Evaluation of equine spermatogonial stem cell viability after cryopreservation

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The establishment of proper conditions for germ cells cryopreservation and storage represents an important biotechnological procedure for studies involving germ cells transplantation and is a very useful tool for the preservation of the genetic stock of valuable animals. In this regard, in the present study we have addressed the effects of different cryopreservation protocols on the viability and survival rates of spermatogonial stem cell in horse. Spermatogonial stem cells (SSCs) were enzymatically isolated from testis of 8 adult horses. In order to evaluate the presence of SSCs in the obtained cell suspension after Percoll gradient enrichment, we performed immunolabeling and western blot for GFRA1 receptor, which is considered so far the best marker for undifferentiated spermatogonia. Because most germ cells present in the obtained suspension were $GFRA1^{+}$, we evaluated the feasibility of several SSCs cryopreservation protocols. Three different cryoprotectants media [dimethyl sulfoxide + DMEM + 10%BFS (1); ethylene glycol (2); dimethyl sulfoxide + sucrose (3)], associated with different methods (vitrification, slow-freezing and fast-freezing) were tested. The cell viability was evaluated before and after thawing by trypan blue staining assay using the Newbauer chamber. Annexin V and propidium iodide staining was used to estimate the rate of apoptotic and necrotic cells, after thawing, by flow cytometry (Facscan, BD Pharmingen). The cell metabolic activity (cell viability) and "stemness" potential were also evaluated post-thawing, maintaining these cells in culture for 24 days. The metabolic activity was measured performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, and the stemness potential was investigated through immunohistochemistry using specific SSCs markers (GFRA1 and NANOS2). Based on the rates of viable SSCs found before and after thawing, as well as the number of recovered cells after the cryopreservation, the best results were obtained utilizing the DMSO-based cryoprotectants using fast (Medium 3) and slow freezing method (Media 1 and 3). In addition, when these cells were kept in culture (24 days), the MTT test data have indicated that the cryopreserved cells were as metabolically active as the fresh cells, and also they were expressing typical stem cell proteins (GFRA1 and NANOS2). Thus, the results obtained so far have indicated that equine SSCs could be cryopreserved without impairment of their metabolic activity and "stemness". Further studies are now being performed, including BrDU immunostaining and germ cell transplantation, in order to evaluate *in vivo* the most efficient SSC cryoprotectant media. (CETEA/UFMG/Protocol#56/2011).

Financial support: FAPEMIG and CNPq.

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