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Research Article

Sperm Specific Two-Step Dye Exclusion Assays to Evaluate Integrity of Plasma and Organelle Membranes-New Approach for Quality Assurance of the Sperm for Intra-Cytoplasmic Sperm Injection

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ABSTRACT

Objective: The comprehensive assessments of the plasma and organelle membranes are new approach to understand human sperm fertility. The present study developed 2 types of the sperm specific 2-step dye exclusion assays to evaluate the plasma and organelle membranes in human sperm.

Methods: Sperm specific 2-step dye exclusion assay: Sperm was incubated with reactive red 195 (RR195) or Cy3-conjugated concanavalin A (Cy3-con A) in isotonic culture medium, they were counterstained with reactive blue 222 or Alexa 488 conjugated concanavalin A after exclusion of the plasma membrane with methanol, respectively. Integrity of the organelle membrane in the mitochondria was observed by retention of Mito Tracker FM. The cellROX Orange produced orange fluorescence upon oxidation by reacted reactive oxygen species.

Results: The sperm specific 2-step dye exclusion assay is composed of permeation and bind of the first dye to the nuclei through damaged plasma and orgnelle membranes, after membrane exclusion, newly exposed nuclei or the inner acrosomal membrane were counterstained with the second dye. The intact in plasma and acrosomal membranes in human motile sperm (live sperm; LS) excluded RR195 and Cy3-con A, but those denatured allowed permeations. LS colored bule or produced green fluorescence, on the one hand, those denatured did red or red fluorescence, respectively. The fluorescence of Mito Tracker FM and cellROX Orange in the mitochondria certified the normality of oxidative phosphorylation in the mitochondria with intact organelle membrane. The derivative single-step RR195 exclusion assay is a viable candidate for intra-operative non-destructive assessment of the motile sperm in clinical ICSI.

Conclusion: The dye exclusion and retention assays contribute as the pre-operative assessments of sperm qualities and the modified single-step dye exclusion assay may be useful for intra-operative sperm selection in clinical intra-cytoplasmic sperm injection, respectively.

Keywords: Human sperm; Apoptosis; DNA fragmentation; Dye exclusion assay; Dye retention assay; Reactive red 195; Reactive blue 222; Mito tracker FM; Cellrox orange; One-dimensional single-cell pulsed-field gel electrophoresis

INTRODUCTION

Denaturation of the plasma and organelle membranes are included in apoptosis, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane [1], and release of cytochrome C from mitochondria are initiative steps in apoptosis [2]. Our previous reports revealed that human semen is heterogeneous population of the sperm in terms of motility and DNA integrity [3-6], we separated the motile sperm with fibrous DNA and those immotile with granular DNA segments in the end stage of DNA fragmentation [5,6]. The comparison of their features suggested that they were corresponded to the sperm that have not yet and already undergone apoptosis, respectively [6]. All the organelles in somatic cells put into one compartment being enveloped with the plasma membrane. As a prime example, trypan blue has been widely used for the dye exclusion assay to observe the integrity of plasma membrane in somatic cells [7]. As is well known, the structure of mammalian sperm is so much different in terms of

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the membrane organization from the somatic cells. Its surface is at least compartmentalized into 4 regions: The acrosomal cap at the anterior region of the head, the posterior region of the head, the midpiece, and the principal and terminal pieces of the tail. The acrosome cap is composed of the plasma membrane and outer and inner acrosomal membranes [8,9], in contrast, the posterior region of the head is enveloped solely with the plasma membrane and lack of the cytoplasm placed the nucleus just below the membrane, of which damages may directly affect DNA integrity.

Our previous report [10], observed that Ascorbic Acid (AA) which acted as a pro-oxidant in the presence of transitional metals dose-dependently cleaved DNA in the membrane excluded human sperm, but not did that in the swimming sperm, and EDTA inhibited the action. The damages of the plasma membrane on the tail as well as the mitochondrial membrane in the midpiece interfere motility. The comprehensive assessments of the plasma and organelle membranes are new approach to understand human sperm fertility. The present study developed 2 types of the sperm specific 2-step dye exclusion assays to evaluate the plasma and organelle membrane in human sperm.

METHODOLOGY

Ethics statement

Ejaculates (n=12) were obtained from volunteers; 8 specimens satisfied the criteria (motility was found to be more than 80% after the separation) were used to develop the dye exclusion assays. The other 4 specimens were provided for Polyacrylamide Gel Electrophoresis (PAGE). The aim of this study and the measurement items were clearly explained to them, and they provided written informed consent for participation in and publication of the study. The ethics committee of the Ichikawa General Hospital approved this study (approval no. I19-68).

Purification of human motile sperm with fibrous DNA and those immotile with granular DNA segments

Sperm concentration and motility were determined according to the WHO reference manual [11]. Human motile sperm with fibrous DNA and those immotile with granular DNA segments in the end stage of fragmentation were purified according to our previous report [5,6,12]. Briefly, the sperm was fractionated by means of sedimentation equilibrium in the discontinuous Optiprep (apparent density of 1.085/1.17 g/mL, Axis Shield, San Jose, CA, USA), and subsequent differential velocity sedimentation in the isotonic 90% Percoll (GE Healthcare, Chicago, IL, USA) density gradient. The progressively motile sperm was recovered in the interface layer of OP/sediment of Percoll. The immotile sperm was recovered in the sediment of OP/intermediate layer of Percoll.

Extraction of protamines from human sperm and polyacrylamide gel electrophoresis

Extraction of protamines from human sperm and Polyacrylamide Gel Electrophoresis (PAGE) were performed as described previously [12]. Briefly, human semen specimens (Volume: 1.0, Sperm concentration: 60 ± 15 × 10^6/mL, Motility: 68% ± 6.9%, n=4) were processed as described above. An aliquot (80 × 10^6 sperm in 0.2 mL) was mixed with an equal volume of the lytic medium (0.1 M Na2CO3-NaHCO3, 2.0 mol/L guanidine sulfate, 5.0 mmol/L Dithiothreitol (DTT), pH 10.0), the extracted protamines was electrophoresed by means of the common protocol for acid PAGE (18% polyacrylamide) [13], according to the previous report [12]. The gel was stained with 0.1% trypan blue in pure water for 120 min, the excess dyes were washed out with tap water.

Staining human sperm with trypan blue

Trypan blue exclusion assay [7], was performed as follows; Sperm suspension (10^6 in 100 µl) was adhered on a plane glass slide by centrifugal auto-smear. The present study stained the sperm with 0.05% trypan blue in 20 mmol/L Hepes-NaOH, pH 7.4, for 20 min after exclusion of the plasma membrane with methanol. The head of membrane excluded sperm was swelled by means of 20 mmol/L Hepes-NaOH, pH 7.4, 0.1 mol/L guanidine sulfate, 5.0 mmol/L Dithiothreitol (DTT), for 10 min, then stained in the same manner.

Sperm specific 2-step dye exclusion assay

Sperm suspension (10^6 in 100 µl) was incubated with an equal volume of 0.01% reactive red 195 (RR195) in isotonic 20 mmol/L Hepes-NaOH, Hanks' solution, pH 7.4, at 37°C for 20 min, then the reaction mixture was being adhered on a plane glass slide by centrifugal auto-smear, treated with methanol to exclude the plasma membrane, then mount 0.2 mL of 0.01% reactive blue 222 (RB222) in 0.1 mol/L Na2CO3-NaHCO3, pH 10.0, at ambient temperature for 20 min. The slide was washed out with saline, then the profile of the sperm was observed under bright field microscope. The head of sperm stained to be red indicated that RR195 permeated through the damaged plasma membrane. Those stained to be blue did that the counter-dye (RB222) was permeated after membrane exclusion, suggesting that the plasma membranes were intact. The rate of the undamaged plasma membrane was calculated from the sperm stained to be blue/the sperm observed 100x.

Observation of the plasma and the acrosomal membranes by 2-step concanavalin A labeling

Sperm suspension (10^6 in 100 µl) was incubated with an equal volume of 2.5 µg/mL Cy3-conjugated concanavalin A (Cy3-con A, Molecular Probes, Eugene, OR, USA) in isotonic 20 mmol/L Hepes-NaOH, Hanks' solution, pH 7.4, Hanks' solution, 10 mmol/L methyl α-D-mannopyranoside, a potent antagonist of concanavalin A [14], at 37°C for 20 min, then the reaction mixture was were adhered on a plane glass slide by centrifugal auto-smear, treated with methanol or 0.1% Triton 100x to exclude the plasma and the outer acrosomal membranes, then mount 0.2 mL of 2.5 µg/mL Alexa 488 conjugated concanavalin A (Alexa488-con A, Molecular Probes) in in the same manner with the first step. The slide was washed out with saline, then the same field of view was observed under florescent microscope with green and red filter. The images were merged by the aid of Image J (Ver 1.5.3). The red fluorescence on the merged photograph indicated that the plasma and the outer acrosomal membranes have already been damaged, facilitating the permeation of Cy3-con A. The green fluorescens indicated that Alexa488-con A was bound to the inner acrosomal membrane that newly exposed after methanol treatment, suggesting that the acrosomal membranes were intact. The rate of the undamaged acrosomal membrane was calculated from the sperm with green fluorescence/the sperm observed 100x.

Dye retention assay of mitochondrial organelle membrane

Sperm suspension (10^6 in 100 µl) was incubated with an equal volume of 2.0 µmol/L Mito Tracker green FM (Thermo fisher scientific, MA, USA) [15] in isotonic 20 mmol/L Hepes-NaOH, Hanks' solution, pH 7.4, at 37°C for 20 min, then the reaction mixture was being adhered on a plane glass slide by centrifugal auto-smear. The fluorescence in the midpiece was observed under florescent microscope with green filter.

The endogenous Reactive Oxygen Species (ROS) generated in the mitochondria

Sperm suspension (106 in 100 µl) was incubated with 1.0 µmol/L cellROX Orange (Thermo fisher scientific) [16], in the same manner with Mito Tracker green FM. The orange fluorescence in the midpiece was observed under florescent microscope with red filter.

Hypo-osmotic swelling test

LS in isotonic 20 mmol/L Hepes buffered Hanks' solution, pH 7.4, was diluted with 20 mmol/L Hepes buffer, pH 7.4, to decrease the osmotic pressure by half, then incubated at 370°C for 20 min. Thereafter, the suspensions were retrieved to give 106 sperm to make smear.

Observation of vacuoles in the head of sperm

Sperm suspension (106 in 100 µl) was adhered to a plane glass slide by using centrifugal auto-smear and fixed with methanol for 5 min. The specimens were stained with 0.02% reactive blue 2 (RB2; 0.1 mol/L Na_2CO_3-$NaHCO_3$, pH 10.0) for 10 min, and the excess dye was washed out with pure water [17,18].

RESULTS

Human normozoospermic semen (Volume: 4.0 ± 0.85 mL, Sperm concentration: 78 ± 24 × 10^6/mL, Motility: 60% ± 6.8%, n=12) were used to prepare the sperm specimens. Almost the sperm recovered from the interface layer of OP/sediment of Percoll was progressively motile. Of these, 8 specimens recorded more than 80% motility (1.0 mL, 38 ± 13 × 10^6/mL, 92% ± 3.8%) were termed as the Live Sperm (LS). In contrast, those recovered from the sediment of OP/intermediate layer of Percoll were almost immotile and auto agglutinated, those yielded from the 8 specimens used as LS were termed as the denatured sperm (DS, 1.0 mL, 60 ± 15 × 10^6/mL, 9.6% ± 1.6%). LS and DS were used as the comparative standards to verify the principles and the quantitative performances of the sperm specific 2-step dye exclusion assays.

After acid PAGE, 2 isoforms of extracted protamines were visualized with trypan blue (4 sulfate residues in a molecule) (Figure 1A). The dye unstained almost all the membrane excluded LS (Figure 1B), whereas deeply stained the swollen head to be blue (Figure 1C). We further examined some dyes including sulfate residue such as bromophenol blue (1 sulfate residue) and ponceau 4R (3 sulfate residues), they also gave similar electrophoretograms for the extracted protamines (data not shown), and scarcely stained the membrane excluded LS (Figures 2A and 2C), but deeply stained the swollen head to be blue and red, respectively (Figures 2B and 2D). The features of 3 dyes shown in Figures 1 and 2 were agreed completely.

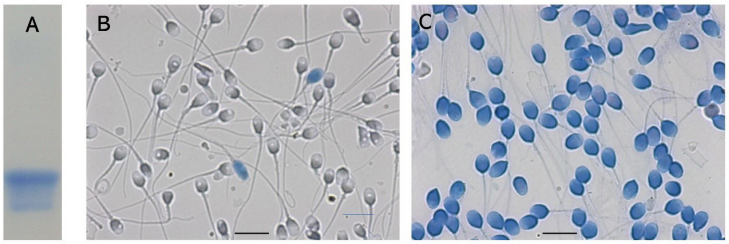

Figure 1: Staining of the Live Sperm (LS) with trypan blue. A) Electrophoretic profile of human sperm extract in acid PAGE, the gel was stained with 0.1% trypan blue; B) Membrane excluded LS was stained with 0.05% trypan; C) The swollen head of membrane excluded LS was stained in the same manner with B. **Note:** Scale bars are 10 µm in length.

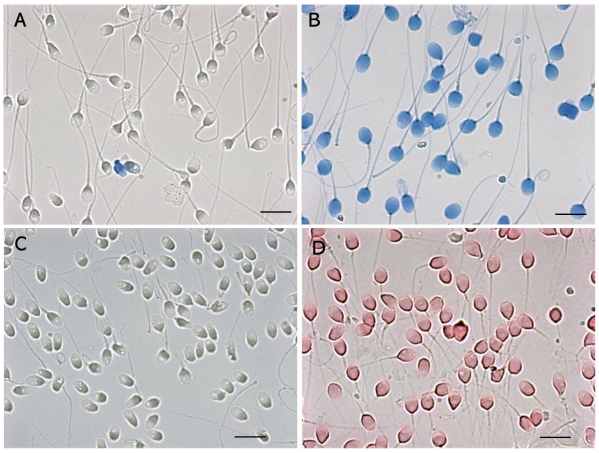

Figure 2: Staining of the Live Sperm (LS) with bromophenol blue and ponceau 4R. The membrane excluded LS or those after swollen were stained with 0.05% bromophenol blue (A,B) and 0.05% ponceau 4R (C,D) in the same manner with Figure 1, respectively. **Note:** Scale bars are 10 µm in length.

Next, we screened some candidates which colored the nuclei in LS brilliantly. Reactive red 195 (RR195, 5 sulfate residues) and reactive blue 222 (RR222, 6 sulfate residues) bound to the extracted protamines as like trypan blue (data not shown), RR195 and RB222 deeply colored not only the swollen LS (Figures 3B and 3D) but also the membrane excluded LS to be red and blue in contrast to the other 3 dyes. The results in Figures 1-3 suggested that the dyes including sulfate residue uniformly bound to the extracted protamines and swollen nucleus regardless of the number of sulfate residue, whereas the sustainability for the tightly packed nucleus was different among them.

The sperm specific 2-step dye exclusion assay is composed of permeation and bind of the first dye to the nucleus through damaged plasma membrane, after membrane exclusion, newly exposed nucleus was counterstained with the second dye. (Figure 4A) exhibited staining profile of LS with RR195 as the first dye, then counterstained with RB222, (Figure 4B) was in the reverse order. The former sequence gave higher contrast rather than the latter, and we thought it preferable to show the damage as red color, the present study employed the sequence shown in Figure 4A. (Figures 4C and 4D) exhibit the features of LS and DS, the rate of undamaged plasma membrane in DS was found to be 29 ± 11% (n=8). The unseparated semen was heterogeneous population in terms of the integrity of the plasma membrane, the present method efficiently separated LS from DS. In contrast, the rate in LS altered among the specimens with mean of 81 ± 7.1%, the specimen shown in Figure 4C gave the highest value of 96% among the sperm examined. The Intra-Cytoplasmic Sperm Injection (ICSI) is major fertilization tool for male infertility with poor semen quality. RR195 exclusion assay without counterstain is a viable candidate for intra-operative non-destructive assessment in clinical ICSI. If the medical technologist recognizes that a motile sperm caught under the microscope is colored red, it should discard. This concept contributes the quality control of the sperm for injection, of course, it must undergo detailed exposure experiments according to the guidelines of reproductive toxicity prior to clinical use.

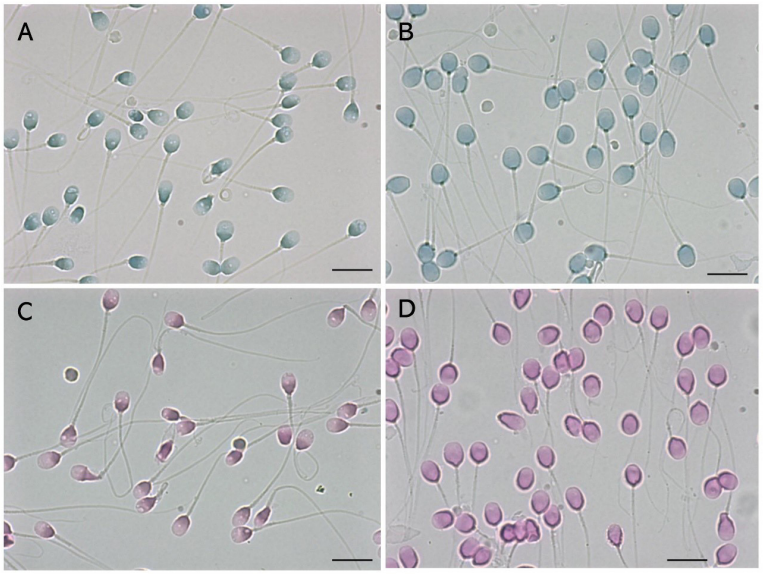

Figure 3: Staining of the live sperm with reactive red 195 and reactive blue 222. The membrane excluded LS or those after swollen were stained with 0.01% RR195 (A,B) and 0.01% RB222 (C,D) in the same manner with Figure 1, respectively. **Note:** Scale bars are 10 μm in length.

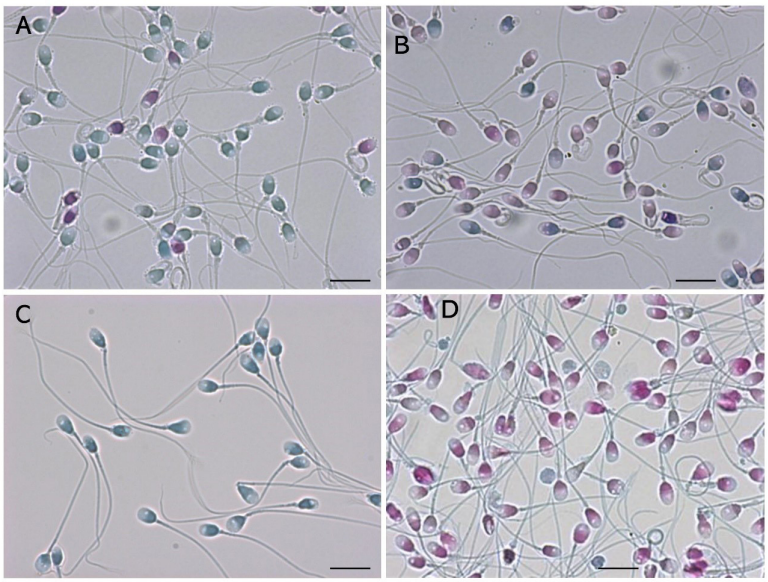

Figure 4: The sperm specific 2-step RR195 exclusion assay. A) Staining profile of LS with RR195 as the first dye, then counterstained with RB222; B) staining in the reverse order; C) Live sperm; D) Denatured sperm. **Note:** Scale bars are 10 μm in length.

Double staining with fluorescent labeled concanavalin A was used to monitor the acrosome reaction [12], the present study applied it to observe the permeability of the plasma and the acrosomal membranes based on the same principle of the dye exclusion assay shown in Figure 4. Concanavalin A had high affinity to high-mannose carbohydrates on the inner acrosomal membrane in the presence of 5.0 mmol/L methyl α-D-mannopyranoside [14], Alexa488-con A produced green fluorescence on the acrosome of LS after membrane exclusion with methanol (Figure 5A), meanwhile, those excluded with 0.1% Triton X-100 rinsed off the carbohydrates, lost the fluorescence (Figure 5B). As like as the dye exclusion assay Figure 4, choice of the first probe is also reversible in this assay, we thought Cy3-con A with red fluorescence is preferable as the first probe to demonstrate the damage and used Alexa488-con A for the counterstain. Almost LS excluded Cy3-con A and produced green fluorescence, the rate of undamaged acrosomal membranes was found to be 87 ± 5.4% (n=8). The specimen in (Figure 5C), which was identical with (Figure 4C), also gave the highest value of 94%. In contrast, almost DS gave red fluorescence, the rate of undamaged acrosomal membranes was found to be 5.6 ± 2.1%.

Next, we employed Mito Tracker FM for dye retention assay of mitochondrial organelle membrane, it permeates and holds in the mitochondria regardless of the membrane potential of the organelle [19]. As shown in (Figure 6A), the swimming LS incubated with 1.0 μmol/L Mito Tracker FM produced green fluorescence in the midpiece. Oxidative phosphorylation in the mitochondria generates the endogenous Reactive Oxygen Species (ROS), cellROX Orange permeated in the mitochondria produced orange fluorescence upon oxidation by ROS [16]. The midpiece of LS produced the fluorescence in consequence of the metabolite of TCA cycle (Figure 6B). On the one hand, those excluded membrane with methanol produced no fluorescence in both methods (Figures 6C and 6D). The retention of Mito Tracker FM and generation of ROS certified the normality of oxidative phosphorylation in the mitochondria with intact organelle membrane.

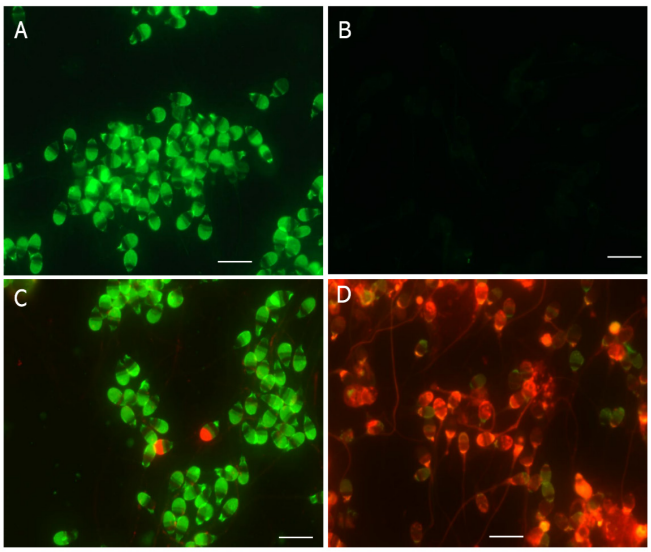

Figure 5: Observation of the acrosomal membranes by means of the sperm specific 2-step Cy3-con A exclusion assay. A) Live sperm was labelled with Alexa 488-con A after membrane exclusion with methanol; B) With 0.1% Triton-X; (C and D) Represent the staining profiles of LS and DS by 2-step Cy3-con A exclusion assay. **Note:** Scale bars are 10 μm in length.

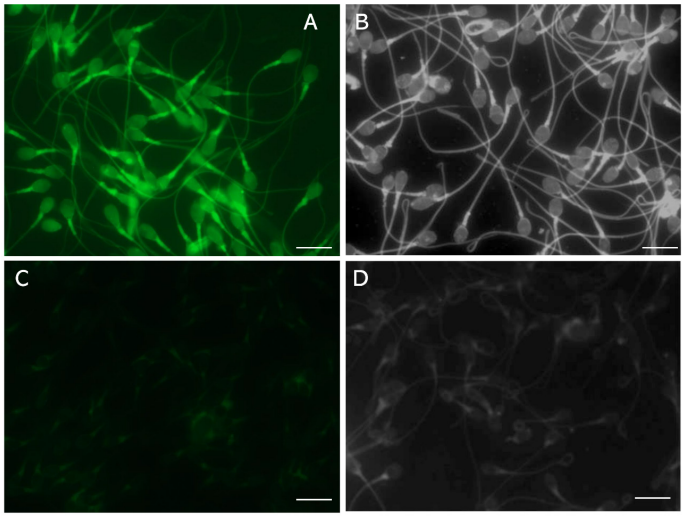

Figure 6: Observation of mitochondrial functions with Mito Tracker FM and cellROX Orange. A) Mito Tracker FM retention assay; B) The endogenous reactive oxygen species in the mitochondria with cellROX Orange, the feeble fluorescence was recorded with highly sensitive monochrome camera; (C and D) Were those for membrane excluded LS with methanol. **Note:** Scale bars are 10 μm in length.

Hypo-osmotic pressure of extracellular fluid damages the plasma and the organelle membranes, the Hypo-Osmotic Swelling (HOS) test has been widely used to predict integrity of the plasma membrane of human sperm [20]. The fibrous sheath is a cytoskeletal structure surrounding the axoneme and outer dense fibers at the principal piece of the sperm tail [21]. HOS test observes swelling of the terminal piece without covering with the fibrous sheath and defined that the sperm with swollen terminal piece have intact plasma membrane [20]. LS used in Figure 4A (the rate of undamaged plasma membrane 78%) was provided for re-evaluation of HOS test by means of the present assay methods (Figure 7). By decreasing the osmotic pressure by half, all the sperm was immobilized (data not shown), and the terminal piece became coiled form (Figure 7A). RR195 exclusion assay suggested that the rate of the sperm with undamaged plasma membrane was decreased to be 64% (Figure 7B). In both the dye retention and ROS generation assays, their fluorescent intensities were decreased from those in the isotonic environment, the functions of mitochondria did not degenerate completely (Figures 7C and 7D). The plasma membrane on the head and the organelle membrane in the mitochondria were more tolerant for hypo-osmotic pressure rather than those on terminal piece of the tail. The outcome of HOS test represents the regional feature of terminal piece of the tail but not those of whole membranes.

Our previous reports visualized the vacuoles in the head of human sperm by means translucent staining with RB2 (3 sulfate residues), it faintly stained the head to reveal a translucent bluish body and the vacuoles were visualized as toneless spots [17,18]. RR195 and RB222 are family of RB2. RR195 exclusion assay was modified for simultaneous observation of the plasma membrane and the vacuole, the concentration with RB222 was decreased to 0.0025%. (Figure 8A) showed the staining profiles with RB2. Although a specimen satisfied the criteria with the motility of more than 90%, almost all the sperm involved a variety of vacuoles, (Figure 8B) showed the modified RR195 exclusion assay with diluted BR222, the specimen scarcely involved the sperm staining to be red, similar profile of the vacuoles was observed with (Figure 8-A). The simultaneous observation suggested that the intact plasma membrane uncertified absence of the vacuole.

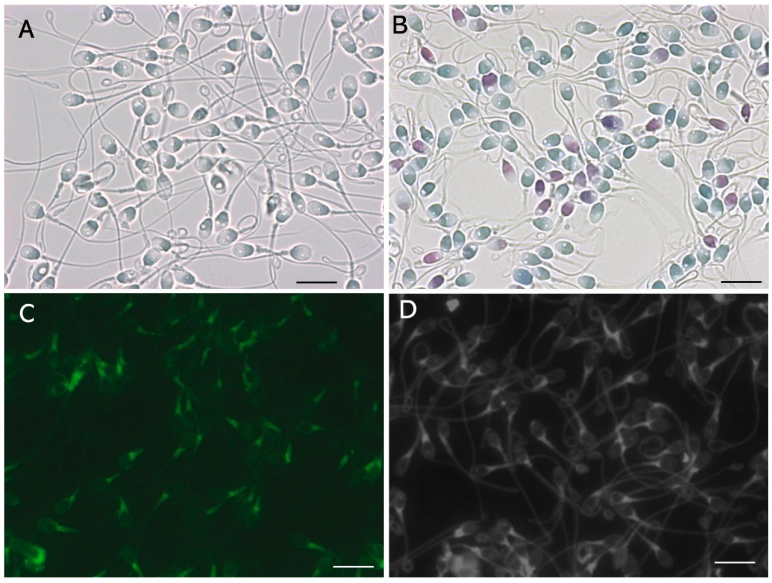

Figure 7: Influences of hypo-osmotic pressure of extracellular fluid for live sperm. After decrease the osmotic pressure by half, LS was incubated at 37°C for 20 min. A) Bright field optics; B) RR195 exclusion assay; C) Mito Tracker FM retention assay; D) The endogenous reactive oxygen species with cellROX Orange. **Note:** Scale bars are 10 μm in length..

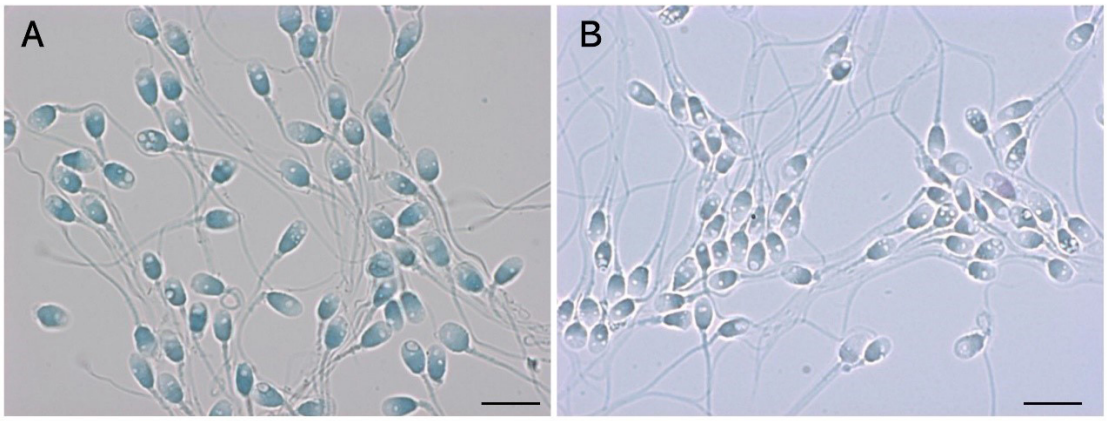

Figure 8: Modified RR195 exclusion assay with dilute RB222 for simultaneous observation of the plasma membrane and the vacuole. A) Reactive blue 2 staining; B) modified RR195 exclusion assay with dilute RB222. **Note:** Scale bars are 10 μm in length.

DISCUSSION

As is well known, major component of the protamines is arginine with guanidyl residue [22], which strongly bind electrostatically with phosphoric residue in DNA, neutralize the electric charge and disulfide cross-linking among protamines packed DNA-nucleoprotein complex [23]. Guanidine sulfate (Figure 1C) is a potent dissociating agent for DNA-protamine complex, after reduction of disulfide cross-linking among protamines with DTT, guanidyl residue in guanidine competitively binds with DNA and sulfate residue formed a salt with dissociated protamines. The high (1.0 mol/L) and low (0.1 mol/L) concentrations of the agent extract protamines for PAGE (Figure 1A) and swelled the head by electrostatic repelling of DNA fibers (Figures 1-3) respectively.

The organic structure of dyes closely relates not only to their color tones but also to the binding specificity to cellular components through the intermolecular forces such as Vander waals', hydrophobic, hydrogen, and electrostatic forces. The results in Figures 1-3 suggested that all the dyes with sulfate residue uniformly bound to the extracted protamines regardless of the organic structures, they might interact through electrostatic forces. They also permeated and stained the swollen nucleus. Tightly packed DNA-nucleoproteins complex physically blocked permeation of the dye shown in Figures 1 and 2, whereas RR195 and RB222 stained deeply, there found no significant correlation between number of sulfate residue in a molecule and the permeability, it remains obscure whether RR195 and RB222 can permeate in the nucleus due to their organic structures or binding to the surface of nucleus gave enough color intensities.

Denaturation of human sperm during waiting the ejaculation in epididymis accompany with immobilization and DNA fragmentation, our previous reports suggested that LS and DS were corresponded to those have not yet and already undergone apoptosis [5,6,12], the 2 types of sperm specific dye exclusion assays shown in Figures 4 and 5 distinguished them based on the features of the plasma and the acrosomal membranes, the counterstain promoted the viewability. The permeation path of Cy3-con A, a protein with molecular weight of 105, was not transmembrane passive diffusion, but some physical gaps passing through 3 layers of the plasma and acrosomal membranes. RR195, a hydrophilic organic compound (Molecular weight: 1136) with 5 sulfate residues, also might bind to the nucleus through similar pathway with Cy3-con A, but not through transmembrane passive diffusion.

Processing of the normozoospermic semen by means of the present method efficiently separated LS from DS, almost LS was motile with fibrous DNA [5,6,12], intact plasma membranes in terms of dye exclusion, retention of Mito Tracker FM and generation of ROS in the mitochondria and swelling the terminal piece by hypo-osmotic pressure. In contrast, DS gave the opposite outcomes in all the examinations. Overall results strongly suggested the above comments that LS and DS were corresponded to those have not yet and already undergone apoptosis.

The dye exclusion assays as well as HOS test to validate the status of the plasma membrane on human sperm require careful interpretation, with the test specimen being rigorously limited to the separated motile sperm fraction, the unseparated semen is the mixture of LS and DS, and often includes immotile sperm with abnormal tails even in isotonic environment. Trypan blue unstained the tightly packed nuclei in LS regardless of the membrane exclusion (Figure 1B), it is unfitted for examination of human sperm. HOS test advocated that the swollen terminal piece represents the integrity of entire plasma membrane in the sperm [7], as stated above, the plasma membrane of sperm is at least compartmentalized into 4 regions, the tolerance against hypo-osmotic pressure were different among the regions (Figure 7). The plasma membrane enveloping the head was found to be more tolerant for hypo-osmotic pressure rather than the tail. HOS test did not represent feature of the whole membranes.

The posterior region of the head is enveloped solely with the plasma membrane and lack of the cytoplasm placed the nucleus just below the membrane, of which damages may directly affect DNA integrity. DNA in DS with damaged plasma membrane have granular DNA segments [5,6,12]. AA is produces hydroxy radical in the presence of transitional metals and cleaved DNA double strands in the membrane excluded human sperm but not did that in the swimming sperm [10], the fact that chelating metals inhibited the action generates a hypothesis, if the plasma membrane was damaged, AA in the seminal plasma [24] contact with DNA-transitional metals complex in nuclei, causing DNA fragmentation.

In ICSI practice, the sperm parameters available under common bright microscope are only motility and outline of head and tail. The separated motile sperm fraction still involves various dysfunctions, we therefore developed some pre-operative examinations such as one dimensional single-cell pulsed-field gel electrophoreses for detection of early stage DNA fragmentation [4-6,10], reactive blue 2 staining for detection of the vacuoles in the head [17,18], the sperm specific RR195 and Cy3-con A exclusion assays (Figures 4 and 5), the retention of Mito Tracker FM and ROS generation assays (Figure 6). RR195 and Cy3-con A exclusion assays are destructive inspections, and the sperm retained Mito Tracker FM or cellROX Orange in the mitochondria cannot be injected clinically. The single-step RR195 exclusion assay without counterstain may be useful as intra-operative non-destructive assessment in clinical ICSI. Another modified RR195 exclusion assay with dilute RB222 counterstain achieved simultaneous visualization of integrity of the plasma membrane and the vacuoles. Our previous report [18], indicated that at least in human sperm, local failure of the disulfide cross-linkage might play a critical role in determining the head morphology as well as vacuole formation. Figure 8 suggested that even in the separated motile sperm with intact plasma membrane, the vacuoles were involved, motility do not come with a full warranty for other sperm functions.

CONCLUSION

The present study developed 2 types of the sperm specific 2-step reactive red 195 or Cy3-conjugated concanavalin A exclusion assays to evaluate the plasma membrane. The assay is composed of permeation and bind of the first dye to the nuclei through damaged plasma and acrosomal membranes, after membrane exclusion, newly exposed nuclei or the inner acrosomal membrane were counterstained with the second dye.

Integrity of the organelle membrane in the mitochondria was observed by the combination of 2 types of dye retention assays. Green (Mito Tracker FM) and orange (cellROX Orange) fluorescence in the mitochondria certified the normality of oxidative phosphorylation in the mitochondria with intact organelle membrane.

The dye exclusion and retention assays contribute as the pre-operative assessments of sperm qualities in clinical infertile therapy.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

Authors declare the absence of any conflicting interests.

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