# Flow Cytometric Chromosomal Sex Sorting of Stallion Spermatozoa Induces Oxidative Stress on Mitochondria and Genomic DNA

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# Flow Cytometric Chromosomal Sex Sorting of Stallion Spermatozoa Induces Oxidative Stress on Mitochondria and Genomic DNA.

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**ABSTRACT**

To date, the only repeatable method to select spermatozoa for chromosomal sex is the Beltsville sorting technology using flow cytometry. Improvement of this technology in the equine species requires increasing awareness of the modifications that the sorting procedure induces on sperm intactness. Oxidative stress is regarded as the major damaging phenomenon, and increasing evidence regards handling of spermatozoa -including sex sorting- as basic ground for oxidative damage. The aim of this study was to disclose whether the flow cytometric sorting procedure increases the production of reactive oxygen species (ROS), and to identify if ROS production relates to DNA damage in sorted spermatozoa using specific flow cytometry-based assays. After sorting, oxidative stress increased from 26% to 33% in pre- and post-incubation controls, to 46% after sex sorting ($p<0.05$). Proportions of DNA fragmentation index post-sorting was approximately 10% higher (31.3%); an effect apparently conduced via oxidative DNA damage as revealed by the oxyDNA assay. The probable origin of this increased oxidative stress owes the removal of enough seminal plasma due to the unphysiological sperm extension, alongside a deleterious effect of high pressure on mitochondria during the sorting procedure.

**Key words:** flow cytometry, sex sorting, oxidative stress, oxidative DNA damage, stallion.

## INTRODUCTION

To date, the only repeatable method for selecting spermatozoa by chromosomal sex is the Beltsville sorting technology using flow cytometry (Johnson 1995, Johnson 2000). This method has reached commercial status in the bovine industry (Frijters et al. 2009). Despite the birth of foals of the desired sex (Buchanan et al. 2000) the technology lags behind for equine compared with other species including ovine (de Graaf et al. 2007a, de Graaf et al. 2007b) and porcine (Vazquez et al. 2002, Vazquez et al. 2008, Vazquez et al. 2009,). Yet considerable advances in the understanding of the damage induced by the sorting procedure have recently occurred (Gibb et al. 2011, Balao da Silva et al. 2012, Gibb et al. 2013b, Gibb et al. 2013a). In spite of this, major problems preventing widespread use of this technology in the equine industry are: limited throughput of current flow cytometers, high waste at sorting and high sperm numbers needed for AI compared with other species (Samper 2012). Moreover, poor resolution of the X and Y bearing equine sperm populations is also a major constraint (Gibb et al. 2011) presumably due to the sperm head shape (Garner, 2006) that differ equine from other livestock species (Mari et al. 2010, Gibb et al. 2011). As a result, stallion spermatozoa have a low sorting index (59), in contrast to bulls with a sorting index of 131 (Garner 2006). A high stallion-to-stallion variability causes further difficulties in the development of standardized protocols. Improvement of this technology in the equine species requires an increase in the knowledge of the modifications that the sorting procedure induces in the intactness of the stallion spermatozoon. Oxidative stress plays a major role in sperm pathology and in the damage induced by many sperm biotechnologies (Ortega Ferrusola et al. 2009b, Balao da Silva et al. 2011, Koppers et al. 2008a), and recent research suggests that oxidative damage may be simply induced by the sorting procedure (Klinc et al. 2007, Gibb et al. 2013a). It is plausible that the sorting procedure removes loosely attached seminal plasma components, that play a role as antioxidant sperm defense (Ortega Ferrusola et al. 2009b). Oxidative stress causes lipid peroxidation (LPO), which increases membrane permeability allowing enzymes, substrates and nucleotides to pass through the membrane, impairing the metabolic status of the cell (Storey 1997). In view of all this evidence, the aim of this study was to disclose whether the flow cytometric sorting procedure increases the production of reactive oxygen species (ROS), and to identify if ROS levels relate to oxidative DNA damage in sorted spermatozoa. Moreover, since malfunction of sperm mitochondria may be a major source of oxidative stress the effect of sorting in the mitochondrial membrane potential and production of mitochondrial ROS was also investigated.

## Material and methods

### Reagents and Media

YO-PRO-1, CellROX, Ethidium homodimer, and 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolylcarbocianyne (JC-1) were from Molecular Probes (Molecular Probes, Leiden The Netherlands), OxyDNA assay kit was from Calbiochem (San Diego, CA), all other chemicals were purchased from Sigma Aldrich (St Louis MO).

### Semen collection and processing

Semen was obtained from one Quarter horse, four Pure Spanish (PRE) horses, one Anglo-Arabian horse and one Lusitanian horse, each individually-housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. All individuals were of proved fertility, and were cared for according to institutional and European regulations. Semen collection was performed regularly, with a media of two to three collections per week, during the 2012 and 2013 breeding seasons.

The stallion´s penis was cleansed with warm water and thoroughly dried to avoid contamination. All ejaculates were collected using a Missouri model artificial vagina, filled with non-spermicidal lubricant and warmed to 45-50ºC, to which an inline nylon micromesh filter was added to separate both debris and the gel fraction. Semen was immediately transported to the laboratory for evaluation and processing. Each filtered ejaculate was then extended 1+0.5 (v/v) in INRA96-modified Tyrode's (Balao da Silva et al, 2013). Semen was then processed through a double layer of colloid adapted from a protocol previously described (Balao da Silva et al., 2013). The resulting sperm pellet was re-extended in INRA modified Tyrode's to a final concentration of $100x10^6$ spz/mL.

One aliquot was split and analyzed as T0 pre-sorting control. Aliquots of 4 ml of semen were then stained with 6µL of Hoechst 33342 (16,2mM), and 4µL of Food Dye solution was added. Samples were then filtered with a mesh of 30µm. The samples were incubated during 90 minutes at 34ºC in a water bath. Sex-sorting took place in a MoFLo SX-DP (Beckman Coulter Inc., Miami, FL, USA) using PBS Puraflo 8x as sheath fluid (Beckman Coulter Inc., Miami, Fl, USA). Samples were analyzed using the option "Bulk Sorting". Catch fluid was composed of 2mL of INRA-T with 4% of centrifuged egg yolk (1000xg for 10 minutes). The obtained sperm suspension (S) was centrifuged at 800xg for 20 minutes in order to re-concentrate the sample for analysis. An aliquot (control after incubation at 34ª C in a water bath for 90 minutes, T1) was also separated and analyzed at the same time.

**Sperm motility proportions and kinematics**

Proportions of motile spermatozoa and sperm kinematics were assessed using a CASA system (ISAS® Proiser Valencia Spain) (Ortega-Ferrusola et al. 2009, Nunez-Martinez et al. 2007a, Pena et al. 2005a). Semen was loaded in a 20 μm depth Leja chamber (Leja Amsterdam, The Netherlands) and placed on a warmed (38ºC) stage. The analysis was based on the examination of 60 consecutive digitalized images obtained from a single field using x10 negative phase contrast objective. Images were taken with a time lapse of 1 second. The number of objects incorrectly identified as spermatozoa was minimized on the monitor by using the playback function. With respect to the setting parameters for the program, spermatozoa with a VAP <15 μm/s were considered immotile, while spermatozoa with a velocity >15μm/s were considered motile. Spermatozoa deviating <45 % from a straight line were designated linearly motile and spermatozoa with a circular velocity (VCL) > 45μm/s were designated rapid sperm. Sperm motion absolute and re-calculated kinematic parameters measured by CASA included the following: Curvilinear Velocity (VCL) μm/s, measures the sequential progression along the true trajectory; Linear Velocity (VSL) μm/s, measures the straight trajectory of the spermatozoa per unit time; Mean Velocity (VAP) μm/s, measures the mean trajectory of the spermatozoa per unit of time.

**Flow Cytometry**

Flow cytometric analyses were carried out with a MACSQuant Analyzer 10 (Miltenyi Biotech) flow cytometer equipped with 3 lasers emitting at 405, 488 and 635 nm and 10 photomultiplier tubes (PTM): V1 (Ex 405 Em 450/50) V2 (Ex 405 filter 525/50), B1 (Ex 488 filter 525/50) B2 (Ex 488 filter 585/40) B3 (Ex 488 filter 655-730 (655LP + split 730)) B4 (Ex 499 filter 750 LP), R1 (Ex 635 filter 655-730 (655LP+split 730) and R2 (Ex 635 filter 750 LP). The system is equipped with the MACSQuantify software. Sperm subpopulations were divided by quadrants, and the frequency of each subpopulation quantified. Forward and sideways light scatter were recorded for a total of 50,000 events per sample. Non-sperm events were eliminated by gating the sperm population after Hoechst 33342 staining. The instrument was calibrated daily using specific calibration beads provided by the manufacturer, and compensation overlap performed before each specific experiment, using unstained and single stained controls.

**Simultaneous flow cytometric assessment of subtle membrane changes, viability and oxidative stress**

The following stock solutions were prepared in DMSO: Yo-Pro-1 (25 μM), Ethidium Homodimer-1 (1.167 mM); and CellRox (5mM). Hoechst 33342 was used to identify spermatozoa and eliminate debris from the analysis. One mL of a sperm suspension containing $5x10^6$ spermatozoa/mL was stained with 1 μL of Yo-Pro-1, 1μL of CellRox and when necessary 0.3μL of Hoechst 33342. After thorough mixing, the sperm suspension was incubated at RT in the dark for 25 minutes, the spermatozoa were then washed in PBS and 0.3 μL of ethidium homodimer added and incubated further 5 minutes before reading in the flow cytometer. This staining is modified from previous protocols and distinguishes four sperm subpopulations and the oxidative stress simultaneously (Nunez-Martinez et al. 2007b, Ortega Ferrusola et al. 2009b, Pena et al. 2005b). The first is the subpopulation of only Hoechst 33342 positive spermatozoa, considered alive and without any membrane alteration. Another subpopulation is the Yo-Pro-1 positive cells emitting green fluorescence. This subpopulation are those spermatozoa showing early damage or a shift to another physiological state, since membranes become slightly permeable during the first steps of damage, enabling Yo-Pro-1 but not ethidium homodimer to penetrate the plasma membrane. None of these probes enters intact cells. Finally, two subpopulations of dead spermatozoa are easily detected, either apoptotic (spermatozoa stained both with Yo-Pro-1 and ethidium homodimer, emitting both green and red fluorescence), or necrotic spermatozoa (cells stained only with ethidium homodimer emitting red fluorescence). Spermatozoa suffering oxidative stress emit fluorescence in the far red spectrum, while Hoechst 33342 emit blue fluorescence. Positive controls for oxidative stress were samples supplemented with 800 μM $SO_4Fe$ and 200 μL of $H_2O_2$ (Sigma) to induce the Fenton reaction.

**Evaluation of mitochondrial membrane potential (ΔΨm)**

The lipophilic cationic compound 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocianyde iodine (JC-1) has the unique ability to differentially label mitochondria with low and high membrane potential (Ortega Ferrusola et al. 2009a, Pena et al. 2003, Ortega-Ferrusola et al. 2009). In mitochondria with high membrane potential, JC-1 forms multimeric aggregates emitting in the high orange wavelength of 590 nm, when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers, that emit in the green wavelength (525 to 530 nm) when excited at 488 nm. For staining, a 3mM stock solution of JC-1 (Molecular Probes Europe, Leiden, The Netherlands) in dimetylsulfoxide (DMSO) was prepared. From each sperm sample, 1 mL of a sperm suspension in PBS (5 $x10^6$/mL) was stained with 0.5 μL JC-1 stock solution. The samples incubated at 37 °C in the dark for 40 minutes before flow cytometric analysis.

**Determination of DNA oxidation, 8-oxoguanine assay**

This assay is based in the direct binding of a fluorescent probe to the DNA adduct 8- oxoguanine (OxyDNA Assay Kit, Calbiochem), a major oxidation product and an important indicator of free radical-induced DNA damage and oxidative stress. The assay was performed according to the manufacturer´s instructions and following previously published protocols (De Iuliis et al. 2009b, Koppers et al. 2011). In brief, spermatozoa (5 x $10^6$/mL) were separated from each sample, washed in PBS and fixed in a 2% paraformaldehide solution in 0,1M PBS (pH 7.6) for 15 minutes at room temperature. Cells were then washed twice in PBS and once in PBS 1% BSA. Permeabilization was made by incubation in PBS 1% BSA supplemented 0.1% saponin for 30 minutes. Samples were washed with 1mL of wash solution (1:25 dilution in water of Wash Concentrate provided by the manufacturer). Staining with 100µL of 1X FITC-Conjugate (1:10 dilution of FITC-Conjugate with wash solution) was done for 60 minutes in the dark, at room temperature. Finally, cells were washed twice and resuspended in 1 mL of PBS for flow cytometry analysis. The amount of 8-oxiguanosine formed (excitation at 495 nm and emission at 515 nm), was measured in the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany), as green fluorescence being proportional to the oxidative damage caused to the DNA. Positive controls were made after incubation of additional samples in 800µM $Fe^{2+}$ and 200µM $H_2O_2$

**Sperm chromatin structure assay (SCSA)**

In order to determine DNA fragmentation levels, an aliquot of spermatozoa (2 x $10^6$/mL) was snap frozen in liquid nitrogen and stored at -20ºC until analysis. After thawing at 37ºC for 1 minute, 1mL of TNE (0,001M EDTA disodium, 0,001M Tris HCl, 0,15M NaCl, pH 6.8) was added. After, to 200µL of this solution, 400µL of a Triton solution (0,15N NaCl, 0,08N HCl, 0.1% Triton X-100, pH 1.4) was added and incubation made at room temperature for 30 seconds. Finally, 1.2mL of an Acridine Orange solution (0.1M Citric Acid, 0.2M $Na_2HPO_4$, 1mM EDTA disodium, 0.15M NaCl, pH 6) was added, the sample was incubated for 3 minutes at room temperature in the dark, and measurements performed with the flow cytometer (Ex 502nm Em 525/650nm).

**Statistical analysis**

Data were first examined using the Kolmogorov-Smirnov test to determine their distribution. As most of the gathered data had a non-Gaussian distribution, a multivariate analysis of variance was performed (ANOVA). When significant differences were found, the non-parametric Mann-

Whitney U-test was used. All analyses were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). Power analysis was set at 0.8, and statistical significance at $P<0.05$.

### Experimental design

Motility, viability, sperm membrane permeability, mitochondrial membrane potential, oxidative stress, DNA oxidation and DNA fragmentation were determined shortly after collection (control T0), after incubation for 90 minutes in a water bath at 34º C (T1) and after sex-sorting (SS). Experiments were reviewed and approved by the Ethical committee of the University of Extremadura, Spain, ref AGL201020758, the only manipulation of animals being semen collection using standard procedures. Experiments were replicated three times (three ejaculates) for each of the seven stallions used.

## RESULTS

### Motility and kinematic parameters

Although the incubation period had no effect, percentages of both total and progressive motility decreased significantly on the sorted spermatozoa (Figure 1A and 1B), indicating that the detrimental effect of sex sorting occurred during and after the pass through the flow cytometer. Total motility of control samples before and after incubation was 90% and 65% after sex sorting ($p<0.05$). Moreover, circular velocity (VCL) and straight-line velocity (VSL) also decreased significantly after the sorting procedure, while average velocity (VAP) remained unchanged (Figure 1 C).

### Assessment of subtle membrane changes, viability and oxidative stress

The percentage of spermatozoa with intact membranes did not change after sex sorting, with 79, 70 and 70% of sperm having intact membranes in controls, after incubation and after sex sorting respectively. However sex sorting induced an increase in the percentage of YoPro+ spermatozoa with no changes in the percentages of ethidium positive spermatozoa (Figure 2A). The production of ROS was measured simultaneously and in this case major changes were observed after sorting, when oxidative stress increased from 26 and 33% in controls pre and post-incubation to 46% after sex sorting ($p<0.05$) (Figure 2B).

**Evaluation of mitochondrial membrane potential (ΔΨm)**

The subpopulation of spermatozoa depicting lower mitochondrial membrane potential significantly increased after sorting (Figure 3). The subpopulations with higher and intermediate mitochondrial membrane potential remained unchanged through all the sorting procedure.

**Determination of DNA oxidation and fragmentation**

The genomic integrity of the sorted samples was lower after the sorting process than before (%DFI being approximately 10% higher (31.3%) , as seen on Figure 4 (A). Apparently much of this DNA fragmentation was due to oxidative DNA damage as revealed by the oxyDNA assay that revealed the same effect of sorting, increasing the oxidative DNA damage (figure 4 B).

**DISCUSSION**

We studied changes induced by high-speed/pressure flow cytometric sex sorting on stallion sperm intactness, paying special attention to oxidative stress induced damage as a potential cause of the reduced lifespan of sex sorted spermatozoa in this species. We found a significant increase in the production of ROS resulting in oxidative DNA damage and fragmentation after the sorting procedure. The percentage of stallion spermatozoa with decreased mitochondrial membrane potential also increased after the procedure. Additionally, when comparing with previous work (Balao da Silva et al. 2013, Mari et al. 2010), similar results were obtained for kinematic parameters, viability and membrane permeability of sorted cells.

Documented ROS-sources in spermatozoa are the mitochondria, through electron leakage from complexes I and III of the mitochondrial electron transport chain and the sperm membrane, through a NAD(P)H-dependent oxidase (Sabeur and Ball 2006). Also aromatic amino acids released from dead sperm may be a source of ROS (Aitken et al, 2014), and the presence of dead sperm has been recently demonstrated to reduce the fertilizing ability of the boar ejaculate through oxidative mechanisms (Roca et al, 2013). Production of ROS by mitochondria is not dependent on the maintenance of mitochondrial membrane potential (Koppers et al. 2008b, Aitken et al. 2012). This could justify the results found on our work: the significant increase on the subpopulation of spermatozoa with low ΔΨm and the parallel increase of ROS production. Oxidative stress is posteriorly followed by peroxidative damage (Aitken 1995) resulting in impairments in motility and increased membrane permeability (Ortega-Ferrusola et al 2009). The high proportion of unsaturated fatty acids on the sperm membrane has been related to

susceptibility to LPO (Storey 1997), with LPO triggering also mitochondrial dysfunction and increased ROS production thus amplifying oxidative damage in a self-perpetuating cycle (Ortega Ferrusola et al, 2009; Aitken et al; 2013). Although LPO was not assessed in thisstudy, we certainly registered a decrease in total and progressive motility and an increase in membrane permeability, which would support this theory. As sperm motility on the equine species is highly dependent on mitochondrial ATP produced by oxidative phosphorylation (Ortega Ferrusola et al. 2010), the decreased motility found correlates with previous data on lower ATP content of sorted sperm (Balao da Silva et al. 2013). In human sperm, the relation between increased ROS production by the mitochondria, with absent increase on the $\Delta\Psi m$ has been related to a decrease on the motility (Aitken et al. 2012). A similar decrease in sperm motility has been found in stallion spermatozoa after induction of oxidative stress, hydrogen peroxide being the main ROS responsible (Baumber et al. 2000). Interestingly in relation to flow cytometric sex sorting, oxidative stress also increases when seminal plasma content is reduced by excessive extension and washing of the sperm suspension, since much of the antioxidant capacity relies on seminal plasma (Ball 2008). Therefore, the increased oxidative status of sorted spermatozoa may result from increased production of ROS and from a decrease on the antioxidant capacity of the medium. Previous reports on the increase of oxidative damage after sorting on other species support our results (Leahy et al. 2010, Klinc and Rath 2007). Another factor that may lead to increased ROS production during sex sorting, and directly related to mitochondrial dysfunction, is the increased hydrostatic pressure occurring during the procedure. Previous works in our laboratory indicates that the mitochondria of the stallion spermatozoon are especially sensitive to this physical stress (Macias García et al, 2012, Gonzalez Fernández et al, 2012) and that mechanical damage inflicts injury to the cells (Garner 2006). This damage is clearly affecting the plasmalemma, increasing membrane permeability (Balao da Silva et al. 2013), but our new data indicate that it should probably be damaging also the mitochondria. Mitochondria of stallion spermatozoa are highly susceptible to changes in the media, such as osmolality levels (Gonzalez-Fernandez et al. 2012). Increased hydrostatic pressure could induce a similar type of mitochondrial effect, which would impair mitochondrial function and increase formation of ROS.

We detected a significant increase on %DFI after the sorting procedure. In human spermatozoa, most DNA damage may be caused by oxidative stress (De Iuliis et al. 2009a). Membrane permeable ROS, like hydrogen peroxide or non-permeable ROS that pass through ion channels, such as superoxide anion, are generated at the mitochondria and could be wielding an oxidative effect over the sperm DNA (Aitken and Baker 2013, Fisher 2009). Moreover, in the equine species, a negative correlation between the antioxidant capacity of seminal plasma and DNA damage has been established (Wnuk et al. 2010). To gather information on the source of DNA damage, we evaluated DNA damage using two techniques simultaneously, the SCSA and the

oxyDNA assay and our results suggest that the source of DNA damage is oxidative; moreover recent attempts to reduce DNA damage after sorting using quercetin support this theory (Gibb et al, 2013), although in the mentioned study the degree of DNA damage was much higher than in our work.

Genotoxic stress commonly causes impaired pre-implantation development, abortion or disease of the offspring (Zini and Sigman 2009), especially when more than 30% of the DNA in human spermatozoa is damaged (Agarwal and Said 2003), which mimicked our findings despite the low number of intended conceptions. Apparently the flow sorting procedure is not impairing early embryonic development (at least until day 16) since the fertility data obtained (40% conceptions after first cycle of hysteroscopic inseminations of sex-sorted stallion spermatozoa) is within the range reported in the literature (Samper et al. 1991, Buchanan et al. 2000, Lindsey et al. 2002).

In conclusion, the major findings of our study are that sorted stallion spermatozoa experience increased oxidative stress during high pressure flow cytometric sex sorting. The probable origin of this increased oxidative stress relies most likely on the removal of seminal plasma due to high sperm extension rates during the sorting procedure albeit mitochondrial dysfunction could also be caused by high-pressure mechanical stress. This work underlines the importance of oxidative stress and mitochondrial dysfunction on stallion spermatozoa and stresses the need for further research aiming to alleviate the impact of oxidative stress during sex sorting.

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**FIGURE LEGENDS**

**Fig. 1.** Percentages of total and progressive motility (A and B, respectively) after colloidal centrifugation at the beginning of the incubation period (T0), at the end of the incubation period (T1) and after (S1) flow cytometric sex-sorting (mean±SEM) of stallion spermatozoa. Evaluation by computer assisted sperm analysis (CASA). * $P<0.05$. C, Velocities of stallion spermatozoa after colloidal centrifugation at the beginning of the incubation period (T0), at the end of the incubation period (T1) and after (S1) flow cytometric sex-sorting. Circular velocity (VCL), average velocity (VAP) and straight line velocity (VSL) were calculated by computer assisted sperm analysis (CASA) in µm/s. *$P<0.05$.

**Fig. 2.** A) Representation of viability and membrane permeability of control samples, after colloidal centrifugation (T0), just before entering the sorting machine at the end of the incubation period (T1), as well as the sorted sample after re-concentration (S1) (mean±SEM). Intact spermatozoa do not allow entry of any of the dyes; YO-PRO-1 positive sperm have increased membrane permeability emitting green fluorescence; double positive cells are in a late apoptotic status, emitting green and red fluorescence; and finally necrotic spermatozoa only depict red fluorescence (EthD positivity). ** $P<0.01$, B) Assessment of reactive oxygen species production (ROS) of live and dead cells after staining with CellROX Deep Red reagent and Ethidium Homodimer. The only subpopulation represented is the one composed by live cells with high emission of infrared fluorescence (mean±SEM). Samples T0 were analyzed after centrifugation, samples T1 just before entering the cytometer and samples S1 consist on sorted and re-concentrated spermatozoa. * $P<0.05$.

**Fig. 3.** Percentages of the three subpopulations with different mitochondrial membrane potential stained with JC-1: mitochondria with high membrane potential depict orange fluorescence; with low mitochondrial membrane potential emits green fluorescence and when both potentials are present both colors are emitted. Sample T0 consists on spermatozoa after colloidal centrifugation at the beginning of the incubation period; T1 represents spermatozoa just before entering the flow cytometer and S1 is the sorted sample just after re-concentration (mean±SEM). * $P<0.05$.

**Fig. 4.** DNA fragmentation index (DFI)(A) and OxyDNA assay (B) of control T0 after colloid centrifugation, control T1 before entering the flow cytometer and S1 after the sorting procedure and re-concentration (mean±SEM). Sperm Chromatin Structure Assay (SCSA) was accomplished by staining with acridine orange, which emits green fluorescence when intercalated into double stranded DNA and red fluorescence when single stranded DNA is present; DNA Oxydation was determined using a fluorescent antibody against to the DNA adduct 8- oxoguanine *P<0.05.