UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

TRANSPLANTE DE ESPERMATOGÔNIAS TRONCO EM PEIXES TELEÓSTEOS, UTILIZANDO COMO MODELO EXPERIMENTAL A CARPA COMUM (*Cyprinus carpio*)

Angel Andrés Arias Vigoya

Doutorando

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Prof. Dr. Rafael Henrique Nóbrega

Orientador

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AUTOR: ANGEL ANDRÉS ARIAS VIGOYA
ORIENTADOR: RAFAEL HENRIQUE NÓBREGA

Aprovado como parte das exigências para obtenção do Título de Doutor em AQUICULTURA, pela Comissão Examinadora:

Prof. Dr. RAFAEL HENRIQUE NÓBREGA
Departamento de Morfologia / Instituto de Biociências de Botucatu - UNESP

Profa. Dra. ROSICLEIRE VERISSIMO SILVEIRA
Departamento de Biologia e Zootecnia / Faculdade de Engenharia de Ilha Solteira

Prof. Dr. SERGIO RICARDO BATLOUNI
Laboratório de Reprodução de Peixes / Centro de Aquicultura da UNESP, CAUNESP, Jaboticabal-SP

Prof. Dr. LUÍS ANTONIO JUSTULIN JUNIOR
Depto. de Morfologia / IB/Botucatu - Unesp

Profa. Dra. MARICY APPARICIO FERREIRA
UNIFRAN / Universidade de Franca

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RESUMO GERAL

Nos vertebrados, a espermatogênese é um processo de desenvolvimento celular altamente conservado e organizado, no qual uma pequena população de espermatogônias tronco produz continuamente milhões de espermatozóides, os quais são os responsáveis pela propagação do genótipo dos machos. Este processo é sustentado pelas espermatogônias tronco através da sua capacidade de auto-renovação e diferenciação. Vários aspectos relacionados com a regulação da atividade e fisiologia das espermatogônias tronco são ainda desconhecidos nos peixes teleósteos. Portanto, a técnica de transplante de células germinativas torna-se uma poderosa abordagem para melhorar o conhecimento da biologia das espermatogônias tronco e da espermatogênese. Resumidamente, a técnica envolve a transferência de células germinativas isoladas de um doador fértil para os testículos de um receptor estéril, e as células transplantadas são capazes de restaurar a gametogênese do receptor. Neste contexto, realizamos uma caracterização morfológica e estereológica dos diferentes tipos de células encontradas na espermatogênese da carpa comum (*Cyprinus carpio*). Também caracterizamos fenotipicamente as potenciais espermatogônias tronco. Portanto, descrevemos cinco tipos espermatogôniais na carpa comum: espermatogônias indiferenciadas do tipo A (A_{und}* - A_{und}), espermatogônias diferenciadas do tipo A (A_{diff}) e espermatogônias do tipo B (inicial e final). Nesta espécie, o processo espermatogênico durou aproximadamente uma semana. Nossos resultados demonstraram que a população espermatogonial expressa as proteínas c-Kit, Gfrα-1 e POU2. Neste estudo, também foi padronizada a técnica de transplante de espermatogônias na carpa comum. Assim, células germinativas isoladas de kinguios sexualmente maduros foram transplantadas através da papila urogenital de carpas macho quimicamente esterilizadas. As células germinativas derivadas dos doadores foram capazes de colonizar e se desenvolver nos testículos dos receptores. Em geral, nossos resultados reforçam a compreensão da biologia das células germinativas, em especial das potenciais células tronco. Além disso, o transplante, padronizado aqui, é uma abordagem com implicações significativas para a conservação e manejo das espécies valiosas e/ou ameaçadas de extinção.

Palavras chave: Biotecnologia, células germinativas, espermatogênese, reprodução de peixes.

ABSTRACT

In vertebrates, spermatogenesis is a highly conserved and organized developmental process, in which a small population of spermatogonial stem cells (SSC) continuosly produce millions of spermatozoa, which are responsible for spreading the male genotype. Likewise other stem cells, SSC are capable of either self-renew or differentiate. Several aspects related to the regulation of SSC activity and physiology are still unknown in teleost fish. Thus, the germ cell transplantation technique becomes a powerful approach to improve the knowledge of SSC biology and spermatogenesis. Briefly, the technique involves the transfer of germ cells isolated from a fertile donor into the testes of a sterile recipient, and transplanted donor germ cells are able to restore the recipient gametogenesis. Taking advantage of this background, we performed morphological and stereological characterization of the different cell types found in the common carp (*Cyprinus carpio*) spermatogenesis. We also characterized the putative SSC candidates by morphology. Therefore, we described five spermatogonial types in common carp: type A undifferentiated spermatogonia (A_{und}* - A_{und}), type A differentiated spermatogonia (A_{diff}) and type B spermatogonia (early and late). In this species, the spermatogenic process lasted approximately one week. Our findings demonstrated that the spermatogonial population expressed the c-Kit, Gfrα1 and POU2 proteins. Donor germ cells isolated from goldfish were transplanted non-surgically through urogenital papilla into the sexually mature cytoablated common carp recipients. Donor transplanted germ cells were able to colonize and develop in recipients' testes. Overall, our results strengthens the knowledge of germ cell biology, focusing on stem cells. Finally, transplantation, standardized here, is an approach with significant implications for the conservation and management of endangered and valuable fish species.

Keywords: Biotechnology, fish reproduction, germ cells, spermatogenesis.

I. INTRODUÇÃO GERAL

1. Espermatogênese: uma visão geral

A espermatogênese é um processo altamente complexo, no qual a partir de espermatogônias tronco diplóides são produzidas células germinativas masculinas haplóides (Hess & França, 2008; Hogarth & Griswold, 2010; Leal et al., 2009; Nóbrega et al., 2009; Schulz, et al., 2010). O processo pode ser dividido em fases que ocorrem através de ciclos organizados (Hess & França, 2008). Estes ciclos são indispensáveis para a produção continua de espermatozóides, e dependem tanto de fatores intrínsecos (células somáticas e germinativas) quanto extrínsecos (hormônios sexuais, fatores de crescimento), além de ter especificidade de espécie (Hess & França, 2008; Schulz et al., 2010).

A espermatogênese pode ser agrupada em três fases: proliferativa ou espermatogonial, na qual as espermatogônias passam por rápidas e sucessivas divisões mitóticas; meiótica ou espermatocitária, na qual o material genético dos espermatócitos é duplicado, recombinado, e segregado formando-se as espermátides (células haplóides); e de diferenciação ou espermiogênica, na qual as espermátides passam por mudanças morfo-funcionais para originar os espermatozoides completamente funcionais (De Rooij & Russell, 2000; Schulz & Miura, 2002; Nóbrega et al., 2009; Schulz et al., 2010). Durante o desenvolvimento das células germinativas, a partir de divisões incompletas, formam-se grupos celulares ou cistos conectados por pontes citoplasmáticas (De Rooij & Russell, 2000).

A seqüência a seguir indica o desenvolvimento linear das células espermatogênicas em roedores, desde a espermatogônia isolada (Ais) até espermátides: Ais (isolada) → Apr (pareada) → Aal (alinhadas com várias gerações) → A1 → A2 → A3 → A4 → In → B → espermatócitos primários → espermatócitos secundários → espermátides. As espermatogônias A do tipo isolada, pareada e alinhada são funcionalmente consideradas indiferenciadas, enquanto as do tipo A1-4, intermediária (In) e do tipo B são diferenciadas. O esquema na próxima página ilustra o "modelo Ais", desenvolvido em 1971 (Hunckins, 1971), que propõe que as espermatogônias isoladas (Ais) atuam como células tronco, e as espermatogônias conectadas por pontes citoplasmáticas (Apr, Aal, A1) são terminalmente já diferenciadas e comprometidas com a formação de espermatozóides (Figura 1). Este modelo tem servido de base conceitual para compreender a biologia das espermatogônias tronco em diversas espécies de animais. No entanto, estudos recentes têm demonstrado que a diferenciação espermatogonial proposta pelo modelo de Hunckins (1971), não é unidirecional e irreversível como foi sugerido (Barroca et al.,

2009; Nakagawa et al., 2010; Yoshida, 2010), mas sim de caráter multidirecional e reversível dependendo das necessidades fisiológicas.

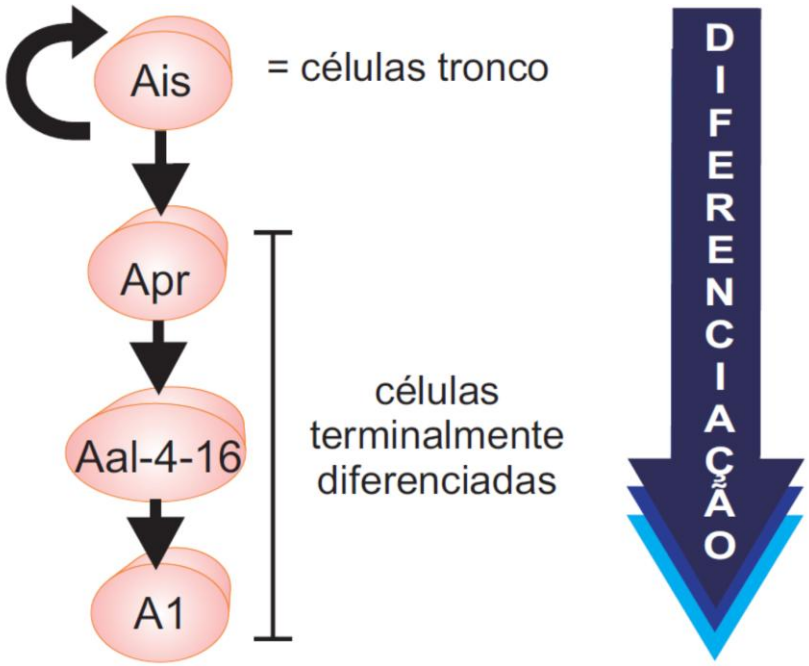


Figura 1. Ilustração esquemática do "modelo Ais". As espermatogônias A isoladas (Ais) atuam como células tronco, e as gerações subseqüentes conectadas por pontes citoplasmáticas; espermatogônias pareadas (Apr), alinhadas (Aal) e A1, são espermatogônias terminalmente já diferenciadas. Neste modelo, o potencial tronco é único e exclusivo das espermatogônias isoladas, e a diferenciação é sempre unidirecional e irreversível (Huckins, 1971).

A espermatogênese é um processo indispensável na manutenção da fertilidade dos machos, já que constantemente são produzidos milhões de espermatozóides. Neste contexto, em termos de reposição celular, a espermatogênese é considerada como um dos processos de renovação celular mais eficientes do corpo (França & Chiarini-Garcia, 2005). O processo espermatogênico é sustentado pelas espermatogônias tronco, em função da sua capacidade de auto-renovação, proliferação e diferenciação (De Rooij & Russell, 2000; De Rooij & 2001, 2006 a,b; Ehmcke et al., 2006a; Hofmann, 2008; Yan, 2006).

A duração do ciclo espermatogênico é determinada pelas espermatogônias tronco, enquanto que as células de Sertoli são responsáveis pela manutenção da dinâmica do processo através da produção de fatores, e por proporcionar estrutura adequada para manter as associações celulares no epitélio germinativo (França, et al. 1993; Hess & França, 2008; Ye, et al. 1993).

Ultimamente as pesquisas têm sugerido que as espermatogônias indiferenciadas, incluindo as espermatogônias isoladas (Ais) ou tronco, encontram-se em determinados locais do epitélio seminífero, denominados nichos (Chiarini-Garcia, et al. 2001, 2003; De Rooij, 2001, 2006a,b;

Yan, 2006; De Rooij, 2009; Ryu, et al. 2006). O nicho é uma região onde as células tronco permanecem em seu estado indiferenciado, e onde é regulada sua atividade celular (Hofmann, 2008). Geralmente os nichos, localizam-se em áreas estratégicas do epitélio seminífero, próximos de vasos sangüíneos e das células de Leydig (Chiarini-Garcia et al., 2001, 2003; Yoshida et al., 2007).

Múltiplos fatores intra e extracelulares estão envolvidos na regulação da atividade do nicho espermatogonial (fatores de crescimento, hormônios, c-Kit, vitamina A), os quais são importantes na diferenciação espermatogonial. Recentes pesquisas também revelam a existência de outros fatores relacionados com a regulação das espermatogônias tronco, como as seguintes proteínas: GDNF (*glial cell line-derived neurotrophic factor*) e seus receptores GFRα1 (*GDNF family receptor alpha-1*) e c-RET (*RET proto-oncogene*), Oct-4 (*octamer-binding transcription factor 4*), NGN-3 (*Neurogenin-3*), Notch-1 (*Translocation-associated Notch protein TAN-1*), Sox-3 (*Transcription factor SOX-3*), RBM (*RNA binding motif proteins*), Ep-CAM (*Epithelial cell adhesion molecule*), STRA-8 (*Stimulated by retinoic acid 8 gene*), EE-2 (*protein binding of ethinylestradiol*); fatores de transcrição ERM (ETV5) e PLZF (*promyelocitic leukemia zinc finger*), FGF-2 (*fibroblast growth factor 2*) e seu receptor FGFR2, o TAF4b (*TATA-Box Binding Protein Associated Factor 4b*), Activina A, BMP4 (*Bone morphogenetic protein 4*) e SOHLH1 (*Spermatogenesis and oogenesis specific basic helix-loop-helix 1*) dentre outros (Aponte et al., 2005; Chen et al., 2005; Hofmann et al., 2005 McLean 2005; De Rooij, 2006b; Yan, 2006; Cooke et al., 2006; Hess et al., 2006; Brinster, 2007; He et al., 2007, 2008; Hofmann, 2008; Lucas et al., 2009).

Dentre os mencionados acima, o fator GDNF e seu complexo de receptores GFRα1 e c-RET, são importantes na manutenção das espermatogônias tronco em mamíferos (Meng et al., 2000; Yomogida et al., 2003; Naughton et al., 2006). Este fator secretado pelas células de Sertoli atua por meio do complexo de receptores GFRα1/c-RET localizado na superfície das espermatogônias tronco (De Rooij, 2006b; Hess et al., 2006; Cooke et al., 2006). Os estudos demonstram que o GDNF é fundamental para manter a viabilidade das espermatogônias tronco, além de regular a expressão de um complexo de genes e fatores de transcrição que estão envolvidos na sobrevivência destas células (Chen et al., 2005; Hess et al., 2006; Oatley et al., 2006). Adicionalmente, as recentes pesquisas apontam que os genes Oct-4, Gfrα1, CD-24, Nanos2,3, Egr-3, Plzf, Sox-3, Taf4b, Bcl6b, Ret, Sohlh-2, Cdh-1, Gpr-125, Utf-1 e Lin-28, expressam-se unicamente pelas espermatogônias indiferenciadas, e são identificados como marcadores das células tronco germinativas masculinas (Phillips et al., 2010).

2. Espermatogênese em teleósteos

A espermatogênese nos peixes apresenta um arranjo do tipo cístico. O processo ocorre em estruturas denominadas espermatocistos, os quais são formados quando as espermatogônias indiferenciadas são envolvidas pelos processos citoplasmáticos das células de Sertoli (Grier, 1993; Pudney, 1993, 1995; Schulz et al., 2010). Após sucessivas divisões mitóticas das espermatogônias indiferenciadas, formam-se clones de espermatogônias diferenciadas que são envolvidos pelas células de Sertoli (Vilela et al., 2003). Deste modo as células de Sertoli unicamente estão em contato com um tipo de célula germinativa (Figura 2) (Nóbrega et al., 2009; Schulz et al., 2010).

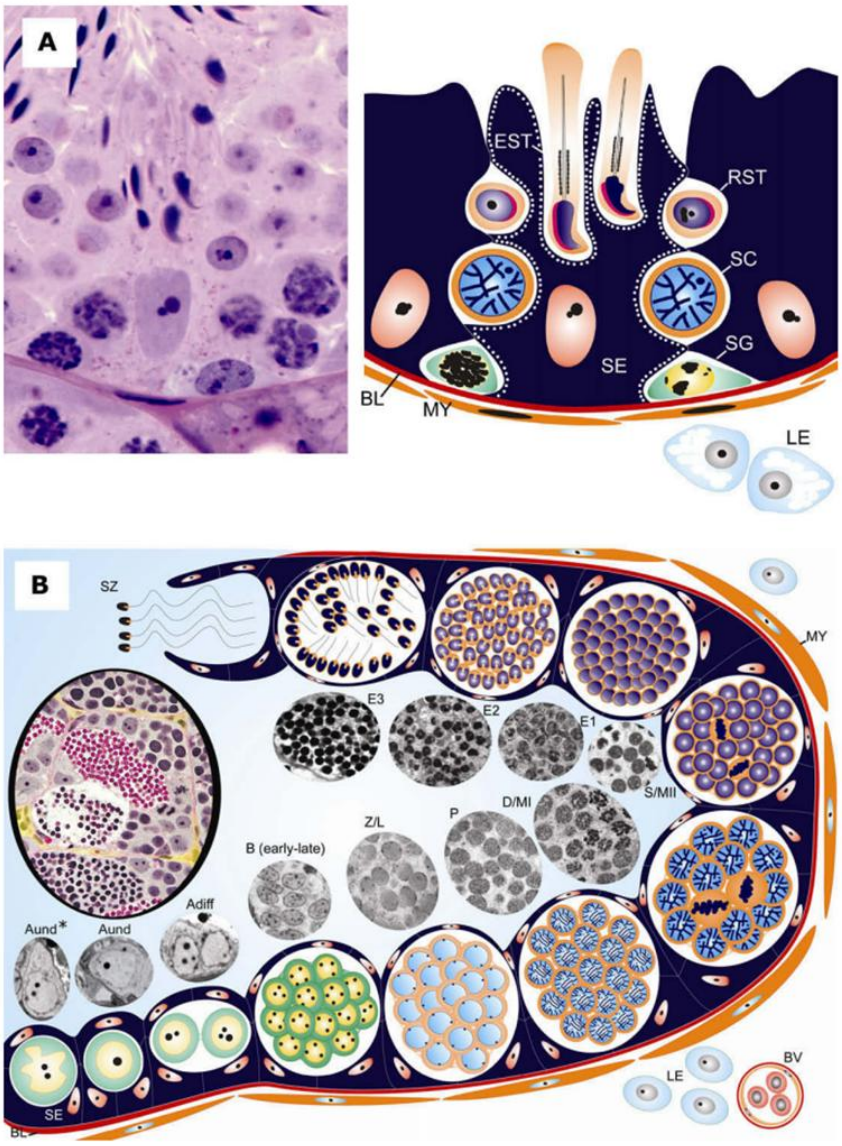


Figura 2. Comparação entre a espermatogênese não cística de amniotas (répteis, aves, mamíferos) (A) e anamniotas (peixes, anfíbios) (B). A figura ilustra as diferenças entre a relação célula de Sertoli/célula germinativa na espermatogênese não cística e cística. Em A, a célula de Sertoli suporta ao mesmo tempo diferentes "clones" de células germinativas em diferentes fases de desenvolvimento. Enquanto que em B, a célula de Sertoli suporta apenas

um "clone" em uma mesma fase de desenvolvimento por vez. Células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig (LE), espematogônia (SG); espermatócito (SC); espermátide arredondada (RST); espermátide alongada (EST); espermatogônia do tipo A indiferenciada* (Aund*) (célula tronco?); espermatogônia do tipo A indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); espermatogônia do tipo B (B early-late); espermatócitos primários em leptóteno/zigóteno (L/Z), paquíteno (P), diplóteno/metáfase I (D/MI); espermatócitos secundários/metáfase II (S/MII); espermátides iniciais (E1); intermediárias (E2); finais (E3); espermatozóides (SZ); e vasos sangüíneo (BV). Retirado de Schulz & Nóbrega, 2011.

Em teleósteos, durante a espermatogênese, os grupos de células germinativas permanecem conectados através das pontes citoplasmáticas (Figura 2) e se desenvolvem sincronicamente até a formação dos espermatozóides, do seguinte modo: espermatogônias indiferenciadas → espermatogônia diferenciadas → espermatócitos primários (pré-leptóteno, leptóteno/zigóteno, paquíteno, diplóteno) → espermatócitos secundários → espermátides (iniciais, intermediárias, finais) → espermatozóides. Finalmente os cistos se abrem e os espermatozóides são liberados no lúmen dos túbulos seminíferos, evento conhecido como espermiação (Schulz & Miura, 2002; Schulz, 2003; Batlouni et al., 2009; Grier & Uribe-Aranzábal, 2009; Leal et al., 2009; Shulz et al., 2010; Uribe et al., 2015).

Nos peixes, o processo espermatogênico é de menor duração quando comparado com os mamíferos, e é influenciado pela temperatura (Koulish et al., 2002; Vilela et al., 2003), sendo que o processo é acelerado a temperaturas elevadas e diminuído a temperaturas baixas (Vilela et al., 2003; Nóbrega et al., 2010). Além disso, a espermatogênese nos peixes é mais eficiente que a espermatogênese nos mamíferos, devido em grande parte à maior proporção entre o número de células germinativas por célula de Sertoli, que leva a uma maior produção de fatores de crescimento derivados das células de Sertoli, resultando em altas taxas de proliferação e diminuição da apoptose das células germinativas (Matta et al., 2002; Vilela et al., 2003; Leal et al., 2009; Schulz et al., 2005).

As espermatogônias indiferenciadas são consideradas como as celulas tronco da linhagem germinativa masculina nos peixes, e são classificadas como as células de maior tamanho no epitélio germinativo (Schulz & Miura, 2002). A continuidade do processo espematogênico é dependente da atividade e sobrevivência das espermatogônias tronco, estas células encontram-se em locais específicos no epitélio germinativo denominados "nichos". O nicho espermatogonial é o microambiente molecular e celular responsável por regular as propriedades tronco das espermatogônias (auto renovação, pluripotência, capacidade de diferenciação) e manter estas células em seu estado indiferenciado (Nóbrega et al., 2010). Os nichos são

microambientes dinâmicos e com grande capacidade adaptativa, já que estão sujeitos à influência de fatores internos quanto externos, os que provavelmente determinam os ciclos reprodutivos das espécies de regiões temperadas e tropicais (Schulz et al., 2010; Nóbrega et al., 2010).

3. Transplante de espermatogônias

O transplante de espermatogônias tronco é uma técnica que consiste na transferência de células tronco do testículo de um doador fértil para o testículo de um receptor infértil. As células do doador tem a capacidade para colonizar os testículos do receptor e formar espermatozóides com as características genotipicas do doador. Esta técnica foi desenvolvida em roedores por Brinster e colaboradores (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994) conforme ilustra a Figura 3. Esta metodologia é uma ferramenta importante para verificar a atividade das espermatogônias tronco, além da dar subsídios para o conhecimento da biologia das espermatogônias tronco, e o funcionamento dos nichos espermatogoniais (Orwig & Schlatt, 2005; McLean, 2005). Esta linha de pesquisa também tem possibilitado grandes avanços na compreensão da espermatogênese, e no estudo da relação entre células de Sertoli e germinativas. Ao mesmo tempo tem um enorme potencial para a preservação de espécies de interesse econômico e ameaçadas de extinção, medicina reprodutiva e produção de animais transgênicos (McLean, 2005; Orwig & Schlatt, 2005; Hill & Dobrinski, 2006; Dobrinski, 2006; Kanatsu-Shinohara et al., 2008).

O transplante interespecífico ou xenogênico também é uma abordagem promissora desta metodologia. O transplante xenogênico consiste na utilização de doadores e receptores de diferentes espécies (Clouthier et al., 1996; Ogawa et al., 1999). O transplante xenogênico considera-se viável quando existe uma proximidade filogenética entre os indivíduos. Estudos sugerem que algumas fases da espermatogênese possuem uma regulação espécie-especifica, assim, o transplante é inviável entre espécies filogeneticamente distantes (McLean, 2005; Hill, Dobrinski, 2006; Dobrinski, 2006).

A eficiência do transplante está relacionada à quantidade de células transplantadas e também do ambiente testicular do receptor. Neste último caso, deve-se garantir a existência de nichos disponíveis para a colonização das células do doador. Neste contexto, os receptores adequados são aqueles que não apresentam espermatogênese, e seu epitélio seminífero seja formado unicamente por células de Sertoli (Brinster et al., 2003). Os métodos mais comuns para depletar a espermatogênese e disponibilizar nichos para a colonização das células dos doadores são: 1) uso de quimioterápicos, como o agente citostástico busulfan; 2) irradiação dos testículos, e 3)

aumento da temperatura testicular. O tratamento com busulfan é o procedimento mais utilizado (Brinster et al., 2003; Honaramooz et al., 2005).

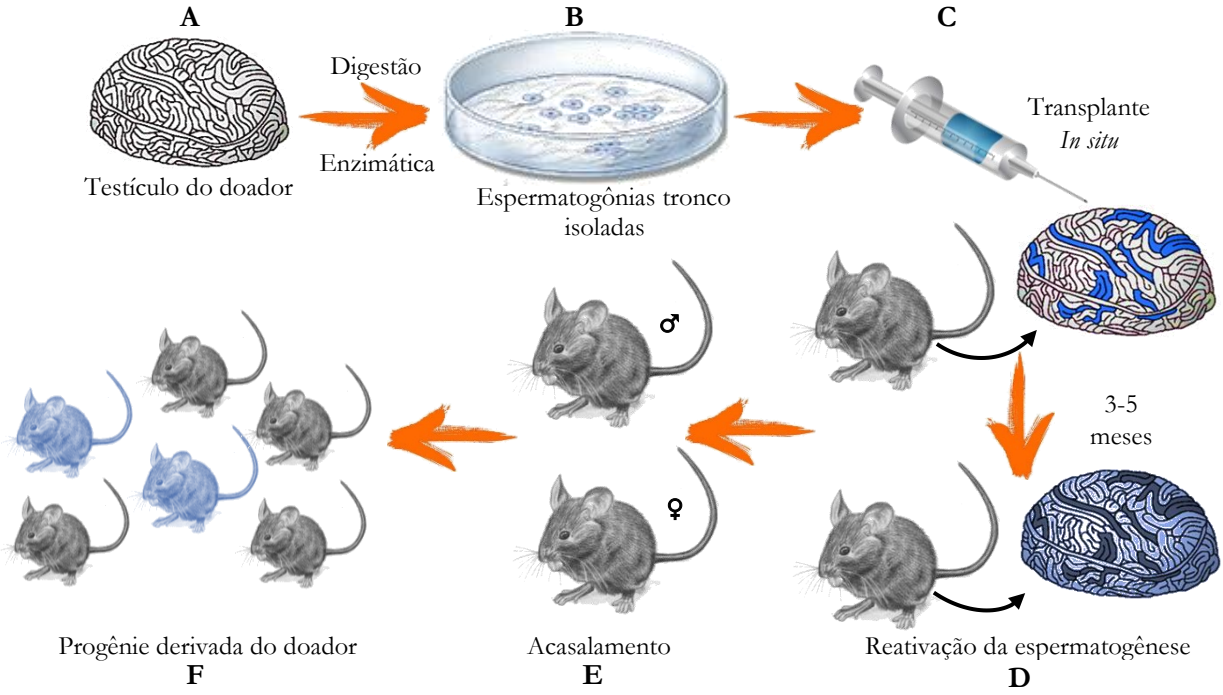


Figura 3. Principais fases do transplante de espermatogônias em camundongos. **A:** testículo do animal transgênico que expressa o gene da β-galactosidase de *Escherichia coli* (lacZ); **B:** suspensão de células germinativas obtidas através de digestão enzimática; **C:** transplante das células germinativas pelos túbulos seminíferos e dúctulos eferentes; **D:** testículo transplantado com células germinativas do doador; **E:** cruzamento do animal transplantado com fêmea normal; **F:** progênie do receptor portando características genéticas do camundongo doador transgênico. Adaptado de Kubota & Brinster (2006).

Outro importante aspecto sobre o transplante é a preparação das células do doador. O número de espermatogônias tronco no testículo é muito reduzido, estimando-se em 0,03% do total de células germinativas, aproximadamente 35.000 células; ou 1,3% de todas as espermatogônias no camundongo (Tegelenbosch & De Rooij, 1993). Como o número de espermatogônias é baixo, é indispensável fazer uma seleção para aumentar a quantidade destas células antes de serem transplantadas. A seleção incrementa o número de espermatogônias e diminui a quantidade de células somaticas e germinativas "contaminantes" (McLean, 2005; Hill & Dobrinski, 2006; Dobrinski, 2006). A seleção pode ser feita através de: 1) seleção por fluorescência (FACS) (Nóbrega et al., 2010) ou campo magnético (MACS) com base nos marcadores (positivos e negativos) das espermatogônias tronco, como scatterlow, α6-integrina+, β1-integrina+, CD24+, Thy-1+, αv-integrina-, Kit-, MHC-I-, CD9+, Rholow, Ep-CAM+, EE2+ (McLean, 2005); 2) velocidade de sedimentação seguido de plaqueamento diferencial com componentes da matriz extracelular como laminina (Luo et al., 2006); e 3) gradiente de percoll (Lacerda et al.,

2006, 2010). O maior número de espermatogônias tronco gerado pelo enriquecimento *in vitro* tem propiciado novas abordagens ao transplante de espermatogônias, como por exemplo, o cultivo das espermatogônias tronco com a finalidade de avaliar os fatores que atuam na sua auto-renovação/diferenciação; a criopreservação das espermatogônias e sua posterior utilização no transplante; a possibilidade de preservação do genótipo de espécies ameaçadas de extinção ou de interesse comercial; a manipulação gênica das espermatogônias tronco, com perspectivas de produção de transgênicos ou melhoramento genético, dentre outras aplicações (McLean, 2005; Ehmcke et al., 2006b; Hill & Dobrinski, 2006; Dobrinski, 2006).

4. Transplante de espermatogônias em teleósteos

Embora o transplante de espermatogônias seja uma técnica desenvolvida e bem estabelecida em mamíferos, apenas recentemente a metodologia tem sido aplicada em peixes teleósteos. O transplante de células germinativas e/ou espermatogônias tronco em peixes teleósteos apresenta três abordagens (Lacerda et al., 2013a):

A. transplante de células germinativas primordias (CGPs) em embriões (Figura 4A). Esta abordagem consiste no transplante intra-específico de CGPs em embriões no estágio de blástula. Através de micromanipulação as CGPs derivadas dos doadores são coletadas e microinjetadas nos embriões receptores no estágio de blástula, diretamente no blastodisco, onde estas células se dividem antes de se estabelecer na crista gonadal (local das futuras gônadas). Assim, os peixes quiméricos produzidos são capazes de produzir gametas e progênie derivada dos doadores (Ciruna et al., 2002; Giraldez et al., 2005; Saito et al., 2008).

B. transplante de células germinativas em larvas (Figura 4B). Neste método, células germinativas isoladas do doador, são transplantadas na cavidade celomática de larvas recém eclodidas mediante um microinjetor, durante o período no qual as CGPs endógenas dos receptores ainda estão em migração. As células transplantadas são capazes de migrar e colonizar as cristas genitais dos receptores. As larvas recém eclodidas podem ser utilizadas como receptores, devido ao fato de que não possuem um sistema inmune funcional. A carência de um sistema imunológico ativo evita a rejeção das células transplantadas (Lacerda et al., 2013a). Os estudos realizados com larvas demostraram que as células germinativas derivadas dos doadores e transplantadas nas larvas receptoras tem o potencial para se diferenciar em gametas com características genéticas do doador (Okutsu et al., 2006a,b, Yoshizaki et al., 2011a,b). Além disso, a partir do cruzamento de indivíduos transplantados é possível gerar progênie com o genótipo do doador, demonstrando que o transplante xenogênico de espermatogônias pode ser utilizado para

produzir indivíduos de uma espécie utilizando outra espécie correlata (Okutsu et al., 2006a,b, 2007; Yoshizaki et al., 2011a,b).

C. transplante de células germinativas em peixes adultos (Figura 4C). Esta metodologia é caracterizada pela utilização de receptores adultos e sexualmente competentes, os quais tem sua espermatogênese suprimida com auxílio de fármacos citotóxicos e altas temperaturas. As células germinativas isoladas dos doadores são diretamente transplantadas nos testículos dos receptores, através da papila urogenital (mediante um procedimento não cirúrgico), onde posteriormente restabeleceram a gametogênese (Lacerda et al., 2006, 2010).

Independentemente da metodologia utilizada no transplante de celulas germinativas, as celulas derivadas dos doadores, tem a capacidade de migrar, colonizar, diferenciar e produzir gametas viáveis nas gônadas dos receptores, e inclusive mediante cruzamentos programados podem gerar progênie com as caracteristicas genotípicas dos doadores (Lacerda et al., 2013a).

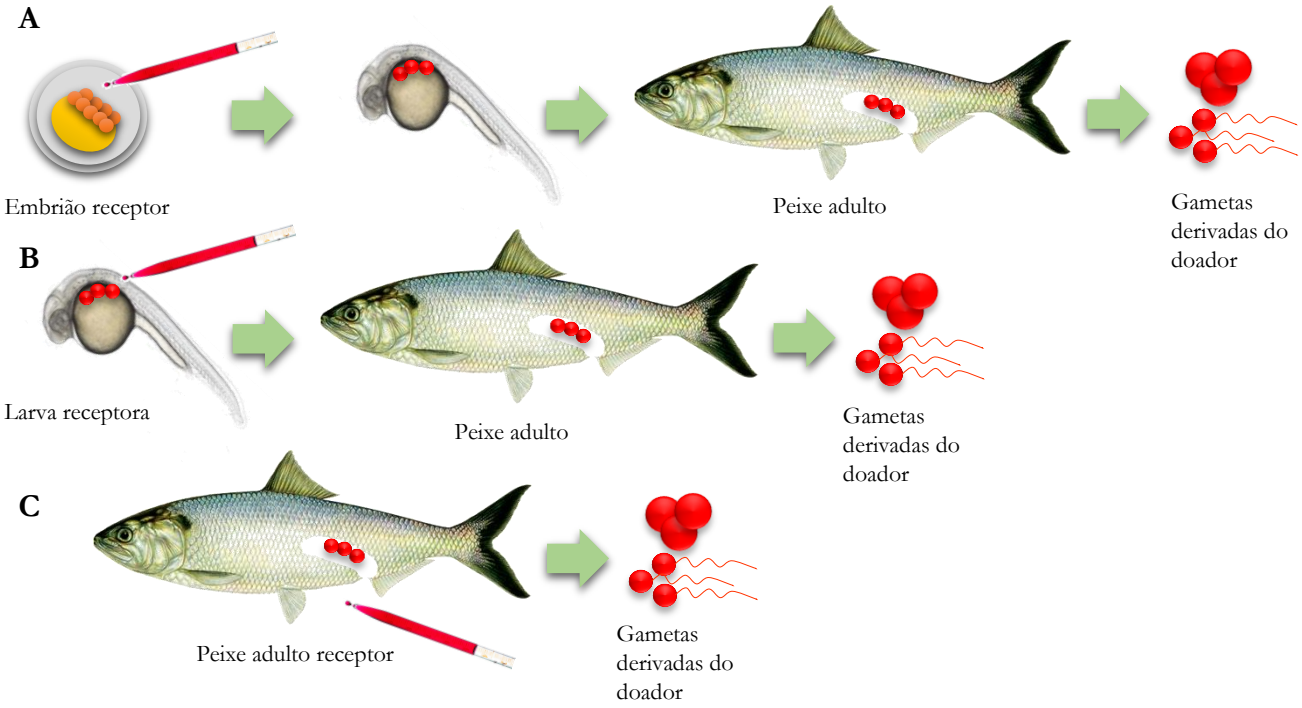


Figura 4. Diferentes técnicas de transplante de células germinativas em peixes que utilizam receptores em diferentes estágios do desenvolvimento. **A:** Transplante de células germinativas primordiais (CGPs) em embriões. As CGPs dos doadores são microinjetadas em embriões no estágio de blástula, no blastodisco, no local onde futuramente serão formadas as gônadas. **B:** Transplante de celulas germinativas em larvas. As células dos doadores são microinjetadas na cavidade celomática das larvas receptoras e são capazes de migrar e colonizar as cristas genitais dos receptores. **C:** Transplante de celulas germinativas em peixes adultos. Neste método as celulas germinativas são transplantadas nos testículos dos receptores e restauram a gametogênese. Adaptado de Lacerda et al. (2013a).

Desta forma, o transplante de espermatogônias pode ser usado para preservar espécies em risco de extinção ou até mesmo ser aplicado em espécies que possuem problemas ou limitações reprodutivas. Neste último caso, podemos citar o atum do Pacífico (*Thunnus orientalis*), espécie de grande valor econômico que atinge a maturidade sexual em torno de 3-5 anos de vida. A produção de gametas do atum é cara em função do longo período de maturação dos animais. No entanto, se as espermatogônias do atum fossem transplantadas em espécies correlacionadas, como o mackerel (*Scomber japonicus*) que atinge a maturidade sexual em 1 ano, gametas do atum poderiam ser facilmente produzidos em um curto período de tempo (Yoshizaki et al., 2011a).

Portanto, as técnicas de transplante de espermatogônias criam um novo cenário no campo da biotecnologia para preservar espécies ameaçadas em extinção, conservar o genótipo de espécies de interesse zootécnico, resolver problemas reprodutivos e até mesmo gerar transgênicos se as spermatogônias transplantadas tiverem seu material genético alterado (Yoshizaki et al. 2011a,b).

5. Transplante de células germinativas em peixes adultos

A utilização de indivíduos maduros para transplante tem sido limitado, já que as pesquisas focam-se principalmente no uso de peixes em estágios iniciais do desenvolvimento (Yoshizaki et al., 2011a; Lacerda et al., 2013a). Portanto, o uso de animais sexualmente maduros é uma alternativa viável para o transplante de células germinativas, em comparação com o emprego de embriões ou larvas que exige uma metodologia sofisticada e um prazo maior para a produção de gametas derivadas do doador (Majhi et al., 2009; Lacerda et al., 2013a).

Os experimentos pioneiros foram desenvolvidos em truta arco-íris (*Oncorhynchus mykiss*). Os resultados obtidos foram promissórios, os indivíduos transplantados foram capazes de produzir esperma derivado do doador. Porém, células isoladas ainda não foram utilizadas no transplante. (Nagler et al., 2001; Bouma et al., 2005). Um grande avanço no aperfeiçoamento da técnica foi alcançado por Lacerda e colaboradores, os autores desenvolveram um eficiente método de transplante de espermatogônias tronco, utilizando como modelo experimental a tilápia do Nilo, *Oreochromis niloticus* (Lacerda et al., 2006, 2010, 2013b). Assim, espermatogônias isoladas dos doadores foram transplantadas através da papila urogenital dos receptores. As células transplantadas colonizaram as gônadas e regeneraram a espermatogênese dos receptores, formando espermatozoides completamente viáveis. Até mesmo espermatogônias crio-preservadas tem o potencial para restaurar a espermatogênese e produzir espermatozóides nos receptores após o tratamento (Lacerda et al., 2010) (Figura 5).

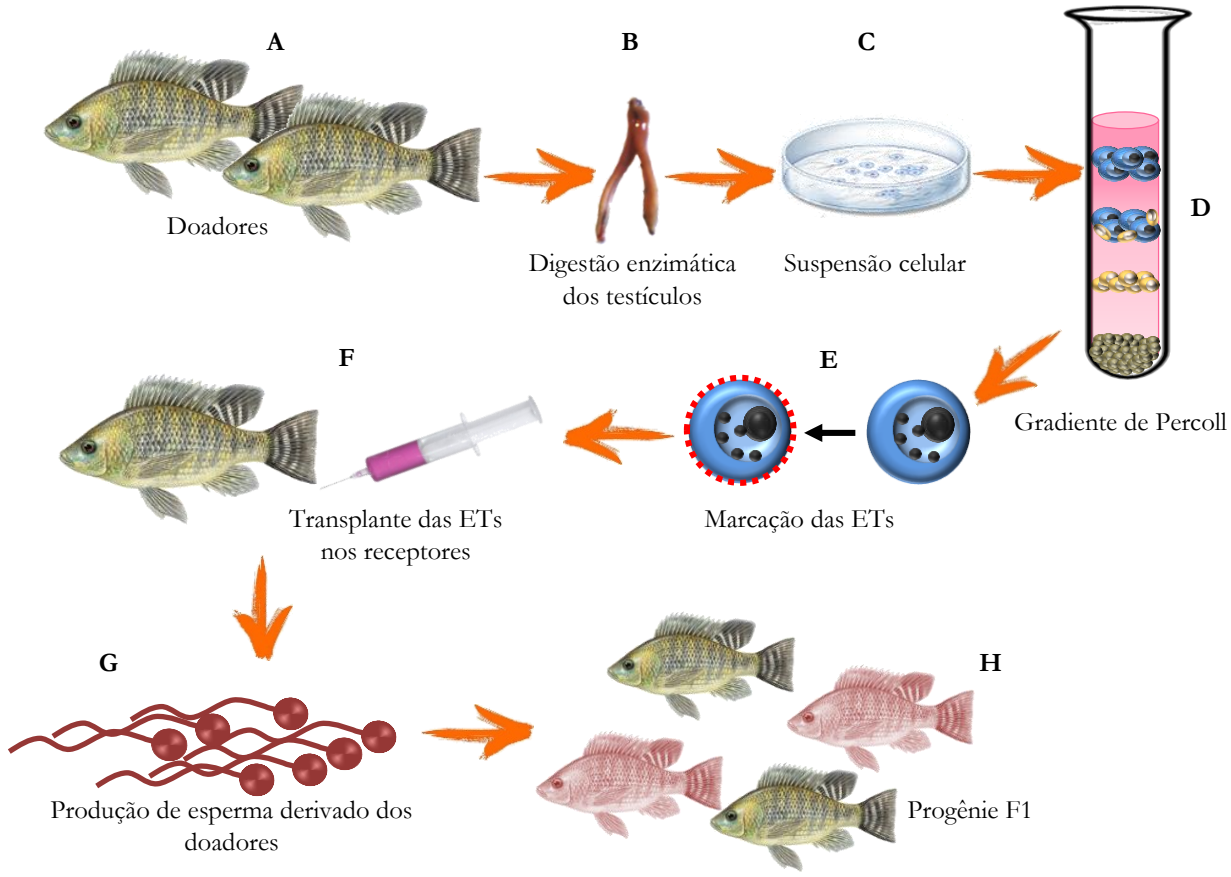


Figura 5. Principais etapas do transplante de espermatogônias tronco (ETs) em tilápia-nilótica (*Oreochromis niloticus*), adaptado de Lacerda e colaboradores (2010). **A:** remoção dos testículos dos doadores; **B**: digestão enzimática; **C**: suspensão celular; **D**: centrifugação em gradiente de percoll, para obter as ETs isoladas; **E**: marcação das ETs com PKH26 (fluorescente vermelho). **F**: transplante via papila urogenital. Os receptores tiveram sua espermatogênese suprimida com busulfan associada a temperatura de 35ºC; **G**: algumas semanas após o transplante, foram observados cistos espermatogênicos marcados e produção de espermatozoides marcados (9 semanas pós-transplante); **H**: progênie derivada dos doadores.

Nóbrega e colaboradores (2010), avaliaram o transplante de espermatogônias utilizando como modelo o zebrafish. Nestes estudos, as espermatogônias de zebrafish transgênicos (vasa::egfp), foram isoladas mediante FACS (Fluorescent Activated Cell Sorting), e transplantadas nos testículos ou ovários dos receptores que tiveram sua gametogênese suprimida com auxílio do busulfan e elevadas temperaturas. Interessantemente, as células transplantadas colonizaram as gônadas dos receptores e formaram oócitos dependendo do microambiente onde foram implantadas.

O sucesso obtido no transplante deve-se a preparação dos receptores mediante a inibição da sua espermatogênese, em resposta a associação da alta temperatura da agua e a utilização do agente citotóxico busulfan (Lacerda et al., 2006, 2010; Majhi et al., 2009, 2014; Nóbrega et al., 2010; Silva et al., 2016). O busulfan tem sido usado para suprimir a espermatogênese em várias

espécies dentre elas roedores (Ogawa et al., 1999; Zhang et al., 2003), primatas (Hermann et al., 2007, 2012), suínos (Honaramooz et al., 2005; Mikkola et al., 2006) e ruminantes (Honaramooz et al., 2005).

Embora o tratamento seja eficaz, o mesmo pode gerar sérios efeitos colaterais, toxicidade sistêmica e inclusive pode ser letal devido à destruição das células da medula óssea. Os efeitos associados ao tratamento podem limitar a eficiência do transplante e ameaçar o homeostase dos animais receptores, o que é realmente crítico quando os animais são espécies em perigo ou destinados para o consumo humano (Ogawa et al., 1999; Brinster et al., 2003; Honaramooz et al., 2005; Dobrinski, 2006; Hill & Dobrinski 2006; Ma et al., 2011).

O transplante xenogênico de espermatogônias também foi desenvolvido com sucesso em peixes sexualmente competentes. Nos estudos feitos por Majhi et al. (2009, 2014), espermatogônias isoladas a partir de juvenis de Pejerrey (*Odontesthes bonariensis*), foram transplantadas diretamente nos testículos de exemplares adultos de Pejerrey patagônico (*Odontesthes hatcheri*), os quais tinham sua espermatogênese depletada. As células transplantadas colonizaram as gônadas dos receptores e retomaram a espermatogênese seis meses após o transplante. Além disso foi gerada progênie derivada dos doadores usando peixes adultos como receptores. Mais recentemente, Silva e colaboradores (2016) conseguiram realizar com sucesso o transplante de espermatogônias entre especies filogeneticamente distantes. Mediante o transplante interespecífico, espermatogônias de Jundiás (*Rhamdia quelen*) sexualmente maduros, foram transplantadas nos testículos de tilápias do Nilo adultas. Nos animais que receberam o transplante, a espermatogênese foi reativada e foram produzidos espermatozoides a partir das células de Jundiá.

É importante ressaltar o enorme potencial e plasticidade exibido pelas células germinativas. Assim, nos estudos desenvolvidos por Nóbrega et al. (2010) e Majhi et al. (2014), as espermatogônias e/ou oogônias transplantadas em machos ou fêmeas, mostraram um elevado nível de plasticidade sexual e demonstraram que esta plasticidade é mantida mesmo quando as células são transplantadas num microambiente adulto diferenciado. Assim, dependendo do nicho no qual foram transplantadas, as células germinativas podem se diferenciar tanto em gametas masculinos quanto em gametas femininos.

Num recente estudo, Lacerda et al. (2013b) utilizando tilápia do Nilo, desenvolveram um sistema funcional de cultura de espermatogônias indiferenciadas, com a capacidade para manter sua sobrevivência, a atividade mitótica e a auto-renovação. Interessantemente, o transplante de espermatogônias cultivadas in vitro é viável. As espermatogônias isoladas foram mantidas

durante diferentes períodos de tempo, e passados 30 dias de cultura foram transplantadas em peixes sexualmente maduros que tiveram sua espermatogênese suprimida previamente. As células transplantadas mantiveram sua capacidade para colonizar os testículos dos receptores. Analisando as gônadas dos receptores observou-se que as células transplantadas formaram colônias nos túbulos seminíferos, e após três ou quatro semanas após o transplante o número de células/colônias aumentou. Isto prova a capacidade de auto-renovação e o potencial tronco das espermatogônias cultivadas.

Recentemente a técnica de transplante de espermatogônias tem adquirido uma enorme importância para se estudar os aspetos básicos da biologia das células tronco (Brinster et al., 2003). Igualmente, são notáveis os avanços na compreensão do desenvolvimento da linhagem germinativa no testículo, a dinâmica do nicho espermatogonial e suas interações com as células somáticas, e a manipulação *in vitro* das espermatogônias (Brinster et al., 2003; Orwig & Schlatt, 2005; McLean, 2005). Por outro lado, a técnica de transplante além de ampliar o conhecimento da biologia e fisiologia das espermatogônias, contribuiu importantemente no avanço da ciência animal e da zootecnia (Lacerda, 2013a). Adicionalmente, oferece múltiplas aplicações na biotecnologia, além do grande potencial na reprodução e produção animal e produção de animais transgênicos (Brinster et al., 2003; McLean 2005; Orwig & Schlatt 2005; Hill & Dobrinski 2006; Dobrinski 2006, 2008; Kanatsu-Shinohara et al., 2008; Phillips et al., 2010; Honaramooz & Yang, 2011; Zheng et al., 2014).

A técnica é altamente útil no estabelecimento de bancos de germoplasma, na preservação e propagação de linhagens portadoras de fenótipos desejados e também dos recursos genéticos de espécies valiosas ou ameaçadas (Majhi et al., 2009; Yoshizaki et al., 2011a,b; Lacerda et al., 2013a). A preservação dos recursos genéticos é apenas uma potencial aplicação do transplante de espermatogônias. A técnica poderia também oferecer novos enfoques, por exemplo, estabelecer um novo sistema de genes knockout para as espécies de peixes desejadas (Yoshizaki et al., 2011a,b; Lacerda et al., 2013b).

Embora a técnica apresente um grande potencial de aplicação pratica e rapidez na produção de gametas e progênie derivada dos doadores, um ponto crítico é sua baixa eficiência na transmissão das características da linha germinativa dos doadores (Majhi et al., 2009; Lacerda et al., 2010; Honaramooz & Yang 2011). Neste sentido, a eficiência do transplante pode ser afetada pelas mudanças causadas pelo tratamento com farmacos citotóxicos (Nóbrega et al., 2010). Além disso, algumas espermatogônias residuais do receptor persistem após o tratamento, e concorrem com as espermatogônias transplantadas pelos nichos disponíveis nos túbulos

seminíferos (Shinohara et al., 2002; Brinster et al., 2003). Assim, o receptor "ideal" seria um indivíduo com a espermatogênese suprimida e ausência de células germinativas, mas, com células somáticas intactas. Uma boa alternativa poderia ser o uso de receptores triplóides (Okutsu et al., 2007). Não obstante, o longo tempo necessário para a produção de animais triplóides, sua fisiologia reprodutiva e as técnicas envolvidas, poderiam limitar sua utilização como receptores do transplante de espermatogônias (Majhi et al., 2009, 2009b).

Neste contexto, a presente tese, apresentada em capítulos, abordará os seguintes assuntos:

I. No primeiro capítulo será apresentada a descrição morfológica e estereológica das células germinativas, e a caracterização dos diferentes cistos espermatogênicos na carpa comum. Além disso, a duração do processo espermatogênico será estimado através da metodologia de "label-retaining cell", usando 5´bromo-2´-deoxiuridina (BrdU).

II. No segundo capítulo, será abordada a caracterização fenotípica das espermatogônias tronco da carpa comum mediante técnicas de microscopia eletrônica de transmissão, imunohistoquímica e western blot. Simultaneamente, a depleção da espermatogênese da carpa comum, mediante um protocolo que combina alta temperatura da agua e o uso do agente quimioterápico busulfan será padronizada na carpa comum. Ao mesmo tempo, será apresentada a adaptação do protocolo de purificação, isolamento e enriquecimento das espermatogônias tronco da carpa comum utilizando o gradiente descontinuo de densidade de percoll.

III. Finalmente, no terceiro capítulo, será testada a viabilidade do transplante xenogênico de células germinativas entre duas espécies diferentes, utilizando o Goldfish (*Carassius auratus*) como doador das células germinativas e a carpa comum (*Cyprinus carpio*) como receptor do transplante.

Os capítulos estão organizados na forma de manuscritos, porém, os mesmos não estão formatados de acordo com as normas específicas de publicação e o periódico será escolhido posteriormente para submissão. Não obstante, para fins de melhor compreensão e facilitação da leitura, os capítulos não obedecem à norma de manter as figuras e tabelas no final do manuscrito e são então apresentados ao longo do texto.

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MANUSCRITO I

SPERMATOGENESIS IN COMMON CARP (*Cyprinus carpio*).

Angel Andrés Arias Vigoya[1,2]**, Marcos Antonio de Oliveira[1,2], Emanuel Ricardo Monteiro Martinez[2], Arno Juliano Butzge[2], Rafael Henrique Nóbrega[2]**.

[1] Aquaculture Center of São Paulo State University – CAUNESP - Jaboticabal, São Paulo – Brazil

[2] Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu – IBB/UNESP, São Paulo – Brazil.

**E-mail address: biorhn@yahoo.com.br aaariasv1@gmail.com

ABSTRACT

Common carp (*Cyprinus carpio*, family Cyprinidae, order Cypriniformes) is an important teleost fish, originally from Eurasia. This species is commonly used for world aquaculture and as experimental model in different research fields. Despite their importance, there remains a paucity of evidence regarding to testis structure, cell composition and spermatogenesis. In addition, nowadays no studies have been found addressing morphological and stereological approaches in this species. In the present work was performed a morphometric study and characterization of the different germ cell types, and was determined the spermatogenesis duration (meiotic and spermiogenic stages). We found five spermatogonial types (type A undifferentiated spermatogonia, A_{und}* - A_{und}; type A differentiated spermatogonia, A_{diff}; type B early and type B late spermatogonia), spermatocytes in different meiotic stages and three spermatid types. The number of germ cells and Sertoli cells per cyst increased markedly from A_{und}* to initial spermatids. Spermatogenesis duration was determined by evaluation of the proliferative activity of the BrdU-possitive germ cells during the differentiation process. Based on the BrdU retention capacity of the cells was possible to estimate that the spermatogenesis lasted approximately one week. Our data provide a novel information about common carp reproductive biology and the mechanisms involved in spermatogenesis. Thus, this work contributes to a better understanding of spermatogenic process in teleost fish and can be used in comparative studies among different groups of vertebrates.

Key words: BrdU, germ cells, morphometry, reproductive biology, teleost fish.

1. INTRODUCTION

Among vertebrates, teleost fish are the most numerous group with a high diversity of species. The evolutionary success of teleost derived from their plasticity and adaptive capacity, which is expressed in their diversity of biological, physiological and morphological patterns, and even in the most diverse reproductive strategies of the group (Nelson, 2006; Grier and Aranzábal, 2009). Sexual reproduction is the typical mode of reproduction in teleost fish. In males, testes produce large amounts of small gametes (sperm), while in females the ovaries produce large gametes with abundant reserves (oocytes) (Schulz and Nóbrega, 2011a).

In teleost fish, spermatogenesis is a highly organized and complex process, in which diploid spermatogonia constantly generate millions of haploid spermatozoa. Fish spermatogenesis present a cystic arrangement and occurs in structures called spermatocysts and these structures are formed when a type A undifferentiated spermatogonia is completely surrounded by Sertoli cell cytoplasm projections (Schulz and Miura, 2002; Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010). Spermatogenesis in fish is classified into three stages: proliferative, in which spermatogonia undergo successive mitotic divisions; meiotic, in which the genetic material is duplicated, recombined and segregated and spermiogenesis, in which spermatids undergo drastic morphofunctional changes, originating structurally mature spermatozoa. In this biological model, germ cells remain interconnected by cytoplasmic bridges and Sertoli cells are in contact with only one germ cell type (Schulz and Miura, 2002; Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010).

Histological, stereological and/or histomorphometric analysis are powerful tools to understand the spermatogenic process and testicular functions in fish. These studies allow to evaluate interactions between somatic and germ cells providing information about the spermatogenic efficiency for each species, through morphometrical data, such as number of spermatogonial generations, germ cell loss percentage and Sertoli cell efficiency, among others. Thus, these procedures constitute an effective tool on interpretatining fish testis function (Vilela et al., 2003; Schulz et al., 2005; Leal et al., 2009; Schulz et al., 2010).

Common carp (Cyprinus carpio, Cyprinidae, Cypriniformes) is a freswater fish originally from Eurasia with a huge socioeconomic importance and represents a valuable resource for global aquaculture (Balon, 1995; Chistiakov and Voronova, 2009; FAO, 2010; FAO, 2014). Common carp has been used as a biological model in basic and applied research fields (Henkel et al., 2012; Ji et al., 2012; Zhang et al., 2013; Goodbred et al., 2013; Cejko et al., 2014). This species shows interesting and attractive reproductive features, such as early puberty and continuous

spermatogenesis (Cejko et al., 2014). Despite the usefulness of histological and stereological studies (Schulz et al., 2005; Leal et al., 2009), few quantitative and/or qualitative data are available about the basic aspects of testis morphology, spermatogenesis and cellular kinetics in common carp. Thus, the aim of this work was to perform a morphological and stereological description and characterization of the different spermatogenic cysts in common carp. Additionally, we determined the duration of the spermatogenic process through 5´bromo-2´-deoxyuridine (BrdU).

2. MATERIALS AND METHODS

2.1. Ethics statement

All procedures were consistent with Brazilian national regulation and followed the guidelines for the ethical animal treatment, approved by the National council for the control of animal experimentation (CONCEA) and the Ethics committee on animal care and experimentation of São Paulo State University (CEUA), protocol n° 02248/14.

2.2. Animals, sampling and testis stereology

Thirty sexually mature common carp males (mean body weight 150 ± 10g) were used for histology, stereology/morphometry and analysis of the duration of spermatogenesis. Animals were kept in 200L fiber tanks, at a temperature of 26°C, at 26°C, under natural photoperiod (12L:12D) and feed with pelleted commercial diet three times per day. For histological and stereological analysis, the animals (n=5) were anesthetized with 1% benzocaine and euthanized. Whole testes were sampled, fixed at least for 24 h in paraformaldehyde solution at room temperature, dehydrated and embedded in methyl methacrylate (Leica Microsystems). Testes were sectioned at 3μm thickness and the histological sections obtained were stained with 1% toluidine blue. Histological slides were analyzed and photographed using Leica DMI 4000 B microscope. The total number of germ cells and Sertoli cells per spermatogonial, spermatocyte, and spermatid cyst was determined on serial sections using ten sexually mature animals. This evaluation was performed after selecting cysts entirely encompassed by serial sections. At least five cysts were analyzed for each germ cell type in each fish. Because all germ cells and their nucleus investigated in common carp are spherical or nearly rounded, their volume was estimated using the mean cell and nuclear diameter. One hundred nuclei were quantified for each germ cell type per fish. Cell and nuclear volume was expressed (in cubic micrometers) using the formula $4/3\pi R^3$, where R= nuclear diameter/2. The cyst volume, excluding Sertoli cells, was calculated according to the germ cell volume and the total number of germ cells per cyst.

The Sertoli cell efficiency was estimated from the ratio of germ cells:Sertoli cells for each cyst type. Measurements of cell nuclei diameter were performed using Image-Pro Plus 6.0 image analysis software.

2.3. Spermatogenesis duration (proliferative, meiotic and spermiogenic phases)

To estimate the spermatogenesis duration, animals (n=20) were injected intracoelomatically with BrdU for three consecutive days at a concentration of 150 mg/BW, protocol modified from Nóbrega, et al. (2010). Animals (n=5 per evaluated period) were weighed and anesthetized by benzocaine immersion 1%, and sampled (testes dissection) at 4 hours, 1, 2 and 3 weeks after the last BrdU injection. BrdU incorporation was detected using a protocol modified from Van de Kant and de Rooij (1992). Testes were fixed for 2 hours at room temperature in methacarn (60% absolute methanol, 30% chloroform and 10% glacial acetic acid), processed and embedded in methyl methacrylate (Technovit 7100-Heraeus Kulzer, Wehrheim, Germany) and were obtained 3 μm-thick histological sections. Sections were subjected to antigen retrieval (1% periodic acid in water at 60°C for 30 min) and endogenous peroxidase blocking (1% H_2O_2 in PBS for 10 min). Slides were incubated for 1 hour (at room temperature) with mouse anti-BrdU 1:80 (BD Biosciences – San Jose, CA) diluted in PBS containing 1% bovine serum albumin (BSA - Sigma-Aldrich, St. Louis, MO), and then for 30 minutes with PowerVison (EasyLink One – HRP polymer [Easypath©]), diluted in PBS containing 1% BSA. Immunostaining revelation was done with avidin-biotin complex (ABC), incubated for 1 h (Vector Laboratories) and diaminobenzidine (Dako, Denmark) substrate for 3-6 min. Counterstaining was done with Harris hematoxylin (Sigma-Aldrich) for 30 sec. Spermatogenesis duration was determined by analysing BrdU-positive germ cells found in the most advanced stage of the differentiation process (meiotic phase).

2.4. Statistical analysis

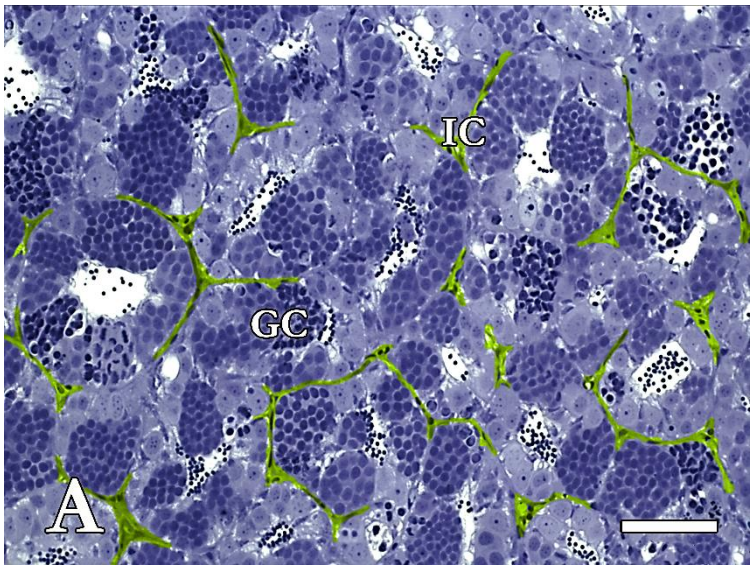
Results were expressed as mean values ± SEM. Significant differences between two groups were identified using Student test (paired and unpaired) ($p<0.05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($p<0.05$). Data analysis were carried out using Graph Pad Prism 6.0.

3. RESULTS

3.1. Testis structure

Common carp testis are a paired organ located dorsally in the coelomic cavity under the swim bladder and are connected to the dorsal body wall by a mesorchium. Testes are composed by two compartments: the germinative, formed by germ cells and Sertoli cells, and the interstitial one, constituted by connective tissue elements and Leydig cells. These compartments are separated by a basement membrane (Figure 1). According to the morphological organization of the germinal epithelium, testis is classified as anastomosing tubular testis. In this arrangement, the germinal epithelium consist of loops and branched tubules that are interconnected peripherally and end in the main testicular duct. Regarding the spermatogonia distribution, testes are classified as spermatogonial unrestricted, where spermatogonia are distributed throughout the length of the seminiferous tubules.

In the germinal epithelium of common carp were found spermatocysts in different stages of spermatogenesis, from cysts of spermatogonia to late spermatids, and spermatozoa in the testicular lumen. The different cell types founded in the tubules were characterized according to morphological criteria as cell size, nuclear features, nucleoli quantity, and chromatin condensation. We propose five basic spermatogonial types based on the results of Leal et al. (2009): type A undifferentiated spermatogonia (A_{und}*), type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B early spermatogonia and type B late spermatogonia. Thus, in the proliferative phase were found five spermatogonial types. In the meiotic phase were identified three primary spermatocytes (leptotene/zigotene, pachytene, diplotene) and one secondary spermatocytes types. Finally, in the spermiogenic phase were distinguished three spermatid forms: initial, intermediate and final (Figure 2).



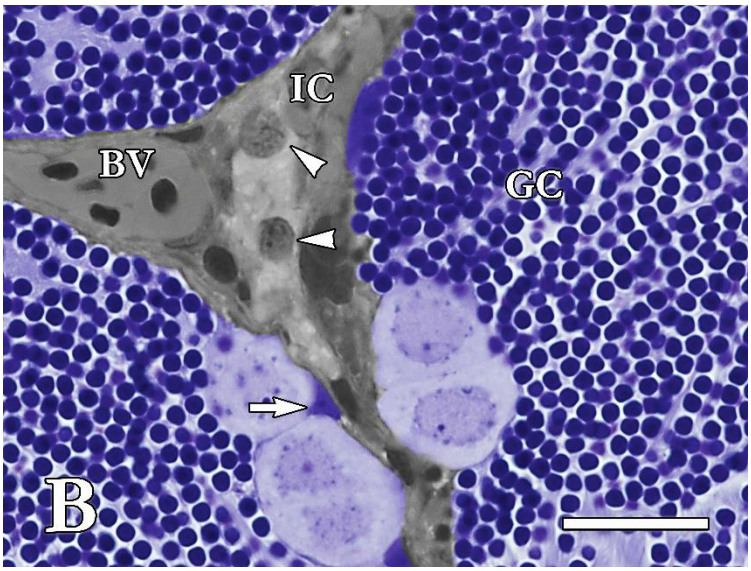


Figure 1. Common carp testes are composed of two main compartments, the interstitial and the germinal compartment. **(A-B)** The interstitial compartment (light green in A and grey in B) is composed of Leydig cells (arrowheads), blood vessels and connective tissue. The germinative compartment houses the germinal epithelium, formed by somatic Sertoli cells (arrow) and germ cells in different stages of development. Interstitial and germinal compartment are separated by a basal lamina. BV: blood vessel; GC: germinal compartment; IC: interstitial compartment Scale bars in A: 50μm. Scale bars in B: 25μm

3.2. Germ cell morphology during the proliferative, meiotic and spermiogenic phases

3.2.1. Proliferative phase

All morphometric data associated to spermatogenic cysts are presented in Table 1. Type A undifferentiated spermatogonia – A_{und}* are the largest germ cells (volume, 755μm^3) and appear isolated throughout the germinal epithelium. This cell type is preferentially distributed close to the interstitial region (Figure 2A,B). These cells present a large and irregular nucleus (volume, 333μm^3), one or two nucleoli, finely granular chromatin, large amounts of nuages and are completely surrounded by a single Sertoli cell (Figure 2A,B). Type A undifferentiated spermatogonia – A_{und} are smaller than A_{und}* (volume, 660μm^3), they are found isolated along the germinal epithelium. A_{und} presented a well-defined, large and rounded nucleus (volume, 288μm^3), with rounded nuclei, condensed chromatin and few nuages (Figure 2B,C).

Morphologically, type A differentiated spermatogonia (A_{diff}) resemble the undifferentiated type, although they are smaller than A_{und}* and A_{und} (volume, 394μm^3). A_{diff} can be found grouped in cysts from two to eight cells, show a denser and smaller nucleus (volume, 137μm^3), with two or three nucleoli and present discretely condensed chromatin (Figure 2C).

Type B early spermatogonia (B early) are smaller cells than type A spermatogonia (volume, 195μm^3), show rounded shape and well-defined spherical nucleus with one or two nucleoli (volume, 82μm^3), the heterochromatin becomes denser and is distributed throughout the nucleus forming clusters (Figure 2D).

Type B late spermatogonia (B late) are slightly smaller than type B early spermatogonia (volume, 119μm^3), present rounded and smaller nucleus with denser heterochromatin (volume, 62μm^3). At this point of spermatogenesis, mitotic divisions are concluded because type B late spermatogonia replicate their genetic material and enter in meiosis (Figure 2E,F).

3.2.2. Meiotic phase

Spermatocytes at different stages of meiosis were categorized in accordance with nuclear features such as size, chromosome condensation and organization.

Primary spermatocytes show similar size and shape to type B late spermatogonia, and show homologous chromosomes in different stages of meiotic prophase I: leptotene, zygotene, pachytene and diplotene. Leptotene/zygotene spermatocytes (L-Z) show a comparable size to type B late spermatogonia (volume, 137μm^3), rounded nucleus (volume, 74μm^3), clear chromatin and small heterochromatin spots (Figure 2G). Pachytene spermatocytes (P) are the largest cell type found during the meiotic stage (volume, 221μm^3). These cells have a denser nucleus (volume, 102μm^3) and chromosomes are more visible in this cell type (Figure 2H). Cysts of diplotene spermatocytes (D) are typically found together with metaphase I figures, and their chromosomes exhibited the highest degree of condensation, easily identified as strongly marked filaments (Figure 2I). These cells present a similar size than leptotene/zygotene spermatocytes (volume, 144μm^3) with a rounded and highly dense nucleus (volume, 74μm^3). Secondary spermatocytes (SS) are rounded small cells (volume, 73μm^3) with spherical and heterochromatic nucleus(volume, 36μm^3) (Figure 2J). These cells are rarely found in testes because they immediately entry into the second meiotic division (Figure 2K).

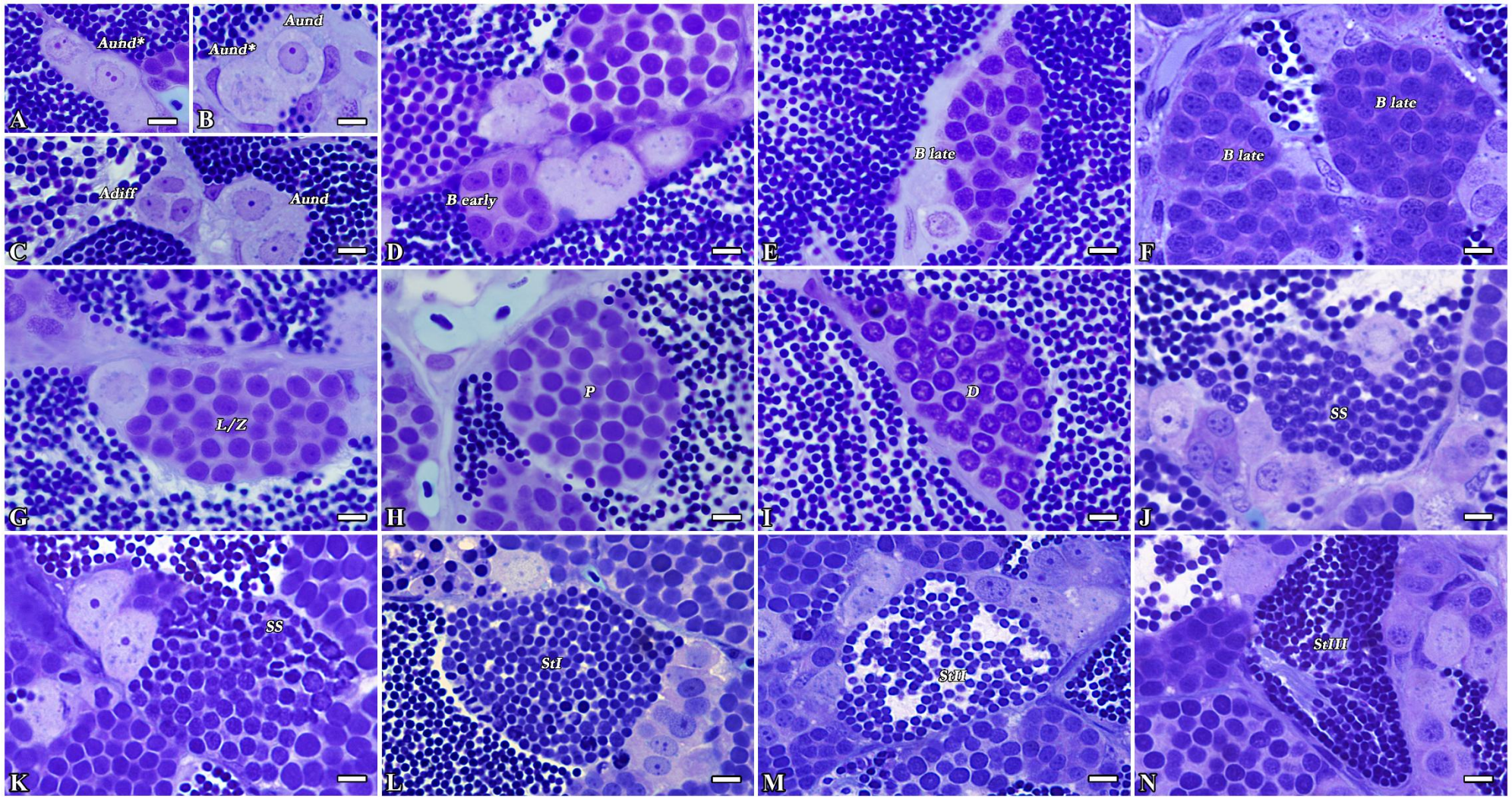


Figure 2. Different germ cell cysts observed during the spermatogenic process in common carp from a type A undifferentiated* spermatogonia to final spermatids. **(A)** The most undifferentiated type A spermatogonium (A_{und*}; probable stem cell). **(B)** Type A undifferentiated spermatogonium (A_{und*}) and type A undifferentiated spermatogonium (A_{und}; possibly limited stem cell capacity). **(C)** Type A differentiated spermatogonia (A_{diff}). **(D)** Early type B spermatogonia (B early). **(E)** and **(F)** Late type B spermatogonia (B late). **(G)** Primary spermatocytes in leptotene to zygotene (L/Z). **(H)** Primary spermatocytes in pachytene (P). **(I)** Primary spermatocytes in diplotene (D). **(J)** Secondary spermatocytes (SS). **(K)** Secondary spermatocytes (SS) entry into the second meiotic division. **(L)** Spermatids initial (StI). **(M)** Spermatids intermediate (StII). **(N)** Spermatids final (StIII). Bars= 10 μm.

3.2.3. Spermiogenic phase

This phase begins after the second meiotic division of secondary spermatocytes and is characterized by a significant reduction in cell size, resulting in the spermatid production. Spermatids are very small cells found in large numbers within the spermatocysts. Three types of spermatids were described according to nuclear condensation, cell size, space between cells and flagellum formation.

Initial spermatids (StI) are spherical small cells (volume, 29μm^3) with highly condensed spherical nucleus (volume, 13μm^3) and dense chromatin (Figure 2L). Intermediate spermatids (StII) are smaller cells than the previous ones (volume, 20μm^3), with rounded shape and denser nucleus (volume, 10μm^3) (Figure 2M). Final spermatids (StIII) undergo through changes in shape and size reduction. They are rounded small cells (volume, 11μm^3) with a highly condensed chromatin and smaller nucleus (volume, 6μm^3) (Figure 2N). The intracellular space in the cysts become larger, cells do not divide anymore, however undergo morphological changes to form spermatozoa throughout the spermiogenic process. This process is associated to important morphological alterations related with the spermatozoa formation of the head and their highly condensed nucleus, the mid-piece, and the flagellum.

3.3. Germ cell number and Sertoli cell efficiency

During spermatogenesis of common carp, the total number of Sertoli cells per cyst increased significantly from type A differentiated spermatogonia to pachytene spermatocyte cysts, and was stabilized from pachytene/diplotene spermatocytes to mature spermatids (Table 1; Figure 3). The number of germ cells per cyst increased strikingly from type A undifferentiated spermatogonia to early spermatids and showed a decrease in intermediate and final spermatids cysts (Table 1; Figure 3). In contrast, nuclear/cellular diameter and volume decreased gradually, however these parameters showed an increase from leptotene/zigotene to pachytene spermatocytes (Table 1). The data obtained for the number of spermatogonia and early spermatocytes per cyst allowed us to estimate 9 spermatogonial generations in common carp from type A undifferentiated spermatogonia – A_{und*}, type A undifferentiated spermatogonia – A_{und}, type A differentiated spermatogonia – A_{diff}, type B early spermatogonia and type B late spermatogonia. Due to the morphological similarity among the generations of type B spermatogonia, these cells were distinguished through the cell size and the number of spermatogonia per cyst. In the current work was found a Sertoli cell efficiency of approximately 94 mature spermatids per each Sertoli cell (Table 1; Figure 3).

			Germ cells					
Cyst type*	N° of Sertoli cells per cyst	N° of germ cells per cyst	Cell diameter (μm)	Cell volume (μm^3)	Nuclear diameter (μm)	Nuclear volume (μm^3)	Cyst volume (μm^3)**	N° of germ cells per Sertoli cell
A_{und}*	1.1 ± 0.1^{a}	1.0 ± 0^{a}	11.3 ± 0.1^{a}	755 ± 28^{a}	8.6 ± 0.1^{a}	333 ± 13^{a}	805 ± 29^{a}	0.9 ± 0.1^{a}
A_{und}	2.0 ± 0.1^{a}	1.0 ± 0^{a}	10.8 ± 0.1^{a}	660 ± 18^{a}	8.2 ± 0.1^{a}	288 ± 10^{a}	660 ± 20^{a}	0.5 ± 0.1^{a}
A_{diff}	$2.3 \pm 0.1^{a,b}$	2.2 ± 0.2^{b}	$9.1 \pm 0.1^{a,b}$	394 ± 11^{b}	6.4 ± 0.1^{b}	137 ± 6^{c}	786 ± 21^{a}	1.0 ± 0.1^{a}
B-early	5.2 ± 0.2^{c}	15 ± 0.4^{c}	7.2 ± 0.1^{c}	195 ± 8^{c}	5.4 ± 0.1^{b}	82 ± 2^{c}	2631 ± 71^{a}	2.9 ± 0.1^{b}
B-late	11.4 ± 0.3^{d}	218 ± 10^{d}	6.1 ± 0.1^{d}	119 ± 3^{c}	$4.9 \pm 0.1^{b,c}$	$62 \pm 2^{c,d}$	25227 ± 803^{b}	19 ± 0.5^{c}
L/Z	12.4 ± 0.2^{e}	404 ± 14^{e}	$6.4 \pm 0.1^{c,d}$	137 ± 3^{c}	$5.2 \pm 0.1^{b,c}$	$74 \pm 2^{c,d}$	53314 ± 1223^{c}	33 ± 0.3^{c}
P	12.9 ± 0.2^{e}	392 ± 12^{e}	$7{,}5 \pm 0.1^{c}$	221 ± 5^{c}	$5.8 \pm 0.1^{b,c}$	102 ± 1^{c}	84006 ± 3386^{d}	30 ± 0.4^{c}
D	$13.3 \pm 0.3^{e,f}$	448 ± 14^{e}	$6.5 \pm 0.1^{c,d}$	144 ± 4^{c}	$5.2 \pm 0.1^{b,c}$	$74 \pm 1^{c,d}$	56788 ± 1758^{c}	34 ± 0.4^{c}
SS	13.4 ± 0.4^{f}	812 ± 18^{f}	5.2 ± 0.1^{e}	73 ± 3^{d}	$4.1 \pm 0.1^{b,d}$	36 ± 1^{e}	53455 ± 1465^{c}	61 ± 0.3^{d}
StI	13.8 ± 0.3^{f}	1348 ± 44^{g}	3.8 ± 0.1^{f}	29 ± 2^{e}	2.9 ± 0.1^{e}	13 ± 0.2^{e}	37423 ± 1288^{e}	98 ± 0.9^{e}
StII	13.6 ± 0.3^{f}	$1292 \pm 36^{g,h}$	3.4 ± 0.1^{f}	20 ± 2^{e}	2.7 ± 0.1^{e}	10 ± 0.2^{e}	24108 ± 906^{b}	95 ± 0.9^{e}
StIII	13.3 ± 0.3^{f}	1250 ± 22^{h}	$2.8 \pm 0.1^{f,g}$	11 ± 0.4^{e}	2.3 ± 0.1^{e}	6 ± 0.1^{e}	13808 ± 248^{f}	94 ± 0.7^{e}

Table 1. Morphometric data of the different germ cell cysts in common carp (mean ± SEM). *Type A undifferentiated spermatogonia (A_{und}*); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); type B early spermatogonia (B-early); type B late spermatogonia (B-late); leptotene/zygotene primary spermatocytes (L/Z); pachytene primary spermatocytes (P); diplotene primary spermatocytes (D); secondary spermatocytes (SS); initial spermatids (StI); intermediate spermatids (StII); final spermatids (StIII). **Sertoli cells not included. Different superscript letters indicate that mean values differ significantly ($p < 0.05$).

Theoretically, the expected number of mature spermatids is 2048; however, the observed number was 1208 cells, indicating that the total germ cells loss during spermatogenesis was approximately 41% (Table 1).

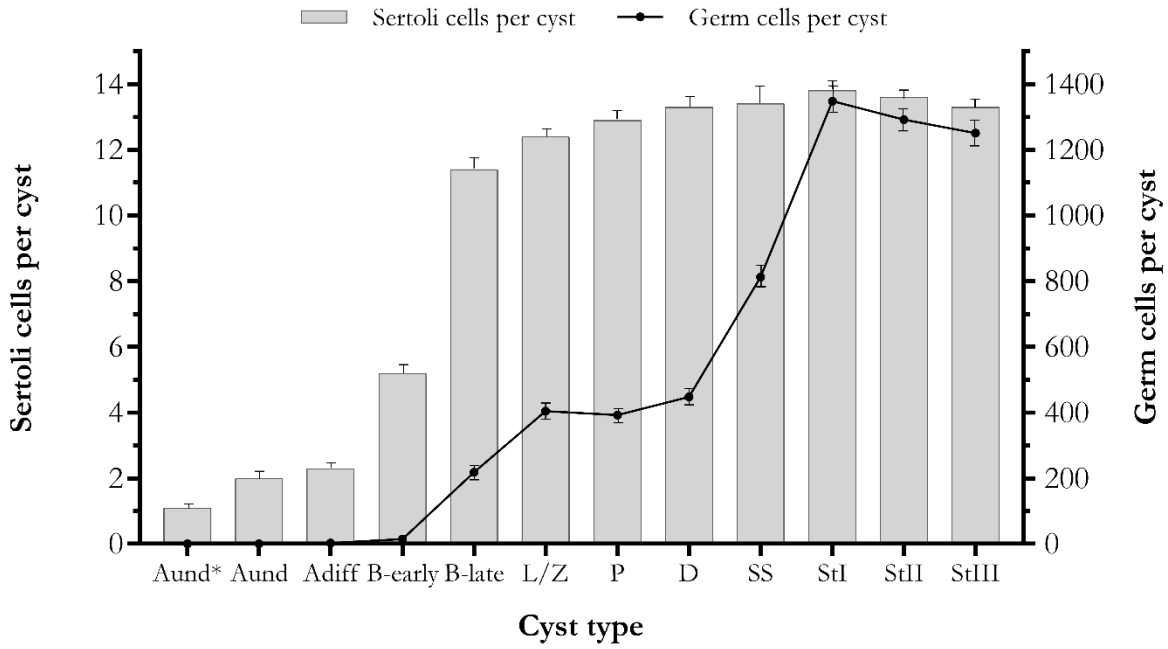


Figure 3. Total number of Sertoli and germ cells per different cyst type in common carp (*C. carpio*). Type A undifferentiated spermatogonia (A_{und}*); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); type B early spermatogonia (B-early); type B late spermatogonia (B-late); leptotene/zygotene primary spermatocytes (L/Z); pachytene primary spermatocytes (P); diplotene primary spermatocytes (D); secondary spermatocytes (SS); initial spermatids (StI); intermediate spermatids (StII); final spermatids (StIII).

3.4. Immunohistochemistry and spermatogenesis duration

Using BrdU exposure was possible to estimate the spermatogenesis duration in common carp. This estimation was done through identification of the most advanced germ cell populations that retained BrdU, allowing to estimate the duration of the spermatogenic process from the meiotic phase. Four hours after the last BrdU exposure, the more advanced BrdU-positive germ cells were type B late spermatogonia (Figure 4D), also were found A_{und*} (Figure 4A), A_{und} (Figure 4B) and A_{diff} spermatogonia (Figure 4D), and type B early spermatogonia (Figure 4C). One week after exposure, spermatocytes (Figure 4E), spermatids in different stages (initial, intermediate and final) (Figure 4F,G) and spermatozoa showed a positive BrdU reaction (Figure 4H). Based on these observations we can estimate that the spermatogenesis duration (combination of meiotic and spermiogenic stages) is approximately one week (7 days), from the meiotic stage until the spermatozoa formation.

One week after the last BrdU injection, BrdU-labeled spermatids and spermatozoa were found in the lumen of the seminiferous tubules.

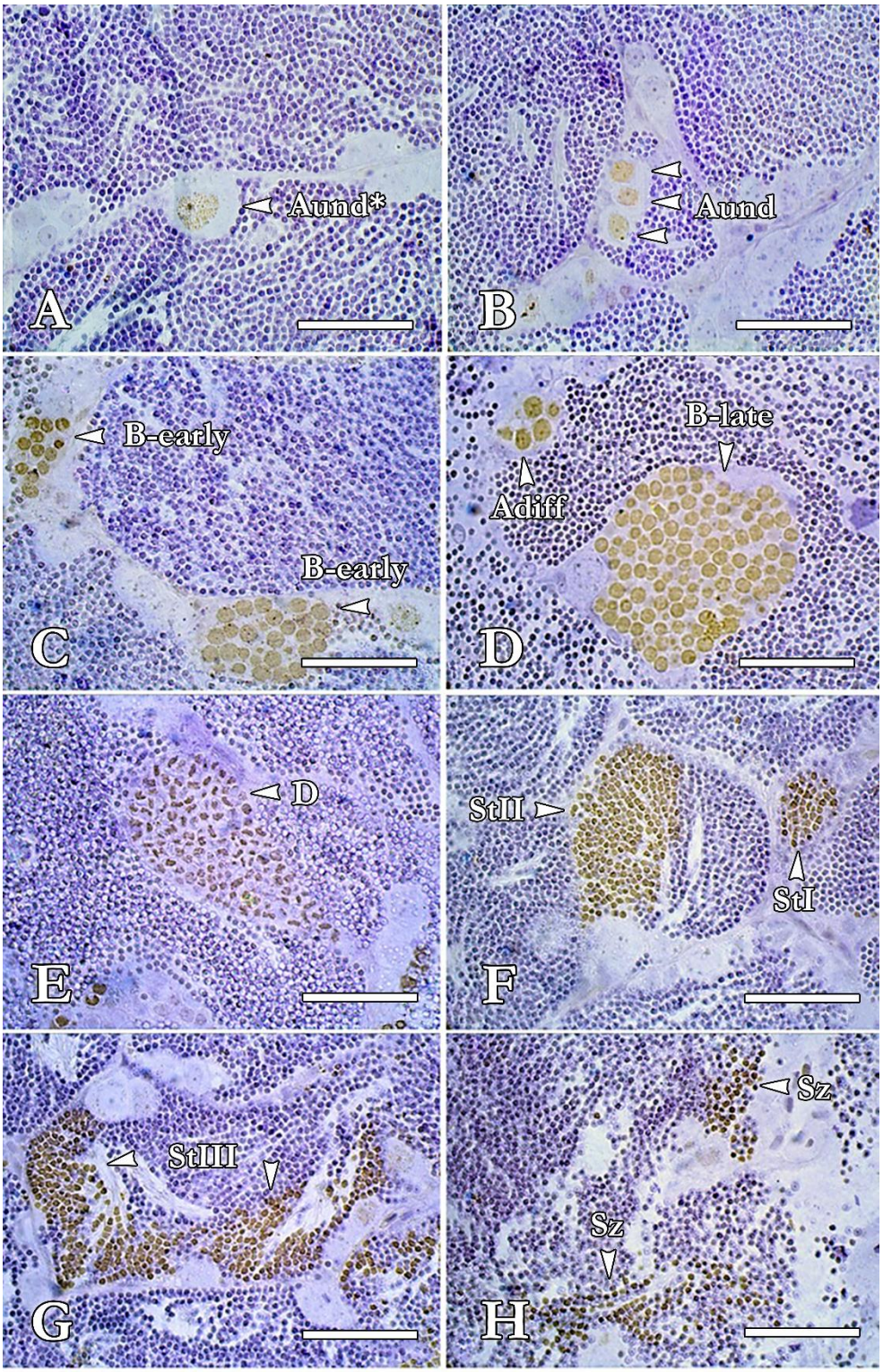


Figure 4. Proliferating (BrdU-positive) germ cells and estimation of the spermatogenic process in adult common carp (*C. carpio*) testis. The following labeled cells were observed at different time periods after BrdU injection: **(A – D)** at four hours type A undifferentiated spermatogonia (A_{und}* - A_{und}), type A differentiated spermatogonia (A_{diff}), type B early and late spermatogonia (B-early - B-late); **(E – H)** at one week diplotene spermatocytes (D), initial, intermediate and final spermatids (StI - StII - StIII) and spermatozoa in the lumen (Sz). A_{und}*, type A undifferentiated

spermatogonia; A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiated spermatogonia; B-early, type B early spermatogonia; B-late, type B late spermatogonia; DSc, diplotene spermatocytes; StI, initial spermatids; StII intermediate spermatids; StIII final spermatids. Scale bars 50μm.

4. DISCUSSION

4.1. Common carp testis morphology

The anatomical, structural and functional conformation of testes is highly conserved among vertebrates. In common carp as in other vertebrates, testes are formed by two main compartments, the germinative and interstitial. Germinal compartment is formed by germ cells (spermatogonia, spermatocytes, spermatids and spermatozoa) and Sertoli cells. Connective tissue, Leydig cells, peritubular myoid cells, blood vessels, lymph vessels, fibroblast, collagen and amyelinic fibers form the interstitial compartment. These two compartments are separated by a basement membrane (Parenti and Grier, 2004; Kerr et al., 2006; Nóbrega et al., 2009; Grier and Uribe-Aranzábal, 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a; Uribe et al., 2015).

In teleost, testicular germinal epithelium is classified into two types, anastomosing tubular and lobular testis. In the anastomosing tubular testis arrangement, the tubules form loops along the testicular parenchyma, creating a strongly anastomosing and branched structure. The tubules are interconnected peripherally and end in the main testicular duct. On the other hand, lobular testis is found in neoteleost except in order Atherinomorpha. In this testis the germinal compartment present digitiform shape and form lobules, which extend to the periphery of the testes and end in a blind bottom. (Parenti and Grier 2004; Grier and Uribe-Aranzábal 2009; Uribe et al., 2015). Morphologically common carp showed an anastomosing tubular testis structure, this organization is an ancestral feature found in basal groups as characiforms, cyprinids, lepisosteids and salmonids.

Based on the spermatogonia distribution in the germinal epithelium testes can be classified in two types, restricted and unrestricted (Parenti and Grier 2004; Grier and Uribe-Aranzábal 2009; Uribe et al., 2015). In the restricted type, spermatogonia are located in the distal regions of the seminiferous tubules, close to the tunica albuginea. This type of arrangement is a derived feature found in orders such as Atheriniformes, Beloniformes and Cyprinodontiformes. In the unrestricted type, spermatogonia are distributed along the seminiferous tubules throughout the testis. This testicular structure is a primitive pattern found in basal orders such as Characiformes, Cypriniformes and Salmoniformes (Parenti and Grier 2004; Grier and Uribe-Aranzábal 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a; Uribe et al., 2015). In common carp, spermatogonia were found along the germinal compartment, thus, testes are classified as spermatogonial

unrestricted. Summarizing, the common carp testes are characterized as anastomosing tubular type, with unrestricted spermatogonial arrangement.

4.2. Spermatogenesis and germ cell types

Spermatogenesis is a highly organized, complex and conserved process among vertebrates, in which diploid spermatogonia proliferate, differentiate and undergo morphofunctional changes to form haploid spermatozoa. Spermatogenesis in fish can be divided in three different phases: proliferative, meiotic and spermiogenic. During these phases, spermatogonia divide actively (mitotic divisions), subsequently, the genetic material is duplicated, recombined and segregated (meiotic divisions), and finally the spermatids undergo drastic morphofunctional changes that lead to mature sperm formation. Interestingly, the meiotic and spermiogenic phases of spermatogenesis show a high degree of similarity among vertebrates. Nevertheless, the mitotic phase exhibits a large diversity among orders. The number of spermatogonial generations is variable and is a distinctive genetic feature among species (Ando et al., 2000; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011).

Spermatogenesis in common carp as in other anamniote vertebrates is cystic. In this model, several Sertoli cells surrounding and keeping one synchronously developing germ cell clone (Batlouni et al., 2009; Grier and Uribe-Aranzábal, 2009; Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a). Despite the huge variability between species and the lack of studies addressing the mitotic phase of spermatogenesis in fish, this process has the following cell progression: *Proliferative phase*: type A undifferentiated spermatogonia (stem cells) → type A differentiated spermatogonia → type B spermatogonia (several generations) → *Meiotic phase*: spermatocytes in leptotene/zygotene → spermatocytes in pachytene → spermatocytes in diplotene → metaphase I → secondary spermatocytes → metaphase II → *Spermiogenic phase*: early spermatids → intermediate spermatids → final spermatids → spermatozoa (Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a). Spermatogenesis in common carp presents a similar pattern to that described above, which is basically the same for other teleost fish.

The number of spermatogonial generations is highly variable among teleosts and is genetically determined (Ando et al., 2000; Nóbrega et al., 2009; Schulz et al., 2005, 2010; Schulz and Nóbrega, 2011). In this context, stereological analysis is a valuable approach to quantify the approximate number of germ cells per cyst, which allows determining the number of spermatogonial generations (Schulz et al., 2005; Leal et al., 2009). In the current study, we found at least nine spermatogonial generations in common carp. These results are similar with those of Leal et al. (2009) who reported the same number of spermatogonial generations in *Danio rerio*, which is another cyprinid (same

family of common carp). One of the issues that emerges from these findings is that the species belonging to the same family probably have a certain degree of similarity in terms of the number of spermatogonial divisions. Thus, we can infer that the similarity found between the common carp and zebrafish, in relation to the number of spermatogonial generations, it may be due to these two species are phylogenetically related.

Despite the great diversity of teleost species, few studies have addressed the stereological analysis to understand the cell kinetics in the testicular germinal epithelium. For example, in *Liza dumerili* were found three spermatogonial generations, six in *Umbra limi*, *Sebastodes paucispinis* and *Oncorhynchus mykiss*, ten to twelve in *Gambusia holbrooki* and fourteen in *Poecilia reticulata* (Geiser, 1924; Foley, 1927; Moser, 1967; Billard, 1969; Van der Horst, 1978; Loir, 1999). Recent studies have described six to eight spermatogonial generations in Salmoniformes, seven to ten in Perciformes, eight in Characiformes, eight to nine in Cypriniformes, ten in Beloniformes, eleven in Gadiformes and fourteen in Cyprinodontiformes (Miura 1999; Ando et al. 2000; Fishelson 2003; Vilela et al. 2003; Fishelson et al. 2006, 2007; Bizzotto and Godinho 2007; Almeida et al. 2008).

Spermatogonial types in fish have been determined based on morphological characteristics such as cell and nuclear size, chromatin arrangement and the number of cells per cyst (Leal et al., 2009; Schulz et al., 2010). Similarly, to that reported by Vilela, et al. (2003), Leal et al. (2009), Schulz et al., (2005, 2010), and Schulz and Nóbrega (2011a), in this study we found five spermatogonial types: type A undifferentiated spermatogonia (A_{und}* and A_{und}), type A differentiated spermatogonia and type B (early and late) spermatogonia. These cells undergo several mitotic divisions and showed different intermediary cell types. Undifferentiated spermatogonia (A_{und}* and A_{und}) are the largest germ cells type founded in common carp. These cells are isolated and completely enclosed by Sertoli cells cytoplasmic extensions, having a prominent clear nucleus with one or two electron-dense nucleoli, poorly condensed chromatin and an irregular (A_{und}*) or regular (A_{und}) nuclear envelope. The chromatin is thin, slightly condensed and distributed homogeneously. Their cytoplasm is clear, large, scarcely differentiated and show high-density of nuages (Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a).

Morphologically, differentiated spermatogonia are similar to the undifferentiated type, although they are smaller. These cells are usually found in cysts from two to eight cells connected by cytoplasmatic bridges; exhibiting a rounded dense and small nucleus, with two or three nucleoli and poorly condensed chromatin (Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a).

In fish, type B spermatogonia are smaller cells than type A_{diff} spermatogonia. They are rounded shape cells with well-defined spherical nucleus with one or two nucleoli and gradually the chromatin becomes denser and distributed throughout the nucleus. Type B spermatogonia has several intermediate generations between type B early spermatogonia and type B late spermatogonia. The number of generations is species-specific and occur in spermatocysts with 16 or more cells (~300) (Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a). In mammals and fish, type B spermatogonia divide faster than type A spermatogonia (De Rooij and Russell, 2000).

Spermatogenesis progression depends on spermatogonia, specifically the spermatogonial stem cells, due to their capacity for self-renewal and differentiation. From the successive divisions of type A undifferentiated spermatogonia (A_{und}*) are originated the subsequent cell generations that determine the functional characteristics of the testis and the transmission of male genotype (Leal et al., 2009; Nóbrega et al., 2009, 2010; Schulz et al., 2010; Schulz and Nóbrega, 2011a).
The latest generation of type B spermatogonia differentiate into primary spermatocytes and enter meiosis (meiotic phase). During this phase are produced two cell cycles, in which the parental genetic material is reorganized during the first meiotic division and the genome is reduced during the second meiotic division.

In meiosis I, homologous chromosomes of primary spermatocytes are segregated, while in the meiosis II the sister chromatids segregate and are formed four haploid cells with a copy of each chromosome (Schulz et al., 2010; Schulz and Nóbrega 2011a). Common carp primary and secondary spermatocytes showed similar morphological features to those found in zebrafish and tilapia. During the meiotic phase, spermatocytes are distinguished mainly by their size, chromatin condensation and the arrangement of chromosomes in the nucleus. On the other hand, secondary spermatocytes resulting from meiosis I are a cell type that has a shorter cell cycle because they rapidly enter in meiosis II. (Leal et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011a).

Spermiogenesis begins with the formation of spermatids after the second meiotic division of secondary spermatocytes. In common carp and in most of teleost fish, during spermiogenesis the spermatids differentiate into spermatozoa through a series of morpho-functional changes. These morphological modifications include nuclear condensation, elimination of cytoplasm and organelles, flagellum formation and reorganization of the remaining organelles in the spermatozoa cytoplasm. As spermiogenesis advances, spermatids show a decrease in size and increases the chromatin condensation.

Thus, it is possible to identify three types of spermatids according to the nuclear condensation: early spermatids, intermediate spermatids and final spermatids (Leal et al., 2009; Schulz et al., 2005, 2010; Schulz and Nóbrega, 2011a; Uribe et al., 2015). In the current study was found a significant decrease of cysts volume during spermiogenesis, the same trend was also observed in mammals, Nile tilapia and zebrafish. This occurred due to the nuclear and DNA condensation, elimination of organelles and cytoplasm (França and Russell 1998; Vilela et al., 2003; Schulz et al., 2005; Leal et al., 2009; Schulz and Nóbrega, 2011a).

This study found that changes in germ cell size during meiotic and spermiogenic phases of common carp were similar to those observed in studies with tilapia, zebrafish and mammals. However, common carp germ cells showed a smaller size compared to tilapia (França and Russell 1998; Schulz et al., 2005; Leal et al., 2009). Despite the great reproductive diversity, spermatogenesis is a highly conserved process between the different groups. Some functional and structural aspects of this process are quite similar in mammals and fish (Schulz and Miura, 2002; Miura and Miura, 2003; Schulz, 2003). The evidence from this study suggests that morphological changes during spermatogenesis show common features between common carp, zebrafish, tilapia and presumably among the different teleost species (Schulz et al., 2005; Leal et al., 2009).

4.3. Germ cell number, Sertoli cell efficiency and germ cell loss

In this work was found a large quantity of Sertoli cells associated to the spermatogenic cysts. We found that the number of germ cells and Sertoli cells population increased considerably. In Nile tilapia, zebrafish and common carp (this study) the number of Sertoli cells increases significantly, the stabilization was observed during the spermiogenic phase and showing a ratio of ~94 spermatids per Sertoli cell in common carp, ~91 in Nile tilapia and ~96 in zebrafish (Schulz et al., 2005; Leal et al., 2009). Thus, the support capability of Sertoli cells seemed to be conserved among fish (~100 spermatids per Sertoli cell) (França et al., 2015). In the same way, the ratio between the number of germ cells per Sertoli cell shows that teleost fish have a higher Sertoli cell efficiency compared to mammals (<10 in most of mammalians and 4 in humans). These relationships suggest that the cystic arrangement in fish is more efficient than the tubular arrangement found in mammals (França and Russell 1998; De Rooij and Russell, 2000; Schulz et al., 2005; Leal et al., 2009). Despite the changes in the number of germ cells per cyst observed in carp, tilapia and zebrafish, we can infer that Sertoli cell efficiency in these species is quite similar, indicating that some morphofunctional features of spermatogenesis are highly conserved in teleost fish (Schulz et al., 2005; Leal et al., 2009).

In mammals, the total number of Sertoli cells is determined during puberty, when at this stage of development these cells suspend their proliferative activity and differentiate definitely (Hess et al., 2005). In teleost as in mammals, the number of Sertoli cells found in the germinal epithelium associated with spermatogenic cysts is a determining factor to ensure the sperm production and male fertility. This suggests that germ cell development is highly dependent on regulatory factors (hormones and growth factors) produced by Sertoli cells (Leal et al 2009; Schulz et al., 2005, 2010; Schulz and Nóbrega, 2011b; França et al., 2016).

In mammals, a programmed germ cells loss occurs normally in some stages of spermatogenesis, and it is performed to eliminate abnormal germ cells or cells with DNA damage (França and Russell 1998; Baum et al., 2005; Schulz et al., 2010). Thus, considering cell loss in mammals, only two to three spermatozoa (of 10 expected cells) are generated from each differentiated spermatogonia (França and Russell 1998; De Rooij and Russell, 2000). Conversely, fish spermatogenesis is more efficient than mammals; in this regard approximately 60% of the germ cells differentiate into spermatozoa (Vilela et al., 2003). Thus, in common carp (present study), Nile tilapia and zebrafish approximately 5 to 6 of the 10 possible expected mature spermatids were formed from each mature spermatogonia. Suggesting that in these teleost species the process is 100% more efficient than in mammals (Schulz et al., 2005; Leal et al., 2009). This suggests that the high fish spermatogenesis efficiency, viability of germ cells and sperm production is strongly influenced by Sertoli cell proliferation. Consequently, the increase of germ cell number and cyst volume is associated with the rise in the number of Sertoli cells per cyst. In the cystic spermatogenesis, the Sertoli cells can be more efficient in supporting the germ cell development (e.g. managing growth factors required for each stage), resulting in a low rate of germ cell loss and high efficiency of the spermatogenic process (Leal et al., 2009; Schulz et al., 2005, 2010).

In mammals, germ cell apoptosis occurs usually during spermatogonial and meiotic stages of spermatogenesis, in contrast, in fish, this process is found during the spermatogonial stage and especially during spermiogenesis. In Nile tilapia, zebrafish and common carp (present work), the main germ cell loss occurred during the spermiogenic phase (Schulz et al., 2005; Leal et al., 2009). On the other hand, in guppies and Atlantic cod, the germ cell loss occurred in the spermatogonial phase (Billard, 1969; Almeida et al., 2008). In this context, the germ cell loss during spermatogenesis is quite similar among different fish species, indicating that spermatogenesis is highly conserved in this group of vertebrates, however the process shows subtle differences and is highly regulated in each species (Leal et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011b).

4.4. Spermatogenesis duration

Knowledge of spermatogenesis duration is a tool to understand the physiological and regulatory mechanisms of the spermatogenic process (Leal et al., 2009; Nóbrega et al., 2009). The spermatogenesis in mammals is determined according to the stages of the seminiferous epithelium, which are specific associations formed by germ cells. Thus, estimating the frequency of each stage enables the duration of spermatogenic process from undifferentiated spermatogonia to spermatozoa (França and Russell 1998; Leal and França, 2006). In fish, due to the cystic arrangement of spermatogenesis is not possible to use this approach, however, is possible to determine the duration of the meiotic and spermiogenic stages (Nóbrega et al., 2009; Leal et al., 2009). During this period, it is possible to use a cell proliferation marker as BrdU, which is incorporated into cell DNA at the S-phase of the cell cycle and is very useful to characterize the cell cycle kinetics and determine the cell proliferation (Leal et al., 2009). Using this methodology, we found that the duration of meiotic and spermiogenic stages is approximately 7 days in the common carp. Similar results were reported for banded gourami *Trichogaster fasciata* and zebrafish, where the duration of the meiotic and spermiogenic phases was 6 days (Sinha et al., 1979; Leal et al., 2009). The length of spermatogenesis was determined in few fish species, such as 10 days in mosquito fish *Gambusia affinis*, 12 days for medaka *Oryzias latipes*, 14.5 days for guppies *Poecilia reticulata*, 21 days for black mollies *Poecilia shenops* and 14 days for Nile tilapia (Nagahama, 1983; De Felice and Rasch, 1969;Vilela et al., 2003; Koya and Iwase, 2004).

In general, the duration of the spermatogenic process in fish is shorter than in mammals and fish spermatogenesis is strongly influenced by water temperature. In fish, there is an inversely proportional relationship between the spermatogenesis length and the temperature. Thus, high temperatures accelerates the process, while low temperatures delay the process (França and Russell 1998; Vilela et al., 2003; Lacerda et al., 2006; Nóbrega et al., 2009).

5. CONCLUSION

In summary, we performed for first time a detailed study of common carp spermatogenesis using morphological and stereological approaches. In addition, we used the classification of the different germ cell types proposed for zebrafish, which is a closely related species. Moreover, we estimated that the duration of spermatogenic process (meiotic and spermiogenic stages) was about 7 days in common carp. This study provides basic information about the reproductive biology of the common carp, which can be used in comparative studies and contributes to better understand the spermatogenic process in teleost fish.

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MANUSCRITO II

SPERMATOGENESIS DEPLETION, SPERMATOGONIAL STEM CELLS CHARACTERIZATION AND ISOLATION IN COMMON CARP (*Cyprinus carpio*)

Angel Andrés Arias Vigoya[1,2]**, Emanuel Ricardo Monteiro Martinez[2], Melanie Digmayer[2], Marcos Antonio de Oliveira[1,2], Arno Juliano Butzge[2], Rafael Henrique Nóbrega[2]**.

[1] Aquaculture Center of São Paulo State University – CAUNESP - Jaboticabal, São Paulo – Brazil

[2] Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu – IBB/UNESP, São Paulo – Brazil.

**E-mail address: biorhn@yahoo.com.br aaariasv1@gmail.com

ABSTRACT

Spermatogonial stem cell (SSC) is a subtype of undifferentiated spermatogonia, which sustain spermatogenesis throughout adult male life of vertebrates. Although important, molecular markers to characterize SSC are not available in several vertebrate species. This represents a limiting factor for an accurate identification of these cells and their use for biotechnological purposes. Herein we characterized SSC using specific molecular markers and transmission electron microscopy. Moreover, we also described a simple method to suppress common carp spermatogenesis using a thermo-chemical treatment, and finally we isolated and enriched undifferentiated spermatogonial fraction from common carp cell suspension. To achieve the first purpose, immunohistochemistry, western blot analysis and transmission electron microscopy were carried out to characterize phenotypically the presumptive SSC in common carp. Undifferentiated spermatogonial population preferentially expressed Gfrα1 and POU2. Using a combination of high temperature (35°C) and busulfan (40 mg/kg/BW), we supressed spermatogenesis in sexually mature common carp males. This approach is a useful tool to prepare the recipients for spermatogonial transplantation assays. The *amh* expression analysis showed differences between the controls (26ºC) when compared to 35°C with a single o two busulfan doses, confirming that association of busulfan at high temperature depleted the testes. In an attempt to isolate the undifferentiated spermatogonial fraction, we used the percoll discontinuous density gradient. Thus, we successfully dissociated the carp whole testes in different cellular fractions; subsequently we isolated and enriched the undifferentiated spermatogonial population. In summary, the common carp SSC candidates were characterized phenotypically, its endogenous spermatogenesis was effectively depleted by a thermo-chemical treatment and the SSC fraction was enriched through the percoll density gradient.

Keywords: fish, spermatogenesis, depletion, germ cells, molecular markers, *amh* expression, percoll gradient.

1. INTRODUCTION

Spermatogenesis is a complex and highly organized process by which a subtype of type undifferentiated spermatogonia, the spermatogonial stem cells (SSC), maintain the continuous sperm production. This is possible due SSC capability of self-renewal to generate more stem cells in the testis, and also differentiation to form commited cells engaged to sperm production. SSCs role is vital to ensure male fertility and for transmitting their genotype to the offspring (Valli et al., 2014; Nagano and Yeh, 2013; Yoshida, 2012). In this context, SSC transplantation is a promising methodology successfully tested in farm animals and fish (Honaramooz and Yang, 2011; Lacerda et al., 2013a; Zheng et al., 2014), which consists in the transference of isolated SSCs from a fertile donor into the testis of an infertile recipient, thus, transplanted cells restore recipient spermatogenesis and generate donor-derived gametes (Lacerda et al., 2013a). Despite their great biotechnological potential for aquaculture, transplantation technique present a limiting factor: a lack of suitable molecular markers for phenotypic/genotypic identification and isolation. In fact, unlike in mammals, in fish there are few markers for spermatogonial phenotypic identification (Lacerda et al., 2012, 2013a,b, 2014). Nowadays, few studies have addressed the identification of undifferentiated spermatogonia using male germ cells markers such c-Kit, Gfrα1 and POU2 (fish homologue of mammalian POU5f1/Oct4) was reported in blue catfish, (*Ictalurus furcatus*) (Shang et al., 2015), channel catfish (*Ictalurus punctatus*) (Shang et al., 2015), medaka (*Orizias latipes*) (Hong et al., 2004; Sánchez et al., 2010; Froschauer et al., 2013), Nile tilapia (*Oreochromis niloticus*) (Lacerda et al., 2013b), rohu carp (*Labeo rohita*) (Panda et al., 2011), dogfish (*Scyliorhinus canicula*) (Loppion et al., 2008; Bosseboeuf et al., 2013) and rainbow trout (*Oncorhynchus mykiss*) (Nakajima et al., 2014; Bellaïche et al., 2014a,b; Farlora et al., 2014). A proper spermatogonial identification, isolation and enrichment using specific markers is critical for the advance of the spermatogonial transplantation and to develop new biotechnologies in aquaculture (Lacerda et al., 2012, 2013a,b, 2014).

Germ cell transplantation has three main approaches: the transplantation of primordial germ cells in embryos, the transplantation of germ cells into the intracoelomatic cavity of newly hatched larvae and the direct injection of spermatogonial stem cells into the testes of sexually mature recipient through of their common spermatic duct (reviewed by Lacerda et al., 2013b). The effectiveness of the SSCs transplant in sexually competent fish is determined by the suppression of recipient spermatogenesis. To this end, in fish the use of a treatment associating

water hyperthermic conditions with the application of busulfan (1,4-Butanediol dimethanesulfonate), is effective to eliminate most of endogenous germ cells. Busulfan acts on cells with high proliferative rate, such as germ cells in their early stages, suppressing endogenous spermatogenesis in treated animals. Thus, the treatment generates a privileged microenvironment for donor cells to colonize and develop in the recipient germinative epithelium (Lacerda et al., 2006, 2010; Nóbrega et al., 2010; Majhi et al., 2014).

SSCs population in fish testes is very low (~1%), since fish germinative epithelium present a higher number of differentiated spermatogonial generations when compared to mammals (Lacerda et al 2006). Several methods for spermatogonia purification and isolation have been employed in fish, including discontinuous percoll or ficoll density gradient centrifugation (Lacerda et al., 2010; Panda et al., 2011), differential plating (Lacerda et al., 2010), magnetic activated cells sorting (MACS) (Panda et al., 2011), fluorescence activated cells sorting (FACS) (Nóbrega et al., 2010), flow cytometry (Kise et al., 2012) and centrifugal elutriation (Bellaïche et al., 2014b). Among them, percoll is highlighted as a simple, inexpensive and feasible technique that not require high-priced or special equipment (Lacerda et al., 2006; Koh et al., 2004).

Thus, the aim of this study was to characterize SSC candidates performing immunohistochemistry, western blot analysis and transmission electron microscopy. We also develop a protocol for common carp spermatogenesis depletion, through the combination of hyperthermic conditions and the use of chemotherapeutic agent busulfan. Finally, we established a suitable protocol for spermatogonial isolation and enrichment by sorting these cells using a percoll discontinuous density gradient.

2. MATERIALS AND METHODS

2.1 Ethics statement

All procedures were consistent with Brazilian national regulation and followed the guidelines for the ethical animal treatment, approved by the National council for the control of animal experimentation (CONCEA) and the Ethics committee on animal care and experimentation of São Paulo State University (CEUA), protocol n° 5229/15.

2.2 Experimental animals and rearing procedures

Sexually mature common carp males (mean body weight 150 ± 10g) were kept in 200-liter tanks, at 26°C, under natural photoperiod (12L:12D) and feed with pelleted commercial diet three times per day.

2.3. Spermatogonial stem cell characterization

2.3.1 Immunohistochemistry

In order to characterize the presumptive SSCs, we evaluate the c-Kit, GFRα-1 and POU2 proteins expression, through the immunoperoxidase technique. Testes samples (n=5) were fixed overnight in 4% paraformaldehyde and embedded in paraplast (Sigma Aldrich). 5μm-thick sections were mounted in silane-coated slides. After xylene-dewax and hydration, sections were subjected to antigen retrieval in 1000 ml of buffer-citrate (pH 6.0) for 30 min in Pascal pressure chamber (DakoCytomation). Non-specific binding sites were blocked by incubating the sections with Protein Block Serum-Free (Spring Bioscience, Pleasanton, CA, USA) for 20 min. Endogenous peroxidase was inactivated with BLOXALL blocking solution (Vector Laboratories), incubating the sections for 15 min at room temperature. Subsequently, the slides were incubated overnight at 4°C with the primary antibodies rabbit anti-c-Kit (1:500, CD117, Dako, A4502), rabbit anti-GDNF receptor alpha-1 (1:200, Abcam, ab84106, Cambridge, MA, USA) and mouse anti-POU2 (1:100, Oct 3/4, Santa Cruz, sc-5279). After washing, slides were incubated for 60 min at room temperature with the secondary biotinylated antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Thereafter, sections were revealed using DAB (3,3´-diaminobenzidine) reaction and counterstaining with Harris's hematoxylin. Histological slides were analyzed and photographed using Leica DMI 4000 B microscope. For anti-GDNF receptor alpha-1 immunofluorescence, antigens were detected by incubation with Dylight® 488 (1:500, Abcam, ab96891) for 1h at room temperature. After nuclear counterstaining using DAPI (Sigma Aldrich), sections were mounted with Mowiol 4-88 solution (Sigma Aldrich). In order to quantify the percentage of undifferentiated spermatogonia that expressed c-Kit, GFRα-1 and POU2 proteins, 200 cells were counted per animal (n=5). Undifferentiated spermatogonia were classified based on morphological criteria.

2.3.2 Western blotting

To evaluate the qualitative expression of c-Kit, GFRα-1 and POU2 proteins, immunoblots were performed using the total protein from common carp testes (n=5). Thus, 200 mg of testes were immersed in a solution containing 50 mM Tris–HCl buffer (pH 7.5) - 0.25% Triton-X 100 and mechanically homogenized by Polytron for 30 s at 4°C. Afterwards, the lysates were centrifuged and the protein was extracted from the supernatants, quantified and stored at -80°C. Protein samples (70 μg) were loaded in a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel) and, then was performed electrophoresis under reducing conditions. Successively, the proteins

were transferred into a nitrocellulose membrane (Sigma-CO™, St. Louis, MO, USA). The membranes were blocked using 5% BSA (bovine serum albumin) in TBST (10 mM Tris–HCl, pH 7.5; 150 mMNaCl; 0.1% Tween-20) for 1h at room temperature. Subsequently, the membranes were incubated with 3% BSA in TBST containing the primary antibodies rabbit anti-c-Kit (1:1000, CD117, Dako, A4502), rabbit anti-GDNF Receptor alpha 1 (1:1000, ab84106, Cambridge, MA, USA) and mouse anti-POU2 (1:1000, Oct 3/4, Santa Cruz, sc-5279) overnight at 4°C. Thereafter, the blots were washed five times for 20 min in TBST and then incubated with the corresponding secondary peroxidase-conjugated antibodies (1:5000, goat anti-mouse IgG-HRP, Santa Cruz – 2005 for GFRα-1 and POU2, and 1:5000, goat anti-rabbit IgG-HRP, Santa Cruz – 2030 for c-Kit), for 60 min at room temperature. After washing, a chemi-luminescence substrate kit (Pierce ECL Western Blotting Substrate-GE Healthcare) was used for detection. Substrate was removed from the membranes, and ECL signals were captured by a CCD camera (ImageQuant LAS 4000 mini®, GE Healthcare™). Integrated optical density (IOD) of the target protein bands was measured using Image J software downloaded from the NIH website (http://rsb.info.nih.gov.ij/) to compare the protein levels.

2.3.3 Transmission electron microscopy (TEM)

In order to characterize morphologically the undifferentiated spermatogonia, small fragments of common carp testes (n=5) were fixed in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer (0.1M, pH 7.2) for at least 24 hours and post-fixed in 1% osmium tetroxide buffer phosphate (pH 7.2) for 2 hours. The material was contrasted collectively in 5% aqueous uranyl for 2 hours, dehydrated in acetone, embedded in araldite resin (502 Polyscience Inc®), sectioned and contrasted with saturated uranyl acetate solution in 50% alcohol and lead citrate. The material was examined and documented using a transmission electron microscope (Tecnai Spirit - FEI Company).

2.4. Spermatogenesis depletion protocol

To deplete the recipient endogenous spermatogenesis, a protocol combining high water temperature with cytostatic agent busulfan (Sigma-Aldrich) was used. Before treatment, fishes (n=40) were subjected to an acclimation period of two weeks, when water temperature was gradually increased until reach 35°C. In this study, animals (n=40) received one or two intracoelomatic busulfan injections (40mg/kg/BW), with a two-week interval between injections. The animals (n=40) were divided in four groups: (I) control group, 26°C; (II) high temperature group, 35°C; (III) 35°C with a single busulfan dose and (IV) 35°C with two

busulfan doses. I and II groups received only an intraperitoneal injection of vehicle solution (dimethyl sulfoxide diluted in phosphate-buffered saline solution - PBS).

2.4.1 Sampling and testis histology analysis of busulfan-depleted testis

For biometric and histological analysis, animals were anesthetized with 1% benzocaine and euthanized. Testes were sampled (n=40) and were weighed to determine the gonadosomatic index (GSI= testes weight/body weight ×100). Next, testes were fixed at least 24 h in paraformaldehyde solution at room temperature, dehydrated and embedded in methyl methacrylate (Leica Microsystems). Testes were sectioned at 5μm thickness and the histological sections obtained were stained with 1% toluidine blue. Histological slides were analyzed and photographed using Leica DMI 4000 B microscope. A stereological analysis were performed in order to address the cellular composition in all groups mentioned above (I, II, III and IV). Thus, at least 10 fields were randomly selected and analyzed for each fish in each group. To this end, cysts of undifferentiated and differentiated type A spermatogonia, type B spermatogonia, spermatocytes, spermatid, spermatozoa and Sertoli cells (when alone) were counted. Results were expressed as percentage of the total number of counted structures.

2.4.2 *amh* expression in busulfan-depleted spermatogenesis

Total testis RNA was extracted according to the TRIzol method (Invitrogen, Carlsbad, CA, USA) and FastRNA Pro Green Kit (MP Biomedicals, Solum, OH, USA), following the manufacturer's recommendations. To avoid genomic DNA contamination, RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) prior to cDNA synthesis. The cDNA synthesis was performed with random hexamers using Supercript II (Invitrogen, Carlsbad, CA, USA). qPCR were conducted using specific primers for common carp amh (NCBI: M24113.1): CarpFw4144amh: 5'-ACAAAAAACGAAGTGGCCTCAA-3' and CarpRv4145amh: 5'-TCCTTCTCAGCATCCCAAATATCT-3' amplified a product of 100 bp. For qPCR, Cq values for amh were determined using SYBR Green kit (Invitrogen) while Cq values for the reference gene (eukaryotic 18S rRNA) expression were determined with an Invitrogen assay in combination with an Universal TaqMan kit (Invitrogen). All qPCR reactions (20 μl) were performed in a StepOne system (Life Technologies) following the manufacturer's instructions, and relative gene expression profiles were calculated, according to the ΔΔCt method as described previously by Vischer et al. (2003). amh expression was evaluated in animals of the following three groups: (I) control group, 26°C; (II) high temperature group, 35°C - temperature effect only; and (III) 35°C with a single or two busulfan doses.

2.5. Percoll gradient and SSC candidate isolation

To perform spermatogonia isolation we used the protocol described for Nile tilapia (Lacerda et al., 2006, 2010). Briefly, testes (n=5) of sexually mature common carps were collected, finely cut, rinsed in HBSS (Hank´s buffered salt solution), and dissociated with 0,2% collagenase in DMEM/F12 medium for 3h at 26°C and 80 rpm. The solution was centrifuged for 5 min at 1200 rpm, re-suspended in 10 ml trypsin/1mM EDTA and incubated for 30 min at 26 ° C and 80 rpm. To inactivate trypsin was used 10% of fetal bovine serum. Next, the solution was filtered using a 70μm mesh, centrifuged for 10 min at 1200 rpm and re-suspended in DMEM-BSA 0.75% medium. percoll discontinuous density gradient technique was used to isolate a large amount of type A undifferentiated spermatogonia. Percoll solution was diluted in density gradients 15%, 25%, 30% and 40% and placed in a 15ml falcon tube from the higher (40%) to lower (15%) gradient. Subsequently, the solution obtained from the enzymatic digestion was added on the top and centrifuged for 30 min (1200rpm) at 25°C. After centrifugation were identified six different bands containing the germ cells. For morphological analyses the bands were collected separately and placed in Eppendorf tubes, rinsed in DMEM-BSA 0.75%, centrifuged, re-suspended and fixed for 24h at 4°C in 5% buffered glutaraldehyde (0.05M phosphate buffer, pH 7,3). The material was dehydrated and embedded in methyl methacrylate (Leica Microsystems), sectioned at 3μm thickness and stained with 1% toluidine blue. Histological slides were analyzed and the germ cells of each percoll layer were photographed using Leica DMI 4000 B microscope. The cellular concentration of each percoll layer was determined microscopically by counting the cells number, employing a Neubauer hemocytometer containing 5 μl cell solution and 5 μl of 0.4% trypan blue, at 400× magnification under a Leica DMI 4000 B microscope. The number of cells was counted in five squares of the Neubauer chamber with two repetitions. The cell content was expressed as the absolute number of cells obtained from each percoll layer by multiplying the number of cells by the volume of each cell sample (10 μl).

2.6. Statistical analysis

Results were expressed as mean values ± SEM. Significant differences between two groups were identified using Student test (paired and unpaired) ($p<0.05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($p<0.05$). Data management and analysis were carried out using Graph Pad Prism 6.0.

3. RESULTS

3.1 SSC putative candidates characterization

3.1.1 Immunohistochemistry analysis

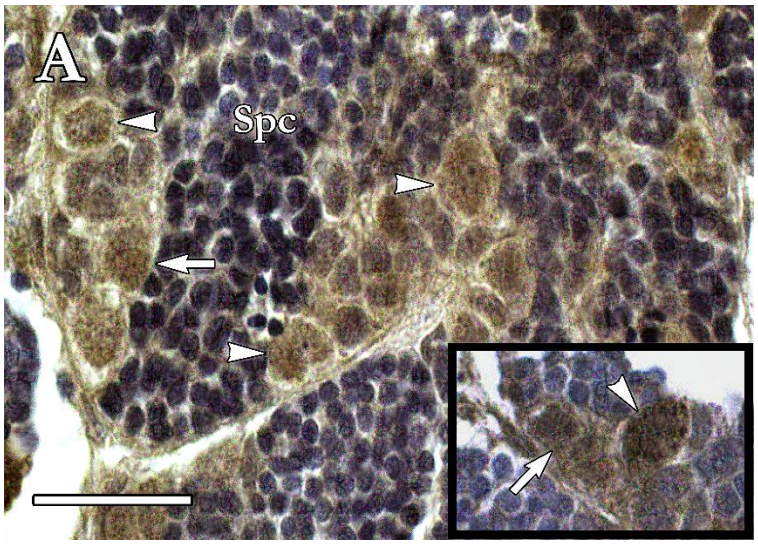
Immunohistochemistry analyses showed that c-Kit, GFRα-1 and POU2 proteins were expressed in germ cells. A strong immunoreactivity was observed for the three evaluated molecular markers. GFRα1 was detected in the cell membrane of the labeled cells, while c-Kit and POU2 immunostaining were observed mainly in cytoplasm (Figure 1A,B,C,D). Our results showed that GFRα1 and POU2 proteins were predominantly expressed in type A undifferentiated spermatogonia populations (Figure 1B,C,D). On the other hand, we observed that type A differentiated spermatogonia and other advanced germ cells showed less or no expression of these proteins (Figure 1B,C,D). In contrast, c-Kit protein expression was detected not only in undifferentiated spermatogonia but also in more advanced cell generations such type A differentiated spermatogonia and type B spermatogonia (Figure 1A).

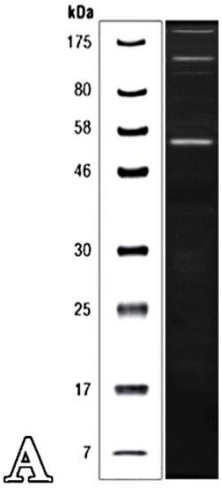
Further quantitative analysis performed through morphological criteria showed that at least 94,11% ($p<0.05$) of type A undifferentiated spermatogonia presented a positive immunostaining for c-Kit (Figure 2). For GFRα1 and POU2 was founded that 95,38 and 96,46% ($p<0.05$) of type A undifferentiated spermatogonia showed positive immunoreactivity for these markers respectively (Figure 2).

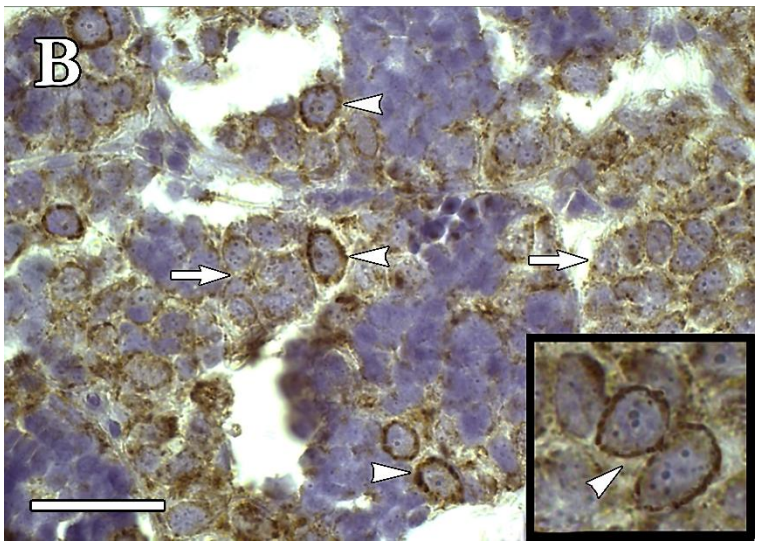
3.1.2 Western blot analysis

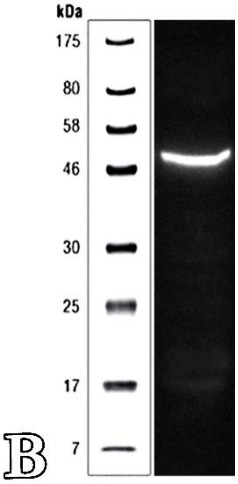
The specificity of c-Kit, GFRα-1 and POU2 antibodies to recognize common carp c-Kit, GFRα-1 and POU2 proteins was demonstrated by western blot analysis using total lysates from adult testes. The analysis of c-Kit, GFRα-1 and POU2 proteins expression showed a band pattern at the expected molecular weight of approximately 190, 145, 114 and 56 kDa for c-Kit; 50 kDa for GFRα1; 52 and 45 kDa for POU2 (Figure 1A, B, C).

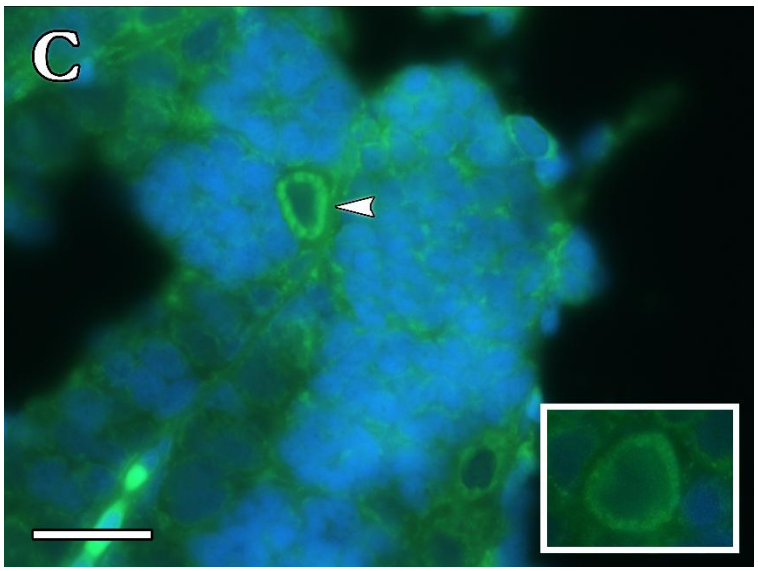
According to our results, the band localization showed a single and specific band for GFRα1 and two specific bands for POU2 proteins (corresponding to A and B protein isoforms) (Figure 1B, C). On the other hand, c-Kit showed a pattern of four specific bands (Figure 1A). These results suggest that GFRα-1 and POU2 can be considered as potential specific molecular markers to identify undifferentiated spermatogonia.


A
Spc


kDa
175
80
58
46
30
25
17
7
A


B


kDa
175
80
58
46
30
25
17
7
B


C

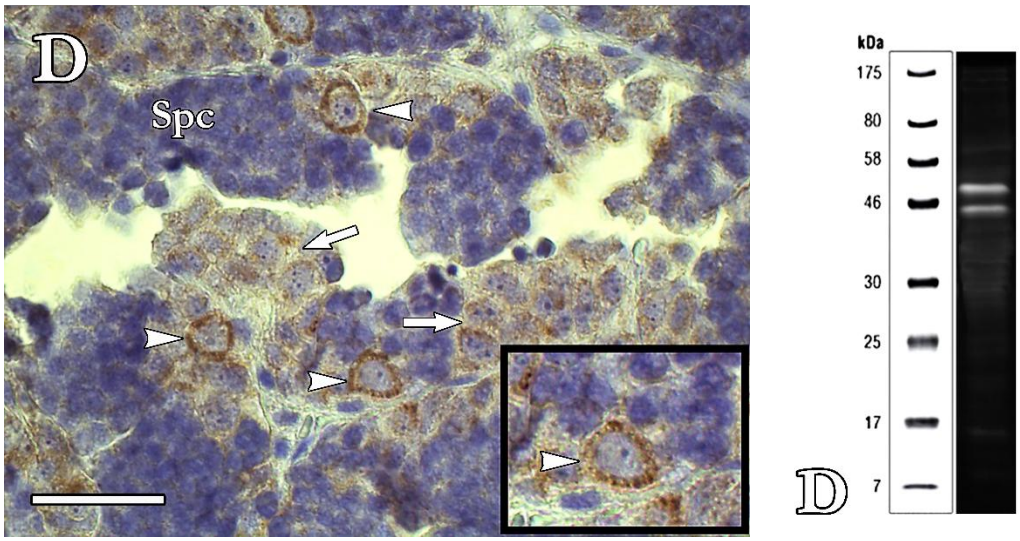


Figure 1. Immunohistochemical localization of c-Kit, Gfrα1 and POU2 proteins in spermatogonial cells in common carp testes. **(A)** c-Kit immunolabeling was detected by both undifferentiated and differentiated spermatogonia (arrowheads and arrows respectively). The inset in A at higher magnification shows a c-Kit-positive undifferentiated and differentiated spermatogonia. Immunoblotting analysis validating the c-Kit protein expression (~190, 145, 114 and 56 kDa) in common carp testes. **(B)** Immunoreactivity of Gfrα1 protein was founded preferentially in undifferentiated spermatogonia (arrowheads). In contrast, Gfrα1 immunoreactivity was less detected in cysts of differentiated spermatogonia (arrows). The inset in B illustrates at higher magnification Gfrα1-positive undifferentiated spermatogonia (arrowheads). Specific expression of Gfrα1 protein (~50 kDa) confirmed through immunoblotting analyses. **(C)** Immunofluorescence staining of the membrane receptor Gfrα1 in undifferentiated spermatogonia. Inset in C at higher magnification showing a Gfrα1-positive undifferentiated spermatogonia. **(D)** POU2 immunolabeling was founded in undifferentiated spermatogonia (arrowheads), whereas POU2 immunoreactivity was less detected in differentiated spermatogonia (arrows). Inset in D at higher magnification showing a POU2-positive undifferentiated spermatogonia. Specific POU2 protein expression (~45 and 52 kDa) validated by immunoblotting analysis. (Spc) spermatocytes. Scale bars: 25μm.

Conversely, due to the banding pattern founded in c-Kit, probably this molecular marker is less specific to identify undifferentiated spermatogonia, because other more advanced germ cell types express this protein. In this study, c-Kit protein was mainly expressed by undifferentiated and differentiated spermatogonia.

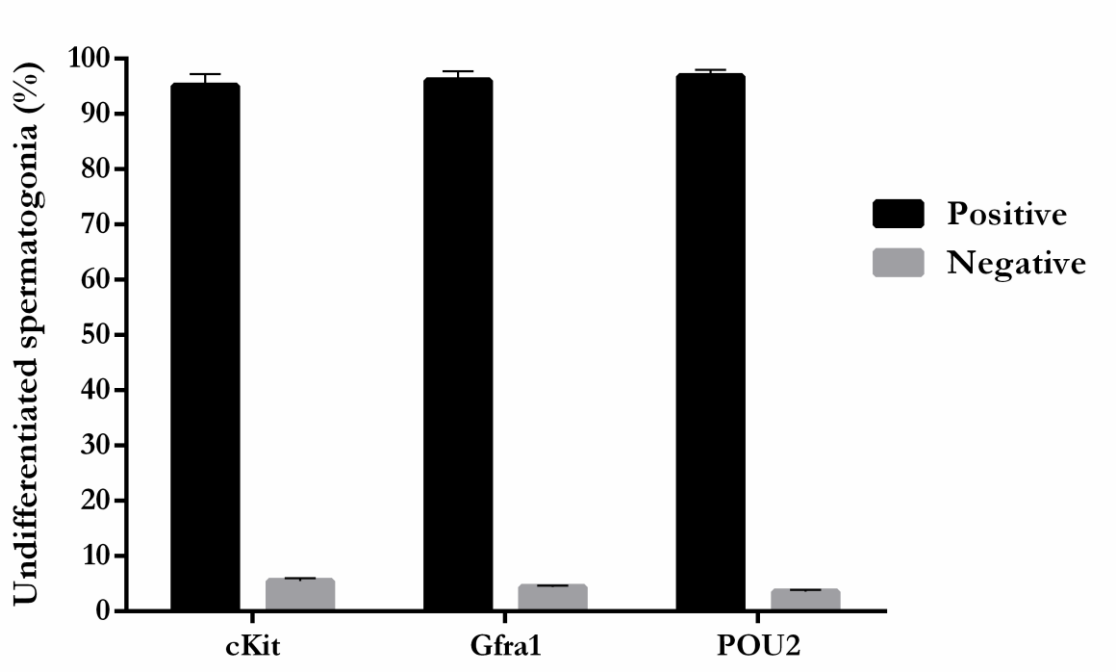


Figure 2. Quantitative analysis demonstrated that at least 94,11%, 95,38% and 96,46% of undifferentiated spermatogonia showed a positive signaling for c-Kit, Gfrα1 and POU2, respectively ($p<0,05$).

3.1.3 Transmission electron microscopy (TEM)

Type A undifferentiated spermatogonia (A_{und}) is the largest germ cell founded in common carp germinal epithelium (Figure 3A, B). These cells were found isolated and completely surrounded by Sertoli cells cytoplasmic extensions (Figure 3A). Aund is not linked by cytoplasmic bridge to other germ cell. These cells show a large and pale nucleus, irregular nuclear envelope with one or two electron-dense nucleoli (Figure 3A, B). The chromatin is thin, slightly condensed and distributed homogeneously (Figure 3A, B). The cytoplasm is clear, volumous, scarcely differentiated and showed high density of mitochondria associated to an electron-dense material (nuage) (Figure 3A, B). Generally, the mitochondria was surrounded by smooth endoplasmic reticulum (Figure 3C, D).

3.2 Spermatogenesis depletion, hyperthermia and busulfan effects on testes

As shown in Figure 4, testes from groups III and IV presented macroscopically a drastic size reduction and a clear coloration as compared to testes from groups I and II. Interestingly GSI analysis of animals from III and IV groups revealed a significant decrease when compared with I and II groups (Figure 5). Nevertheless, we found that GSI decrease was more pronounced in the animals of group IV, which corresponds to those that received two busulfan doses (Figure 5). Histological analysis showed a clear temperature and busulfan effect (Figure 6).

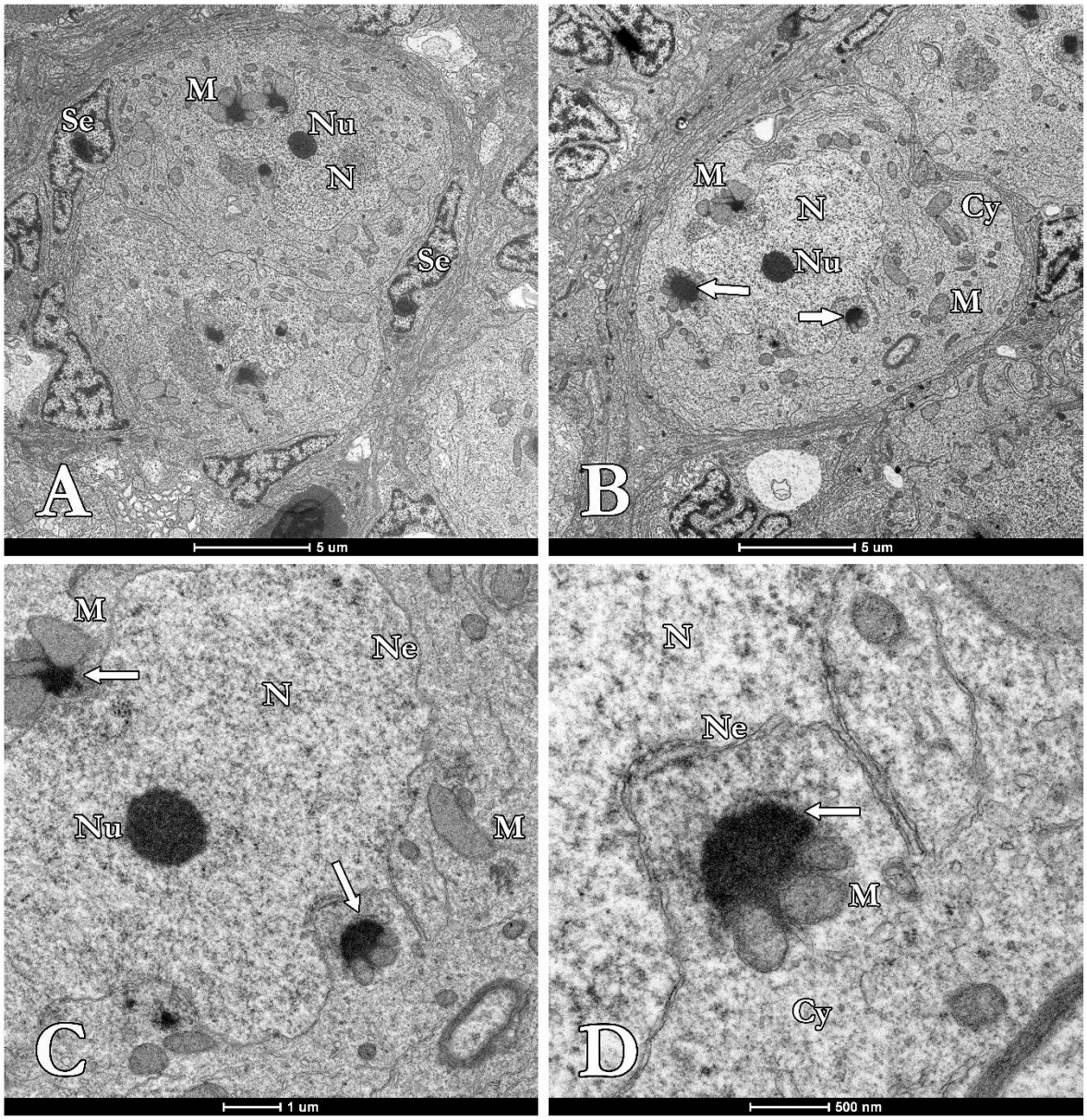


Figure 3. Transmission electron microscopy of common carp testis sections. **(A)** Type A undifferentiated spermatogonia (Aund) surrounded by cytoplasmic extensions of Sertoli cells. **(B)** Aund ultrastructure showing nucleus, nucleoli, cytoplasm, mitochondria and nuages close to the nucleus (white arrows). **(C-D)** Detail of Aund nucleus, showing nucleoli, nuages (white arrows) and mitochondria. (Cy) cytoplasm, (M) mitochondria, (N) nucleus, (Ne) nuclear envelope, (Nu) nucleoli, (Se) Sertoli cells.

Histological analysis showed normal spermatogenic progression in the testes of group I (26°C). In the seminiferous tubules of these animals, spermatogenic cysts at different stages of developmente were seen (Figure 6A). Interestingly, in testes of group II, the exposure to higher temperatures (35°C) did not affect the spermatogenesis process (Figure 6B). Thus, these animals showed all cellular types in the germinal epithelium (Figure 6B). Another striking result emerging from this data is that high temperatures were associated to cellular abnormalities, such as, apoptotic regions and a perceptible GSI decrease (Figures 5, 6B).

Surprisingly, some animals treated with a single busulfan doses (group III) were less affected by the treatment (Figure 6C). However, we found that generally most of animals treated with a single dose of busulfan exhibited several areas with massive apoptosis in their germinal epithelium (Figure 6C). On the other hand, in males of group IV (two busulfan doses), the treatment was more effective on inducing a severe cell apoptosis and tissue degeneration (Figure 6D). Interestingly, the association of higher temperature and two doses of busulfan was able to suppress completely endogenous spermatogenesis (Figure 6D).

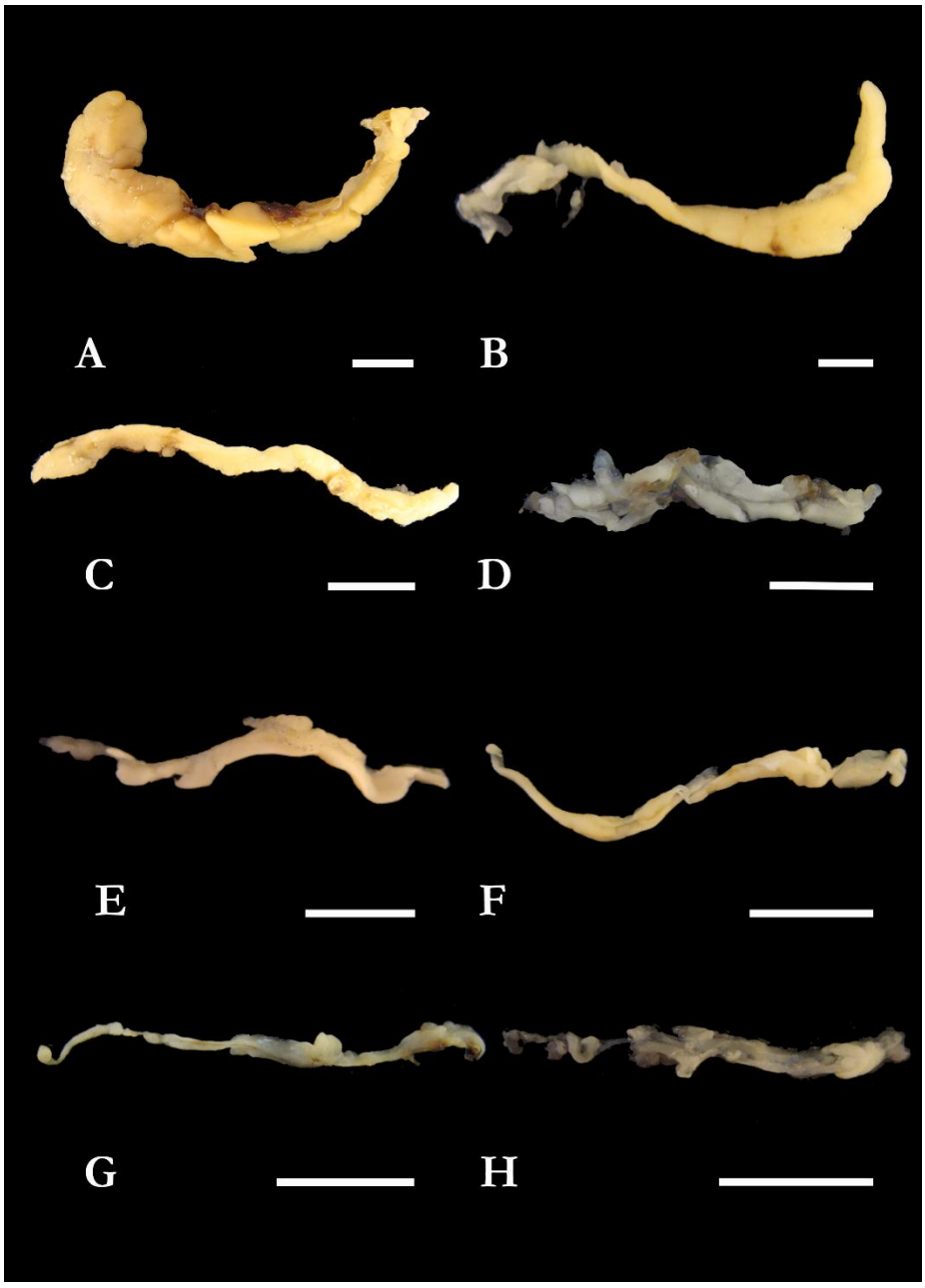


Figure 4. Macroscopic photographs of common carp (*C. carpio*) testes subjected to the different treatments. **(A - B)** Control group I, 26°C; **(C - D)** High temperature group II, 35°C; **(E -F)** busulfan 1 dose group III, association of busulfan and high temperature 35°C; **(G - H)** busulfan 2 doses group IV, association of busulfan and high temperature 35°C. Scale bars: 1cm.

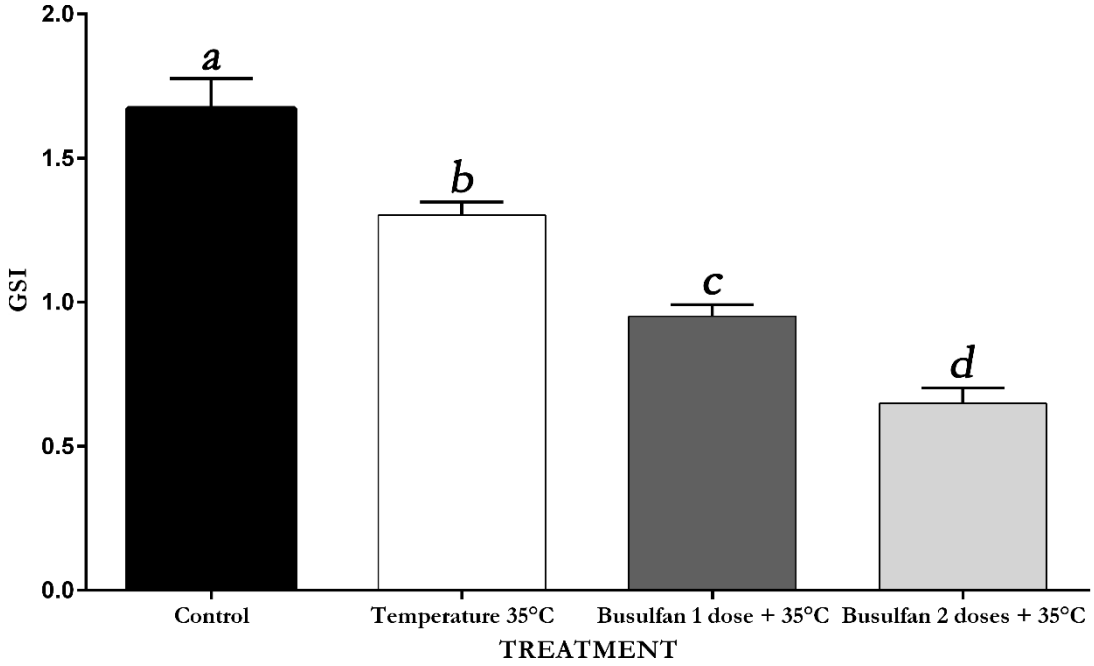


Figure 5. Effects of busulfan and high temperature on common carp gonadosomatic index (GSI). Bars represent mean ± SE. Different letters indicate significant differences ($p<0.05$) among groups.

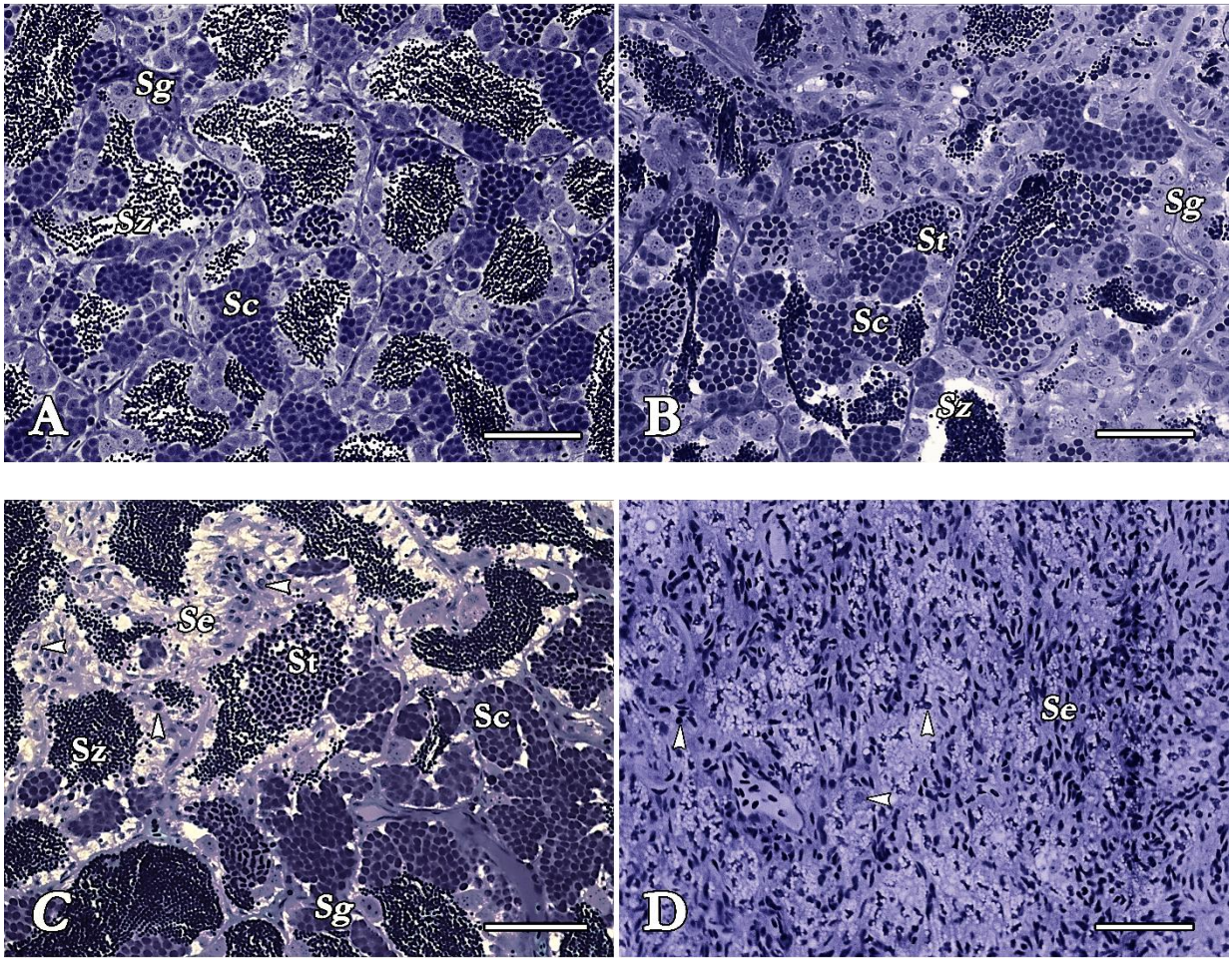


Figure 6. Histological sections of common carp testes collected at 28 days after a single or two busulfan doses (40 mg/Kg/BW) at 35°C and untreated controls at 26°C. The association of busulfan and high temperatures induced

germ cell loss in the treated individuals. **(A)** control group I, 26°C; **(B)** high temperature group II, 35°C; **(C)** busulfan 1 dose group III, in association with high temperature 35°C; **(D)** busulfan 2 doses group IV, in association with high temperature 35°C. Germ cell apoptosis was observed in busulfan-treated testes (**C-D**, arrowheads) Sertoli cell only (Se), spermatocytes (Sc), spermatogonia (Sg), spermatids (St), spermatozoa (Sz). Scale bars: 50μm.

Thus, those animals that received the treatment presented in their spermatogenic tubules only somatic cells, virtually no germ cells were found (Figure 6D). Taken together, these results suggest that there is a clear spermatogenic suppressive effect caused by the association between temperature and the chemotherapeutic action of busulfan.

Stereological analysis showed that only the busulfan-depleted testes presented a marked gonadal reduction, with a significant germ cell loss compared to control. The animals treated with a single dose of busulfan showed a perceptible germ cell reduction of 48,7% (Figure 7). However, the treatment with two busulfan doses resulted in a drastic gonadal depletion of 98,2%, and the seminiferous tubules exhibited a Sertoli cell only phenotype (Figure 7). Moreover, in control group, no sign of depletion was observed and the animals showed normal spermatogenesis (Figure 7).

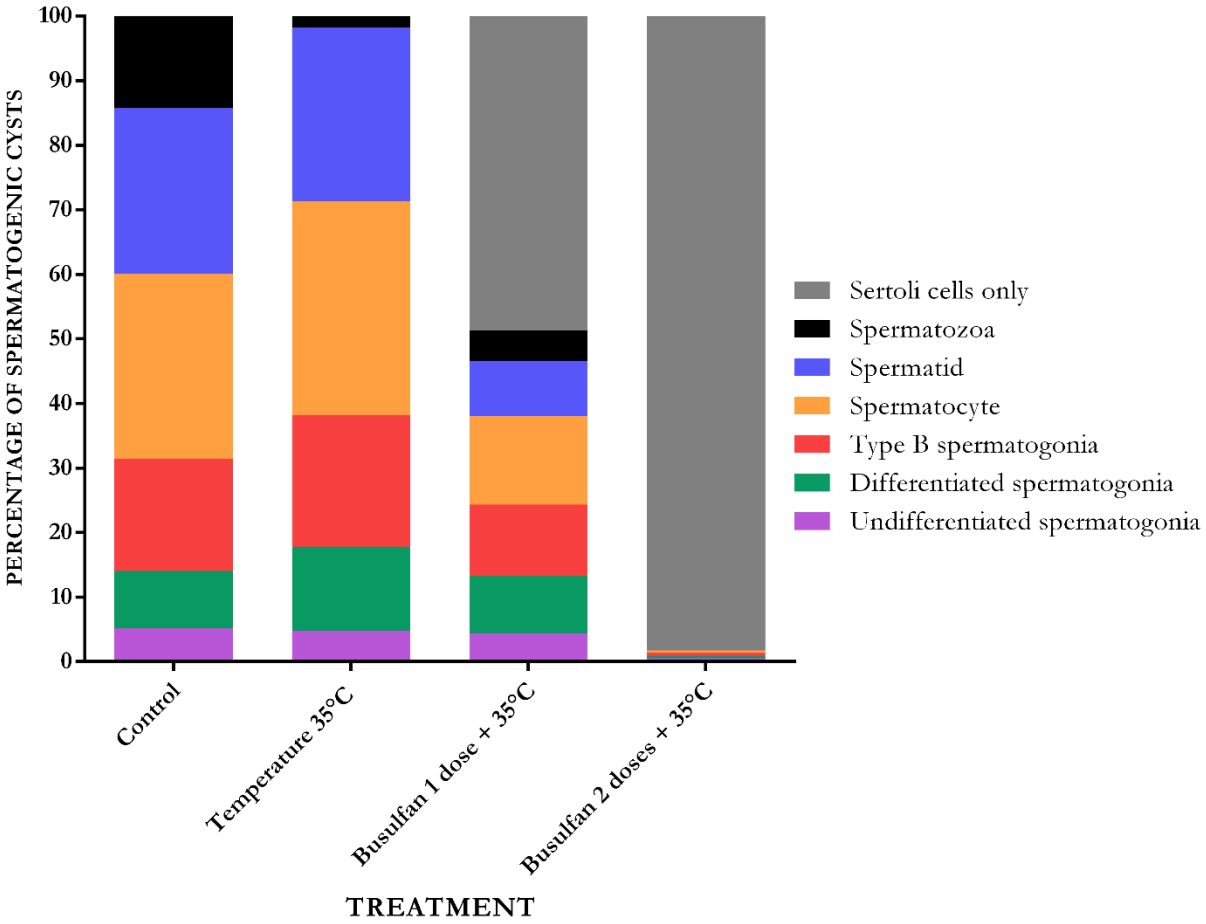


Figure 7. Frequency of spermatogenic cysts after a single or two busulfan doses (40 mg/Kg/BW). Bars represent means expressed as percentage.

3.2.1 *amh* expression in busulfan-depleted spermatogenesis

As shown in the figure 8, *amh* gene expression analysis showed significant differences in mRNA expression between group I (control, 26°C) and III (35°C with a single or two busulfan doses). Downregulation of *amh* confirmed endogenous spermatogenesis depletion when using high water temperatures with busulfan. Depleted testes showed a substantial decrease of *amh* mRNA, since spermatogonia are present in very low quantities (Figures 6, 7).

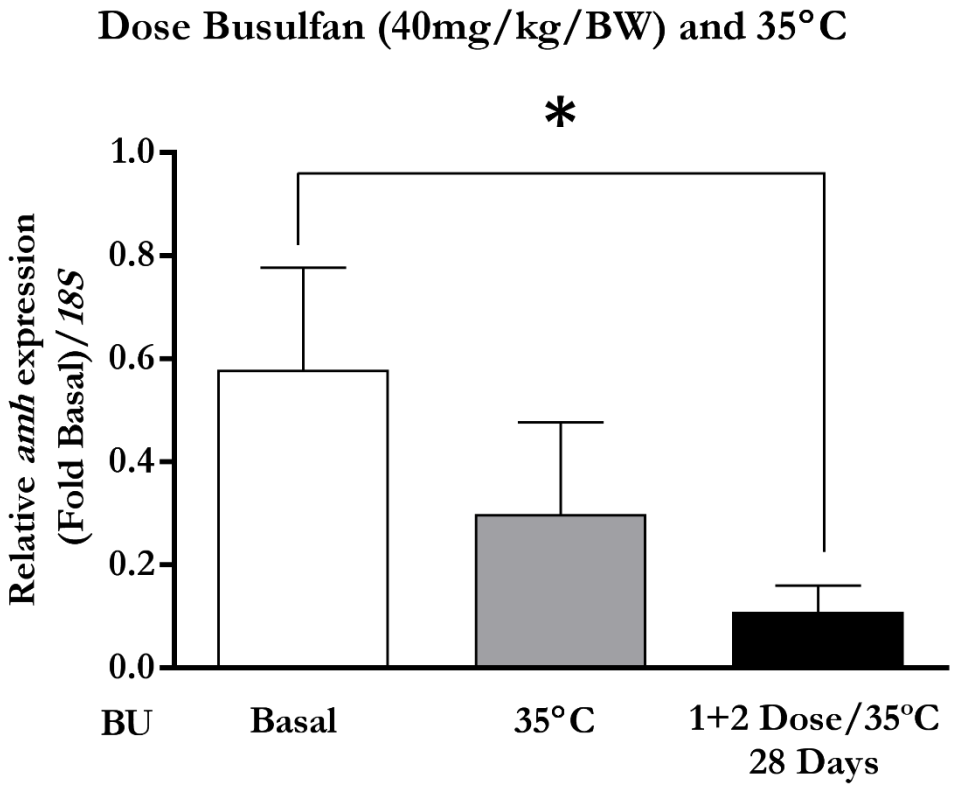


Figure 8. *amh* expression in spermatogenesis depletion. The expression was normalized by *18S* reference gene and the values are expressed in relation to basal levels. Bars represent the mean ± SE of relative gene expression. Asterisks (*) denote significant differences ($p < 0.05$) between treatment and basal.

3.3 Isolation and enrichment of SSC putative candidates

Due to the low amount of type A undifferentiated spermatogonia in carp testis, we tried to remove the somatic cells and mature germ cells from the enzymatically digested testes suspension through a percoll discontinuous density gradient. As result of enzymatic digestion, we obtained a heterogeneous cell suspension, which was observed under optical microscope. Further analysis identified six bands between percoll interfaces. The concentration of undifferentiated spermatogonia in the second and third bands were significantly higher than in

the other bands. According to morphological analysis, in the second and third band were observed a higher concentration of viable undifferentiated spermatogonia, included type A undifferentiated and differentiated spermatogonia (Figure 9B,C).

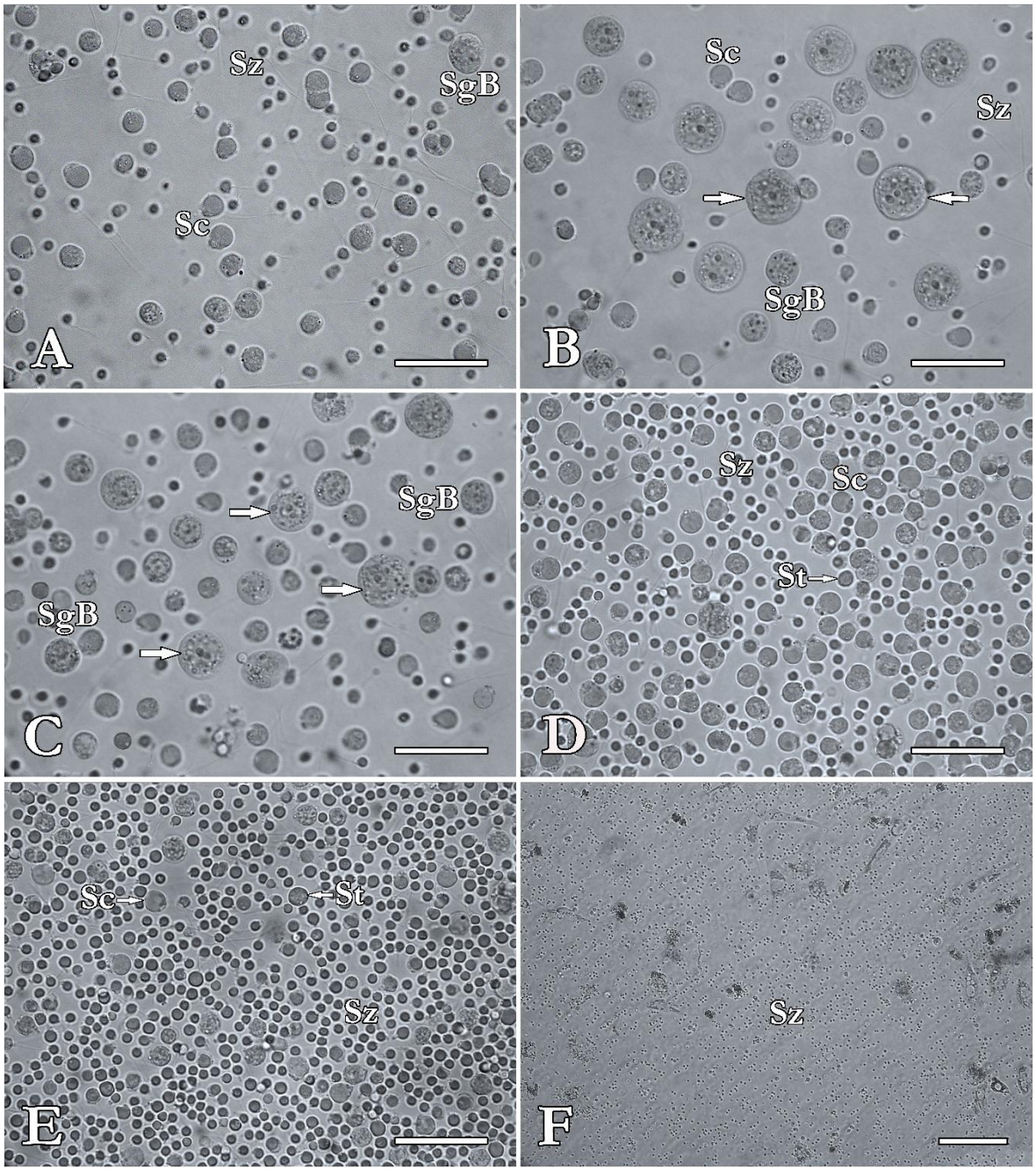


Figure 9. Selection and isolation of common carp germ cells through percoll discontinuous density gradient. Six distinct bands are present in the Falcon tube. The material of each band was fixed in 5% buffered glutaraldehyde and embedded in glycol methacrylate. First, second and third band were formed by spermatogonia type A and B, spermatocytes, spermatids and spermatozoa **(A-B-C)**. Second and third bands showed a higher concentration of undifferentiated spermatogonia **(B-C, arrows)** than other bands. Fourth and fifth band were predominantly formed by type B spermatogonia, spermatocytes, spermatids and spermatozoa **(D-E)**. Sixth band was formed by

spermatozoa, somatic cells and cellular residues **(F)**. Spermatocytes (Sc), spermatogonia type A (SgA), spermatogonia type B (SgB), spermatids (St), spermatozoa (Sz). Scale bars: 25μm. Scale bar in F: 100μm**.**

First band contained approximately 9% undifferentiated spermatogonia, 25% type B spermatogonia, 44% spermatocytes, 11% spermatids and spermatozoa (Figure 9A, 10). Second band was formed approximately by 19% undifferentiated spermatogonia, 12% type B spermatogonia, 28% spermatocytes, 41% spermatids and spermatozoa (Figure 9B, 10). Third band was formed of about 17% undifferentiated spermatogonia, 11% type B spermatogonia, 41% spermatocytes, 31% spermatids and spermatozoa (Figure 9C, 10). Fourth band contained 6% type B spermatogonia, 39% spermatocytes, 55% spermatids and spermatozoa (Figure 9D, 10). Fifth band contained approximately 2% type B spermatogonia, 9% spermatocytes, 89% spermatids and spermatozoa (Figure 9E, 10). Finally, sixth band was formed approximately by 95% spermatozoa, also was identified spermatids, somatic cells and cellular residues (Figure 9F).

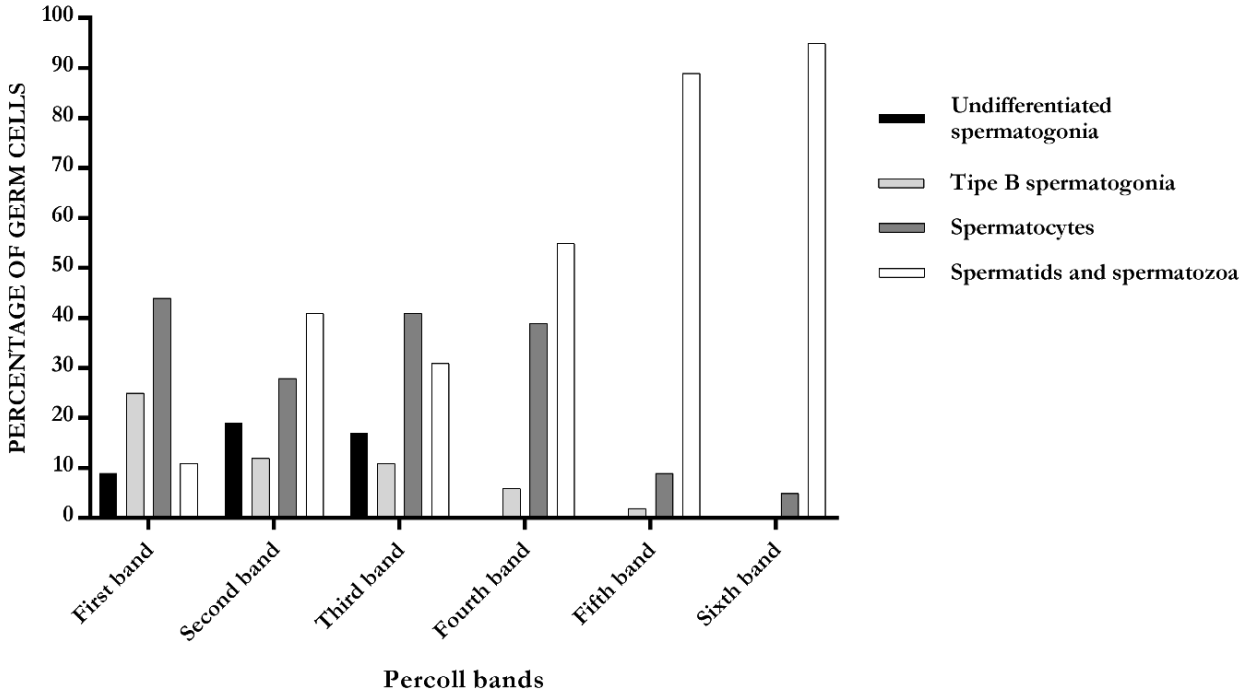


Figure 10. Frequency of germ cells found in the percoll bands. Bars represent means expressed as percentage.

4. DISCUSSION

4.1 SSCs characterization

Spermatogonial identification in teleost fish is currently based on morphological criteria (Leal et al., 2009; Nóbrega et al., 2009; Schulz et al., 2010; Schulz and Nóbrega, 2011). According to the literature, the primary or type A spermatogonia contain putative stem cell elements. These

cells are the responsible to sustain the spermatogenic process through their potential of self-renewal and differentiation.

In fish, spermatogenesis shows a cystic arrangement; cysts are formed when a single, type A undifferentiated spermatogonia (Aund) is completely surrounded by one or two Sertoli cells (Schulz et al., 2010; Schulz and Nóbrega, 2011). Morphologically Aund is the largest cell of the germinal linage in the germinative epithelium (Schulz et al., 2010; Schulz and Nóbrega, 2011). These cells have a prominent clear nucleus with one or two electron-dense nucleoli, poorly condensed chromatin and an irregular nuclear envelope. Their cytoplasm is volumous, scarcely differentiated and showed high density of mitochondria associated to a large amount of electron-dense material called "nuage" and the mitochondria was usually surrounded by smooth endoplasmic reticulum (Schulz et al., 2010; Schulz and Nóbrega, 2011). These electron-dense material or nuages are located between the nucleus and the mitochondria. Presumably nuages are composed by ribonucleoproteins and long half-life time mRNA. Remarkably, these nuages are a hallmark shared by primordial and early germ cells (Schulz et al., 2010; Schulz and Nóbrega, 2011). In this context, some morphological criteria such nuclear features, heterochromatin and cytoplasmic organelles distribution, were used to identify Aund in common carp testis.

In mammals, several molecular markers such as cell surface proteins, cytokines, transcription factors and others have been used to identify phenotypically the SSCs specially in rodents and domestic species (Kolasa et al., 2012; Abid et al., 2014; Zheng et al., 2014).

Among the multiple detected markers, c-Kit, GFRα-1 and POU5f1 are proteins known to be closely involved with the regulation and fate of undifferentiated germ cells. c-Kit is a transmembrane tyrosine kinase receptor and its ligand stem cell factor (SCF) are involved in the development and differentiation of germ cells. This receptor has an important function in the regulation of differentiated spermatogonia (Ohta et al., 2000; Rossi et al., 2000). The glial cell-derived neurotrophic factor (GDNF) and their membrane receptor GFRα-1 form the GDNF–GFRα-1 pathway, that has a major role in the control, regulation and signaling of the spermatogonial self-renewal and maintenance. GFRα-1 receptor is considered an important marker of undifferentiated spermatogonia in mammals (Hofmann et al., 2005, 2008; Oatley and Brinster, 2008). Oct-4/POU5f1 protein is a class V POU domain transcription factor (PouV) involved in the regulation of pluripotency activity and self-renewal expressed in primordial germ cells and early germ cells (Sánchez et al., 2010; Froschauer et al., 2013).

These proteins have been studied in different species such as primates (Gassei et al., 2010; He et al., 2010; Hermann et al., 2010), rodents (Von Schönfeldt et al., 2004; Dann et al., 2008),

ruminants (Mahla et al., 2012), felines (Powell et al., 2012; Vansandt et al., 2012) and equids (Costa et al., 2012). In contrast, there is a lack of specific markers for phenotypic and/or genotypic identification and isolation of SSCs in fish, limiting their use in aquaculture and biotechnological applications (Lacerda et al., 2012, 2013b, 2014).

Based on this, the current have characterized SSCs in the common carp testis. To this end, we used the c-Kit, GFRα-1 and POU2 (fish homologue of mammalian POU5f1/Oct4) proteins as potential SSC molecular markers. Through immunohistochemistry and western blot analysis, we found the expression of these markers in early germ cells. Likewise, studies in teleost species, such as blue catfish (Shang et al., 2015), channel catfish (Shang et al., 2015), medaka (Hong et al., 2004; Sánchez et al., 2010; Froschauer et al., 2013), Nile tilapia (Lacerda et al., 2013b), rohu carp (Panda et al., 2011), dogfish (Loppion et al., 2008; Bosseboeuf et al., 2013) and rainbow trout (Bellaïche et al., 2014a,b; Nakajima et al., 2014) have demonstrated the expression of the same markers in early germ cells.

Moreover, we showed in common carp testis that GFRα-1 and POU2 proteins are expressed in the most undifferentiated germ cells, preferentially type A undifferentiated spermatogonia. In fact, at least 95,38 and 96,46% of type A undifferentiated spermatogonia showed positive immunolabeling for GFRα-1 and POU2 respectively. These results are in agreement with those obtained in medaka (Hong et al., 2004; Sánchez et al., 2010; Froschauer et al., 2013), Nile tilapia (Lacerda et al., 2013b), dogfish (Loppion et al., 2008; Bosseboeuf et al., 2013) and rainbow trout (Bellaïche et al., 2014a,b; Nakajima et al., 2014).

GFRα-1 and POU2 protein expression was founded mainly in undifferentiated spermatogonia. According to these data, we can infer that GFRα-1 and POU2 are presumably expressed by the same cell type, which can be considered as potential stem cells in common carp. In the current study, we found that GFRα-1 and POU2 expression decreasing dramatically in more advanced germ cells stages (e.g. type B spermatogonia labeling is completely undetectable), these observations match those observed in the abovementioned teleost species. We confirmed the expression of these factors through western blot technique, validating the specificity of the results obtained in the immunohistochemistry for GFRα-1 and POU2 proteins, showing also an approximate molecular weights of 50 kDa GFRα-1 and 52 - 45 kDa for POU2 (A and B protein isoforms respectively). A recent study in Nile tilapia confirmed our observations, in which the immunoblot analysis showed the same band pattern of ~50 kDa described for common carp (Lacerda et al., 2013b). Several studies have shown that these molecules are key factors involved physiologically in spermatogonial niche regulation in vertebrates.

To date, the expression profiles of GFRα-1 and POU2 transcripts or proteins, the regulatory mechanisms that controlled the fate of the early germ cells and their role in the gonadal functions have been focused primarily in mammals, mainly in rodents (Ohta et al., 2000; Rossi et al., 2000; Dann et al., 2008; Hofmann et al., 2008).

Despite their importance in spermatogenesis, there are few studies addressing this topic in teleost fish. Studies shows that GFRα-1 and POU2 expression can be found in the same area of the germ compartment of phylogenetically distant species. An interesting fact is that in medaka, Nile tilapia and common carp (current work) was observed a similar expression pattern for GFRα-1 and POU2 to that found in rodents (Sánchez et al., 2010; Lacerda et al., 2013B). These results provide further support for the hypothesis that GFRα-1 and POU2 may be considered as relatively well-conserved functional factors among vertebrates (mammals and teleosts). These elements and their evolutionarily conserved functions are fundamental in niche regulation, signaling pathways and control of early spermatogenesis stages (Loppion et al., 2008; Sánchez et al., 2010; Panda et al., 2011; Bosseboeuf et al., 2013; Lacerda et al., 2013b; Bellaïche et al., 2014a,b; Nakajima et al., 2014).

In rodents, the tyrosine kinase receptor c-Kit is known to stimulate primordial germ cell development, and spermatogonial proliferation, differentiation and maintenance (Ohta et al., 2000; Rossi et al., 2000; Von Schönfeldt et al., 2004; Dann et al., 2008). Currently few studies have addressed the role of c-Kit in fish spermatogenesis, showing that this factor displayed the same functional characteristics as related in mammals. c-Kit expression in fish germ cells was recorded in medaka (Hong et al., 2004), rohu carp (Panda et al., 2011) and dogfish (Loppion et al., 2008; Bosseboeuf et al., 2013). Regarding to c-Kit protein expression, our results are in line with dogfish (Loppion et al., 2008), in which c-Kit was expressed in both undifferentiated and dfferentiated spermatogonia. Additionally, we found a positive signal for c-Kit protein through western blot. Immunoblot analysis revealed a pattern of four specific bands with a molecular weight of ~190, 145, 114 and 56 kDa, which presumably correspond to different forms of the same protein expressed by different germ cell types.

In the current study, the quantitative morphological analysis showed that 94,11% ($p<0.05$) of type A undifferentiated spermatogonia showed c-Kit positive immunostaining. Our results corroborate the ideas of Loppion et al. 2008, who found that this protein showed five forms (41, 52, 56, 83, 114, and 190 kDa). In this context, c-Kit in rodents presented multiple forms and the labelled bands are rather broad, from 33 to 190 kDa (Dym et al., 1995; Feng et al., 1997). As well as in rats, proteins of ~114 and 190 kDa found in this study presumably correspond to immature and mature forms of c-Kit (Dym et al., 1995).

4.2 Spermatogenesis depletion

In teleost fish, spermatogenesis was successfully depleted, through association of high temperatures and the use of chemotherapeutic agents as busulfan. This approach has been scarcely used particularly in juvenile or adult individuals of few species (Lacerda et al., 2006, 2010; Nóbrega et al., 2010; Majhi et al., 2009a, 2009b, 2014; Silva et al., 2016).

In contrast, the use of chemotherapeutic drugs has been widely reported as treatment to suppress spermatogenesis in rodents (Brinster et al., 2003; Ogawa et al., 2003; Zhang et al., 2003), monkeys (Hermann et al., 2007, 2012), pigs (Honaramooz et al., 2005; Mikkola et al., 2006) and goats (Honaramooz et al., 2005).

However, unlike mammals, chemotherapy alone seems to be ineffective to suppress the spermatogenic activity in fish. Thus, rainbow trout busulfan-treated individuals did not show signs of testicular tissue depletion. The reason for this is that in mammals the body temperature acts synergistically with the drug to exert their suppressive effect in the testes (Billard, 1982).

It is well known that physiological processes in fish are significantly influenced by temperature, thus, in some teleost fish species high temperature appears to have a harmful effect on testicular germ cells (Lacerda et al., 2006, 2010; Nóbrega et al., 2010; Majhi et al., 2009a,b, 2014).

In our work, individuals subjected to high temperatures (35°C) showed an apparent normal spermatogenic cycle, although germ cell loss was seen in the seminiferous tubules, similarily with data obtained in Nile tilapia (Lacerda et al., 2006, 2010).

On the other hand, Majhi et al. (2009a) observed that after 8 weeks high temperature (25°C) caused gonadal tissue regression in Patagonian pejerrey *Odontesthes hatcheri*, in both males and females. In this species, the major morphological changes included a lack of all intermediary germ cell types from spermatogonia to spermatozoa in males; and ovarian regression, tunica thickening and ovigerous lamella hypertrophy in females.

Ito et al. (2008a,b) reported similar results, thus, elevated water temperatures caused gonadal degeneration in a related species, pejerrey *Odontesthes bonariensis*. Fishes exposed to high temperatures showed partially or completely loss of their germinal cells.

In contrast, we found that hyperthermic conditions were not effective to suppress the common carp spermatogenesis. Nevertheless, we found several apoptotic areas in the germinal epithelium. On the other hand, the association of high temperature and busulfan was effective to deplete the common carp spermatogenesis. The tissue degeneration observed in carp testes was noticeably dose-dependent. In this way, the busulfan treated groups were clearly affected by the association between drug concentration, number of doses and the hyperthermic effect. Indeed, animals treated with two busulfan doses the gonads showed an intense degeneration of

the germinal epithelium, characterized by an increased lack of germ cells and a significant GSI decrease. In addition, the histological analysis revealed that the seminiferous tubules are composed mainly by somatic cells. Unlike to previous findings, single dose of busulfan had lesser degree of abnormalities and germ cell loss, indicating that this dosage is not able to keep the pharmacological effects observed with two doses.

Our findings are similar to those found in zebrafish, wherein high temperatures accelerate the spermatogenesis and optimize the busulfan treatment. Zebrafish males treated with busulfan (40mg/kg/BW) showed testicular degeneration, GSI decrease and spermatogenic tubules containing only Sertoli cells (Nóbrega et al., 2010). Same deleterious effects were found in pejerrey, where the animals of both sexes had a marked loss of germ cells and epithelium degeneration (Majhi et al., 2009a,b, 2014).

Lacerda et al. (2006, 2010) also demonstrated that in busulfan-treated Nile tilapia, two busulfan injections in association with high temperatures resulted in germ cells loss and consequently in spermatogenesis depletion. Similar to mammals, hyperthermia creates a stressful microenvironment in common carp testes. Thus, high temperature increased the vulnerability of germ cells to busulfan, facilitating their effect and causing a further spermatogenesis depletion, due to the synergistic effect of busulfan-hyperthermic treatment (Setchell, 1998; McLean et al., 2002).

In fish, busulfan depletion doses seems to be species and lineage specific. In this regard, the treatment can cause serious side effects, including systemic toxicity and hematopoietic suppression that can even be lethal due to destruction of bone marrow cells. Treatment associated effects may be threaten to corporal homeostasis, because the dose used is close to lethal dose, this is really critical when animals are valuable and/or endangered species (Brinster et al., 2003; Dobrinski, 2006; Hill and Dobrinski, 2006; Ma et al., 2011).

In Patagonian pejerrey, the highest busulfan dose caused ulcerations and mortalities in some animals after treatment. In this species, mortalities derived from busulfan-hyperthermic treatment were more frequent in females than males (Majhi et al., 2009a,b, 2014). Thus, busulfan side effects may be due to the specific susceptibility observed in some individuals as response to the drug action. Although treatment is effective to suppress spermatogenesis, some spermatogonial stem cells are able to survive to the unfavorable testicular conditions (Bucci and Meistrich, 1987).

In this way, we found that busulfan-induced depletion was reversible and the spermatogenesis can be restored two weeks post-treatment (data not shown). In fact, in busulfan-treated individuals were observed a rapid recovery of germinal epithelium after the treatment exposure.

This observation suggests that, not all cells are destroyed and these surviving cells have the ability to resume spermatogenesis. To this end, residual SSCs can repopulate the testes based on their self-renewal and proliferative capacity. Under these conditions somatic cells (specifically Sertoli and Leydig cells) plays a main role in spermatogenesis recovery. Surprisingly, these cells are capable to maintaining spermatogonial niche functions and structure for support the further activity of survival SSCs in terms of self-renewal, proliferation and differentiation.

In the same way, in mammals, busulfan is not able to eliminate all spermatogonia and few endogenous cells are able to reinitiate the spermatogenesis (Brinster and Zimmermann, 1994; Russell et al., 2000). More recently, it has been demonstrated that zebrafish spermatogenesis could be quickly resumed after busulfan depletion treatment, due to the proliferative activity of few surviving undifferentiated spermatogonia (Nóbrega et al., 2010).

The *amh* expression analysis showed decreased mRNA levels in bulsulfan-depleted common carp males. An implication of this is the possibility that in the depleted testes the decrease of *amh* mRNA was associated to the germ cell loss. Probably, the lack of germ cell resulting from the treatment influences the activity of somatic cells. This effect is especially critical for Sertoli cells, in which a negative feedback on *amh* production was generated.

In zebrafish testis, SSC maintenance and activity depends on their interaction with Sertoli cells and interstitial tissue (Nóbrega et al., 2010). More recently, it has been shown that Fsh (follicle-stimulating hormone) is an important extrinsic signal on modulating the production of Sertoli cell growth factors that are relevant either for SSCs self-renewal, or for differentiation (Skaar et al., 2011; Sambroni et al., 2013a,b; Nóbrega et al., 2015). Among these factors, anti-müllerian hormone (Amh) seems to play an important role on SSC fate (Miura et al., 2002; Skaar et al., 2011; Sambroni et al., 2013a,b). Amh, a TGF-β (transforming growth factor β) family member, has been discovered in tetrapods as a Müllerian inhibiting substance (MIS) due to its role on inducing regression of the Müllerian ducts in male embryogenesis (Josso et al., 2006).

Although Müllerian ducts are not present in teleost fish, *amh* orthologous have been found in Iberian chub (Pala et al., 2008), Japanese eel (Miura et al., 2002), medaka (Klüver et al., 2007), pejerrey (Fernandino et al., 2008), pufferfish (Kamiya et al., 2012), rainbow trout (Vizziano et al., 2007), sea bass (Halm et al., 2007), tilapia (Shirak et al., 2006) and zebrafish (Rodríguez et al., 2005), with differential expression in testis than in ovaries. Thus, fish Amh might have different functions than the one found in tetrapods. Studies in Japanese eel have demonstrated that Amh prevented the initiation of spermatogenesis in pre-pubertal animals (Miura et al., 2002). In adult zebrafish, Amh inhibited Fsh-stimulated androgen production and blocked

androgen-induced spermatogonial proliferation and differentiation (Skaar et al., 2011). Based on the inhibitory action of Amh and the fact that *amh* transcripts are commonly found in Sertoli cells surrounding undifferentiated spermatogonia, it is suggested that Amh may have an important regulatory role on teleost SSC niche.

4.3 SSC putative elements enrichment and isolation

We adapted a successful protocol for cell sorting including techniques of tissue enzymatic dissociation and percoll discontinuous density gradient, in order to isolate and enrich common carp type A undifferentiated spermatogonia. This protocol is very effective to separate different cellular populations.

Similar protocols were tested in bovines (Herrid et al., 2009; De Barros et al., 2012), buffalos (Rafeeqi and Kaul, 2013), chickens (Wu et al., 2009), goats (Kaul et al., 2012; Heidari et al., 2014), pigs (Han et al., 2009) and rodents (Koh et al., 2004), showing their effectiveness for cellular sorting from different fractions.

Due to density differences in percoll solutions, cells were separeted by size, forming visible bands along percoll gradient. Thereby, through cell sorting we were able to select putative SSCs for posterior use in transplantation procedures for instance. Interestingly in the current study, the methods described did not affect negatively the cells. This method showed to be simple, easy and did not require expensive or special equipment (Lacerda et al., 2006; Koh et al., 2004). In this work, we isolated an enriched SSC putative elements from common carp testicular suspension. Percoll gradient separated the undifferentiated spermatogonia in the upper layers while the remaining cell components were located in the lower layers and in the bottom of the tube. The results obtained from this analysis allows us to identify the most undifferentiated spermatogonia according to morphological criteria. Thus, similarily to zebrafish, we classified the SSCs candidates as the largest germ cells, with a large nucleus and one or two prominent nucleolus (Leal et al., 2009).

According to morphological analysis, upper bands showed a higher concentration of viable undifferentiated spermatogonia, including type A undifferentiated and differentiated spermatogonia. However, in these bands we also found some contamination with other cell components, mainly type B spermatogonia, spermatids and spermatocytes. In the middle bands a mixture of differentiated germ cells, predominantly spermatocytes, spermatids and spermatozoa was found. In the bottom, mainly spermatozoa, somatic cells and cellular residues formed the lower band. Based on these criteria, we were able to confirm the presence of

presumptive SSC in the upper layers. In the same line, studies performed with catfish (Shang et al., 2015), Nile tilapia (Lacerda et al., 2006, 2010), pejerrey (Majhi et al., 2009b, 2014), rohu carp (Panda et al., 2011), sturgeon (Pšenička et al., 2015) and tench (Linhartová et al., 2014) obtained similar results, suggesting that discontinuous density gradient protocols (percoll or ficoll) are suitable techniques for germ cell sorting and enrichment.

5. CONCLUSION

In conclusion, we showed c-Kit, GFRα-1 and POU2 protein expression in common carp testes. These molecular markers are expressed in early germ cells, while GFRα-1 and POU2 were preferentially expressed in type A undifferentiated spermatogonia, suggesting that both GFRα-1 and POU2 are highly conserved and SSC putative markers in common carp testis. We have demonstrated that combination of high water temperature and busulfan were able to suppress common carp endogenous spermatogenesis. Thus, this approach is an economic, simple and practical option to prepare mature recipients for transplantations assays in a short time. The *amh* expression confirmed that the concomitant action of busulfan associated to high temperatures was efficient to suppress the common carp spermatogenesis. Finally, we used an efficient protocol to isolate and enrich common carp undifferentiated spermatogonia through percoll method. This procedure is a practical and inexpensive alternative for spermatogonial purification and isolation. These approaches provide subsidies for understanding the SSC biology, and additionally are useful tools to develop studies in biotechnology with practical implications in aquaculture, such as SSC transplantation techniques, which can contribute in the management of valuable genetic resources.

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MANUSCRITO III

INTRAPAPILLARY XENOGENEIC GERM CELL TRANSPLANTATION FROM GOLDFISH (*Carassius auratus*) INTO ADULT COMMON CARP (*Cyprinus carpio*) TESTES

Angel Andrés Arias Vigoya[1,2]**, Emanuel Ricardo Monteiro Martinez[2], Melanie Digmayer[2], Marcos Antonio de Oliveira[1,2], Arno Juliano Butzge[2], Rafael Henrique Nóbrega[2]**.

[1] Aquaculture Center of São Paulo State University – CAUNESP - Jaboticabal, São Paulo – Brazil

[2] Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu – IBB/UNESP, São Paulo – Brazil.

**E-mail address: biorhn@yahoo.com.br aaariasv1@gmail.com

ABSTRACT

Germ cell transplantation in fish is a promising technique with wide applications in aquaculture, for instance, for the conservation and propagation of endangered and valuable genetic resources. In addition, this technique also provides tools to improve the understanding of germ cell biology and physiology. Herein, we describe the establishment of an intrapapillary xenogeneic transplant of germ cells from sexually mature goldfish (*C. auratus*) males into the testes of sexually mature common carp (*C. carpio*) males, whose testes were cytoablated by a thermo-chemical treatment (two doses of busulfan at 40mg/kg, at 35°C). Thus, in order to analyze the presence and development of donor germ cells in recipient testes, donor germ cells were labeled with PKH26, a fluorescent cell membrane dye. Ours results demonstrated that transplanted spermatogonial cells were able to colonize the recipients' testes, resume spermatogenesis and generate spermatozoa within eight weeks after germ cell transplantation. Taken together, these findings suggest that recipient testes provided suitable conditions for the survival, colonization, proliferation and differentiation of the donor spermatogonia from a related species. This approach have significant implications on assisted animal reproduction, biotechnology, conservation and production of valuable genetic resources and endangered fish species.

Keywords: biotechnology, spermatogonial stem cells, spermatogenesis, teleost fish.

1. INTRODUCTION

Several reproductive technologies were developed to produce functional gametes, progeny and to manage the genetic resources of endangered species and farm animals with high commercial value (Dobrinski and Travis, 2007; Dobrinski, 2008; Honaramooz and Yang, 2011). In fact, cryopreservation of gametes and embryos, multiple ovulation induction, embryo transfer, *in vitro*

gametogenesis and germ cell transplantation, are widely studied and well established approaches in mammals and more recently in fish (Pukazhenthi et al., 2006; Dobrinski and Travis, 2007; Honaramooz and Yang, 2011; Majhi et al., 2014; Zheng et al., 2014; Kawasaki et al., 2015).

Germ cell transplantation is an important and promising reproductive technique developed in rodents by Brinster and colleagues in 1994 (Brinster and Avarbock, 1994a; Brinster and Zimmermann, 1994b). This technique consists of isolating and transplanting germ cells from a fertile donor into the testes of a sterile recipient. Transplanted germ cells are able to resume recipient spermatogenesis and produce viable donor-derived spermatozoa. The technique provides a powerful approach to studying the biology of the male germline cell and its microenvironment (Brinster, 2002; Dobrinski and Travis, 2007; Dobrinski, 2008). In recent years, the interest in cell transplantation has increased, due to its remarkable potential for application in reproductive medicine and assisted animal reproduction (Hill and Dobrinski, 2006; Dobrinski and Travis, 2007; Honaramooz and Yang, 2011; Zheng et al., 2014).

Although widely studied in rodents and large animals, germ cell transplantation has recently been applied to fish (Honaramooz and Yang, 2011; Lacerda et al., 2013a; Zheng et al., 2014). Germ cell transplantation using adult and sexually competent fish has been developed in pejerrey, Nile tilapia, zebrafish, although few species were studied, this approach improves the understanding and feasibility of germ cell transplantation in teleost fish (Lacerda et al., 2010; Nóbrega et al., 2010; Majhi et al., 2009b, 2014; Silva et al., 2016). The success of germ transplantation in adult fish is determined by the availability of the receptors to host the donor cells. Thus, suitable recipients for transplantation should have testes partially or completely devoid of germ cells (Lacerda et al., 2010; Nóbrega et al., 2010; Majhi et al., 2009b, 2014; Silva et al., 2016). In mammals, testicular irradiation, heat shock and treatment with cytotoxic drugs such as busulfan are commonly used methodologies to remove the endogenous germ cells (Brinster et al., 2003; Honaramooz et al., 2005; Honaramooz and Yang, 2011; Ma et al., 2011). In this context, thermo-chemical treatments are effective to eradicate endogenous germ cells and suppress spermatogenesis in adult fish. Thus, through association between high rearing temperatures and busulfan was achieved experimental suppression of spermatogenesis in sexually competent animals (Lacerda et al., 2010; Nóbrega et al., 2010; Majhi et al., 2009a, 2009b, 2014; Silva et al., 2016).

In fish, surgical and non-surgical procedures have been performed to transplant donor germ cells into the recipient testes (Lacerda et al., 2010; Nóbrega et al., 2010; Majhi et al., 2009b, 2014; Silva et al., 2016). Nagler et al. (2001) conducted the first report of germ cell transplantation in

fish and specifically in sexually immature animals. In this study, pieces of testes were grafted by surgical procedure into isogenic rainbow trout (*Oncorhynchus mykiss*) males, resulting in donor-derived spermatogenesis. This work was followed by the development of new and more advanced approaches. Majhi et al. (2009b) conducted an effective intra-gonadal surgical intervention for xenogeneic germ cell transplantation between two species of pejerrey. An important advance for the improvement of the technique was obtained by Lacerda et al. (2006, 2010), who developed and standardized a successfully non-surgical method for transplanting germ cells using Nile tilapia. In this model, donor germ cells were injected into recipients' testes through the urogenital papilla and along the common spermatic duct.

Based on these results, Nóbrega et al. (2010), and more recently Majhi et al. (2014) and Silva et al. (2016), performed syngeneic and xenogeneic transplantation assays, respectively. Regardless the system used in germ cell transplantation, the receptor males were sterilized through thermo-chemical treatments (busulfan at high temperatures). In addition, the studies showed that transplanted cells were able to colonize and develop in the recipients testes, restoring gametogenesis and producing donor-derived functional gametes (Lacerda et al., 2010; Nóbrega et al., 2010; Majhi et al., 2009b, 2014; Silva et al., 2016).

Taking advantage of the great sexual plasticity exhibited by fish, xenogeneic germ cell transplantation is emerging as an important tool for the management and conservation of genetic recourses. This model allows germ cell transplantation between different species, which may be related species or even phylogenetically distant species (Yoshizaki et al. 2011; Lacerda et al., 2013a; Majhi et al., 2009b, 2014; Silva et al., 2016). In this context, the aim of the current study was to determine the feasibility of xenogeneic germ cell transplantation between two different species, using goldfish (*C. auratus*) as a germ cell donor and the common carp (*C. carpio*) as a recipient.

2. MATERIALS AND METHODS

2.1. Ethics statement

All procedures were consistent with Brazilian national regulation and followed the guidelines for the ethical animal treatment, approved by the National council for the control of animal experimentation (CONCEA) and the Ethics committee on animal care and experimentation of São Paulo State University (CEUA), protocol n° 5229/15.

2.2. Experimental animals and rearing procedures

Twenty sexually mature common carp males (mean body weight 150 ± 10g) were kept in 200-liter tanks, at 26°C, under natural photoperiod (12L:12D) and feed with a commercial diet three times per day.

2.3. Recipient preparation

In this study, we used a protocol that combines high water temperature with the cytostatic agent busulfan (Sigma-Aldrich) in order to suppress the recipient spermatogenesis. Before treatment, fishes were subjected to an acclimation period of two weeks, where water temperature was gradually increased until reach 35°C. Subsequently, the animals received two intracoelomic busulfan injections (40mg/kg/BW) dissolved in vehicle solution (dimethyl sulfoxide diluted in phosphate-buffered saline solution - PBS), with a two-week interval between injections.

2.4. Isolation of donor germ cells for transplantation

Donor germ cells from adult goldfish (*Carassius auratus*) were isolated based on protocol described by Lacerda et al. (2006, 2010). Briefly, sexually mature males (n=5) were sacrificed by anesthetic overdose and testes were excised, finely cut and rinsed in HBSS (Hank´s balanced salt solution). Testicular tissue was finely minced and incubated in a dissociating solution containing 0,2% collagenase in DMEM/F12 medium for 3h at 26°C and 80 rpm. The solution was filtered using a nylon membrane (mesh size 70 μm) to eliminate the non-dissociated cell clusters, centrifuged for 10 min at 1200 rpm and re-suspended in DMEM-BSA 0.75% medium. The solution was suspended in a discontinuous percoll density gradient, from the higher (40%) to lower (15%) gradient and centrifuged at 1200rpm for 30 min at 25°C. Bands enriched of spermatogonial stem cells (SSC) were collected, the cells were washed and their viability was assessed through Trypan blue (0.4%) exclusion assay.

2.5. Germ cell labeling and transplantation

Before transplantation donor cells were labeled with a fluorescent membrane dye PKH26 (Sigma-Aldrich, St. Louis, MO, USA) to track their localization and development inside the recipient testes. Germ cells were stained following the manufacture's guidelines, at the concentration of 8μL per mL for 5 min at room temperature. Then, labeled germ cells were rinsed three times with DMEM/F12, suspended in DMEM-BSA 0.75%, and stored on ice until transplantation. Thus, two weeks after the second busulfan injection, recipient common carps (n=20) were anesthetized with benzocaine (75mg per L of water) and the donor germ cells were

injected through the urogenital papilla using a micropipette (70μ diameter) under a stereomicroscope (Zeiss 475052, Germany). Each individual was injected with 1 mL of cell suspension containing DMEM and 0.4% trypan blue solution (1:10) at a concentration of approximately $1x10^7$ cells/mL. After transplantation, animals were reared in tanks in which water temperature was decreased 1-2 °C per day until reach 26°C.

2.6. Germ cell transplantation analysis

To assess transplantation efficiency (colonization of recipient testes), the fate and the post-transplantation development (proliferation and differentiation) of donor germ cells, testes transplanted with PKH26-labeled germ cells were collected at specific periods, from one to 8 weeks. To this end, testes from transplanted animals (n=20) were removed, embedded in Jung Tissue Freezing Medium (Leica Instruments, Nussloch, Germany), frozen in liquid nitrogen and stored at -80°C until actual sectioning. Tissue samples were sectioned at 10μm thickness through cryostat (Leica CM 1850, Germany) and stained with DAPI (targeting DNA in the cell nucleus). The obtained sections were observed, analyzed, and photographed under fluorescent Leica DMI 4000 B microscope.

3. RESULTS

3.1. Recipient preparation

In general, the treatment to deplete endogenous spermatogenesis (combination of high temperature and busulfan) was tolerated by the animals and the survival rate was 100% (no deaths occurred during the trial period). The busulfan-treated animals reduced their food intake after drug administration, however, the animals normalized their behavior once the treatment was suspended and males were returned to a temperature of thermal comfort (26°C). External injuries or pathologies were not detected during the treatment. In this study, the treatment that combines high temperature and busulfan was effective to suppress the recipient endogenous spermatogenesis. Histologically, fish exposed to busulfan showed acute atrophy of the germinal epithelium, and virtually no spermatogenic cysts after two weeks after treatment, resulting in spermatogenic tubules showing Sertoli cell only phenotype (Figure 1). In contrast, control testes showed a normal spermatogenesis progression showing spermatocysts at different developmental stages (Figure 1).

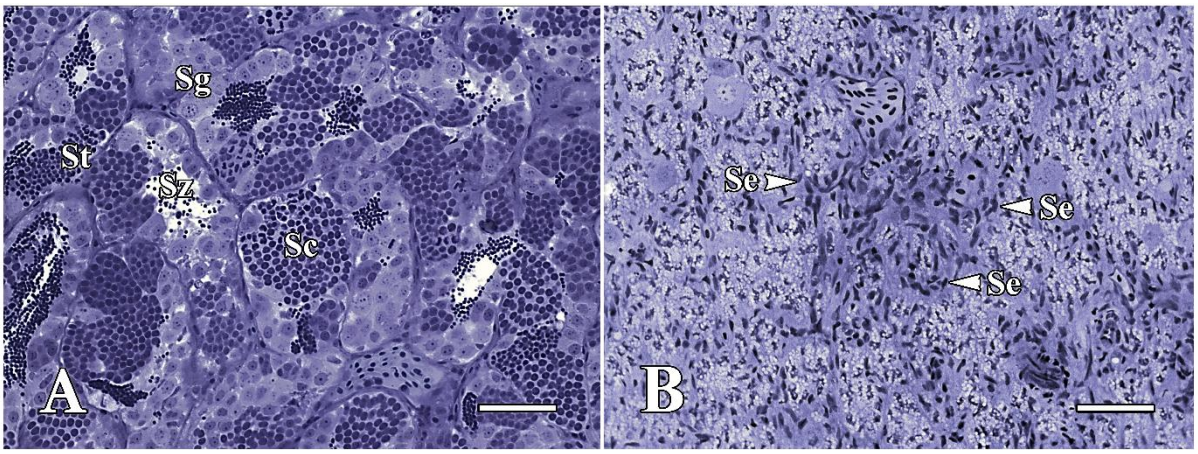
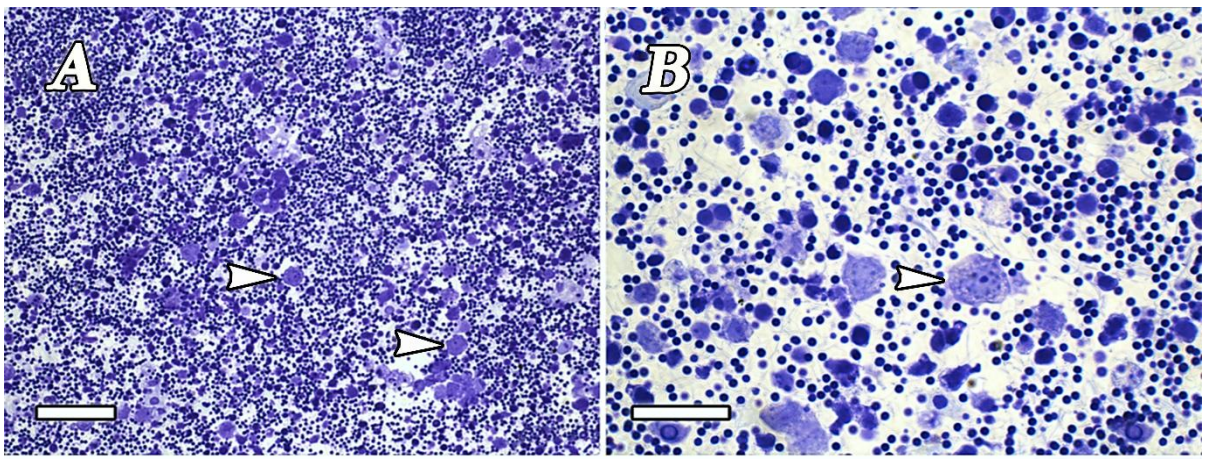


Figure 1. Histology of sexually mature common carp testis kept at 35°C, control **(A)** and treated with busulfan at high termperature, 35°C **(B)**. The seminiferous tubules of control fish show spermatogenic cysts at different phases of development, whereas Sertoli cells-only (Se) are present in most of seminiferous tubule of busulfan-treated common carp. Sertoli cell only (Se), spermatocytes (Sc), spermatogonia (Sg), spermatids (St), spermatozoa (Sz). Scale bars: 50μm.

3.2. Isolation of donor germ cells for transplantation

In order to isolate and enrich the SSC fraction used for transplantation, protocol developed by Lacerda et al. (2006, 2010) has been employed. This protocol allowed to remove successufully the somatic and mature germ cells and enrich SSC. After enzymatic digestion, a heterogeneous cell suspension was submitted to a discontinuous percoll density gradient. Six bands between percoll interfaces were found. Spermatogonia enrichment was found in the upper percoll bands, whereas mature spermatids, spermatozoa and cell debris, were pelleted down at the bottom of the tube. Further morphological analysis confirmed that the second and third layers contained a higher concentration of viable undifferentiated spermatogonia, including putative SSCs (Figure 2). These bands were selected for transplantation and the cell concentration present in these two bands was estimated at approximately $1x10^7$ cells.



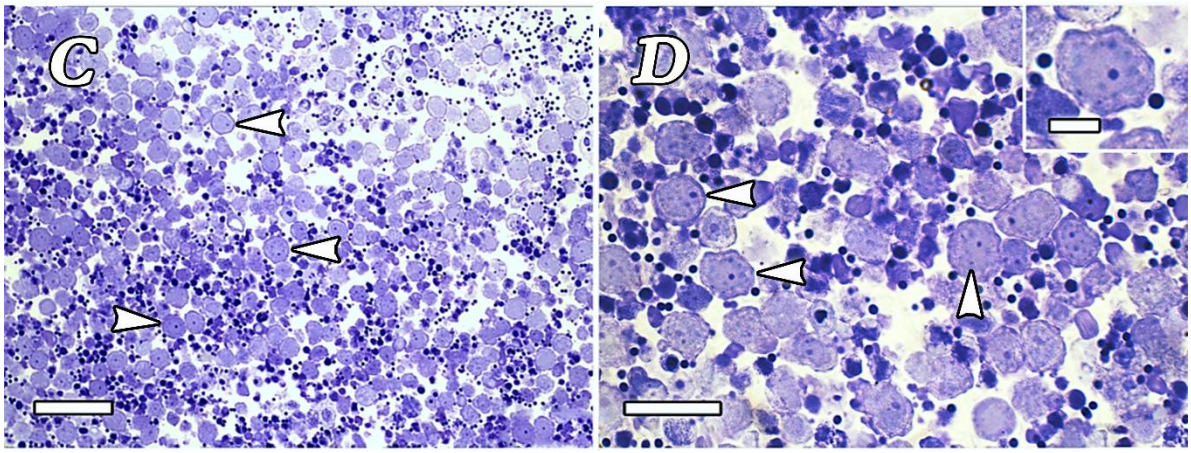


Figure 2. Heterogeneous donor germ cell suspension before percoll gradient centrifugation. A mixture of different germ cell types and somatic cells formed this suspension **(A-B)**. After density gradient centrifugation (percoll) was obtained a donor germ cell suspension enriched with type A undifferentiated spermatogonia (arrow heads). However, spermatocytes, spermatids and spermatozoa were also observed **(C-D)**. Inset in **(D)** show a presumptive spermatogonial stem cell. Scale bars: A and C = 50 μm; B and D = 25 μm. Inset in D = 10 μm.

3.3. Germ cell labeling and transplantation

In the present study, an enriched donor SSC suspension was transplanted into the receptor testes using a micropipette (non-surgical procedure), through the urogenital papilla and the spermatic duct of common carp males. To assess the transplantation efficiency, a trypan blue solution was added to the cell suspension. After transplantion, recipient testes were found labeled with trypan blue solution, indicating that cell suspension was completely incorporated into the seminiferous tubules (Figure 3).

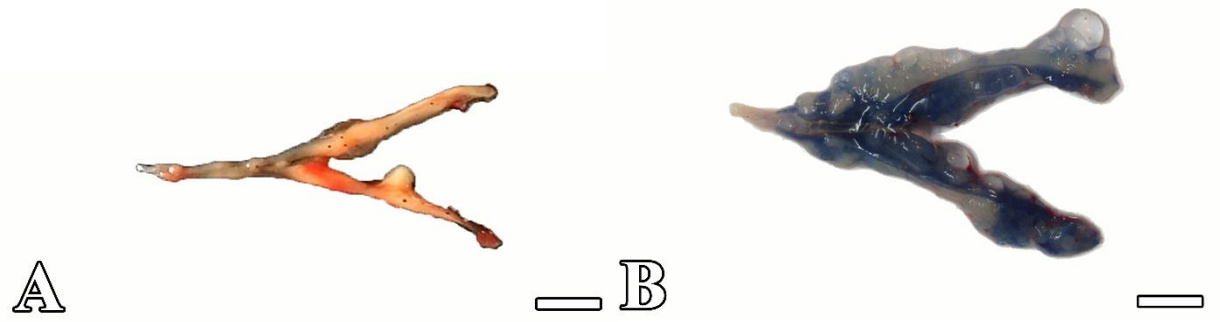


Figure 3. Control and transplanted testes of sexually mature common carp. **(A)** Appearance of the control testes treated with busulfan at 35°C, which did not receive germ cell transplantation. **(B)** Testes of recipients completely filled with the solution after transplantation. Scale bars: 1cm.

The analysis of recipient testes demonstrated that one week after transplantation, donor PKH26 labeled germ cells (presumably SSC) were found distributed in the lumen of seminiferous tubules (Figure 4A,B,C,D). After two weeks, the transplanted cells migrate, colonized and were

able to form clusters in the recipient germinal epithelium (Figure 4E,F). Donor germ cells then proliferated and were organized in a cystic arrangement. PKH26 labeled cells forming spermatocysts of different size and at different developmental stages were seen in the recipient testes (Figure 4E,F). Three to seven weeks after transplantation, cysts increased in size and number (Figure 4G-Q). After eight weeks post-transplantation, cysts in advanced stages of development and presumably spermatids and spermatozoa were found (Figure 4R,S).

4. DISCUSSION

In the current study, goldfish germ cells (including putative SSCs) were transplanted into busulfan-depleted common carp males. It has been suggested that the efficiency of germ cell transplantation improved when recipient testes are devoid of endogenous cells, since there is a higher availability of niches for transplanted cells. In this way, the competition between transplanted and endogenous germ cells also affects the success/efficiency of this procedure (Brinster, 2002; Shinohara et al., 2002; Brinster et al., 2003). Busulfan has been used as pre-treatment to suppress endogenous spermatogenesis in several species including mice (Brinster et al., 2003; Ogawa et al., 2003), rats (Ogawa et al., 1999; Zhang et al., 2003), monkeys (Hermann et al., 2007, 2012), pigs (Honaramooz et al., 2005; Mikkola et al., 2006) and goats (Honaramooz et al., 2005). This approach has also been used in sexually mature fish.

Thus, one or two busulfan doses in association with high water temperature, results in a high rate of germ cells loss; however, busulfan-treated individuals are not completely sterile (Nóbrega et al., 2010; Lacerda et al., 2006, 2010; Majhi et al., 2009a, 2014; Silva et al., 2016).

Similar as in Nile tilapia, Patagonian pejerrey (*Odontesthes hatcheri*) and zebrafish, this study demonstrated that spermatogenesis of common carp was influenced by the thermo-chemical treatment, resulting in seminiferous tubules with Sertoli cells only phenotype one week after the second busulfan injection. Surprisingly, spermatogenesis can be restored relatively quickly after treatment, through SSC self-renewal and differentiation (Nóbrega et al., 2010; Lacerda et al., 2006, 2010; Majhi et al., 2009a, 2014; Silva et al., 2016).

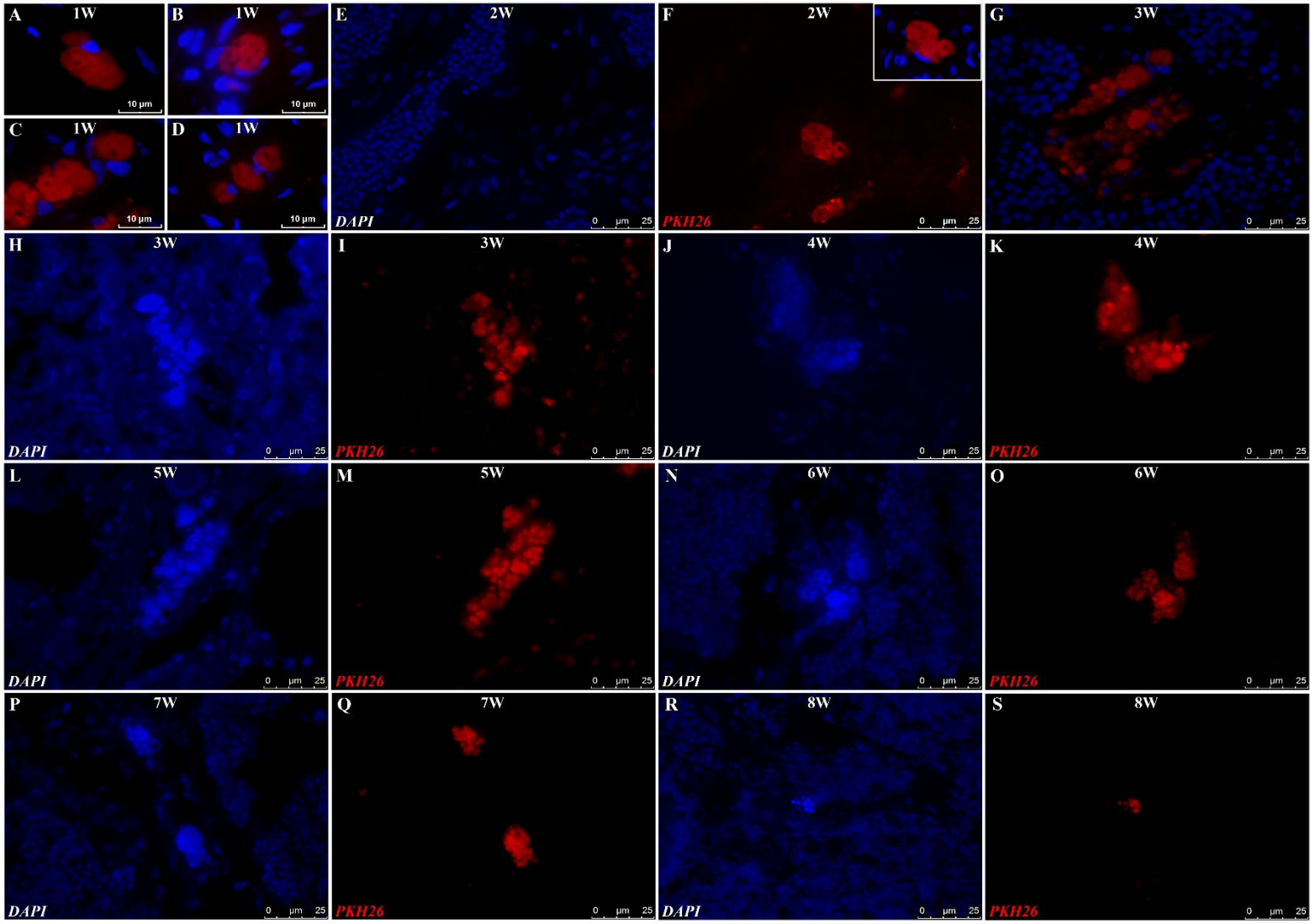


Figure 4. Microscopic evaluation of recipient common carp testis following 1 to 8 weeks after goldfish spermatogonial cells transplantation. The post-transplantation interval (in weeks) is shown at the top of each figure. At one-week **(A–D)** post-transplantation, PKH26 labeled spermatogonial cells (labeled in red) were observed in the common carp seminiferous tubules epithelium. From two to eight weeks after transplantation **(E-S)**, these PKH26 labeled germ cells (in red), also labeled with DAPI (cell nuclei labeled in blue; E, H, J, L, N, P, R), formed spermatogenic cysts of different sizes. The insert in **(F)** represents the merged image from "F". Eight weeks after transplantation, donor-derived spermatozoa labeled with DAPI (in blue) and also labeled with PKH26 (in red) were observed in the recipient seminiferous tubule lumen.

The results of this study confirmed that treatment with busulfan creates suppressed endogenous spermatogenesis, being as suitable model/recipient for SSC transplantation in these species. In this work, type A undifferentiated spermatogonia (including putative SSC) was isolated and enriched through a discontinuous density gradient of percoll. This protocol has been successfully used in experimental and farm animals, including rodents, ruminants, avian and pigs (Koh et al., 2004; Herrid et al., 2009; Han et al., 2009; Wu et al., 2009; De Barros et al., 2012; Kaul et al., 2012; Rafeeqi and Kaul, 2013; Heidari et al., 2014).

Likewise, this technique is suitable for spermatogonial isolation in some teleost fish species, prior to germ cell transplantation or spermatogonial biology study (Panda et al., 2011; Lacerda et al., 2010, 2013a, 2013b; Linhartová et al., 2014; Majhi et al., 2009b, 2014; Pšenička et al., 2015; Shang et al., 2015; Silva et al., 2016). In this work, the discontinuous percoll gradient decontaminated somatic and mature germ cells from the enzymatically digested testes suspension, and enriched the spermatogonial fraction. Although the technique isolated efficiently the spermatogonial population, the total elimination of "contaminants" was not possible (Panda et al., 2011).

Prior transplantation, donor germ cells can be subjected to different protocols of cell labeling (*in vivo* or *in vitro*), which enables the monitoring of the activity and subsequent location of transplanted cells (Saito et al., 2008; Nóbrega et al., 2010; Higuchi et al., 2011; Lacerda et al., 2010, 2013b; Majhi et al., 2009b, 2014; Pšenička et al., 2015; Silva et al., 2016). In this work, we used the fluorescent lipophilic cell membrane dye PKH26, in order to label the germ cell pool isolated from goldfish testes. Our results demonstrated that the dye stained effectively the germ cells and the labeling remained stable several weeks after transplantation. Besides its effectiveness and simplicity of application, this labeling protocol is safe and not affect the survival and viability of the labeled germ cells (Lacerda et al., 2010, 2013b; Majhi et al., 2009b, 2014; Pšenička et al., 2015; Silva et al., 2016).

In the current work, donor cells isolated from sexually mature goldfish testes were successfully transplanted into busulfan-depleted testes of sexually mature common carp by non-surgical procedure. Previous studies conducted in rodents showed that transplanted germ cells were able to colonize the seminiferous epithelium, self-renew, proliferate, differentiate and reestablish the host spermatogenesis (Brinster et al., 1994a, 1994b; Ogawa et al., 1999, 2000; Brinster et al., 2003). Despite the suitability of germ cell transplantation, few studies were performed using sexually mature fish (adults) as experimental models. Nonetheless, the studies showed the striking ability of the isolated type A spermatogonial cells to restore spermatogenesis and

generate gametes in the induced infertile recipients (Nóbrega et al., 2010; Lacerda et al., 2010, 2013b; Majhi et al., 2009b, 2014; Silva et al., 2016). In this context, we approached the intrapapillary xenogeneic germ cell transplantation using the goldfish (belongs to Carassius genus) as a donor and the common carp (belongs to Cyprinus genus) as a recipient. Although belonging to different genus, these two species are classified in the Cypriniformes order and are phylogenetically related fish species. Our results showed that despite their exogenous origin, PKH26-labeled germ cells were able to migrate and colonize the seminiferous tubules, among the recipient germ cells. These results are in agreement with Majhi et al. (2009b, 2014) findings, which developed a successfully xenogeneic transplantation in adults of *Odontesthes bonariensis* (donors) and *O. hatcheri* (recipients). Thus, through surgical and non-surgical procedures, spermatogonia and oogonia from *O. bonariensis* were isolated and subsequently transplanted in males and females of *O. hatcheri*. Following transplantation, donor germ cells were able to migrate and colonize the germinal epithelium of recipients. Recently, Silva et al. (2016) reported the effectiveness of xenogeneic transplantation between two phylogenetically distant species. In this study, germ cells from sexually mature Jundia catfish (*Rhamdia quelen*, Siluriformes order) were intrapapillary transplanted into Nile tilapia (*O. niloticus*, Perciformes) testes. One week after transplantation, Jundia germ cells were found in the seminiferous tubules of recipient Nile tilapia, demonstrating their potential to colonize the host testes. In addition, studies developed by Lacerda et al. (2006, 2010, 2013b) and Nóbrega et al. (2010) demonstrated the migration-colonization of recipient testes by donor spermatogonia, through an intrapapillary syngeneic transplantation in Nile tilapia and zebrafish, respectively.

After different time intervals post-transplantation, we found that donor germ cells were able to colonize, proliferate and differentiated in the recipient testis, generating spermatocysts and spermatozoa in a short period (8 weeks). This data showed full donor-derived spermatogenesis and confirmed the presence of SSC elements in the type A undifferentiated spermatogonial suspension. Similar results were obtained by Lacerda et al. (2006, 2010), where donor-derived spermatozoa were obtained after 8 to 9 weeks post-transplantation. Interestingly, cryopreserved spermatogonia also exhibited the same potential for proliferate and differentiate into spermatozoa in recipient testes (Lacerda et al. 2010). In addition, Lacerda et al. (2013b) observed that cultured spermatogonia maintained their ability to survive, colonize and proliferate in the recipient testes three to four weeks post-transplantation. On the other hand, a long timeframe post-transplantation were required for donor-derived gamete production in Jundia catfish (17 weeks) and Patagonian pejerrey (24–34 weeks) (Majhi et al., 2014; Silva et al., 2016). However, gametogenesis in adult fish as recipients is faster when compared to the onset of gamete

production in embryos or larvae as recipients (Saito et al., 2011, 2010, 2008; Yazawa et al., 2010; Yoshizaki et al., 2010, 2011).

Overall, these studies suggested that the recipient gonads provided somatic elements and factors (hormones, growth factors etc.) to sustain a suitable microenvironment for the development of the transplanted germ cells and the resumption of the gametogenesis. Additionally, germ cell transplantation in teleost fish demonstrate a remarkable potential and plasticity of somatic elements (i.g. Sertoli cells) to support exogenous germ cells (Silva et al., 2016).

Germ cell transplantation using adult fish as recipients reduce the time needed to produce donor-derived gametes and offspring. However, nowadays, a critical point is its low efficiency to transmit the donor germ line (Majhi et al., 2009b; Lacerda et al., 2010; Nóbrega et al., 2010). In this context, the efficiency of transplantation is affected by competition between transplanted and recipient germ cells for the available niches in the germinal epithelium (Shinohara et al, 2002; Brinster et al, 2003). On the other hand, in cytoablated recipients the treatment with busulfan may affect the properties for self-renewal, pluripotency and differentiation of xenogenic germ cells. In addition, the microenvironment formed in busulfan-depleted gonads suffered an imbalance of growth factors that regulate the activity of transplanted germ cells (Nóbrega et al., 2010). Finally, the use of adult recipients in germ cell transplantation has potential applications in the conservation of genetic resources, the propagation of valued or endangered fish species, and in the development of new biotechnologies in aquaculture (Yoshizaki et al, 2011; Lacerda et al., 2013a, 2013b).

5. CONCLUSION

In summary, we conclude that the thermo-chemical treatment caused an effective spermatogenesis suppression and a pronounced germ cell loss, which is suitable as recipients for germ cell transplantation. The gonads of cytoablated recipients remained sexually competent and able to host donor-derived spermatogenesis after a non-surgical xenogeneic germ cell transplantation through an intrapapillary injection. Thus, goldfish germ cells were able to migrate, colonize, proliferate and differentiate into common carp recipient gonads, producing gametes 8 weeks after transplantation.

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III. CONCLUSÃO GERAL

1. No primeiro capítulo foram descritas morfológica e estereológica as células germinativas, e foram caracterizados os diferentes cistos espermatogênicos na carpa comum. Assim, o epitélio germinativo da carpa comum esta constituído por 5 tipos espermatogôniais (espermatogônias indiferenciadas do tipo A, A_{und}* - A_{und}; espermatogônias diferenciadas do tipo A, A_{diff}; espermatogônias do tipo B inicial e final), espermatócitos em diferentes fases da meiose, espermátides (iniciais, intermediarias e finais) e espermatozoides. Além disso, a duração do processo espermatogênico (fases meiótica e espermiogênica) foi estimada em aproximadamente 7 dias.

2. No segundo capítulo, as espermatogônias tronco da carpa comum foram caracterizadas fenotipicamente. Simultaneamente, foi padronizada a depleção da espermatogênese e foi adaptado um protocolo de purificação, isolamento e enriquecimento das espermatogônias tronco da carpa comum. Neste estudo, as proteínas c-Kit, GFRα-1 e POU2 foram expressas pelas células germinativas iniciais, porém GFRα-1 e POU2 foram preferencialmente expressas pelas espermatogônias indiferenciadas do tipo A. O uso do busulfan associado à condições de hipertermia causaram uma acentuada perda de células germinativas e a concomitante supressão da espermatogênese nos animais tratados. A utilização da técnica do gradiente de densidade de percoll possibilitou o isolamento e enriquecimento da fração espermatogonial indiferenciada a partir da população celular heterogênea, produto da digestão enzimática dos testículos da carpa comum.

3. Finalmente, no terceiro capítulo, foi realizado com sucesso o transplante xenogênico de células germinativas entre o Goldfish (*Carassius auratus*) como doador das células germinativas e a carpa comum (*Cyprinus carpio*) como receptor do transplante. Assim, os receptores permaneceram sexualmente competentes após o tratamento com busulfan e os testículos ofereceram as condições funcionais para o desenvolvimento das células germinativas dos doadores. As células transplantadas foram capazes de migrar, colonizar, proliferar, se diferenciar e gerar gametas 8 semanas após o transplante.

4. A técnica transplante é uma importante abordagem para o estudo da fisiologia e biologia das células germinativas, e dos mecanismos envolvidos na regulação da espermatogênese. Além disso, apresenta um grande potencial para o desenvolvimento da aquicultura, biotecnologia, medicina reprodutiva, a preservação do germoplasma de especies em perigo e/ou recursos geneticamente valiosos.