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Original Research

# Isolation of Germ Cells From Testes of Stallions Using Collagenase and Trypsin-Ethylenediaminetetraacetic Acid

Heejun Jung [a], Minjung Yoon [a,b,*]

[a] Department of Animal and Biotechnology Science, College of Animal Science, Kyungpook National University, Sangju, Republic of Korea
[b] Department of Horse, Companion and Wild Animal Science, Kyungpook National University, Sangju, Republic of Korea

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ABSTRACT

A germ cell isolation technique with two-enzyme digestion has been widely used to disaggregate testicular tissues for the preparation of individual germ cells. The main objective of this study was to assess the applicability of the two-enzyme germ cell isolation procedure to stallion testes. The testicular samples were obtained via field castration of prepubertal and postpubertal stallions. Then, equal amounts (10 g) of testicular tissue from each stallion were subjected to the two-enzyme germ cell isolation procedure with collagenase and trypsin-ethylenediaminetetraacetic acid. After the germ cells were isolated, viability and recovered germ cells were evaluated using trypan blue staining and a hemocytometer. Immunocytochemistry with UTF1 antibody was performed to evaluate the rate of undifferentiated spermatogonial stem cells. In addition, VASA antibody was used to measure the recovery rate for spermatogonia, spermatocyte, and round spermatid. The viability and recovery rate of the germ cells from prepubertal and postpubertal stallions were not statistically different. However, greater numbers of UTF1-positive germ cells (i.e., undifferentiated spermatogonia) were obtained from the testes of prepubertal stallions than from those of postpubertal stallions. In addition, the viability of germ cells isolated 2 days after castration did not differ from that of cells isolated on the same day that horses were castrated, and germ cells were successfully cryopreserved with 10% dimethyl sulfoxide for 1 month, although the viability was significantly reduced. In conclusion, the two-enzyme germ cell isolation procedure is applicable to the isolation of high-purity spermatogonia, spermatocyte, or round spermatid from stallion testes.



## 1. Introduction

Spermatogenic cells are surrounded by the epithelia of seminiferous tubules, which, in turn, are surrounded by interstitial fibromuscular tissue [1]. The structural integrity of the interstitium is maintained by a complex network of collagen, elastin, and cross-linked proteins [1]. In previous studies, mechanical shredding of testicular tissue, using scissors, has been used to isolate germ cells from seminiferous tubules; however, this procedure results in contamination of the spermatogenic cells with interstitial cells and Sertoli cells [1]. As an alternative, enzymes that break down the complex network of interstitial fibromuscular tissue have been used to isolate spermatogenic cells from testicular tissue with little contamination from somatic cells [1,2].

The germ cell isolation technique that uses two-enzyme digestion has been widely used for the preparation of individual germ cells. The enzymatic digestion of testicular tissue has been used in several species, including mouse [2], goat [3], cow [4], buffalo [5], rhesus macaque [6], and pig [7]. The isolation technique has been used, along with a germ cell culture system, to explore various factors of

* Corresponding author at: Minjung Yoon, Department of Horse, Companion and Wild Animal Science, Kyungpook National University, Sangju 742-711, Korea.
*E-mail address:* mjyoonemail@gmail.com (M. Yoon).

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spermatogenesis [8,9]. In addition, this technique has been used for the preparation of single germ cells to transplant into the rete testis of recipient animals in goat [10], boar [11], dog [12], and sheep [13]. The two-enzyme digestion procedure was applied for stallion germ cell isolation [14]. However, the efficiency of the procedure for germ cell isolation, such as the recovery rate, viability, and purity of specific stage of germ cells, remains to be evaluated, and the reproductive stage-dependent recovery rate and viability should be tested. In addition, the testicular samples of stallions are usually collected at horse farms and may require several hours to reach the laboratory or may even be shipped using overnight delivery services. Thus, conditions for germ cells isolated not immediately after castration should be tested.

Cryopreservation is a technique used to preserve the genetic value of germ cells, and 10% dimethyl sulfoxide (DMSO) has been used as a standard medium for germ cell cryopreservation [15]. Thus, the objectives of this study are (1) to evaluate the viability and the recovery rate of undifferentiated spermatogonia (UTF1 positive) or spermatogonia, spermatocyte, and round spermatid (VASA positive) using the general two-enzyme germ cell isolation procedure, (2) to compare these values between prepubertal and postpubertal stallions, (3) to compare the viability of germ cells isolated on the same day that horses were castrated to that of germ cells isolated from testes stored in a refrigerator for 2 days after castration, and (4) to test the efficiency of the cryopreservation of stallion germ cells with 10% DMSO.

## 2. Materials and Methods

### 2.1. Animals for Sampling

Stallion testicular samples were obtained from private horse farms and a subtropical animal experiment station on Jeju Island in the Republic of Korea. The testes were collected from various horse breeds, including Thoroughbred, Lusitano, and Jeju horses. Castration was performed as a result of the owners' decision to use these horses for pleasure riding. Stallions were sedated by intervenes injection of domosedan (Orion Pharma Animal Health, Sollentuna, Sweden). The standard standing castration procedure was performed by veterinarians. The reproductive stages of the horses were categorized according to the histology of seminiferous tubule cross sections, such as the presence or absence of lumen and the organization of germ cells [16].

### 2.2. Transportation of Sample Testes

Testes were collected into presterilized Ziploc bags and were transported using iceboxes to maintain their temperature at 4°C. In the laboratory, the testes were stored in a refrigerator until used.

### 2.3. Germ Cell Isolation From Testes

Individual germ cells were isolated from the testes of prepubertal (n = 5) and postpubertal (n = 5) stallions using a two-step enzyme procedure, as used for rhesus macaques [6] but with slight modification. A chunk of testicular tissue (10 g) was sliced into 1-$cm^3$ pieces. For the initial enzymatic digestion procedure, the sliced tissues were incubated with collagenase type IV (1 mg/mL; Sigma) dissolved in Hank's balanced salt solution (HBSS; Invitrogen) for 10–15 minutes with vigorous shaking at 37°C in a shaking incubator (Vision Scientific Co, Ltd, Daejeon, Korea). After the initial enzymatic digestion, the sample was centrifuged at 200*g*, and the supernatant, containing Leydig cells, was removed [17].

For the second enzymatic digestion, the pellets that contained dispersed seminiferous tubules were digested using trypsin-ethylenediaminetetraacetic acid (EDTA) (2.0 mg/mL trypsin in 1.04 mM EDTA; Invitrogen) and DNase I (1.4 mg/mL; Sigma) in HBSS for 15 minutes. After quenching with fetal bovine serum (FBS, 10%), a 70-μm cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ) was used to filter the testicular cell solution, to remove clusters of germ cells with Sertoli cells from the cell preparation. Individual filtered germ cells were then resuspended in minimum essential medium α (MEMα) that was supplemented with 10% FBS.

The viability of germ cell preparations was evaluated using trypan blue staining (Life Technologies, Grand Island, NY), and the population of germ cells was assessed using a hemocytometer (MARIENFELD, Am Wollerspfad, Lauda-Konigshofen, Germany). Spermatozoa were not counted for the number, viability, and purity of germ cells during the procedures. Approximately $5 \times 10^4$ germ cells in MEMα with 10% FBS were loaded onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific Company, Ottawa, Canada) and subjected to ice-cold methanol for individual germ cell fixation. After overnight air-drying, the slides were kept frozen until performing immunocytochemistry.

### 2.4. Cryopreservation and Thawing of Germ Cells

Another study was performed to examine the effect of cryopreservation of germ cells. For cryopreservation, single germ cells were isolated from the testes of prepubertal (n = 5) and postpubertal (n = 5) stallions. To make a basic solution for cryopreservation (cryosolution), MEMα was mixed with both 20% DMSO and 20% FBS. The pelleted germ cells were reconstituted with MEMα at a concentration of $40 \times 10^6$ germ cells/mL and mixed with cryosolution at 1:2 dilutions (50:50, final concentration of $20 \times 10^6$ germ cells/mL) in 1.8 mL cryovials (Corning, Midland, MI). After inverting, the cryovials were placed in a Nalgene freezing container (Nalgene, Rochester, NY) filled with Iso-Propyl Alcohol (DUKSAN, Gyeonggido, Korea) and frozen at −80°C overnight. The following day, the frozen samples were transferred to a liquid nitrogen tank, where they were kept for a freezing period of 1 month.

For thawing the cryopreserved germ cells, the vials were immersed in a 37°C water bath for approximately 2 to 3 minutes until the cell solutions were completely thawed. The germ cell solutions were then transferred to 15 mL tubules, and the doubled volume of HBSS was added dropwise. After centrifugation at 600*g* for 10 minutes, the pellets were resuspended in MEMα with 10% FBS, and the