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Developmental Potential of Mouse Single Blastomere Derived from Isolated 2-, 4- and 8- Cell Embryos into Blastocyst and Inner Cell Mass (ICM) Outgrowths

(Potensi Perkembangan Sel Mencit Blastomer Tunggal daripada Pencilan Sel Embrio 2-, 4- dan 8- kepada Blastosista dan Pertumbuhan Luar Jisim Sel Dalam (ICM))

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ABSTRACT

The application of blastomere separation (BS) technique to produce embryonic stem cell (ESC) from inner cell mass (ICM) is not yet perfected due to low cleavage efficiency of single blastomere. Therefore, the aims of this study were to evaluate the developmental potential of single blastomere (SB) derived from embryos at different stages to blastocyst and the potential of ICM outgrowth. The female mice of ICR strain (age: 8-12 weeks; n=47) were superovulated using gonadotrophins and mated with ICR strain males (age: 10-14 weeks; n=20). The 2-cell embryos (n=366) were flushed from the oviduct of the treated females and cultured in vitro before assigned into the following groups: a) 2-cell, b) 4-cell, c) 8-cell and d) control prior blastomere separation. The SBs were cultured in vitro and daily observations were conducted to record the cleavage rates up to Day 5. Developmental rate of 2-cell derived SB (77.28 ± 6.77) was greater than 4-cell (63.70 ± 5.35) and 8-cell (55.73 ± 3.35), corresponding to the results of ICM outgrowth at 2-cell (69.29 ± 4.13), gave higher rate followed by 4-cell (55.73 ± 7.81) and 8-cell (41.85 ± 3.58). Diameter of blastocyst decreased as the SB parent embryo stage increased, with the respective ratio of 5 (diameter of SB blastocyst): 3 (diameter of SB) (2-cell: 92.55 ± 1.59 vs. 56.48 ± 0.40 ; 4-cell: 78.71 ± 1.37 vs. 44.02 ± 0.49 and 8-cell: 64.13 ± 2.20 vs. 35.68 ± 0.34) as well as total cell number in blastocyst (2-cell: 43.00 ± 1.48 ; 4-cell: 28.33 ± 1.15 ; 8-cell: 8.80 ± 0.58). In conclusion, SB at different stages of mouse embryos successfully develop to blastocysts in vitro that can be used as ICM source, which is a prerequisite for establishment of ESC outgrowth.

Keywords: Diameter of blastocyst; embryonic stem cell; inner cell mass isolated single blastomere; mouse embryos

ABSTRAK

Penggunaan aplikasi teknik pemisahan blastomer (BS) untuk menghasilkan sel stem embrio (ESC) daripada jisim sel dalam (ICM) masih belum sempurna kerana kecekapan belahan blastomer tunggal masih rendah. Tujuan kajian ini adalah untuk menilai potensi perkembangan blastomer tunggal (SB) yang dihasilkan daripada embrio pada pelbagai peringkat blastosista dan potensi pertumbuhan luar ICM. Strain ICR mencit betina (umur: 8-12 minggu; n=47) telah disuperovulasi menggunakan gonadotropin dan disenyawakan dengan strain ICR jantan (umur: 10-14 minggu; n=20). Embrio 2-sel (n=366) dikeluarkan daripada oviduktus betina yang dirawat dan dikultur secara in vitro sebelum diumpukkan ke dalam kumpulan berikut: 2-sel, 4-sel, 8-sel dan kawalan sebelum pengasingan blastomer. SB telah dikultur secara in vitro dan pemerhatian harian telah dijalankan untuk merekod kadar belahan sehingga hari ke-5. Kadar perkembangan diperolehi daripada 2-sel SB (77.28 ± 6.77) adalah lebih besar daripada 4-sel (63.70 ± 5.35) dan 8-sel (55.73 ± 3.35), sama seperti keputusan hasil pertumbuhan luar ICM pada 2-sel (69.29 ± 4.13), memberikan kadar yang lebih tinggi diikuti dengan 4-sel (55.73 ± 7.81) dan 8-sel (41.85 ± 3.58). Diameter blastosista menurun apabila peringkat SB induk embrio meningkat dengan nisbah 5 (diameter blastosista SB): 3 (diameter blastomer SB) (2-sel: 92.55 ± 1.59 lawan 56.48 ± 0.40 ; 4-sel: 78.71 ± 1.37 lawan 44.02 ± 0.49 dan 8-sel: 64.13 ± 2.20 lawan 35.68 ± 0.34) dan juga jumlah sel dalam blastosista (2-sel: 43.00 ± 1.48 ; 4-sel: 28.33 ± 1.15 ; 8-sel: 8.80 ± 0.58). Kesimpulannya, SB pada pelbagai peringkat embrio mencit berjaya dibangunkan ke peringkat in vitro blastosista yang boleh digunakan sebagai sumber ICM, yang merupakan pra-syarat bagi penubuhan pertumbuhan luar ESC.

Kata kunci: Diameter blastosista; jisim sel dalam; pencilan blastomer tunggal; sel stem embrionik; embrio mencit

INTRODUCTION

The embryonic stem cell (ESC), derived from the inner cell mass (ICM) of the blastocyst, have special characteristics: self-renewal which is the cells can divide to duplicate themselves for a prolonged period of time without differentiating and pluripotent in which the cell can differentiate to different types of cells. The ESC research has

the potential to be applied in regenerative medicine which essentially cures serious degenerative diseases such as heart disease, Parkinson's disease and leukemia. However, derivations of ESC that involved destruction of pre-implantation human embryos have raised many ethical issues.

Blastocysts derived from isolated single blastomere at early stage of embryos *inter alia* were commonly used

for multiplication of genotypes and pre-implantation genetic diagnosis (PGD) (Bielanska et al. 2003; Katayama et al. 2010). Earlier studies reported that mouse single blastomere derives from 2-cell embryo could develop to term (Tarkowski 1959). Subsequently, it led to the establishment of mouse ESC (Wakayama et al. 2007). Blastomere separation techniques had been successfully developed for various mammalian species. Developmental potential of the isolated rabbit blastomere was reported to be decreasing with as the SB derived embryo stages increased stage (Tao & Niemann 2000). In monkey, 35% of blastocysts was obtained after blastomere separation and led to pregnancy (Mitalipov et al. 2002). In pigs, the number of blastocysts derived from single blastomere of 4-cell was higher versus single blastomere of 8-cell with more total cell but lower ratio of ICM to total cell (Eckert et al. 1997).

Several reports had been described on the establishment of ESC from isolated single blastomere. Monolayer cell was obtained from isolated single blastomere cultured *in vitro* on different extracellular matrix components (Wilton & Trounson 1989). High percentage of blastocyst and subsequent ICM outgrowth derived from mouse 2-cell single blastomere was obtained after culture in KSOM and mES medium (Lorthongpanich et al. 2008) and high number of pluripotent ESC lines were obtained from single blastomere of 8-cell (Delhaise et al. 1996).

Therefore, this research was aimed to evaluate the developmental competence of single blastomere separated from early stages of embryos with the emphasis on the number of blastocysts produced that subsequently being used as a source for establishment of ESC line.

MATERIALS AND METHODS

EMBRYO FLUSHING

Adult ICR females (8-12 weeks old; n=49) were superovulated using 10 IU of pregnant mare serum gonadotrophin (PMSG, Folligon; Intervet) via intraperitoneal injection followed by 10 IU of human chorionic gonadotrophin (hCG, Chorulon; Intervet) 48 h later. The female mice were directly mated with ICR males (10-14 weeks old; n=20). The vaginal plug was examined on the following morning at 0800 hours for mating confirmation. The 2-cell embryos collected from oviduct of mated females were sacrificed by cervical dislocation at 48 h post-hCG injection. The 2-cell embryos were flushed with the hepes buffered KSOM and cultured in 50 μ L droplets KSOM supplemented with 4 mg/mL BSA, 5 μ L/mL MEM non-essential amino acid and 10 μ L/mL BME amino acid (Sigma-Aldrich, USA) under silicone oil (Sigma-Aldrich, USA) at 37°C in CO₂ (5%) incubator until reaching 4- and 8-cell stages before blastomere isolation. The experiment with mice was conducted in accordance and approved by Institutional Animal Care and Use Committee, University of Malaya.

BLASTOMERE SEPARATION

The 2-, 4- and 8-cell embryos were transferred into a 20 μ L droplet of pronase (0.5%) (Sigma-Aldrich, USA) to digest the zona pellucida (ZP) before isolated mechanically by gently repeat pipetting in hepes KSOM medium.

BLASTOMERE *IN VITRO* CULTURE

The isolated blastomeres of each embryos and control group (ZF without separation at 2-cell) were washed and cultured *in vitro* in a droplet of KSOM medium (20 μ L) at 37°C in a humidified atmosphere with CO₂ (5%). The developments of the single blastomeres were observed daily under inverted microscope until blastocyst stage.

PREPARATION OF MOUSE EMBRYONIC FIBROBLAST (MEF) AS FEEDER CELL LAYER

MEF were prepared from 14.5 days post-coitus mouse foetuses. All limbs, head, tail and internal organ of the foetuses were removed in phosphate buffered saline free-Ca/Mg, [PBS (-)]. Then, the foetuses were transferred into trypsin-EDTA (25%) (Sigma-Aldrich, USA) solution and minced into small pieces. An optimal trypsin-EDTA medium (5-10 mL) was added into a beaker containing the small pieces of foetuses and stir on magnetic stirrer for 15 to 20 min. Culture medium supplemented with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, USA), 10% of foetal bovine serum (FBS) (Gibco BRL, USA) and 1xpenicillin/streptomycin (Sigma-Aldrich, USA) was added into the conical tube with ratio 1.5-fold of the trypsin-EDTA containing minced foetus and centrifuged at 3000 rpm for 5 min. The supernatant was removed and the remaining pellet was gently pipetted with culture medium to make single cell. The cells were seeded and cultured on agelatine coated culture dish in CO₂ (5%) incubator at 37°C the cell reaching 80-90% confluency. The MEF was inactivated by using mitomycin C (Sigma-Aldrich, USA) before being used as feeder cell layer for ICM outgrowth.

DIAMETER OF BLASTOCYST

Diameters of the isolated single blastomere and its subsequent blastocyst production were measured with XYClone measurement tools. Next, the blastocysts were stained with Hoechst 33342 staining for cell counting.

BLASTOCYST CELL COUNT

The blastocysts derived from isolated blastomere were stained for cell counting. The blastocysts were washed three times in PBS (-) and transfer in 10 μ L droplet of fixative solution for 5 min. Then, the blastocysts in a small volume of solution of PBS (-) were placed onto the slides and covered with a coverslip. About 5 μ L solution of Hoechst 33342 (Sigma-Aldrich, USA) was dispense at the edge of cover slip and let it spread into the blastocyst. The slides were visualized under a fluorescent microscope using DAPI filter and number of cell were counted.

STATISTICAL ANALYSIS

All the data were analysed using ANOVA test. All the statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 16.0 (SPSS, IL, USA).

RESULTS

DEVELOPMENT POTENTIAL OF ISOLATED SINGLE BLASTOMERE FROM DIFFERENT STAGES OF PARENT EMBRYOS

Table 1 shows that development of isolated single blastomere decreased as the stage of parent embryos increased (2-, 4- and 8-cell) with cleavage rates of 80.88 ± 19.59 , 69.82 ± 17.65 and 56.36 ± 6.70 , respectively. However, control group gave the highest results (98.50 ± 4.74). Single blastomere derived from different stages of embryos could develop until blastocyst as shown in Figure 1(a)-1(d).

DEVELOPMENT OF MOUSE ICM OUTGROWTH ON MEF FEEDER LAYER OF BLASTOCYST DERIVED FROM SINGLE BLASTOMERE

Whole blastocyst developed from single blastomere derived at different stages were cultured on MEF feeder layer for ICM outgrowth. Table 2 shows that blastocyst derived from control group gave the highest development (76.90 ± 19.21) followed by 2-cell SB (69.29 ± 10.92) 4-cell SB (55.73 ± 15.62) and 8-cell SB (41.85 ± 6.19) (Table 2). Visible clump of ICM outgrowth from blastocyst derived SB could be observed after attachment on MEF (Figure 1(f)).

DIAMETER OF SINGLE BLASTOMERE AND THE SUBSEQUENT BLASTOCYST AND THE TOTAL NUMBER OF CELL

The size of individual single blastomere and its subsequent blastocyst at different stage of parent embryos were measured (Table 3). Figure 2 shows that the diameter of redundant single blastomere was approximately 3 (diameter of SB)/ 5 (diameter of SB blastocyst) smaller than its subsequent blastocyst. The size of blastocysts derived from SB at different stages of parent embryos were decreasing from 2- to 8-cell (92.55 ± 6.56 to 64.13 ± 6.95). The blastocysts derived from SB and control group have been stained for cell counting (Figure 3). Total number of cell in blastocyst derived from control group gave the highest (177.50 ± 17.68), followed by 2-cell (44.67 ± 3.72), 4-cell (28.33 ± 2.80) and 8-cell (8.80 ± 1.30).

DISCUSSION

We have demonstrated that all isolated single blastomeres from each different stages of 2-cell, 4-cell and 8-cell embryo could develop until blastocyst stage. As the stages of parent embryos increased (2- to 8-cell) the developmental potential of single blastomere into blastocyst was decreased. This was probably due to the intercellular junction between blastomeres where it becomes tighter (Becker et al. 1992) with increasing embryo age; therefore, blastomere separation using mechanically gentle pipetting of the ZP-free embryo at later stage is more prone to blastomere injury. We also observed that high abnormal blastocysts formed from 8-cell parent embryos which developed into the characteristic of 'trophoblastic vesicle' as shown in Figure 1 (e) contains no embryonic inner cell mass (ICM) (Tarkowski & Wroblewska 1967).

TABLE 1. Effect of different cell stages on the *in vitro* development of isolated single blastomere

Stage of embryo	No. of isolated blastomere used	<i>In vitro</i> development of isolated blastomere				
		% 2-cell (mean \pm SD)	% 4-cell (mean \pm SD)	% 8-cell (mean \pm SD)	% morula (mean \pm SD)	% blastocyst (mean \pm SD)
2-cell	172	80.88 ± 19.59^b	78.84 ± 20.62^b	78.08 ± 20.81^b	77.28 ± 20.30^{bc}	77.28 ± 20.30^{bc}
4-cell	252	69.82 ± 17.65^{ab}	69.83 ± 17.65^{ab}	69.83 ± 17.65^{ab}	69.83 ± 17.65^{ab}	63.70 ± 16.91^{ab}
8-cell	152	56.36 ± 6.70^a	56.36 ± 6.70^a	56.36 ± 6.70^a	55.73 ± 7.00^a	55.73 ± 6.98^a
Control*	198	98.50 ± 4.74^c	96.83 ± 6.69^c	95.72 ± 7.02^c	91.39 ± 9.22^c	91.39 ± 9.22^c

*ZF 2-cell without separation.

^{abc}Means with different superscript in same column differ significantly ($p < 0.05$)

TABLE 2. Development of mouse ICM outgrowth on MEF after culturing blastocyst obtained from isolated SB of early stage embryos

Stage of embryos	No. of blastocysts derived-SB	No. of ICM outgrowth (mean \pm SD)
2-cell	145	69.29 ± 10.92^b
4-cell	73	55.73 ± 15.62^{ab}
8-cell	60	41.85 ± 6.19^a
Control*	157	76.90 ± 19.21^b

*ZF 2-cell without separation.

^{ab}Means with different superscript in same column differ significantly ($p < 0.05$)

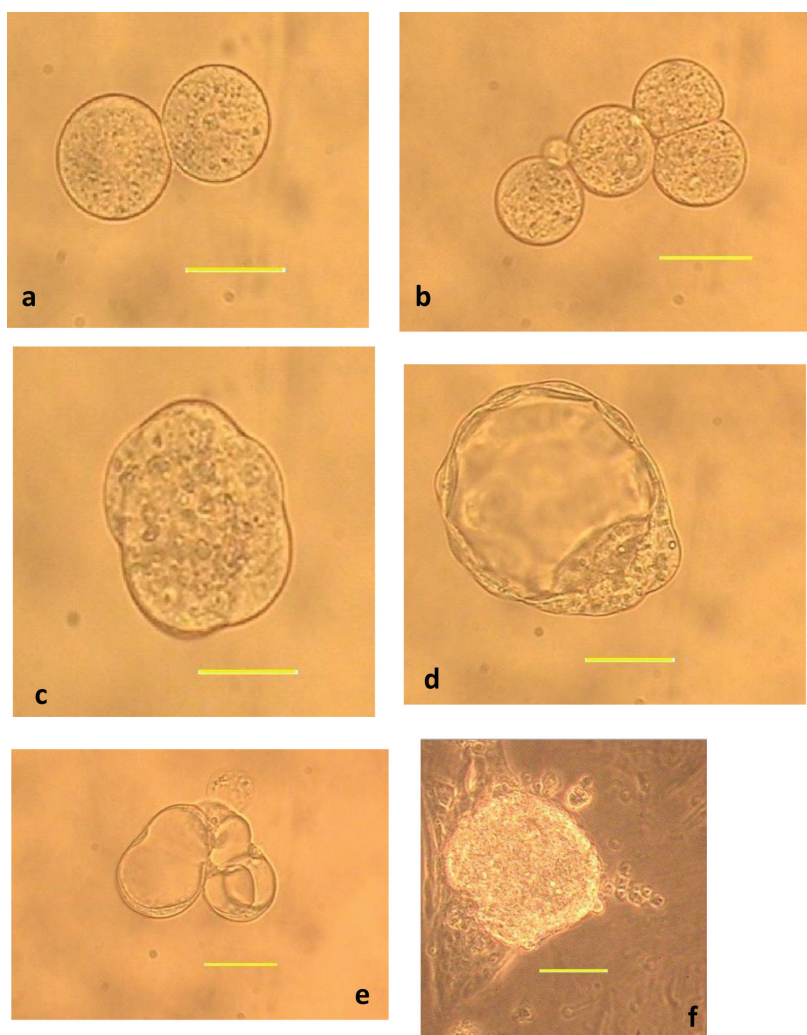


FIGURE 1(a)-1(f): Development of isolated single blastomere from 2-cell stage embryos (a) 2-cell (b) 4-cell (c) morula (d) blastocyst (e) abnormal blastocyst and (f) ICM outgrowth; Bar. 60 μm

TABLE 3. The diameter and cell number of blastocyst derived from isolated blastomere

Stage of embryo	Diameter of SB after isolation (μm) (mean \pm SD)	Diameter of blastocyst derived-SB (μm) (mean \pm SD)	No. of cell in blastocyst (mean \pm SD)
2-cell	56.48 \pm 1.37 ^c	92.55 \pm 6.56 ^c	44.67 \pm 3.72 ^c
4-cell	44.02 \pm 1.62 ^b	78.71 \pm 5.48 ^b	28.33 \pm 2.80 ^b
8-cell	35.68 \pm 1.08 ^a	64.13 \pm 6.95 ^a	8.80 \pm 1.30 ^a
Control*	-	108.28 \pm 4.26 ^d	177.50 \pm 17.68 ^d

*ZF 2-cell without separation.

^{abcd}Means with different superscript in same column differ significantly ($p < 0.05$)

One explanation for the low blastocyst rate obtained in this study could be due to the absence of zona pellucida. It has important roles in order to protect the embryo, regulate cell division and cell allocation for the formation of ICM and trophectoderm in mouse (Suzuki et al. 1995) and pig embryos (Eckert et al.1997; Tao et al.1995).

In vitro culture (IVC) medium has been thought as one of the factors which can affect the development potential of isolated single blastomere. KSOM medium

was used as IVC medium in this study shows that single blastomere can develop up to blastocyst with relatively high developmental rate (77%) which is similar to results reported by Lorthongpanich et al. (2008) where high blastocyst formed when single blastomere derived from 2-cell cultured in KSOM compared to mES medium. In contrast, low blastocyst rate was obtained when blastomere cultured in T6 culture medium even though growth factor was added into the medium (Sheikholslami

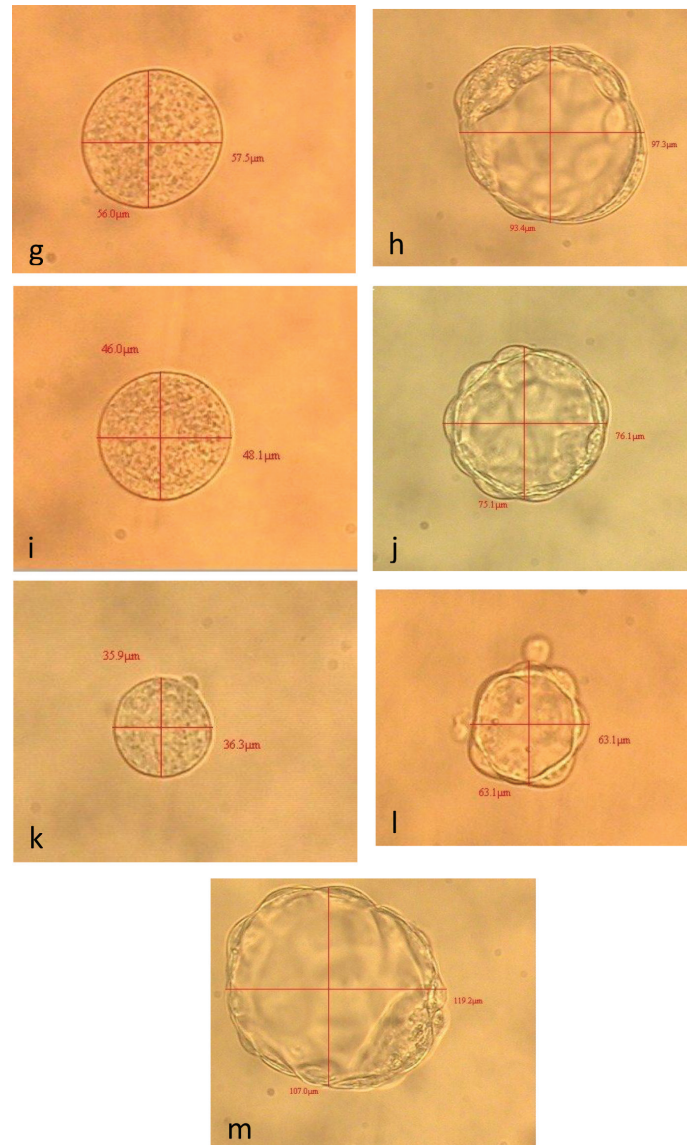


FIGURE 2(g-m): Diameter of isolated SB from different stage of embryos and its subsequent blastocyst; (g and h) 2-cell, (i and j) 4-cell, (k-l) 8-cell and (m) control

et al. 2008). Therefore, we were suggested to culture the single blastomere in KSOM medium up to blastocyst stage before transferred on inactivate MEF feeder layer for ICM outgrowth using mESC medium which contain LIF for maintaining pluripotent state of ESC cell.

In order to do diverging observation on blastomere development, the isolated blastomeres from the same parent embryo were cultured in groups. In addition, it is more effective culture system because growth factors were secreted during development of the blastomeres which have stimulatory effect on its development. It was suggest that single blastomere from 2- to 8-cell embryo can develop as normal embryo but the developmental potential was limited by non-optimal culture system. The present results showed that isolated SB could develop to blastocyst with the size of the later was approximately 3/5 bigger than the former where this was in agreement

that intact sister blastomere was capable of compensating for lost or damaged blastomere in an embryo and thus has the potential to sustain development (Avis & Anderson 1988).

CONCLUSION

Blastomere separation technique could be used to increase the number of blastocyst where in other words, we are targeting that blastocyst derived from single blastomere could be used as a source of ICM for production of embryonic stem cell (ESC). From the results of our study, we suggest that single blastomeres from mouse embryos could be isolated at 2-, 4- and 8-cell stage to produce many blastocysts after *in vitro* culture, subsequently facilitating the use of ICM for production of mouse ESC in future research.

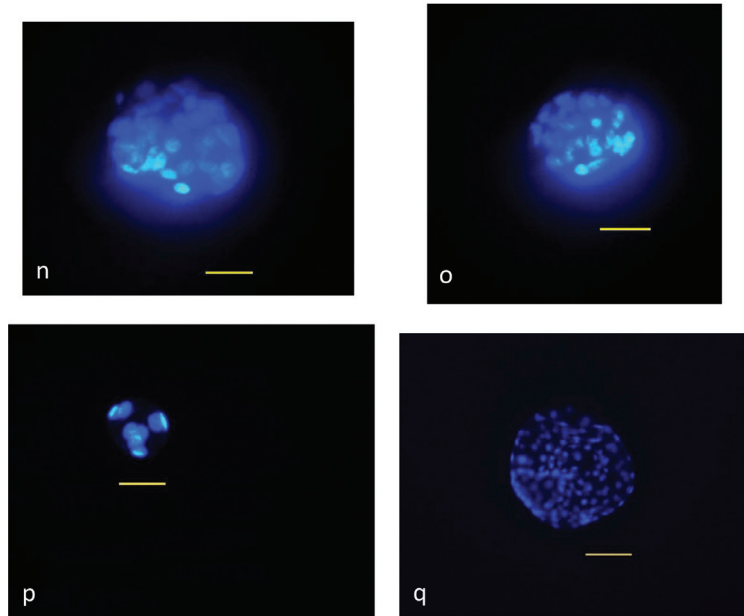


FIGURE 3(n-q): Hoechst 33342 staining of blastocyst derived from SB at different stage of embryos; (n) 2-cell (o) 4-cell (p) 8-cell and (q) control; Bar, 60 μ m

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