

Hydrogen Peroxide Has Adverse Effects on Human Sperm Quality Parameters, Induces Apoptosis, and Reduces Survival

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ABSTRACT

Background: One of the causes of male fertility disorders is the exposure of oxidative stress on the human sperm. Understanding the mechanism of disturbance is important to develop a better treatment for infertile or subfertile patients. **Aims:** The aim of this study was to analyze the effects of hydrogen peroxide (H₂O₂) on human sperm quality parameters and cell survival. **Settings and Design:** This study used an experimental design. **Materials and Methods:** Sperm cells from 15 donors were washed in a Percoll gradient and dissolved in Biggers, Whitter, and Whittingham medium. Cells were incubated with H₂O₂ at various concentrations from 0 to 250 µM for 2 h. Sperm viability was examined by eosin assay, sperm kinetic by computer-assisted sperm analyzer, sperm penetration by cervical mucus penetration assay, and membrane integrity by hypo-osmotic swelling test. Sperm capacitation, apoptosis, and cell survival were analyzed using western immunoblotting. **Statistical Analysis Used:** One-way ANOVA on SPSS 21 combined with *post hoc* LSD test was used to analyze differences among the groups. A *P* < 0.05 was considered significant. **Results:** Sperm viability and kinetic were significantly reduced at H₂O₂ concentrations of 200 and 250 µM. H₂O₂ reduced sperm capability to penetrate cervical mucus and also damage cell membrane integrity at all concentrations used. H₂O₂ significantly inhibited sperm capacitation, indicated by reduced total tyrosine phosphorylation. H₂O₂ exposure stimulated activation of caspase 3 and significantly reduced phosphorylated AKT at all concentrations used. **Conclusions:** H₂O₂ comprehensively inhibits sperm qualities related to the capacity to fertilize oocyte, stimulates caspase activity, and inhibits cell survival.

KEYWORDS: *AKT, capacitation, caspase, hydrogen peroxide, sperm*

INTRODUCTION

Infertility is a clinical problem experienced by approximately 15% of reproductive age couples. Causes of the infertility can be from male factors that takes approximately 45% of the cases, whereas female and idiopathic factors contribute 40% and 15%, respectively.^[1] The etiology of male infertility is multifactorial such as hormonal, genetic abnormalities, immunology, urogenital trauma, and inflammation on male reproductive system, which will affect sperm function.^[2] Furthermore, other factors in particular

lifestyle and environmental factors that are considered as idiopathic causes often related to oxidative stress.^[3,4] Oxidative stress is defined as a condition that occurs when there is an imbalance between free radicals or pro-oxidant with antioxidants in the human body.^[5]

Reactive oxygen species (ROS) are radical compounds because they tend to attract electrons. When the

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presence of ROS such as hydrogen peroxide (H_2O_2) is excessive, it can cause disruption of sperm cell interactions with oocyte. H_2O_2 is a reactive molecule that is often associated with decreased sperm function due to oxidative stress.^[6] The susceptibility of human sperm towards oxidative stress is due to the fact that this cell only have very little cytoplasm with minimum amount of antioxidant enzymes. Moreover, human sperm membrane contains lot of polyunsaturated fatty acid (PUFA) that can react with ROS to form lipid peroxidation.^[7]

Another report showed that oxidative stress can affect sperm motility,^[8] capacitation process,^[9] and apoptosis.^[10] However, most of the previous studies did not analyze sperm parameters comprehensively. Here, in our study, we examined the effect of oxidative stress, in the form of H_2O_2 , on sperm viability, kinetics, penetration assay, membrane integrity, capacitation, apoptosis, and survival of the sperm cells. In light of investigating the causes of idiopathic male infertility, this comprehensive study is expected to give a complete understanding of the effect of H_2O_2 on male fertility.

MATERIALS AND METHODS

Human sperm preparation

This study was approved by Research Ethical Committee of the Faculty of Medicine Universitas Indonesia (No. 0480/UN2. F1/ETIK/2018) for the use of human semen sample. A total number of 15 semen samples from different individuals with normal criteria (concentration ≥ 20 million cells/ml) were obtained from volunteers with prior informed consent. Sample calculation to determine minimum number of volunteers for sperm donor was not performed because it was not relevant to the characteristic of the study, which focuses on sperm cells with normal criteria. Samples were collected in sterilized containers and left at room temperature for 20 min for liquefaction. Semen was then overlaid on 50% Percoll (v/v in Biggers, Whitten, Whittingham [BWW] medium). Semen in 50% BWW was centrifuged at 1900 rpm for 30 min. Pellet-containing sperm cells was washed with 3 ml BWW medium and centrifuged again at 1900 rpm for 15 min. Pellet was resuspended in 1 ml of BWW, and sperm cell concentration was determined using a counting chamber. Data for all donors participating in this study are available.

Treatment of hydrogen peroxide

H_2O_2 (Sigma, St Louis, USA) was used to induce oxidative stress on human sperm. Thirty percent stock of H_2O_2 (9.8 M) was diluted to 50 μ M, 100 μ M, 150 μ M, 200 μ M, and 250 μ M (final concentration) in 0.5 mL

of sperm suspension containing 10×10^6 cells for each H_2O_2 concentration. Sperm were incubated at 37°C for 2 h. At least two aliquots of samples were used for each concentration of H_2O_2 . Sperm suspension without the addition of H_2O_2 was served as a control.

Malondialdehyde evaluation

Malondialdehyde (MDA) measurement was performed to confirm oxidative stress had occurred in the treatment groups. After H_2O_2 incubation, samples were centrifuged at 10,000 rpm for 5 min; then, the supernatant was removed, and the pellet was dissolved in the sodium dodecyl sulfate (SDS) extraction buffer and heated for 5 min followed by centrifugation at 13,000 rpm for 5 min. Supernatant was discarded and pellet was added 0.67% solution of thiobarbituric acid and heated for 10 min. After cooling down, MDA was read using a spectrophotometer at a wavelength of 532 nm.

Examination on viability of spermatozoa

Sperm viability was examined using eosin supravital staining. A hundred microliter of semen was mixed with an equal amount of eosin Y solution on an object glass and covered with deck glass. Observation was carried under a light microscope with $\times 400$ magnification. Sperm cells with white head were considered as live cells, whereas those with pink head were considered as dead cells. The percentage of live cells was calculated from total 100 cells count.

Sperm kinetic examination

Kinetic of sperm was examined by using a computer-assisted sperm analyzer (CASA, Hamilton Thorne, USA). Ten microliters of sperm samples was added into CASA slide chamber (Leja, the Netherlands) and observed under the CASA microscope. Observation was performed at frame capture speed of 60 Hz, maximum cell size of 50 μ m², minimum cell size of 5 μ m², and minimum brightness of 170. The kinetic parameters of sperm that were tested were average path velocity (VAP), straight-line velocity (VSL), and curvy linear velocity (VCL).

Cervical mucus penetration assay

Human cervical mucus obtained from mother and child clinic was inserted into the capillary tube with a reservoir on the one end and plasticine on the other end. Capillary tube was placed in horizontal position. Semen was put into the reservoir and distance migration reached by sperm cells was observed under microscope. The observation was carried out during 2 h incubation at 37°C.

Sperm membrane integrity

Sperm membrane integrity was examined by hypo-osmotic swelling test (HOST). Sperm cells that had been diluted in BWW were divided into separated microtubes according to treatment groups. Cell suspensions were mixed with an equal amount of hypo-osmotic solution (7.35 gr sodium citrate, 2H₂O; 13.51 gr fructose in 1-l ddH₂O) and then incubated for 30 min at 37°C. Swollen (indicating intact cell membrane) and un-swollen (indicating leaky cell membrane) cells were counted under a light microscope.

Sperm protein extraction

After treatment with H₂O₂, sperm cells were centrifuged at 10,000 rpm for 5 min and pellet was dissolved in SDS extraction buffer (2% SDS, 10% sucrose, and 0.1875 M Tris pH 6.8). Sample was boiled for 5 min and centrifuged again at 13,000 rpm for 10 min. Supernatant was transferred to a new tube for western blot analyses. Protein concentration was determined using a spectrophotometer.

Western immunoblotting

Western immunoblotting analyses were performed to examine tyrosine phosphorylation, caspase-3 activity, and phosphorylation of AKT on sperm after being treated with H₂O₂. Twenty-five micrograms of sperm protein were separated on 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Science, UK). The membrane was subsequently blocked in 3% bovine serum albumin (BSA) for 1 h at room temperature. Membrane was then incubated with the following primary antibodies: Phosphotyrosine antibody (Sigma, USA): 1:2000; caspase 3 antibody (Cell Signaling Technology, USA): 1:1000; AKT antibody (Cell Signaling Technology, USA): 1:1000 overnight at 4°C. The next day, the membrane was washed with tris-buffered saline, 0.1% Tween (TBST) 3 × 5 min and then incubated with secondary antibodies, namely goat antimouse IgG or donkey antirabbit IgG (Santa Cruz Biotechnology, USA) at 1:2500 for 2 h at room temperature. The membrane was washed again with 1 × TBST for another 3 × 5 min and visualization was carried out using enhanced chemiluminescence (ECL) plus western blot detection system (GE Healthcare Life Science, UK). Signal was detected using Imagequant LAS 4000 (GE Healthcare Life Science, Sweden). Band intensity was measured using ImageJ (NIH, USA).

Data analysis

All experiments were repeated at least three times and analyzed using one-way ANOVA on SPSS 21 program

(IBM, Chicago, IL, USA). The *post hoc* differences between groups were analyzed using LSD test. $P < 0.05$ was considered as significant.

RESULTS

Malondialdehyde levels

To confirm that the use of doses of H₂O₂ from 50 to 250 μM did increase lipid peroxidation (LPO) on sperm cells, the level of MDA after treatment was measured. Our data showed that MDA levels in the control group was 1.35 ± 0.31 nmol/ml, and with increasing concentration of H₂O₂ from 50 to 250 μM, the MDA levels were 2.05 ± 0.51; 2.15 ± 0.54; 2.45 ± 0.42; 3.30 ± 0.27; and 3.70 ± 0.36 nmol/ml, respectively [Figure 1]. This indicates that LPO had occurred in the sperm cells used in this study.

Sperm viability

To determine whether sperm viability was affected by incubation in H₂O₂, we examined percentage of viability by using eosin Y solution. The data showed that viability in the control group was 68.20% ± 3.63% and with increasing concentration of H₂O₂ percentage of sperm viable decreased in a concentration-dependent manner. At 50 μM, H₂O₂ sperm viability decreased to 61% ± 1.87%, at 100 μM was 58.20% ± 1.64%, at 150 μM was 55.80% ± 2.16%, at 200 μM was 54.0% ± 3.39%, and at 250 μM was 48.40% ± 6.98% [Figure 2]. Significant changes were observed at the concentration of 200 ($P < 0.05$) and 250 μM ($P < 0.01$).

Sperm kinetics

Sperm kinetic was analyzed using CASA to observe sperm motility in detail. Three sperm kinetic parameters were selected in this study, namely VAP, VSL, and

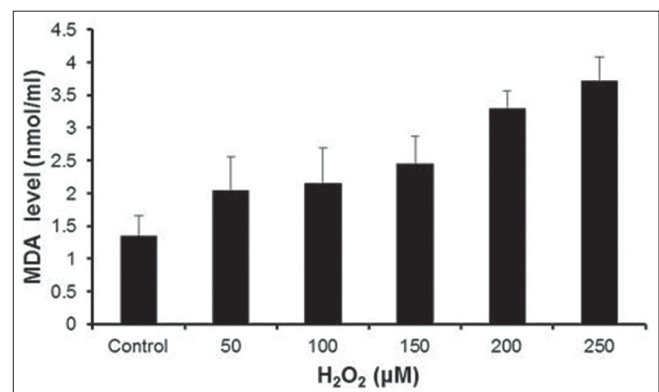


Figure 1: Analysis of malondialdehyde by spectrophotometer on sperm cells extract after treatment with hydrogen peroxide to confirm intrusion of the oxidative stress into the cell. All the treatment groups from 50 to 250 μM of hydrogen peroxide show increasing levels of malondialdehyde, suggesting reliability of the treatment. Error sign on top of each bar represents SEM. $n = 3$. SEM: Standard error of the mean

VCL. Our study showed that treatment with H₂O₂ decreased all three parameters of CASA. Sperm VAP decreased significantly from 41.01 μm/s at control group to 30.35 μm/sec at concentration of 200 μM (*P* < 0.05) and 21.19 μm/s at 250 μM (*P* < 0.01) [Figure 3, black bars]. Sperm VSL decreased significantly only at the concentration of 250 μM from 33.58 μm/s at the control to 13.11 μm/s (*P* < 0.01) [Figure 3, white bars]. Sperm VCL decreased significantly from the control 69.62 to 49.65 μm/s at 200 μM (*P* < 0.05) and 37.53 μm/s at the 250 μM of H₂O₂ (*P* < 0.01) [Figure 3, diagonally striped bars].

Sperm cervical mucus penetration

Decrease in sperm kinetics made us curious about sperm cell ability to penetrate cervical mucus. Human cervical mucus was collected from three volunteers at mother and child clinic and used in the cervical mucus penetration assay. Sperm incubated with increasing concentration

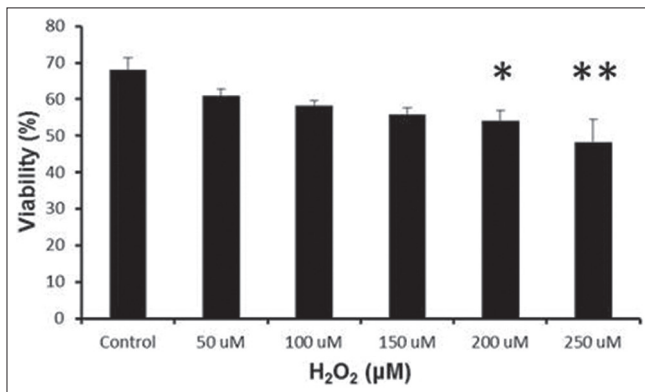


Figure 2: Sperm viability assay using eosin Y solution. Significant differences were observed at hydrogen peroxide concentration of 200 μM (*P* < 0.05) and 250 μM (*P* < 0.01). Error sign on top of each bar represents SEM. *n* = 3. *Significant; **Very significant. SEM: Standard error of the mean

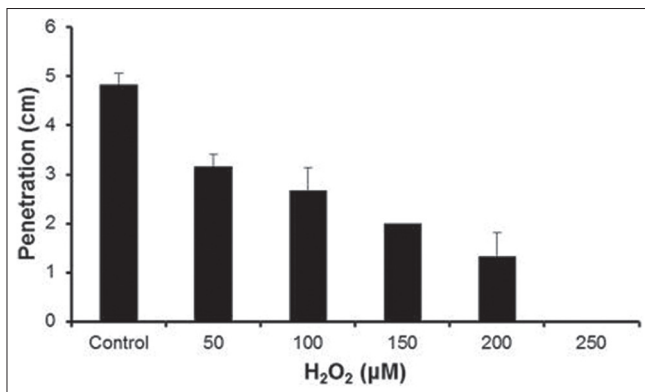


Figure 4: Sperm cervical mucus penetration assay. After treatment with hydrogen peroxide, sperm cells were put into a capillary glass tube filled with human cervical mucus. Distance reached by sperm cells was observed under a microscope. Significant changes were observed at all concentrations (*P* < 0.01). Error sign on top of each bar represents SEM. *n* = 3. SEM: Standard error of the mean

of H₂O₂ showed inhibition in the migration along the capillary filled with the mucus. Throughout increasing concentrations from control, 50, 100, 150, 200, and 250 μM of H₂O₂, the distance migration reached by sperm samples from each group were 4.83, 3.17, 2.67, 2.0, 1.33, and 0 cm, respectively. All these decreases were significantly different compared to the control group (*P* < 0.05) [Figure 4].

Sperm membrane integrity

Sperm membrane integrity is important for molecules transport and signal transduction in and out of the sperm cells to induce capacitation and also other processes required for the cell activation. Our data showed decrease in the sperm membrane integrity after treatment with various concentrations of H₂O₂. The percentage of sperm cells with curly tails, indicating intact membrane, went down from the control throughout concentrations of

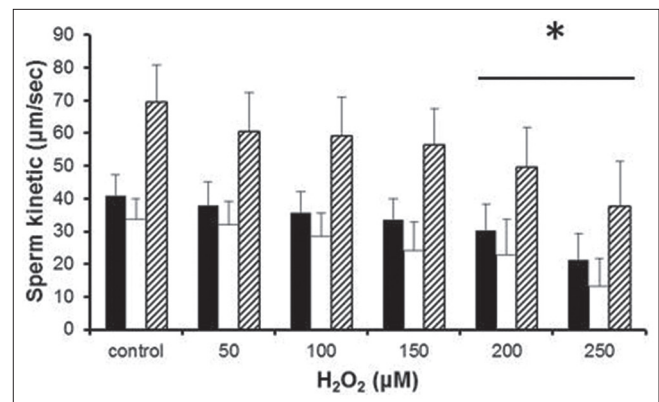


Figure 3: Analysis of sperm kinetics using computer-assisted sperm analyzer. Black bars represent average path velocity, white bars represent straight-line velocity, and diagonally-striped bars represent curvilinear velocity. Significant changes were observed at hydrogen peroxide concentration of 200 μM (*P* < 0.05) and 250 μM (*P* < 0.01). Error sign on top of each bar represents SEM. *n* = 3. *Significant. SEM: Standard error of the mean

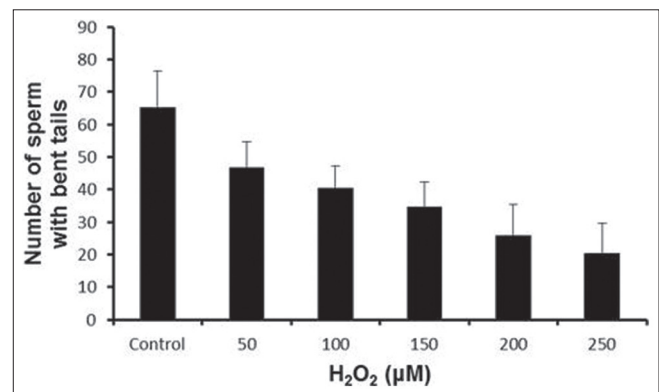


Figure 5: Analysis of hypo-osmotic swelling test to assess sperm membrane integrity after treatment with hydrogen peroxide. Sperm with swollen or bent tail, indicating intact membrane, were counted in at least three observation fields. All data represent mean from three independent experiments. All changes are significantly different (*P* < 0.01). Error sign represents SEM. *n* = 3. SEM: Standard error of the mean

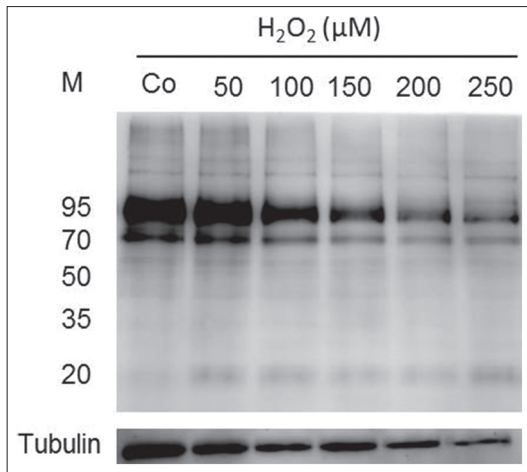


Figure 6: Western immunoblotting using antibody recognizing general phosphorylated tyrosine as an indicator of sperm capacitation after hydrogen peroxide treatment. The result shows reduced band intensity along with increasing concentration of hydrogen peroxide from 50 to 250 μM . The major bands detected in this analysis were AKAP4 (82 kDa) and AKAP3 (110 kDa). Membrane was stripped and re-probed with tubulin antibody as a loading control. The experiment was repeated at least three times with the same result. M = Protein marker, kDa

50, 100, 150, 200, and 250 μM with 65.17% (control), 46.92%, 40.59%, 34.93%, 25.95%, and 20.47%, respectively. All the changes were significant compared to the control ($P < 0.05$) [Figure 5].

Sperm tyrosine phosphorylation

One of the indicators of sperm capacitation is increase of global tyrosine phosphorylation in many proteins located in the sperm tail. Our western immunoblotting data showed that exposure of sperm cells with increasing concentration of H_2O_2 from 50 to 250 μM gradually decreased global tyrosine phosphorylation detected in the total sperm protein [Figure 6]. A significant decrease was observed starting at the concentration of 100 μM and continued to the highest concentration of 250 μM . The major bands detected in the western immunoblotting analyses were AKAP3 at 110 kDa and AKAP4 at 82 kDa.

Sperm caspase 3 levels

Caspase activation is one of the early indicators of programmed cell death. We measured the activation of caspase 3 protein in the human sperm after being treated with H_2O_2 using western immunoblotting. The data showed that H_2O_2 treatment activated caspase 3 indicated by band with a size of 32 kDa which increased in intensity along with increase in the H_2O_2 concentration [Figure 7a]. The upregulation of the caspase 3 was confirmed by measurement of the band intensity using ImageJ program (National Institute of Health, USA) in which relative band intensity against α -tubulin, as a loading control, increased steadily in line with the increase in H_2O_2 concentration from 50 to

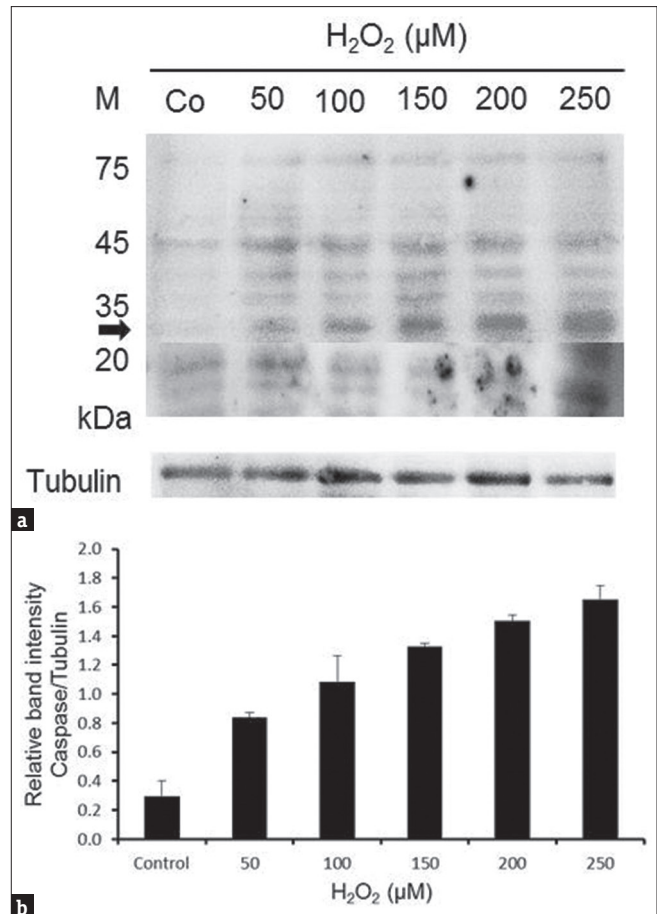


Figure 7: Effect of hydrogen peroxide on sperm caspase 3 activity analyzed using western immunoblotting. (a) Caspase 3, indicated by arrow at 32 kDa, shows increased band intensity as concentration of hydrogen peroxide increased. Membrane was stripped and re-probed with tubulin antibody as a loading control. (b) Caspase 3 band intensity was analyzed using ImageJ to confirm the increase in caspase 3 signal. All changes were significant ($P < 0.01$). Error sign on top of each bar represents SEM. $n = 3$. M = Protein marker, kDa. SEM: Standard error of the mean

250 μM [Figure 7b]. All the increase was statistically significant ($P < 0.05$).

AKT phosphorylation

The obvious sign of adverse effect of oxidative stress on cells is the inhibition of the survival protein AKT. The activation of caspase 3 after treatment of H_2O_2 was followed by decrease in AKT phosphorylation (Ser473). This study showed that H_2O_2 treatment at various concentrations from 50 to 250 μM reduced AKT activation indicated by decrease in band intensity of phosphorylated AKT (pAKT) at 50 kDa [Figure 8a]. This reduced phosphorylation was confirmed by densitometry analysis using ImageJ program which showed decrease in band intensity of pAKT at all treatment groups [Figure 8b]. All the changes were statistically significant ($P < 0.05$).

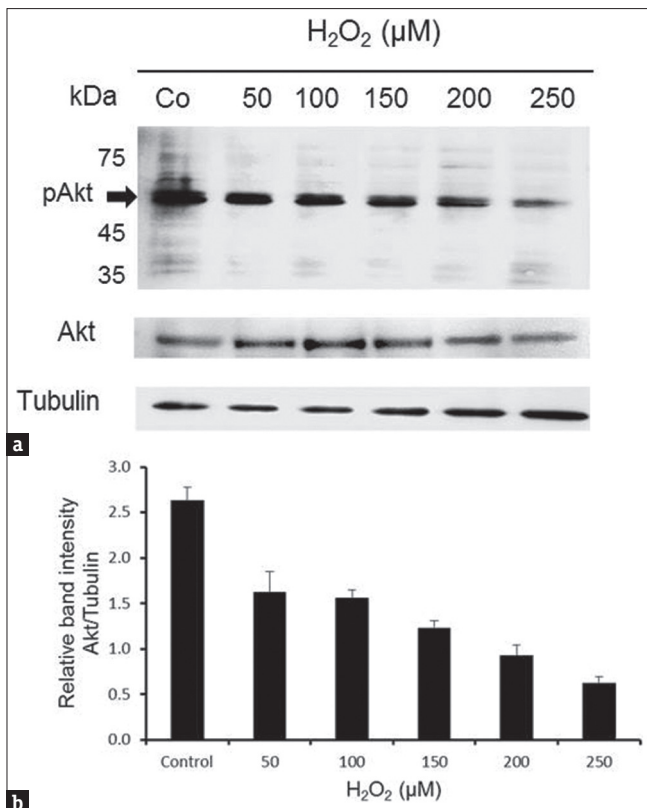


Figure 8: Detection of phosphorylated AKT in human sperm after hydrogen peroxide treatment. (a) Phosphorylated AKT, indicated by arrow at 50 kDa, was very strong in the untreated sample (control) and gradually decreased as hydrogen peroxide concentration increased. Membrane was stripped and re-probed with AKT and tubulin as a loading control. (b) Decrease in the phosphorylated AKT was confirmed by ImageJ analysis showing significant decrease in the intensity of phosphorylated AKT signal. Experiment was conducted at least 3 times with the same result. Error signs on top of each bar represent SEM. $n = 3$. SEM: Standard error of the mean

DISCUSSION

Oxidative stress on male germ cells has been suspected as one of the causes of male subfertility to infertility. Understanding the mechanism of oxidative stress in interfering the human sperm will help an andrologist in designing an effective therapy for subfertile or infertile males. There have been studies on the effect of H₂O₂ on sperm qualities both in human and animals such as motility,^[11] capacitation,^[8] sperm–oocytes interaction, and embryo development.^[12] This study provides comprehensive effects of oxidative stress in the form of H₂O₂ towards human sperm.

Oxidative stress is a condition that reflects an imbalance between ROS and antioxidant defenses. MDA is a marker of oxidative stress which is the result of a chain reaction of LPO. We could make sure that H₂O₂ treatments in this study produced dose-dependent increase in MDA levels. There was a previous study by Rhemrev *et al.* showed that progressive motile sperms

are protected from LPO.^[13] Our data did show that all the sperm kinetic parameters related to the progressive motility (VAP, VSL, and VCL) were inhibited by H₂O₂ treatments with significant different compared to control at concentration of 200 μM ($P < 0.05$) and 250 μM ($P < 0.01$).

The mechanism of H₂O₂ inhibition on sperm motility is the susceptibility of PUFA, which is abundant in the sperm membrane, causing a cascade of chemical reactions so-called LPO.^[14] This LPO produces lipid aldehydes that will attack mitochondrial proteins involved in the ATP production in the mitochondria, leading to loss of sperm motility and cell death. Furthermore, it was reported that the loss of sperm motility due to oxidative stress can also be consequence of reduced activity of protein that functions as ROS eliminator such as uncoupling protein 2 in the human sperm.^[15] This is confirmed by cervical mucus penetration assay in which H₂O₂-treated sperm cells were unable to penetrate cervical mucus as good as untreated control. The cervical mucus contains proteins such as catalase that are important for sperm survival that can bind to the sperm cell to eliminate oxidative attack.^[16]

LPO can also affect membrane structure and function such as fluidity, ion gradient, receptor function, and transport through membrane.^[17] Our data showed that H₂O₂ significantly reduced integrity of sperm membrane measured by HOST.^[18] This method had been shown to be useful to indicate functional integrity and fertility potential of the sperm. HOST is also used to select better sperm for intracytoplasmic sperm injection.^[19] H₂O₂ was reported to increase membrane phosphatidylserine externalization, although the change was not significant.^[20] This discrepancy may be due to different in the method used. Annexin V is better suit for apoptotic detection.

Disturbance in sperm membrane integrity affects signal transductions required for capacitation. Sperm capacitation requires intake of bicarbonates, calcium ion, and efflux of cholesterol.^[7] Disruption of the sperm membrane integrity by oxidative stress such as H₂O₂ will affect sperm membrane transport. Previous studies showed that intake of Ca²⁺ and HCO³⁻ will stimulate soluble adenylyl cyclase that converts ADP into cAMP, leading to activation of protein kinase A that drives global tyrosine phosphorylation, which is the hallmark of sperm capacitation.^[21] This global tyrosine phosphorylation activates proteins that are responsible for sperm hyperactivation^[22] and opening receptor for interaction with oocyte.^[23,24] Our data showed that incubation with increasing concentration of H₂O₂ decreased global tyrosine phosphorylation, which is in

agreement with previous studies. AKAP3 and AKAP4 are the most noticeable phosphorylated proteins during capacitation. These proteins are components of fibrous sheath in the axoneme of mammalian spermatozoa and responsible for sperm motility.^[25]

The current study also emphasized on the effect of H₂O₂ on the survival of the sperm cell. Human sperms have a mechanism of entering default pathway of apoptotic when it fails to fertilize the oocyte.^[9] Several molecules such as prolactin^[26] and insulin^[27,28] had been shown to have pro-survival effect on human sperm or other cells. It seems that there is a mechanism in the sperm to prevent premature apoptotic before reaching the oocytes. Our data clearly showed that H₂O₂ activates caspase 3 as an indicator of apoptosis. Interestingly, we observed that the activation of caspase 3 was followed by decrease in pAKT. This AKT is in the PI3K signaling pathway which is involved in sperm cell survival.^[26] The AKT protein when it is phosphorylated will liberate apoptosis inhibitory protein which is previously held in inactive state by bad protein. The role of AKT in the survival of sperm cell against oxidative stress was shown in previous studies in which treatment of sperm cell with melatonin^[29] and peroxiredoxin,^[30] both have antioxidant property, can improve sperm viability by maintaining AKT phosphorylation.

It is important to mention that our study used H₂O₂ from lower (10–100 µM) to higher concentration (150–250 µM) to observe in which level inhibition start and study its mechanism. Human sperms need low amount of ROS for normal functions such as motility, capacitation, and hyperactivation although at higher amount will be detrimental to sperm.^[31] Report also suggest that low concentrations of H₂O₂ (10 and 100 µM) activate defense system and motility of human sperm.^[32] Our study focused on by which mechanism H₂O₂ disrupt sperm function; therefore, higher concentrations were used. This study suggests examination of ROS levels as a screening for patients suffering from “unexplained infertility” to see whether ROS is a contributing factor. Besides, sperm cells undergoing preparation for assisted reproductive techniques such as differential gradient centrifugation also need to be screened for possible increase in ROS levels.

CONCLUSIONS

This study has given more comprehensive data on the effect of oxidative stress on human sperm qualities. H₂O₂ as one the most common ROS that cause oxidative stress on human sperm was used in this study. H₂O₂ decreased sperm viability, sperm kinetics, ability to penetrate cervical mucus, membrane integrity, and capacitation.

Furthermore, we provide evidence that H₂O₂ activated caspase 3 as an indicator of apoptotic stimulation and at the same time also inhibited phosphorylation of the AKT protein as pro-survival mediator. Thus, oxidative stress exposure to the human cell has adverse effects not only on human sperm parameters but also on sperm longevity while traversing along reproductive tract. In relation with etiology of male infertility, especially idiopathic or unexplained male infertility, this study should give caution on how to treat male subfertility or infertility.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient/donor consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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As above.

Conflicts of interest

There are no conflicts of interest.

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