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Effect of age on expression of spermatogonial markers in bovine testis and isolated cells

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ABSTRACT

Spermatogonial stem cells (SSC) are the most undifferentiated germ cell present in adult male testes and, it is responsible to maintain the spermatogenesis. Age has a negative effect over stem cell, but the aging effect on SSC is not elucidated for bovine. The present study aim to evaluate the effect of age on the expression of undifferentiated spermatogonial markers in testis and in enriched testicular cells from prepubertal calves and adult bulls. In this matter, testicular parenchyma from calves (3–5 months) (n = 5) and bulls with 3 years of age (n = 5) were minced and, isolated cells were obtained after two enzymatic digestions. Differential platting was performed for two hours onto BSA coated dish. Cell viability was assessed by Trypan Blue solution exclusion method and testicular cells enriched for SSC was evaluated by expression of specific molecular markers by qRT-PCR (*POU5F1, GDNF, CXCR4, UCHL1, ST3GAL, SELP, ICAM1 and ITGA6*) and flow cytometry (GFRA1, CXCR4 and ITGA6). CXCR4 and UCHL1 expression was evaluated in fixated testes by immunohistochemistry. We observed that age just affected the expression of selective genes [SELP (Fold Change = 5.61; p = 0.0023) and UCHL1 (Fold Change = 4.98; p = 0.0127)]. By flow cytometry, age affected only the proportion of ITGA6+ cells (P < 0.001), which was higher in prepubertal calves when compared to adult bulls. In situ, we observed an effect of age on the number of UCHL1+ (p = 0.0006) and CXCR4+ (p = 0.0139) cells per seminiferous tubule. At conclusion, age affects gene expression and the population of cells expressing specific spermatogonial markers in the bovine testis.

1. Introduction

Normally, a bull produces about 5–6 million sperm cells daily during the first ten years of its life (Amann et al., 1974). Spermatogenesis depends on SSC self-renewal and differentiation during the male reproductive life. In non-primates mammalian, just type A single spermatogonia (A_S) are considered true SSC (de Rooij, 2001). SSC have the ability to colonize testes and promote spermatogenesis after transplantation (Brinster and Avarbock, 2004; Brinster and Zimmermann, 2004). SSC have been considered

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as a powerful biotechnological tool for reproductive biology in large animals but the gap between basic research and potential applications need to be overcome (Zheng et al., 2014). Studies on bovine SSC often use prepubertal calves as cell sources, including SSC cryopreservation (Izadyar et al., 2002a; Oatley et al., 2004), in vitro culture (Aponte et al., 2006; Fujihara et al., 2011), molecular marker characterization (Reding et al., 2010), differentiation (Qasemi-Panahi et al., 2011), homologous transplant (Izadyar et al., 2003) and heterologous transplant (Izadyar et al., 2002b; Oatley et al., 2002). However, the reproductive performance and generation of offspring with superior genetic merit can only be proved in proven bulls.

Adult stem cells support tissues and organs homeostasis, but tissue regeneration dramatically decrease with age (Boyle et al., 2007). In most strains of mice, aging is associated with hematopoietic stem cell loss of function and number (Chambers et al., 2007; Geiger and Rudolph, 2009). Also, reduced differentiation of hematopoietic stem cells to lymphoid lineage cell might be strong correlated with senescence (Chambers et al., 2007; Geiger and Rudolph, 2009). In the human male, aging affects sperm DNA damage, chromatin integrity, gene mutations and aneuploidies (Wyrobek et al., 2006). However, few studies were performed to understand the effect of aging on mammalian SSC. In mice, some genes are expressed specifically in SSC from oldest mice such as *SELP* and *ICAM1* (Kokkinaki et al., 2010). However, the molecular mechanisms involved in age influences of SSC functional and properties are still unclear for mouse and other mammalian species.

In domestic animals, SSC characterization and properties are not as elucidated as in rodents and few SSC markers were studied. In bovine, UCHL1 (PGP9.5) was previously reported to be only expressed by type A spermatogonia in adult testis (Fujihara et al., 2011). In domestic animals, UCHL1 is considered an optimal marker for spermatogonia as it does not show affinity for somatic cells and is expressed by pre-meiotic male germ cell (Kon et al., 1999). The number of positive cells per seminiferous tubule for UCHL1, OCT4 or for affinity to *Dolichos Biflorus* agglutinin (DBA; indirect marker for undifferentiated germ cells in bovine) is higher in adult bulls than in prepubertal calves, reveling that bovine undifferentiated germ cells have different characteristics during the development of testis from neonatal to adult life (Fujihara et al., 2011).

In almost all tissues, stem cells generally have adhesive contact to the basement membrane and are surrounded by stromal cells producing a microenvironment called niche (Spradling et al., 2001). Beta-1 integrin (ITGB1) and alpha-6 integrin (ITGA6), help SSC attachment on the basement membrane by binding to laminin (Shinohara et al., 1999). In the spermatogonial niche, Sertoli cells express GDNF and CXCL12. These factors bind respectively to receptor GFRA1 and CXCR4 in stem cell membrane promoting SSC self-renewal and maintenance (Meng et al., 2000; Kokkinaki et al., 2009; Yang et al., 2013). In bovine, the number of As and Apr is higher in GDNF treated in vitro culture (Aponte et al., 2005).

In livestock breeding programs, just adult bulls have proven genetic merit due to offspring assessment. Homologous transplantation of SSC from proven breeding bull donors to low genetic merit bulls could be an interesting tool to improve breeding systems. However, effect of age on the testicular niche and on SSC is not clear for domestic animals. Thus, we aimed to evaluate the effect of age on the expression of undifferentiated spermatogonial markers in testis and in enriched testicular cells from prepubertal calves and adult bulls. We observed that age affected the expression of some markers but not all, indicating that age influenced some particular characteristics of bovine undifferentiated spermatogonia.

2. Material and methods

All animal procedures were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo. All chemicals were supplied by Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise stated.

2.1. Animals and immunohistochemistry

Testes from prepubertal bull calves (PUSP-P, University of Sao Paulo, Pirassununga, Sao Paulo, Brazil) with 5 months of age (n = 5) were surgically removed Testes from adult bulls with 3 years of age (n = 5) were obtained in a commercial slaughterhouse. Testes were weighted and decapsulated prior processing in the laboratory. Testicular fragments (0.3 mm^3) from each sample were fixed in Methacarn for 24 h for immunohistochemistry analysis. After fixation, external edges were removed and all fragments were transferred to absolute ethanol until paraffin embedding. Sections (5 μm thick) were dried over silanized glass slides and stored at room temperature until staining. Expression of UCHL1 and CXCR4 was evaluated by immunohistochemistry in testis from prepubertal calves (n = 5) and adult bulls (n = 5).

Sections were rehydrated (xylol for 20 min, xylol for 20 min, absolute ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 5 min and distillated water for 5 min). Peroxidase blocking was performed with incubation for 30 min at room temperature with 30% (v/v) hydrogen peroxidase in ethanol. Slides were washed with PBS (three times of 3 min) and non-specific reaction was blocking with 5% (w/v) non-fat powdered milk in PBS for 1 h at room temperature. Slides were washed with PBS (three times of 3 min) and incubated overnight with primary antibody 1:500 anti-CXCR4 (Ab7199, Abcam, Cambridge, MA, EUA) and 1:200 anti-PGP9.5- (Ab72911, Abcam) at 4 °C. After incubation, slides were washed and incubated with ADVANCE =HRP Link (ADVANCE™ HRP, DAKO, Carpinteria, CA, EUA) for 30 min at room temperature without light exposition. After wash, slides were incubated with ADVANCE HRP Enzyme (ADVANCE™ HRP, DAKO,) for 30 min. Visualization of specific immunolocalization by DAB were obtained by Liquid DAB+ Substrate Chromogen (DAKO,). Morphology and specific staining was analyzed in ten representative images that were obtained by optical microscopy (Olympus IX81) at 400 x magnification by Image Plus Software (Olympus) for each animal. We obtained the number of positive cells per seminiferous