like nuclear condensation and cell shrinkage. This method enables the detection of early phase of apoptosis prior to loss of cell membrane integrity. At initial stages of apoptosis, phosphatidylserine migrates from the inner cell membrane to their outer part; this translocation is a primary feature of cells affected by apoptosis. Annexin V, by the binding to phosphatidylserine, reveals the cells with early membrane alterations and the cells dead due to apoptosis (Muratori et al., 2004). Therefore, annexin V labelling can be used as a marker of early stage apoptosis prior to morphological changes occur (Hardy, 1999). Annexin V was successfully used for the detection of apoptosis on mammalian embryos (Levy et al., 1998; Mateusen et al., 2005; Fabian et al., 2007) as well on sperm cells (Anzar et al., 2002; Martin et al., 2004; Makarevich et al., 2008)

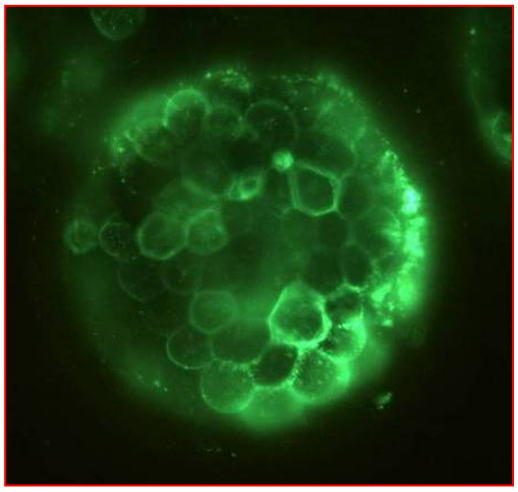

Fig. 3: Detection of apoptotic cells in rabbit blastocyst using fluorescently labelled annexin V

The presence of apoptotic changes may be analyzed using electrophoresis or immunocytochemical detection of **caspase activity**, an important group of enzymes involved in the activation and triggering the apoptotic process (Thornberry and Lazebnik, 1998). The major wave of apoptosis in embryos of mice (Handyside and Hunter, 1986), rabbits (Makarevich et al., 2005), cattle (Byrne et al., 1999) and human (Hardy, 1997) is activated during the blastocyst formation and declined at the passage up to advanced stage – expanded blastocyst, eventually when the embryo cell number grows (reviewed by Brison, 2000). Most of apoptotic nuclei in bovine in vitro produced blastocysts is localized in the area of embryoblast (Byrne et al., 1999; Makarevich and Markkula, 2002), whereas in human (Hardy, 1999), mice (Jurisicova et al., 1996) and rabbits (Makarevich et al.,

2005) apoptotic cells are randomly distributed through the whole embryo.

Frequency of apoptosis in mammalian embryos can be decreased by the addition of different **growth factors** to embryo medium. In particular, insulin-like growth factor –I (IGF-I) and insulin (Herrler et al., 1998; Spanos et al., 2000; Makarevich and Markkula, 2002; Byrne et al., 2002; Augustin et al., 2003), epidermal growth factor (EGF) (Makarevich et al., 2005; Sirisathien and Brackett, 2003) and transforming growth factor α (TGF- α) (Brison and Schultz, 1997; Brison and Schultz, 1998) suppressed apoptosis in rabbit, human, bovine and mouse preimplantation embryos. Anti-apoptotic effect of growth factors is probably mediated by specific receptors on the cell surface, since reduction in the number of the IGF-I receptors induced extensive apoptosis, whilst IGF-I receptor overexpression protected embryo cells against apoptosis in vivo (Resnicoff et al., 1995).

APOPTOSIS AND QUALITY OF EMBRYOS FOLLOWING GENE MICROINJECTION

Manipulations on embryos have been widely used for the formation of transgenic or cloned animals. A main problem at in vitro manipulations on embryos is a decreased viability and subsequent abnormalities of the foetus. Genetically modified (transgenic) embryos, produced by the **microinjection of the gene construct into pronuclei** of fertilized eggs, are often unevenly divided and their development may be arrested at earlier stages (Makarevich et al., 2005; Makarevich et al., 2006). As a result, an efficiency of the gene transmission is very low (Chrenek et al., 1998).

There are several causes for embryo damages and their low viability. Mostly these embryos are exposed to culture conditions off the mother's body, which are suboptimal. Differences in viability between in vivo recovered and in vitro derived embryos are well-known. Factors which mostly influence embryo viability at micromanipulations are: light exposure during the procedure, a mechanical injury by the microinjecting pipette (Mann and McMahon, 1993), single or double microinjection (Chrenek et al., 2005) and, not the least, factors related to the integration of transferred gene into the genome of the cell (Covarrubias et al., 1987). All mentioned influences may therefore induce an initiation of apoptotic process exhibited in cell fragmentation (uneven division) and developmental arrest of the embryo (Makarevich et al., 2005).

In our study we aimed at the question, whether a cause for low quality and developmental potential of micromanipulated embryos is an apoptosis occurrence, as a response to detrimental effect of the gene microinjection or microinjection-related factors. We compared ratio of apoptotic cells detected by TUNEL technique in embryos following microinjection either with the gene construct or blank solution (without the gene) or without microinjection (Makarevich et al., 2005). Results of this study showed that both gene-microinjected and blank solution (gene-free) - microinjected blastocysts had similarly higher ratio of apoptotic cells and lower total cell number than intact embryos. A detrimental effect was obviously caused by factors associated with the microinjection procedure itself rather than with the gene construct or the integration. Moreover, apoptosis was defined as one of reasons of lower quality and viability of micromanipulated embryos. Apoptosis detection may therefore be a proper parameter for determination of the quality and survivability of such embryos.

APOPTOSIS AND QUALITY OF EMBRYOS EXPOSED TO HYPERTHERMIA

Early preimplantation embryos are very sensitive to various forms of stress like temperature changes, oxidative stress or toxic substances (Sakatani et al., 2004). Ultrastructural alterations caused by heat shock may result in a translocation of organelles toward the centre of the embryonic cell (Rivera et al., 2004). Damages occurred due to heat stress during preimplantation development may negatively influence developmental program decreasing proteosynthesis and survivability of embryos (Putney et al., 1988, Ealy et al., 1993). Such damages may include destruction of cytoskeleton and subsequently cell death (apoptosis or necrosis).

Cultivation of animal preimplantation embryos in **hyperthermia** environment is a proper model for the study of the stress action (Makarevich et al., 2007). Response of embryos on stress factors can be influenced by animal species, embryo origin (*in vivo, in vitro)* and developmental stage. Unfavourable conditions of embryo milieu force the embryo to activate different adaptation mechanisms. One of processes involved in acquisition of resistance against heat shock may be a **stress-induced apoptosis** (Paula-Lopes and Hansen, 2002). It is proved that increased occurrence of apoptosis in cells exposed to heat stress can be reduced due to **heat-shock protein 70 (Hsp70)** formation induced by moderate heat shock (Mosser et al., 1997; McMilan et al., 1998).

Apoptosis frequency in embryos depends on the intensity of heat shock. Moreover, response of embryos on temperature elevation is species-specific and critical temperature for each embryo species is different. Thus, in rabbit embryos, hyperthermic exposure to 41.5ºC for 6 hours did not affect a rate of apoptosis, whilst at 42.5ºC the embryos were arrested at early preimplantation stages (Olexikova et al., 2007) and showed an increased rate of apoptosis (Makarevich et al., 2007). In bovine blastocysts (Paula-Lopes and Hansen, 2002), apoptotic cell percentage was increased with an elevation of the temperature of

cultivation. At minimum of 41ºC, apoptosis occurrence depends on the duration of heat shock.

Despite permanently accumulating data about heat stress influence on embryos a mechanism of thermotolerance acquisition in embryos is still unclear. Generally, more information is available on the physiological responses of the cell to heat stress, compared to those of mammalian oocytes and embryos (Ju, 2005). To better understand the mechanisms of thermal injuries or tolerance, more work on cellular and molecular changes in oocytes and embryos in response to heat shock is necessary.

CONCLUSION

Fertility of farm animals is an important factor determining the efficiency of the breeding process. Future fertility is substantially influenced by viability of gametes at fertilization as well as subsequent embryos at preimplantation development. Approaches, identifying decreased developmental potential, could reveal possible causes for early embryonal mortality. Stress factors and unfavourable external conditions surrounding the embryo force it to trigger adaptation mechanisms. One of them is a programmed cell death or apoptosis, by which the embryo may get rid of damaged or genetically abnormal cells. On the other hand, increased apoptosis caused by environmental factors may negatively influence subsequent embryo development. Detection and quantification of apoptosis occurrence may help to determine consequences of these influences and to find out factors inhibiting apoptosis. Exact knowledge of processes leading to damages and embryonal loss, as well as protective mechanisms preventing these damages, may find out approaches for the improvement of embryo quality and survivability. Finally, it may contribute to increase the efficiency of manipulations on animal embryos in frames of biotechnology.

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Authors' addresses: Alexander V. Makarevič, Peter Chrenek, Juraj Pivko, Slovak Agricultural Research Centre, Research Institute of Animal Production, Hlohovska 2, 949 92 Nitra, Slovak Republic;
Peter Chrenek, Peter Massanyi, Norbert Lukač, Slovak Agricultural University, Trieda A.Hlinku, 94901 Nitra, Slovak Republic