Original Article

Novel Approach of Differential Staining to Detect Necrotic Cells in Preimplantation Embryos

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Abstract

Background: This novel approach describes a rapid and simple method for identification of necrotic vs. viable cells within a mammalian blastocyst.

Materials and Methods: Hatched bovine blastocysts produced in vitro were first incubated for 30 min in pre-equilibrated culture medium containing propidium iodide (PI; 300µg/ml) and bisbenzimide (Hoechst: H33342; 5µg/ml) fluorescent dyes. Embryos were then freed from residual dyes by thoroughly washing in warm phosphate buffer saline free of calcium and magnesium (PBS^-), fixed in 2.5% glutharaldehyde and washed again in PBS^-. Stained embryos afterwards were mounted in a drop of glycerol over a microscopic slide. Prepared samples were examined under an epifluorescent microscope using the same excitation wavelength (330-385nm) and barrier filter (400nm) to distinguish necrosed vs. viable blastomers as being appeared in red and blue, respectively.

Results: Obtained results showed that in cells with altered cell membrane such as late apoptotic or necrotic cells, PI and H33342 readily enter through the cytoplasmic barriers and so the chromatin materials are stained by both, but since PI quenches bisbenzimide fluorescence, necrotic blastomeres are seen in red to pinky red, while live cells are seen just as blue.

Conclusion: Obtained results clearly indicated that this novel approach can be used as a simple, feasible and precise method for every embryology lab and with all the mammalian blastocysts produced either in vitro or in vivo. The basic assay can be completed in 60 min, and valuable and reliable information can be obtained about the quality of the embryos.

Keywords: Mammalian Blastocyst, Viability, Hoechst 33342, Propidium Iodide

Introduction

Livestock production has significantly changed over the past decades with industrial systems and their associated techniques such as in vitro embryo production (IVP) and embryo transfer (ET) is becoming increasingly important (1). The large–scale production of mammalian embryos in vitro is dependent on the production of large number of high quality embryos (2). In this regard, the success rates of IVP systems in terms of blastocyst yield remain modest and ranges 30 to 40%, which is still lower than that obtained from embryos produced in vivo (2, 3). Furthermore, the quality of IVF embryos is inferior to that of embryos produced in vivo as judged by morphology, increased susceptibility to cryo–injury and poor implantation and viability (3). The low rate of successful pregnancies achieved following transfer of the IVP blastocysts indicated that qualitative assessment of the IVP blastocysts is a crucial factor determining the success of IVP and ET programs (3-5). Therefore, intensive focus has been paid to provide valuable means for determining the quality of the embryos produced in vitro not only for livestock animals but also for human embryos (5). Accordingly, a wide range of techniques have been presented for assessment of the total cell number (TCN) as well as viable and dead cells of the blastocysts such as morphological examination (6), differential staining (5, 6), tunnel assay (7), metabolic assays (8) and etc. Most of these techniques are time consuming, elaborative and need expensive equipments or reagents and high technical skills. Here, we describe a feasible qualitative approach for determining TCN as well as the viable vs. necrotic cells of the blastocysts.

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The basic conception on which this novel technique was proposed is based on the natural function and permeability of the cell membrane in viable and dead cells in response to propidium iodide (PI) and bisbenzimide (H33342) flourochromes. Viable cells do not permit PI to enter the cell and stain the nuclear chromatin (8, 9); hence it merely stains the cells with altered membrane integrity such as necrotic cells or cells with ultra damaged cell membrane (9). H33342, on the other hand, has a complete potency to enter all cells. By having this in mind, this novel approach is proposed as a feasible technique for assessment of cell viability in blastocysts.

Materials and Methods

Chemicals were purchased from Sigma chemical Co. (St. Louis, Mo, USA) unless other types indicated. The basic point considered obligatory is that before embryo fixation, all the media and procedures used for embryo manipulation should be performed in preincubated optimum culture media, otherwise, the number of dead cells will increase.

Bovine in vitro embryo production was performed as described elsewhere (10). Day 7 to 9 hatched embryos were first washed two times in prewarmed Ca^{+2} and Mg^{+2} free phosphate buffer saline (PBS^{-}) and then were incubated in the freshly prepared preincubated staining solution (propidium iodide (PI Cat No: P 4127; 300µg/ml) and hoechst (H33342: Cat No H33342: 5µg/ml) for 30 min at the optimum incubation conditions of the embryos (for example the optimum culture conditions of bovine blastocysts in which embryos were stained was: 38.5ºC, 5% CO_2, 5% O_2 and maximum humidity). Embryos were then washed three times by warm PBS^{-} to remove residual dyes and embryos were quickly fixed in the 2.5% glutharaldehyde for at least 5 mins at room temperature. Fixed embryos were washed thoroughly in PBS^{-} before being mounted in a drop of glycerol between two lines of paraffin wax. A coverslip was placed on top of the embryos and gently pressed until the embryos were slightly flattened. The edge of the slide was sealed with fingernail polish. Prepared samples were examined under a common epifluorescent inverted microscope (Olympus BX51) at 100X and 400X magnifications using the same excitation wavelength (330-385nm) and barrier filter (400nm) to visualize both dyes. H33342 fluorescent dye readily enters all the cells, either viable or dead, while PI can merely enter the cells with altered membrane integrity, therefore; it was expected that late apoptotic and early to completely necrosed cells to be appeared as red while alive cells with intact cell membrane to be appeared as blue and dark with this excitation wavelength. In cells with altered cell membrane, although both PI and H33342 stain the chromatin, PI quenches H33342 fluorescence, so necrotic blastomeres are seen just in pinky red to red, while live cells are seen as blue. Embryos were also examined for red fluorescent using excitation wavelength (460-490 nm) and barrier filter (505 nm). Under this condition only necrotic cells or PI positive cells can be observed.

Results

Figure 1.A shows excellent quality blastocysts with symmetrical and spherical embryo mass with individual cells that are uniform in size, color, and density. This embryo is consistent with its expected stage of development (day 7 of embryo development). Under fluorescence microscopy, with excitation wavelength (330-385nm) and barrier filter (400nm) this blastocyst does not show any necrotic cells (Fib 1.B). No necrotic cell was observed using excitation wavelength (460-490 nm) and barrier filter (505 nm) which further confirmed that all the cells of the stained blastocyst were alive.

Figure 1D shows another good quality blastocyst similar to figure 1A while figure 1E shows fluorescence microscopy of this blastocyst when it was exposed to liquid nitrogen and thawed three times in absence of any cryo-protectant and then was observed with excitation wavelength (330-385nm) and barrier filter (400nm). As expected, due to cryo-injury all the cells appear as red. All the cells of the blastocysts also appear red (Fig 1F) when observed under excitation wavelength (460-490nm) and barrier filter (505nm).

Figure 1G shows a grade 3 to 4 blastocyst with major irregularities in overall shape of the embryonic mass or in size, color and density of individual cells. Under fluorescence microscopy with excitation wavelength (330-385nm) and barrier filter (400nm), 31.1% and 61.8% of cells appears as blue and red, respectively (Fig 1H); while under excitation wavelength (460-490nm) and barrier filter (505nm) only red cells can be observed (Fig 1).

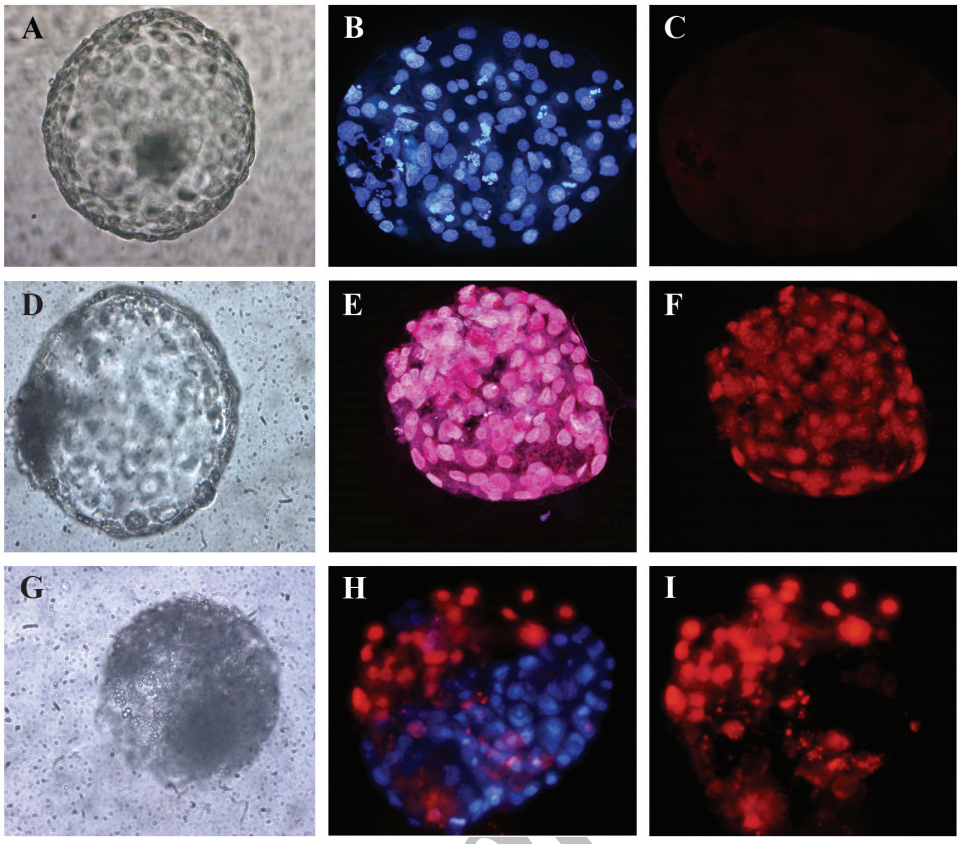

Fig 1: Differential viable staining of the embryos as a valuable approach for assessment of the blastocyst quality. (A) Excellent quality bovine blastocysts produced in vitro (IVP). This embryo is consistent with its expected stage of development (day 7 of embryo development) with symmetrical and spherical embryo mass with individual cells that are uniform in size, color, and density. (B) Under fluorescence microscopy, with excitation wavelength (330-385nm) and barrier filter (400nm) this blastocyst does not show any necrotic cells. (C) No necrotic cell was observed using excitation wavelength (460-490 nm) and barrier filter (505 nm) which further confirmed that all the cells of the stained blastocyst were alive. (D) Another good quality bovine blastocyst similar to figure (A) while figure (E) shows fluorescence microscopy of this blastocyst when exposed to liquid nitrogen and thawed three times in absence of any cryo-protectant and then was observed with excitation wavelength (330-385nm) and barrier filter (400nm). As expected, due to cryo-injury all the cells appear as red. (F) All the cells of the blastocysts also appear red when observed under excitation wavelength (460-490 nm) and barrier filter (505 nm). (G) A grade 4 bovine blastocyst with major irregularities in overall shape of the embryonic mass or in size, color and density of individual cells. (H) Under fluorescence microscopy with excitation wavelength (330-385nm) and barrier filter (400nm), 31.1% and 61.8% of cells appears as blue and red, respectively while under excitation wavelength (460-490 nm) and barrier filter (505 nm) only red cells can be observed (I). (This Figure has also been printed in full-color at the end of the tissue)

Discussion

This novel approach describes a rapid and simple method for the identification of necrotic and/or dead cells within a mammalian blastocyst. Owing to changes in membrane permeability, late apoptotic (11), early necrotic and necrosed (9, 11) blastomeres show an increased uptake of the vital DNA dye propidium iodide. Unlike these cells, live or possibly early apoptotic are not permeable to this vital dye (9). The method appears to be applicable to all the mammalian blastocysts produced in vitro or in vivo, including human blastocysts. However, it is of note that this procedure merely distinguishes necrosed cells or the cells with altered cell membrane (late apoptotic cells) and not the live or none apoptotic cells or the cells which have an intact cell membranes.

The results of staining with an excellent blastocyst (Fig 1A, B, C) showed no red cell (necrosed or dead cell), while in another good quality blastocyst exposed to liquid nitrogen in absence of cryo-protectant (Fig 1D, E, F), all the cells were dead as appeared in red. A low quality blastocyst (Fig 1G, H, I) showed 31.1% dead (red) and 68.8% live (blue) cells.

Apoptosis and necrosis are two major processes by which cells die (12). Apoptosis is an active, genetically regulated disassembly of the cell from within (13-15). Disassembly creates changes in the phospholipid content of the cytoplasmic membrane outer leaflet (13). Phosphatidylserine

(PS) is translocated from the inner to the outer surface of the cell for phagocytic cell recognition (12, 13). Necrosis, on the other hand, normally results from a severe cellular insult; both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosolic and organellar contents into the surrounding environment (13, 14). Although apoptosis has been characterized by morphological changes including cell shrinkage and chromatin condensation (15), but the integrity of the cell membrane is yet conserved in contrast to cell necrosis in which the integrity of the cell membrane is interrupted and a free transmission of the molecules is evident between the inner and outer cell environment (15, 16). Therefore, necrotic cells are not able to regulate the transmission of the molecules in either intra or extra cell environment and a freely state of molecule transmission is evident (15). In this regard, PI which originally is not able to penetrate the cells with intact cell membranes readily enters the cells with altered or altering plasmalemal integrity such as late apoptotic and necrosed cells (9). It is of note that although the early apoptotic cells are in the verge of progressive degenerative events, the intact integrity of the cell membrane does not permit PI to enter the cell (9, 15, 16). This simple concept was the base for introducing this approach for determining viable vs. dead cells of the blastocysts produced in vitro or in vivo.

Conclusion

The results of this technique might be very useful for examination of cryo-preserved blastocyst, since during cryo-preservation embryos are exposed to temperature insults which may lead to cell necrosis.

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