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RESEARCH ARTICLE

Effect of sucrose on cryopreservation of pig spermatogonial stem cells

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Abstract

Sucrose is known to play an important role in the cryopreservation of sperm and female gonads; however, its effect on the cryopreservation of pig spermatogonial stem cells (pSSCs) has not been tested. The aim of this work was to study the effect of sucrose during pSSC cryopreservation and to find the most effective concentration in freezing medium. pSSCs were cryopreserved with freezing media containing different concentrations of sucrose (70, 140, 210, and 280 mmol L^{-1}) and a control group without sucrose. The survival rates, plasma membrane integrity, and mitochondrial membrane potential of thawed cells were detected by trypan blue (TB) staining, SYBR-14/propidium iodide (PI) dual staining, and JC-1 staining, respectively. All the staining results showed an obvious increase in cell survival in the sucrose-treated groups as compared to that in the control group, with the exception of 280 mmol L^{-1} sucrose. Moreover, the 210 mmol L^{-1} sucrose group yielded the highest survival rate among all the groups (P<0.05). The results of SYBR-14/PI dual staining and JC-1 staining were consistent with those of TB staining as above described. Quantitative real-time PCR (qRT-PCR) indicated that the mRNA levels of three apoptosis-promoting genes (*BAX*, *APAF1* and *CASPASE9*) were significantly higher in thawed cells than in cells before freezing (P<0.05). Moreover, the mRNA level of one anti-apoptotic gene (*XIAP*) was significantly lower in thawed cells than in cells before freezing (P<0.05). When comparing the mRNA expression of apoptosis-related genes in thawed cells, the mRNA level of the anti-apoptotic genes in the control group was significantly lower than that in the sucrose-treated groups (P<0.05). Western blot analyses showed that the expression levels of cleaved CASPASE9, CASPASE3 and PARP-1 in the sucrose-treated groups were lower than those in the control group and were the lowest in the 210 mmol L^{-1} sucrose group. Both qRT-PCR and Western blot analyses suggested that sucrose inhibited cell apoptosis during freezing and thawing. Briefly, sucrose promoted pSSCs survival after freezing and thawing, especially at a concentration of 210 mmol L^{-1}, which possibly assisted pSSC dehydration and inhibited cell apoptosis. These findings hold great promise for further studies of the regulatory mechanism of proliferation and differentiation of pSSCs.

Keywords: spermatogonial stem cells (SSCs), pig, cryopreservation, sucrose, apoptosis, slow-freezing

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1. Introduction

As the only stem cell found in the germ line, spermatogonial stem cells (SSCs) are capable of transferring genetic infor-

mation to the next generation and are also the foundation of spermatogenesis and male fertility (Zohni *et al.* 2012; Song and Wilkinson 2014). Because of their capacity for self-renewal and differentiation, SSCs are useful for commercial and clinical applications (Kanatsu-Shinohara and Shinohara 2013). SSC transplantation can not only provide an effective treatment approach, but also preserve genetic resources and reverse male infertility (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Honaramooz *et al.* 2003; Aliakbari *et al.* 2016). Recently, SSCs have become a popular research subject in developmental biology and regenerative medicine.

However, SSCs are extremely rare, comprising only 0.02–0.03% of all germ cells in the rodent testis (Aponte *et al.* 2005; Zhang *et al.* 2014; Manku and Culty 2015). Not only are the typical quantities of SSCs small, but also long-term culture of SSCs from large livestock has proven to be difficult. Studies showed that mouse or rat SSCs could be maintained for at least 1 year or 5 months, respectively, with *in vitro* culture systems (Kanatsu-Shinohara *et al.* 2008, 2014; Wu *et al.* 2009). However, studies on the SSCs of large livestock, especially pig, had encountered many setbacks. It was demonstrated that pig SSCs (pSSCs) could only survive in culture for 2 months using a 31°C culture system; apparently, the time is much shorter and the conditions are stricter than those for rodent cells (Lee W Y *et al.* 2013). Moreover, to date, a pSSC cell line has yet to be established. Pigs share many physiological and biochemical characteristics, as well as many anatomical structures with humans, which could make them an ideal mammalian model for human reproductive biology and regenerative medicine (Jiang and Rothschild 2007). In this context, obtaining large populations of pSSCs from the testicular tissues is a difficult but indispensable method to study the characteristics of pSSCs. Thus, it is important to enrich pSSCs by utilizing long-term culture systems and preservation. Cryopreservation is an essential step in the long-term preservation of SSCs, allowing for the characteristics of SSCs to be maintained after thawing (Lee Y A *et al.* 2013; Goossens and Tournaye 2014; Cai *et al.* 2016). For example, previous studies indicated that mouse SSCs maintained stem cell potential and the ability to reestablish spermatogenesis more than 14 years after being cryopreserved (Wu *et al.* 2012).

An efficient cryopreservation method would provide a useful way to preserve SSCs for a long term, which is needed for SSC transplantation in clinical applications (Wu *et al.* 2012). A slow-freezing method is the most common long-term preservation approach for SSCs (Avarbock *et al.* 1996; Kim *et al.* 2015); however, it is known that the ice crystals, which form during cryopreservation, can produce mechanical damage to the cell plasma membranes (Lee *et al.* 2014a). Cryoprotective agents (CPAs), consisting of permeable cryoprotective agents (PCPAs) and non-permeable cryoprotective agents (non-PCPAs), are helpful to minimize the formation of ice crystals and other cryodamage (Meryman 1971; Lee *et al.* 2014a). CPAs can prevent extracellular and intracellular ice crystals formation and desiccation (Meryman 1971; Lee *et al.* 2014a; Zhao *et al.* 2014). It has been suggested that non-PCPAs, such as saccharides, could improve the glass transition temperature and cell viability (Oldenhof *et al.* 2013; Tian *et al.* 2015). As one type of commonly used non-PCPA, sucrose and trehalose, two isomerides of a non-reducing disaccharide, could balance extra- and intra-cellular osmotic pressure during freezing and thawing cycles (Garcia de Castro and Tunnacliffe 2000). Studies on mouse and bovine SSCs proved that sucrose and trehalose were the most effective cryoprotectants among all the disaccharides (Wang *et al.* 2014; Kim *et al.* 2015). Thus, as the most economical and convenient non-reducing disaccharide, sucrose was chosen in order to study its effect on the cryopreservation of pSSCs.

Based on previous studies on SSC cryopreservation in other species, a concentration gradient of sucrose (0, 70, 140, 210, and 280 mmol L^{-1}) was chosen to evaluate its effect on pSSCs cryopreservation and the most effective concentration (Lee *et al.* 2014a; Poels *et al.* 2014). All these data provide a scientific basis for developing cryopreservation technology of pSSCs.

2. Materials and methods

2.1. Collection of porcine testes

Animal procedures were approved by the Animal Care and Use Committee of Northwest A&F University, China in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, China. Testis samples were obtained from 2-month-old male landrace pigs, and then transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS) with 200 U mL^{-1} penicillin and 0.2 mg mL^{-1} streptomycin (P/S) solution (Invitrogen, Carlsbad, CA, USA) within 1 h of harvest.

2.2. Isolation and enrichment of pSSCs

Two-step enzymatic digestion was used to obtain single cell suspensions from the pig testicular tissues, and the pSSCs were enriched by differential plating. In detail, testes were collected and washed three times with DPBS containing P/S solution. The tunica albuginea was removed, and tissues were minced into small pieces. The tissue fragments were