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RESEARCH ARTICLE

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The Effectiveness of Different Diluent Media as the Storage Medium for Human Sperm: To Maintain Its Quality

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The aims of this research is to find the best diluent media as the storage media for human sperm. The sperm sample were divided in the three group to be stored in the three kinds of diluent media (silk-select-stock, Tris-egg yolk, CEP-D). The viability and motility are determined by direct observation under microscope, the number of MDA is counted by computer tools and the genetic stability of the sperm is determined by polymerase chain reaction and DNA sequencing method. The percentage of the viable sperm that were stored in the silk-select-stock, tris-egg yolk, and CEP-D in the 24 hours were 56.31; 34.91 and 51.98, respectively. The percentage of the sperm motility were 43.57 (silk-select-stock), 38.93 (tris-egg-yolk) and 34.45 (CEP-D). The MDA number of the sperm that was stored in the silk-select-stock, tris-egg yolk and CEP-D in the 24 hours were 0.907; 9.237 and 2.586, respectively. The fresh sperm had 2.516 MDA number. The similarity rank of the DNA band pattern between fresh sperm and the sperm that was stored in the three kinds of diluent media was that the sperm which was stored in the silk-select-stock have the best similarity to the fresh sperm, while the second best was the sperm which was stored in the CEP-D and the last was the sperm which was stored in the tris egg yolk. Based on the data of viability, motility, MDA number, and the genetic stability in this research, it can be concluded that silk-select-stock is the best diluent media, CEP-D in the second and the tris egg yolk is the worse than the other two as a medium for human sperm storing.

Keywords: Viability, Motility, Genetic Stability, Number of MDA, Diluent Medium, Human Sperm.

1. INTRODUCTION

Human sperm are usually stored at very low temperature until −20 °C.[16] Therefore, the storage methods of human sperm needs very expensive and sophisticated equipment and also needs sophisticated skills for practice.[17] Some kinds of cell organelle of the sperm that is stored in the freeze condition are damaged.[17] The cool shock can cause the motility decreasing, achrosome enzyme releasing and disturbancing of the bilayer.[22,23] The temperature has influenced sperm viability dan motility.[19]

Recently, some kinds efforts have done to get the best spermatozoa quality which is stored in freezing conditions. One kind of effort is vitrification method which has successed when it is widely applied in the egg cell of a cow,[14] horse[13] and sheep embryo.[6] The basics of vitrification method is to raise the viscosity of the solution or solve medium by increasing cooling and warming rate within determined limits. In the other hand is adding or using cryoprotectant medium that will repress the increasing of viscosity at low temperatures.[16,21] Some kinds of macromolecules like specific lipids and carbohidrates act as the cryoprotectant. Choosing the proper diluent medium can minimize genetic material damage.

Solve medium is one of the aspects which define the sperm survival during storage. The solving solution usually used involve egg yolk Tris 20%[2,4,8,9] and Andromed[20] which is produced by the producers. Both kinds of diluents are commonly used for frozen storage. The purpose of this study is to discover the solve which keep the sperm quality. The viability, motility, genetic stability of spermatozoa and the MDA number during storage are the parameter of sperm quality.

In the first step of this research is found three kinds of solve media called the tris-egg yolk (tris aminomethane with the egg yolk[4,7–9,11] the silk-select-stock and the CEP-D. The tris-egg yolk, and the CEP-D are usually used as a solve medium for storing mammals' sperm groups[8–10] and the silk-select-stock usually used as solve media for storing human spermatozoa.[20] The novelty of this research was the use human sperm as the research sample, while another research use animal sperm as the sample.

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2. METHODOLOGY

Human semen is collected from a number of volunteers by non aseptic masturbation process.[15] The semen is observed by the semen volume, viscosity, motility, viability, color, pH, and odor. The cylinder glass is used to measured the semen volume. The microscope is used to observed the semen motility and viability.[9] The drop time of semen is used to know the semen viscosity.[8] Accepted sample has volume of >2.5 mL, with percentage of motility and viability are >50% and >70%, respectively, the drop time is >2 minutes, with the white-light yellow color, the number of pH is 6–7 and has the semen odor characteristic.[9]

This study used three kinds of solve media: (a) silk-select-stock, (b) tris-egg yolk and (c) CEP-D. Media is prepared by diluting some kinds of a chemical compound. Silk-select-stock is prepared by diluting the commercial media with the distillated water. The CEP-D diluent media is prepared by diluting some kinds of a chemical compound: NaCl 8.8 g; KCl 5.2 g; $CaCl_2(H_2O)_2$ 4.4 g; $MgCl_2(H_2O)_6$ 8.1 g; $NaHCO_3$ 1 g; NaH_2PO_4 9.6 g; KH_2PO_4 2.72 g; Fructose 9.9 g; sorbitol 1 g; BSA 0.2 g/100 ml; Tris 9.9 g; penicylin 1000 IUI; streptomycin 1 g; citric acid 9 g, the eosin-negrosin staining agents. Tris egg yolk diluent media is prepared by adding 20% the egg yolk to trisamino methane buffer.[9]

The sperm storing process is done by mixing the diluent media (silk-select-stock, CEP-D, and the tris egg yolk) and semen until 10^6 spermatozoa/ml. Then mixing is stored in the refrigerator in temperature of 5 °C. The mixture is observed every 6 hours to know its motility and viability.[9]

The liquida semen (50–100 μL) are diluted in buffer KCl + HCl (300 μL) and TBA (300 μL). Then, the solution is boiled indirectly in the eppendorf, cooled in the refrigerator. The sample is counted by the number of MDA and analyzed the genetic stability by the DNA band pattern.[20]

There are some data got in this research

(a) the sperm viability that is stored in the three kinds of diluent media

(b) the sperm motility that is stored in the similar diluent media

(c) the MDA number of sperm that is stored in the three kinds of diluent media, and

(d) the genetic stability of sperm that is stored in the three kinds of diluent media.

The descriptive qualitative analysis was employed to analyze the data in order to get the answer for the problems in this research.

3. RESULTS AND DISCUSSION

The sperm viability that is stored in the silk-select-stock, CEP-D, and tris egg yolk is displayed in Figure 1.

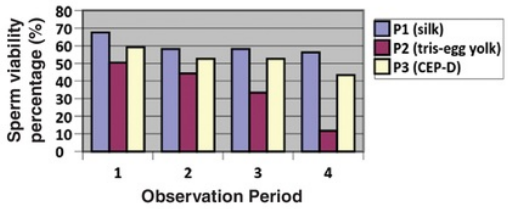

Fig. 1. The viability of the sperm that is stored in the three kinds of diluent media.

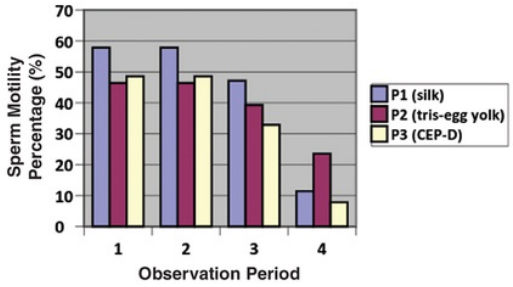

Fig. 2. Motility of the sperm that is stored in the three kinds of diluent media.

Based on Figure 1, it can be stated that the silk-select-stock is the best solve media to preserve the viability of the sperm. Figure 2 explain the sperm motility which is stored in the silk-select-stock, CEP-D, and tris egg yolk. Based on Figure 2, it can be stated that the silk-select-stock gave the highest percentage of the sperm motility and the tris egg yolk can maintain sperm motility in a long time. Meanwhile, based on Figure 3, it can be stated that the silk-select-stock is the medium that gave the smallest sperm MDA number.

Sperm genetic stability is observed by the DNA band pattern. The DNA band pattern of those three samples that has more similarity with band pattern of the fresh sperm is stated as having a good genetic stability. The performance of the band pattern of this DNA can be seen in Figure 4.

The determination of genetic stability of the spermatozoa is based on the similarity of the DNA band pattern between the sperm that is stored in the three kinds of media and that of the fresh sperm. Based on Figure 4, can be stated that samples 1, 2 and 4, have more similarity for DNA band pattern with the DNA band pattern of the fresh sperm (number 6) than that of others sample. Second range related to the similarity of this research is placed by the sperm that is stored in the CEP-D medium. The sample related to the sperm that storage in the CEP-D medium are number 7 and number 8. The DNA band pattern of this sample rather same with the DNA band pattern of the fresh sperm (number 6). Unfortunately, the DNA band pattern of sperm that is stored in the tris-egg-yolk (number 3) has no much similarity with the fresh sperm.

Spermatozoa viability is observed by staining with negrosin-eosin. The dead spermatozoa will be full of red color, while the living spermatozoa is colorless. The spermatozoa performance after staining process shown in Figure 5. The determination of spermatozoa viability is based on the ratio of live

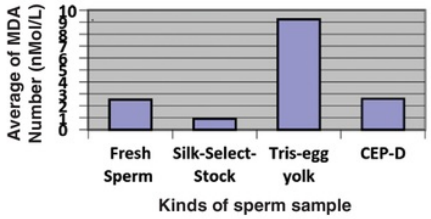

Fig. 3. The average of MDA number of sperm that is stored in the three kinds of diluent.

Fig. 4. The performance of DNA band pattern of the sperm samples (1) sperm in the sil-select-stock 12F, (2) sperm in the sil-select-stock 12G, (3) sperm in the Tris-egg-yolk 24F, (4) sperm in the sil-select-stock 1F, (5) sperm in the CEP-D 24F, (6) sperm in the fresh sperm, (7) sperm in the CEP-D 12F sperm in the CEP-D 1F.

and dead spermatozoa. The spermatozoa viability depend on the mechanism of ion pump in and out of the cell.[3] Observations of spermatozoa viability that has been stained in negrosin-eosin are performed by a microscope under 400× magnification. The viable spermatozoa had colorless look due to the selective process to absorb the external materials including the color, while the dead spermatozoa had full color look because of it's inability to select the external materials that come into the cell. The viable spermatozoa have the selective permeability of the membrane. However, the dead spermatozoa have high membrane permeability.[1] The spermatozoa viability is observed every 6 hours on each treatment. The observation of the spermatozoa viability shown in Figure 1 above. The results showed that silk and CEPD diluting solution can preserve the viability of spermatozoa that is stored in it. The viability of spermatozoa highly depends on the stability of the cell membrane. Stable cell membrane will ensure normality of metabolic processes inside the cell. In the silk, the medium contains HEPES buffer that preserve the stability of the solution pH, even under temperature-changing condition. This state triggers the enzyme to work optimally. So the spermatozoa viability can be preserved. Other components such as human serum and albumin that are contained in the silk medium are also very helpful to spermatozoa viability. This ingredient supplies the nutrient for the spermatozoa.

The other good spermatozoa buffer is CEPD medium. The medium contains useful ingredients to preserve the cell membrane integrity, to trigger the enzyme working optimally, and to provide crucial nutrients to the spermatozoa. This state is very crucial to preserve the viability of spermatozoa. Resemble to the tris-egg yolk media, the spermatozoa that is stored in CEPD have the subdued viability.

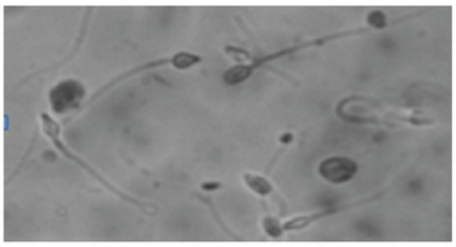

Fig. 5. The performance of DNA band pattern of the sperm samples (1) sperm in the sil-select-stock 12F, (2) sperm in the sil-select-stock 12G, (3) sperm in the Tris-egg-yolk 24F, (4) sperm in the sil-select-stock 1F, (5) sperm in the CEP-D 24F, (6) sperm in the fresh sperm, (7) sperm in the CEP-D 12F sperm in the CEP-D 1F.

Cell membrane integrity and stability are very crucial for the spermatozoa viability. Damaged cell membrane makes increasing of membrane permeability. This state will make the important ingredients inside cells will be released. And the toxic ingredients will enter the cell. Then, the life of the spermatozoa will be disrupted.[18]

Cell membranes can be damage by some factors. The existence of the dead spermatozoa will cause toxic presence for alive spermatozoa. The presence of these toxic substances will trigger level increasing of free radicals which will distrube the cell plasma membrane integrity.[23] The pH and temperature change during storing period can also increase the occurrence of lipid peroxidation. The result of free radicals with a lipid bilayer membrane a chain reaction that makes the damaged cell membrane is lipid peroxidation. This condition is prove by fully colored of died spermatozoa because of the damaged membrane. In the damaged membrane, the dye can be absorbed and stored in it freely. On the other hand, on the alive cell, the dye will be absorbed by the selective mechanism. The components inside solve media can keep the cell membrane from destruction.

The three kinds of diluent media gave the different influence to the sperm motility. For 6 hours storing period, the silk select stock gave the best result. For 12 hours storing period the silk-select-stock and the tris egg yolk gave almost same yields with the motility percentage. After storing period CEP-D gave the best result for 18 hours, three kinds of diluent media gave almost the same results. For 24 hours storing period the CEP-D and the tris egg yolk gave a result better than the silk-select-stock.

The energy source in the CEP-D is fructose, tris egg yolk contains lactose and raffinose, albumin is the dominant component in the silk-select-stock. The kind and the number of energy sources in the diluent medium influent the sperm motility. Albumin in the silk-select-stock can be metabolized quickly by the sperm to supply the energy for cell motility for 12 hours. Fructose as an energy source in the CEP-D can supply energy for cell motility longer than the silk-select-stock and tris egg yolk. Consequently, the CEP-D can maintain the sperm motility until 24 hours. The similar result for tris egg yolk that has more kinds of energy sources, so this medium can give a good result. Egg yolk as an energy supply can trigger the cell motility because the albumin contains glucose.

The sperm that is stored in the silk-select-stock have respiration process rapidly and have too much energy for sperm motility. So the nutrient containing in diluent medium deplete rapidly and the diluent medium can not supply sperm motility for long period.

Lactic acid as a result from metabolism process can cause pH decreasing of the diluent medium. In the low pH, the sperm can not motil or move normally. That is the others factor that causes the silk-select-stock can not maintain the sperm motility for long periode.

Metabolism process release ROS (*reactive oxygen species*) also.[12] The high contain ROS influence the DNA stability in the sperm. This condition affects the sperm motility. Cell membrane integrity is very important to maintain normality metabolism process in the cell. The damaged cell membrane

can block the ATP production. And the cell can not move normally.

There is no antibiotics compound in the silk-select-stock. Thus, there is no defect mechanism to microbials contamination in this diluent medium. Presenting the contaminant is very dangerous for cell activity. Some kinds of contaminant can release the toxic agent that can "kill" the cell. Fortunately, the CEP-D and the tris egg yolk contain penicillin and streptomycin as an antibiotic compound.

Based on Figure 3, it can be stated the human sperm that is stored in the tris egg yolk shown the highest MDA number (9.237 nmol/L), in the second is the sperm that is stored in the CEP-D (2,586 nmol/mL) and the sperm that is stored in the silk-select-stock shown the smallest MDA number (0.907 nmol/L). This number is lower than the fresh sperm (2.516 nmol/L). The facts indicated that too much sperm cells that is stored in the tris egg yolk have the damaging cell membrane due to the peroxidase reaction in its bilayer lipids. The cell membrane is built by some components: phospholipids, glicolipids, and cholesterol. Unsaturated fatty acid as a phospholipids component react with the free radicals easier and then create the lipids free radicals. In the aerob-conditioned lipids, free radicals react with an oxygen molecule to build hyperoxyde lipids that damage all of the cell components.

Inside the cells, the lipids-type free radicals break to become some kinds of a compound including the MDA (malondialdehide). Therefore, presenting the malondialdehide indicate the damaging process in the cells membrane. Its means the higher MDA number, the more cells membrane damaged. In the other word, the number of MDA indicate the presenting free radicals in the body. Free radicals influence to membrane fluidity. Therefore the intermembane transportation mechanism disturbance. Free radicals can block the enzyme sytem and rupture the receptor system. This condition creates metabolism disturbance. The other negative effects of free radicals in the body are these compounds react with cell or tissue protein and create the protein damaging. But, if this protein in the component of cell structure, the cell will be a rupture, if this protein is the enzyme component, the blocking of chemicals reaction will happen. Finally, the cells will die.

Free radicals can be caused by DNA mutation from the hydroxylation reaction on tymin and cytosin, opening process of purin and pyrirmidin ring and cutting process of phosphodiester chains of DNA. The yields are similar with the conclusion of former research which stated that tris egg yolk is the poor diluent media for human sperm storing.[20] On the other hand the CEP-D gave the good yields. The MDA number of the sperm that is stored in it have the MDA number almost same with that of human fresh sperm (CEP-D 2,586 nmol/L and the fresh spermatozoa 2,516 nmol/L). It means that the number of free radicals in the two kinds of sperms almost same. In the early research gave yields that the CEP-D is one kind of good diluent medium for human sperm storaging.[20] The sperm sample that is stored in the silk-select-stock, shown the lower MDA number than that of fresh sperm. Chemicals coumpound in the silk-select-stock can block the free radicals activity and give the high vitality to the sperm so the sperm can fight the free radicals activity by its self.

Some factors affect the genetic stability of the spermatozoa. First, the genetic stability depends on the sperm viability. To maintain the viability, it is very important to keep the mechanism of ion pump into and out of the cell.[13] The viability of spermatozoa which has stained in negrosin-eosin are observed by a microscope under 400× magnification. The viable spermatozoa had colorless look due to the selective process to absorb the external materials including the color. The viable spermatozoa have the selective permeability of the membrane. However, the dead spermatozoa have high membrane permeability.[1]

The crucial factors for the spermatozoa viability are cell membrane integrity and stability. Increasing of membrane permeability is caused by the damage cell membrane. This state will make the important ingredients inside cells will be released. And the toxic ingredients will enter the cell. Then, the life of the spermatozoa will be disrupted.[18]

The existence of the dead spermatozoa will cause toxic presence for alive spermatozoa. The presence of these toxic substances will trigger level increasing of free radicals that will distrube the cell plasma membrane integrity.[23] The pH and temperature change during storing period can also increase the occurrence of lipid peroxidation.[19] The result of free radicals with a lipid bilayer membrane a chain reaction that makes the damaged cell membrane is lipid peroxidation. This condition is prove by fully colored of died spermatozoa because of the damaged membrane. In the damaged membrane, the dye can be absorbed and stored in it freely, opposite on the alive cell. The components inside solve media can keep the cell membrane from destruction.[24]

The genetic stability of the sperm that is stored in the three kinds of diluent media in this research is determined by observe the similarity DNA band pattern of the sperms with that of the fresh sperm. Based on Figure 4 above the DNA band pattern number 1, 2, 4 and 6 are almost similar. DNA band number 6 is the fresh sperm. The DNA band pattern number 1, 2 and 4 are DNA band pattern from the sperm that is stored in the silk-select-stock. The other groups that have the similarity of DNA band pattern with that of number 6 are number 8, 7 and 5. These numbers are the DNA band pattern for the sperms that is stored in ther CEP-D diluent media. On the other hand, the DNA band pattern number 3 unsimilarity with that of number 6. The DNA band number 3 is the DNA band from the sperm that is storaged in the tris egg yolk.

Based on the previous data, it can be stated that silk-select-stock is the best human sperm diluent media which can preserve the genetic stability of the sperm. In the second rank is CEP-D diluent medium and the tris egg yolk is poor to maintain the genetic stability.

The DNA band pattern shown in Figure 4 matches the result of MDA number counting. The MDA number of the sperm that is stored in the silk-select-stock is the lowest, The MDA number the sperm in the CEP-D is higher than in the silk-select-stock. And the sperm that is stored in the tris egg yolk have the MDA number is the highest number.

The DNA band pattern in this research is extracted from the gene that control cell motility (from the DNA mitochondria). The sperm have good motility, while others have a good viability too.

4. CONCLUSION

In summary, we found the exectly diluent media for human sperm storaging. The silk-select-stock is the best diluent medium to maintain viability, cell membrane integrity and genetic stability of the human sperm that is storaged in it. The CEP-D and Tris

egg yolk are the best media to maintain human sperm motility that is storaged in it.

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