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VARIABILITY IN APOPTOSIS INCIDENCE IN MOUSE BLASTOCYSTS IN RELATION TO THEIR AGE AND THE TYPE OF THEIR DERIVATION

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ABSTRACT

This study was undertaken to obtain information about apoptotic cell death incidence during embryo development up to the blastocyst stage under standard *in vivo* or *in vitro* conditions.
Mouse embryos were conceived in maternal body and isolated at early or late blastocyst stage. Portion of these blastocysts was additionally cultured *in vitro* for 24 h. The presence of apoptotic cells in embryos was evaluated by cell death assay, based on the triple fluorescence staining, which enabled morphological assessment of nuclei (DNA staining), detection of specific DNA degradation in the nucleoplasm (TUNEL-labeling) and assessment of membrane integrity / cell viability (vital staining by propidium iodide).
In our experiments, only 23 % of freshly isolated early blastocysts contained blastomeres with apoptotic features and the incidence of apoptotic cells in them was relatively low (1.19 % at average). On the contrary, groups of advanced blastocysts, derived both *in vivo* or by the additional culture *in vitro*, showed significantly higher frequency of embryos with at least one apoptotic cell (over 68 %, $P<0.001$) and significantly higher percentage of apoptotic cells (over 4.46 %, $P<0.001$). Moreover, the *in vitro* conditions apparently slowed down blastocysts growth and caused further elevation in cell death incidence.
Our results show that the incidence of spontaneously appearing apoptosis in mouse blastocysts increases during their growth and this increase is more apparently by culture under *in vitro* conditions.

Key words: mouse; blastocyst; apoptosis

INTRODUCTION

The development of preimplantation embryo is characterized with intense cell proliferation, cell differentiation and relatively frequent appearance of apoptotic cell death (Huppertz and Herrler, 2005). Apoptosis is a physiological process occurring in the majority of cell populations. In normally developing mouse embryos, sporadic appearance of cells with apoptotic features can be observed even at the 4-cell stage (Fabian et al., 2007). Nevertheless, as in other species, the highest incidence of apoptosis appears at the blastocyst stage (reviewed in Fabian et al., 2005). The main function of apoptosis is to provide normal embryonic development by the elimination of abnormal, detrimental or superfluous cells (Pampfer and Donnay, 1999).

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Apoptosis is a self-directed process based on a genetic mechanism. Although apoptotic cells appear to self-destruct from within, the determination whether the cell will survive or die depends on various internal and external factors, which affect the expression of various pro- and anti-apoptotic proteins. Majority of these proteins (e.g. Bcl-2 family members) is localized permanently or transiently to mitochondrial membranes, where they are believed either to regulate, or to form mitochondrial pores. Opening of these pores results in the release of several death-inducing factors from the mitochondria into the cytoplasm and activation of death-specific enzymes (Fadeel et al., 1999).

Apoptosis has received increasing attention mostly because of its potential role in cellular response to suboptimal developmental conditions and stress (reviewed in Betts and King, 2001; Makarevich et al., 2008). Increased incidence of cell death is an important indicator of inadequate *in vivo* or *in vitro* environments for preimplantation embryos (Pomar et al., 2005). Moreover, after reaching inadequate level, the massive dying of blastomeres might probably decrease the quality of derived blastocysts and lower their abilities to implant.

The aim of this study was to expand knowledge on the physiology of apoptotic processes in blastocysts. We aimed to found out if there is any relation between apoptosis incidence and progressive blastocyst growth or if the percentage of apoptotic cells remains at the same level during the whole blastocyst stage period. Moreover, we determined if the proportion of spontaneously appearing apoptotic cells can be affected by relatively low external stress, induced experimentally by the change of developmental conditions from *in vivo* (maternal body) to *in vitro* (standard culture).

MATERIALS AND METHODS

Female mice (ICR strain, Velaz, Prague, Czech Republic; 4 weeks old) underwent superovulation treatment with pregnant mare's serum gonadotropin (eCG 5 UI i.p.; Folligon, Intervet International), followed 47 h later by administration of human chorionic gonadotropin (hCG 5 UI i.p.; Pregnyl). Females were mated with males of the same strain overnight. Mating was confirmed by identification of a vaginal plug. Females were killed by cervical dislocation. Embryos were recovered by flushing of the oviduct and the uterus using a flushing–holding medium (FHM) (Lawits and Biggers, 1993). The developmental stages of isolated embryos were classified by stereomicroscopical evaluation. Isolation of early blastocysts was performed at 92 h after hCG treatment and isolation of late blastocysts was performed at 116 h after hCG. Isolated morulas and blastocysts were pooled, washed in KSOM culture medium (Lawits and Biggers, 1993) and divided into two groups. First group was stained immediately after isolation and second was stained after 24 h culture *in vitro*. Selected embryos were cultured in drops of KSOM (1 embryo/1 µl approximately) in a humidified atmosphere with 5% CO_2 at 37 ºC.

All blastocysts (from fresh isolations and additional cultures *in vitro*) were first stained with propidium iodide (PI, 10 µg/ml; Sigma–Aldrich; stains dead cells only). Then they were washed in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA, Sigma–Aldrich), fixed in 4% paraformaldehyde (Merck) in PBS at room temperature for 1 h and optionally stored in 1% paraformaldehyde in PBS at 4 ºC. Nuclei with degraded DNA were detected using a cell death-detection technique based on the TUNEL principle using fluorescein-conjugated dUTP (Fabian et al., 2007). Fixed blastocysts were washed, permeabilized for 1 h in PBS with 0.5% Triton X-100 (Sigma–Aldrich) and again washed in PBS (+BSA).Then they were incubated in 10 µl of terminal deoxynucleotidyl transferase and 90 µl of fluorescein-conjugated dUTP (TUNEL, In Situ Cell Death Detection Kit; Roche) for 1 h at 37 ºC in the dark. After TUNEL reaction, all embryos were counterstained with Hoechst 33342 (20 µg/ml; Sigma–Aldrich; stains all nuclei, shows nuclear morphology) for 5 min at 37 ºC, washed, mounted on a slide, covered with a coverslip and observed under fluorescence microscope (BX50 Olympus) at ×400 magnification.

Average number of nuclei per blastocyst was determined as the main indicator for embryo growth. According to their nuclear morphology (M), the presence of specific DNA fragmentation (T) and PI positivity/negativity (PI±) nuclei were classified as: normal (M–T–P–; oval, without morphological changes, without TUNEL labeling and able to exclude PI), apoptotic (M+T±P–; PI negative, with typical fragmented or condensed morphology, usually containing degraded DNA), secondary necrotic (M+T±P+; with typical apoptotic morphology, usually containing degraded DNA, reaching terminal stages of apoptotic process characterized by PI positivity) and necrotic (M–T±P+; PI positive, mostly without specific morphological changes and usually without TUNEL labeling). The percentages of normal, apoptotic and secondary necrotic blastomeres were calculated as the numbers of normal, apoptotic and secondary necrotic nuclei in relation to the total number of evaluated nuclei.

Statistical analysis was performed using Statistica (StatSoft). Standard chi-square tests were used to detect differences in the portion of blastocysts with at least one apoptotic blastomere, in cell death incidence and in the profiles of apoptotic cell death. Student's t-test was used to detect differences in average cell numbers. The results are expressed as mean values ±SD. Values $P<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Developmental capacities

Stereomicroscopic evaluation of pooled embryos isolated at 92 h post hCG from 48 mice showed that 62 % of them reached blastocyst stage, 24 % reached morula stage and the rest arrested at the lower stages of development or degenerated. At 116 h post hCG (isolates from 44 mice), the percentage of *in vivo* derived blastocysts was much higher (88 %), there were almost no morulas (less than 1 %) and only few arrested or degenerated embryos (up to 11 %).

Freshly isolated early blastocysts contained at average 49.48 cells per embryo (Table 1). During additional 24 h culture, all morulas and early blastocysts reached the stage of expanded blastocyst or higher and their average cell number increased to 85.80 ($P<0.001$).

Although showing relatively good growth capabilities, cell numbers of these embryos did not reach the level of the cell numbers of freshly isolated advanced blastocysts ($P<0.001$), evaluated at the same time (at 116 h post hCG). This proved the negative effect of *in vitro* developmental conditions on this parameter. Advanced blastocysts contained at average 115.05 cells and they showed very low ability to grow *in vitro*. During additional culture their development was arrested and their average cell number remained approximately at the same level ($P>0.05$).

Developmental arrest of late (advanced) blastocysts proved that standard culture conditions are not sufficient for the development of mouse blastocyst outgrowths. As documented by other authors, more complex media, often containing some type of serum, are necessary for this purpose (Hunter et al., 1988; Lawitts and Biggers, 1993; Drakakis et al., 1996).

Apoptosis incidence

Approximately 23 % of freshly isolated early blastocysts contained at least one apoptotic cell. In this group, average incidence of apoptosis was relatively low (around 1.19 %) and both types of evaluated dying cells (apoptotic – showing majority of typical apoptotic features, and secondary necrotic – of apoptotic origin, but showing the loss of membrane integrity) were presented equally.

Twenty-four hours later, after both culture *in vitro* or continual *in vivo* development, the percentage of blastocysts with apoptotic cells and the incidence of apoptosis were increased ($P<0.001$ for all cases). Furthermore, in the case of cultured embryos, numbers of blastocysts with apoptotic cells were significantly higher than in freshly isolated advanced blastocysts (93 % vs. 68 %, $P<0.001$). This proved the negative effect of artificial developmental conditions.

Although a great progress has been achieved in the field of developmental biotechnologies during past years, standard culture *in vitro* is apparently still not able to substitute ideally environment of maternal body. Increased apoptotic incidence in *in vitro* produced mouse blastocysts has been documented several times (Brison and Schultz, 1997; reviewed in Levy 2001). When compared to their *in vivo* counterparts, a higher level of apoptosis has been documented also in cultured bovine, porcine and equine blastocysts (Gjorret et al., 2003; Pomar et al., 2005).

In our experiment no further increase in any of cell death parameters was recorded in late blastocysts, which were additionally cultured for another 24 h ($P\geq0.05$). In this group, the relatively low frequency of apoptotic processes could be probably related to general embryonic arrest, which was accompanied with lowered cellular and division activity and consecutively lowered need for the activation of repair mechanisms. Thus, these results might not reflect a physiological status of normally developing late blastocysts.

In all groups of blastocysts, which were older than 116 h, higher numbers of observed apoptotic cells were at the stage of secondary necrosis, which is in accordance with previous findings. Phagocytosis, cell extrusion and secondary necrosis are characteristic for terminal stages of apoptotic processes in blastomeres (Fabian et al., 2005).

Table 1: Apoptosis incidence in early and late blastocyst

Development	Early blastocysts		Late blastocysts	
	92 h *in vivo*	92 h *in vivo* +24 h *in vitro*	116 h *in vivo*	116 h *in vivo* +24 h *in vitro*
Blastocysts (n)	195	107	122	82
Blastocysts with apoptotic cells (%)	22.56%	92.52%[a]	68.03%[b]	80.49%[b]
Average cell number	49.48±9.23	85.80±16.78[a]	115.05±21.51[b]	112.83±21.30[b]
Apoptotic cells (%)	1.19%	4.98%[a]	4.46%[a]	4.65%[a]
Apoptosis (%)	0.56%	1.20%	0.78%	0.92%
Sec. necrosis (%)	0.63%	3.78%	3.68%	3.73%

Footnote: Values with different superscripts differ significantly. Differences at $P<0.05$ were considered as significant

From literature, percentage on apoptosis incidence in mouse blastocysts widely differs. Several authors documented similar results to ours (Brison and Schultz, 1997; Jurisicova et al., 1998), others rather different (reviewed in Fabian et al., 2005). This wide variability is probably affected by various internal (different mouse strain, mothers age) and external factors (different culture media, culture density of embryos, methodology of cell death assay, etc.). Furthermore, in these experiments, the age of evaluated blastocysts usually differs too. This is a reason why the knowledge of physiological processes in particular embryos (developing both *in vivo* and *in vitro*) used in the laboratory is very important for appropriate interpretation of obtained results. From this point of view, our findings could help during arranging of appropriate model of experiment, using apoptosis as a marker of blastocyst quality or a quantitative output of toxicological test.

In conclusion, our results proved previous findings documenting that apoptosis is a physiological event occurring during normal preimplantation development both *in vivo* and *in vitro*. Moreover, we showed, that at blastocyst stage the incidence of spontaneously appearing apoptotic processes is increased during the progressive growth of the embryo and that it can be further elevated in inadequate developmental conditions. Our results demonstrate that even well-standardized *in vitro* environment might significantly affect both cell division and cell death predominantly during early cleavage period of blastocyst development.

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