Introduction

Infertility is the inability to achieve a pregnancy after one year of unprotected intercourse. Oxidative stress is considered as one of the important causes of male infertility, affecting more than 18 million people around the world. It is mediated by ROS (Reactive Oxygen Species), a group of highly reactive oxygen-based molecules. Oxidative stress is directly correlated with sperm dysfunction causing genomic and mitochondrial DNA fragmentation, lipid peroxidation in sperm membrane and loss of protein function, and negatively affecting sperm motility and thus male reproductive potential.

The negative action of ROS is normally counterbalanced by different antioxidant systems that can limit the harmful effects of ROS. Therefore, a proper balance between oxidants and antioxidants in the system is necessary in order to attain the physiological cellular homeostasis. ROS acts as a mediator in many physiological processes such as capacitation, acrosome reaction, hyperactivation and/or in regulating the oxidative stress. Oxidative stress inducer cumene hydroperoxide (CH) (1M, Sigma, St. Louis, MO, USA), was used in Sperm Wash Medium (SWM) to get final concentrations of 313 µM and 963 µM, equivalent to ORP values of 1.48 and 2.75 mV/10^6 sperm/mL, respectively (Fig 1). ORP was measured using the galvanostat-based MIOXSYS system (Ayhu Bioscience, Englewood, CO, USA) (Fig 2).

Materials and Methods cont.

Calibration of ORP to SWM

Oxidative stress inducer cumene hydroperoxide (CH) (1M, Sigma, St. Louis, MO, USA) was diluted in Sperm Wash Medium (SWM) to get final concentrations of 313 µM and 963 µM, equivalent to ORP values of 1.48 and 2.75 mV/10^6 sperm/mL, respectively (Fig 1). ORP was measured using the galvanostat-based MIOXSYS system (Ayhu Bioscience, Englewood, CO, USA) (Fig 2).

MMP assay

Mitochondrial membrane potential (MMP) was analyzed using MitoScreen kit (JC-1, BD Pharmingen™) in samples (n=8) exposed to 1.48 and 2.75 mV/10^6 sperm/mL in SWM for 0, 60 and 120 mins. Sperm (1 X 10^6) were incubated with 0.5 ml JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolcarbocyanine iodide) for 30 mins at 37°C. A positive control, pre-incubated with 25 mM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) for 30 minutes at 37°C and a non-stained negative control were included in the analysis. Each sample was analyzed in duplicate using a BD Accuri C6 Flow Cytometer. MMP results under the oxidative stress conditions were normalized against sperm results at different time points in order to determine the relative decrease or increases in the MMP values using the formula:

\[
\text{MMP of sperm at 0 mins} - \text{MMP of sperm at 120 mins} / \text{MMP of sperm at 0 mins} \times 100
\]

Vitality Staining

The sperm vitality was assessed in samples (n=8) exposed to ORP values of 1.48 and 2.75 mV/10^6 sperm/mL for 0, 60 and 120 minutes by Eosin-Nigrosin staining and expressed as percentage of vital cells. The vitality results at different ORP values were normalized against the SWM result at the respective time-point in order to determine the relative decrease or increase in vitality using the formula:

\[
\text{Sperm vitality when exposed to 1.48 mV/10^6 at 60 mins} - \text{Sperm vitality in SWM at 60 mins} / \text{Sperm vitality in SWM at 60 mins} \times 100
\]

Statistical Analysis

Statistical analysis was performed using MedCalc Statistical Software version 16.8.3 (MedCalc Software bvba, Ostend, Belgium). Different groups were compared using Kruskal-Wallis test and pairwise comparison analysis was done to determine the statistical significance. A p-value of less than 0.05 was considered significant.

Results

MMP remained unchanged following sperm exposure for 60 minutes, but decreased significantly by 2.5% (P=0.0014) and by 61.1% (P=0.0001) at 120 minutes when sperm was exposed to ORP values of 1.48 mV/10^6 sperm/mL and 2.75 mV/10^6 sperm/mL, respectively (Fig 4). Vitality decreased by 21.2% (P=0.001) at 60 minutes and by 41.1% (P<0.0001) at 120 minutes when sperm were exposed to ORP value of 2.75 mV/10^6 sperm/mL (Fig 5).

Materials and Methods

Subjects

In this pilot study, semen samples were obtained from 8 healthy donors of proven and unproven fertility after 2-7 days of ejaculatory abstinence according to WHO 5th edition guidelines. In order to select the best motile sperm fraction, all normozoospermic semen samples were prepared by double-density gradient centrifugation (PureCeption SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA).

Conclusions

1. This is the first study to evaluate the effect of ORP levels on sperm functions within a normal sperm population.
2. Spermatozoal MMP and vitality were affected at ORP levels of ≥ 2.75 mV/10^6 sperm/mL.
3. We suggest that oxidative stress can affect sperm motility through impairment of mitochondrial function.
4. High seminal ORP may have a negative effect on sperm function and therefore, on the fertilizing ability of spermatozoa.