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# Cryopreservation of chicken primordial germ cells by vitrification and slow freezing: A comparative study

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ABSTRACT

In the present study, we compare a classical slow freezing (SLF) method and an aseptic vitrification (Vitrif) technique to cryopreserve a stable primordial germ cell (PGCs) line issued from the *Ardennaise* chicken breed. Viability immediately after warming was close to 80% and did not differ between the two cryopreservation methods. Proliferation tended to be slower for both cryopreservation methods compared with controls, but the difference was significant only for Vitrif. No difference was found between the two methods after flow cytometry analysis of stage-specific embryonic antigen-1 expression and reverse transcription–polymerase chain reaction on several factors related to PGC phenotype. After 1 week in culture, all cryopreserved cells reached controls' main morphologic and expanding (viability/proliferation) features. However, SLF generated more unwanted cells clusters than Vitrif. After injection of the PGCs into recipient embryos, vitrified PGCs reported a clear, yet not significant, tendency to colonize the gonad at a higher rate than slow frozen PGCs. SLF in cryovials remains simple, inexpensive, and less technically demanding than Vitrif. Nevertheless, the intrinsic advantages of our aseptic Vitrif method and the present study suggest that this should be considered as safer than classical SLF for cryopreserving chicken PGCs.



## 1. Introduction

Cryobanking of either animal germplasm or embryos enables preservation of genetic resources for research, for the livestock industry and for preserving the genetic diversity of extant but endangered populations. In the context of avian genetic resources preservation, laid chicken eggs appear as refractory to cryopreservation as a consequence of a huge yolk mass. Rooster semen freezing remains the sole commonly used method for cryobanking chicken genetic diversity [1–3], omitting female-sided germplasm and mitochondrial and W chromosome contents. It is also challenging because its efficiency is highly variable across breeds and individuals [4,5]. As an alternative, gonadal tissue cryopreservation and transplantation were developed to ensure preservation of the female-sided germplasm [6–8]. Because it involves surgery and use of immunosuppressants, this option appears highly technical, costly, and raises welfare issues.

Recently, chicken primordial germ cells (PGCs) have been reported as a valuable starting material for cell-based

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genetic engineering, germplasm expansion, and genetic preservation. Indeed, chicken PGCs can be expanded in culture and cryopreserved without irreversibly altering their biologic properties [9–11]. Moreover, their subsequent injection in recipient embryos can provide a means for reviving the germplasm, whatever its sexual origin, ending up with functional gametes [12]. Therefore, PGCs appear as the best candidates to date for preserving the chicken germplasm. However, only 100 to 300 PGCs in total are present in the circulatory system of the chicken embryo [13] at Hamburger and Hamilton [14] stages 13 to 18, which requires robust and efficient methods of cryopreservation, especially if no *in vitro* expansion step is intended.

Two main methods are commonly used for cryopreserving live biologic material: slow freezing (SLF) and vitrification (Vitrif). Since 1994 and the first success in obtaining germline chimeras from cryopreserved chicken PGCs [15], studies involving cryopreservation of avian PGCs were usually on the basis of SLF techniques [11,16–21]. SLF of PGCs is usually performed using serum-containing media with addition of 5% or 10% DMSO [11,15,20,21] or ethylene glycol (EG) [16]. Commercially available premixed media were also used successfully [17–19]. To date, few data have been published on PGCs Vitrif, although this approach is deemed as more efficient than SLF for mammalian oocytes [22,23], embryos [24–26], and stem cells' cryopreservation [27–29] considering cell survival and stability. In 2008, Kohara et al. [20] reported the first attempt to vitrify chicken gonadal germ cells, using the protocol established by Nakao et al. [30] for mouse embryos. Viability after Vitrif was significantly lower than after SLF, but gonadal colonization was equivalent for the two methods. Kim et al. [31] also observed a lower viability of vitrified PGCs compared with SLF. More recently, Sawicka et al. [32] reported that viability of chicken blastodermal cells was lower after Vitrif than after SLF. These results are in contradiction with the claimed superiority of Vitrif for cryopreserving mammalian embryos and stem cells.

In the present study, we challenge a classical SLF method against an aseptic Vitrif technique on a stable PGCs line issued from a Belgian endangered breed called *Ardennaise* [10]. Effects on the morphology, survival, proliferation, expression of specific markers, and gonadal colonization of PGCs are compared.

## 2. Materials and methods

### 2.1. Cell culture

Chicken PGC cell line AR111012 [10] was used throughout this study. This line, derived from a Belgian endangered chicken breed (*Ardennaise*), was cultured for 6 months and characterized before cryopreservation.

PGCs were cultured as described by Tonus et al. [10]. Briefly, cells were grown in a Biopore cell culture insert (Merck Millipore, Billerica, MA, USA), in six-well plates seeded with irradiated buffalo rat liver feeder cells. The culture medium was composed of KnockOut DMEM, including 50% buffalo rat liver conditioned medium, and was supplemented with 7.5% embryonic stem (ES) cells qualified fetal bovine serum (FBS), 2.5% chicken serum, 2 mM glutamine, 1 mM pyruvate, 1× nucleosides (Sigma Aldrich, St Louis, MO, USA), 1× nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 units/mL penicillin and 100 μg/mL streptomycin, 2.5 mg/mL amphotericin B, 6 ng/mL stem cell factor, 4 ng/mL human fibroblast growth factor, 5 ng/mL mouse leukemia inhibitory factor (Sigma Aldrich), and 10 ng/mL human insulin-like growth factor 1 (PeproTech, Rocky Hill, NJ, USA). All reagents were purchased from Life Technologies (Carlsbad, CA, USA), unless otherwise indicated.

### 2.2. Cryopreservation and warming

Before cryopreservation experiments, reagents toxicities were tested by successively exposing the cells to cryoprotectants solutions and thawing/warming solutions but omitting the cooling step.

One million cells were cryopreserved per vial or straw; 50 SLF and 11 Vitrif were performed in four independent experiments. All samples were stored in liquid nitrogen (LN2) for 2 to 10 days before warming.

SLF was performed according to the protocol developed and optimized in our laboratory [10]. Cells were centrifuged at ×200*g* for 8 minutes and resuspended in 1 mL cold (4 °C) freezing medium containing 50% (v:v) FBS and 5% (v:v) DMSO (Sigma Aldrich) in KnockOut DMEM supplemented with glutamine, pyruvate, nonessential amino acids, nucleosides, antibiotics, and antimycotics. Cell suspensions were transferred in cryovials, and samples were cooled in a commercial freezing device (Mister Frosty; Nalgene, Rochester, NY, USA) placed overnight in a −80 °C freezer before storage in LN2. Warming was performed by placing the cryovials in a water bath at 37 °C until the disappearance of ice crystals. Cryoprotectants were diluted immediately afterward in 10 mL prewarmed (37 °C) culture medium. Cells were harvested after 8-minute centrifugation at ×200*g* and resuspended in complete culture medium before counting.

The Vitrif protocol has been adapted from Vanderzwalmen et al. [24] and optimized for mouse ES cells cryopreservation (F. Ectors, unpublished data). Cells were harvested as described for SLF and resuspended in 140 μL of phosphate-buffered saline (PBS) containing 36 mg/L sodium pyruvate, 50 mg/L streptomycin sulfate, 100 mg/L kanamycin monosulfate, and 1 g/L glucose (Sigma Aldrich, ref D4031) supplemented with 10% (v:v) FBS (PBS + 10% FBS). The same PBS was used in the non-vitrifying (nVS) and vitrifying (VS) solutions. All manipulations were performed at room temperature. One hundred forty microliters of nVS1 (10% [v:v] DMSO and 10% [v:v] EG [Sigma Aldrich] in PBS + 10% FBS) were added to the sample, followed by 3 minutes incubation. Two hundred eighty microliters of nVS1.5 (15% [v:v] DMSO and 15% [v:v] EG in PBS + 10% FBS) were added to the mix, to obtain a final concentration of 10% DMSO and 10% EG. Cells were incubated for three more minutes, including 30 seconds of centrifugation at ×2000*g*. The pellet was resuspended in 260 μL VS solution (20% [v:v] DMSO, 20% [v:v] EG, 1 M sucrose, and 10 mg/mL Ficoll in PBS + 10% FBS). Each sample was loaded in a 250-μL semen straw (Minitube International, Tiefenbach, Germany) that was immediately sealed at both ends with an ultrasonic straw sealer (Ultraseal 21; Minitube International) and