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Supplementary Material

Effects of various physical stress factors on mitochondrial function and reactive oxygen species in rat spermatozoa

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Supplementary Data

MIFlowCyt standard compliant information for submitted flow cytometric data.

1. Experiment Overview

1.1. Purpose

The purpose of the experiment was to evaluate the effects of various physical interventions on the function of epididymal rat sperm and determine if there are correlations among these functional parameters. Rat sperm are well known to be very sensitive to various physical interventions (pipetting, centrifugation, and osmotic stress). However, effects of these stress factors on the function of epididymal rat sperm have not been systematically studied. We hypothesized that physical stress factors would affect MMP and intracellular ROS of epididymal rat sperm as well as motility and plasma membrane integrity. This study was performed to give useful information for selecting proper rat sperm handling and condition for biomedical research.

1.2. Keywords

mitochondrial membrane potential, osmotic condition, physical interventions, reactive oxygen species, rat sperm.

1.3. Experiment Variables

The sperm have to be well washed after treatment of Percoll gradient separation. Percoll may affect the intensity of fluorescence and flow rate when the samples are run.

1.4. Organization (name and address)

1.4.1. Name

Agca Lab, Department of Veterinary Pathobiology

College of Veterinary Medicine
University of Missouri-Columbia

1.4.2. Address

Veterinary Medicine Building
1600 East Rollins Street, Room W191
Columbia, MO 65211

1.5. Primary Contact (name and email address)

1.5.1. Name

Yuksel Agca

1.5.2. Email address

agcay@missouri.edu

1.6. Date (or time period)

Experiments were set up from 2/24/12 to 3/3/12 and performed from 3/11/12 to 4/13/12 and from 10/10/2012 to 12/7/2012.

1.7. Conclusions

The physical stresses (pipetting, centrifugation, and anisosmotic condition) excepting for Percoll gradient separation decreased total motility, plasma membrane integrity, and MMP. The sperm treated with the mechanical stresses (pipetting, centrifugation, and Percoll gradient separation) showed a higher susceptibility to the external source of ROS and hyposmotic stress increased basal ROS, indicating possibility of oxidative stress.

1.8. Quality Control Measures

The flow cytometer was calibrated by Cell and Immunology Core Facility of University of Missouri once per week using fluorescent beads. Unstained controls and single stains were set up for each condition tested. Single stains (SYTO 10, PI, and JC-1) were checked. In addition, a mitochondrial membrane potential disrupter, CCCP stain as control on JC-1 evaluation was checked.

2. Flow Sample and Specimen Details

2.1. Sample/Specimen Material Description

2.1.1. Biological sample

2.1.1.1. Biological Sample Description

Rat spermatozoa from cauda epididymis

2.1.1.2. Biological Sample Source Description

Rat, Outbred Sprague-Dawley [SD] strain, Cauda epididymis, Cauda epididymal spermatozoa

2.1.1.3. Biological Sample Source Organism Description

2.1.1.3.1. Taxonomy

Rattus norvegicus

2.1.1.3.2. Age

12-15 weeks old

2.1.1.3.3. Gender

Male

2.1.1.3.4. Phenotype

Healthy (none)

2.1.1.3.5. Genotype

None; not applicable

2.1.1.3.6. Treatment

The cauda epididymides were placed in 35-mm petri dishes containing HEPES-buffered Tyrode lactate (TL-HEPES) solution (300 mOsm, pH 7.4) supplemented with 3 mg/mL bovine serum albumin and 0.11 mg/mL pyruvic acid. Each cauda epididymis was cut at several places and the diffused sperm suspension was transferred to 1.5-mL tubes.

2.1.2. Environmental Samples

Not applicable

2.2 Sample Characteristics

Samples collected from cauda epididymides was almost spermatozoa without other cells and contamination when the samples were observed using microscope. Therefore, the sperm suspension was proper samples to perform our study.

2.3. Sample Treatment(s) Description

Samples were aliquot for treatment with each physical intervention (pipetting, centrifugation, Percoll gradient separation, and anisosmotic condition) at 37°C. The groups without any treatment were control. The control groups were compared to groups treated by each physical intervention for motility, plasma membrane integrity, MMP, and ROS of sperm. The concentration of sperm samples was adjusted to 2×10^6/mL sperm for flow cytometry evaluation.

2.4. Fluorescence Reagent(s) Description

Characteristics being measured	Analyte	Analyte Detector	Reporter	Manufacturer	Catalog #, Concentration used
Cell viability	DNA in all cells	SYTO 10	SYTO 10	Molecular Probe Inc.	L7013, 1:1000
Cell viability	DNA in membrane compromised Cells	Propidium iodide (PI)	PI	Molecular Probe Inc.	L7011, 1 μM or 2 μM
Mitochondrial membrane potential (MMP)	Differentiation of cells with high or low MMP	JC-1	JC-1 aggregate and monomer	Molecular Probe Inc.	M34152, 0.5 μM

Oxidative burst	Reactive oxygen species (ROS)	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA)	DCF	Molecular Probe Inc.	I36007, 10 µM

PI was used at the final concentration of 1 µM for counterstain of SYTO 10 and DCF, and 2 µM for counterstain of JC-1.

3. Instrument Details

3.1. Instrument Manufacturer

Becton Dickinson (BD) Biosciences (http://www.bdbiosciences.com/home.jsp)

3.2. Instrument Model

BD FACSCalibur, Serial number F 0920

3.3. Instrument Configuration and Settings

All lasers, filters and mirrors were manufactured by BD Biosciences. All filters and mirrors came with the machine and were installed August 2005.

3.3.1. Flow Cell and Fluidics

3.3.1.1. Flow Cell Type

stream-in-air

3.3.1.2. Other Relevant Flow Cell and Fluidics Information

General operation

Front key panel control in three modes: RUN, STNDBY, and PRIME; automatic standby mode for conserving sheath fluid by stopping sheath flow when no sample tube is installed.

Fluid reservoirs

Easily accessible 4-L capacity sheath and waste containers housed in a convenient pull-out drawer; level detectors for automatically indicating low levels of sheath or high levels of waste.

Sample flow rates

Three selectable flow rates of 60 µL/min, 35 µL/min, and 12 µL/min; regulated and monitored pressure difference between sheath and sample; particle velocity in flow cell: approximately 6 meters/second.

Quartz cuvette

Internal cross-section: rectangular 430 × 180 µm; antireflection coated external surfaces for maximum transmission of laser light.

Sample concentration

Single-cell suspension of 10^5 to 2×10^7 particles/mL recommended range.

3.3.2. Light Source(s)

3.3.2.1. Light Source Type

Two lasers: an air-cooled argon laser and a red diode laser

3.3.2.2. Light Source Excitatory Wavelength

Dual excitation lasers at the 488 nm (argon laser) and 635 nm (diode laser) wavelengths

3.3.2.3. Light Source Power at the Excitatory Wavelength

15 mW; life expectancy >5,000 hours

3.3.2.4. Light Source Beam

Prismatic expander and achromatic spherical lens providing 22 × 66 µm elliptical beam for argon-ion laser. 15 × 61 µm elliptical beam for red-diode laser.

3.3.3. Optical Filters and Detectors

Laser	Excitation Laser Line (nm)	Fluorescence Channel	Fluochromes detected
Argon (L1)	488	FL1 Green	FITC, Alexa Fluor 488
		FL2 Yellow	PE
		FL3 Red	PE-Cy5, PerCP, PerCP-Cy5.5, PE-Cy7
Red Diode (L2)	635	FL4 Red	APC, Alexa Fluor 647

3.3.4. Optical Paths

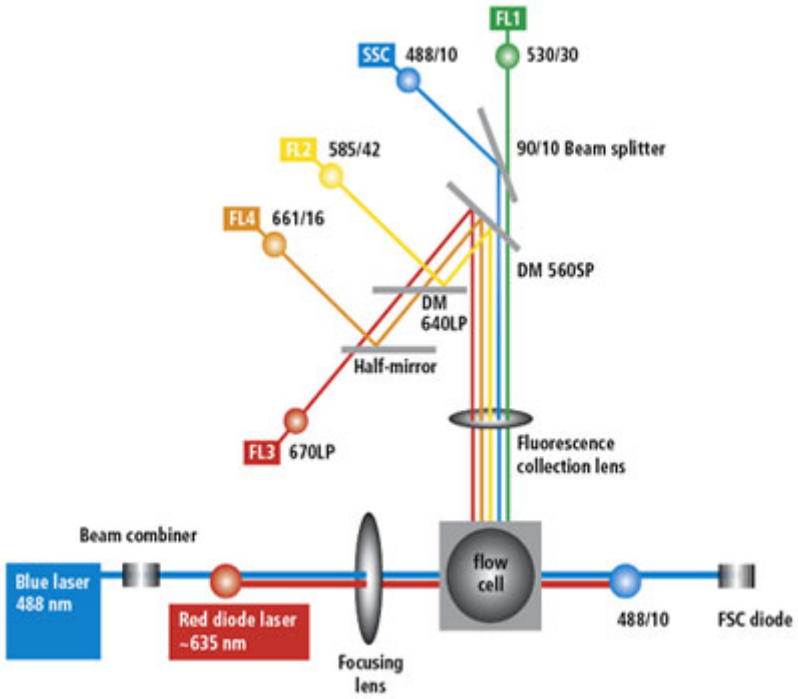

Figure S1. BD FACSCalibur Optical Path Configuration.

Forward scatter detector and filter

High-performance solid state silicon detector with 488 nm band pass filter for clear signal detection and red-diode (635 nm) laser signal rejection

Side scatter detector

High-performance photomultiplier using Brewster-angle beam splitter in the emission optical train

Fluorescence detectors and filters

Four high-performance, high dynamic range photomultipliers with bandpass filters: 530 nm (FITC), 585 nm (PE/PI), and > 670 nm (PerCP) with base unit, and 661 nm (APC) with FL4

Fluorescence Sensitivity

Estimated detection limit: 750 molecules of equivalent soluble fluorescein

Fluorescence resolution

Coefficient of variation in FL2-Area of < 3%, full peak for propidium iodide-stained chicken erythrocyte nuclei

Workstation resolution

1,024 channels on all parameters

Dynamic range

Logarithmic amplifiers for SSC, FL1, FL2, FL3, and FL4 providing four log decade range

Fluorescence compensation networks

Compensation for fluorescence spectral overlap between FL1 and FL2, between FL2 and FL3, and between FL3 and FL4 channels

Pulse processing

Width and area measurements for discriminating doublets; available for all fluorescence parameters

4. Data Analysis Details

4.1. List-mode Data File

To request raw data, please contact Prof. Yuksel Agca (agcay@missouri.edu)

4.2. Compensation Details

Signal and compensation of fluorescence were done in Cell Quest Pro software.

Signal information on each parameter

Parameter name	Parameter voltage	Amplification mode	Amplification Gain
FSC	E00	Linear	3.10
SSC	315	Linear	3.77

Staining combination	Parameter name	Parameter voltage	Amplification mode	Amplification Gain
SYTO 10/PI	FL1	585	Log	1.0
	FL2	529	Log	1.0
	FL3	649	Log	1.0
JC-1/PI	FL1	445	Log	1.0
	FL2	444	Log	1.0
	FL3	693	Log	1.0
DCF/PI	FL1	505	Log	1.0
	FL2	504	Log	1.0
	FL3	690	Log	1.0

Compensation information on each staining combination

	SYTO 10/PI	JC-1/PI	DCF/PI
FL1-%FL2	6.6	0	6.6
FL2-%FL1	45.4	65.9	43.5
FL2-%FL3	47.2	52.4	47.2
FL3-%FL2	33.9	22.8	38.1

4.3. Data Transformation Details

4.3.1. Purpose of Data Transformation

Selection of sperm on the basis of FSC/SSC distribution may occur misestimation of percentages because the sizes of sperm and debris are very similar. Therefore, application of mathematical formulae and equation is needed for inclusion of minimal debris (< 3%) or removal of debris (Theriogenology. 2010;73(7):839-47, Theriogenology. 2010;73(7):995-1000 and J Immunol Methods. 2011;368(1-2):71-9.)

4.3.2. Data Transformation Description

The sperm population was first gated on the basis of the forward and side scatter (FSC/SSC) properties (Figure S2*a*). In order to assess and exclude non–DNA-containing alien particles such as cytoplasmic droplets, cell debris, or diluent components, material unstained by the nuclear probes SYTO 10 and PI was back gated to a light scatter plot, where it distributes within the sperm light-scatter region. Using the data obtained from the procedure, the percentages of all data were then corrected for non-sperm particles (Figure S2*d*). Data was recalculated using FCS express, version 4 (De Novo Software, Thornthill, Ontario, Canada).

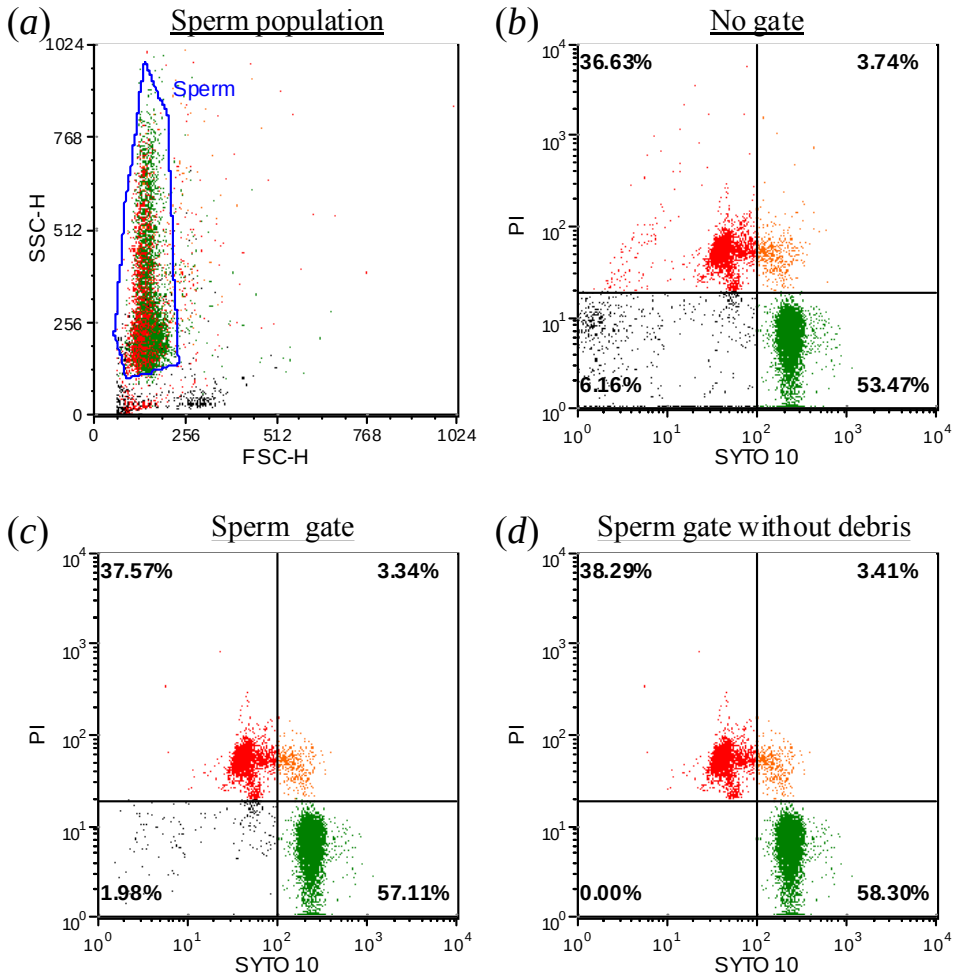

Figure S2. Sperm population setting for flow cytometry analysis and data recalculation. To correctly set the region on the sperm and avoid debris or fragments of cells, sperm population was selected using SYTO/PI staining (*b*) as well as FSC/SSC (*a*). In (*b*, *c*, and *d*), lower left quadrant (SYTO 10−/PI−) represents debris (population unstained by the nuclear probes SYTO 10 and PI), lower right quadrant (SYTO 10+/PI−) represents sperm with intact plasma membrane, and upper quadrants (PI−) represent sperm with damaged plasma membrane. The percentage of debris was 6.16% in total population that was not gated (*b*) and 1.98% in sperm population (*c*) that was gated by FSC/SSC and SYTO 10/PI staining. The debris was removed from sperm population and corrected percentage of SYTO 10+/PI− (*d*) was used for evaluation of sperm with intact plasma membrane.

4.4. Gating (Data Filtering) Details

Gate Description	Description of the Subpopulation	Gate Statistics for non-treated normal sample (control, %)
Sperm on the basis of FSC/SSC	Sperm population with debris	100

SYTO 10−/PI−	Debris	2.96
Not SYTO 10−/PI−	Sperm population without debris	97.04
SYTO 10+/PI−	Viable sperm with intact plasma membrane	65.95
J_{agg}+	Sperm with high and intermediate MMP	70.72
Bright J_{agg}+	Sperm with high MMP	62.35
Faint J_{agg}+	Sperm with intermediate MMP	8.37
J_{agg}− (or J_{mono})	Sperm with low MMP	29.28
J_{agg}+/PI−	Viable sperm with high and intermediate MMP	62.29
Bright J_{agg}+/PI−	Viable sperm with high MMP	59.65
Faint J_{agg}+/PI−	Viable sperm with intermediate MMP	2.63
Ratio bright J_{agg}+ to PI−	Ratio for viable sperm with high MMP of viable sperm	90.66
Gate Description	**Description of the Subpopulation**	**Gate Statistics for control (Mean Fluorescence intensity; MFI)**
J_{agg} MFI/Total sperm	MMP level in total sperm	442.41
J_{agg} MFI/Viable sperm	MMP level in viable sperm	696.21
Basal DCF MFI/Total sperm	ROS level in total sperm non-treated with TBHP (ROS inducer)	5.28
Basal DCF MFI/Viable sperm	ROS level in viable sperm non-treated with TBHP	4.58
Stimulated DCF MFI/Total sperm	ROS level in total sperm treated with TBHP	15.34
Stimulated DCF MFI/Viable sperm	ROS level in viable sperm treated with TBHP	13.38

Gate Images

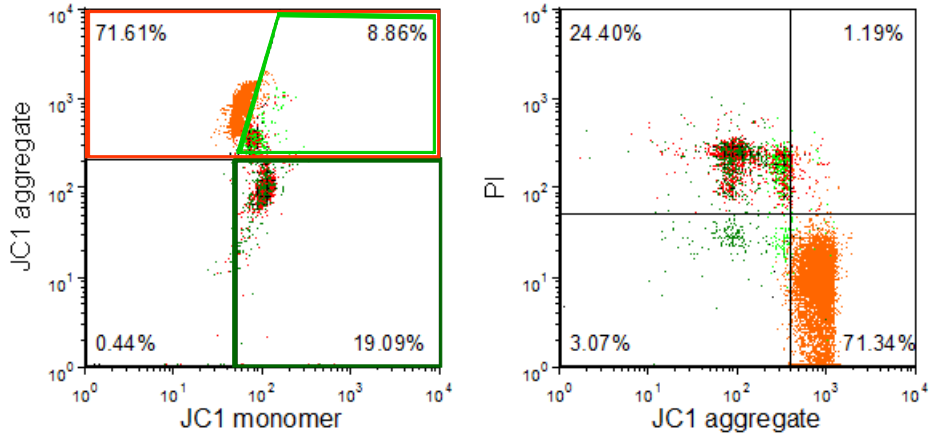

Figure S3. Classfication of sperm mitochondrial membrane potential (MMP) by JC-1/PI fluorescence staining. $J_{agg}-$ (dark green color dots) represents sperm with low MMP, faint $J_{agg}+$ (bright green color dots) represents sperm with intermediate MMP, and bright $J_{agg}+$ (orange color dots) represents sperm with high MMP. PI− (red color dots; upper quadrants in right picture) represents dead sperm.

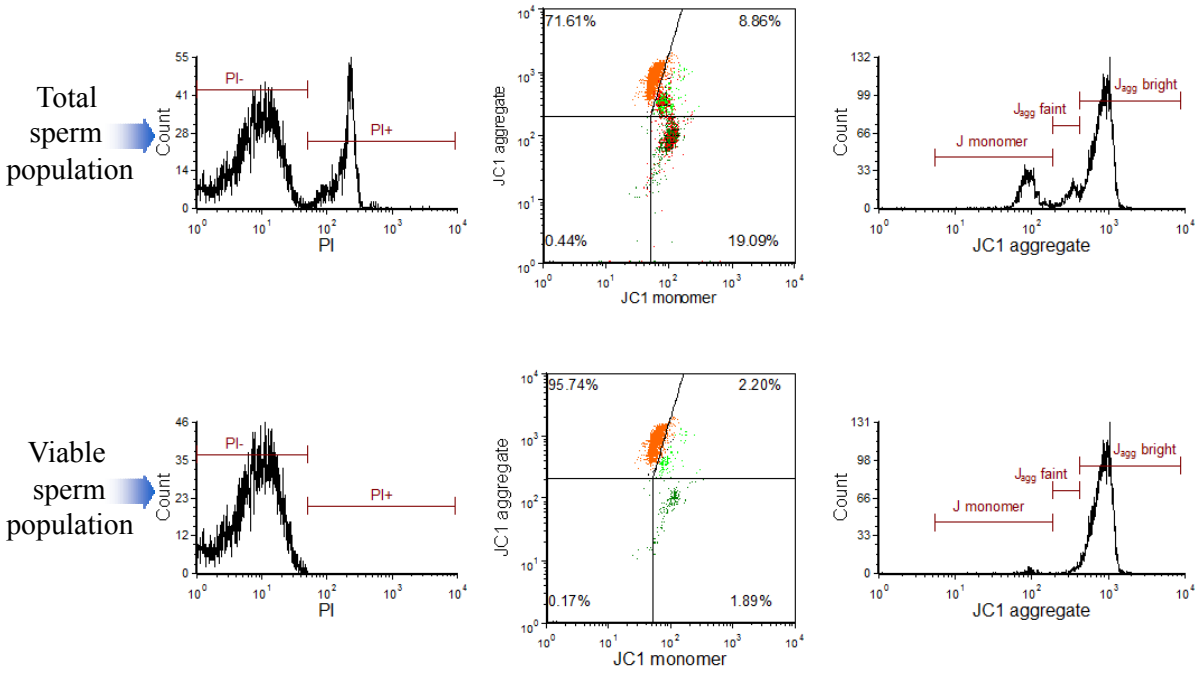

Figure S4. Determination of ratio of bright J_{agg}+ to PI−. The PI− and PI+ peaks represent viable and nonviable sperm, respectively. The dot plots showing the bright J_{agg}+ fluorescence distribution of viable sperm (orange color dots) were derived by gating on the PI− populations in lower panel.

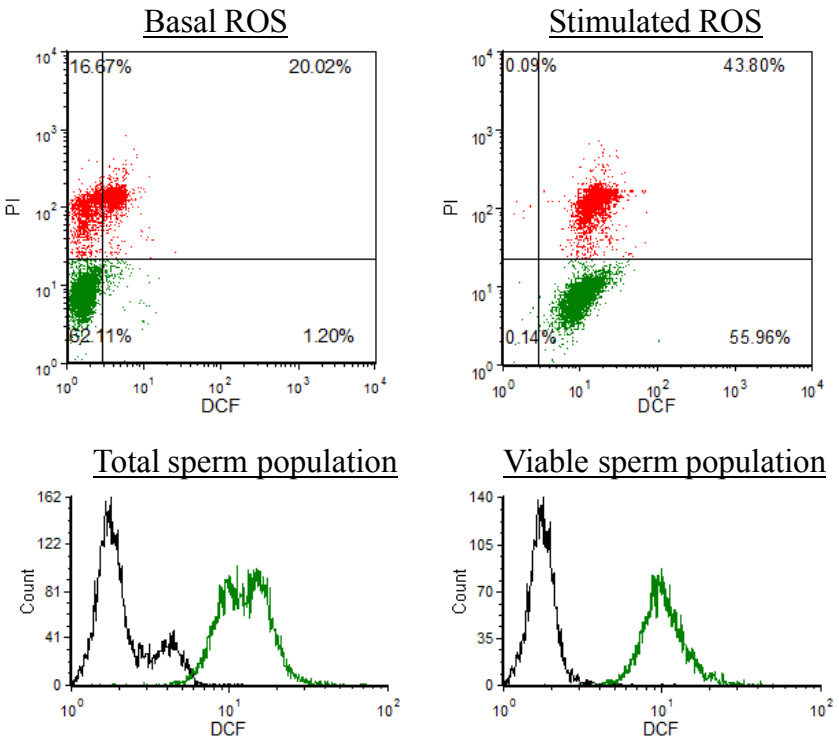

Figure S5. Determination of ROS level in total and viable sperm populations. ROS was evaluated by DCF/PI fluorescence staining before and after TBHP treatment in sperm, respectively. Mean DCF fluorescence intensity (DCF MFI) was analyzed in total and viable sperm populations (lower panel). In upper panel, lower left quadrant (DCF−/PI−) represents viable sperm with low ROS, lower right quadrant (DCF+/PI−) represents viable sperm with high ROS, upper left quadrant (DCF−/PI+) represents dead sperm with low ROS, and upper right quadrant (DCF+/PI+) represents dead sperm with high ROS. In lower panel, black lines represent basal ROS before TBHP treatment and green lines represent stimulated ROS after TBHP treatment.