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Application of saponin on differential staining examination in animal blastocysts

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ABSTRACT

Although there are several ways such as karyotyping to evaluate the quality and normality of embryos, the counting of total cell in blastocyst after the differential staining has been used as a simple indicator for quality of culture systems and normality of embryo itself. This differential staining method was regarded as a basic technique of early developmental biology of mammals, and it helps the scientific community to understand the signals regulating morphological events of early developmental process. The present study was undertaken to develop a simple and fast differential staining method for inner cell mass (ICM) and trophectoderm (TE) cells of mammalian blastocysts using saponin as a permeabilizing agent without using species-specific antibodies and complements. The pre-stained blastocyst with SYTO-13 (green) was exposed to saponin solution for propidium iodide (PI) permeation into TE cells and examined for the differential staining patterns. Three dimensional confocal microscopy was used to demonstrate the process of successful staining and showed the high impact on saponin treatment. Although the fluorescent images of blastocysts showed that one or two cell of TE stained to yellowish green, ICM was protected from saponin/PI mixture with the short exposure time of SYTO-13 pre-stained blastocysts. The total staining procedure did not exceed 30 min before examination under epi-fluorescence or confocal microscope. These results clearly demonstrate that saponin could be used as substituent molecule instead of species-specific antibodies and complements in differential staining examination for the first differentiation of mammalian embryos.

Key words: Blastocysts staining, Bovine, Inner cell mass, Saponin, Trophectoderm cells

The development of mammalian zygote, a new life from oocyte and sperm, starts the first cellular differentiation of embryo to the compacted morula stage, in which small group of internal cells become inner cell mass (ICM) and large group of external cells become trophectoderm (TE) by its location (Barlow *et al.* 1972). Although there are several ways such as karyotyping to evaluate the quality and normality of embryos, the counting of total cell in blastocyst after the differential staining of ICM and TE cells (Ebert *et al.* 1985) has been used as a simple indicator for quality of culture systems and normality of embryo itself. This differential staining method was regarded as a basic technique of early developmental biology of mammals, and it helps the scientific community to understand the signals regulating morphological events of early developmental process. The efficiency of differential staining has been improved by many researchers who used a micromanipulator to isolate ICM (Gardner *et al.* 1973) and selective immunosurgery method (Solter and Knowles 1975, Handyside and Hunter 1984, Leppens *et al.* 1996) where the action of complement involved to damage the membrane of TE cells. By using confocal microscopy, the cytoskeleton specific dye and a monoclonal antibody against oocyte specific capsule were also used to label the boundary cells of equine (Tremoleda *et al.* 2003). Immuno-surgery method with selective antibody and complement to obtain ICM of mouse blastocysts (Solter and Knowles 1975) has been modified and successfully stained with TE cells on mice (Leppens *et al.* 1996), bovine (Iwasaki *et al.* 1990, Van Soom *et al.* 1996), porcine (Papaioannou and Ebert 1988, Machaty *et al.* 1998), rabbit (Giles and Foote 1995, Tao and Niemann 2000) and human (Hardy *et al.* 1989) with some minor modifications. The efficiency of immuno-surgery method is highly dependent on the specificity of antibody and chemical stability of the complement which was used widely by reproductive biologists (Summers *et al.* 2000, Thouas *et al.* 2001, Koo *et al.* 2002). Therefore, the assay conditions such as incubation time with complement solution or concentration of the biochemical

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materials in each experiment could be a major problem for repetitions of differential staining. To overcome micro-surgery methods of complement, two chemicals, calcium ionophore (Fuente and King 1997) and Triton X-100 (Thouas *et al.* 2001), were reported as permeant on the membrane of TE cells, so could be a substituent of antibody and complement. Fuente and King (1997) firstly reported that calcium ionophore could be used for differential staining of mouse embryos with 85% success rate. They also reported the kinetics of membrane permeation induced by calcium ionophore and optimized the exposure time (50–60 min) to achieve proper staining of mammalian blastocysts from mouse, porcine and bovine with PI and Hoechst dyes. Thouas *et al.* (2001) also reported that Triton X-100, one of the most common chemicals of permeation, could be used for staining of mouse and bovine blastocyst. According to the protocols, the nuclei of ICM were stained by Hoechst dye in simultaneous fixative/staining solution of 100% ethanol. Because of its clear image of nuclei of ICM, Triton X-100 treatment which was modified from rapid staining methods reported by Ebert *et al.* (1985) has been used widely by many researchers to stain bovine or porcine nuclear transfer (Hyun *et al.* 2003), intracellular sperm injected embryos (Abdalla *et al.* 2009, Giritharan *et al.* 2010) or mouse parthenogenetic/ *in-vitro* fertilized blastocysts (Giritharan *et al.* 2007).

Saponin from *Qullaja* bark, another chemical agent used for permeabilization/ emulsification on biological samples, is known to be a heterogenous mixture of molecules varying in sugar moieties that usually contains glucose, galactose, glucuronic acid, xylose, rhamnose or methyl pentose which are linked through glycosidic bond to a hydrophobic aglycone, named sapogenin, a triterpenoid or steroid in nature (Francis *et al.* 2002). It has been widely used for permeation of various cell types like cultured human intestinal epithelial cells (Jalal *et al.* 1992) to examine cell surface molecules like receptors or cytokines by immunohistochemical analysis (Kardel *et al.* 2003) or for a lysis of outer membranes in Rous sarcoma viruses and cell membrane of chicken liver and erythrocytes (Dourmashkin *et al.* 1962).

With these backgrounds of well established documents of saponin on various cell membranes specifically on staining examination, we investigated the possible usage of saponin for differential staining of mammalian blastocyst and optimized the successful experimental conditions on mouse, porcine and bovine blastocyst in the present study.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louise, MO, USA), unless otherwise indicated and plastic wares from Nunc (Roskilde, Denmark) and BD Bioscience (NJ, USA).

Super-ovulation regimen and embryo collection in mice: All animal treatment protocols were reviewed and approved by the National Institute of Animal Science Animal Ethical Committee (2010 - 006). The experimental procedures used for animal management, reproduction and embryo manipulation followed the standard program of our laboratory. For embryo collection, 6 to 9 week old ICR mice were used. Females were super ovulated by intraperitoneal injection of 5 unit of pregnant mare serum gonadotropin (PMSG) followed by 48 h with 5 unit of human gonadotrophin (hCG). The hormone-treated mice were mated naturally and checked for the vaginal plug next morning. After 48–76 h of HCG injection, the mated females were sacrificed by cervical dislocation, 4-cells or morula stage embryos were collected from dissected oviduct by flushing with HEPES-buffered Tyrode's medium supplemented with 0.1% polyvinyl alcohol (HEPES TLP-PVA) and cultured to blastocyst stage for 2–3 days onto 50 ml drops of 0.4% BSA-CZB medium under mineral oil in a humidified incubator at 37°C with 5% CO_2.

Culture of in-vitro *fertilized bovine blastocyst:* Immature bovine oocytes with cumulus cells (COCs) were aspirated from 2–6 mm sized antral follicles of ovaries that were delivered from abattoir and collected in HEPES TLP-PVA medium. After washing with HEPES TLP-PVA, pooled COCs were matured into 0.5 ml HEPES buffered TCM 199 medium of 4 well dish supplemented with 0.5 mM sodium pyruvate, 40 mg/ml gentamycin, 1.0 ml FSH, 0.1 IU/ml hCG and 10% fetal bovine serum (FBS) for up to 20–22 h under mineral oil in a humidified incubator adjusted to 38.5°C and 5% CO_2. The matured COCs were washed twice in Bracket and Oliphant's (BO) fertilization medium which was supplemented with 10 mg/ml fatty acid free bovine serum albumin (BSA) and 10 mg/ml heparin. The motile sperm was prepared by means of a modified percoll gradient preparation from frozen semen in 0.5 ml straw and the concentration of sperm was adjusted to 2×10^7 cells/ml with BO sperm solution containing 10 mM caffeine (Brackett and Oliphant 1975, Parrish *et al.* 1986). The sperm suspension was diluted two-fold into 25 ml BO fertilization drops with 20 COCs and cultured for 5–6 h at 38.5°C in a humidified atmosphere of 5% CO_2 incubator. After *in-vitro* fertilization, embryos were co-cultured with cumulus cells in CR1 medium supplemented with 1% essential and non-essential amino acids and 5% FBS at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 (Rosenkrans *et al.* 1993).

Culture of porcine blastocysts from oocyte activation: Porcine gilt ovaries were collected from the abattoir and stored in saline at 25–30°C during transportation. Immature COCs derived from aspiration of 3–8 mm sized antral follicles were cultured for 44 h in TCM199 supplemented with 0.1% PVA, 10 ng/ml EGF, 0.57 mM cysteine, 0.5 mg/ml LH, 0.5 mg/ml FSH, 75 mg/ml penicillin-G and 50 mg/ml streptomycin (Abeydeera *et al.* 2000). The matured oocytes were released from COCs by repeated pipetting in 1 mg/ml hyaluronidase and exposed to 70 mM calcium ionophore for 5 min at 25°C and followed by subsequent exposure to 7.0 mg/ml cytochalasin B for 4 h in NCSU 23 medium at 38.5°C in a humidified atmosphere of 5% CO_2 incubator used for the activation of porcine MII oocytes.

After activation, the oocytes were cultured in NCSU 23 medium supplemented with 0.4% fatty acid-free BSA (NCSU 23-BSA) and 1% MEM non-essential amino acid for seven days.

Culture of in-vivo *fertilized porcine blastocysts:* Large White gilts were treated with a combination of 400 IU of eCG and 200 IU of hCG (PG600, Intervet International B.V., Boxmeer, The Netherlands) to induce puberty to obtain *in-vivo* fertilized blastocysts (Iwamura *et al.* 1999). A week later, the gilts were synchronized by feeding with 12.5–15 mg/day active altrenogest orally (Regu-mate, Hoechst Pharm. Inc.) for 9 days by inhibiting natural estrus (Webel and Day 1982). Sixteen days after PG600 treatment, super-ovulation was induced by injections of 1500 IU of eCG (Peamex, Sankyo) followed by injection of 1500 IU of hCG 72 h later (Puberogen, Sankyo). Sexually matured boar was used for fertilization by natural mating 24 h after hCG injection. At 52–54 h after hCG injection, the oviducts were flushed by the surgical operation to recover embryos with 40 ml of phosphate buffer saline (PBS) supplemented with 0.1% BSA. The pooled embryos with sperm head were cultured for 7 days with NCSU23-BSA medium in the same conditions that were used for a culture of activated porcine oocytes.

Differential staining of mouse, pig and bovine blastocysts: HEPES buffered TLP medium with 0.1% BSA (HEPES-TLP) was used as a basic medium for dilutions of fluorescence dyes and saponin. The zona pellucida of blastocysts was partially removed by repeated pipetting with 0.1% pronase solution to remove unwanted nuclei stains of spermatozoa. The total nuclei of the blastocysts were stained with 0.5 mM SYTO-13, live stain green dye from Molecular Probes, for 5~10 min in the incubator. After washing three times in HEPES-TLP, total blastocysts were preserved for 20~30 min in 500 ml of TCM 199 medium under mineral oil in 4 well dish at the incubator to reduce nonspecific cytosolic stains. The SYTO-13 pre-stained blastocyst was transferred into 50 ml drop for staining with 100 mg/ml saponin and 100 mM PI mixture for porcine or bovine blastocyst whereas 50 mg/ml of saponin solution for mouse. After 3–5 min, slightly shrunken blastocyst was transferred immediately to 500 ml of the fresh basic medium in 4 well dish and washed three times. A completely washed blastocyst was examined under an epifluorescent microscope. Fresh saponin solution was used for permeation and made PI infiltrate into the nuclei of TE cells, where the green color of SYTO-13 was exchanged to red within 3–5 min when the sample was examined under an inverted epi-fluorescence microscope (Olympus IX71) using an FITC/TRITC dual filter at room temperature. The permeation was confirmed by red in TE and the blastocyst was immediately rinsed 3 times with basic medium and carefully compressed under 22 × 22 mm glass cover slips with paraffin wax supports at the corners and 5 ml of anti-fading gel mount (Biomeda) for epifluorescence imaging. The fluorescence images were taken immediately by a digital camera and the number of permeated cells stained by PI and intact cell by SYTO-13 were counted.

Visualization of differentially stained blastocysts by confocal microscopy: The localization of TE and ICM was confirmed by scanning of 3–7 mm on Z sections with a confocal microscope. After PI staining with saponin, the blastocyst was transferred into a small drop (about 2 ml) of HEPES TLP-PVA under mineral oil on a hand-made acryl chamber in which a cover slip was attached by spreading vacuum grease (Beckman, USA). The samples were excited with 488 nm argon laser and 543 nm He-Ne laser in Carl Zeiss LSM510 and resulting images were reconstructed from a range of band path 505–530 nm for SYTO-13 and long path 560 nm for PI respectively.

RESULTS AND DISCUSSION

Whole blastocyst staining with cell permeant SYTO-13: A small group of porcine parthenogenetic blastocysts were used for pre-staining test of TE and ICM cells by live cell stain of SYTO-13. As shown in Fig. 1a, total nuclei of the whole blastocyst were stained green by cell-permeant SYTO-13 dye, and the nucleoli of each cell remained brighter green than those of nuclei marked with white arrow.

Chemical treatment of blastocyst with saponin: The permeation on mouse blastocysts was easily induced by a concentration of 50 mg/ml saponin and examined under a confocal microscope for judgment of successful stains. As shown in Figs. 2b and b', when mouse blastocyst in green was treated with saponin/PI mixture for 3–5 min and the

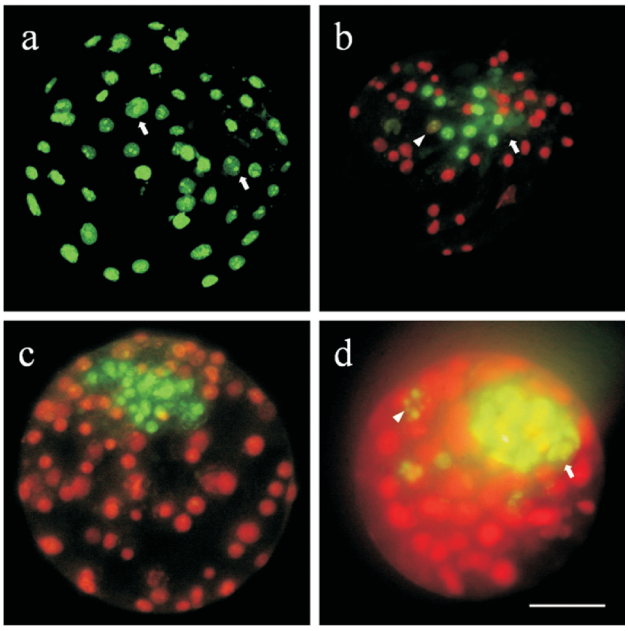

Fig. 1 (a-d). Differential staining of porcine and bovine blastocyst with SYTO 13 and PI dyes. Parthenogenetic porcine blastocyst was stained by SYTO 13 dye alone (a). Differential staining of *in-vivo* derived hatching porcine blastocyst (b) was examined after 6 days of culture. Parthenogenetic porcine blastocyst (c) and IVF derived bovine blastocyst (d) after 7 days of culture was prepared by the same procedures. Scale bar was 50 μm.

cell permeant SYTO-13 in TE cells could be replaced by PI. On the other hand, green colour in ICM nuclei illustrated that membranes have not been permeabilized by saponin treatment (Figs 2b', c' and d'). The 8 fluorescence (Figs 2a ~ h) and respective transmission (Figs. 2a' ~ h') images from the whole 20 Z-sections were selected to show ICM and TE cell loci of blastocysts. Three dimensional reconstructed images (Figs 2i and i') from total sections also depicts the loci of TE/ICM cells.

The transmission and fluorescence images of serial z-sections with 6.3 µm gaps in mouse blastocyst were scanned for judgment of successful stains. Red or green colour in B' depicts nuclei of TE and ICM cells respectively. The scanning distance between images was 6.3 mm for ICM region and 12.6 mm for TE region. Scale bar was 50 µm.

To permeate porcine and bovine embryos, 100 mg/ml saponin was used to prepare second staining solutions. When *in-vivo* porcine or IVF derived bovine blastocysts were transferred into second solution of saponin and PI for 5~6 min, permeation of PI into TE cells induced and so green colour of TE nuclei turned to red (Figs 1b-d).

In this study, brief treatment of saponin within 5–6 min on the membranes of TE cells could be useful procedure for differential staining of the mouse, pig, and bovine blastocysts. Because of the higher sensitivity of SYTO dye series on DNA and RNA, the image of nucleus stained by SYTO 13 had the brighter green colour with clear detection of nucleoli. When mammalian blastocysts were pre-stained by SYTO 13, the total cell number of whole cell could be measured. Even more, the brilliant green colour of SYTO 13 of nucleoli would be helpful for judging the number of cells in blastocyst. Interestingly, staining patterns of nuclei of parthenogenetic porcine blastocyst were more variable in size than those of *in-vivo* derived porcine blastocyst.

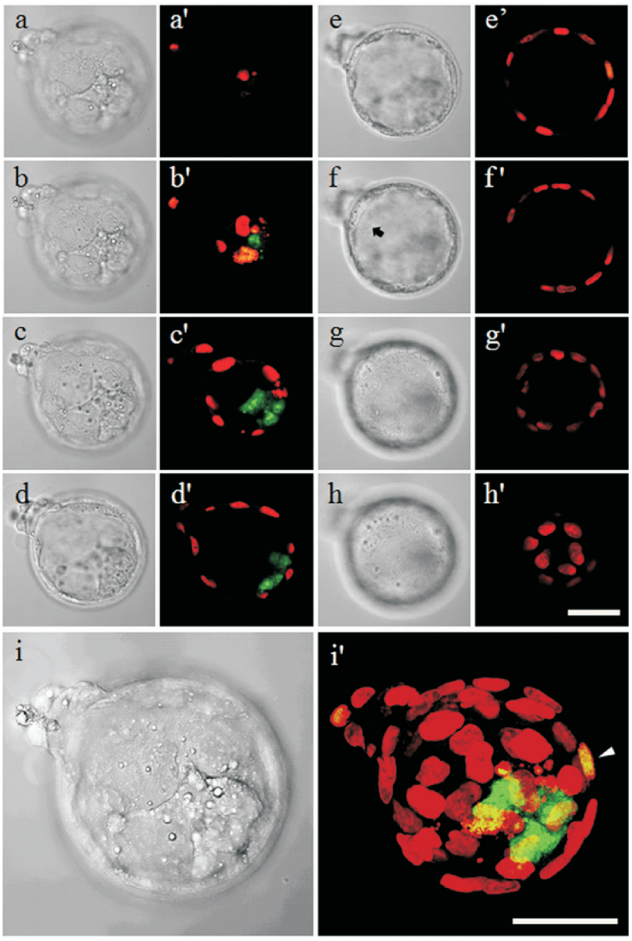

Fig. 2. Images of differentially stained mouse blastocyst with 3D confocal microscopy.

Because the permeation treatment on the membrane of TE could be one of the key steps for successful results of our differential staining, the pattern of ICM and TE nuclei were highly dependent on saponin concentration in second staining solution. So, embryo treatment time into the mixture should not be longer than 10 min for staining of porcine and mouse blastocysts. Occasionally, the yellowish green or green colour of TE nuclei was observed in porcine blastocysts from early blastocysts as in Fig. 1D marked with arrowhead. These may not be permeated by saponin during treatment, but these could be rectified individually by more accurate confocal microscopy like 3D imaging techniques marked as an arrow head in Fig 2i'. When the blastocoelic cavity of mouse blastocyst was examined during permeation with saponin, the inner membrane of TE cells made a blebbing pouch (Fig. 2f, black arrows). These indicate that SYTO 13 dye in mouse ICM was protected from chemical treatment of lower concentration of saponin and mouse blastocyst was not collapsed by the current methods.

In the case of porcine and bovine blastocyst, cytosolic background colour of SYTO 13 chromophore of ICM cells discriminated itself from the cellular compartment of TE cells, so made clear differential staining images (Figs 1c and d) (white arrows). The incomplete differential staining of TE into red colour, examined as a non-specific scattering pattern of green and red colour in TE cells, was also found at lower concentration of 100 mg/ml saponin treatment in porcine and bovine embryos (Figure not shown). These suggested that 100 mg/ml of saponin or slightly higher concentration would be a proper condition for differential staining of bovine and porcine. However, the same treatment on the membrane of mouse TE cells within 5 min exposure made some of ICM to be stained into red colours. The distinction between green and red colour images of successfully stained blastocysts from porcine and bovine are presented in Fig. 1d where the result revealed the permeation for 5–6 min was effective on differential staining of porcine and bovine blastocyst.

In conclusion, the staining procedures described with saponin and SYTO-13 live fluorescent dye may be used for rapid differential staining methods of the mouse, porcine and bovine blastocysts produced *in-vivo* or *in-vitro*. Another membrane permeable staining dye, like DAPI or Hoechst, also could be used with saponin treatments that were described in this study, but SYTO-13 had some merits like intensity of quantum yield of fluorescent chromophore or staining property of cytoplasm of ICM. When the blue staining dyes were used for differential staining, this method

may have a particular benefit for assessment of chimera blastocysts which was produced by the injection of GFP stem cells or fused chimera blastocysts with GFP producing iPS cells.

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