

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



---

# **The Role of Cytometry for Male Fertility Assessment in Toxicology**

---

Maria de Lourdes Pereira, Helena Oliveira,  
Henrique M.A.C. Fonseca,  
Fernando Garcia e Costa and Conceição Santos

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/62965>

---

## **Abstract**

Infertility is nowadays a major concern, affecting approximately 8–12% of the couples and the male factor accounts for about 50% of the cases. Occupational and/or environmental exposure to heavy metals and other pollutants is the main cause of male infertility. Lead, cadmium and chromium are heavy metals widely used in industry and quite persistent in the environment, raising major concerns over the possible effects on the reproductive health of workers and the general population. Sperm DNA integrity is essential for the accurate transmission of paternal genetic information, and normal sperm chromatin structure is important for sperm fertilizing ability. Flow cytometry can be to characterize multiple physical characteristics of the population of spermatozoa in the sperm, including sperm concentration, viability, mitochondrial mass and function, acrosome integrity, capacitation, membrane fluidity, DNA content and status, etc. This chapter elucidates the role of cytometry in the study of male fertility under toxicological insult by pollutants such as chromium, cadmium and lead. Some representative examples are presented using in vivo studies with rodents. In addition, complementary techniques to cytometry and future perspectives will be mentioned in an interdisciplinary point of view to gain knowledge on this subject.

**Keywords:** cytometry, male fertility, sperm analysis, heavy metals, toxicology

## 1. Introduction

Extensive use of heavy metals in some sectors of industry and its long persistence in the environment has been taken as an important factor affecting male fertility. The male infertility results from a combination of factors that include sperm DNA integrity. Spermatozoa may contain a variety of nuclear alterations including chromosome aberrations, DNA sequence modifications, DNA chemical modifications and DNA strand breaks. DNA breaks can arise spontaneously during chromatin remodeling on spermiogenesis and are usually repaired under normal circumstances. However, multifactorial pressures may lead to the occurrence of abnormal sperm DNA breaks.

Flow cytometry (FCM) is a powerful technique which allows the simultaneous measurement and analysis of multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light tuned at a particular wavelength. FCM is used to determine sperm concentration, viability, mitochondrial mass and function, acrosome integrity, capacitation, membrane fluidity, DNA content and status, etc. DNA fragmentation assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay and chromatin integrity evaluated by sperm chromatin structure assay (SCSA) are extremely important flow cytometry-based tools to evaluate the impact of toxicants on male fertility.

In the field of male reproductive health, the role of cytometry, among other techniques for semen analysis, was reported by several authors [1–3]. Some elegant reviews focus on the relevant role of FCM on animal and human spermatology [4, 5]. They give an insight into FCM as a useful tool in the analysis of semen so indispensable for the artificial insemination of livestock and breeding programs in industry, as well as its application in assisted human reproductive technology. These authors not only discuss the potential of FCM in the diagnosis of fertility potential, but also elaborate on the development of current technologies of reproduction, such as sperm freezing, sperm selection and sperm sorting.

This chapter discusses the role of cytometry in the study of male infertility exposed to toxicants such as chromium, cadmium and lead. Representative example using *in vivo* rodent studies will be presented to demonstrate the applicability of flow cytometry techniques.

## 2. General concepts of analytical cytology

Analytical cytology, or quantitative cytometry, is a discipline that allows an innovative vision of the cell. Despite being recent, it is closely linked to the long history of microscopy and to the historical development of FCM and image cytometry. The evolution of cytometry (and cytomics) relies on advances of disciplines such as dye chemistry (allowing the development of new fluorochromes), electronics and software (allowing more powerful computer-based analyses).

The study of cytomes within an -omics perspective combines a multiparametric and statistics-based approach. Eventually, cytomics may be considered an ultimate expression of functional

genomics. This emerging and multidisciplinary discipline can and should bridge the gap between quantitative disciplines of molecular/-omics biology and other disciplines of cytology, cell biology and physiology.

Analytical cytology is strongly based on two major approaches: FCM and microscopy-associated cytometry (image cytometry/analysis and imaging flow cytometry). In each of these research techniques, cells can be measured one at a time in an automated device. In FCM, measurements are taken in a flow cytometer, as particles pass one by one in a narrow liquid stream through an illuminated flow chamber. Thus, FCM offers unique advantages, particularly its statistical potential, as it allows multi-parametric analysis of a large number of individuals in a given population and the objective detection and characterization of (sub)populations. However, when using FCM, one loses the three-dimensional perspective of the structure where the cells are inserted. Recently, new technology has been developed by combining in a single instrument the speed and sample size of FCM with the resolution and sensitivity of microscopy. This hybrid technology, often called of image stream, offers the advantages of both image and flow cytometry, particularly in studies, for example, of cell morphology or internalization and trafficking.

In the image cytometry/analysis method, slides containing cells are scanned and each cell image is analyzed on an electronic imaging and analysis system, which with appropriate software allows the measurement of physical/chemical cell features of interest. These analytical imaging techniques offer huge potential for dimensional and spatial organization, but they usually allow only restricted number of sampling.

Imaging flow cytometry introduced a quantum leap to cytometry flow analysis by adding the resolution and sensibility of light microscopy with robust statistical analysis of FCM. This technology allows the development of gating strategies where morphological characteristics (including those enhanced by multicolor staining), fluorescence, advanced fluidics control and the power of statistical analysis are used to resolve details that normally fall within the noise of FCM machines. In the literature, there are many examples of the use of imaging flow cytometry applied to diverse areas of research showing gains compared with the FCM [6–8].

Equipping a FCM laboratory requires high initial investment, but nowadays more affordable flow cytometers can be found in the market. In addition to the general advantages described above for FCM (e.g., speed, statistical potential, rare subpopulation detection), sample preparation is extremely simple and rapid, the cost of consumables is very low, and the rapidity of analyses allows that several samples to be run within a short period. Furthermore, small amounts of material are required (e.g., 50 mg or less), different tissues are suitable for analyses (e.g., for DNA analyses, there is no need for mitotically active cells), and although fresh samples are always recommended, often good results can be achieved with few days old samples or even using fixed or dried material.

### **3. Flow cytometry and flow cytometers**

The first primitive equipment for FCM was developed in the years 1940–1950 by Wallace Coulter initially for aerosol analyses or blood cell counting. Later, Mark Fulwyler (US Los

Alamos National Laboratories) added to the Coulter principle for cell size analyses, an ingenious idea of sorting electric charged droplets that contained the desired cells [9].

The advances in fluorescence from the late 1960s broadened the research applications enabling multiparametric analysis of cells, and the first commercial fluorescence flow cytometers appeared in 1970s. Since then, several and increasingly powerful and sensitive flow cytometers have been developed (for example, BD Biosciences® CA, USA; Coulter Beckman Coulter CA, USA; Sysmex Partec GmbH, Germany), together with devices with specific applications, such as portable and marine flow cytometers. Simultaneously, the first commercial cell sorter (fluorescence-activated cell sorter) was developed.

A FCM is composed of several components, namely optical, fluidic, electronic, computational and mechanical systems. In the fluidic system, the suspension of particles flowing at high speed are hydrodynamically focused to flow one by one in the flow chamber (or flow cell) where they are intersected by the light source. This hydrodynamic focusing is achieved by one of two methods: using another fluid (sheath fluid) that surrounding the sample stream at a different speed but does not mix with the sample (laminar flow); or by acoustic focus (acoustic-assisted hydrodynamic focusing) using the ultrasonic radiation pressure (>2 MHz) to transport the particles into the center of the sample stream.

An alternative to the enclosed stream and hydrodynamic focus is the “jet-in-air” design, common in most cell sorters, where the sample stream exits the flow chamber into open air through narrow orifices.

The optical system consists of narrow very stable laser beams that illuminate the particles, and each particle response (light scatter and/or fluorescence) is used to measure their physical-chemical characteristics. The most common light sources are argon ion lasers (emitting at 488 nm) and helium-cadmium lasers usually emitting in the blue (442 nm) or in the UV (325 nm) regions. More recent cytometers are equipped with more efficient light-emitting diodes and solid-state lasers, which give greater accuracy within a wider spectral range [10, 11].

In response to the incidence of the laser beam, particles scatter the light both as *forward scatter*, FS (light scattered at narrow angles to the laser beam axis) and *side scatter*, SS (light scattered at approximately a 90° of the laser beam). These are two important characterization parameters: FS gives rough information of the particles size, while the SS informs on the internal granularity and the surface roughness.

Particles may also emit fluorescent light at specific wavelengths that can be separated by appropriate optical filters. Several filters may be used: the *long-pass* and *short-pass* filters transmit light, respectively, above and below a specific wavelength, while the *band-pass* filters transmit light within a certain range of wavelengths. Dichroic mirrors are also used to selectively pass light within a small range of wavelengths while reflecting others.

Light emitted from particles is collected by photodiodes (typically used for forward scatter channel detection) and photomultiplier tubes (they are two different photon collection devices), which convert the light they receive into a proportional voltage pulses.



These pulses are then amplified, integrated, and analyzed by a computer-based electronic system. Amplified signals are linearly or logarithmically transformed and then converted from analog continuous voltages into discrete digital data through an analog-to-digital converter. Typically, conventional instruments had 10-bit ADCs, but more recent instruments are equipped with higher 14-bit ADCs, resolution that is divided into 1024 channels scale. Data are then displayed, analyzed and stored by the computational system in list modes that contain all measurements of each particle. The resulting histograms or cytograms provide a global and easy-reading information of all particle (sub)populations; diverse software are also available for further FCM data analysis (e.g., gating, histogram/cytogram overlay) and statistics.

FCM is constantly evolving. For example, to circumvent the problematic correlation of FS with particle size, which often is not true, new FCM equipment provide an *electronic volume* parameter, instead of the classical FS parameter, which is more accurate for the particle size range. Also, in order to combine the statistical advantages of FCM and the image analyses of microscopy systems, new equipments have CCD cameras integrated.

Currently, most flow cytometers compute and directly provide, for example, histograms (univariate for single parameters or bivariate histograms/cytograms when multiparametric parameters are simultaneously analyzed); and the ratios of a distinct subpopulation of cells in relation to others and/or to the total. If suitable markers are used, resolving specific types of fluorescence, rare subpopulations may be separated in a proportion as low as 1 cell in  $10^7$ .

## 4. Most relevant parameters in toxicological assays

The main parameters in toxicological assays are DNA content, DNA ploidy and cell cycle analyses; cell viability and death; mitochondrial membrane potential and oxidative stress and ROS.

### 4.1. Quantification of DNA, ploidy and cell cycle analyses

Both ploidy and genome size determinations are important in clinical and biological sciences. In general, FCM genome sizes are usually expressed in picogram of DNA (pg) per 2C (or C) or as the number of base pairs (Mbp) assuming that  $1 \text{ pg} = 0.978 \times 10^9 \text{ bp}$ . DNA ploidy and, less often, the quantification of DNA are one of the most widely used applications in FCM. The quality of a DNA histogram, given by the coefficient of variation which depends on the peak width of the  $G_0/G_1$  peak of DNA cycle, is crucial to estimate the DNA content.

Several aspects must be considered when performing DNA analyses by FCM, as they may condition the quality of the results. Particularly important are the source and preservation of the biological material, the use of a reliable standard, the selection of an adequate buffer solutions, the selection of suitable fluorochromes and the proper exclusion of clumped cells/nuclei.

Fluorochromes such as propidium iodide (PI, stoichiometrically intercalates with nucleic acids; excitation at 530 nm and emitting at 620 nm) or DAPI (diaminophenylindole, stains

regions rich in A/T; excitation 360 nm in the ultraviolet region, and emission 460 nm) are the most widely used DNA fluorochromes, not only to quantify DNA content (only PI is suitable as a stoichiometric stain), but also for studies on DNA ploidy and cell cycle dynamics.

#### 4.2. Cell viability and death

Viability vs. apoptotic vs. necrotic cells are among the most used analyses in flow cytometry. A main difference between apoptotic and necrotic cells is that the former have intact plasma membrane, whereas the latter have lack of membrane integrity. Therefore, PI fluorochromes, which are excluded by integral membranes, as in cells undergoing apoptosis can enter the necrotic cells. Other fluorochromes are retained by intact membranes, as, for example, the fluorescein diacetate (FDA, excitation 488 nm, emission 530 nm) that penetrates the membrane and inside the cell is converted by viable cells to fluorescein. Thus, the combination of multiple fluorochromes will allow separate different subpopulations according to their staining profiles.

FCM also allows to discriminate between early and late apoptotic subpopulations in addition to necrotic cells. A particularly useful technique for assessing early apoptosis is the use of phosphatidylserine (PS) residues (typical of the cytoplasmic side of the plasma membrane) that during apoptosis are exposed to the external side of the membrane. By adding fluorescently labeled Annexin V, which binds to PS, only those cells with externalized PS will stain as positive. It is common to combine Annexin V with PI and/or with other fluorochromes.

It should be noted that in late apoptosis, cells become increasingly permeable to fluorochromes as PI or Hoechst 33342 (excitation 352 nm, emission 461 nm), such as with necrotic cells, late apoptosis can be marked as positive for these staining (which, however, will decrease as DNA is also degraded). Other parameters include detecting proteins as caspases, using fluorochrome-conjugated probes (e.g., anti-PARP antibodies, fluorogenic substrate for the detection of activated caspases).

Another parameter widely used to monitor apoptosis, DNA fragmentation, is determined by TUNEL assays. This technique uses an enzyme, terminal deoxynucleotidyl transferase (TdT) that adds labelled nucleotides to the 5' ends of the fragments of DNA in apoptotic cells. These labeled nucleotides, often BrdUTP, are proportional to the amount of fragmented DNA present in the apoptotic cells.

#### 4.3. Mitochondrial membrane potential

Mitochondrial assays by flow cytometry allow to determine mitochondrial changes often associated with apoptotic pathways. Several fluorochromes are retained by mitochondria, being the most widely used the rhodamine 123 (common excitation at 507 nm and emission 529 nm) and MitoRed (common values of excitation around 560 nm, and of emission around 580 nm). When the mitochondrial membrane potential decreases by loss of integrity, the fluorochrome redistributes resulting in signal loss. Alternatively, some fluorochromes, for example, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), may shift from red (in healthy cells; excitation ~535 nm, emission ~595 nm) to green (excitation

~485 nm, emission ~535 nm) depending on the state of aggregation of the fluorochrome (e.g., [12]).

#### 4.4. Oxidative stress and ROS

Another process closely associated with various events in the cell (e.g., stress, aging, apoptosis, etc.) is oxidative stress. Normal cells have basal levels of reactive oxygen species (ROS) in a complex homeodynamic balance. Using this oxidative potential, compounds such as 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) are used to study these key events. The cell-permeable DCFH-DA (nonfluorescent when reduced) is retained inside the cells and, when hydrolyzed by cellular esterases, emits fluorescence when oxidized by ROS.

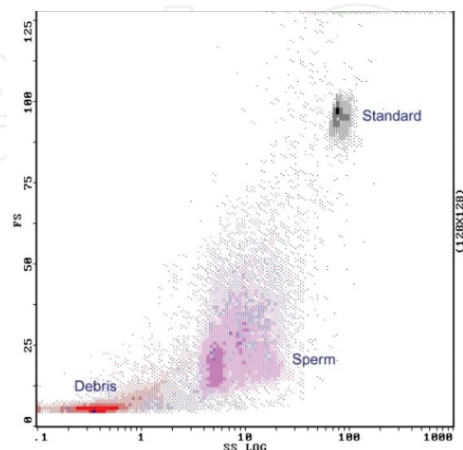
### 5. Flow cytometry and other techniques: the case study of sperm analyses

Potential applications of flow cytometry for the evaluation of sperm functions and genetic integrity were reviewed by Oliveira et al. [13].

The following section outlines some examples of the application of flow cytometry to study the toxicity of chromium, cadmium and lead on mice spermatozoa.

#### 5.1. Counts

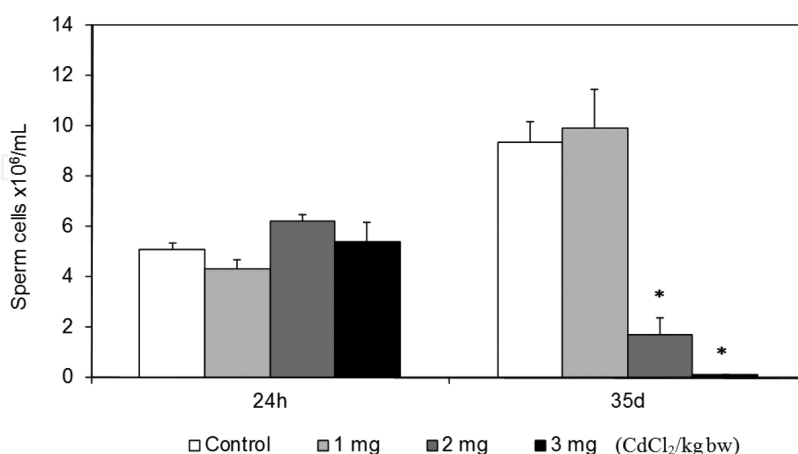
FCM can be used to automatically evaluate sperm concentrations based on sperm suspension mixture with a known concentrations of fluorospheres [14]. Another approach combines gating sperm cells based on light scattering proprieties, forward scatter and side scatter, which are related with cell size and granularity, respectively (**Figure 1**), and DNA staining [15]. Sperm concentration is then calculated as the ratio of sperm cells to fluorosphere counts and fluorosphere concentration. This methodology allows a more accurate evaluation of sperm concentration than usual counts performed with a hemocytometer. Based on Oliveira



**Figure 1.** Flow cytometry cytogram showing the identification of a sperm cells population of ICR-CD1 mice based on light scatter. FS, particle size; SS LOG, internal granularity and surface roughness.



et al. [16], **Figure 2** shows an example of the effect of cadmium chloride exposure on male mice, obtained by FCM.



**Figure 2.** Effect of a single subcutaneous administration of cadmium chloride at 1, 2, or 3 mg/kg body weight (bw) to ICR-CD1 mice on the sperm concentration. Animals were sacrificed 24 h or 35 days after the administration. Values represent mean  $\pm$  standard deviation. Symbol \* indicates significant difference between the control and treated animals to  $p < 0.001$ .

## 5.2. Viability

Sperm viability is usually evaluated by assessing the status of the plasma membrane, based on the differential membrane permeability of damaged sperm cells to fluorescent probes. For a review on used dyes such as PI, ethidium bromide (EtBr), ethidium homodimer-1 (EthD-1) and YOPRO-1, see Oliveira et al. [13].

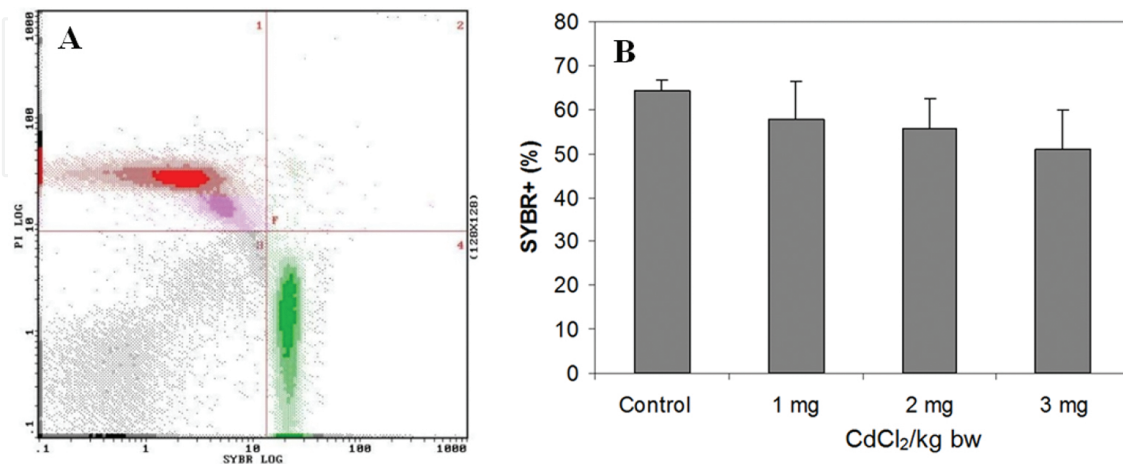
The fluorescent probes are excluded by intact cell membranes but can enter and stain nuclei of cell with damaged membranes. Dyes such as Hoechst 33342 and 33258 are cell permeant (the first has a slightly higher permeability than the latter) and are also used to assess membrane status [17].

Acylated dyes are amphipathic probes that can pass the intact membrane and enter live sperm. These probes are readily deacylated inside the cell by intracellular non-specific esterases producing a fluorescent compound. If the cell membrane is intact, these fluorescent probes are entrapped within cells, and as membrane damage allows leakage of the probes and signal loss [13].

SYBR-14 [18] is an example of acylated membrane permeant DNA fluorochrome, which labels viable cells with functional ion pumps.

Cell viability can be estimated in a more accurate way using membrane impermeant and permeant stains in combination, for example, the LIVE/DEAD® sperm viability kit that combines SYBR-14 and PI. The SYBR-14 stains living spermatozoa green, whereas the PI stains dead or membrane-damaged spermatozoa red. A third population is often detectable, which is stained with both fluorochromes, and represents dying spermatozoa [13]. **Figure 3A** shows

an example of a flow cytometry cytogram of mice sperm cells stained with SYBR-14/PI, and **Figure 3B** shows the effect of cadmium chloride exposure on sperm viability. Our results show that despite the apparent tendency to a decline in sperm viability after exposure to cadmium chloride, the differences did not reach statistical significance.



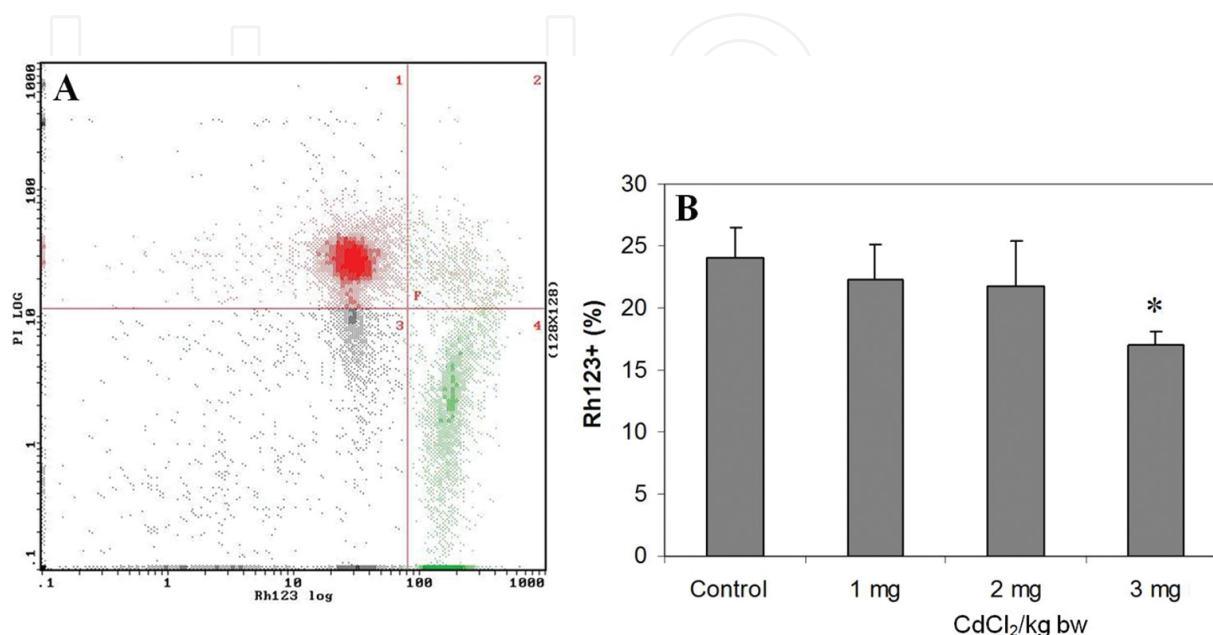
**Figure 3.** (A) Logarithmic flow cytometry cytogram of mice sperm stained with LIVE/DEAD sperm viability kit (SYBR-14/PI). SYBR-14 stains living spermatozoa green, whereas the PI stains the dead or membrane damaged. A third population of dying sperm cells is represented in the cytogram in purple. (B) Effect of a single subcutaneous administration of cadmium chloride at 1, 2, or 3 mg/kg body weight (bw) to ICR-CD1 mice on the sperm viability. Mice were sacrificed 24 h after the administration. Values represent mean  $\pm$  standard deviation.

### 5.3. Mitochondrial function

In sperm cells, mitochondria are arranged helicoidally in the midpiece of the sperm tail and are responsible for generating energy for the flagellar beat. Therefore, changes in mitochondrial membrane potential are a good indicator of functional impairment.

Mitochondria can be stained by specific fluorochromes for these organelles. Rhodamines and carbocyanines are the most usual compounds that are sensitive to mitochondria membrane potential (MMP). Rhodamine 123 (Rh123, excitation 507 nm, emission 529 nm) is a cationic fluorescent dye frequently used to indicate MMP [19]. However, Rh123 is not capable of distinguishing mitochondria with high and low membrane potential. The lipophilic cationic probe JC-1 is a special type of multimer of carbocyanine that in contrast to Rh123 is able to distinguish mitochondria with high and low membrane potential. In mitochondria with high membrane potential JC-1 forms multimers of J-aggregates that fluoresce orange (590 nm) when excited at 488 nm. In mitochondria with low membrane potential, JC-1 forms monomers that when excited at 488 nm emit in the green range (530 nm). MitoTracker fluorochromes, namely MitoTracker Green, MitoTracker Orange, MitoTracker Red 580, and MitoTracker Deep Red 633, are also used to evaluate sperm mitochondrial function [13]. Finally, 10-n-Nonyl Acridine Orange chloride (NAO), a mitochondrial-specific fluorochrome, which is MMP independent, is used to estimate mitochondrial mass in male germ cells [20]. An example is shown in **Figure 4A**, where mice sperm stained with Rh123/PI showed

that cells with functionally active mitochondria appear intensely stained in green for Rh123, whereas dead or membrane-damaged sperm cells stain red for PI. **Figure 4B** demonstrates the effect of a single subcutaneous administration of cadmium chloride at 1, 2 or 3 mg/kg body weight to ICR-CD1 mice on mitochondrial function. A significant decrease in sperm mitochondrial function was observed for the highest cadmium chloride dose.



**Figure 4.** (A) Logarithmic flow cytogram of mice sperm stained with Rh123/PI. Functionally active mitochondria stain intensely for Rh123, whereas the PI stains red the dead or membrane-damaged sperm cells. (B) Effect of a single subcutaneous administration of cadmium chloride at 1, 2, or 3 mg/kg body weight (bw) to ICR-CD1 mice on mitochondrial function. Mice were sacrificed 24 h after the administration. Values represent mean  $\pm$  standard deviation. Symbol \* indicates significant difference between the control and treated animals to  $p < 0.001$ .

#### 5.4. Flow cytometry for the evaluation of sperm DNA damage

Sperm DNA integrity is essential for the accurate transmission of paternal genetic information, and normal sperm chromatin structure is important for fertilizing capacity of spermatozoa [21–25].

Chromatin of mature sperm cells is highly condensed during spermatogenesis due to the replacement of nucleosomal histones by intermediate proteins and finally, by protamines [26]. Defects in sperm chromatin structure can be associated with abnormal nucleoprotein content and/or DNA strand breaks. However, it has been demonstrated that sperm with damaged DNA is able to fertilize the oocyte and form pronuclei [27], but embryonic development is dependent on the degree of DNA damage [28].

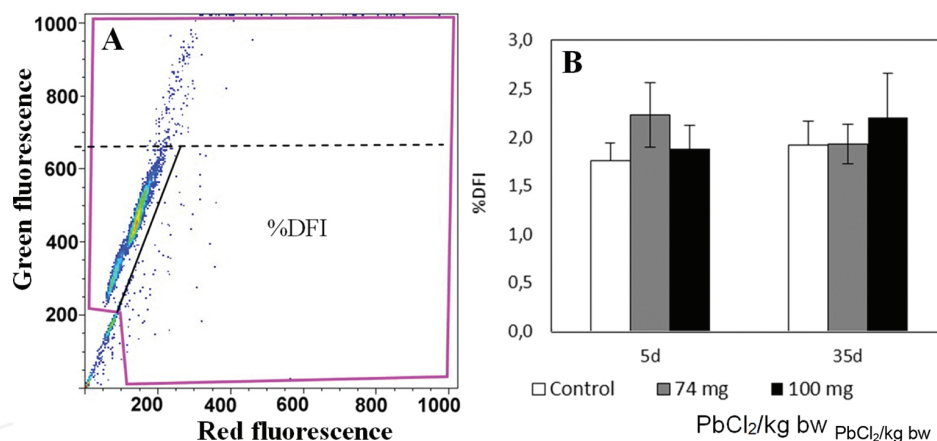
Several tests can be performed in order to evaluate sperm DNA integrity. The chromatin structure can be evaluated by the SCSA, AOT, CMA3, or toluidine blue assays. DNA fragmentation can be assessed by the TUNEL assay, the *in situ* Nick Translation assay, SCD or the comet assay as revised by Oliveira et al. [29].

## 5.5. Sperm chromatin structure assay—SCSA

### 5.5.1. *PbCl<sub>2</sub>/kg bw*

Normal sperm development leads to a chromatin structure, which confers DNA resistance to denaturation; by contrast, DNA in regions within abnormal chromatin structure is susceptible to acid denaturation. The extension of DNA denaturation can be quantified by SCSA, which is a FCM assay developed by Evenson and co-workers [30] that measure the sperm DNA susceptibility to acid denaturation *in situ* [31].

SCSA is based on metachromatic proprieties of acridine orange that emits green fluorescence (515–530 nm) when intercalated to double-stranded native DNA and red fluorescence (630–640 nm) when bound to single-stranded denatured DNA. The DNA fragmentation index (DFI), which is the ratio of red fluorescence/(red + green) fluorescence, is calculated for each measured cell (usually 5–10,000). **Figure 5A** shows a typical SCSA cytogram of a sperm samples with low % DFI. Green fluorescence is from native DNA, whereas red fluorescence is from fragmented DNA. The effect of a daily administration of lead chloride at 74 and 100 mg/kg bw for four consecutive days to ICR-CD1 mice on % DFI is presented in **Figure 5B**. Results indicate that lead chloride treatment did not induce significant changes in DNA fragmentation assessed by the SCSA.

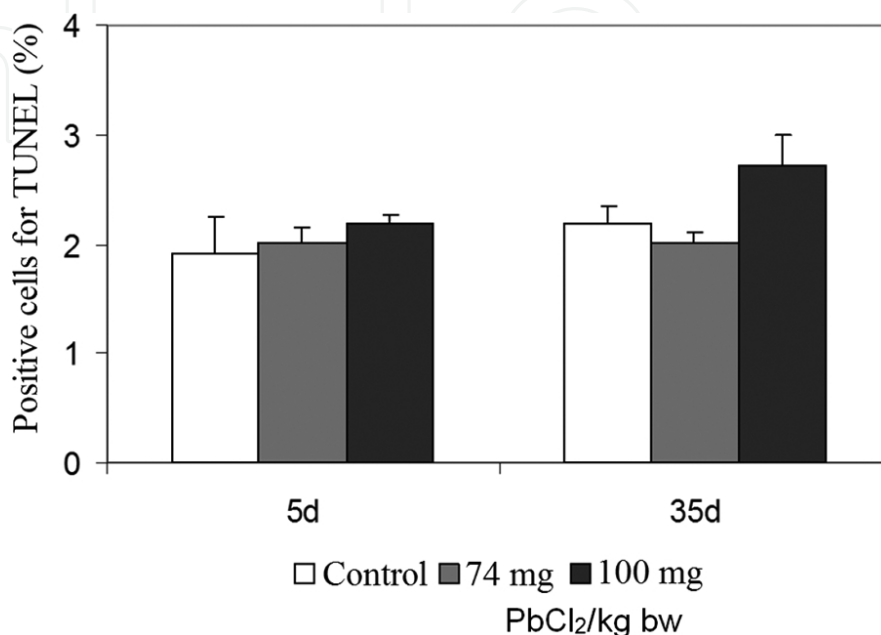


**Figure 5.** (A) Typical SCSA cytogram of a sperm samples with low % DFI. (B) Effect of a daily administration of lead chloride at 74 and 100 mg/kg body weight (bw) for four consecutive days to ICR-CD1 mice on sperm % DFI. Mice were sacrificed at day 5 and 35 of the assay. Values represent mean  $\pm$  standard deviation.

## 5.6. Evaluation of DNA fragmentation-TUNEL assay

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at free 3'-OH termini of single- and double-stranded DNA breaks in a reaction catalyzed by the template-independent enzyme, TdT. This assay allows simultaneous detection of both single- and double-strand breaks. The incorporated dUTP is chemically labelled so that breaks can be quantified either by fluorescence microscopy, light microscopy or flow cytometry, where sperm with DNA breaks shows positive after being subjected to TUNEL

reaction [32]. **Figure 6** shows an example of the effect of a daily administration of lead chloride at 74 and 100 mg/kg bw for four consecutive days to ICR-CD1 mice on percentage of sperm DNA fragmentation assessed by the TUNEL assay. Although the results show a trend towards an increase in sperm DNA damage in the presence of lead chloride, the differences did not reach statistical significance.



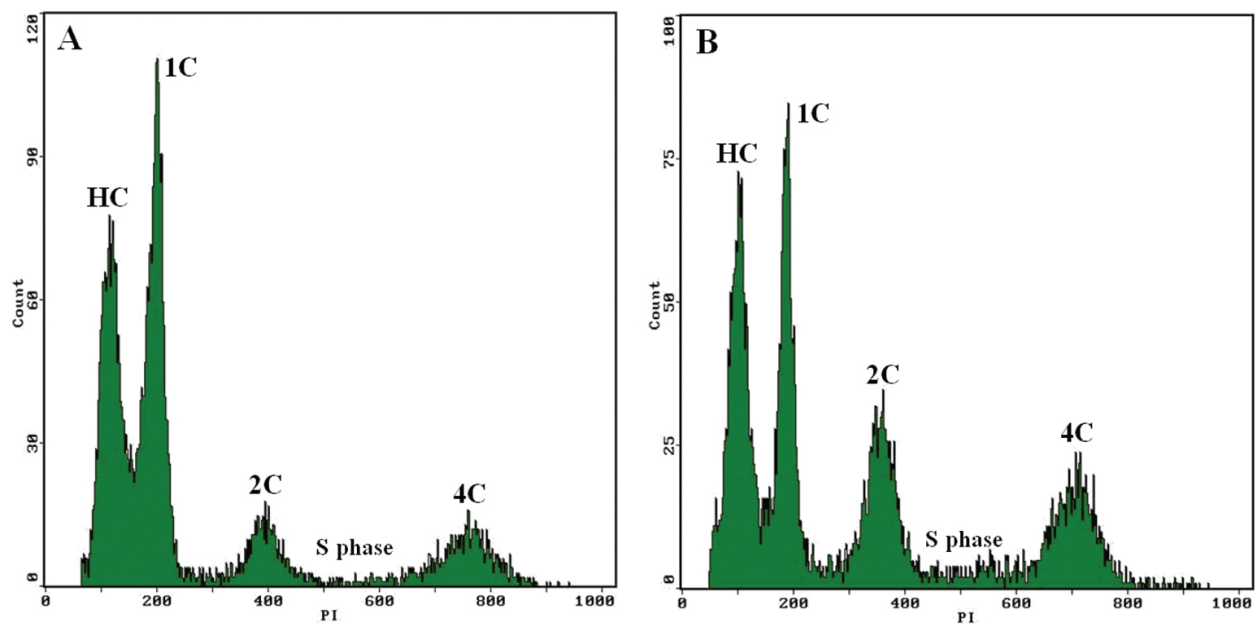
**Figure 6.** Effect of a daily administration of lead chloride at 74 and 100 mg/kg body weight (bw) for four consecutive days to ICR-CD1 mice on sperm % DNA fragmentation assessed by the TUNEL assay. Mice were sacrificed at days 5 and 35 of the assay. Values represent mean  $\pm$  standard deviation.

### 5.7. Flow cytometry for the evaluation of the testicular subpopulations

The different populations of cells of testes can be isolated from fresh biopsies or from fixed and paraffin-embedded samples as described by Oliveira et al. [33]. Permeabilized cells or isolated nuclei are then stained with PI, or other DNA-staining dye, and then analyzed by flow cytometry. **Figure 7** represents a histogram of testes of control and exposed mice to  $\text{CdCl}_2$  showing the distribution of subpopulation of cells. DNA histograms revealed four main peaks corresponding to different ploidy levels: the sub-haploid peak (HC) that consists of elongated spermatids and the haploid peak (1C) refers to round spermatids.

The discrimination of haploid nuclei into two peaks is due to differences in chromatin condensation. Elongated spermatids have highly condensed chromatin; therefore, PI intercalation with DNA is lower. Somatic cells, spermatogonia and secondary spermatocytes are recorded in the diploid peak (2C). Cells in the G2/M phase of the cell cycle and primary spermatocytes are included in the tetraploid peak (4C). The region between the diploid and the tetraploid peaks corresponds to cells actively synthesizing DNA (S phase). Administration of 3 mg  $\text{CdCl}_2$ /kg body weight decreased the number of haploid cells (round spermatids) and increased the percentage of diploid, tetraploid and cells in S phase (**Figure 7**).





**Figure 7.** Flow cytometry histograms of nuclei isolated from ICR-CD1 mice testis from paraffin embedded tissue as described by Oliveira and co-workers [33]. Initially, to set up the instrument's acquisition conditions, chicken red blood cells were used as standards (not shown); thereafter, samples were run without the standard. (A) control, (B) exposed to 3 mg CdCl<sub>2</sub>/kg body weight by subcutaneous injection for 24 h. PI, propidium iodide assay. HC, sub-haploid; 1C, haploid; 2C, diploid; and 4C, tetraploid peaks. Doublets were excluded in the cytogram linear fluorescence pulse integral versus linear fluorescence pulse height as described in Oliveira and co-workers [16, 33].

## 6. Conclusions and final remarks

In this chapter, we address some of the latest advances in functional cytometry applied to the analysis of sperm quality. Structural and functional cytometry has been a robust support for cellular functional analysis in numerous fields of biology and particularly in animal and human reproductive technologies. As outlined above, FCM comprises a set of technologies that enhance the potential of other fields and methods, namely does related to the reproductive management in animal husbandry, in specialized andrology laboratories, and in areas of reproductive toxicology.

FCM has enormous potential to explore complex biological matrices, such as semen through consistent evaluation of gamete quality. This will be achieved with the advances in hardware/instrumentation, reagent development and bioinformatics, allowing increasing automation and sophistication of polychromatic/multiple parameter data set analysis.

We reviewed here some recent applications of this technique in assessing fundamental endpoints in toxicological assays (including quantification of DNA, ploidy, cell cycle dynamics, cell viability and apoptosis/necrosis, mitochondrial membrane potential, or oxidative stress parameters). Moreover, we also demonstrated the validity of FCM in comparison with other techniques in assessing sperm analyses (counting, viability, sperm DNA damage, chromatin

structure, DNA fragmentation) and testicular subpopulations. Finally, we also stress that, in the foreseeable future, the expected developments in cytometry will produce major gains in the understanding of mechanisms of action of toxicants in animal models and in the field of toxicological diagnosis applicable to human clinical and will consolidate its value in providing cytological analytical data essential in a multidisciplinary approach (from genomics to functional biology).

## Acknowledgements

This chapter was developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (Foundation for Science and Technology—FCT Ref. UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement; and FCT Ref. UID/GEO/04035/2013. Also FEDER/COMPETE/POCI—Operacional Competitiveness and Internacionalization Programme, (POCI-01-0145-FEDER-006958) and FCT, UID/AGR/04033/2013. FCT awarded grant to Helena Oliveira (SFRH/BPD/111736/2015) is also acknowledged.

## Author details

Maria de Lourdes Pereira<sup>1\*</sup>, Helena Oliveira<sup>2</sup>, Henrique M.A.C. Fonseca<sup>3</sup>,  
Fernando Garcia e Costa<sup>4</sup> and Conceição Santos<sup>5</sup>

\*Address all correspondence to: [mlourdespereira@ua.pt](mailto:mlourdespereira@ua.pt)

1 Department of Biology and CICECO – Aveiro Institute of Materials, University of Aveiro, Aveiro, Portugal

2 Department of Biology and CESAM, University of Aveiro, Aveiro, Portugal

3 Department of Biology and GeoBioTec, University of Aveiro, Aveiro, Portugal

4 Department of Morphology and Function, CIISA Interdisciplinary Centre of Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

5 Department of Biology, GreenUP/Citab UP, University of Porto, Rua Campo Alegre, 4169-007 Porto, Portugal

## References

- [1] Nizański W, Partyka A, Rijsselaere T. Flow cytometry and state of the art in semen analyses in research and breeding management. Proceedings of the 7th International Symposium on Canine and Feline Reproduction—ISCFR, 2012. Whistler, Canada.
- [2] Marchiani S, Tamburrino L, Olivito B, Betti L, Azzari C, Forti G, Baldi E, Muratori M. Characterization and sorting of flow cytometric populations in human semen. *Andrology* 2014; 2: 394–401.
- [3] Sutovsky P. New Approaches to Boar Semen Evaluation, Processing and Improvement. *Reproduction in Domestic Animals* 2015; 50 (Suppl. 2): 11–19.
- [4] Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian Journal of Andrology* 2011; 13: 406–419.
- [5] Peña FJ. Multiparametric flow cytometry: a relevant tool for sperm function evaluation. *Animal Reproduction* 2015; 12(3): 351–355.
- [6] George TC, Basiji DA, Hall BE, Lynch DH, Ortyn WE, Perry DJ, Seo MJ, Zimmerman CA, Morrissey PJ. Distinguishing modes of cell death using the ImageStream multi-spectral imaging flow cytometer. *Cytometry A* 2004; 59(2): 237–45.
- [7] Basiji DA, Ortyn WE, Liang L, Venkatachalam V, Morrissey P. Cellular image analysis and imaging by flow cytometry. *Clinics in Laboratory Medicine* 2007; 27(3): 653–70.
- [8] Headland SE, Jones HR, D'Sa ASV, Perretti M, Norling LV. Cutting-edge analysis of extracellular microparticles using ImageStreamX imaging flow cytometry. 2014; *Scientific Reports* 4: 05237-05237.
- [9] Shapiro HM. *Practical Flow Cytometry*. New York: Wiley, 2005.
- [10] Kapoor V, Karpov V, Linton C, Subach FV, Verkhusha VV, Telford WG. Solid state yellow and orange lasers for flow cytometry. *Cytometry Part A* 2008; 73A: 570–577.
- [11] Lawrence WG, Varadi G, Entine G, Podniesinski E, Wallace PK. Enhanced red and near infrared detection in flow cytometry using avalanche photodiodes. *Cytometry Part A* 2008; 73A: 767–776.
- [12] Christensen ME, Jansen ES, Sanchez W, Waterhouse NJ. Flow cytometry based assays for the measurement of apoptosis-associated mitochondrial membrane depolarisation and cytochrome c release. *Methods* 2013; 61(2): 138–145.
- [13] Oliveira H, Spanò M, Santos C, Pereira ML. Flow Cytometric Analyses of Sperm Functions and Genetic Integrity. In: Glantz B. and Edquist K. (eds.) *Male and Female Infertility: Genetic Causes, Hormonal Treatments and Health Effects*, Edition: 1, Chapter: Nova Publishers, New York, 2009; pp. 1–32.

- [14] Evenson DP, Parks JE, Kaproth MT, Jost LK. Rapid determination of sperm cell concentration in bovine semen by flow cytometry. *Journal of Dairy Science* 1993; 76: 86–94.
- [15] Eustache F, Jouannet P, Auger J. Evaluation of flow cytometric methods to measure human sperm concentration. *Journal of Andrology* 2001; 22: 558–567.
- [16] Oliveira H, Spanò M, Santos C, Pereira Mde L. Adverse effects of cadmium exposure on mouse sperm. *Reproductive Toxicology* 2009; 28(4): 550–555.
- [17] Sutkeviciene N, Riskeviciene V, Januskauskas A, Zilinskas H, Andersson M. Assessment of sperm quality traits in relation to fertility in boar semen. *Acta Veterinaria Scandinavica* 2009; 51: 53.
- [18] Garner DL, Thomas CA, Joerg HW, DeJarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of Reproduction* 1997; 57: 1401–1406.
- [19] Gravance CG, Garner DL, Miller MG, Berger T. Fluorescent probes and flow cytometry to assess rat sperm integrity and mitochondrial function. *Reproductive Toxicology* 2001; 15: 5–10.
- [20] Petit JM, Ratinaud MH, Cordelli E, Spanò M, Julien R. Mouse testis cell sorting according to DNA and mitochondrial changes during spermatogenesis. *Cytometry* 1995; 19: 304–312.
- [21] Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertility and Sterility* 2008; 89: 823–831.
- [22] Lewis SE, Agbaje I, Alvarez J. Sperm DNA tests as useful adjuncts to semen analysis. *Systems Biology in Reproductive Medicine* 2008; 54: 111–125.
- [23] Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. *Journal of Andrology* 2009; 30: 219–229.
- [24] Bungum M, Bungum L, Giwercman A. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. *Asian Journal of Andrology* 2011; 13: 69–75.
- [25] Oleszczuk K, Giwercman A, Bungum M. Sperm chromatin structure assay in prediction of in vitro fertilization outcome. *Andrology* 2016. doi:10.1111/andr.12153
- [26] Rathke C, Baarends WM, Awe S, Renkawitz-Pohl R. Chromatin dynamics during spermiogenesis. *Biochimica and Biophysica Acta* 2014; 1839(3): 155–168.
- [27] Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Human Reproduction* 1998; 13: 1864–1871.
- [28] Ahmadi A, Ng SC. Developmental capacity of damaged spermatozoa. *Human Reproduction* 1999; 14: 2279–2285.

- [29] Oliveira H, Spanò M, Santos C, Pereira ML. Lead chloride affects sperm motility and acrosome reaction in mice. *Cell Biology and Toxicology* 2009; 25: 341–353.
- [30] Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980; 210: 1131–1133.
- [31] Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. *Methods in Cell Science* 2000; 22: 169–189.
- [32] Takeda K, Uchiyama K, Kinukawa M, Tagami T, Kaneda M, Watanabe S. Evaluation of sperm DNA damage in bulls by TUNEL assay as a parameter of semen quality. *Journal of Reproductive Development* 2015; 61(3): 185–190.
- [33] Oliveira H, Loureiro J, Filipe L, Santos C, Ramalho-Santos J, Sousa M, Pereira Mde L. Flow cytometry evaluation of lead and cadmium effects on mouse spermatogenesis. *Reproductive Toxicology* 2006; 22(3): 529–535.



