THE ROLE OF OXIDATIVE PHOSPHORYLATION IN THE MECHANISM OF HUMAN SPERM MOTILITY REGULATION

Sudjarwo*, Aucky Hinting, Noor Cholies Zaini***
* Faculty of Pharmacy, Airlangga University
** Faculty of Medicine, Airlangga University

ABSTRACT

Energy synthesis for sperm motility in the mitochondria with enzymatic reactions in nucleus and mitochondria is called Oxidative Phosphorylation (OXPHOS), in which the respiratory process is coordinated in nucleus and mitochondria. Enzymes in OXPHOS process are complex enzymes I, III, IV and V in the mitochondria and complex enzyme II in the nucleus. If there is OXPHOS dysfunction, the mutation of mitochondria DNA (mtDNA) will decrease energy (ATP, Adenosine Triphosphate) production and increase Reactive Oxygen Species (ROS). The characteristics of ROS are oxidant for lipid, protein and DNA, and all these reactions lead to the dysfunction of OXPHOS and, in the spermatozoa, they will decrease motility. The detection of ROS, Malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OH-dG), and sperm motility can use Chemiluminicense method, Spectroflourometric method, High Performance Liquid Chromatography (HPLC) method and Markler method, respectively, as World Health Organization (WHO) Procedures Laboratory. These results indicated there was significant indirect correlation between ROS with sperm motility and direct correlation between ROS with MDA, 8-OH-dG.

Key words: *ROS, MDA, 8-OH-dG, sperm motility*

INTRODUCTION

Respiration of eucaryote cell occurs in the mitochondria through oxidative phosphorylation (OXPHOS), involving enzymes in the nucleus and mitochondria. Cells respiration chain is complex enzymes I, III, IV, and V in the mitochondria, as well as complex enzyme II in the nucleus. At the early respiration chain, oxidative phosphorylation is produced by electron from complex enzymes I and II, and were transferred to complex enzyme III through ubiquinone compound (C_0Q_{10}), and, subsequently, from complex enzyme III to complex enzymes IV and V. In the complex enzyme V proton pump force occurs and, with P (Phosphor and ADP (Adenosine Diphosphate), it forms ATP (Adenosine Triphosphate) as energy cells. If dysfunction OXPHOS occurs, for example, by electron transfer inhibition to decrease proton pump force, Reactive Oxygen Species (ROS) will increase (Sohal and Brunk, 1992).

ROS is an oxidant compound, such as free radical. The target of ROS are lipid, protein, and Deoxyribonucleic Acid (DNA). Lipid is a primary compound of the membrane, and if oxidation occurs, it will produce Malon Dialdehyde (MDA) (Suleiman *et al.*, 1996). Protein is also a primary membrane compound, and if oxidation occurs, the consequence is the decrease of membrane integrity. DNA is composed from nucleotides as well as adenine, tymine, guanine, and cytosine. In high ROS, nucleotide guanine is susceptible for oxidation. The end product oxidation of guanine is 8-hydroxydeoxyguanosine (8-OH-dG) (Xu *et al*., 1992). If all oxidation process occurs in the sperm, sperm motility may decrease.

In addition, the presence of mitochondrial DNA (mtDNA) mutation has correlation with energy synthesis (ATP), and energy needed for sperm motility, so that energy need for sperm motility is not enough and, consequently, it decreases sperm motility. This research was intended to detect sperm motility from OXPHOS dysfunction as indicated from ROS, MDA, and 8-OH-dG.

MATERIALS AND METHODS

Sperm sample was taken from patients with infertility problem and visited routinely to Infertility Laboratory, Budi Mulia Hospital, Surabaya. Sample was taken using random sampling and two-time collection. The number of first collection was 33 samples and the second collection was 49 samples.

Analysis of Sperm Quality

The estimation of sperm count was done using counting chamber from *Markler,* in which the sperm was dropped on Markler chamber with thickness of 10 lm and cells were counted in the column and converted to concentration million/ml (WHO, 1992)

The estimation of sperm motility used object glass, onto which sperm of $10-15\ \mu$l were dropped and covered with cover glass. Detection was done using microscope

with magnification of 400−600 times. Classification of motility (WHO, 1992) was as follows: a) fast and straight motile sperm; b) slow and not straight motile sperm; c) not straight motile sperm; and d) not motile sperm.

Reactive Oxygen Species (ROS) and Malondialdehyde (MDA) Assays

Sperm suspension (0.2 ml) was added with 4 μl luminol [25 mM in dimethyl sulfoxide (DMSO) with final concentration 250 μM], 8 μl horseradish peroxides (2 mg/ml PBS), and detection of chemiluminisence for one minute (Aitken *et al*., 1994)

Sperm was separated from protein by adding 20% trichloroacetic acid and centrifugated for 10 minutes at 5000 rpm. Supernatant was taken and added with 0.1 ml 1% sodium thiobarbiturate and 1N hydrochloride acid to 10 ml, and were incubated in the waterbath for 135 minutes. The emerged coloured compound (3.0 ml) was extracted with 5.0 ml isobutanol and detected with Spectrofluorometer on wavelength excitation and emission in 522 nm and 575 nm (Rice – Evans *et al.*, 1991)

Isolation of Sperm DNA

Isolation of sperm DNA reffered to Kao *et al.* method (1995). Sperm (2.0 ml) in Percoll solution was washed with twice volume buffer phosphate solution pH 8.0 three times. Subsequently, osmotic shock was carried out by incubating in 8 °C for 20 minutes. Cell lysis was done by adding 0.5 ml lysis buffer containing 2 μl PCR buffer (10 mM Tris-HCl pH 8.3 containing 50 mM KCl; 0.01% b/v gelatin); 0.6 μl $MgCl_2$ 50 mM; 1 μl Proteinase-K 20 mg/ml; 5.0 μl Dithiothreitol (DTT) 20 mM and 10 μl Sodium Dodecyl Sulfate (SDS) 0.1%. The mixed solution was incubated in 37 °C overnight. Then, inactivation was done using Proteinase-K in waterbath 95 °C for 10 minutes, and centrifugation for three minutes at 4000 rpm. DNA extraction was done by adding phenol-chloroform. The mixture solution was added one time volume phenol solution (in buffer Tris-HCl solution pH 8.0) and mixed by means of vortex, and centrifugatied at 12.000 rpm (Sorvall @ MC 12 V, Dupont, USA) for three minutes. Water phase was removed to other tube and added with one time phenol: chloroform (25:1), vortexed and centrifugated at 12.000 rpm for three minutes. The following process was the removal of water phase into other tube and added with one time volume chloroform, vortexed and centrifugated again at 12.000 rpm for three minutes. The resulting water phase was moved into other tube and added with 2.5 time alcohol (100%), mixed and kept at – 20 °C for 10 minutes. DNA was precipitated at 4 °C, 14.000 rpm for 10 minutes. DNA result were dried Vacuum SPEEDVAC (DNA Speed Vac, DNA 110, Savant) and diluted in TE buffer solution (Tris-EDTA, pH 8.0).

8-hydroxydeoxyguanosine (8-OH-dG) Assays

For detection of 8-OH-dG as indicator of DNA damage, DNA product from isolation was fragmented by adding 200 μl sodium acetic 20 mM (pH 8.0). Mixed solution was digested by adding 20 μg Nuclease-P1 (Sigma) and were incubated at 37 °C for 60 minutes. The next fragmentation product was filtrated with Whatmann paper and detected with High Performance Liquid Chromatography (HPLC) method (Xu *et al*., 1994)

Data Analysis

The estimation of correlation ROS, MDA, 8-OH-dG and sperm motility was done using linear regression in MedCalc Program, Cut-Off value with ROC Curve in MedCalc Program and for significant value with t-test in Excel Program.

RESULTS

Sample was randomly collected from infertile donor sperm who routinely came for controlling sperm quality in Infertility Laboratory, Budi Mulia Hospital, Surabaya. The first collection was 33 samples for ROS and MDA detection. The second collection was 49 samples for the detection of 8-OH-dG as indicator DNA damage. The analysis of sperm quality was carried out according to the guidelines of WHO Laboratory (1992). From the first collection it was found that 16 were normozoospermia (Spermatozoa count $\geq 20.\ 10^6$/ml) and 17 were asthenozoospermia (motility of spermatozoa, a and b motility criteria $\geq$ 50%), while at the second collection 34 were normozoospermia and 15 were asthenozoospermia.

ROS Assays

Mt DNA mutation is influenced by OXPHOS process to produce energy (ATP), and to increase ROS. The normal mtDNA has small ROS, so that increased ROS may result in high OXPHOS dysfunction and decreased ATP. If it occurs in spermatozoa, the consequence will be immotility.

Characteristic ROS as oxidation, if it occur in the cells can be lipid peroxidation with the end product of MDA, as well as DNA oxidation with the end product of 8-OH-dG.

MtDNA mutation influences OXPHOS process to produce energy (ATP), and increases ROS. Normally, mtDNA has few ROS, so that its presence will increase ROS

to act to influence high OXPHOS process and to decrease ATP. If it is in the spermatozoa, it can act to decrease motility or to immotilize spermatozoa.

The characteristic of ROS is oxidation. If it is in the cell, it undergoes lipid peroxidation with the end product of MDA, DNA oxidation with the end product 8-OH-dG. Lipid peroxidation causes the decrease of cell integrity and DNA damage, causing dysfunction energy (ATP) needed, that will finally lead to cellular death.

ROS (CPM/10^6 spermatozoa) in lipid peroxidation is 7.51 ± 5.97 (normozoospermia) and 18.98 ± 13.73 (asthenozoospermia).

MDA Assays

High MDA concentration can be an indicator of lipid peroxidation and causes the decrease of sperm motility. MDA concentration of nmol/10^6 spermatozoa on lipid peroxidation were 0.62 ± 0.49 (normozoospermia) and 2.60 ± 0.51 (asthenozoospermia).

DNA Oxidation

In the cells such as spermatozoa, DNA in the nucleus and mitochondria arrange nucleotides as adenine, guanine, tymine and cytosine. Nucleotide sensitive of oxidation is guanine, and the end product of 8-hydroxydeoxyguanosine compound can be detected with High Performance Liquid Chromatography (HPLC) method (Xu *et al*., 1992)

Indicator of DNA damage can be shown from high 8-OH-dG concentration. High 8-OH-dG concentration has correlation with the decrease of sperm motility.

Detection of 8-OH-dG concentration (fmol/ng DNA) showed 3.68 ± 2.68 in normozoospermia, and 8.91 ± 5.89 in asthenozoospermia.

Table 1. Correlation ROS, MDA and 8-OH-dG in the sperm

Diagnosa	ROS (CPM/10^6 sp)	MDA (nmol/10^6 sp)	8-OH-dG (fmol/ngDNA)
Normozoo-spermia	7.51 ± 5.97*	0.62 ± 0.49**	3.68 ± 2.68**
Asthenozoo-spermia	18.98 ± 3.73*	2.60 ± 0.51**	8.91 ± 5.89**

*) P<0.05; ** P< 0.01

DISCUSSION

Concentrations of ROS (CPM/10^6 spermatozoa) in lipid peroxidation were 7.51 ± 5.97 (normozoospermia), 18.98 ± 13.73 (asthenozoospermia).

ROS have indirect correlation with sperm motility in the asthenozoospermia, r = −0.5176 (p = 0004; P < 0.01). In the asthenozoospermia, ROS rather of normozoospermia.

Sperm motility dependent for energy/ATP produced by mtDNA through OXPHOS process. The dysfunction of mtDNA, for example, mtDNA mutation, can cause the decrease of ATP production. If it occurs in the spermatozoa, it can decrease sperm motility, so that sperm motility is a phenotype of spermatic mtDNA mutation, as reported by Ruiz-Pesini *et al.* (2000).

The phenotypic consequence of mtDNA variants influences sperm quality. Sperm motility depends highly on the function of OXPHOS chain. Electron transfer chain for producing energy arranged is by complexes enzymes I, III, IV, and V in the mitochondria and complex enzyme II in the nucleus. The characteristic of mtDNA variant is highly individual (Moore and Reijo-Pera, 2000)

The major factor of mitochondrial dysfunction is caused by dysfunction OXPHOS which may stimulate ROS to produce and to induce DNA damage. Indication of mitochondrial dysfunction are two pathophysiological effects from OXPHOS deficiency: first, to decrease energy (ATP) production, and second, to increase of production and toxicity of ROS. Endogenous ROS is produced primarily in the mitochondria as superoxide anion radical, hydrogen peroxide and hydroxyl radical. The increase of ROS occurs if respiration chain is inhibited. First, electron accumulation occurs particularly in complex enzyme I and Coenzyme Q, so that the inhibition of OXPHOS can increase ROS.

The cut-off value of ROS with sperm motility 5,8282 CPM/10^6 spermatozoa, showing that ROS value above the cut-off needs treatment to decrease of ROS to return into normal.

MDA concentration (nmol/10^6 spermatozoa) on lipid peroxidation were 0.62 ± 0.49 (normozoospermia) and 2.60 ± 0.51 (asthenozoospermia). ROS has direct correlation with MDA (r = 0.78; p = 0,00). MDA correlates with motile spermatozoa (criteria a and b), r = −0.41; P: 0.018 (P < 0,05); MDA with immotile spermatozoa (criteria c and d), r = 0.49; P:0.003 (P < 0.01). Reported MDA (nmol/10^6 spermatozoa) 5.72 ± 0.84 (normozoo-spermia), 9.12 ± 1.23 (oligospermia) and 9.75 ± 1.41 (asthenozoospermia) (Suleiman *et al*., 1996). MDA concentration 10 nmol/10^6 spermatozoa may cause spermatozoal death (Alvarez *et al*., 1987).

Sperm motility depends on the integrity of mitochondria, in which phospholipid is the first component. If fatty acid in the phospholipid is oxidized by ROS, it will damage spermatozoa and decrease sperm motility. It is reported that

human spermatozoa becomes immotile in five minutes after being added with 30−60 nmol lipid peroxide (Suleiman *et al*., 1996). Lamirande and Gagnon (1992) reported that ROS can cause sperm immotility in 5−30 minutes and depends on sperm concentration.

The cut-off value of MDA with sperm motility is 1.4308 nmol/10^6 spermatozoa, showing that if the MDA is higher than this value, treatment is needed to decrease MDA concentration back to normal.

ROS has direct correlation with 8-OH-dG (r) = 0.4321; p = 0.0019 (P < 0.01), showing that high ROS concentration may result in high 8-OH-dG concentration. Whereas, 8-OH-dG concentration with spermatozoa motile has correlation (r) = −0.4903; p = 0.0003 (P < 0.01), indicating that high 8-OH-dG concentration has correlation with the decrease of sperm motility. The cut-off value of 8-OH-dG is 9.6071 ng/μDNA. It indicates that normal 8-OH-dG concentration is under the cut-off value, while the abnormal one is on the contrary.

In aerobic organism according to damage by ROS, which may cause mutagenesis or cell death if there is no repair system. One of the product of DNA damage is 8-OH-dG, and steady-state condition, which is 10 times higher than that in the mtDNA as to DNA, and dramatically increase in aging process (Souza-Pinto *et al*., 1999). It is very important to indentify factors that cause the risk of genetic dysfunction, which is responsible for genetic variations. 8-OH-dG compound is promutagenic DNA dysfunction produced from deoxyguanosine by oxygen radical. 8-OH-dG form in DNA induces G:C change into T:A, primarily in DNA replication (Shimoda *et al*., 1994). The accumulation of endogeneous DNA damage can influence RNA transcription process (Holmes *et al*., 1992)

The correlation of ROS, MDA and 8-OH-dG in the sperm is showed Table 1. 8-OH-dG concentration is reported to have significant different (P < 0.05) between normozoospermia and asthenozoospermia. 8-OH-dG concentration with ROS has direct correlation (r = 0,431; P = 0,002) and ROS can cause the decrease of sperm motility through DNA damage mechanism by oxidation (Hinting and Sudjarwo, 2001*)*

Asthenozoospermia has a high ROS, MDA and 8-OH-dG concentration compared normozoospermia. Using t-test to analyze the change of normozoospermia to asthenozoospermia, significant correlation was found in ROS concentration (p = 0.04; P < 0.05); MDA (p = 0.009; P < 0.01) and 8-OH-dG (p = 0.004; P < 0.01). It was showed that ROS had direct correlation with MDA and 8-OH-dG, so that asthenozoospermia condition have correlation with the increase of ROS, MDA and 8-OH-dG concentration, although 8-OH-dG concentration is below cut-off value.

However, normozoospermia with ROS = 7.51 ± 5.97 CPM/10^6 spermatozoa is not yet capable to induce membrane and DNA damage. It can be seen from MDA and 8-OH-dG concentrations, which are relatively small or under cut-off value. It is suggested to conduct study on ROS that is capable to induce membrane and DNA damage.

Mechanism of sperm motility has correlation with the system of OXPHOS process. The dysfunction of OXPHOS process can cause the decrease of energy production needed for sperm motility and the increase of ROS can cause oxidation of lipid, protein and DNA. The decreases of sperm motility has correlation with the increase of ROS. The high ROS concentration leads to lipid oxidation with the end products MDA and guanine as DNA components with the end product of 8-OH-dG. ROS, MDA and 8-OH-dG concentrations have indirect correlation with sperm motility.

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