Oligoasthenoteratozoospermia and Necrozoospermia: A Study of Sperm Na\(^+\), K\(^+\)-ATPase \(\alpha4\) and Plasma Membrane Ca\(^{2+}\)-ATPase 4 Regulation

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**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 \(\alpha4\) 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 \(\alpha4\) 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 \(\alpha4\) 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 \(\alpha4\), PMCA4

**Introduction**

Male factors are responsible for more than forty percent of the infertility cases (Gurfinkel et al., 2003). Male factors are determined by the existence 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 homeostasis is regulated by integral enzymes in the sperm plasma membrane, namely Na⁺, K⁺-ATPase and Ca²⁺-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; Mobasher 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 Ca²⁺-ATPase retains calcium ion homeostasis (Takahashi and Yamaguchi, 1999). Calcium ion homeostasis plays a role on sperm motility (Dragileva et al., 1999). There are numerous Ca²⁺-ATPase types, including Plasma Membrane Ca²⁺-ATPase (PMCA), Sarcoplasmic-Endoplasmic Ca²⁺-ATPase (SERCA) and Secretory Pathway Ca²⁺-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 flagellum 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 Ca²⁺-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 Ca²⁺-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 Ca²⁺-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: oligozoosperma, asthenozoospermia and teratozoospermia (Cavallini, 2006; Singh et al., 2012). The oligozoosperma was determined as sperm concentration <15 million/ml, whereas asthenozoosperma was determined as progressive motility ≤32% (grade a) or ≤40% (grade a + b) and teratozoospermia was determined as normal sperm morphology <4% (WHO, 2010). Furthermore, necrozoosperma was defined as a condition in which the spermatocytes in seminal fluid are dead or motionless, approximately more than 60% (Merriam-Webster, Medical Dictionary, 2016; Fang and Baker, 2003). The necrozoosperma 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 × 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× 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 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 × 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× 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× 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 MgCl₂, 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 Na₂ATP 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× 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 Ca²⁺-ATPase

The analysis of the activity of Ca²⁺-ATPase were performed based on previous studies (Davis et al., 1989; Vivenes et al., 2009). A medium consisting of 3 mmol/l MgCl₂, 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 Na₂ATP 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× g for 10 min was performed 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 CaCl₂ was defined as the Ca²⁺-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 (Amerham, 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) was set at 1:200 dilution at 4°C overnight for incubation. After that, the horse radish peroxidase-conjugated donkey anti-goat IgG antibody (Santa Cruz, California, USA) at a 1:200 dilution was prepared at 4°C overnight 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× 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× g for 5 min was performed. The 0.2% Triton X-100 was defined as the 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. Secondary antibody (donkey anti-goat IgG 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 Ca²⁺-ATPase and the expression of Na⁺, K⁺-ATPase α4 and PMCA4 were compared in the control normozoospermia and oligoasthenoteratozoospermia or necrozoospermia 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

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viability assay, the intact cytoplasmic membrane of live sperm is the basis, which sperm refrain the absorption of eosin dye. Moskvitsev 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 (Ndudwayo 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 \(\mu\)mol Pi/mg protein/h in OAT group and 0.559±0.160 \(\mu\)mol Pi/mg protein/h in necrozoospermia group, whereas it was 1.962±0.56 \(\mu\)mol 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 \(\mu\)mol Pi/mg protein/h in OAT group and necrozoospermia group at 0.928±0.248 \(\mu\)mol Pi/mg protein/h, whereas in normozoospermia group at 2.657±1.329 \(\mu\)mol 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.

| 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            |
| Motility (%)               | 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

| ATPase activity (\(\mu\)M 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: Oligoaesthenoteratozoospermia
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 extracellularly. 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 normozoospermia 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.

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 necrozoospermia, whereas it was 4207.63±580.02 in the normozoospermia group (Fig. 2B and Table 3). The PMCA4 isoform band also seemed less frequency 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 than 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 were 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 in OAT samples, 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. 3A, B). 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). A negative control of unincubated sperm showed in Fig. 4D.
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 retrain 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 addition, 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 a4 and PMCA4 isoforms (Nduwayo et al., 1995; Coutinho et al., 1984; El-Gotheyam 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 (Bramhem 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.

**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.
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 \(\alpha4\) 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 extracellularly 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 extracellularly. 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 \(\alpha4\) and Plasma Membrane Ca\(^{2+}\)-ATPase 4 (PMCA4) Isoforms**

These results are in line with the activity of the Na\(^{+}\), K\(^{-}\)-ATPase \(\alpha4\) isoform of the sperm that were inhibited by ouabain and lead to declined sperm motility. Hence, this study confirmed that proved Na\(^{+}\), K\(^{-}\)-ATPase \(\alpha4\) isoform has dissimilar elements and plays a major role in sperm motility (Sanchez et al., 2006). The Na\(^{+}\), K\(^{-}\)-ATPase \(\alpha4\) 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 \(\alpha4\) was due to the change of membrane potential, whether depolarized or hyperpolarized (Mobasher 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 \(\alpha4\) and Plasma Membrane Ca\(^{2+}\)-ATPase 4 (PMCA4) isoform**

This study confirmed other studies that stated the Na\(^{+}\), K\(^{-}\)-ATPase \(\alpha4\) isoform is expressed in the proximal portion of the sperm flagellum, which relate to the midpiece of the sperm’s tail (Mobasher 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 \(\alpha4\) and PMCA4 isoforms. Surely, further studies are required to reveal the other cellular mechanism that control the sperm motility.

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**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.

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