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Busulfan administration produces sublethal effects on somatic tissues and inhibits gametogenesis in Senegalese sole juveniles

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Summary. Busulfan, a cytotoxic alkylating agent used for treatment of chronic myeloid leukemia has effects in mammalian germ cells. In fish species, the use of this compound is of special interest in intra and interspecies germ cell transplants. To determine the effects of busulfan in fish a previous range finding experiment was designed. Survival and growth rate of 150-days after hatching (150DAH) Senegalese sole (*Solea senegalensis*) juveniles was determined. In a second experiment, the effects of a sublethal busulfan dose in fish germ cell depletion and in somatic tissues were analysed. Sublethal effects of several busulfan treatments (B10-10 days after injection, B20-20 days after injection, B20+-20 days after injection with double injection) were determined in somatic and gonadal tissues. Alterations were registered through histopathological techniques, TUNEL (cell apoptosis) and quantified at molecular level (Q-PCR analyses) using the *vasa* mRNAs (*Ssvasa1-2* and *Ssvasa3-4* mRNAs) as molecular markers for germinal cells in Senegalese sole juveniles. Several sublethal effects were observed with 40 mg kg^{-1} busulfan, a non-lethal dose, such as: pyknosis in liver, increase of melano-macrophage centres and blood stagnation in spleen and interruption of gonadal development. Females were more affected by busulfan treatments than males in terms of germ cell disruption, since a significant decrease in the expression of both *Ssvasa1-2* and *Ssvasa3-4* markers was found in the gonad of treated females rather than males. At 10 days post-treatment (B10), females already presented a decrease in germ cell proliferation, as confirmed by Q-PCR. *Ssvasa* expression proved to be a reliable tool for the direct evaluation of the effects of busulfan on Senegalese sole gonadal development, proving that busulfan can be a suitable treatment for causing transient sterility in recipient gonads for germ cell transplantation.

Key words: *Ssvasa* expression, Busulfan sublethal effects, Gametogenesis inhibition, *Solea* senegalensis

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Introduction

In higher vertebrates, several methods have been used to inhibit gametogenesis and germ cell proliferation, (Schlatt et al., 1999; Shinohara et al., 2000; Zhang et al., 2004; Jahnukainen et al., 2007; Trefil at al., 2010; Drumond et al., 2011; Gao et al., 2012; Zohni et al., 2012). In fish, techniques such as triploidy (Yoshizaki et al., 2011), hyperthermia (Strussman et al., 1998; Ito et al., 2008a,b), radiation (Chmilevsky et al., 1997), antiandrogens (Billard, 1982) or chemical compounds, have been used to obtain the same results. Recent studies in some fish species demonstrated the efficiency of chemical methods in the sterilization of recipient species (Majhi et al., 2009a,b; Lacerda et al., 2013). Busulfan is the most common drug used for this purpose.

In marine teleosts, germ cell transplantation is a valuable technique for assisted reproductive strategies to improve broodstock management (Morita et al., 2012).

However, efficient colonization of transplanted cells depends on several factors, such as the degree of recipient sterilization, since transplanted cells will compete with endogenous spermatogonia for available stem cell niches (Shinohara et al., 2001). Although busulfan treatment could be very useful for the preparation of fish recipients for spermatogonia transplantation, few reports have used busulfan as a reproductive disruptor agent in fish (Majhi et al., 2009a,b; Lacerda et al., 2010) and its effect has not been well documented.

In reproductive organs, the sterilizing dose of busulfan is species-specific. In mice, a single injection of 15-45 mg kg^{-1} busulfan produced sterilization after 4 weeks post-treatment, although results were obvious after 3 days treatment (Zohni et al., 2012). In other species these concentrations were higher (pigs, 40-100 mg kg^{-1}) or lower (coyotes, 4-12 mg kg^{-1}) (Stellflug et al., 1985; Kim et al., 1997), not only showing a species-specific interaction, but also a dose-dependent and time-dependent effect. In Patagonian pejerrey (*Odontesthes hatcheri*) (Majhi et al., 2009a) and in rainbow trout (*Onchorynchus mykiss*) (Billard, 1982), some mortalities have been reported for busulfan doses of 20-40 mg kg^{-1} and 10 mg kg^{-1} respectively.

Adverse effects of busulfan on the animals' health have been mentioned in mammalian spermatogonia transplantation studies (Clouthier et al., 1996; Dobrinski et al., 2000), but few reports have elucidated these effects in fish at molecular level.

The aim of this study was to identify busulfan sublethal treatments capable of producing transient sterilization in Senegalese sole, *Solea senegalensis*, a species with high commercial value whose massive production still has several reproductive problems (Cabrita et al., 2006, 2011; Garcia-López et al, 2007; Guzman et al., 2010) and which could be a good candidate for germ cell transplantation programs. Histopathological analyses and TUNEL assay were performed to describe the effects of busulfan on the somatic and gonadal tissues. Furthermore, the quantification of specific germ cell molecular markers for Senegalese sole, the Ssvasa mRNAs (Pacchiarini et al., 2013), were used to determine the effect of busulfan on Senegalese sole early gonads at molecular level.

Material and methods

Biological material

Senegalese sole juveniles from a commercial fishfarm (A. Coelho e Castro, Povoa do Varzim, Portugal), were acclimatized in two 200 l seawater tanks with a photoperiod of 12 h light/12 h dark and 19°C temperature before the onset of the experiments. They were fed 4 times daily (Aqua Gold 3, Sorgal, Portugal), corresponding to approximately 3% of their body weight. Water flow rate was maintained at 100 l/h. After a three-week-period, the sole juveniles were redistributed in small subunits (4.5 liter) at a density of 5.4±0.2 g dm^{-3}. For each treatment, 30 fish (2.53±0.47 g) were used, redistributed equally in 3 subunits (n=10 in each subunit) maintained in the main tanks. A total of 29 fish batches were used. Fish handling was in accordance with the European Union Directive (2010/63UE) and REGA-ES110280000311 for the protection of animals used for experimental and other scientific purposes.

Experimental procedures

Two experiments were designed, one to evaluate the effects of busulfan concentration and the period after injection on the survival and growth rate of Senegalese sole (experiment 1) and another to analyze the effects of a sublethal busulfan dose in fish germ cell depletion and in somatic tissues (experiment 2). In experiment 1, the effect of five busulfan doses (5, 10, 20, 40 and 80 mg kg^{-1}) was tested in 150 DAH (days after hatching) Senegalese sole juveniles. Busulfan concentrations were prepared from a stock solution (4 mg of busulfan dissolved in 1 ml of DMSO). Each dose was prepared in PBS. For each treatment 30 juveniles were anesthetized with 250 ppm phenoxyethanol for 2 min. Fish were injected intraperitoneally with a microsyringe (Hamilton, Spain) using different busulfan amounts previously diluted in PBS to reach a final volume of 50 μl of each concentration. Two controls were used, one group injected with DMSO+PBS (dose vehicle) and another group was left untreated. All treatments were performed in triplicate. The fish were incubated in subunits and maintained as described before for 10 and 20 days post-treatment. Busulfan doses were selected according to Majhi et al. (2009a).

For experiment 2, the dose of 40 mg kg^{-1} was chosen according to the results of experiment 1. In this case, the effects of busulfan were analyzed in 3 different conditions: B10-after 10 days injection; B20-after 20 days injection; and $B20^{+}$- at day 20, after a second booster, ten days after the first injection. As in experiment 1, the individuals were anesthetized with 250 ppm of phenoxyethanol and a dose of 40 mg kg^{-1} busulfan was injected intraperitoneally as described before. The experiment was performed in triplicate (n=30, 10 fish for each subunit) and the control subunits (injected with the vehicle solution) were maintained under the same conditions described before.

At the end of the experiments fish were sacrificed with a lethal dose of phenoxyethanol (2000 ppm). Half of the samples from each treatment (n= 15) were immediately frozen in liquid nitrogen after dissection. Samples were stored at -80°C for vasa quantification until total RNA extraction. The other half were dissected for histological analysis and fixed in 4% paraformaldehyde in PBS for 48 h at 4°C, and washed three times with PBS before being placed in methanol at -20°C.

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (Spain) and were reagent

grade or higher. The experimental procedures were in accordance with the OECD guidance document for the diagnosis of endocrine-related histopathology of fish gonads (2009).

Survival and growth rate

The survival rate was determined by counting the number of fish in each subunit with regard to the number of fish present at the beginning of the experiment. Survival rates were calculated at 24 h, 48 h, 10 and 20 days after treatment. The growth rate was determined by weighing the fish at the beginning of the experiments and on days 10 and 20 post-treatment, using the formula: $GW=W_f - W_i t^{-1} \times 100$, where W_f is the final weight, W_i is the initial weight and t is the period of experimentation.

Q-PCR Ssvasa transcripts expression

Samples for RNA extraction were collected from half of the fish (n=15) used in each treatment and control group. The sex of the individuals was checked during necropsy, separating females from males. Approximately 100 mg of tissue were added to 1 ml of Trizol and homogenized at room temperature (RT). Then 200 μl of chloroform was added and after 10 min the samples were centrifuged for 15 min at 14,000 rpm, 4°C. The supernatants were transferred into new tubes and 0.5 ml of isopropanol was added. After 10 min of incubation, the samples were centrifuged as before and 1 ml of 75% ethanol was added to each sample, and centrifuged for a third time (14,000 rpm, 5 min, 4°C). The pellets were dried before re-suspending the RNA in RNase free water. Total RNA concentration was assessed spectrophotometrically (A260nm/A280nm ratio>1.8) and integrity verified by electrophoresis in a 1.2% agarose/formaldehyde gel.

Before cDNA synthesis, RNA samples were treated with 1 μl of DNase I, RNase Free (Fermentas, Madrid, Spain) for 15 min and DNase inactivated at 65°C for 10 min in the presence of EDTA.

Total RNA (1 μg) was reverse transcripted in a final volume of 20 μl using the iScript™ cDNA Synthesis Kit (Bio-Rad, Madrid, Spain). PCR products from *Ssvasa1-2* (JN564594, JN564595) and *Ssvasa3-4* (JN564596, JN564597) were obtained using specific primers previously described by Pacchiarini et al., (2013) (Table 1). Genomic interference was tested by performing Q-PCR using total RNA in the absence of cDNA. Relative mRNA expression levels were determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2011).

Three biological replicates of each treatment (males and females collected from treatments B10, B20, B20$^+$ and control) were analyzed and each PCR was performed in parallel with a technical duplicate. Q-PCR reactions were performed using the SsoFast™ EvaGreen® Supermix Kit (Bio-Rad, Madrid, Spain) in a Mastercycler® Realplex real time PCR (Eppendorf, Germany). The program used had three steps: denaturalization (2 minutes at 94°C) followed by 40 cycles composed of 15 s at 94°C, 30 s of annealing at 65°C and 45 s of extension at 72°C. The melting curve ran for 20 minutes from 65°C to 94°C. For normalization of cDNA loading, all samples were run in parallel using the eEF1A1 (AB326302.1) housekeeping gene chosen according to Infante et al., (2008). Standard curves (1:10, 1:100; 1:1000 and 1:10000) were generated for each primer pair as described by Gil-Salas et al., (2007) and the efficiency (100±5%) was determined as described by Pacchiarini et al., (2013). Negative Q-PCR controls using double distilled water instead of cDNA were included in the assays for each primer pair.

Histological procedure

To decalcify ossified structures, a dissolution of 10% EDTA and 2% formaldehyde at pH=7 was used for 7 days. The samples were washed three times for 30 min in PBS and after this a standard inclusion protocol for paraffin embedding was used. The pieces were cut into 5 μm thick sections in a microtome (LEICA, SPAIN). Haematoxylin-Eosin staining was performed on dewaxed slides for 6 min in Harris Haematoxylin, followed by 1 min in Eosin solution. Finally, the slides were mounted and sections were observed and recorded using light microscopy (LEICA DM 2000, Germany) and a digital camera (LEICA DFC 420C, Germany). Quantification of histological alterations was performed in 60 non-consecutive slide sections for each treatment and control. For that, 15 slides containing 4 sections of each organ (gonads, liver and spleen) were observed for each individual (n= 3), registering the presence of histological alterations (gametogenesis depletion, pyknosis, blood stagnation and increase of melanomacrofage centres).

Cell apoptosis analysis

Cell apoptosis (TUNEL assay) was determined in gonad slide sections using the *in situ* cell death detection Kit (Roche, Spain), which identifies DNA damage at 3'-OH endings (Gillan et al., 2005). The slides were treated with proteinase K (10 μg ml^{-1} in 10 mM Tris/HCl, pH

Table 1. Oligonucleotide primers used in Q-PCR analyses.

Transcripts	Primers	Sequence (5'→3')
Ssvasa1-2	Ssvasa-3'-I-Rev	TCCTGGAGTCTGTGGAGGCAAA
	Ssvasa-3'-I-For	TGACTCGCTCCTTGGTTTCAAT
Ssvasa3-4	Ssvasa-3'-II-Rev	CAGCAGGTCTCGTCCTTGGTGA
	Ssvasa-3'-II-For	CCGTGTGCGGCAGTGATGGAG
eEF1A1	Ss-eEFIAI-Rev	GGCAAAGCGACCAAGGGGAGCAT
eEF1A1	Ss-eEFIAI-For	GATTGACCGTCGTTCTGGCAAGAAGC

7.4) for 30 min at 37°C, washed with PBS and dried. Each slide was incubated with 5 μl of enzyme solution and 45 μl of labeling solution (BrdU FITC) for one hour at 37°C in the dark. Two controls were used: a positive control where slides were treated with DNase I (3 U ml-1 in 50 mM Tris/Hcl, pH 7.5) for 10 min, previously to the labeling, and a negative control where slides were incubated without the enzyme solution. All the slides were mounted with Vectashield and observed in a fluorescent microscope (LEICA DM 2000, Germany) with 450-490 nm excitation and 515 nm emission. Apoptosis effect were registered only in ovary using (-), (+) and (++) symbols according to the different levels observed: (-) absent (+), slightly present and (++) highly present. A total of 60 slide sections were analyzed for each treatment and control.

Statistical analysis

The data were analyzed with a one way analysis of variance (ANOVA) using the multiple comparison Student-Newman-Keuls test (S-N-K) with SPSS (version 17.0). Percentile data were normalized using arcsine transformation. Data are shown as mean± SD and significant differences between groups were signed when $P \leq 0.05$.

Results

Effects of busulfan on survival and growth rate

For all the treatments tested, mortality was only registered with 80 mg kg^{-1} busulfan dosage. For this concentration and in both post-treatment periods, we obtained similar values of survival at 24 and 48 hours after injection. Survival rate decreased significantly in the first 24 hours (43.3% for both periods after treatment). Survival rates were 20% and 23.3%, 10- and 20- days post-treatment, for this concentration (Fig. 1). After 10 days of treatment, the growth rate was negative in most cases. Under these conditions, we did not observe a relationship between administered dose and growth rate, since all dosages, from the lowest (5 mg kg^{-1}) to the highest (80 mg kg^{-1}) produced similar results. At 20 days post-treatment there was still a significant decrease in the growth rate of animals treated with 40 mg kg^{-1} in comparison with the control fish (Fig. 2).

Gametogenesis Inhibition

The single injection of busulfan (B10 and B20) caused different effects on gonadal tissues, but in general the effects were more evident in Senegalese sole treated with the double dose ($B20^{+}$). Q-PCR results showed no significant differences between relative expression of both *Ssvasa1-2* and *Ssvasa3-4* transcripts in the testis (Fig. 3A,B) of treated juveniles when compared with the control group (calibrator). Nevertheless, in females (Fig. 3C,D) a significant decrease in the expression of the *Ssvasa3-4* was registered in treated individuals compared with the controls (Fig. 3C). The decrease was 3.5 times for B10, 3 times for B20 and 2 times for $B20^{+}$. However, no significant differences were found between treatments. In the expression of *Ssvasa1-2* we observed a similar pattern to the *Ssvasa3-4* transcript (Fig. 3).

By using Haematoxylin-Eosin staining it was possible to identify germinal epithelium, oogonia and basophilic previtellogenic oocytes which followed the normal response pattern corresponding to the development for the age of the female juvenile fish (Fig. 4A). Males from the control group shown a delay in gametogenesis when compared with females (data not shown). Differences in gonadal developmental stages

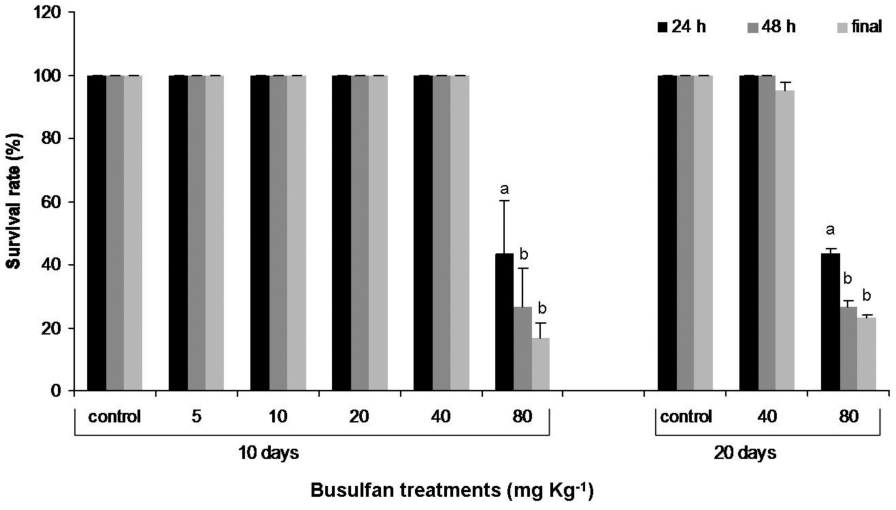

Fig. 1. Survival rate (%) of juveniles after 10 and 20 days post-treatment with 5, 10, 20, 40 and 80 mg kg^{-1} busulfan. Significant differences are shown by different letters ($p \leq 0.05$; n=30).

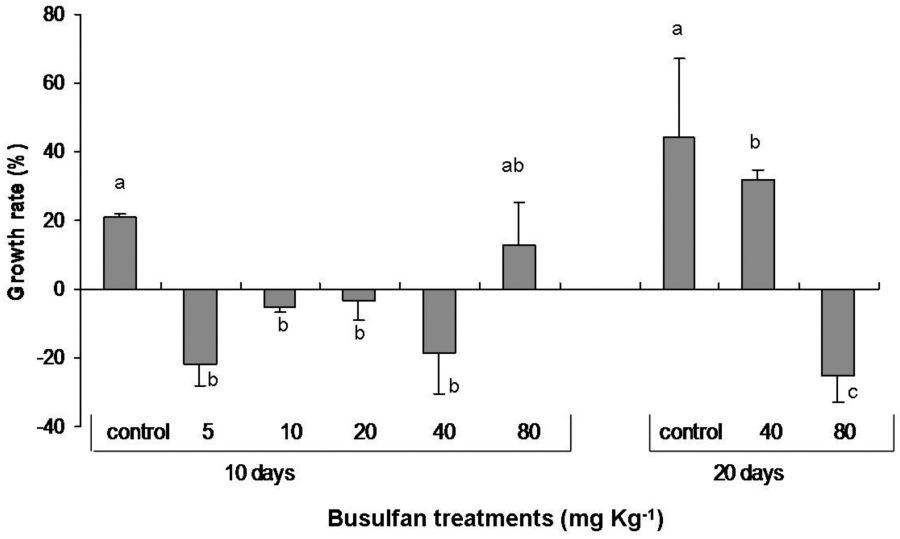

Fig. 2. Juveniles' growth rates (%) obtained 10 and 20 days post-treatment with 5, 10, 20, 40 and 80 mg/kg busulfan. Significant differences registered separately for 10 and 20 days are shown by different letters (p≤0.05; n=30).

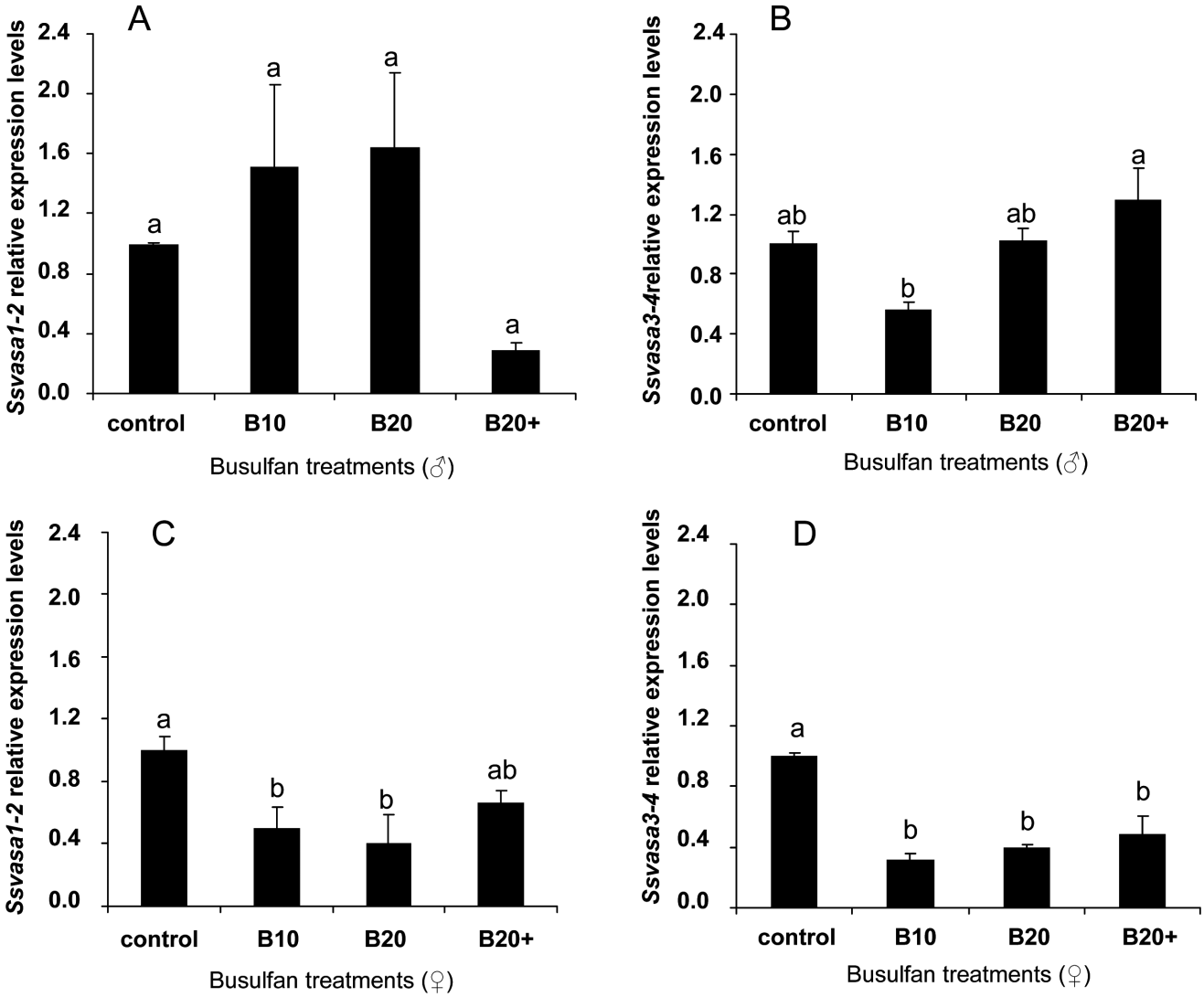

Fig. 3. Relative expression of *vasa* in juvenile males (**A, B**) and females (**C, D**) treated with B10, B20 and B20⁺. Control data (untreated fish) was used as calibrator. Significant differences are shown by different letters (p≤0.05; n=3).

were observed between control and treated sole juveniles. Gonadal development was more delayed in the fish corresponding to B20 (Fig. 4B) and B20^{+} treatments than in B10 treatment (Fig. 4C). In treated females, a gradual and progressive effect related to busulfan concentration was observed, a lower number of

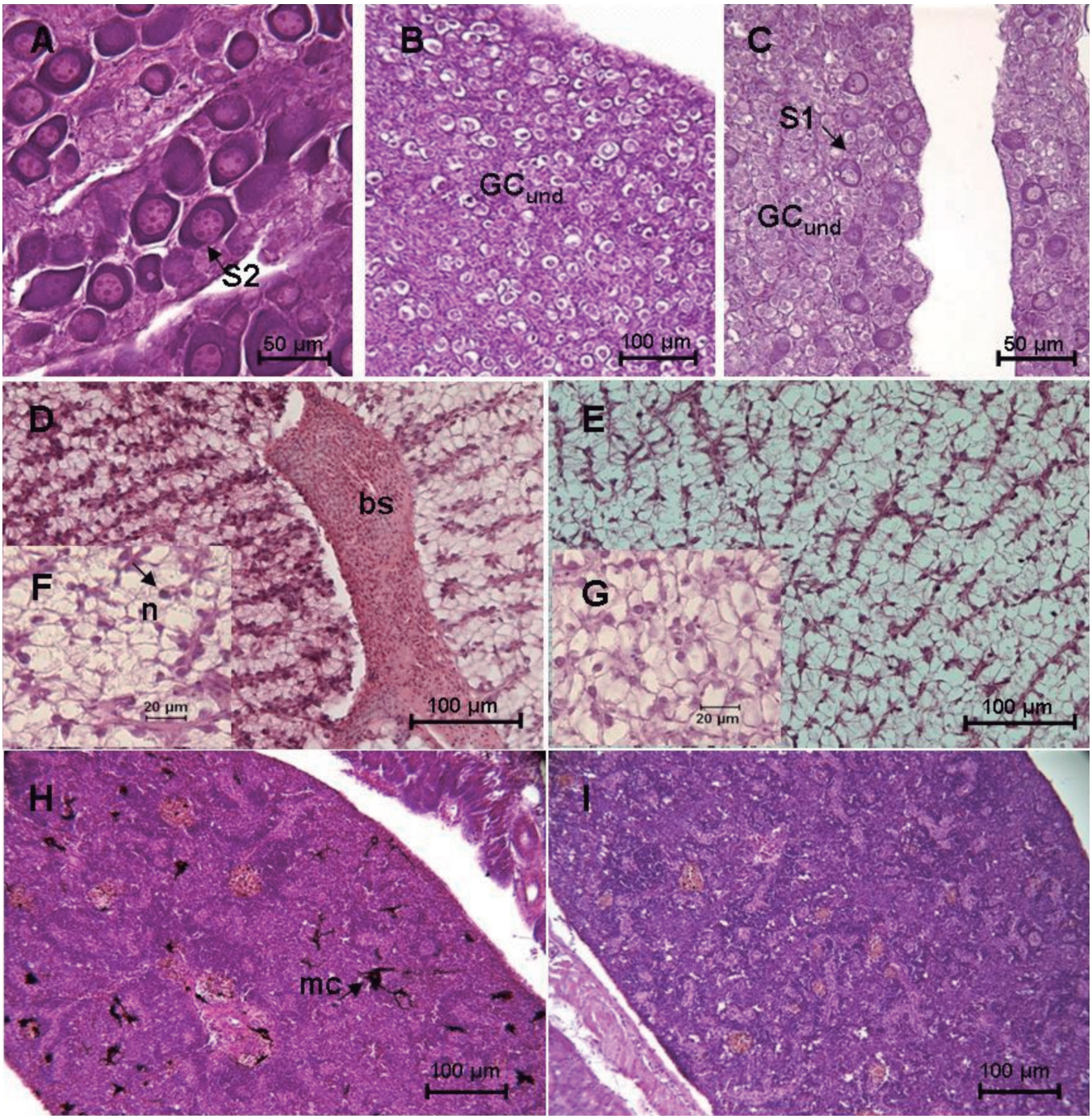

Fig. 4. Hematoxylin-Eosin staining of sections from gonad (**A-C**), liver (**D-G**) and spleen (**H, I**) of Senegalese sole juveniles. Gonad sections are from control group (**A**) and from treated fish (40 mg kg^{-1} busulfan) B20 (**B**) and B10 (**C**) groups. Early perinucleolar oocytes (S2) and chromatin nucleolar oocytes (S1) could be observed in figures **A** and **C** and are absent in **B**. Undifferentiated germ cells, GCund could be observed in delayed/depleted gonads are shown in figures **B** and **C**. Histological alterations such as nuclear darkness (n), blood stagnation (bs) are indicated in liver (**D, F**). Untreated control sections from liver are shown in figure **E** and **G**. In spleen (**H**) an increase of melanomacrophage centres (mc) is shown when compared with untreated control sections (**I**) (Scale bars are shown in each micrograph).

Table 2. Sublethal damage observed in juveniles treated with 40 mg kg^{-1} busulfan.

Tissue	Alterations	Quantification of alterations (%)			
		Treatments			Control
		B10	B20	$B20^+$	DMSO
Ovary	Gametogenesis depletion	85.4±12	93.4±7	100	0
Liver	Pyknosis	91.6±11	100	100	0
	Blood stagnation	100	100	100	35.2±10
Spleen	Increase of melanomacrophage centres	58.8±26	83.3±23	100	27.2±4

Table 3. Degree of apoptosis in gonads of fish exposed to 40 mg kg^{-1}busulfan treatments.

Tissue	Treatment			Control
	B10	B20	$B20^+$	DMSO
Ovary	+	++	++	-
Testis	+	+	+	-

previtellogenic oocytes and a higher proportion of undifferentiated cells and oogonia were detected.

Using TUNEL technique, cell apoptosis was observed in ovary (Fig. 5A) in all treatments (B10, B20 and $B20^+$) when compared with controls, but cellular death was more evident 20 days post-treatment (both with a single or a double dose) (Fig. 5B, Table 3).

Histopathological alterations

The single injection of busulfan (B10 and B20) showed different effects in somatic tissues, but in general the effects were more evident in Senegalese sole treated with the double dose ($B20^+$) (Figs. 4, 5). In the liver, some hepatocytes showed nuclear darkness and pyknosis, and blood stagnation was observed in all treated fish (Fig. 4D,F) compared with the control fish (Fig. 4E,G). A progressive increase in melano-macrophage centres (Fig. 4H) appeared in the spleen of treated animals, especially at 20 days post-treatment, compared with the control animals (Fig. 3I).

Discussion

To our knowledge, there are no reports on the establishment of the busulfan lethal dose in fish species, although some mortalities have been reported in Patagonian pejerrey (*Odontesthes hatcheri*) when a second dose of this compound (20-40 mg kg^{-1}) was administered (Majhi et al., 2009b), and in rainbow trout (*Onchorynchus mykiss*) with 10 mg kg^{-1} busulfan (Billard, 1982). In mammals, busulfan LD50 (25 mg kg^{-1}) was established for rats (Santos and Tutsehka, 1974) and 125 mg kg^{-1} was determined for mice (Bishop and Wassom, 1986). Sternberg et al., (1958) observed

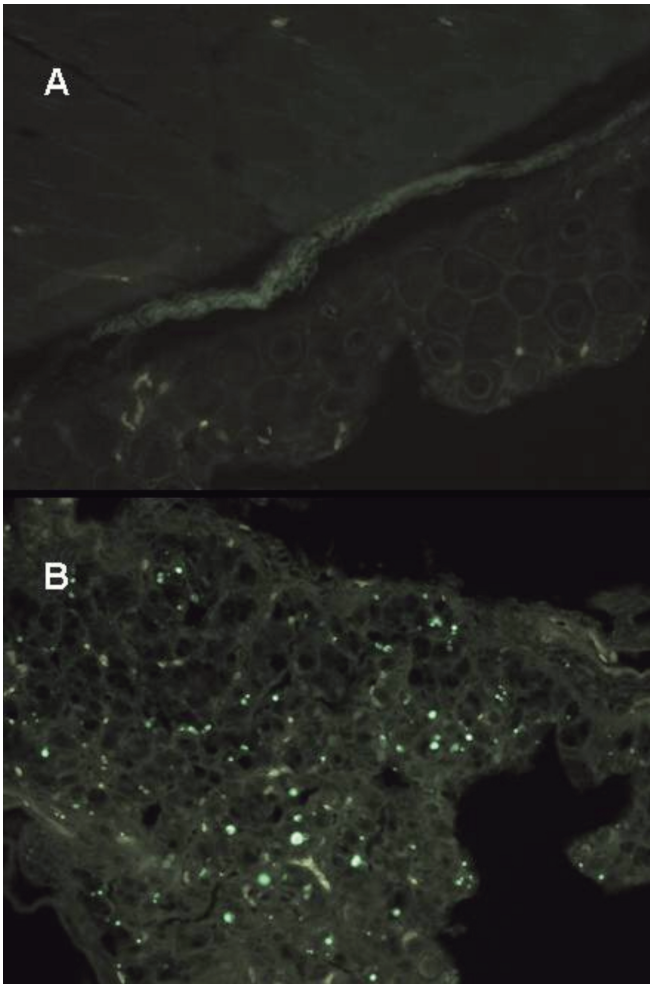

Fig. 5. TUNEL staining of sections (400 x) from gonads of 40 mg kg^{-1} busulfan untreated **(A)** and treated fish **(B)** ($B20^+$). Apoptosis was detected in germinal cells (B).

that the peak of mortality happened 24 hours post-administration, as in our study, demonstrating immediately the acute effects of busulfan.

In the present study, on Senegalese sole juveniles, we observed gametogenesis depletion using 40 mg kg^{-1}

of busulfan, administrated in one or two injections, without producing mortalities in fish. However, we detected high mortality when the busulfan dose was 80 mg kg^{-1}.

For the first time we quantified the direct effects of busulfan in Senegalese sole germ cell arrestment using a specific molecular marker, the *Ssvasa* gene. We showed a decrease in *vasa* transcripts expression associated with the depletion of germ cells in gonads. The *Ssvasa* marker is a useful tool because this gene is predominantly expressed in undifferentiated germ cells and also in previtellogenic oocytes. *Ssvasa1-2* and *Ssvasa3-4* transcripts were characterized previously by our group, demonstrating the presence of both transcripts in juvenile immature gonads (Pacchiarini et al., 2013). Our results agree with the previous ones since both *Ssvasa* transcripts were detected in juveniles (150 DAH). From the quantification of *Ssvasa* expression we did not obtain significant differences between treated and control males for either transcripts. Our results disagree with those of Majhi et al. (2009a,b), who found a higher response to busulfan treatment in males than females in Patagonian pejerrey. However, Billard (1982) found irregular results in rainbow trout male sterility when busulfan was used (10 mg kg^{-1}), recording inhibition of spermiation in some individuals but not in others. On the other hand, the results obtained for Senegalese sole females treated with busulfan indicated a statistically significant decrease in *vasa* expression (*Ssvasa1-2* and *Ssvasa3-4*) compared with control females, meaning that germ cells were affected by the treatment. These results were in accordance with the ones observed in female gonads by TUNEL assay as well as by histological analysis.

According to *vasa* expression levels, this study failed to attribute a dose-time effect on germ cell depletion, since for both transcripts the expression levels obtained by the different treatments were similar during all experimental procedures.

Busulfan may also produce secondary symptoms and sublethal effects (Stellflug et al., 1985). In our study we observed a decrease in fish growth rate, but no relationship between dose and effect was evidenced. Secondary effects caused by busulfan were detected in the liver, with the presence of some pyknotic nuclei and blood stagnation areas. In the spleen an increase in the number of melanomacrophage centers was also registered, representing, among others, breakdown products of hemoglobin from degenerated red blood cells, a mechanism of defense as described by Agius and Roberts (2003) in trout spleen.

The damage produced by busulfan in the DNA (DNA crosslinks or strand breaks) could not be repaired by cellular mechanisms, and thus some apoptotic events were observed in gonads. TUNEL positive cells were detected in germ line, especially in the 20-days treatment, although the effects were already evident at 10 days post treatment. The induced regression of gametogenesis was due to the inhibition of germ cell differentiation and proliferation, because busulfan blocks the mitotic process and brings more cells to apoptotic stages. According to Majhi et al. (2009a,b) working on Patagonian pejerrey, spermatogonia and ovogonia depletion occurred 2 weeks after treatment with busulfan. Other authors detected a decrease in gonadosomatic index in trout (Billard, 1982) or in the gonad weight of mice (Anjamrooz et al., 2007; Minyoung et al., 2007). In the present study, Senegalese sole was already depleted from germ cells at 10 days post-treatment and a prolongation of time (20 days) only allowed an increment of depleted germ cells and oogenesis delay.

A gonad recovery period was not reached in our study since apoptosis was more pronounced in treatments B20 and $B20^{+}$ with longer exposure than B10. TUNEL results together with Q-PCR data also confirmed the lack of gonad recovery throughout the experiment, furthermore at the end of the experiment we expected *vasa* levels similar to the controls and this did not happen. In Patagonian pejerrey, gonad recovery was established at 8 weeks for the 40 mg kg^{-1} busulfan injection, registering a repopulation of germ cells in the gonad (Majhi et al., 2009b). Similar studies on mice have demonstrated that a period of 54 days was necessary to observe a recovery of the testis for the same dose (Anjamrooz et al., 2007)

Although a decrease in the expression of both *Ssvasa* transcripts was observed 10 days post-treatment of busulfan in females, the same was not observed for males. Probably a treatment with more days of exposure or even a combination of other factors such as temperature or other compounds able to deplete Leydig and Sertoli cells (Koh et al., 2006) could produce similar effects as those observed in females. In the control group gametogenesis in males was more delayed than in females. This observation could explain our Q-PCR results and suggests that busulfan can produce different effects at germinal level between males and females regarding gonad developmental stage. This observation supposes an interesting issue for more investigation in the future.

In conclusion, busulfan treatment affected germ cell proliferation in females as the expression of *Ssvasa1-2* and *Ssvasa3-4* transcripts decreased when compared with control fish. This effect was more significant in female juveniles than in males, probably because of delayed development of the testis of juveniles (150 DAH). The quantification of *Ssvasa* expression by quantitative PCR proved to be a useful technique for studying the effects of busulfan in the Senegalese sole gonad. Although B20 and $B20^{+}$ produced higher germ cell apoptosis, at 10 days post-treatment (B10) females already presented a decrease in germ cell proliferation, as confirmed by Q-PCR. Busulfan sublethal effects could be counteracted by the organism defense mechanisms as shown by the different types of alterations registered in spleen, demonstrating that 40 mg kg^{-1} of busulfan can be a suitable treatment for causing transient sterility in recipient gonads for further

studies on germ cell transplantation.

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