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Germ cells transplantation in fish: the Nile-tilapia model[1]

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Abstract

Spermatogonial transplantation, developed in the past decade by Brinster and colleagues, is a fascinating and powerful technique utilized to investigate male reproductive biology, mainly the aspects related to spermatogenesis and the stem cell biology. This technique also offers tremendous potential for studies involving biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species. Although germ cell transplantation is well characterized for mammals, there is no study utilizing this approach for fish. Due to its fast growth, relative small size when it reaches sexual maturity, good adaptability to different environmental conditions and economical importance, the tilapia (*Oreochromis niloticus*) is an excellent experimental model. In the present study, we investigated the viability of adult tilapias as a recipient model for germ cells transplantation in fish. For this purpose, all approaches utilized in the present study for spermatogonial transplantation, such as endogenous spermatogenesis depletion, obtention, selection and spermatonia labeling, and the transplantation through the common urogenital papilla, were standardized in our laboratory. The preliminary results found for the recipient tilapia testes, evaluated by light and fluorescence microscopy, showed the presence of PKH26 labeled germ cells cysts in the seminiferous tubules. These findings obtained, for the first time in fish, suggest that spermatogonial germ cells can be successfully transplanted directly into the testis of this teleost. Therefore, tilapias might be utilized as an experimental model to investigate the germ cell biology and the testis function in teleosts. Moreover, this technique could be also utilized as a potential approach for fish bioengineering, preservation of genetic stock of endangered fish species or fish strains carrying commercially valuable traits.

Keywords: spermatogonia, transplantation, testis, tilapia (*Oreochromis niloticus*).

Introduction

The teleosts, composed by more than 20,000 species, are probably the oldest and most diverse group of vertebrates. The habitat of these vertebrates is highly variable suggesting that during their evolution they developed a very efficient adaptative communication with the environment, and were able to colonize and to reproduce in a great variety of environment (Billard, 1986). The Nile-tilapia (*Oreochromis niloticus*, Cichlidae, Perciformes) is now considered one of the most economically important freshwater fish, mainly due to the good flavor of its meat, and has been cultivated for more than 3,000 years. In contrast to carnivorous teleosts, being omnivorous tilapias grow very fast, are very resistent to different water conditions, reach sexual maturity at approximately 4-5 months of age, and reproduce at each two months if the water temperature is around 25ºC (Beamish, 1970; Stickney, 2000). Besides that, tilapias larvae present very high viability. Taken together, all these positive attributes make the tilapia an excellent fish model for studies developed in laboratory conditions, including those related to reproductive biology (Stickney, 2000).

As in other fish, tilapia testes are located in the celomic cavity, dorsally to the digestive tube, ventrally to the mesonephros, and ventro-laterally to the swim-bladder. The seminiferous tubules in tilapias present a blind ending located just below the tunica albuginea and are arranged radially in relation to the intratesticular spermatic ductule system. These ductules are located in the dorsal aspect of the testis, where the spermatic duct is formed (Silva, 1987; Schulz *et al*., 2005). The two spermatic ducts (one from each testis) join to each other and form the commom spermatic duct that opens in the urogenital papilla (Silva, 1987).

Different from mammals, spermatogenesis in teleost fish takes place in cysts within the seminiferous tubules (Pudney, 1995; Schulz *et al*., 2005). The spermatogenic cyst is formed when Sertoli cells enclose a single primary spermatogonium. The germ cells derived from a single primary spermatogonium then divide synchronously to constitute an isogenic germ cell clone that is bordered by the cytoplasmic extensions of a single layer of Sertoli cells (Schulz *et al*., 2005). Apart from this cystic arrangement, the spermatogenic process in teleosts is very similar to that of mammals (Vilela *et al*., 2003). Usually, in fish the undifferentiated and early differentiated spermatogonia are the biggest germ cells present in the seminiferous tubules, and the number of differentiated spermatogonia generations are much higher in comparison to mammals (Billard, 1969; Vilela

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et al., 2003; Schulz *et al*., 2005).

The duration of spermatogenesis is a species-specific biological constant that is under the control of the germ cell genotype (França *et al*., 1998). Compared to mammals (Russell *et al*., 1990; França and Russell, 1998; França *et al*., 2005), the total duration of spermatogenesis in fish is much shorter (Egami and Hyodo-Taguchi, 1967; De Felice and Rasch, 1969; Billard, 1968; Sinha *et al*., 1982; Silva and Godinho, 1983; Sinha *et al*., 1983; Billard, 1990; Koulish *et al*., 2002, Vilela *et al*., 2003), is influenced by the temperature, being much faster at higher temperatures (Egami and Hyodo-Taguchi, 1967; Billard, 1968; Vilela *et al*., 2003; França *et al*., 2005). Particularly in tilapias, the meiotic and spermiogenic phases of spermatogenesis lasts 10-11 days, 6-7 days, and 5-6 days in fish kept at 25ºC, 30ºC, and 35ºC, respectively (Vilela *et al*, 2003; Lacerda, 2006) (Fig. 1).

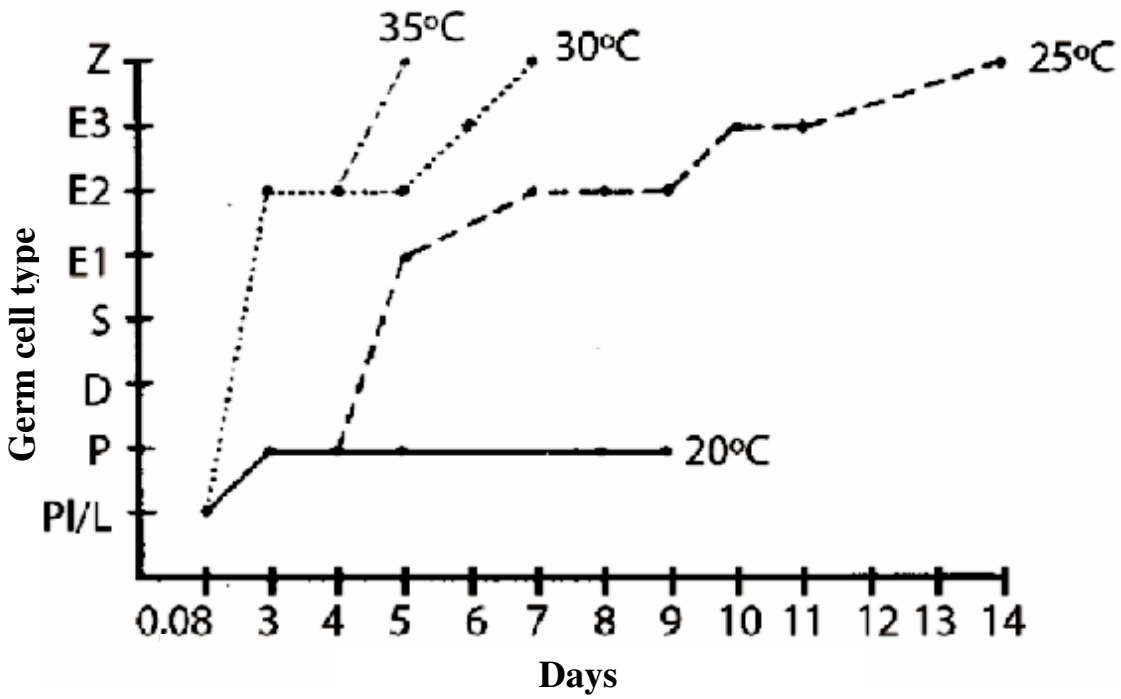
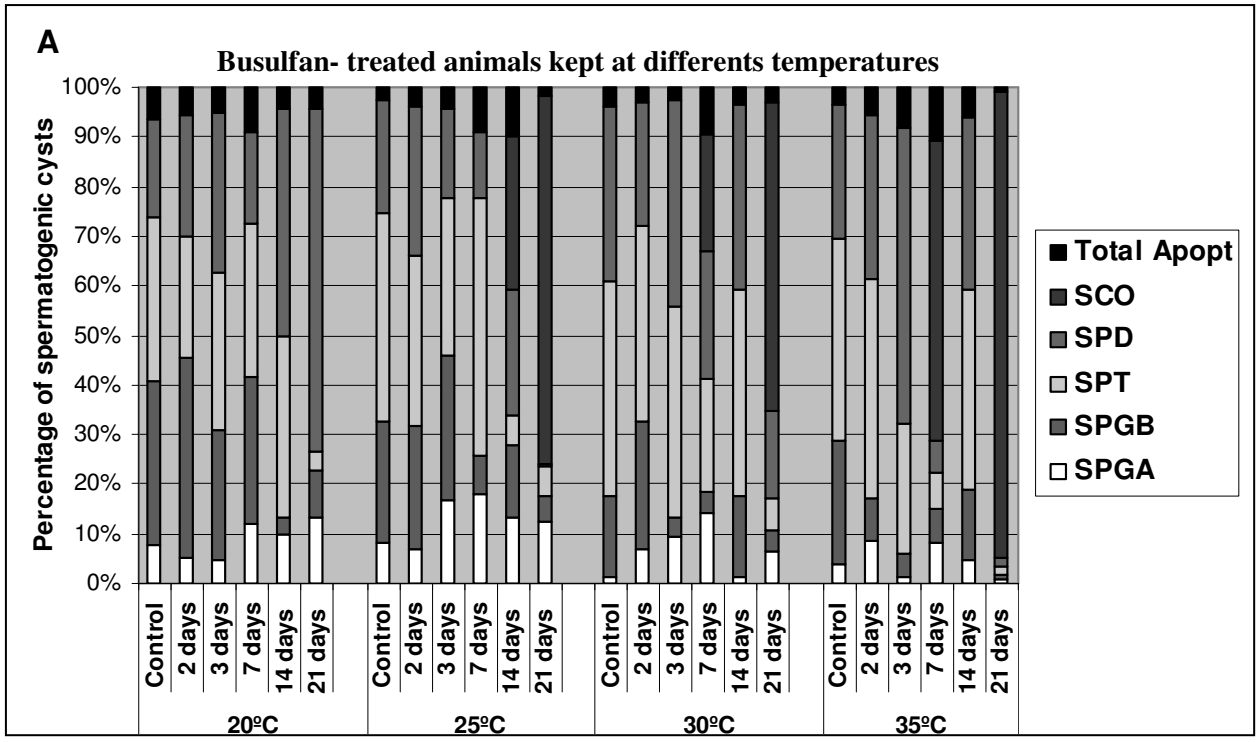


Figure 1. Most advanced germ cells in sexually mature Nile tilapias, sacrificed at different time periods (days) following tritiated thymidine injection and kept at different temperatures. Observe that, compared to 25ºC, the formation of sperm is much faster in fish kept at the temperatures of 30ºC and 35ºC. Abreviations for the different type of germ cells: Pl/L = primary spermatocytes in preleptotene/leptotene; P = pachytene; D = diplotene; S = secondary spermatocytes; E1, E2, and E3 = initial, intermediate, and final spermatids, respectively; Z = sperm.

Germ cell transplantion is a fascinating and powerful technique pioneered by Brinster and colleagues in 1994 and consists in the isolation of spermatogonial stem cells from a donor animal and the transplantation of these cells into a recipient testis, where they will develop and form mature fertile sperm with the donor genetic characteristis (Brinster, 2002; Shinohara *et al*., 2006). This technique has been largely utilized in the past decade in mammals, aiming to investigate spermatogenesis and the stem cell biology (Brinster, 2002; Dobrinski, 2005a; b; Khaira *et al*., 2005; McLean, 2005). It also offers great potential for studies involving biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species (Brinster, 2002; Dobrinski, 2005a; b; Khaira *et al*., 2005; McLean, 2005).

Although germ cell transplantation is well characterized for mammals, there is no study utilizing this approach for fish. This probably occurs due to the lack of a good model in which endogenous spermatogenesis is absent or experimentally depleted, a necessary condition to allow the availability of niches and the development of spermatogenesis in the donor testis (Brinster *et al*.,, 2003). In the few studies developed recently in this area of research for teleosts (trout and salmon), primordial germ cells or spermatogonial cells from adult fish, were transplanted into the celomic cavity of fish larvae, being able to generate fertile gametes (Takeuchi *et al*., 2003; Okutsu *et al*., 2006).

Taking adavantage of tilapias as a very appropriate experimental model, our main objectives in the present investigation were to standardize the tecniques and approaches necessary to perform spermatogonial transplants in this important teleost species.



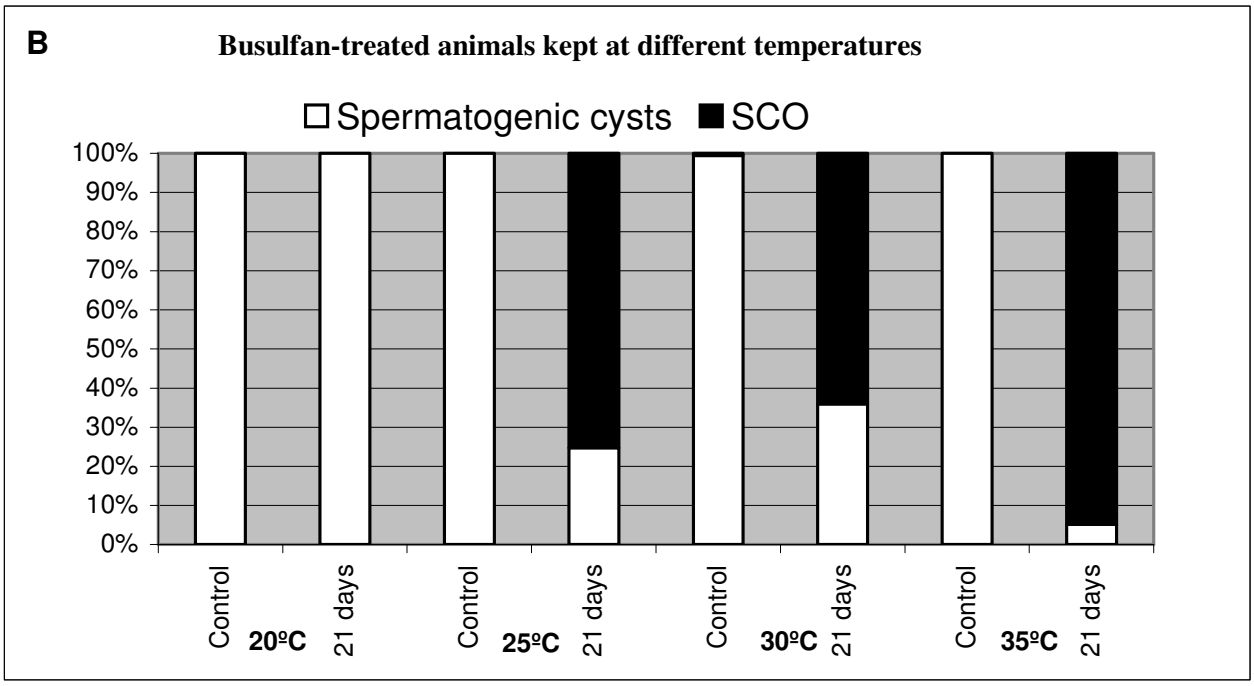


Figure 2. Histological and quantitative analyses of spermatogenic cysts in the seminiferous tubules of sexually mature tilapias treated with busulfan and kept at different temperatures, and sacrificed at different time periods (days) after treatment. Observe that, based on the number of tubules/cysts containing Sertoli cell-only (SCO; Fig. 2A), two busulfan injections in tilapias kept at 35ºC is the most appropriate approach to deplete endogenous spermatogenesis in tilapias. Figure 2B summarizes the degree of endogenous spermatogenesis depletion in tilapias kept at different temperatures and sacrificed 21 days after busulfan treatment. Abbreviations: TAp = total number of cysts with apoptotic germ cells; SCO = Sertoli cell-only cysts/seminiferous tubules; SPD, SPT, SPGB, and SPGA = cysts with spermatids, spermatocytes, and type B and type A spermatogonia, respectively.

Endogenous spermatogenesis depletion

The ideal receptor for germ cells transplantation is the animal in which the seminiferous tubules contains only normal Sertoli cells (Brinster, 2003). Therefore, the success and efficiency of germ cells transplantation depends on a recipient animal in which endogenous spermatogenesis is naturally absent or experimentally deprived (Brinster, 2002; Dobrinski, 2005a; b; Honaramooz *et al*., 2005; McLean, 2005), which provides the availability of niches for germ cells colonization and development (Brinster, 2003). In this regard, several approaches to deplete endogenous spermatogenesis in mammals, such as chemotherapy and X-ray or gamma-ray local radiation have been utilized, being the effective dosage species or strain specific (Ogawa *et al*., 1999; Brinster 2003). However, even when they are very efficient, with these treatments not all germ cells are destroyed and some endogenous spermatogenesis might persists after the treatment (Bucci and Meistrich, 1987; Brinster and Zimmerman, 1994; França *et al*., 1998).

The fish testis is located in the celomic cavity, making it impossible to utilize local radiation without surgical approaches. In order to deplete endogenous spermatogenesis in tilapias, in the present investigation, different concentrations of the chemotherapeutic drug busulfan, in association with different temperatures, were tested. These protocols were based on studies developed in our laboratory (Vilela *et al*, 2003; Lacerda, 2006), showing that higher temperatures are able to accelerate the duration of spermatogenesis in tilapias (Fig. 1). Thus, allowing us to make the assumption that this condition would speed up the germ cells mitotic activity and facilitate the busulfan effect. Therefore, the tilapias investigated received two busulfan (Sigma, MO, USA) injections (18mg/kg/BW and 15mg/kg/BW) with two weeks interval between the first and second injection, in association with temperatures of 20ºC, 25ºC, 30ºC, and 35ºC. As it can be observed in Fig. 2 to 4, endogenous spermatogenesis was drastically depleted three weeks after the first busulfan injection, in sexually mature tilapias kept at 35ºC. Also, besides being very eficient, very low mortality rate was observed due to the busulfan treatment.

As it can be noticed in Fig. 3 and 4, sperm already present before and during the treatment with busulfan were frequently found in the seminiferous tubules of busulfan treated tilapias. Also in these fish, similar to the literature, stereological investigation performed showed that Leydig cells morphology and volume were not affected ($p>0.05$) by the busulfan treatment (Krause, 1975; Aich and Manna, 2001) in association with the temperature of 35ºC (Fig. 5).

Germ cells preparation

Germ cells isolation

The protocols utilized to obtain germ cells in tilapias were an adaptation from those developed by Bellvé *et al* (1977) and Chaves-Pozo *et al* (2004). Thus, the testes of sexually mature tilapias were properly collected, cut in small pieces (~2mm^3), washed in HBSS, and enzymatically digested with collagenase (type IA, Sigma), trypsin and DNAse I (Sigma). After this step, the germ cells isolation was performed utilizing a percoll (Cultilab) gradient, aiming to select the most immature spermatogonia to be transplanted. According to the protocol developed, after the obtained solution was centrifuged for 30 minutes (800g) at 25ºC, four different bands containing testis cells were obtained (Fig. 6), and the testis cells viability in tilapias, tested with trypan blue, was very high.

In order to visualize histologically the cells present in each band, these cells were fixed in glutaraldehyde and the pellets obtained were embedded in glycol methacrilate and routinely prepared for light microscopy. The results suggested that our protocol was very efficient in separating the germ cells population containing early spermatogonia, that were located in the two upper bands (Fig. 6). Although this aspect was not quantified, based on the cell morphology, few Sertoli, peritubular myoid, and Leydig cells were present in the pool of selected germ cells.

Several indirect (positive and negative) markers are utilized to enrich the stem cells population in mammals (ex: integrins β_1, α_6 and αv^-, $CD9^+$, $CD24^+$, Thy-1^+, MHC-I^- and Ep-CAM^+) (Shinohara *et al*., 1999; Kubota *et al*, 2003, Kanatsu-Shinohara *et al*, 2004, Ryu *et al*, 2004; Dobrinski, 2005a; Khaira *et al*., 2005; McLean, 2005). However, it should be mentioned that, similar to mammals, there is no specific marker for spermatogonial stem cells in fish (Takeuchi *et al*., 2003).

Germ cells labeling

In laboratory rodents, germ cells markers such as LacZ (ZflacZ, ROSA26, and MtlacZ) and green fluorescent protein (GFP) are used to identify these cells after transplantation (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994; Ohta *et al*., 2000; Dobrinski 2005a). Because such markers are not available for tilapias, in the present investigation we have used red fluorescent cell Linker (PKH26; Sigma) that has been utilized successfully to label germ cells in domestic mammals (Dobrinsnki *et al* 1999, Schlatt *et al*, 1999, Honaramooz *et al* 2002, 2003). This cell surface marker, developed by Horan and Slezak (1989) and apparently not toxic to the cells (Horan *et al*., 1990; Samlowski *et al*. 1991), is a lipofilic dye that intersperses between the cell membrane lipid bylayer, lasting for several months (Hass *et al*, 2000; Honaramooz *et al*., 2002).

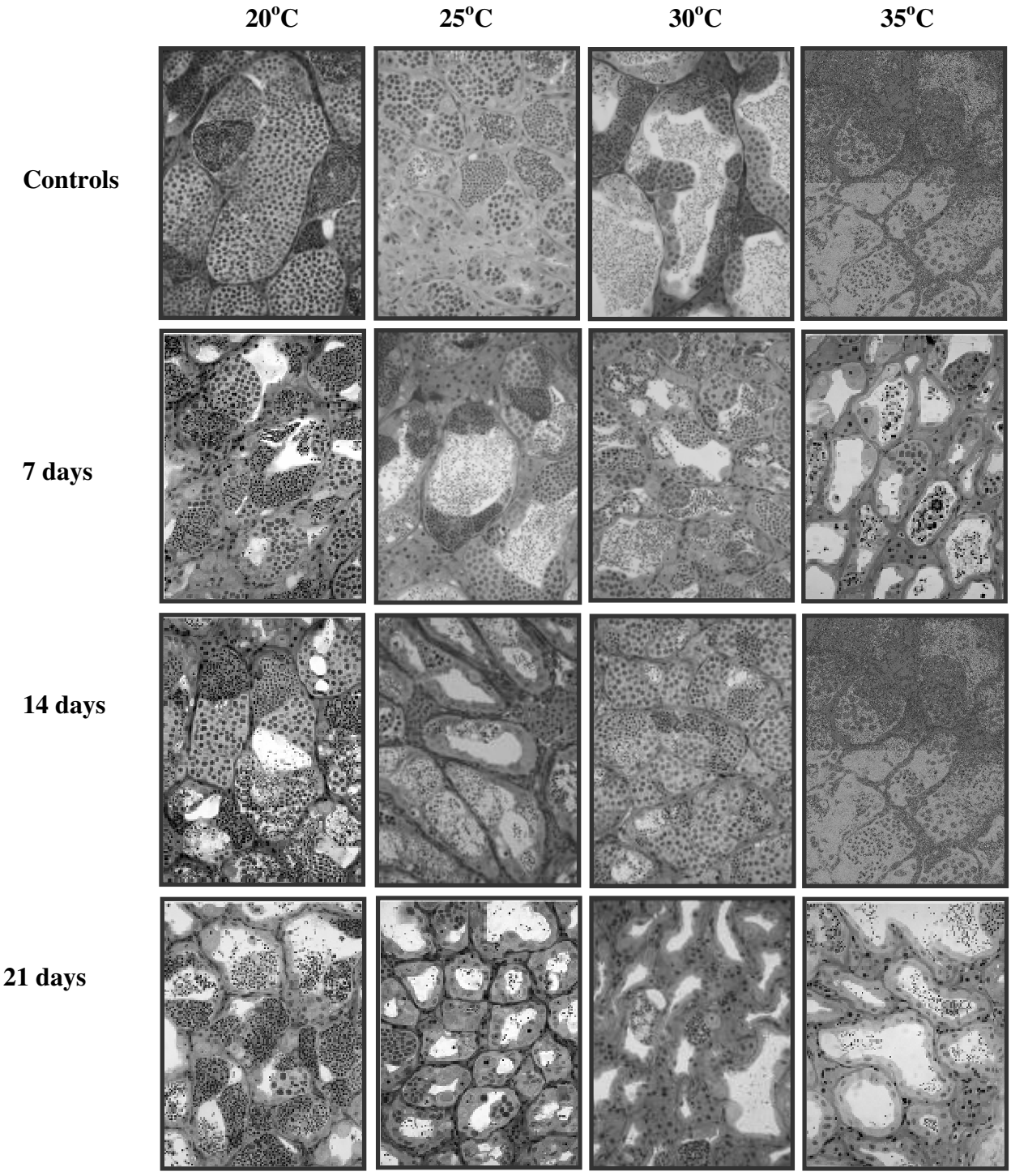


Figure 3. Seminiferous tubules of sexually mature tilapias treated with busulfan and kept at the temperatures of 20ºC, 25ºC, 30ºC, and 35ºC. These fish were sacrificed at 7, 14 and 21 days post-busulfan injections. The tilapias sacrificed at 21 days received a second busulfan injection on day 14. Observe that seven days post-busulfan injection endogenous spermatogenesis was considerably depleted in fish kept at 35ºC. However, probably due to the acceleration of spertmatogenesis (see Fig. 1), at 14 days post-treatment the remaining spermatogonial germ cells were able to promote the recovery of endogenous spermatogenesis in tilapias kept at the temperature of 30ºC and, particularly, at 35ºC. At 21 days post-busulfan treatment, the endogenous spermatogenesis was fairly well depleted in tilapias kept at 35ºC. Probably because the evolution of spermatogenesis is slower in fish kept at 20ºC (see Fig. 1), the busulfan-treatment was less effective in this condition. All pictures were taken at the same magnification and the bar present in the first figure (contol at 20ºC) represents 50µm.

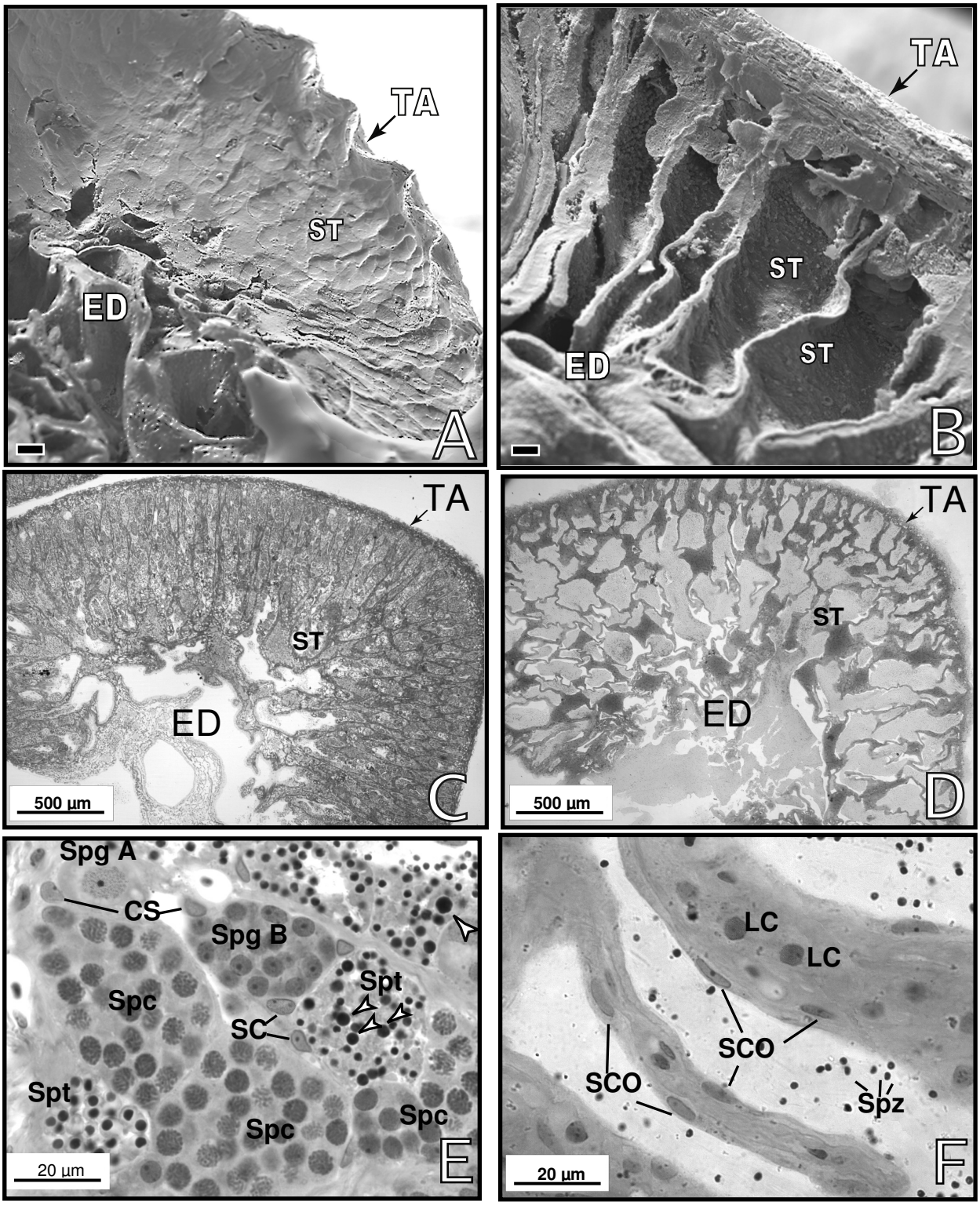


Figure 4. Transversal sections of the testis of sexually mature tilapias kept at 35ºC. These sections were evaluated under scanning electron microscopy (A-B) and light microscopy (C-F). Compared to control fish (A, C, and E), in busulfan-treated tilapias sacrificed 21 days post-treatment (B, D, and F) the endogenous spermatogenesis was severely depleted. Fig. E shows the presence of spermatogenic cysts in different phases of development and apoptotic germ cells (arrow heads) in the seminiferous tubules of control fish. Sertoli cells-only (SCO) were present in most seminiferous tubules of busulfan-treated tilapias (F). However, sperm (Spz) were frequently found in these seminiferous tubules lume. Abbreviations: TA = tunica albuginea; ED = efferent ductules; ST = seminiferous tubules; Spg A = type A spermatogonia; Spg B = type B spermatogonia; Spc = spermatocytes; Spt = espermatids; SC = Sertoli cells; LC = Leydig cells. Bar = 5µm and 2.5µm for figures A and B, respectively.

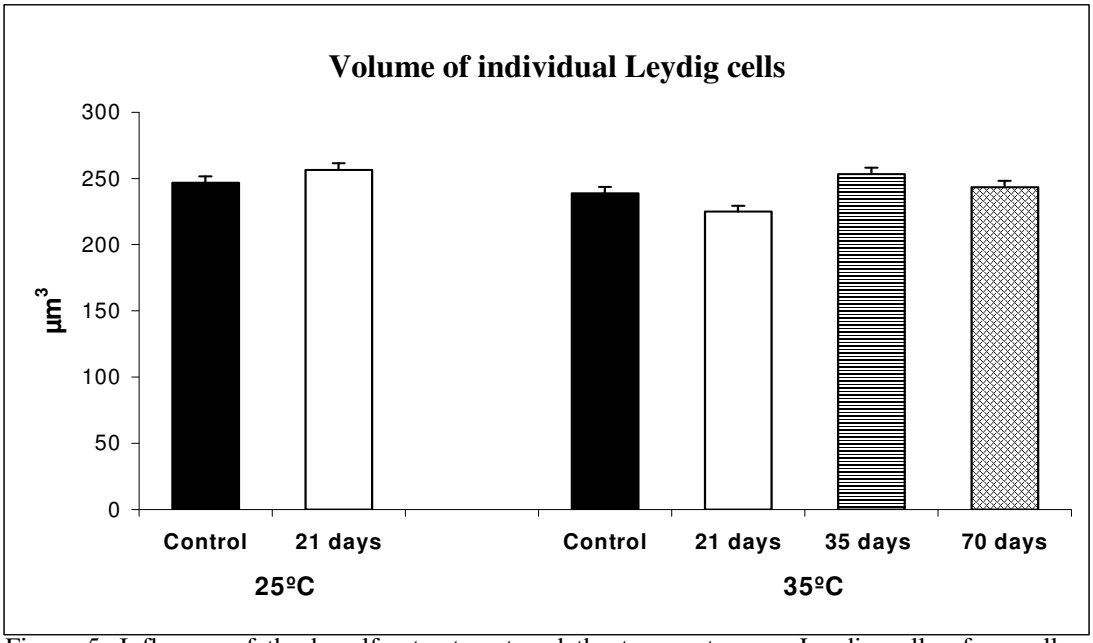


Figure 5. Influence of the busulfan-treatment and the temperature on Leydig cells of sexually mature tilapias kept at 25ºC and 35ºC. Observe that in both the tilapias kept at different temperatures and evaluated several periods (21days, 35 days, and 70 days) after busulfan treatment, no effect ($p>0.05$) was observed in the individual volume of Leydig cells.

In order to label the tilapia germ cells, we tested a protocol in which several concentrations of PKH26, for different time periods, were utilized as follow: a) 32µl of PKH26 per mL of C diluent for 6 minutes; b) 6µl of PKH26 per mL of C diluent, for 15 minutes; c)10µl of PKH26 per mL of C diluent for 10 minutes; and d) 2µl of PKH26 per mL of C diluent for 40 minutes (Fig. 7). From the results obtained and taking into account the cell viability, tested with trypan blue, and the label intensity, we have decided to label tilapia germ cells to be transplanted with 8µl of PKH26 per mL of C diluent, for 10 minutes.

Spermatogonial germ cells transplantation

Transplantation via

In laboratory rodent, germ cells have been successfully transplanted through the seminiferous tubules, *rete testis*, and efferent ductules (Ogawa *et al*., 1997; Dobrinski, 2005b), whereas in large mammals ultrasound guided transplantation into the *rete testis* is preferentially utilized (Schlatt *et al*., 1999; Dobrinski, 2005b). Due to the testis location and the excurrent ducts anatomical disposition, all these approaches are not feasible in tilapias without surgery. In this regard, knowing that the common spermatic duct in tilapias as in other fish open externally in the urogenital papilla, together with the urethral duct, we tested the possibility of transplanting germ cells in tilapias through this via located caudally to the anus. However, before that, we performed a detailed light microscopy investigation about the size and disposition of these two ducts in sexually mature tilapias. The histological and morphometric analyses in the serial sections (4µm in thickness) investigated showed that mean urethral and spermatic duct diameter was about 310µm and 270µm, respectively, and that the former was dorsally located in relation to the spermatic duct (Fig. 8). It is also important to mention that the common spermatic duct total length was very short (~2mm), meaning that a very narrow space was available for germ cells transplantation through this duct. However, after several attempts, we were able to successfully inject into the tilapia testes a solution containing 0.4% trypan blue (Sigma) as a marker for the injection efficiency (Fig. 9).

Germ cells transplantation

After all necessary approaches were developed, the selected germ cells were transplanted into the testes of six sexually mature Nile-tilapias. For this purpose, micropipettes with 100-300µm in diameter on its tip and attached to a 1-3mL syringe, was used utilizing also an Olympus SZX-ILLB2-100 stereoscope. Each animal received a 2mL cell suspension containing 10^6 germ cells per mL. This 2mL suspension also contained DEMEM and 0.4% trypan blue diluted in saline in the proportion of 1:1. The trypan blue was utilized with the purpose to monitor the transplant efficiency (Ogawa *et al*., 1997). The depletion of endogenous spermatogenesis, following busulfan treatment in association with the temperature of 35ºC, was confirmed by light and scanning electron microscopies in two tilapias sacrificed at the time of transplantation (Fig. 4). After the transplant, the water temperature was gradually decreased (1-2ºC per day) from 35ºC to 25ºC.

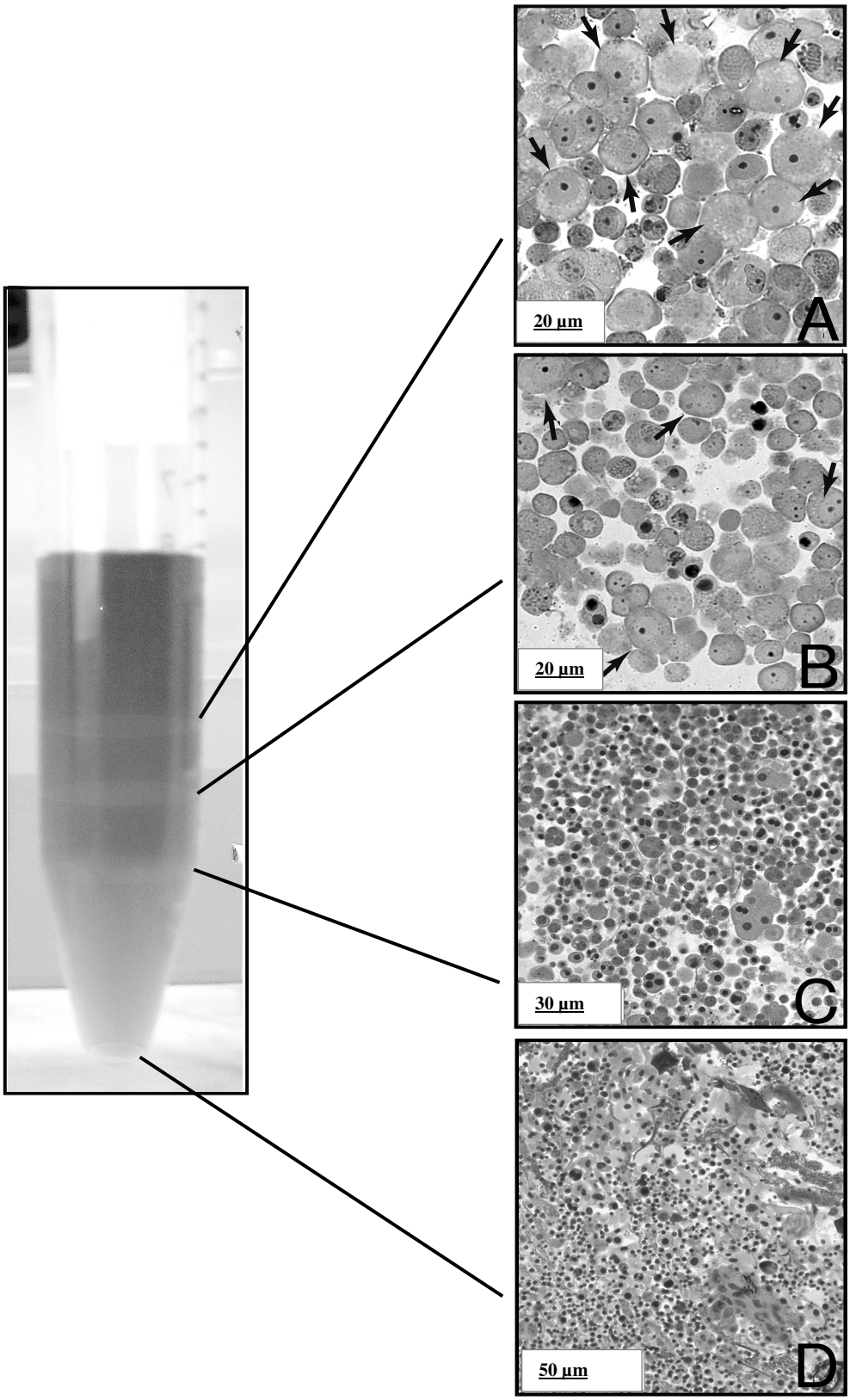


Figure 6. Selection and isolation of germ cells from sexually mature tilapias, utilizing percoll gradient. Observe that three distinct cell bands are present in the Falcon tube. These cells were fixed in 4% glutaraldehyde, ressuspended in agar-agar, and embedded in glycol methacrilate. Because they presented a high concentration of spermatogonia (arrows), the cells present in the two upper bands (A and B) were selected to be transplanted. The lower band (C) presented predominantly spermatyocytes and spermatids, whereas in the pellet (D) red blood cells and sperm were the predominant cells.

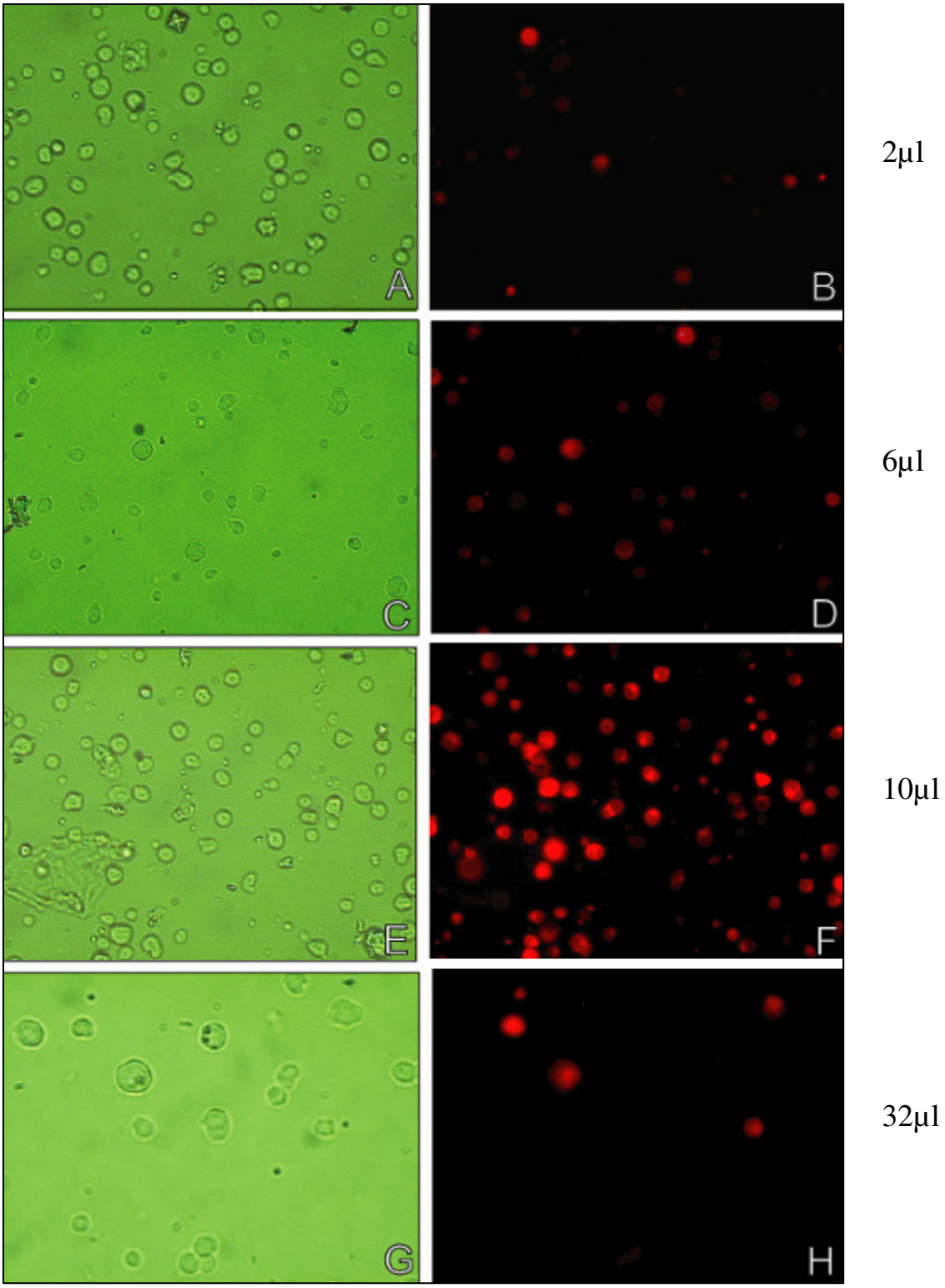


Figure 7. Germ cells labeling with different concentrations (2µl, 6µl, 10µl, and 32µl) of red fluorescent cell Linker (PKH26), at different time periods of incubation. Observe that with 2µl the label intensity was weak and low number of germ cells was labeled (A-B). Although the label intensity was much stronger with 32µl (G-H), the number of labeled germ cells was also low due to the lower germ cell viability. Good to optimum label intensity was observed, respectively, with the concentration of 10µl and 6µl of PKH26 (C-F). The analysis of cell viability allowed to verify that the 6µl concentration was sligtly better than 10µl, suggesting that probably a protocol utilizing an intermediate concentration between 6µl and 10µl of PKH26 would be an efficient protocol to label the germ cells before transplantation.

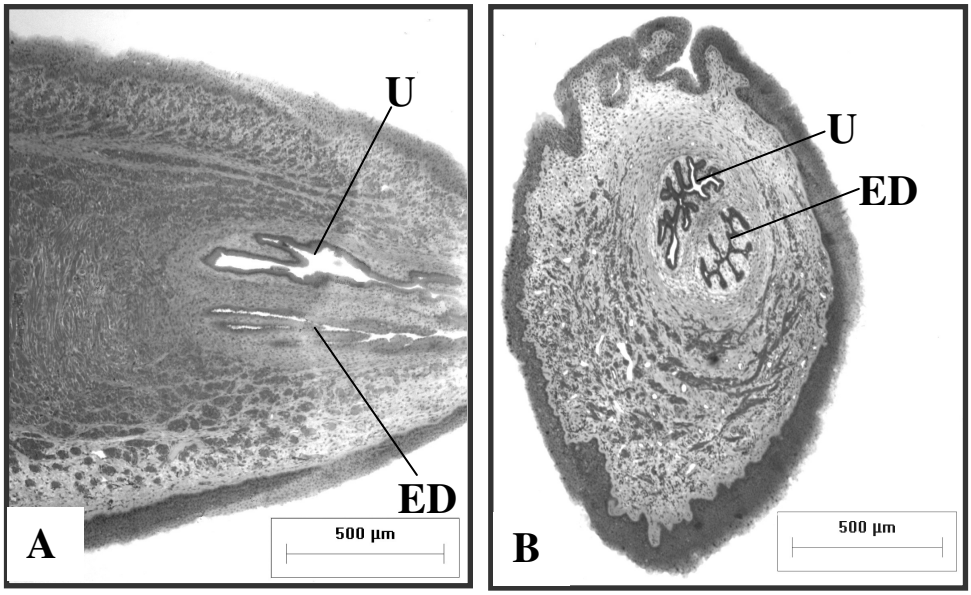


Figure 8. Disposition and relative size of the urethral and commom spermatic duct in sexually mature tilapias, visualized in longitudinal (A) and transversal section (B) of the uregenital papilla. Observe that the urethral duct (U; located dorsally) presents a bigger size in comparison with the commom spermatic duct (ED; ventrally located). The urethral duct is also longer than the commom spermatic duct, making it mistakenly easier to introduce the micropipette in this duct.

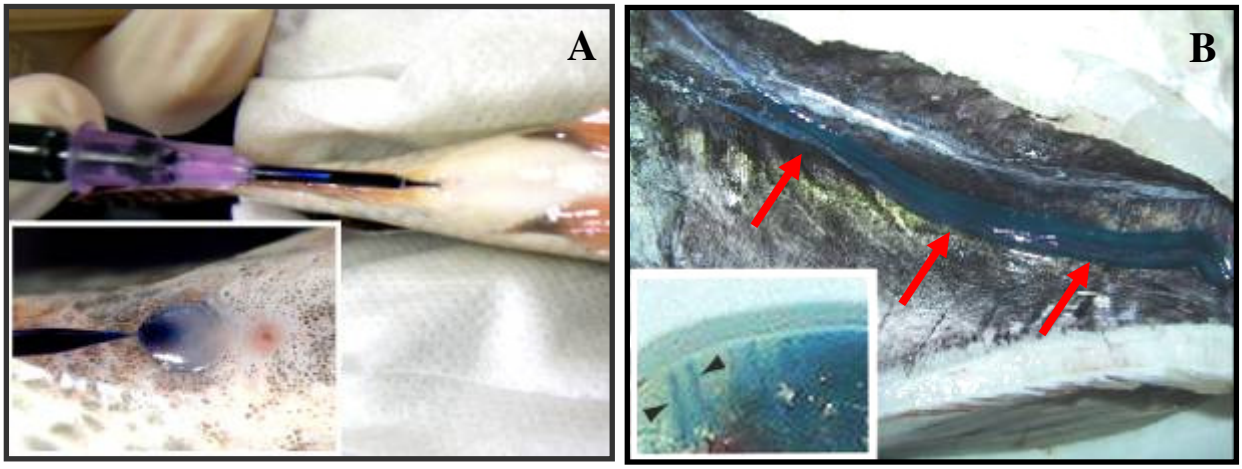


Figure 9. This figure illustrates the procedure utilized the verify the injection efficiency through the commom spermatic duct, utilizing a 0.4% trypan blue solution as a marker (A), and the testis (arrows) of a sexually mature tilapia sacrificed after the trypan blue injection (B). The insert in Fig. A shows the trypan blue injection in the urogenital papilla, whereas the insert in Fig. B depicts, at a higher magnification, the seminiferous tubules lumen (arrow heads) filled with the trypan blue solution.

The transplanted tilapias were sacrificed 14 hours, 2 weeks, and 4 weeks post-transplantation. Immediately after sacrifice, testis fragments were properly embedded (Jung Tissue Freezing Medium; Leica Instruments, Nussloch, Alemanha), frozen in liquid nitrogen (-196ºC) and stored at -80ºC. These samples were then seccioned (5µm in tickness) in a cryostat at -30ºC (LEICA CM 1850; Leica Instruments, Nussloch, Alemanha). These sections were properly prepared to perform light microscopy analyses utilizing a fluorescent Olympus IX-70 microscope, and the photographs were taken utilizing and Olympus IXSPT digital camera. Testis fragments were also fixed in 4% glutaraldehyde and routinely prepared for conventional light microscopy evaluation.

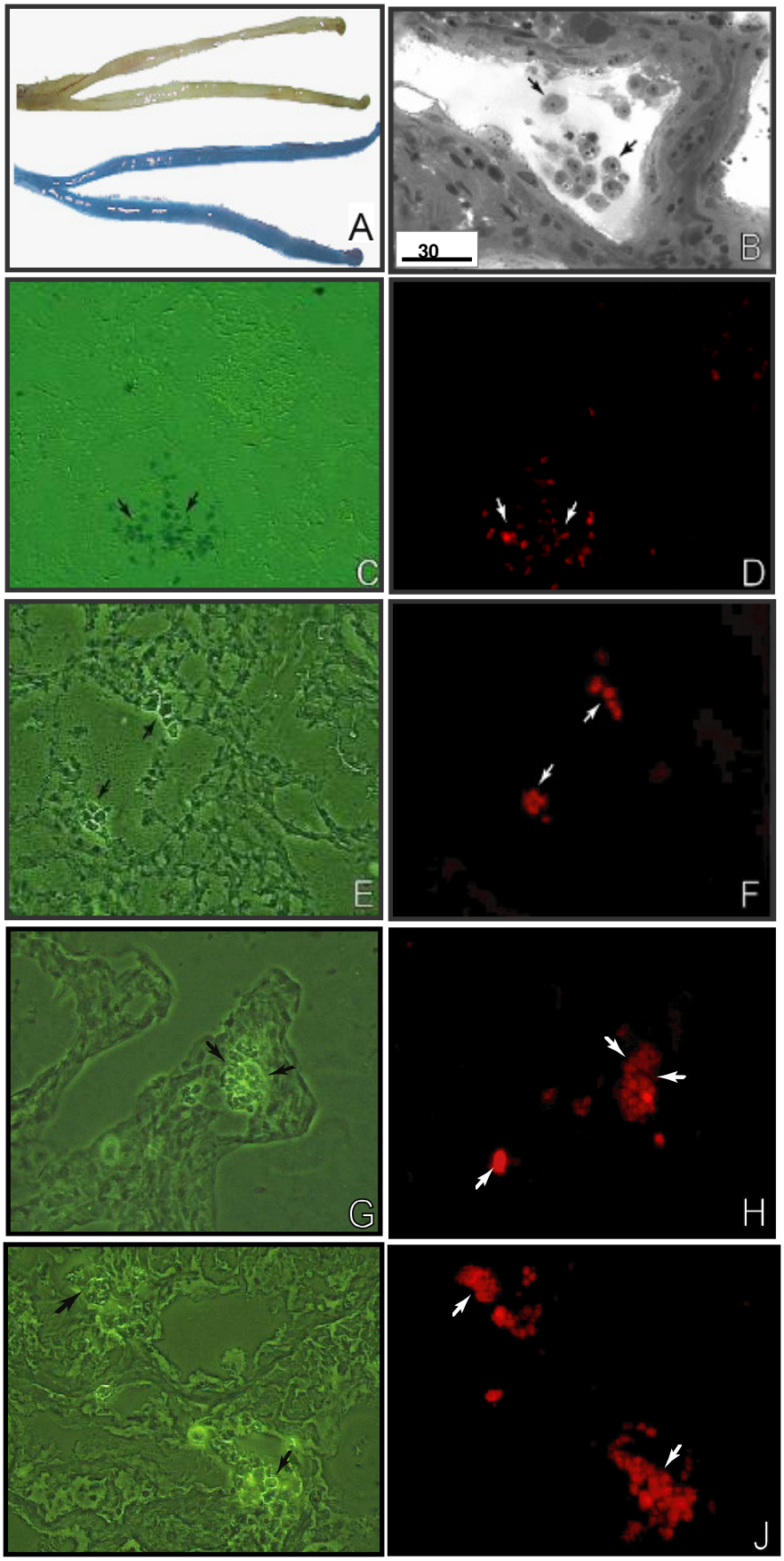


Figure 10. The Figure A depicts the testes of sexually mature tilapias treated with busulfan and kept at 35ºC. Compared to non-transplanted tilapias, the transplant efficiency can be clearly visualized through the blue color (lower figure) present in the tilapias that received germ cells labeled with PKH26 in a solution containing trypan blue. The Figure B illustrates the presence of transplanted germ cells (arrows) in the seminiferous tubule lumen, 14 hours post-transplant. Transplanted PKH26 labeled germ cells present in the seminiferous tubule (arrows), 14 hours post-transplant, can also be observed by fluorescence microscopy (D and F). These cells are found both in seminiferous tubule lumen (C-D) and in the tubule wall (E-F). Apparently, they do not present a cystic arrangement. However, PKH26 labeled germ cells cysts of different sizes were observed at 2 and 4 weeks post-transplant (G to J). These germ cells cysts are probably originated from transplanted spermatogonia stem cells.

As it is shown in Fig. 10, the injection containing germ cells labeled with PKH26 and trypan blue was very efficient. This aspect can be externally visualized through the tunica albuginea of tilapias sacrificed 14 hours pos-transplant, in comparison to the tilapias that were busulfan-treated and did not receive germ cells. This figure also shows, by light and fluorescence microscopy, transplanted germ cells present in the seminiferous tubules lume, 14 hours post-transplant. PKH26 labeled germ cells were also observed in contact with the seminiferous tubule wall. Apparently, they did not show cystic arrangement. At 2 and 4 weeks post-transplantation, PKH26 labeled germ cells cyst were observed in the seminiferous tubules of sexually mature transplanted tilapias (Fig. 10).

Conclusions and perspectives

The main objectives of the present study were to investigate the viability of intra-specific (syngeneic) germ cells transplantation in tilapias. Therefore, several necessary and crucial steps for germ cells transplantation, such as endogenous spermatogenesis depletion, isolation of the germ cells and germ cells labeling, and the investigation of the transplantation via, were developed in our laboratory for tilapias. The presence of PKH26 labeled germ cells in the seminiferous tubules confirmed that the approaches utilized for germ cells transplantation in tilapias were successful. Ongoing studies in our laboratory are showing that more advanced germ cell cysts and even PKH26 labeled sperm can be observed in the seminiferous tubules of transplanted tilapias, several weeks post-transplant. To our knowledge, this is the first report for germ cells transplantation directly into the testis in any teleost species. These very promising results show that the tilapia is emerging as a potential experimental model to investigate the spermatogenic process, the stem cell biology, and the testis function in teleosts. However, we still need to find good markers for spermatogonia stem cells in tilapias, and develop good marker for donor germ cells employing for instance green fluorescent protein. Also, the development of recipient fish lacking endogenous spermatogenesis will be very useful. Other tempting approaches utilizing the transplantation technique would be the investigation of the viability of germ cells after they are frozen and/or cultured. The investigation of the viability of tilapias as a receptor model for germ cells transplantation from other teleost species of economical importance or endangered fish species is also very important. Altogether, we hope that these excitings approaches will provide an entirely new and promissing scenario for fish biotechnology, conservation and production.

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