Original Research Paper

Oligoasthenoteratozoospermia and Necrozoospermia: A Study of Sperm Na+, K+-ATPase α4 and Plasma Membrane Ca2+-ATPase 4 Regulation

[1]Silvia W. Lestari, [2]Dessy Noor Miati and [2]Asmarinah

[1]*Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
[2]*Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

Corresponding Author:
Silvia W. Lestari
Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
Email: finallysilvia@gmail.com

Abstract: The oligoasthenoteratozoospermia (OAT) and necrozoospermia are the extreme sperm abnormalities in male infertility. These sperm abnormalities may be related to the imbalance of ion transport which mediated by the ion-transporting P-type ATPases, such as Na^+, K^+-ATPase and Ca^{2+}-ATPase. Until now, there is limited data about the activity of Na^+, K^+-ATPase and Ca^{2+}-ATPase in sperms in the OAT and necrozoospermia samples. Therefore, this study investigated the activity of Na^+, K^+-ATPase and Ca^{2+}-ATPase and the expression of Na^+, K^+-ATPase α4 and PMCA4 isoforms in both sperm abnormalities. Eighteen semen samples from OAT and necrozoospermia infertile couples were examined in this study. Semen analysis was performed based on WHO 2010, while the enzyme activity was calculated by the released inorganic phosphate. The expression of Na^+, K^+-ATPase α4 and PMCA4 isoform was defined by Western immunoblotting, whereas the localization of the proteins was analyzed by immunocytochemistry. This study showed that the activity of Na^+, K^+-ATPase and the expression of Na^+, K^+-ATPase α4 isoforms in OAT and necrozoospermia group were lower than the normozoospermia group (1.452 ± 0.549 versus 0.559 ± 0.160 versus 1.962 ± 0.56 μmol Pi/mg protein/h, respectively; $p>0.05$). In addition, the activity of Ca^{2+}-ATPase and the expression of PMCA4 of OAT and necrozoospermia group were also lower compared to the normozoospermia group (2.028 ± 0.524 versus 0.928 ± 0.248 versus 2.657 ± 1.329 μmol Pi/mg protein/h, respectively; $p>0.05$). For both ATPases, the necrozoospermia group showed lower values compared to the OAT group. The disruption in ATPase and isoform expression in OAT and necrozoospermia may be responsible for sperm structure and functional damage.

Keywords: Oligoasthenoteratozoospermia, Necrozoospermia, Na^+, K^+-ATPase, Ca^{2+}-ATPase, K^+-ATPase α4, PMCA4

Introduction

Male factors are responsible for more than forty percent of the infertility cases (Gurfinkel *et al*., 2003). Male factors is determined by the existance of semen analysis disturbances such as oligozoospermia (low sperm concentration), asthenozoospermia (low sperm motility) and teratozoospermia (low normal sperm morphology). Besides that, there are also more severe sperm abnormalities such as oligoasthenoteratozoospermia (OAT) and necrozoospermia. OAT as disturbances of sperm concentration, motility and morphology, is a common cause of male factors infertility, but mostly due

to idiopathic issue (Zhu *et al*., 2016). On the other hand, necrozoospermia is rare. Nduwayo reported that necrozoospermia occurs only about 0.2 to 0.5% of the overall male infertility factor (Nduwayo *et al*., 1995; Tournaye *et al*., 1996). Necrozoospermia is defined as a condition where the percentage of live sperm is low and the percentage of sperm immotile is high in semen (WHO, 2010). OAT and necrozoospermia are related to a large variety of etiologies and may involve the disorder of an array of structure, biochemical and functional defects of sperm (Aitken *et al*., 1982).

Sperm motility needs an ion homeostasis which is performed by the ion transport system in the sperm

plasma membrane (Darszon *et al.*, 2006; Jimenez *et al.*, 2012). The ionic homeostatis is regulated by integral enzymes in the sperm plasma membrane, namely Na^+, K^+-ATPase and Ca^{2+}-ATPase (Vignini *et al.*, 2009). The Na^+, K^+-ATPase is an ion transport pump that transduces the energy from the hydrolysis of ATP to catalyze the exchange between intracellular Na+ and extracellular K+ in cells (Kaplan, 2002; Morth *et al.*, 2009). Inhibiting the Na^+, K+-ATPase could interrupt the active transport exchange between Na^+ and K+, which in turn would disturb cell homeostasis and decline sperm motility. In addition, there are some Na^+, K^+-ATPase isoforms, namely α1, α2, α3 and α4, in the mammalian somatic cells, but only the Na^+, K^+-ATPase α4 isoform is expressed in the mammalian testis (Blanco and Mercer, 1998; Blanco, 2005; Mobasheri *et al.*, 2000). The inhibition of Na^+, K^+-ATPase α4 isoform has been proved to decrease sperm motility (Sanchez *et al.*, 2006). While Na^+, K^+-ATPase works as an ion homeostasis regulator, the Ca^{2+}-ATPase retains calcium ion homeostasis (Takahashi and Yamaguchi, 1999). Calcium ion homeostasis plays role on sperm motility (Dragileva *et al.*, 1999). There are numerous Ca^{2+}-ATPase types, including Plasma Membrane Ca^{2+}-ATPase (PMCA), Sarcoplasmic-Endoplasmic Ca^{2+}-ATPase (SERCA) and Secretory Pathway Ca^{2+}-ATPase (SPCA). In addition, PMCA has some isoforms such as PMCA2 and PMCA3 isoforms that are regulated to promote somatic cells, while PMCA 4 isoform is the most common PMCA isoform in sperm. Isoform PMCA4 is present in the principal piece on the flagelum and plays a role in sperm motility, including sperm hyperactivity. Di Leva *et al.* (2008; Strehler *et al.*, 2007; Mata and Sepúlveda, 2005; Boczek *et al.*, 2014; Calamera, *et al.*, 2003; Schuh *et al.*, 2004).

The changes in protein structure and function caused by the decrease of Na^+, K^+-ATPase and Ca^{2+}-ATPase activities and Na^+, K+-ATPase α4 and PMCA4 isoform expression, would disrupt the sperm homeostasis. Performing the inhibition of Na^+, K^+-ATPase α4 and PMCA4 isoforms decreases sperm motility (Jimenez *et al.*, 2012; Woo *et al.*, 2000; Schuh *et al.*, 2004). Nevertheless, the role of Na^+, K^+-ATPase and Ca^{2+}-ATPase activities and the expression of the Na^+, K^+-ATPase α4 and PMCA4 isoforms in human sperm motility disorders still persist unidentified and need further analysis. Thus, this study investigated Na^+, K^+-ATPase and Ca^{2+}-ATPase activity and Na^+, K^+-ATPase α4 and PMCA4 isoform expression in OAT and necrozoospermic men.

Materials and Methods

Semen Collection and Analysis

After the approval for study protocol was obtained from Ethics Committee, Faculty of Medicine,

Universitas Indonesia, Jakarta, Indonesia, eighteen infertile men from Yasmin Infertility Clinic, Jakarta, Indonesia signed the informed consent and provided their semen samples. After 3-5 days of sexual abstinence, semen sample were obtained by masturbation and examined based on WHO reference value (WHO, 2010). Sperm concentration and motility were analyzed using Makler counting chamber, while sperm morphology was analyzed using Papaniculau staining.

Besides standard semen analysis, the sperm function tests such as eosin test for sperm vitality and Hypo-Osmotic Swelling (HOS) test for sperm membrane integrity were also performed. Oligoasthenoteratozoospermia was defined when the three sperm abnormalities are exist: oligozoospermia, asthenozoospermia and teratozoospermia (Cavallini, 2006; Singh *et al.*, 2012). The oligozoospermia was determined as sperm concentration <15 million/ml, whereas asthenozoospermia was determined as progressive motility ≤32% (grade a) or ≤40% (grade a + b) and teratozoospermia was determined as normal sperm morpology <4% (WHO, 2010). Furthermore, necrozoospermia was defined as a condition in which the spermatozoa in seminal fluid are dead or motionless, approximately more than 60% (Merriam-Webster, Medical Dictionary, 2016; Fang and Baker, 2003). The necrozoospermia samples used in this study were 100% motionless or immotile.

Collection of Sperm Membrane Fraction

The modification of Olson and Vignini methods were performed to isolate the sperm plasma membrane and collect membrane fraction (Olson *et al.*, 1985; Vignini *et al.*, 2009). The Olson A solution was prepared in 9 mL of volume and was mixed with membrane fraction and then centrifuged at $1000 \times g$ for 10 min at 4°C. The Olson B solution was prepared in 5 mL of volume and was mixed with the pellet and then centrifuged at $1000 \times g$ for 10 min at 4°C. The Ultra turrax homogenizer was used to homogenize the supernatant, then the resultant was layered over sucrose gradient solution, which composed of 15% sucrose with 25 mmol/l Tris-HCl (pH 7.4) and 50% sucrose with 25 mmol/l Tris-HCl (pH 7.4). After that, the solution was centrifuged again at $17.000 \times g$ for 30 min. The supernatant was removed and the Olson B solution was prepared again to be mixed with the pellet and then centrifuged again at $17.000 \times g$ for 40 min. The supernatant was removed and the pellet was analyzed further.

The Activity of Na^+, K^+-ATPase

The Kitao and Hattori (1983) and Vignini *et al.* (2009) methods were performed to analyze the activity of Na^+, K^+-ATPase. A medium consisting of 5 mmol/l $MgCl_2$, 140 mmol/l NaCl, 14 mmol/l KCl in 40 mmol/l

Tris-HCl, pH 7.7 was prepared to incubate the sperm membrane fraction. Then, 3 mmol/l Na_2ATP was added to start the ATPase reaction and one ml of 15% trichloroacetic acid was supplemented to culminate the ATPase reaction at 20 min later. After that, the centrifugation at $1100 \times$ g for 10 min was performed to centrifuge the reaction and inorganic phosphate (Pi) was determined colorimetrically (Fiske and Subbarow, 1925). The difference in released Pi in the presence or absence of 10 mM ouabain was defined as the Na^+, K^+-ATPase activity. Ouabain is a cardiac glycoside that acts by inhibiting Na^+, K^+-ATPase, which resulting in an increase of intracellular sodium (Dostanic-Larson *et al.*, 2005).

The Activity of Ca^{2+}-ATPase

The analysis of the activity of Ca^{2+}-ATPase were performed based on previous studies (Davis *et al.*, 1989; Vivenes *et al.*, 2009). A medium consisting of 3 mmol/l $MgCl_2$, 80 mmol/l NaCl, 15 mmol/l KCl and 0.1 mmol/l EGTA in 50 mmol/l Tris-HCl, pH 7.4 was prepared to incubate the sperm membrane fraction. Then, the 1 mmol/l Na_2ATP was added to generate the ATPase reaction and one ml of 15% trichloroacetic acid was supplemented to culminate the ATPase reaction at 20 min later. After that, the centrifugation at 1100 xg for 10 min was implemented to centrifuge the reaction and the colorimetric was demonstrated to measure the unconstrained inorganic phosphate (Pi) (Fiske and Subbarow, 1925). The Pi discrepancy in with and without 55 μm $CaCl_2$ was defined as the Ca^{2+}-ATPase activity.

The Expression of Na^+, K^+-ATPase α4 and PMCA4 Isoform

An incubation medium consisting 10% Sodium Dodecyl Sulfate (SDS), 10% sucrose and 0.275 M Tris-HCl (pH 6.8), appended with protease inhibitor cocktail (Roche, Manneheim, Germany) was set at 100°C for 5 min to isolate the sperm protein. The sperm proteins were fractioned by SDS-polyacrylamide gel and PVDF membrane (Amersham, California, USA) was used to moved the protein from the gel (Sanchez *et al.*, 2006; Takahashi and Yamaguchi, 1999). Then, the 3% bovine serum albumin in an incubation medium (10 mM Tris/HCl 7.6, 150 mM NaCl, 0.05% Tween 20) or TBST medium was set at room temperature for one hour to block the membrane. Subsequently, the Na^+, K^+-ATPase α4 (N-19) or PMCA4 (F-20) goat polyclonal antibody (Santa Cruz, California, USA) at a 1:200 dilution was prepared at 4°C overnight for incubation. After that, the horse radish peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz, California, USA) at 1:5000 dilution was prepared at room temperature for one hour for incubation. The Amersham ECL Plus Western blotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK) was demonstrated to detect the antigen-antibody complex, while the Luminescent Image Analyzer (Imagequant™ LAS 4000, GE Healthcare Life Sciences, Uppsala, Sweden) was used to detect the antigen-antibody reaction. Lastly, the ImageJ (NIH) software was used to analyze the Na^+, K^+-ATPase α4 and PMCA4 bands.

The Immunocytochemistry of the Na^+, K^+-ATPase α4 and PMCA4 Isoform

Before the immunofluorescent labelling, the centrifugation at $2407 \times$ g for 10 min was performed to wash the sperm cells. The Biggers, Whitten and Whittingham (BWW) was prepared to suspend the pellet after the supernatant was removed. Then, another centrifugation at $2407 \times$ g for 5 min was performed. The 0.2% Triton X-100 was prepared for permeabilization, while the poly-L-lysine-coated slides and 4% formaldehyde were prepared for fixation of sperm. Primary antibody (Na^+, K^+-ATPase α4 or PMCA4 goat polyclonal antibody) (Santa Cruz, California, USA) was set at 1:200 dilution at 4°C overnight for incubation. Second antibody (donkey anti-goat Ig G antibody) was allied to fluorescein isothiocyanate (FITC) (Santa Cruz, California, USA) to determine the reaction. The Na^+, K^+-ATPase α4 and PMCA4 signals obtained were observed microscopically with confocal microscopy (Zeiss, Vanadia).

Statistical Analysis

The activity of Na^+, K^+-ATPase and Ca^{2+}-ATPase and the expression of Na^+, K^+-ATPase α4 and PMCA4 were compared in the control normozoospermia and oligoasthenoteratozoospermia or necroozoospermia groups using the Mann-Whitney test. The 22nd version of Statistical Package for Social Science (SPSS) with $p<0.05$ was used to analyze the data of this study.

Results

The semen profiles used in this study of OAT, necrozoospermia and normozoospermia control group are presented in (Table 1). In the present study, both the OAT and necrozoospermia group showed lower values of all semen parameters i.e., sperm concentration, motility, morphology, viability and membrane integrity compared to the normozoospermia group, whereas the necrozoospermia group showed lower values than OAT group (Table 1). This study confirmed Kumalic and Pinter (2014) that reported the same lower parameters upon comparing the semen analysis of an infertile group with a normozoospermia group.

Furthermore, in the motility parameter, the necrozoospermia group showed zero progressive motility or 100% immotile values compared to OAT and normozoospermia. In addition, in the viability parameter, the necrozoospermia group showed the lowest of viability values compared to OAT and normozoospermia. In the

viability assay, the intact cytoplasmic membrane of live sperm is the basis, which sperm refrain the absorption of eosin dye. Moskovtsev and Librach (2013) this study confirmed the findings of Samplaski that necrozoospermia have lower viability due to low sperm membrane integrity value (Samplaski *et al.*, 2015). Lastly, the results of this study are concordant with the reports of Lodhi *et al.* (2008) that membrane integrity and sperm motility, morphology, viability and are related.

Although the other etiologies such as infections, toxic substances and seminal plasma has been reported elsewhere, this study is the first study documented the role of Na$^+$, K$^+$-ATPase, Ca^{2+}-ATPase and its isoforms as etiologies of OAT and necrozoospermia (Nduwayo *et al.*, 1995; Coutinho *et al.*, 1984; El-Gothamy and El-Samahy, 1992; Lerda and Rizzi, 1991; Wilton *et al.*, 1988).

The Activity of Na$^+$, K$^+$-ATPase

As demonstrated in Table 2, Na$^+$, K$^+$-ATPase activity in human sperm was decreased in OAT and necrozoospermia, compared to normozoospermia group. In response to ouabain, the Na$^+$, K$^+$-ATPase activity was 1.452±0.549 μmol Pi/mg protein/h in OAT group and 0.559±0.160 μmol Pi/mg protein/h in necrozoospermia group, whereas it was 1.962±0.56 μmol Pi/mg protein/h in the normozoospermia group (p>0.05; Table 2). The activity of Na$^+$, K$^+$-ATPase in necrozoospermia showed lower level than OAT group.

The OAT and necrozoospermia group contains of more abnormal or pathologic sperms, compared to normozoospermia group. In the OAT, the lower sperm count, reduced sperm motility and abnormal morphology are factors that conscientious for the increased of oxidative stress in sperms. Said *et al.* (2005; Kao *et al.*, 2008; Pasqualotto *et al.*, 2008) the abnormal sperm morphology was acknowledged as a cause of ROS

generation, whereas the low sperm count and motility have been conveyed as a cradle of ROS (Dama *et al.*, 2012). The ROS may change the fatty acid composition of membranes, manipulate sperm membrane function and even impair the sperm membrane. Vignini *et al.* (2009) Similar to the OAT, the necrozoospermia group also showed depicts plasma membrane, acrosome loss and damage to microtubule and mitochondrial membrane of the sperms. Talebi *et al.* (2007) the abnormal/pathological conditions may intrude the regulation of membrane permeability, occasioning in disturbed ion pump activity in the sperm cell membrane (Hamamah *et al.*, 1999). In addition, necrozoospermia sperms also showed other pathological process such as the disturbance of membrane integrity which results in uncoordinated release of active enzymes (Brahem *et al.*, 2012) Since the activity of Na$^+$, K$^+$-ATPase in necrozoospermia showed lower level than OAT group, it was assumed that sperms in necrozoospermia group have more pathologic sperms compared to the sperms in OAT group.

The Activity of Ca^{2+}-ATPase

This study showed a similar trend as the activity of Na$^+$, K$^+$-ATPase that the activity of Ca^{2+}-ATPase was lower in OAT and necrozoospermia, compared with the normozoospermia group. The Ca^{2+}-ATPase activity was detected at 2.028±0.524 μmol Pi/mg protein/h in OAT group and necrozoospermia group at 0.928±0.248 μmol Pi/mg protein/h, whereas in normozoospermia group at 2.657±1.329 μmol Pi/mg protein/h (p>0.05; Table 2).

As explained in the above, the OAT and necrozoospermia groups could have pathologic sperm cells, with the assumption that sperms in necrozoospermia group, as the more severe sperm motility disorder, may have more pathologic sperms compared to the sperms in OAT group.

Table 1: Mean of semen analysis in normozoospermia, OAT and necrozoospermia group

Parameter	Normo (n = 14)	OAT (n = 10)	Necro (n = 5)
Volume (mL)	3,4	2,9	2
pH	7,2	7,3	7,3
Colour	White	White	White
Concentration (million.mL^{-1})	52.40±8.81	2.91±1.43	0.12±0.02
Motility (%)	56.07±5.16	18.50±4.87	0
Morfology (%)	21.86±1.52	2.60±0.16	5.0±2.24
Viability (%)	90.79±1.49	56.90±6.64	10.00±4.47
Membrane Integrity (%)	51.71±3.91	36.50±2.78	20.00±8.37

Values are determined as the mean ± SEM; n:number of samples; normo:normozoospermia; OAT:oligoasthenoteratozoospermia; necro:necrozoospermia

Table 2: Activity of Na$^+$, K$^+$-ATPase and Ca^{2+}-ATPase in the normozoospermia, oligoasthenoteratozoospermia and necrozoospermia groups

ATPase activity (μM Pi/mg protein/h)	Normo (n = 14)	OAT (n = 10)	Necro (n = 5)	P
Na$^+$, K$^+$-ATPase	1.962±0.56	1.452±0.549	0.559±0.160	0.379
Ca^{2+}-ATPase	2.657±1.329	2.028±0.524	0.928±0.248	0.457

Values are determined as the mean ± SEM; p: significance level; normo: normozoospermia; necro: necrozoospermia; OAT: Oligoasthenoteratozoospermia

In the pathologic condition, the intracellular calcium concentration may elevate and converse the activity of Ca^{2+}-ATPase. In contrast to our previous study, which proved that the activity of Ca^{2+}-ATPase in asthenozoospermia group was showed higher compared to normozoospermia group, this study demonstrated different trend of Ca^{2+}-ATPase activity which was lower in OAT and necrozoospermia group compared to normozoospermia group.

In the pathologic condition, sperms in the OAT and necrozoospermia group may undergo cell membrane damage that induce calcium deposit, as an outcome of diminished energy metabolism (Trump *et al.*, 1980; Sakaida *et al.*, 1991). It was proved in our previous study that sperms in asthenozoospermia group underwent pathological calcium ion accumulation elevation, therefore Ca^{2+}-ATPase performed cellular defense by extracting calcium ions to extracellular more, to maintain the homeostasis of calcium ions. On the contrary, the sperms in OAT and necrozoospermia group in this study demonstrated lower Ca^{2+}-ATPase activity compared to normozoospermia group. Related to this issue, the authors proposed two assumptions: (1) in the more severe condition such as OAT and necrozoospermia as more severe sperm motility disorders than asthenozoospermia, the sperms are not more struggle to release calcium ion extracelullarly to sustain ion homeostasis and (2) in the OAT and necrozoospermia, sperms do not undergo calcium accumulation, hence do

not have to work harder to release calcium ion extracelullarly. There are still limited data about sperms in the OAT and necrozoospermia, specially the precise mechanism in its pathophysiology.
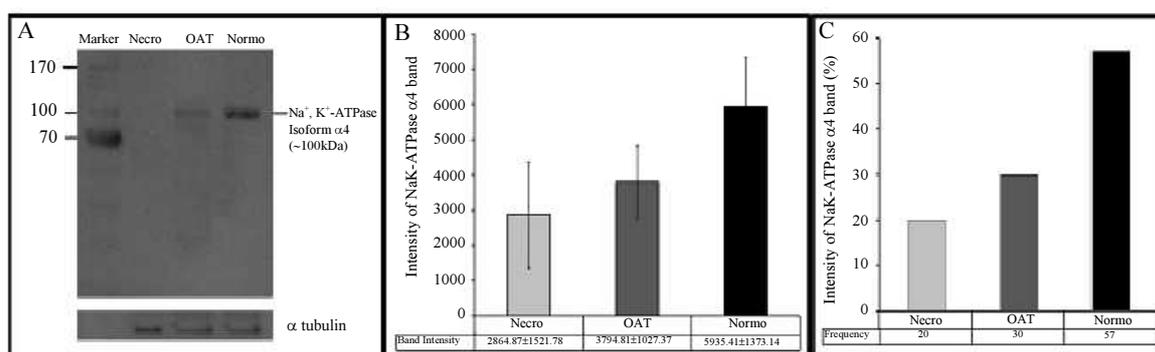
Expression of Na^+, K^+-ATPase α4 and Plasma Membrane Ca^{2+}-ATPase 4 (PMCA4) isoforms

The expression of Na^+, K^+-ATPase α4 isoform and PMCA4 protein in sperm of OAT and necrozoospermia group compared to the normozoospermia group was defined by performing Western blot analysis. The Na^+, K^+-ATPase α4 isoform was detected at a size of ~100 kDa, as shown in Fig. 1A. The band intensity was 3794.81±1027.37 in the OAT and 2864.87±1521.78 in the necrozoospermia group, whereas the band intensity was 5935.41±1373.41 in the normozoospermia group (Fig. 1B and Table 3). Therefore, the expression of the Na^+, K^+-ATPase α4 isoform was lower in the OAT and necrozoospermia (Figure 1B and Table 3) and seemed less frequently (Fig. 1C) than in the normozospermia group. These results are in line with the activity of the Na^+, K^+-ATPase α4 isoform of the sperms that were inhibited by ouabain and lead to declined sperm motility. Hence, this study confirmed Sanchez *et al.* (2006) that proved Na^+, K^+-ATPase α4 isoform has dissimilar modules and pieces a principal role in sperm motility. The Na^+, K^+-ATPase α4 isoform is controlled by the ion gradient, which is essential for ion homeostasis in sperm motility.

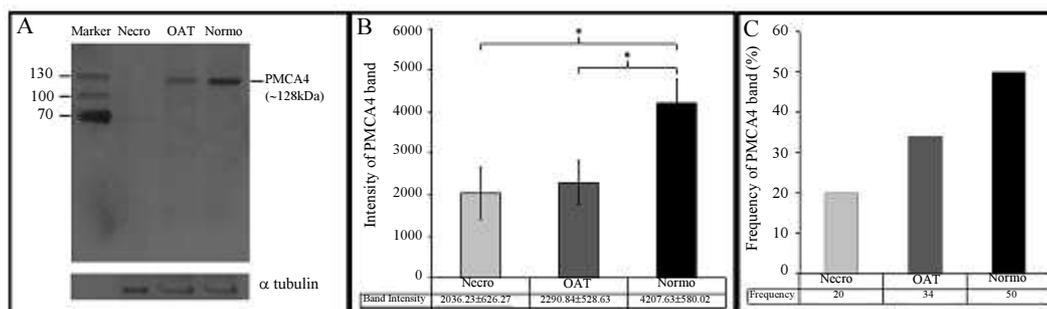**Table 3:** The intensity of Na^+, K^+-ATPase α4 and PMCA4 isoform band of necrozoospermia, oligoasthenoteratozoospermia and normozoospermia group

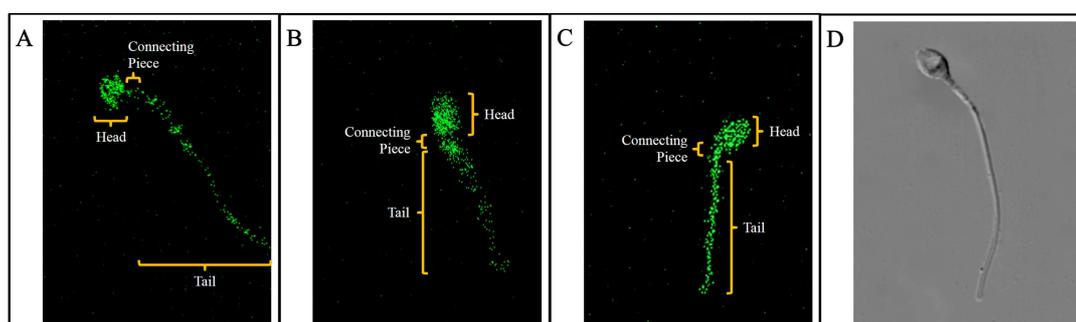| Band intensity (image J)	Normo	OAT	Necro	p
Na^+, K^+-ATPase isoform α4	5935.41±1373.41	3794.81±1027.37	2864.87±1521.78	0.071
PMCA4	4207.63±580.02	2290.84±528.63	2036.23±626.27	0.041*

Note: Value are determined as mean ± SEM; p = significance level; normo:normozoospermia; necro:necrozoospermia; OAT: Oligoasthenoteratozoospermia; p<0.05, (*) significant

Fig. 1: The expression of Na^+, K^+-ATPase α4 isoform of necrozoospermia, oligoasthenoteratozoospermia and normozoospermia group. (A) Western blot analysis showed band at ~100 kDa. (B) Band intensity of Na^+, K^+-ATPase α4 isoform protein in necrozoospermia, OAT and normozoospermia group (mean ± SEM). (C) The Na^+, K^+-ATPase α4 band performed more frequently in the normozoospermia than in the OAT and necrozoospermia group

Fig. 2: The expression of PMCA4 isoform in the necrozoospermia, oligoasthenoteratozoospermia and normozoospermia group. (A) Western blot analysis showed band at ~128 kDa. (B) Band intensity of PMCA4 isoform in necrozoospermia, OAT and normozoospermia groups (mean ± SEM). (C) The PMCA4 band performed more frequently in the normozoospermia than in the OAT and necrozoospermia groups

Fig. 3: Immunocytochemistry analysis showed the localization of Na^+, K^+-ATPase α4 isoform in sperm. (A) Localization of Na^+, K^+-ATPase α4 isoform was less compacted in the head and but almost not detected in the tail of sperm in necrozoospermia sample. (B) Localization of Na^+, K^+-ATPase α4 isoform was compact in the head and but less detected in the tail of sperm in OAT sample. (C) Na^+, K^+-ATPase α4 isoform distribution was compacted in head and tail of sperm in normozoospermia sample. (D) Sperm (without incubation) under the phase contrast was used as a negative control. The images were observed at 1000x magnification
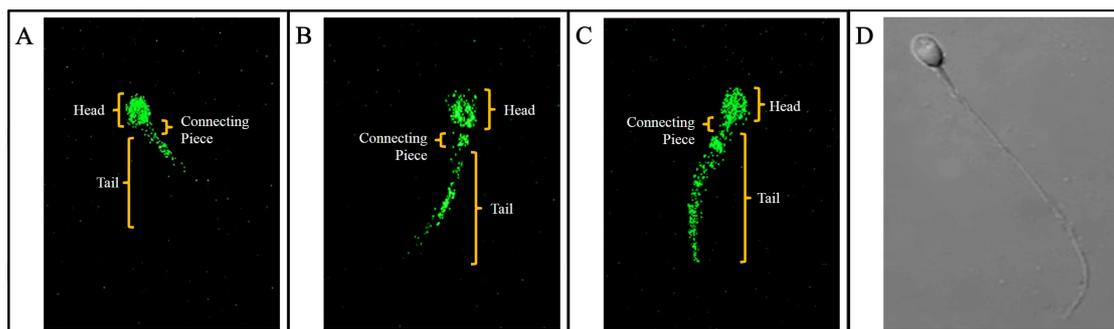
Furthermore, the expression of PMCA4 as the more predominant isoform in sperm compared to PMCA1, was detected at size of ~128 kDa, as shown in Fig. 2A. The band intensity of PMCA4 isoform was 2290.84±528.63 in the OAT and 2036.23±626.27 in the necroozoospermia, whereas it was 4207.63±580.02 in the normozoospermia group (Fig. 2B and Table 3). The PMCA4 isoform band also seemed less frequenty in the OAT and necrozoospermia than normozoospermia (Fig. 2C). These results were in accordance with the Ca^{2+}-ATPase activity which showed lower level in OAT and necrozoospermia compared to normozoospermia group. Different from our previous study in asthenozoospermia group, this study demonstrated that the expression of PMCA4 isoform was in line with the Ca^{2+}-ATPase activity in the OAT and necrozoospermia group.

Distribution of Na^+, K^+-ATPase α4 and Plasma Membrane Ca^{2+}-ATPase 4 (PMCA4) Isoform

The distribution or localization of Na^+, K^+-ATPase α4 isoform and PMCA4 isoform in sperm of OAT and necrozoospermia group compared to the normozoospermia group was defined by performing immunocytochemistry analysis. This study showed the localization of Na^+, K^+-ATPase α4 isoform in necrozoospermia samples less compacted in the head and almost undetectable in the tail of sperm (Fig. 3A), whereas the localization of Na^+, K^+-ATPase α4 isoform in OAT samples was compact in the head and but less detected in the tail of sperm (Fig. 3B). In contrast, the localization of Na^+, K^+-ATPase α4 isoform in normozoospermia samples was compacted in the head and tail of sperm (Fig. 3C). As a negative control, unincubated sperm under phase contrast microscopy was used (Fig. 3D).

Similar to Na^+, K^+-ATPase α4 isoform, the localization of PMCA4 isoform was compacted in the head and almost not detected in the tail of sperm in the necrozoospermia sample (Fig. 4A), whereas the localization of PMCA4 isoform was less compacted in the head and was almost undetectable in the tail of sperm in the OAT sample (Fig. 4B), compared to normozoospermia sample which was located compact in the head and tail of sperm (Fig. 4C). A negative control of unincubated sperm shown in Fig. 4D.

Fig. 4: Immunocytochemistry analysis showed the localization of PMCA4 isoform in sperm. (A) Localization of PMCA4 isoform was compacted in the head and but almost not detected in the tail of sperm in the necrozoospermia sample. (B) Localization of PMCA4 isoform was less compacted in the head and but almost not detected in the tail of sperm in the OAT sample (C) PMCA4 isoform distribution was compact in the head and tail of sperm in normozoospermia sample. (D) Sperm (without incubation) under the phase contrast was used as a negative control. The images were observed at 1000x magnification

This study showed that the Ca^{2+}-ATPase activity was in accordance with the PMCA4 isoform expression and localization in the sperm. Several studies are in line with the results of this study such as Calamera *et al.* (2003) and Prasad *et al.* (2004) that PMCA4 as the most common PMCA isoform in sperm cells is connected to the sperm motility and its dysfunction may cause the infertility in men. In addition, further studies are required to clarify PMCA4 expression as one of the Ca^{2+}-ATPase isoforms in sperm.

Discussion

This study confirmed Khosrowbeygi's that infertile group showed the lower value of semen analysis parameter compared to normozoospermia group (Khosrowbeygi *et al.*, 2004).

In the viability assay, the intact cytoplasmic membrane of live sperm is the basic, which sperm refrain the absorption of eosin dye (Moskovtsev and Librach, 2013). This study confirmed that necrozoospermia has lower viability due to low sperm membrane integrity value (Samplaski *et al.*, 2015). In additon, the results of this study are concordant with the reports that sperm motility, morphology and viability are positively correlated with membrane integrity (Lodhi *et al.*, 2008). At last, the impairment of sperm motility and morphology which caused by the alteration of membrane integrity, may lead to the death of sperm cell (Agarwal and Allamaneni, 2011).

Although infections, toxic substances and seminal plasma have been reported as being causative for OAT, this is the first study documenting the role of Na^+, K^+-ATPase, Ca^{2+}-ATPase and the expression of Na^+, K^+-ATPase α4 and PMCA4 isoforms (Nduwayo *et al.*, 1995; Coutinho *et al.*, 1984; El-Gothamy and El-Samahy, 1992; Lerda and Rizzi, 1991; Wilton *et al.*, 1988). There are still limited data about the precise mechanism of the sperm pathology in the OAT and necrozoospermia.

The Activity of Na^+, K^+-ATPase

Individuals suffering from OAT and necrozoospermia as more severe sperm abnormalities could have more abnormal or pathologic sperm, compared to other minor sperm abnormalities such as asthenozoospermia group. Other studies speculated that the higher level of necrozoospermia indicates a pathological mechanism for sperm cell death (Talebi *et al.*, 2007; Brackett *et al.*, 1998). In the OAT, the lower sperm count, reduced sperm motility and abnormal morphology are factors that responsible for the increased of oxidative stress in sperm (Said *et al.*, 2005; Kao *et al.*, 2008; Pasqualotto *et al.*, 2008). Oxidative stress occurs when there is an excess of ROS (Agarwal and Allamaneni, 2011). The abnormal sperm morphology was acknowledged as a cause of ROS generation, whereas the low sperm count and motility have been also conveyed as a cradle of ROS (Dama *et al.*, 2012). The ROS may change the fatty acid composition of sperm membranes, manipulate sperm membrane function and even impair the sperm membrane (Vignini *et al.*, 2009). Along these lines, the OAT syndrome has usually been related to chromosomal aneuploidy and mitochondrial dysfunction (Agarwal and Allamaneni, 2011). Similar to the OAT, the necrozoospermia group also showed damage plasma membrane, acrosome loss and damage to microtubule and mitochondrial membrane of the sperm (Talebi *et al.*, 2007). The abnormal/pathological conditions may intrude the regulation of sperm membrane permeability, resulting in disturbed ion pump activity in the sperm cell membrane (Hamamah *et al.*, 1999). In addition, necrozoospermia also demonstrated the loss of membrane integrity as a pathological process because it results in unprogrammed release of active enzymes (Brahem *et al.*, 2012). Since the activity of Na^+, K^+-ATPase in necrozoospermia showed lower level than OAT group, the authors assumed that sperm in necrozoospermia group have more pathologic sperm

compared to the sperm in OAT group. The result of this study was in accordance with the findings of Jimenez *et al.* (2011) which proved that Na^+, K^+-ATPase regulates Na+ and K+ ion homeostasis and sperm motility. This study also confirmed the data of Koçak-Toker *et al.* (2002) that the inhibition of Na^+, K^+-ATPase α4 isoform by ouabain reduced the sperm motility in mice.

The Activity of Ca^{2+}-ATPase

As explained above, the OAT and necrozoospermia groups could have pathologic sperm cells, with the assumption that sperm in necrozoospermia group, as the more severe sperm motility disorder, may have more pathologic sperms compared to the sperms in OAT group. In the pathologic condition, sperm in the OAT and necrozoospermia group may undergo cell membrane damage that induces calcium deposit, as an outcome of diminished energy metabolism and converse the activity of Ca^{2+}-ATPase (Trump *et al.*, 1980; Sakaida *et al.*, 1991). In addition, Schuh *et al.* (2004) confirmed that the increase of intracellular calcium concentration declined the sperm motility. It was proved in our previous study that sperm in asthenozoospermia group underwent pathological calcium ion accumulation elevation, hence Ca^{2+}-ATPase performed a cellular defense function by working hard to release calcium ions extracellularly to maintain calcium ion homeostasis (Lestari *et al.*, 2017).

Surprisingly, the sperm in OAT and necrozoospermia group in this study demonstrated different trend compared to asthenozoospermia group, which showed lower Ca^{2+}-ATPase activity compared to normozoospermia group. Related to this issue, the authors proposed two assumptions: (1) in the more severe condition such as OAT and necrozoospermia as more severe sperm motility disorders than asthenozoospermia, the sperms are not struggle anymore to release calcium ion extracelullarly to sustain ion homeostasis and (2) in the OAT and necrozoospermia, sperms do not undergo calcium accumulation, hence do not have to work harder to release calcium ion extracelullarly. The change in sperm structure and function will interrupt the ion homeostasis, which lead to the impaired of sperm motility and lastly, infertility (Lestari *et al.*, 2017).

Expression of Na^+, K^+-ATPase α4 and Plasma Membrane Ca^{2+}-ATPase 4 (PMCA4) Isoforms

These results are in line with the activity of the Na+, K+-ATPase α4 isoform of the sperm that were inhibited by ouabain and lead to declined sperm motility. Hence, this study confirmed that proved Na^+, K^+-ATPase α4 isoform has dissimilar elements and plays a major role in sperm motility (Sanchez *et al.*, 2006). The Na^+, K^+-ATPase α4 isoform is controlled by the ion gradient, which is essential for ion homeostasis in sperm motility. In addition, other studies stated that the expression of Na^+, K^+-ATPase α4 was due to the change of membrane

potential, whether depolarized or hyperpolarized (Mobasheri *et al.*, 2000; Jimenez *et al.*, 2011).

These results were in accordance with the Ca^{2+}-ATPase activity which showed lower level in OAT and necrozoospermia compared to normozoospermia group. Different from our previous study in asthenozoospermia group, this study demonstrated that the expression of PMCA4 isoform was in line with the Ca^{2+}-ATPase activity in the OAT and necrozoospermia group (Lestari *et al.*, 2017). The result of this study confirmed that there was alteration in PMCA expression in the pathologic sperm of asthenozoospermia, as a disorder or sperm motility (Boczek *et al.*, 2014).

Distribution of Na^+, K^+-ATPase α4 and Plasma Membrane Ca^{2+}-ATPase 4 (PMCA4) isoform

This study confirmed other studies that stated the Na^+, K^+-ATPase α4 isoform is expressed in the proximal portion of the sperm flagellum, which relate to the midpiece of the sperm's tail (Mobasheri *et al.*, 2000; Sanchez *et al.*, 2006). The loss expression phenomenon may be an indication of a sperm motility disorder.

This study showed that the Ca^{2+}-ATPase activity was in accordance with the PMCA4 isoform expression and localization in the sperm. Several studies are in line with the results of other studies stated that PMCA4 as the most common PMCA isoform in sperm cells is connected to the sperm motility and its dysfunction may cause the infertility in men (Calamera *et al.*, 2003; Prasad *et al.*, 2004). In addition, further studies are needed to clarify PMCA4 expression as one of the Ca^{2+}-ATPase isoforms in sperm.

Conclusion

In conclusion, the damaged sperm structure and function may alter the Na^+, K^+-ATPase and Ca^{2+}-ATPase activities and the expression of Na^+, K^+-ATPase α4 and PMCA4 isoforms. Surely, further studies are required to reveal the other cellular mechanism that control the sperm motility.

Acknowledgement

Authors' Contribution

Silvia W. Lestari: Experimental design, performance of the semen analysis experiment, data collection, statistical analysis of data and supervisory role of the project.

Dessy Noor Miati: Experimental design and performance of the Na^+, K^+-ATPase and Ca^{2+}-ATPase activity experiments and manuscript review.

Asmarinah: Experimental design and performance of the Na$^+$, K$^+$-ATPase α4 isoform and PMCA4 experiments.

Ethics

This research article is original and contains unpublished material. The corresponding author confirms that all of the authors have read and approved the manuscript and no ethical issues involved.

References

Agarwal, A. and S.S. Allamaneni, 2011. Free radicals and male reproduction. J. Indian Med. Assoc., 109: 184-7.

Aitken, R.J., F.S. Best, D.W. Richardson, O. Djahanbakhch and A. Templeton *et al.*, 1982. An analysis of semen quality and sperm function in cases of oligozoospermia. Fertil Steril, 38: 705-11.

Blanco, G. and R.W. Mercer, 1998. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. Am. J. Physiol., 275: F633-50.

Blanco, G., 2005. Na, K-ATPase subunit heterogeneity as a mechanism for tissue-specific ion regulation. Semin Nephrol., 25: 292-303.

Boczek, T., M. Lisek, B. Ferenc, A. Kowalski and D. Stepinski *et al.*, 2014. Plasma membrane Ca^{2+}-ATPase isoforms composition regulates cellular pH homeostasis in differentiating PC12 cells in a manner dependent on cytosolic Ca^{2+} elevations. PLoS one, 9: e102352.

Brackett, N.L., W.E. Bloch and C.M. Lynne, 1998. Predictors of necrospermia in men with spinal cord injury. J. Urol. 159: 844-7.

Brahem, S., S. Jellad, S. Ibala, A. Saad and M. Mehdi, 2012. DNA fragmentation status in patients with necrozoospermia. Syst. Biol. Reprod. Med., 58: 319-23.

Calamera, J., M. Buffone, M. Ollero, J. Alvarez and G.F. Doncel, 2003. Superoxide dismutase content and fatty acid composition in subsets of human spermatozoa from normozoospermic, asthenozoospermic and polyzoospermic semen samples. Mol. Reprod. Dev., 66: 422-30.

Cavallini, G., 2006. Male idiopathic oligoasthenoteratozoospermia. Asian J. Androl., 8: 143-57.

Coutinho, E.M., J.F. Melo, I. Barbosa and S.J. Segal, 1984. Antispermatogenic action of gossypol in men. Fertil Steril, 42: 424-30.

Dama, M., A.M. Kaleem, A.A. Mahdi, S.S. Narayana and S.P. Jaiswar *et al.*, 2012. A case of oligoasthenoteratozoospermia with AZFc deletion and persistent oxidative stress. Indian J. Hum Genet., 18: 359-62.

Darszon, A., J.J. Acevedo, B.E. Galindo, E.O. Hernández-González and T. Nishigaki *et al.*, 2006. Sperm channel diversity and functional multiplicity. Reproduction, 131: 977-88.

Davis, P.J., F.B. Davis and W.D. Lawrence, 1989. Thyroid hormone regulation of membrane Ca^{2+}-ATPase activity. Endocr Res., 15: 651-82.

Di Leva, F., T. Domi, L. Fedrizzi, D. Lim and E. Carafoli, 2008. The plasma membrane Ca^{2+} ATPase of animal cells: Structure, function and regulation. Arch Biochem. Biophys., 476: 65-74.

Dostanic-Larson, I., J.W. Van Huysse, J.N. Lorenz and J.B. Lingrel, 2005. The highly conserved cardiac glycoside binding site of Na, K-ATPase plays a role in blood pressure regulation. PNAS, 102: 15845-50.

Dragileva, E., S. Rubinstein and H. Breitbart, 1999. Intracellular Ca(2+)-Mg(2+)-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. Biol. Reprod., 61: 1226-34.

El-Gothamy, Z. and M. El-Samahy, 1992. Ultrastructure sperm defects in addicts. Fertil. Steril., 57: 699-702.

Fang, S. and H.W. Baker, 2003. Male infertility and adult polycystic kidney disease are associated with necrospermia. Fertil. Steril., 79: 643-4.

Fiske, C.H. and Y. Subbarow, 1925. The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375-400.

Gurfinkel, E., A.P. Cedenho, Y. Yamamura and M. Srougi, 2003. Effects of acupuncture and moxa treatment in patients with semen abnormalities. Asian J. Androl., 5: 345-8.

Hamamah, S., R. Mieusset, F. Olivennes and R. Frydman, 1999. Male sterility and motility disorders: Etiological factors and treatment, Serono Symposia USA, Springer, New York, USA.

Jimenez, T., G. Sánchez and G. Blanco, 2012. Activity of the Na, K-ATPase α4 isoform is regulated during sperm capacitation to support sperm motility. J. Androl., 33: 1047-57.

Jimenez, T., J.P. McDermott, G. Sánchez and G. Blanco, 2011. Na, K-ATPase α4 isoform is essential for sperm fertility. Proc. Natl. Acad. Sci. USA, 108: 644-49.

Kao, S.H., H.T. Chao, H.W. Chen, T.I. Hwang and T.L. Liao *et al.*, 2008. Increase of oxidative stress in human sperm with lower motility. Fertil. Steril., 89: 1183-90.

Kaplan, J.H., 2002. Biochemistry of Na,K-ATPase. Annu. Rev. Biochem., 71: 511-35.

Khosrowbeygi, A., N. Zarghami and Y. Deldar, 2004. Correlation between sperm quality parameters and seminal plasma antioxidants status. Iran J. Reprod. Med., 2: 58-64.

Kitao, T. and K. Hattori, 1983. Inhibition of erythrocyte ATPase activity by aclacinomycin and reverse effects of ascorbate on ATPase activity. Experientia, 39: 1362-4.

Koçak-Toker, N., G. Aktan and G. Aykaç-Toker, 2002. The role of Na,K-ATPase in human sperm motility. Int. J. Androl., 25: 180-5.

Kumalic, I.S. and B. Pinter, 2014. Review of clinical trials on effects of oral antioxidants on basic semen and other parameters in idiopathic oligoasthenoteratozoospermia. Biomed. Res. Int.

Lerda, D. and R. Rizzi, 1991. Study of reproductive function in persons occupationally exposed to 2,4-dichlorophenoxyacetic acid (2,4-D). Mutat. Res., 262: 47-50.

Lestari, S.W., D.N. Miati, P. Soeharso, R. Sugiyanto and D.A. Pujianto, 2017. Sperm Na$^+$, K$^+$-ATPase α4 and plasma membrane Ca^{2+}-ATPase (PMCA) 4 regulation in asthenozoospermia. Syst. Biolreprod. Med., 63: 294-302.

Lodhi, L.A., M. Zubair, Z.I. Qureshi, I. Ahmad and H. Jamil, 2008. Correlation between hypo-osmotic swelling test and various conventional semen evaluation parameters in fresh Nili-Ravi buffalo and Sahiwal cow bull semen. Pakistan Vet. J., 28: 186-8.

Mata, A.M. and M.R. Sepúlveda, 2005. Calcium pumps in the central nervous system. Brain Res. Brain Res. Rev., 49: 398-405.

Merriam-Webster, 2016. Merriam-webster's medical dictionary, Merriam-Webster, Springfield, Massachusetts, USA.

Mobasheri, A., J. Avila, I. Cózar-Castellano, M.D. Brownleader and M. Trevan *et al.*, 2000. Na$^+$, K$^+$-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. Biosci. Rep., 20: 51-91.

Morth, J.P., H. Poulsen, M.S. Toustrup-Jensen, V.R. Schack and J. Egebjerg *et al.*, 2009. The structure of the Na$^+$, K$^+$-ATPase and mapping of isoform differences and disease-related mutations. Philos. Trans. R Soc. Lond B Biol. Sci., 364: 217-27.

Moskovtsev, S.I. and C.L. Librach, 2013. Methods of sperm vitality assessment. Methods Mol. Biol., 927: 13-9.

Nduwayo, L., C. Barthélémy, J. Lansac, M.J. Tharanne and P. Lecomte, 1995. Management of necrospermia. Contracept Fertil. Sex, 23: 682-5.

Olson, G.E., V.P. Winfrey, D.L. Garbers and T.D. Noland, 1985. Isolation and characterization of a macromolecular complex associated with the outer acrosomal membrane of bovine spermatozoa. Biol. Reprod., 33: 761-79.

Pasqualotto, F.F., A. Sundaram, R.K. Sharma, E. Borges and E.B. Pasqualotto *et al.*, 2008. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. Fertil. Steril., 89: 602-7.

Prasad, V., G.W. Okunade, M.L. Miller and G.E. Shull, 2004. Phenotypes of SERCA and PMCA knockout mice. Biochem. Biophys. Res. Commun., 322: 1192-203.

Said, T.M., N., Aziz, R.K. Sharma, I. Lewis-Jones and A.J. Thomas *et al.*, 2005. Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients. Asian J. Androl., 7: 121-6.

Sakaida, I.A.P.J.L., A.P. Thomas and J.L. Farber, 1991. Increases in cytosolic calcium ion concentration can be dissociated from the killing of cultured hepatocytes by tert-butyl hydroperoxide. J. Biol. Chem., 266: 717-22.

Samplaski, M.K., A. Dimitromanolakis, K.C. Lo, E.D. Grober and B. Mullen *et al.*, 2015. The relationship between sperm viability and DNA fragmentation rates. Reprod. Biol. Endocrinol.

Sanchez, G., A.N.T. Nguyen, B. Timmerberg, J.S. Tash and G. Blanco, 2006. The Na, K-ATPase α4 isoform from humans has distinct enzymatic properties and is important for sperm motility. Mol. Hum. Reprod., 12: 565-6.

Schuh, K., E.J. Cartwright, E. Jankevics, K. Bundschu and J. Liebermann *et al.*, 2004. Plasma membrane Ca^{2+} ATPase 4 is required for sperm motility and male fertility. J. Biol. Chem., 279: 28220-6.

Singh, R., A.M. Kaleem, S.S. Narayana and A.A. Mahdi, 2012. A case of oligoasthenoteratozoospermia with AZFc deletion and persistent oxidative stress. Indian J. Hum. Genet., 18: 359-62.

Strehler, E.E., A.J. Caride, A.G. Filoteo, Y. Xiong and J.T. Penniston *et al.*, 2007. Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. Ann. N Y Acad. Sci., 1099: 226-36.

Takahashi, H. and M. Yamaguchi, 1999. Role of regucalcin as an activator of Ca^{2+}-ATPase activity in rat liver microsomes. J. Cell Biochem., 74: 663-9.

Talebi, A.R., M.A. Khalili, H. Nahangi, A. Abbasi and M. Anvari, 2007. Evaluation of epididymal necrospermia following experimental chronic spinal cord injury in rat. Iran J. Reprod. Med., 5: 171-6.

Tournaye, H., J. Liu, Z. Nagy, G. Verheyen and A. Van Steirteghem *et al.*, 1996. The use of testicular sperm for intracytoplasmic sperm injection in patients with necrozoospermia. Fertil. Steril., 66: 331-4.

Trump, B.F., I.K. Berezesky, K.U. Laiho, A.R. Osornio and W.J. Mergner *et al.*, 1980. The role of calcium in cell injury. A Review Scan Electron Microsc., Pt 2: 437-62.

Vignini, A., E. Buldreghini, L. Nanetti, S. Amoroso and M. Boscaro *et al.*, 2009. Free thiols in human spermatozoa: Are Na+/K+-ATPase, Ca^{2+}-ATPase activities involved in sperm motility through peroxynitrite formation? Reprod. Biomed. Online, 18: 132-40.

Vivenes, C.Y., R.D. Peralta-Arias, M.I. Camejo, K. Guerrero and V.H. Fernandez *et al.*, 2009. Biochemical identification of dynein-ATPase activity in human sperm. Z Naturforsch C, 64: 747-53.

WHO, 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th Edn., World Health Organization, WHO Press, Geneva, Switzerland.

Wilton, L.J., P.D. Temple-Smith, H.W. Baker and D.M. de Kretser, 1988. Human male infertility caused by degeneration and death of sperm in the epididymis. Fertil. Steril., 49: 1052-8.

Woo, A.L., P.F. James and J.B. Lingrel, 2000. Sperm motility is dependent on a unique isoform of the Na, K-ATPase. J. Biol. Chem., 275: 20693-9.

Zhu, J., B. Arsovska and K. Kozovska, 2016. Case Report: Oligoasthenoteratozoospermia, Treatment with Acupuncture. IJIR, 2: 1592-4.