ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 6 (12): 888-891, 2007
ISSN 1682-8356

Suitability of Chicken EPGCS on Different Cryoprotective System

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Abstract: The objective of this study was to evaluate the effect of three cryorotectants (at three concentration each) and two cryopreservation protocols on the preservation of chicken Primordial Germ Cells (PGCs) from gonads at stage 19 and stage 28. The PGCs were cryopreserved using Dimethylsulphoxide (DMSO; 10%, 20%, 30%), glycol (GLY; 10%, 20%, 30%), polyethylene glycol (PEG; 10%, 20%, 30%). In a first series of experiments, we compared viability after three cryorotectants based protocol I and found the viability of PGCs showed very significant percentage (86.53%) ($p<0.01$) in a freezing media IV. We then compared an cryopreserve protocol I with 5% dimethyl sulfoxide (DMSO)+5% Glycol versus a cryoprotectant protocol II and observed a better viability with the former protocol (85.9% versus 67.4%, $p<0.05$). Finally, we compared viability of PGCs at three concentration each cryorotectant and found no significant difference $p>0.05$ between the concentration of 10% and 15% except freezing media III. When the concentration was 20%, the viability of PGCs was the lowest and showed significant difference $p<0.05$ or very significant difference ($p<0.01$) compared to other concentration. In conclusion, 5% DMSO+5%GLY with cryopreserve protocol I was the most effective cryopreservation for chicken primordial germ cells.

Key words: Cryorotectant, cryopreservation, chicken primordial germ cells

Introduction

EPGCs (Embryo Primordial Germ cells, EPGCs) are ancestral cells of sperm and oocyte in avian first found by Waldeyer in 1870, originated from the epiblast (Eyal-Giladi *et al*., 1981) and finally they migrate into the gonadal area via the blood vascular system. These features of EPGCs migration facilitate their isolation and transfer to other chick- or quail- (Il-Kuk *et al*., 1995). A great progress of EPGCs cultured *in vitro* is that they can derive to be ES (embryonic stem cells). Primordial germ cells have, therefore, been considered as a gene carrier for production of transgenic birds. However, so far as we know, PGCs successful culture and cryopreserve have been developed (Naito *et al*., 1994a,b). The Suitability of EPGCs on different freezing media and the protocols are not very clear. In the present study, we try to evaluate various cryopreservate conditions of EPGCs from 19-stage and 28-stage embryos.

Materials and Methods

Experimental materials

Preparation of chicken embryo: White leghorn chickens were maintained at the Jiangsu Institute of Poultry Science, China. Fertilized eggs were collected from 2 to 7 days after artificial insemination, they were then incubated at 38°C and 70% relative humidity. Development stages of chicken embryo were determined according to Hamburger and Hamilton (1951) and Li *et al*. (2003).

Media and reagents: DMEM (Gibco); Ficoll-400 (Pharmacia); FBS (four seasons green of HangZhou); chicken serum; Mitomycin-C (Pharmacia); hSCF (sigma); mLIF (sigma); DMSO (sigma); Glycol (sigma); Polyethylene glycol (sigma); bFGF (sigma); hIL-11 (sigma).

The component of freezing media: Comparing the effects of three common types of cell freezing media (DMSO, glycol, polyethylene glycol) on the survival rate of PGCs.

Cryopreservating media I	:	DMSO+10%FBS+DMEM
Cryopreservating media II	:	glycol+10%FBS+DMEM
Cryopreservating media III	:	polyethylene glycol +10%FBS+DMEM
Cryopreservating media IV	:	DMSO+ glycol +10%FBS +DMEM
Cryopreservating media V	:	polyethylene glycol +glycol+10%FBS+DMEM
Cryopreservating media VI	:	polyethylene glycol + DMSO + 10%FBS+DMEM

PGCs obtained and prepared

Preparation of the chicken genital ridge: embryos collected from stage 19 embryos (incubated for 70-72 hrs) were rinsed with Ca^{+2} and Mg^{+2} free Phosphate-Buffered Saline (PBS) to remove the yolk. The abdomen of the embryo was carefully dissected under a

stereoscope and the genital ridge was removed with sharp forceps and transferred to a culture dish.

Preparation of the chicken gonad: Chicken embryos were collected from stage 28 embryos (incubated for 130-132 hrs). The abdomen of the embryo was carefully dissected under a stereoscope and the gonad, located between the mesonephros and the dorsal cellular kidney, was carefully removed.

Trypsin-EDTA isolation procedure: For trypsin-EDTA isolation of PGCs from stage 19 and 28 embryos, genital ridges or gonads were placed in PBS containing 0.25% (w/v) trypsin and 0.53 mM EDTA at room temperature for 5 min. Then, the cells were dissociated by gentle pipetting. After centrifugation at 200g for 8 min, approximately 1×104 isolated cells were cultured at 38°C with saturated humidity and 5% CO_2 in air, with daily medium changes.

Trypan blue staining: The viability of isolated PGCs was evaluated using 0.4% Trypan blue staining for 2 min, unstained cells were viable cells. The viable PGCs were counted on a counting plate.

Cryoprotectant protocol: The isolation and purifation of EPGCs can consult references. The EPGCs, which were seperatured and refined *in vitro*, were regulated to the density of approx 1-5×10^7 cells/ml by freezing media, the viability was determined by using Trypan blue. Then control the balancing temperature in the freezing process by the refrigerating layer of refrigerator that can regulate temperature and control the temperature of inducing crystallization in the freezing process by the freezing layer of refrigerator that can regulate temperature.

Cryoprotectant protocol I	Cryoprotectant protocol II
2~4°C equilibrium for 45~60min -4~-6°C induce crystall-ization for 45min hang on the liquid nitrogen over 1h then throw into the liquid nitrogen	2~4°C equilibrium for 90~120min hang on the liquid nitrogen over 1h then throw into the liquid nitrogen

EPGCs thawing protocol: After from the liquid nitrogen, then the refrigerant tubes were in the 38°C water-bath immediately and were shaken quickly in the water, which thaw out the EPGCs completely in 1~2min, then added culture medium, centrifugalized, washed 3 times. EPGCs were suspended with culture medium, were counted their vitality and were regulated their density to 1×10^6/ml, then were immigrated to incubator.

The EPGCs culture

Cultural system I: DMEM culture medium, including 10% fetal cattle serum, 2% chicken, 2mmol/L L-glutamine, 1mmol/L pyruvate-Na, 5.5×10^{-5}mol/L β-mercaptoethanol, 100U/mL garasol. The temperature of culture was 38.5°C, the humidity was full and the concentration of CO_2 was 5%.

Cultural system II: DMEM culture medium, including 10% fetal cattle serum, 2% chicken, 2mmol/L L-glutamine, 1mmol/L pyruvate-Na, 5.5×10^{-5}mol/L β-mercaptoethanol, 5ng/mL hSCF, 10UI/mL mLIF, 10ng/mL bFGF, 0.04ng/mL IGF, 100U/mL garasol. The temperature of culture was 38.5°C, the humidity was full and the concentration of CO_2 was 5%.

EPGCs identification

Morphological identification: With invert microscope, we could observe groups of EPGCs forming, their growth and their external character (in the culture medium, PGCs would big or small groups and with the development, EPGCs would augment and become circular, nearly circular or morular. The cells in groups were faint and their alignment are tight, so the single cell was distinguished difficultly).

PAS identification (Li and Guo-Hong, 2001).

Differentiation *In vitro*: The second generation EPGCs were cultured 5d in cultural system II and then in cultural system I. We observed their self-propelled differentiation.

Statistical analysis: Data were analyzed with Student's T-test and the LSD test using the statistical program, SPSS 11.0.

Results

The results of compared three cryoprotectants (at three concentration each) based protocol I.

The EPGCs from embryo gonad of stage 19 and 28 were purified by Ficoll gradient density centrifuging and was frozen in different cryoprotectant. The vitality of the PGCs after frozen in six freeze media and three concentration were showed in Table 1.

The PGCs from stage 19 and 28, the vitality of after frozen in six freeze media showed the best protective effect with the 10% cryoprotectant IV and the vitality were 85.9% (stage 19) and 86.5% (stage 28) respectively. Compared to the others cryoprotectants, the differences were very significant ($p<0.01$). With the 15% cryoprotectant IV, the vitality were 83.9% (stage 19) and 82.9% (stage 28) respectively. Compared to the others cryoprotectants, the differences were very significant ($p<0.01$). With the 20% cryoprotectant VI, the vitality were 58.0% and 58.7% respectively. Compared to the other cryoprotectants, the differences were very significant ($p<0.01$).

Table 1: The vitality of the PGCs after frozen in six freeze media and three concentration (%)

Cryopretectant concentration		Type of freezing media I	II	III	IV	V	VI
10%	Stage 19	63.12±0.86**	64.63±1.08^{**A}	62.37±0.98**	85.93±1.24	71.44±1.19**	70.75±1.13**
	Stage 28	62.34±1.07**	63.16±1.03**	64.02±0.82^{**A}	86.53±1.27^{A}	72.06±1.08^{**A}	71.83±0.93^{**A}
15%	Stage 19	61.20±1.02**	59.66±0.83**	55.76±0.91**	83.94±1.23	69.32±1.11**	70.26±1.07**
	Stage 28	64.62±1.09^{**A}	57.96±1.75^{**B}	59.80±1.17**	82.94±1.31	56.22±1.16^{**C}	62.02±1.08^{B}
20%	Stage 19	47.32±0.99^{**C}	49.04±1.03^{*C}	53.84±1.01^{C}	57.18±1.02^{C}	55.22±0.96^{C}	57.96±1.21^{C}
	Stage 28	44.12±1.12^{**C}	42.04±1.03**	46.18±0.98^{**C}	56.08±1.21^{C}	54.82±0.91^{C}	58.72±1.33^{C}

Note: In the same row, compared to the highest vitality of the PGCs, *p<0.05, **p<0.01. In the same column, A-B, B-C means p<0.05, A-C means p<0.01

Table 2: The vitality of the PGCs frozen using two freezing protocol and six freezing media (%)

Cryopreservation procedure	Stage	Type of freezing media I	II	III	IV	V	VI
I	19	63.12±0.86^{**A}	64.63±1.08^{**A}	62.37±0.98^{**A}	85.93±1.24^{A}	71.44±1.19^{**A}	70.75±1.13^{**A}
	28	62.34±1.07^{**A}	63.16±1.03^{**A}	64.02±0.82^{**A}	86.53±1.27^{A}	72.06±1.08^{**A}	71.83±0.93^{**A}
II	19	56.10±0.79^{**B}	55.82±0.64^{**B}	54.33±0.54^{**B}	67.40±1.09^{C}	64.06±0.97^{B}	63.98±0.78^{B}
	28	57.08±1.02^{*B}	56.52±0.74^{*B}	57.05±0.73^{*B}	65.65±1.11^{C}	65.40±0.84^{B}	63.12±0.79^{B}

Note: In the same row, compared to the highest vitality of the PGCs, *p<0.05, **p<0.01. In the same column, A-B, B-C means p<0.05, A-C means p<0.01

Table 3: The vitality of EPGCs after frozen-thawed cultured for 24h (%)

Cryopreservation stage	Freezing media I	II	III	IV	V	VI
19	36.54±0.79*	38.74±0.93	38.00±1.21	42.26±1.16	35.86±1.08*	38.06±1.33
28	36.34±1.03**	38.14±1.17*	37.04±0.93*	44.98±1.07	37.46±0.89*	40.28±1.01

Note: in the same row, compared with the highest vitality of the PGCs, *p<0.05, **p<0.01. In the same column, A-C means p<0.01

After EPGCs was frozen in the same freezing and protocol, concentration-dependent decreases in the vitality of the EPGCs were demonstrated by culture *in vitro*. When the concentration was 20%, the vitality of the EPGCs was the worst.

The results of two cryopreserve protocols in 10% freezing media: The EPGCs were frozen in the six frozen media at 10% and two freezing protocol. The vitality of the PGCs after thawed were showed in Table 2.

Table 2 showed: the significant differences and very significant differences of the vitality of EPGCs after frozen existed between the freezing protocol I and protocol II. Comparing with the other freezing media, the vitality of EPGCs in freezing media IV with any which of two protocols is the best. There is no significant differences of the vitality of EPGCs between freezing media V and VI, but they are better than that of freezing media I, II,III with any which of two protocols.

The results of PGCs cultured for 24h after they were frozen: The EPGCs were frozen with freezing protocol I in different freezing media, then thawed after frozen for a week, cultured in cultural system II for 24 hours. The results were showed in Table 3.

Table 3 showed: EPGCs from stage 19 after frozen and cultured in cultural system II for 24 hours. The results found : the freezing media IV was the best, the significant differences existed among freezing media IV, I and V ($p<0.05$), but compare with freezing II, III and VI, there is no significant differences ($p>0.05$).

Comparing Table 2 with Table 3, the vitality of EPGCs which were cryopreservated directly after separating from embryo of stage 19 and 28 is very high ($p<0.01$) than that of EPGCs were frozen after cultured for 24h.

From the Table 3, we can also know that the vitality of EPGCs after frozen no difference between EPGCs from the stage 19 and the stage 28.

Discussion

In this research, freezing media I, II, III was consisted of DMSO, glycol, polyethylene glycol alone, but freezing media IV, V, VI was consisted of these three chemical reagents joint. The results showed that the vitality of EPGCs was higher when they were frozen in freezing media IV, V, VI than that of frozen in DMSO, glycol, polyethylene glycol alone at 10% and 15%. Perhaps this is why they have special complementary action when joint used them.

In this research, EPGCs were frozen after cultured for 24 hours, the vitality is lower than that of EPGCs were frozen

directly after separated. The reasons are that the EPGCs may be damaged in the process of digesting and centrifugation, most of them were died due to can not resist to the shock of low temperature.

After EPGCs frozen, the survival time was very short either they were cultured in cultural system I or II. A mortality of EPGCs was higher, that is 70-80%, in cultural system I than that of in cultural system II (65-85%). So we can see that cryopreserve process could harm to EPGCs and destroy the adherence of cell.

There were significant differences of survival time between stage 19 and 28, when they were cultured in cultural system I and were frozen directly after was isolated. The possible reason is that EPGCs be damaged easier from stage 28 than stage 19. This is because of EPGCs had settled gonad at stage 28. They also contact with surround stromal cells. But this link was broken when EPGCs isolated from gonad. However, the EPGCs isolated from stage 19, because of them didn't contact with stromal cells closely, so the damage is lower. The survival time of EPGCs isolated from stage 28 was shouter than stage 19.

In the same freezing media, the viability of EPGCs after frozen had a dose-depend different, from higher to lower in 10%, 15% and 20%. We can see from the results of this study, the freezing media with high concentration do not good to EPGCs.

In the same freezing media, the viability of EPGCs frozen in protocol I was better significantly than that of frozen in protocol II. This is why when protocol II was used, that a quality of heat can't release immediately when water in cytoplasm changed from liquid to material, result in the temperature of cell was sharp changed and made a great damage to cells. Meanwhile, when protocol II was used, the water in cytoplasm neither induced be crystal nor out of cells and cells were damaged.

In this study, we also compared the two kinds of culture media, one culture medium contained some cell factor, such as LIF, SCF and bFGF and the other didn't contained these factors. The results showed EPGCs can't survival for a long time and can't proliferates when EPGCs were cultured in culture medium exogenetic cell factors free. This indicates that exogenetic cell factors are very important to the proliferation of EPGCs.

We also add β-mercaptoethanol to culture medium, β-mercaptoethanol not only can be used to accelerate cell division but also can be used to deoxidize sulfur compound in serum and protect cell from the damage of peroxide.

Acknowledgments

The work was supported by National Natural Science Foundation of China (No. 30671509).

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