Molecular Biology Reports (2020) 47:9609–9614
https://doi.org/10.1007/s11033-020-06004-2

ORIGINAL ARTICLE

A comparison of the effects of fetal bovine serum and newborn calf serum on cell growth and maintenance of cryopreserved mouse spermatogonial stem cells

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Received: 2 October 2020 / Accepted: 11 November 2020 / Published online: 19 November 2020

Abstract

Serum is a common supplement that is widely used to protect various cells and tissues from cryopreservation because it provides the necessary active components for cell growth and maintenance. In this study, we compared the effects of newborn calf serum (NCS) and fetal bovine serum (FBS) on the cryopreservation of mouse spermatogonial stem cells (SSCs). The isolated SSCs were cryopreserved in two groups: freezing medium that contained 10% DMSO (dimethyl sulfoxide) and 10% FBS in DMEM (Dulbecco's Modified Eagle's Medium) (group 1) and freezing medium that contained 10% DMSO and 10% NCS in DMEM (group 2). Real-time PCR was performed for stemness gene expression. The SSCs' viability was performed by trypan blue. We observed that the SSCs had increased viability in the NCS-freeze/thaw group (87.82%) compared to the FBS-freeze/thaw group (79.83%), but this increase was not statistically significant ($P < 0.105$). Promyelocytic leukemia zinc finger (*Plzf)* and *Lin28* gene expression levels in the NCS-frozen/thawed SSCs were not significantly different compared to the FBS-frozen/thawed SSCs; however, *Nanog* gene expression increased considerably, and *Dazl* gene expression decreased significantly. The results in this study demonstrated that the presence of NCS in a solution of cryopreserved SSCs increased their viability after freeze/thawing and might promote the proliferation of cultivated SSCs in vitro by increasing the relative expression of *Nanog*.

Keywords Spermatogonial stem cells · Cryopreservation · Newborn calf serum · Fetal bovine serum

Introduction

Cryopreservation enables spermatogonial stem cells (SSCs) to be stored at very low temperatures for an extended period, prevents cell aging by metabolic stoppage, and maintains the viability of SSCs and their capacity for a prolonged period [20]. This process can be used for fertility preservation in pre-pubertal boys who have testicular dysfunction from chemotherapy and radiotherapy [6]. However, cryopreservation adversely affects the structural, biochemical, and functional attributes of SSCs by intracellular and extracellular ice crystals formation, production of reactive oxygen species (ROS), and causes substantial changes to the expressions of genes group, all of which could affect the quality of these thawed SSCs [8].

Spermatogonial stem cells are rare cells in the epithelium of the seminiferous tubule. A small testicular pre-pubertal patient biopsy might include only a few of SSCs [22]. The success of SSC transplants depends on the number and efficiency of SSC transplants [19]. A study has shown that SSCs comprise just 0.03% of all germ cells in the rodent testis [26]. Therefore, optimization of cryopreservation protocols is necessary to enable maximum functional survival rates for SSCs [21].

The choice of a cryopreservation solution (CP) is crucial to minimize damage during cryopreservation [13]. Bovine serum includes fetal bovine serum, which is obtained from the blood of fetuses of healthy, pre-partum bovine dams, and newborn calf serum, which is the liquid component of

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clotted blood originating from healthy, slaughtered bovine calves under 20 days of age [7]. Both are widely used as main components of CP for testicular tissues and SSCs cryopreservation [12, 29]. The serum is comprised of a mixture of different hormones, growth factors, antibodies, and unknown protein components [7], and provides essential components that include nutrients, pH buffers, and antioxidants [17].

Spermatogonial stem cells, similar to other stem cells, have the ability for self-renewal and differentiation [2] and the success rate of transplants depends on the SSC quality after cryopreservation. In the present study, we compared the impacts of FBS and NCS on viability and stemness related gene expressions of SSCs after cryopreservation.

Materials and methods

Animals

We obtained 6-day-old neonatal male Balb/c mice (n = 80) from original stocks from Razi Laboratory Animal Center (Tehran, Iran). All experimental procedures have been approved and conducted in compliance with the guidelines of the Iranian National Research Council.

Cell isolation

Bilateral testies were collected from the 6-day-old neonatal Balb/c mice to obtain the SSCs. The testes were decapsulated and washed twice in DMEM (Gibco) that contained penicillin (100 IU/ml) and streptomycin (100 µg/ml). Single-cell suspensions of testicular tissue were prepared by enzymatic digestion.

First, the testes were placed in 1 ml of DMEM that contained 2 µg/ml collagenase IV (Sigma) and 5 µg/ml DNase I (Sigma) in an incubator for 15 min at 37 °C and 5% CO_2. The mixture was subsequently centrifuged at 800 rpm at room temperature for 5 min.

The resultant cell pellets were resuspended in 1 ml trypsin/EDTA (Sigma) for 5 min at 37 °C and gently pipetted to generate a testicular cell suspension. We added 2 ml of DMEM that contained 1% FBS (Gibco Leicestershire, UK) to neutralize the trypsin effect and then filtering the cell suspension through a 70 µm nylon strainer (FALCON, USA).

After centrifugation, the medium was drained, and the cells were washed twice and transferred to a fresh medium. Viability was measured by a hemocytometer (Fig. 1a–f).

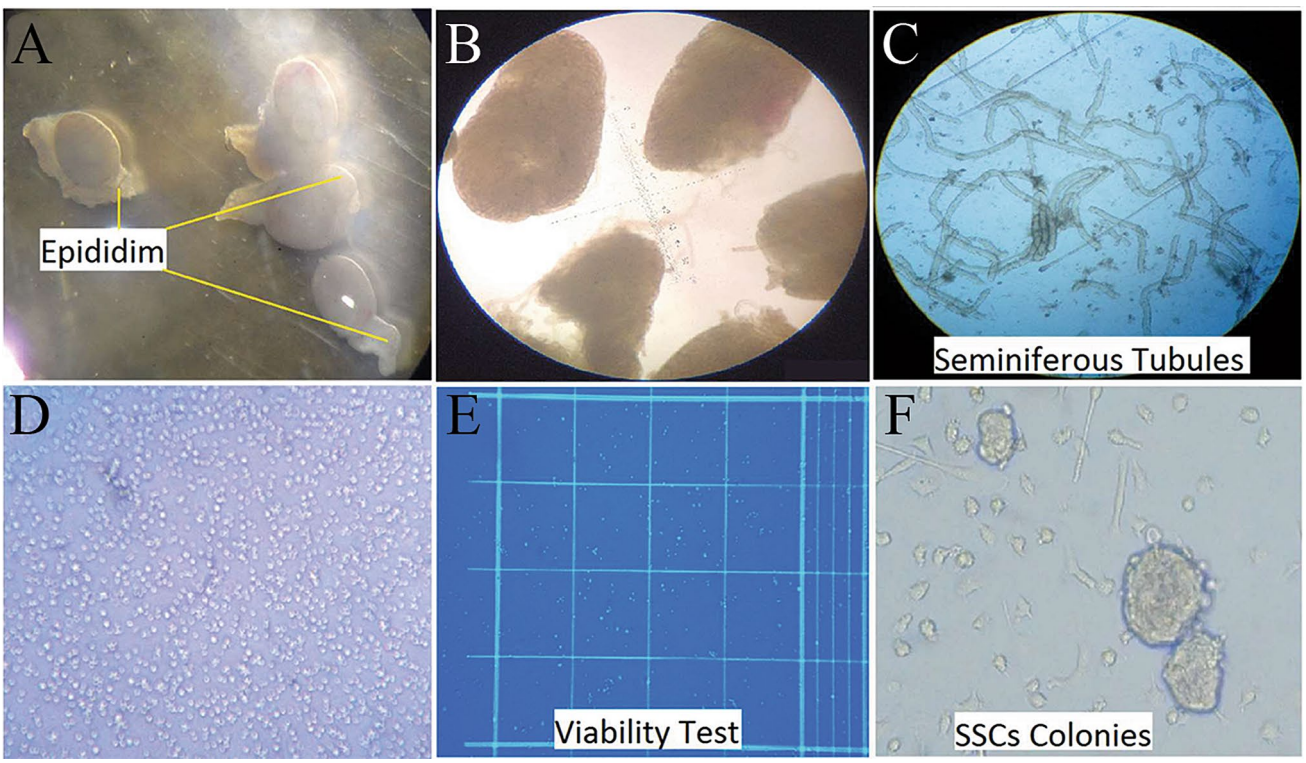

Fig. 1 **a** tunica albuginea testis and epididymis, **b** removal of Tonica albuginea and epididymis from testis, **c** seminiferous tubes, **d** spermatogonial stem cell suspension, **e** Viability assessment by hemocytometer, **f** SSCs colonies resulting from spermatogonial stem cell culture

Spermatogonia cell separation and purification with laminin and MACS

The cells have been placed in a Petri dish (60 mm) covered with a layer of 20 µg/ml laminin and placed in an incubator overnight. Subsequently, the supernatants were removed and the Petri dish laminate was washed with PBS and incubated with 0.5 mg/ml BSA at 37 °C for 1 h, and afterward rinsed using PBS. The MACS method was used to purify the SSCs. A common SSC marker, Thy-1 (MouseCD90.1 MicroBeads, Miltenyi Biotec, 130-094-523) was a positive control and c-Kit (Mouse CD117 MicroBeads, Miltenyi Biotec, 130-091-224) was used as the negative control (Columns: MS, LS, XS, autoMACS Columns). In summary, 10^7 of the total cells have been centrifuged at 300 g for 10 min. The cell pellets were resuspended in 90 µl of a buffer solution that contained PBS, 0.5% BSA, pH 7.2, and 2 mM EDTA. The MACS BSA (# 130-91-376) stock solution was diluted 1:20 with auto MACS Rinsing solution (# 130-091-222). Then, we added 10 µl of the CD90.1 MicroBeads to this buffer solution. The resultant suspension has been refrigerated for 15 min, after which the cells were washed with 1–2 mM buffer and centrifuged for 10 min at 300 rpm. A total of 10^7 cells have been resuspended in 500 µl buffer solution and then the cell suspension was passed through the MACS column to enable cell separation in the magnetic field.

Freezing and thawing of spermatogonial cells

The isolated SSCs were cryopreserved in two groups: freezing medium that contained 10% DMSO (Sigma) and 10% FBS in DMEM medium (group 1) and freezing medium that contained 10% DMSO and 10% NCS (Gibco, Leicestershire, and UK) in DMEM medium (group 2).

The cryovials and freezing medium have been refrigerated at − 20 °C in each experiment. The cooled freezing medium has been added to10^6 cells/ml and the cell mixture was transferred to − 20 °C for 2 h, and then kept at − 80 °C overnight. The next day, the cells were transferred to a liquid nitrogen vapor phase for 10 s before they were plunged into liquid nitrogen. The frozen cryovials have been thawed by placing them in a 37 °C water bath. Just before the entire medium thawed, we gently added 10 ml of DMEM to the cryovials to neutralize the impact of the freezing medium.

Determination of cell viability with trypan blue staining

We used the trypan blue dye exclusion assay for cell viability assessment.

Quantitative real-time PCR (qRT-PCR)

Total RNA has been extracted with a Total RNA Purification Kit (Jena Bioscience). The cDNA has been synthesized using an AccuPower® CycleScript RT PreMix Kit (BIONEER. Quantitative real-time PCR (qRT-PCR) was conducted with a qPCR GreenMaster with lowROX kit (Jena Bioscience). The results were standardized about the expression of the housekeeping gene, *Gapdh*. Table 1 lists the primers used in this experiment.

Statistical analysis

Statistical differences between the percentages of viable cells after freezing/thawing of the SSCs were analyzed by SPSS statistical software with one-way ANOVA. P-values less than or equivalent to 0.05 indicated statistical significance. qRT- PCR data were analyzed by using Rest 2009 software. Tables and graphs were generated by the same software. All data are presented as mean ± SD in the graphs from a minimum of three independent experiments.

Results

The viability rate of the SSCs after freeze/thaw with FBS was 79.83% and it was 87.82% with NCS. The difference between the percentages of viable SSCs between the two experimental groups was not significant (p˂0.105) (Fig. 2).

As shown in Fig. 3 and Table 2, the expressions of *Plzf* and *Lin28* genes in the NCS-frozen/thawed SSCs did not change significantly compared to the FBS-frozen/thawed SSCs; however, there has been a significant increase in expression of the *Nanog* gene and a significant decline in *Dazl* gene expression (Fig. 3; Table 2).

Table 1 Primer sequences used in the present study

Genes	Sequence (5′–> 3′)	Product length (bp)
Nanog	F: GGAGGACTTTCTGCAGCCTT R: ACCTGGTGGAGTCACAGAGT	154
Dazl	F: CTGTCAGGATTGCTCCCTGG R: AGGTAGCAAGGTGCTGTTCC	103
Lin28	F: CTGTCCAGGCATGGGAACAT R: GTGAGGTAGGTTTGTGGCGA	196
Gapdh	F: CAATGTGTCCGTCGTGGATCT R: GTCCTCAGTGTAGCCCAAGATG	208
Plzf	F: ACATGGCTGTCTTCTGTCTG R: GGTGGTCACCTGTATGTGAG	174

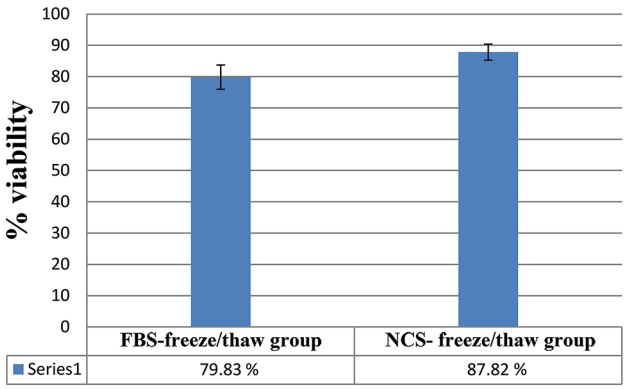

Fig. 2 Cellular viability frequency (%). Comparison of FBS-frozen/thawed and NCS-frozen/thawed SSCs groups. The error bar is SEM. *SEM* standard error of means, *ANOVA* analysis of variance. Values are presented as mean ± SD (n = 3; P < 0.05)

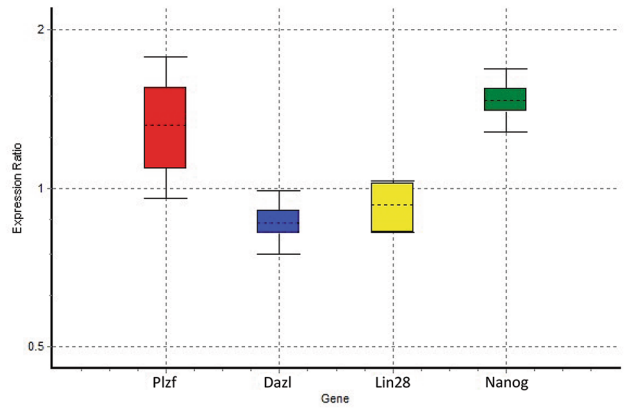

Fig. 3 Relative expression of *Plzf*, *Dazl*, *Lin28*, and *Nanog* genes in the NCS-frozen/thawed SSCs group compared to the FBS-frozen/thawed SSCs group. Values are presented as mean ± SD (n = 3; P < 0.05)

Discussion

The objective of this study was to compare the use of FBS and NCS supplements to the CP for SSCs. In this study, SSCs were obtained from 6-day-old mice testes. Spermatogenesis begins immediately after birth in mice [25]. Only germ cells in the newborn mouse testis can be gonocytes or prospermatogonia, that are located in the seminiferous tubule core [18]. These cells migrate to the basal membrane by six days postpartum and develop into undifferentiated type A spermatogonia, which begins to gradually differentiate [15]. The spermatogonial stem cells were isolated and after enzymatic digestion, we assessed the percentage of SSCs using an undifferentiated germ cell marker, Thy-1 [21].

The trypan blue dye exclusion assay was used to evaluate the viability of the cells after freeze/thawing of the two groups of SSCs.

Fetal bovine serum and Newborn calf serum are widely used in freezing media to preserve spermatogonia and testis tissues from humans and various animal species [1–3, 12, 21, 29]. It has been reported that a freezing solution that contains 10% DMSO and 10–20% NCS maintains the survival of both bovine testicular cells and spermatogonia after cryopreservation [29]. A medium with 10% DMSO and 60% FBS is one of the best cryopreservation conditions of prepubertal mouse SSCs [3]. The fetal bovine serum also improves the functionality after freezing of European eel spermatozoa [10]. Moghaddam et al. have reported that freezing media that contained 10% DMSO and 10% FBS is appropriate for cryopreservation of lamb spermatogonial cells [16].

Our results showed that the post-thaw viability of cryopreserved SSCs preserved in FBS compared with NCS was not significant. Cryopreservation significantly increases ROS production in SSCs and excess ROS has negative effects on SSCs functionality by damaging various cellular components, such as nucleotides, proteins, and membranes [27]. Studies have shown that serum significantly reduces the production of ROS and has antioxidant properties [17].

Table 2 Real-time PCR analysis

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I	P(H1)	Result
Gapdh	REF	0.745	1.000				
Plzf	TRG	0.7575	1.300	1.038–1.639	0.967–1.751	0.344	
Dazl	TRG	0.725	0.861	0.792–0.938	0.756–0.981	0.000	DOWN
Lin28	TRG	0.705	0.922	0.826–1.029	0.825–1.030	0.834	
Nanog	TRG	0.765	1.467	1.348–1.601	1.287–1.673	0.000	UP

Comparison of relative expressions between FBS-frozen/thawed and NCS-frozen/thawed SSCs groups

TRG target, *REF* reference, *Std* standard, *CI* confidence interval

On the other hand, the positive effects of FBS and NCS appear to be from serum proteins and lipids, and they play a significant role in the cycle of cryopreservation as a defense mechanism for plasma membrane instability [28]. Mechanical security at the level of the membrane may be made available through FBS and NCS because they allow for the reactivation of the internal cell structures under reasonable conditions after freezing [10].

Plzf is one of the first reported transcription factors necessary for SSC self-renewal [11]. Studies have shown that the potential role of *Plzf* in spermatogonia may be to maintain an undifferentiated state [9]. *Lin28* is a pluripotency factor that is expressed uniquely in undifferentiated spermatogonia [31]. Numerous researchers have suggested that *Lin28* plays a role in the regulation of cell fate selection [5]. In mammalian cultured cells, it appears that *Lin28* expression is synonymous with "stemness" [23]. In our study, mRNA expression levels of *Plzf* and *Lin28* did not significantly differ between NCS-frozen/thawed SSCs and FBS-frozen/thawed SSCs.

Nanog is considered to be a key transcription factor for the transition of stem cells into pluripotency [4]; however, *Nanog* does not appear in SSCs [30]. In the current study, *Nanog* had significantly higher expression in the NCS-frozen/thawed SSCs. Yung et al. reported that *Nanog* induction might be promoting the proliferation of cultivated SSCs in vitro and partially offset the function of the GDNF growth factor [30]. Results of studies have shown that FBS lacked the necessary factors for the proliferation of SSCs and spermatogenesis progression [24]. *Dazl* serves as the master translation regulator to guarantee correct translation of key sperm factors and, therefore, fertility. *Dazl* also plays a role in the self-renewal of SSC. The level of *Dazl* expression is not well-established, in particular for different stages such as SSCs or round spermatids [14]. Of note, *Dazl* had significantly lower expression in FBS-frozen/thawed SSCs than in NCS-frozen/thawed SSCs.

The results of this study demonstrated that the use of NCS in CP of SSCs increased SSC viability after freeze/thawing. At the gene expression level, we observed an increase in the relative expression of *Nanog*. Therefore, NCS might promote the proliferation of cultivated SSCs in vitro.

Acknowledgements This study was confirmed by the Lorestan University of Medical Sciences. The authors would like to thanks the Lorestan University of Medical Sciences, Khorramabad, Iran The authors also thank the head and staff of the Razi Herbal Medicines Research Center of Lorestan Medical University.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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