Effects of group culture on the development of discarded human embryos and the construction of human embryonic stem cell lines

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Abstract

Purpose To explore the effect of group culture on the developmental potential of discarded embryos in in vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI) cycles and establish the human embryonic stem cell lines for future research.

Methods Fresh discarded embryos were collected from the IVF/ICSI-ET program in the reproductive medical center of the first affiliated hospital of Zhengzhou university in this study. All zygotes were individually cultured from Day 1 to Day 3. On Day 3, discard embryos were then cultured in group of 1–4 embryos per droplet (30 μ l/droplets) with a constant culture medium until Day 5 or 6. Mechanical method was used to isolate the inner cell mass (ICM) of blastocyst from the embryo. Then we inoculated the ICM on feeder layer. After identification of those cells, the human embryonic stem cell lines (hESCs) were established.

Results In this study, we collected 1,223 fresh discarded embryos and they were sequential cultured to the blastocysts (18.07 %, 221/1,223), in which good quality blastocysts were 61(4.98 %, 61/1,223). There was no significant difference in the patients. The embryos from 1PN, 2PN, 3PN were sequential cultured to the blastocyst s(39.31 %,92/234;12.87 %,64/497;13.21 %,65/492),in which good quality blastocysts was 13.6 %(32/92),2.61 %(13/64), 3.04 %(15/65).1PN embryo's

blastulation rate and quality embryo formation rate was significantly higher than the 2PN and 3PN embryos' ($P < 0.05$). Three embryos group cultivation has the highest blastulation rate and quality embryo formation rate ($P < 0.05$). In total, we successfully established 4 hESCs lines.

Conclusion The group culture of human discard embryos can improve the blastulation rate and blastocyst quality to some extent. Three embryos group cultivate is the better culture number. Human discard embryos are good source for establishment of hESCs.

Keywords Group culture · Human discard embryo · Blastocyst · Human embryonic stem cell

Introduction

In 1998, hESCs cell lines were successfully isolated for the first time by Thomson [1]. Human embryonic stem cells (hESCs) have been derived from the inner cell mass (ICM) of day 5–7 blastocysts and have the ability to generate cells from all three embryonic germ layers [2–6]. The study of hESCs will enhance the understanding of early embryo development as well as advance the efficiency of drug discovery and screening [7]. But, the establishment of hESCs cell lines has been hindered by the limited source of human embryos and ethical reasons. While assisted reproductive technology (ART) are now commonly employed tools, a large number of poor quality embryos are discarded because of their low development potentiality, low pregnancy rate, low implantation rate, low survival rate in frozen–thawed cycles and high chromosomal abnormalities [8]. These discard embryos were precious sources of material for human embryonic stem cell lines' establishment.

In ART cycles, there often are some abnormal fertilized eggs that developed into the embryos with 0PN, 1PN, or multi-pronucleus (3PN, mainly 3PN), besides the cleavage-arrested

Capsule To explore the effect of group culture on the developmental potential of discarded embryos in in vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI) cycles and establish the human embryonic stem cell lines for the future research. Then, fresh embryos were collected in the reproductive medical center of the first affiliated hospital of Zhengzhou university in this study. The group culture of human discard embryos can improve the blastulation rate and blastocyst quality to some extent. Three embryos group cultivate is the better culture number. Human discard embryos are good source for establishment of hESCs.

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embryos with 2PN. Those embryos cannot be used in the ART treatment, but they are also the sources to establish the human embryonic stem cell lines. Those hESCs have great value in basic research and a vast prospect in clinical application due to their properties. Firstly, hESCs provide a new method to study human normal development; based on this, we can do research on abnormal pathological development and its mechanism. Secondly, hESCs with different genetic background provide a new research system for detection of human cell gene function.

The embryos were usually cultured individually using two-step in vitro, without blood supply and dependent upon luminal secretions from the oviduct and the uterus for its nutrition. It's that cell division; apoptosis and differentiation are influenced by the environment, including growth factors [9]. The culture conditions in vitro result in low blastulation rate or blastocysts with low cell number and lacking a distinct ICM [10]. Therefore, it is important to increase the efficiency of blastulation rate blastocyst quality from the in vitro culture. Previous studies in mice, sheep, and cattle have demonstrated that group cultures promote embryo development compared with single-culture or small numbers of embryos. It is hypothesized that one reason for benefit of group embryo culture is embryo modification of their microenvironment due to secretion and/or depletion of various factors in the media [11–15]. Autocrine of growth factors by embryos and expression of specific receptors at particular cell stages clearly indicate that growth factors play an important role in the early development of preimplantation embryos. Recent study has also suggested that the density of cultured embryos is important for development [8, 16]. In this study, we investigated systemically the effect of group culture on human discard embryo development.

Materials and methods

All study methods were approved by Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the subjects enrolled into the study gave written formal consent to participate. Fresh discarded embryos were collected from Chinese patients who undergoing their IVF/ICSI cycle at the Center for Reproductive Medicine, The First Affiliated Hospital, Zhengzhou University from December 2012 to July 2013. The fresh discarded embryos include: (1) one pronuclear (1PN) or tri-pronucleus (3PN); (2) the cleavage arrested embryos with 2 pronucleus (2PN) which were not deserved the standards of transplantation or freezing.

Embryo group culture strategies

Oocytes with two pronucleus were individually cultured in 20 μ l droplets of G1 medium (Vitrolife, Sweden) at 37 °C,

6 % CO₂ for 48 h. According to the standard of Peter [17], embryos were examined for cell stage, fragmentation and multinucleation on day 3. We collected the one pronuclear (1PN), tri-pronucleus (3PN) and the cleavage-arrested embryos with two pronucleus (2PN). The group culture principles are: A, embryos in the same droplet were from the same patients; B, embryos in the same droplet were from the same pronuclear. All embryos were grouped by the number (single, two, three and four) and the pronuclear, then cultured in 30 μ l droplets of G2 medium (Vitrolife, Sweden) at 37 °C, 6 % CO₂.

Blastocyst classification

On the Day 5, blastocyst formation was determined. According to scoring system of Gardner and Schoolcraft [18], blastocysts were scored from 1 to 6: 1, early blastocyst, the blastocoel being less than half the volume of the embryo; 2, blastocyst, the blastocoel being half or greater than half of the volume of the embryo; 3, full blastocyst, the blastocoel completely fill the embryos; 4, expanded blastocyst, the blastocoel volume is larger than that of the early embryo and the zona is thinner than before; 5, hatching blastocyst, the trophoctoderm has started to herniate through the zona; 6, hatched Blastocyst, the blastocyst has completely escaped from the zona. The development of the inner cell mass (ICM) was graded as follows: A, tightly packed, many cells; B, loosely grouped, several cells; C, very few cells. The trophoctoderm was assessed as follows: A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; C, very few large cells. Those that did not reached the blastocyst stage on Day 5 were cultured one more day till Day 6. In this study, the blastocysts with grade 3BB or over were regarded as high-quality blastocysts [19].

Establishment of hESC lines

The dishes were covered with Mitomycin C-inactivated mouse embryonic fibroblasts [20] in a density of $3.5 \times 10^5/\text{cm}^2$ for future use. Trophoctoderm cells and ICM were isolated with a syringe needle under a dissecting microscope. Trophoctoderm cells around the ICM were removed as carefully as possible, avoiding damage to the ICM. The ICM was seeded in the feeder layer. After 48 h, the adherence and growth was observed and half of culture medium was changed. Cell passage was performed during 3–7 days in primary clone. Then each cell passage was performed every 4–7 days. These clones were incubated in H-DMEM (Gibco, USA) containing 20% serum replacement (Gibco, USA), penicillin/streptomycin (Gibco, USA), basic fibroblast growth factor (bFGF; Invitrogen, USA), insulin transferrin selenium (ITS; Gibco, USA), non-essential amino acids (Hyclone, USA), β -mercapto-ethanol (Sigma, USA), L-

glutamine(Sigma,USA)at 37 °C,5 % CO₂. Passage was performed with the mechanical method. The pyknotic and solid clones were selected, and then the needle of 1 ml-syringed was used to mechanically cut these clones. The passage ratio was 1:3–1:5 [21].

Identification of hESC lines

Alkalinephosphatase(AKP)staining

The hESCs were fixed with 4%paraformaldehyde for 1–2 min(the time must be controlled in 2 min, avoiding damage to the activity of alkaline phosphatase), washed with TBST, treated with the mixed liquor(Fast Red Violet, Naphthol AS-BI phosphate solution and water 2:1:1) for 15 min, respectively, washed with TBST, and covered with a small account of PBS(Hyclone, USA). The status of coloration was observed under the microscope and photographed.

Immunofluorescence

The hESCs were fixed with 4 % paraformaldehyde for 30 min, washed with PBS three times, and treated with 0.1%Triton-PBS (PBST) at room temperature for 10 min and washed with PBS three times. After that, the clones were placed into PBS containing 5%BSA for 30 min, and then 1:50(v:v) of SSEA-4, SSEA-1, TRA-1-60and TRA-1-81 as first antibody (Millipore) were added for incubation at 4 °C overnight. The next day, hESCs were washed with PBS three times and placed into 5 % BSA for 30 min. The corresponding secondary antibodies (Cy3, invitrogen,USA) 1:300(v:v) were added for incubation at 37°Cfor 60 min. Then, the clones were observed under the laser confocal microscope (LSM700, Zeiss) and photographed.

In vitro differentiation into embryoid bodies

Cells were digested with trypsin or collagenase, blown into single cell suspension, and adjusted at a concentration of 4×10^4 /ml. Every hanging drop containing 30 μ l of suspension was placed on the dish cover, and then incubated at 37 °C, 5 % CO₂. Three days later, simple embryoid bodies were formed, transferred into new dishes for incubation in bFGF-free culture medium, and blown into cell suspension. These simple embryoid bodies were taken out once or twice a day to shake gently and the medium was changed every other day. Five to 10 days later, cyst-shaped embryoid bodies could be seen and photographed.

Differentiate into teratomas in vivo

The clones were mechanically cut into 50–100 small cell aggregates, washed with PBS and blown into 100 μ l of suspension with DMEM (Hyclone,USA). Then, the cell suspension were inoculated into the hind legs or groin of SCID mice (Beijing Vital River Laboratory Animal Co. Ltd., Beijing, China) and observed every 3 or 5 days. Eight to 12 weeks later, when the tumor diameter reached 1 cm, the tumor was taken,washed with PBS three times, fixed with 10 % paraformaldehyde, embedded in paraffin followed by slicing and HE staining. Finally, the samples were observed under microscope [21].

Statistical analysis

Statistical analysis was performed with SPSS statistical package (SPSS version 13.0, Chicago, IL). Analysis of covariance (ANCOVA) was performed to compare the means between

Table 1 Comparison of characteristics in the patients with different embryo sources

Variables	1PN	2PN	3PN	Fvalue	Pvalue
Age(years)	30.75±4.71	31.99±4.63	31.37±4.70	1.60	0.20
Pregnancy history	3.89±2.72	5.08±3.54	5.06±3.59	2.57	0.07
BMI (kg/m ²)	21.90±2.25	22.73±3.07	22.65±3.11	1.57	0.20
Basal FSH levels (IU/L)levels (IU/L)	6.96±1.36	7.33±1.65	7.15±1.56	1.24	0.28
Basal E2 levels (pg/ml)	42.79±18.87	47.39±73.76	41.36±21.30	0.58	0.55
Antral follicle count	5.17±2.58	5.21±2.85	5.43±2.65	0.32	0.72
Starting dose of gonadotropins (IU)	175.96±52.20	186.17±51.42	182.42±54.17	0.76	0.46
Days of stimulation	10.73±1.98	10.90±1.68	10.86±1.48	0.20	0.81
Total gonadotropin dose (IU)	1938.99±864.29	2039.57±785.42	1947.55±657.68	0.69	0.50
Number of good quality embryos	5.85±3.14	5.63±3.56	6.53±4.17	2.34	0.09
Number of embryos cryopreserved	4.21±3.20	3.99±3.48	4.33±3.73	1.51	0.22

$P < 0.05$ was considered significant

Table 2 Blastulation status in different embryos

Group	Number of embryo(n)	Blastulation rate%	High-quality blastulation rate%
1PN	234	39.31(92/234)	13.6(32/234)
2PN	497	12.87(64/497)*	2.61(13/497)*
3PN	492	13.21(65/492)*	3.04(15/492)*
Total	1,223	18.07(221/1,223)	4.98(61/1,223)

*: $P<0.05$, compared with embryos with 1PN

the patients in different groups. Data are expressed as percentage. Chi-square test was used in comparison between blastulation percentages in different group culture groups. All data are reported as the mean with their associated standard deviations and all tests were two-tailed. Test standard was set at $\alpha=0.05$, and $P<0.05$ was considered significant.

Results

General observations

In this study, a total of 1,223 discarded embryos from 372 patients were sequentially incubated into 221 blastocysts containing 92 high-quality blastocysts. All the patients were signed the informed consent. The age of patients varied from 21 to 42 years. And they are without endometriosis and PCOS. There were no statistical differences in the fundamental state of all the patients in inter-group or intra-group (Table 1).

Comparison of blastocyst development

The blastulation rate was the highest in the embryos with 1PN ($P<0.05$). The blastulation rate and the high-quality blastulation rate was significantly higher in the embryos with 1PN than in the embryos with 2PN or 3PN. There were no statistical difference between 2PN and 3PN (Table 2).

Table 3 Effect of group culture methods on blastulation in the embryos with 1PN

Group	Number of embryo(n)	Blastulation rate%	High-quality blastulation rate%
single	60	33.33(20/60)	5(3/60)
two	58	41.37(24/58)	18.96(11/58)*
three	60	50(30/60)	21.66(13/60)*
four	56	32.14(18/56)	8.92(5/56)
Total	234	39.31(92/234)	13.6(32/234)

*: $P<0.05$, compared with the group of single embryo

Effect of group culture methods on blastulation

In the embryos with 1PN, a total of 234 discarded embryos were sequentially incubated into 92 blastocysts containing 33 high-quality blastocysts. Compared with the group of single embryo, the blastulation rate and the high-quality blastulation rate was significantly higher in the group of two or three embryos ($P<0.05$) (Table 3).

In the embryos with 2PN, a total of 497 discarded embryos were sequentially incubated into 64 blastocysts containing 13 high-quality blastocysts. Compared with the group of single embryo, the blastulation rate was significantly higher in the group of two or three embryos ($P<0.05$) and the high-quality blastulation rate was higher in the group of three embryos ($P<0.05$). At the same time, compared with the group of three embryos, the blastulation rate was lower in the group of four embryos. (Table 4)

In the embryos with 3PN, a total of 492 discarded embryos were sequentially incubated into 65 blastocysts containing 15 high-quality blastocysts. Compared with the group of single embryo, the blastulation rate was significantly higher in the group of three embryos ($P<0.05$). Compared with the group of three embryo, the blastulation rate was lower in the group of two or four embryos ($P<0.05$). (Table 5)

Table 4 Effect of group culture methods on blastulation in the embryos with 2PN

Group	Number of embryo(n)	Blastulation rate%	High-quality blastulation rate%
single	126	7.14(9/126)	0(0/126)
two	124	16.12(20/124)*	2.41(3/124)
three	123	18.69(23/123)*	6.5(8/123)*
four	124	9.67(12/124)#	1.61(2/124)
Total	497	12.87(64/497)	2.61(13/497)

*: $P<0.05$, compared with the group of single embryo

#: $P<0.05$, compared with the group of three embryos

Table 5 Effect of group culture methods on blastulation in the embryos with 3PN

Group	Number of embryo(n)	Blastulation rate%	High-quality blastulation rate%
single	122	9.01(11/122)	0.81(1/122)
two	124	12.09(15/124)#	4.03(5/124)
three	126	23.01(29/126)*	5.55(7/126)
four	120	8.33(10/120)#	1.66(2/120)
Total	492	13.21(65/492)	3.04(15/492)

*: $P < 0.05$, compared with the group of single embryo

#: $P < 0.05$, compared with the group of three embryos

Identification of hESCs

Morphology of growth

In this study, we observed the shapes of these hESCs 'colonies' were flat with clear cell boundaries under inverted microscope on D3 (Fig. 1a). These colonies have the large nuclei that were with clear nucleolus and high nucleoplasmic ratio. The hESCs began growing peak on D4–D6 and differentiating on D7.

AKP staining

The hESCs with AKP expression were violet-blue (strongly positive), while the feeder layer cells failed to be stained (Fig. 1b).

Differentiation into embryoid bodies in vitro

After 3 days' hanging drop, simple embryoid bodies were formed. Then, the simple embryoid bodies were to incubate in 10 cm dish for 5–7 days. The sizes of simple embryoid bodies were gradually increased and cyst-shaped embryoid bodies (Fig. 1c) were formed.

Immunofluorescence

The hESCs were positively stained for SSEA-4, TRA-1-60 and TRA-1-81 in red fluorescence, but negatively for SSEA-1 (Fig. 2). Feeder layer cells failed to be stained.

Differentiation into teratomas in vivo

The hESCs cells were injected into the hind legs or groin of SCID mice. 8–12 weeks later, tumor-like tissue was seen and tumor-surrounding tissue was squeezed without marked destruction. The tumor was taken out, fixed with 10 % paraformaldehyde, embedded in paraffin followed by slicing and HE staining. The tumor contained fat cells (Fig. 3a), glands (Fig. 3b), bone, etc., demonstrating that hESCs may developed into three germ layers-derived teratoma in vivo.

Discussion

Since hESCs are defined by unlimited self-renewal and the ability to differentiate—both in vitro and in vivo—into cell types of endodermal, mesodermal and ectodermal origins,

Fig. 1 **a** hESC clones under inverted microscope (4×). hESCs show nest-like growth with clear cell boundaries. **b** AKP staining for hESC clones (4×). The hESCs with AKP expression were violet-blue, while the feeder layer cells failed to be stained. **c** After 3 days' hanging drop,

simple embryoid bodies were formed. Then, the simple embryoid bodies were to incubate in 10 cm dish for 5–7 days. The sizes of simple embryoid bodies were gradually increased and cyst-shaped embryoid bodies were formed

Fig. 2 Immunofluorescence for hESC clones (10×). The hESCs were positively stained for SSEA-4(**a**), TRA-1-60(**c**) and TRA-1-81(**d**) in red fluorescence, but negatively for SSEA-1(**b**)

rendering them a powerful tool for studying the molecular mechanisms of cellular differentiation, for accessing the process of the normal embryo development, as well as for cell replacement therapies [22, 23]. Since the heterogeneous behavior of the distinct lines of hESC regarding their differentiation potential [24] and their epigenetic stability [25], it is very important to establish hESCs from multiple sources [26, 27].

The significance of group culture on establishment of hESCs

In mouse model, there is a high embryonic stem cell establishment rate with a large amount of high-quality embryos. However, there has been considerable debate

about the source of hESCs. The application of human high-quality embryos in researches is restricted by ethics in many countries. Since hESCs easily differentiate spontaneously and hardly maintain normal diploid karyotype during passage, the overall efficiency of hESC establishment is low. Many groups have explored the methods, such as co-culture with decidual cells, to improve the development of the embryos. Safety is the challenge to use hESCs from this source to precede further research and affect the results. In our study, we just cultivate embryos in groups to enhance the quality and quantity, avoiding the external factors.

Fig. 3 hESCs differentiate into teratomas in vivo (20×). The arrows indicate fat cells(**a**), glands(**b**) in the immunohistochemical section

Difference in the development of the different embryos

In IVF/ICSI-ET cycles, there frequently are some abnormal zygotes, which developed into the embryos with 0PN, 1PN, or multi pronucleus (3PN, mainly 3PN) and the cleavage-arrested embryos with 2PN. Due to the sample size; we didn't discuss the embryos with 0PN at this time. To our knowledge, many factors affect the oocyte quality and embryo quality, such as age and BMI [28, 29]. In this study, we excluded the influencing factors from the patients. Feng and coworkers [30] found that more than 50 % of zygotes with 1PN contained Y chromosome, and developed into the embryos with 1PN after cell cleavage in IVF cycles. Then, the cytogenetics indicated these embryos usually possessed diploid karyotype suggesting they had normal fertilization. The data from our study suggests that the embryos with 1PN have better developmental potential to form blastocysts and high-quality blastocysts. Therefore, the embryos with 1PN have the higher rates to have the normal chromosomes and can be used for establishment of hESCs.

Influence of group embryo culture on blastocyst development

At present, some researchers try to use the decidual cells, fibroblasts, granular cells and embryos were co-cultured in order to improve embryo blastocyst formation rate, but heterologous material that they bring can not meet the clinical grade human embryonic stem cell lines established requirements. Recently group culture of embryos has been beneficially applied in mice [31, 32], sheep [33], cow [34–36] and human [37, 38]. It has been reported that group culture may promote embryo development via secretion of embryotrophic factors [39, 40], and embryos cultured in group developed better than those cultured individually [41–43]. Also, group culture of good-quality embryos and poor-quality embryos can improve blastocyst formation rate [44]. The study in the Salahuddin S 'group suggests that specific factors are secreted from the embryos to influence embryo growth in an autocrine or paracrine manner. However, some researchers argue that embryos in group may deplete the media of substrates, and some other factors derived from poor quality embryos may have negative influences on the surrounding embryo development. It has been reported that the accumulation of toxic substances such as ammonia and oxygen-derived free radicals in the culture medium [45–47] may lead to the late developmental anomalies. Whether the modification of the group culture environment is beneficial or detrimental is not clear. The hypothesis generated in this study is that in addition to the positive effects, human embryo development in group culture may also be harmfully affected by negative factors that derived from poor quality of embryos.

The constructions of hESCs with discard embryos

At present, most hESCs come from the remnant embryos after assisted reproductive treatment cycles [48, 49]. In the ARTs, some couples have successfully got children; it is not necessary to store their frozen embryos. Therefore, many scholars believe that under the couples' consent, the remaining frozen embryos may be used for scientific research [50]. At present, the use of clinical discarded fresh or frozen embryos can solve the problems including the lack of embryos and ethic disputes in the establishment of human embryonic stem cell bank. In our Reproductive Center, about 4,000 IVF cycles are performed every year; it provides an abundant embryo resource for the establishment of human embryonic stem cell bank. In this study, a total of 4hESCs have been established, and are all consistent with the current identification criteria of hESCs. When we use discarded fresh embryos to establish hESCs lines, the embryos group culture maybe increase the efficiency of hESCs establishment. This study has important significance for establishing hESCs lines with discarded embryos.

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