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Cryoconservation of Chicken Blastodermal Cells: Effects of Slow Freezing, Vitrification, Cryoprotectant Type and Thawing Method During *In Vitro* Processing*

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Cryoconservation of blastodermal cells (BCs) can preserve genetic material for the future reconstruction of poultry breeds. The aim of our study was to compare the effects of three slow freezing programs and vitrification, different cryoprotectants (5% DMSO, 10% DMSO, or multi-component cryoprotectant (MC) and two thawing methods on the viability of chicken BCs. Significant differences in the survival of slowly frozen BCs using program 3 (2°C/min. to 0.4°C/min.) compared with programs 1 (1°C/min. to 0.3°C/min.) and 2 (4°C/min. to 0.3°C/min.) were observed. The percentage of live BCs was significantly higher after slow freezing in the presence of the MC compared with DMSO. The thawing method did not have a significant effect on the percentage of live BCs. We also observed significant differences in the survival rate of BCs after vitrification (81%) and slow freezing in the presence of 10% DMSO using program 3 (60%). The highest percentage of viable BCs was achieved by slow freezing with the MC using program 2 and thawing with method 1 (94%). The most unfavorable combination for BCs survival was slow freezing in 5% DMSO using program 3 and thawing with method 2 (58.3%). This is the first study to apply MC to the slow freezing of BCs. We also showed successful BCs vitrification.

Key words: Blastodermal cells, cryoprotectants, slow freezing, vitrification, chicken.

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Avian genetic resources are mainly focused on maintaining living collections of birds (*in situ* strategy), which is expensive and does not protect the flocks from the loss of genetic variability, environmental disasters or infectious disease. Therefore, the preservation of avian genetic material should be supported by an *ex situ* strategy (SAWICKA *et al.* 2011). Such a strategy includes the cryoconservation of gametes, embryos or embryonic stem cells to store in a gene bank for future needs. The techniques for cryopreserving the semen of chicken, duck, goose and turkey have been previously described (REEDY *et al.* 1995; MAEDA *et al.* 1984; LUKASZEWICZ 2002; LONG *et al.* 2014). Nevertheless, it is worth noting that male poultry gametes are homogametic. Therefore, semen cryoconservation does not guarantee a reconstruction of the avian species. Due to their large size and high lipid content, the cryoconservation of oocytes and embryos is not possible. The cryopreservation of embryonic cells, namely blastodermal cells (BCs) and primordial germ cells (PGCs), represents one solution that enables the preservation of both female and male genetic material (NAITO *et al.* 2003).

The source of BCs is the blastodisc of the donor chicken embryo at stage X (EYAL-GILADI & KOCHAV 1976). PGCs and their precursors can also be found at this stage. BCs can be injected into the subgerminal cavity and will integrate into both so-

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matic tissues and the germline of recipient embryos, leading to the creation of chicken chimeras (NIEUWKOOP & SUTASURYA 1979; CARCIENCE *et al.* 1993; ETCHES *et al.* 1997). To produce chicken chimeras, cryoconserved BCs can also be used (KINO *et al.* 1997). These chimeras can reconstruct the donor breed after appropriate mating (KINO *et al.* 1997; BEDNARCZYK *et al.* 2002; LI *et al.* 2002).

In previous studies (TERESHCHENKO *et al.* 1993; CHELMONSKA *et al.* 1997; KINO *et al.* 1997; NAITO 2003; PATAKINE VARKONYI *et al.* 2007), the cryoconservation of BCs was performed in the presence of a one-component cryoprotectant using slow freezing. We have demonstrated an effective BCs cryoconservation method using a multi-component cryoprotectant (MC). The aim of this study was to compare the effects of three slow freezing programs, cryoprotectants (5% DMSO, 10% DMSO, or MC) and two thawing methods on BCs viability. This study also reports successful BCs vitrification method.

Material and Methods

Isolation of BCs

BCs were isolated from un-incubated fertilized chicken eggs (Rhode Island Red) at stage X of development according to EYAL-GILADI & KOCHAV (1976). The protocol was approved by the Second Warsaw Local Ethics Committee for Animals Experiments (Permission number 38/2010 of 21/09/2010).

Briefly, eggshells were washed with 70% ethanol, the eggs were opened at the blunt end and the yolk was separated from the white. Subsequently, the yolk was transferred onto a sterile Petri dish (Bionovo, Legnica, Poland) and cleaned from the remains of the white. A paper ring was placed onto the blastodisc, and the vitellin membrane was cut out around the paper ring using sterile microsurgical scissors. Next, the paper ring with the blastodisc was removed from the yolk surface and rinsed in a stream of PBS (-Ca^{2+}, -Mg^{2+}) (Gibco, Invitrogen, Carlsbad, CA, USA) to separate the blastoderm from the vitellin membrane. The blastoderms were suspended in 1 ml of OPTIMEM medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS (Gibco, Invitrogen, Carlsbad, CA, USA) and antibiotics (1x Penicillin--Streptomycin, Sigma, St. Louis, MO, USA) and triturated briefly to obtain a homogeneous cell suspension (Fig. 1). Subsequently, we determined BCs viability using the trypan blue exclusion method (FRESHNEY *et al.* 1987).

Slow freezing of BCs

The BCs (5 x 10^5 cells/ml) were resuspended in OPTIMEM medium supplemented with 5% FBS and were frozen in straws (0.2 ml, IMV, France) in the presence of 5% DMSO, 10% DMSO (Sigma, St. Louis, MO, USA) or the MC cryoprotectant according to the manufacturer's protocol (Blast Freeze, MediCult, Jyllinge, Denmark). The MC cryoprotectant contained sucrose and glycerol as well as other components. Slow freezing of BCs was performed using a CL-8800 temperature controller (CryoLogic, Victoria, Australia) using one of the three programs described below.

Program 1. Slow freezing was initiated at +18°C and continued at 1°C/min until -7°C was achieved. At this temperature, ice nucleation was induced. Subsequently, the cooling rate was decreased to 0.3°C/min until -35°C was reached. Finally, the temperature was reduced through a freefall until -130°C.

Program 2. Cooling of the straws was initiated at +18°C and continued at 4°C/min until 0°C was reached. The straws were maintained at this temperature for 5 min. Next, the temperature was decreased at 1°C/min until ice nucleation was induced at -7°C. The temperature was then reduced at a rate of 0.3°C/min until -37°C was achieved. Finally, the temperature was reduced through freefall to -130°C.

Program 3. The samples were cooled from +24°C at rate of 2°C/min until ice nucleation was induced at -6°C. The cooling rate was then reduced to 0.3°C/min until -32°C was reached. Subsequently, the temperature was decreased to -35°C at a rate of 0.15°C/min. Then, the cooling rate was increased to 0.4°C/min. until -60°C was achieved. Finally, the temperature was reduced through a freefall until -130°C.

After completion of the freezing process, the straws were submerged in liquid nitrogen (-196°C) and stored in deep freezing conditions for two

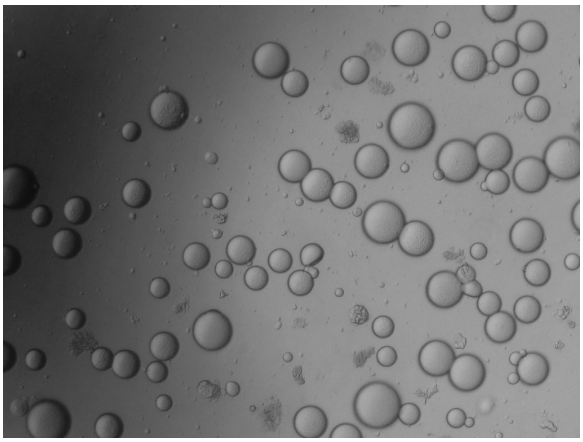

Fig. 1. Chicken BCs isolated from un-incubated fertilized chicken eggs at stage X (EYAL-GILADI & KOCHAV 1976). Magnification: 20x.

months. We performed three replicates of the experiment for every combination of program, cryoprotectant and thawing method.

BCs vitrification

The BCs (~1.5 x 10^4 cells/ml) were vitrified in the presence of 10% DMSO in a total volume of 6 μl on a chilled block using fiber plugs (CVM, CryoLogic, Victoria, Australia). Afterwards, the fiber plugs were placed into the straws (CVM, Cryologic, Victoria, Australia) and stored in liquid nitrogen for two months. We performed five replicates of this experiment.

Thawing of BCs

Method 1. The slowly frozen BCs were thawed using the THAW program (CL-8800 system, CryoLogic, Victoria, Australia). The straws were placed in a cryochamber at -120°C, and the temperature was increased at a rate of 8°C/min until -90°C was achieved. From -90°C to +4°C, the warming rate was 5°C/min. Subsequently, this rate was decreased to 4°C/min until +20°C was reached.

Method 2. Sample thawing was performed at a rate of 40°C/min by placing the straws at 4°C for 5 min. Subsequently, the warming rate was decreased to 4°C/min until +20°C was reached. This method was applied to thaw both the vitrified and the slowly frozen BCs.

The DMSO or MC cryoprotectant was gradually removed from the samples according to a previously described method (KINO *et al.* 1997) or the manufacturer's protocol (Blast Thaw, MediCult, Jyllinge, Denmark), respectively. The thawed BCs were cultured in 500 μl of OPTIMEM medium complemented with 5% FBS and an antibiotic for 24 hours at 37°C under 5% CO_2. The viability of the cryoconserved BCs was examined using a microscope and the trypan blue exclusion method.

Statistical analysis

Significant differences in the viability of BCs frozen using the three different programs, different cryoprotectants and two thawing methods were calculated with a three-factor analysis of variance followed by Bonferroni and Games-Howell tests. To examine the difference between BCs slow frozen or vitrified in the presence of 10% DMSO and thawed by method 2, a one-way analysis of variance was performed. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 17.0 software. Values were considered statistically significant at $P<0.05$. Percentage data are presented as the mean±SD.

Results

The viability of freshly isolated BCs was at high level (97±0.5%). These BCs were slowly frozen using various conditions or vitrified in the presence of 10% DMSO and transferred to liquid nitrogen for 2 months. The survival of cryoconserved BCs was determined 24 h after thawing. There were significant differences ($P<0.05$) in the viability of BCs that were slowly frozen using program 3 compared with programs 1 and 2 (Fig. 2). The per-

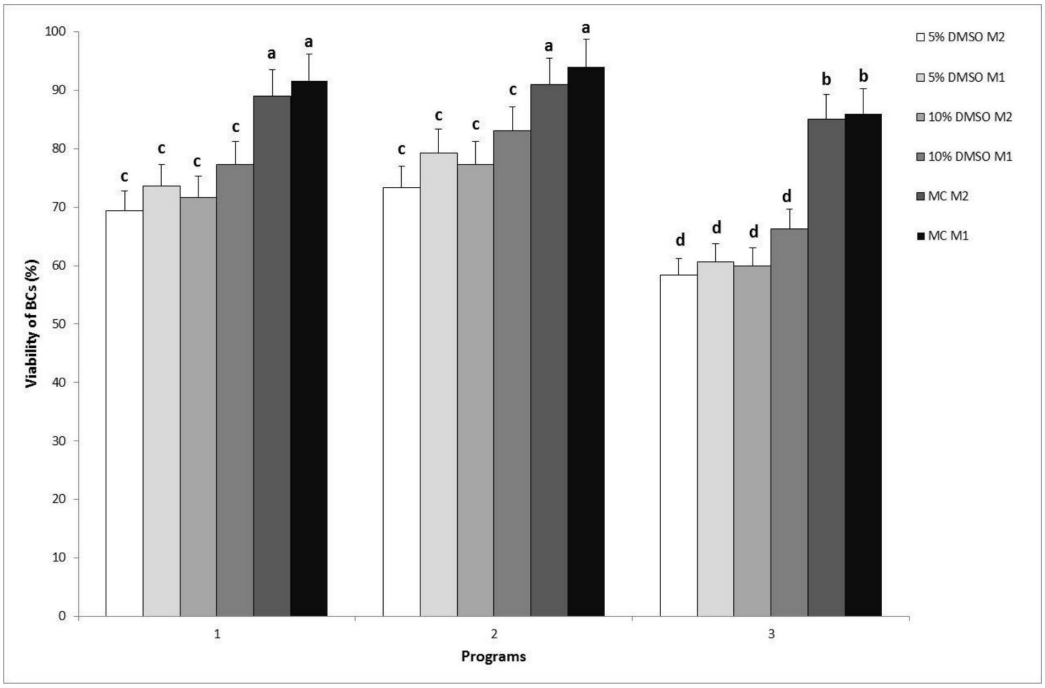

Fig. 2. Percentage of live blastodermal cells (BCs) after slow freezing using one of the three programs in the presence one of the cryoprotectants: 5% DMSO, 10% DMSO, or MC and thawed via method 1 (M1) or method 2 (M2). Letters a-d indicate significant differences at $P<0.05$.

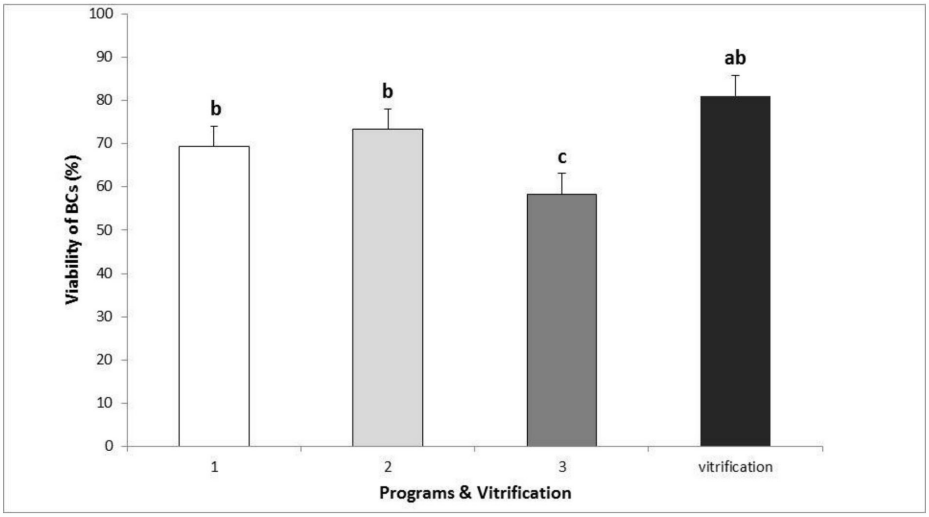

Fig. 3. Percentage of live blastodermal cells (BCs) after slow freezing for the three programs or the vitrification method in the presence of 10% DMSO. Letters a-c indicate significant differences at P<0.05.

centage of live BCs was significantly higher (P<0.05) after slowly freezing in the presence of the MC cryoprotectant compared with 5% or 10% DMSO (Fig. 2). The BCs thawing method did not significantly alter the percentage of viable BCs. However, the highest percentage of live BCs was achieved after slow freezing in the presence of MC cryoprotectant using program 2 and thawing with method 1 (94±1.0%) (Fig. 2). The most unfavorable combination for BCs survival was slow freezing in the presence of 5% DMSO using program 3 and thawing with method 2 (58.33±5.8%) (Fig. 2). We found significant (P<0.05) differences in the survival rate of the BCs after vitrification (81±1.0%) and slow freezing in the presence of 10% DMSO using program 3 (60.0 ±5.5%) (Fig. 3).

Discussion

The first successful application of cryoconserved BCs to reconstitute chicken breeds via chimeric intermediates was reported by KINO *et al.* (1997). This study showed that the cryoconservation of BCs preserved genetic material that can be used to reconstruct endangered chicken breeds in the future. However, the effectiveness of the reconstruction of chicken breeds using cryoconserved BCs is low (6.8%) (KINO *et al.* 1997) compared with freshly isolated BCs (21.1%) (BEDNARCZYK *et al.* 2002). Therefore, future studies are required to improve the effectiveness of cryoconservation techniques. Our research aimed to compare the effects of three slow freezing programs and vitrification, cryoprotectants (5% DMSO, 10% DMSO, or MC) and two thawing methods on the viability of chicken BCs.

The survival of cells subjected to cryoconservation depends on several factors, such as the components and concentration of the cryoprotectant, the cell concentration, the cooling rate, the ice-seeding temperature, and the warming rate, which can expose cells to damage and osmotic stress during the dehydration and hydration processes (MAZUR *et al.* 1963). To date, only one-component cryoprotective agents such as DMSO, glycerol, ethylene glycol or propylene glycol have been used to protect BCs during the cryopreservation process (CHELMONSKA *et al.* 1997; TERESHCHENKO *et al.* 1993; KINO *et al.* 1997). This is the first study to use a MC cryoprotectant for the cryoconservation of BCs. The MC cryoprotectant contained sucrose, which has the ability to decrease osmotic stress, and glycerol, which is routinely applied to BCs cryopreservation (CHELMONSKA *et al.* 1997; TERESHCHENKO *et al.* 1993). We demonstrated that application of the MC cryoprotectant yielded significantly (P<0.05) higher cell viability than DMSO (Fig. 2), suggesting that the MC cryoprotectant is better for BCs cryoconservation compared with DMSO. The viability of cells undergoing cryopreservation also depends on the concentration of the cells (KINO *et al.* 1997). In this study, the BCs were slowly frozen or vitrified at concentrations of 5 x 10^5 or 1.5 x 10^4 cells/ml, respectively. Our results (Fig. 2 and 3) confirmed that the concentration of cells subjected to cryoconservation was appropriate for BCs.

In previous cryoconservation studies (NAITO *et al.* 1992; CHELMONSKA *et al.* 1997; TERESHCHENKO *et al.* 1993; KINO *et al.* 1997), cells were slowly frozen at a constant cooling rate ranging from 0.5°C to 10°C/min. To date, the highest BCs survival (83%) was obtained as a result of slow freezing at a rate of 1°C/min in the presence of 10% DMSO (TERESHCHENKO *et al.* 1993; CHELMONSKA *et al.* 1997). In the current study, we showed that slow freezing in the presence of 10% DMSO using

a variable cooling rate (from 4°C through 1°C until 0.3°C /min, program 2) also yielded 83±3.2% BCs viability (Fig. 2). Furthermore, our study showed that a combination of program 2 with the MC cryoprotectant and thawing with method 1 resulted in the highest percentage of viable BCs (94±1.0%, Fig. 2), implying that the program, cryoprotectant and thawing method were all well suited for the cryoconservation of BCs.

During slow freezing processes, cells undergo dehydration to remove intracellular water and to prevent intracellular ice formation (ZHANG *et al.* 2011). To enhance dehydration, the cells are subjected to an ice-seeding process, which induces extracellular ice crystallization. In most slow cooling protocols, ice-seeding processes are performed at temperatures between -6°C and -9°C (SHAW & JONES 2003). Only KINO *et al.* (1997) reported a seeding process performed during the cryoconservation of BCs. The formation of extracellular ice was induced at -7°C using cold forceps. In our study, we performed seeding at -7°C (programs 1 and 2) or -6°C (program 3) using forceps that were pre-chilled in liquid nitrogen. This might be one reason why programs 1 and 2 were more effective than program 3 in the slow freezing of the BCs (Fig. 2).

The viability of cryopreserved cells also depends on the thawing process. During this process, cells are exposed to damage because melting ice forms areas of pure water that can cause osmotic shock (SHAW & JONES 2003). Cell viability is reduced by the toxic effects of cryoprotectants. KINO *et al.* (1997) reported that thawing BCs at a rate of 70°C/min to +37°C yielded a higher percentage of live BCs than 200 or 2000°C/min. In our study, we thawed BCs to +4°C at a constant rate (40°C/min., method 2) and at a variable rate (from 8°C to 5°C/min, method 1). From +4°C to +20°C, the warming rate was the same for both methods (4°C/min.). After thawing, the cells were gradually diluted in culture medium to protect the BCs from damage due to excessive hydration. The highest survival was observed when the cells were thawed using method 1, although there were no significant differences between the two thawing methods (Fig. 2).

Another method for BCs cryopreservation is the ultra-rapid technique known as vitrification. Vitrification is a process based on the progressive increase in the viscosity of a liquid, which occurs during a rapid decrease of temperature (SAWICKA *et al.* 2011). In our study, we vitrified BCs in the presence of 10% DMSO and obtained a cell survival rate of 81%±1.0% after thawing. Furthermore, the viability of vitrified BCs did not significantly differ from the survival rates obtained after slowly freezing using programs 1 and 2 (Fig. 3), suggesting that our vitrification method is as effective as slow freezing methods in the presence of 10% DMSO.

In conclusion, this is the first study to apply a MC cryoprotectant for the slow freezing of BCs. We demonstrated an improved method for the cryoconservation of chicken BCs using a MC cryoprotectant in combination with program 2 and thawing with method 1. We also demonstrated successful BCs vitrification. Future studies are required to determine whether elaborated methods might contribute to increasing the effectiveness of reconstructing breeds using cryoconserved BCs.

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