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MOTILITIES AND VIABILITIES OF SEPARATION RESULTS OF SPERMATOZOA X AND Y TECHNIQUE OF ANODE-CATHODE BY IN VITRO

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Abstract: Spermatozoa X and Y have different characteristics. Various techniques have been developed to exploit some differences to dissociate spermatozoa carriers of chromosomes X and Y. Technique with cathode-anode *in vitro* can be expected to be more practical, cheap, easy, and efficient compared to other methods. This research is a pure experimental laboratory with anode-cathode treatment at different time treatments and voltages. The research device is used factorial device with a pattern 2 x 4. The research results indicate that the motilities and viabilities of dissociation of spermatozoa X and Y with anode-cathode by *in Vitro* are motilities at 30.33% and viabilities at 67%. Result of spermatozoa Y at the cathode with motilities at 33.25% and viabilities at 65.63%. The best quality of spermatozoa Y dissociation in the cathode was obtained by treatment of voltage 1.0 volt for 10 minutes. Time treatment and voltage do not affect the motilities and viabilities of spermatozoa (P > 0.05).

Keywords: *Motility, Viability, Spermatozoa, Anode, Cathode.*

INTRODUCTION

Based on the characteristics and differences in spermatozoa X with Y spermatozoa in terms of weight, the material content of heredity, motility, and survival, various methods have been developed by researchers to obtain spermatozoa separation with higher accuracy. Various techniques to date have been developed with the aim of exploiting some of these differences to separate spermatozoa carrying X and Y chromosomes, including cefadex columns, swim-up techniques, side migration, H-Y antigen techniques, percoll columns, electrophoresis, and flow cytometry.

Separation by using electric current was carried out by Engelman et al in Puji Sari with a free-flow electrophoresis system and obtained a significant increase in Y spermatozoa in the anode [1]. At the same time, the spermatozoa X in the cathode has decreased. Bhattacharya, argued that X- chromosome spermatozoa were positive and Y-chromosome negative spermatozoa [2]. At the same time, a different opinion is that all spermatozoa as a whole are negative, and spermatozoa carrying X chromosomes have stronger negative currents [3].

From various separation techniques that have been applied before, the Cathode-Anode technique is the most practical, cheaper, easier, and more efficient in its implementation [4]. Spermatozoa X tends to be negatively charged (-), and Y spermatozoa tend to be positively charged (+). The voltage treatment significantly affects the results of spermatozoa X separation on the anode (P <0.05). This study focuses on the percentage of the best motility and viability results of the separation of X spermatozoa and Y spermatozoa using the anode-cathode technique in vitro. And how does the interaction effect of treating voltage and time on the results of the separation?

RESEARCH METHODS

This research is a pure (true) experimental research laboratory with a factorial design cathode-anode treatment at different voltages and times on a number of samples. The first factor is V (voltage) with a DC at level V1 = 0.5 volts; V2 = 1.0 volts. The second factor is T (time) with level T1 = 5 minutes; T2 = 10 minutes.

Sample inclusion criteria: sperm with a concentration of 10 million/ml or more, volume > 2 ml, degree of acidity (pH), normal color and smell, sperm examined after abstinence for 3-5 days, aged between 20-40 years.

Exclusion criteria or criteria for canceling or dropping out of samples for examination if the sperm sample in examination shows abnormalities, including the number of leukocytes in the examination, abnormal pH, abnormal odor, and unusual sperm color, sperm sample brought to the laboratory is more than 60 minutes after ejaculation, the sperm sample is contaminated with other substances before or during the test.

The research procedures include the preparation of materials and laboratory equipment. Preparation materials include human sperm, quinacrine mustard dihydrochloride, pH scale paper 0.1, a set of pH standard solutions, methanol solution, ethanol solution, buffered water solution, copper (Cu) plates, zinc (Zn) plates, pH buffer solution 5.75, paraffin, McIlvaine buffer, 2% eosin solution, 5% Ringer's lactate dextrose (LRD5), immersion oil, distilled water, object glass, deck glass, lens tissue, roll tissue, panel cloth, and special vaseline. Laboratory equipment includes a

fluorescent microscope, light microscope, and adapter. Nelles. Model AD-500, digital multimeter, ammeter, digital pH meter, cable for electrodes, hemocytometer, DC current 0.5 volts; 1.0 volts, freezers, hand counters, stopwatches, measuring cups with several volumes, pipettes, box preparations, Petri dish dishes, rectangular flasks, staining jars and mixing glasses.

The research design used was a factorial design with a 2 x 2 pattern, resulting in 4 treatments. The sample is sperm that meets the requirements of a research sample. From the number of samples that met the inclusion requirements, they were taken and selected and then divided into four treatments, each of which was repeated six times so that the number of samples used became 24.

The variables observed were the motility and viability of spermatozoa which affected the voltage and time with the anode-cathode in vitro.
Measurement of spermatozoa motility and viability, namely:

$$\text{Motility} = \frac{\text{Progressive motile spermatozoa count}}{\text{The number of spermatozoa observed}} \text{x}100\%$$

$$\text{Viability} = \frac{\text{Number of live spermatozoa}}{\text{The number of spermatozoa observed}} \text{ x } 100\%$$

The data were tabulated, and the average score of the percentage of X and Y spermatozoa was calculated in all treatments. The same way on the motility and viability of spermatozoa. To find out the homogeneity of the initial data, Levene`s test was used. To find out the normality distribution of the data, it was tested with the Kolmogorov-Smirnov test. If the data is normally distributed and homogeneous, then a parametric statistical test is performed with One Way ANOVA. In this study, the degree of significance was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

The study of motility and viability of separating X spermatozoa and Y spermatozoa with anode-cathode on human spermatozoa in vitro. The presentation includes data on spermatozoa variables before treatment, variable X spermatozoa at the anode, variable Y spermatozoa at the cathode, results of data analysis on the effect of voltage on X spermatozoa at the anode, results of data analysis on the effect of time on Y spermatozoa at the cathode, analysis of the interaction of voltage and time factors on the motility and viability of spermatozoa at the anode-cathode.

Sperm samples before treatment were examined, which included macroscopic and microscopic examinations. The macroscopic examination consists of abstinence, volume, time, pH, viscosity, liquefaction, odor, and color. The microscopic examination consists of a broad view, motility, concentration, and morphology. The examination results in Table 1 show that the sample meets the requirements for in vitro cathode-anode treatment in this study.

Table 1. The average normal value of sperm examination before treatment

Parameter	Average	Deviation Standard	Normal Value *)
1. Macroscopic:			
▪ Check time (minutes)	37.15	3.71	20-60
▪ Abstinence (day)	4.2	1.23	2-7
▪ Volume (ml)	3.51	0.63	2-5
▪ pH	7.52	0.29	7.2-8
▪ Viscosity (cm)	3.47	0.54	2-5
2. Microscopic:			
▪ Concentration (million/cc)	24.54	2.71	>20
▪ Morphology (%)	40.80	6.63	>14%
▪ Motility	50.61	2.77	>50
▪ Spherical cells	-	-	<1 jt/ml

*) Strasinger KS, Lorenzo SM. (2014).

Variable Spermatozoa X at Anode

Variable data of spermatozoa X at this anode included the effect of treatment with voltage (0.5 volts and 1.0 volts) and time (5 minutes and 10 minutes) on the motility and viability of spermatozoa.

Table 2 shows that the data from the motility variable of spermatozoa X at the anode with a 0.5-volt voltage treatment for 10 minutes on the 4th repetition obtained the highest motility percentage of 44%. The lowest motility of 20% was in the V2T1 treatment (1.0 volts for 5 minutes) on the 1st repetition and V2T2 (1.0 volts for 10 minutes) on the 1st.

The highest percentage of X spermatozoa viability at the anode was obtained by V1T1 treatment (0.5 volts for 5 minutes) in the 4th repetition of 76%, and the lowest occurred in V2T1 (1.0 volts for 5 minutes) in the 2nd repetition of 58%

Table 2. Spermatozoa X at anode

No.	Spermatozoa X at Anode		
	Treatment	Motility (%)*)	Viability (%)*)
1.	V1T1	33	75
2.	V1T1	30	70
3.	V1T1	25	70
4.	V1T1	31	76
5.	V1T1	32	72
6.	V1T1	30	73
7.	V2T1	20	66
8.	V2T1	28	58
9.	V2T1	25	66
10.	V2T1	25	61
11.	V2T1	23	63
12.	V2T1	21	60
13.	V1T2	39	74
14.	V1T2	30	68
15.	V1T2	25	73
16.	V1T2	44	63
17.	V1T2	35	67
18.	V1T2	40	70
19.	V2T2	20	69
20.	V2T2	41	66
21.	V2T2	25	60
22.	V2T2	35	62
23.	V2T2	34	63
24.	V2T2	37	65
Average		30.33	67.08
Minimum		20	58
Maximum		44	76

*) Source: Primary Data.
Information:
V1 = 0.5 volt; V2 = 1.0 volt; T1 = 5 minute; T2 = 10 minute.

Variable Spermatozoa Y at the Cathode

Variable data of Y spermatozoa at the cathode included the effect of voltage treatment (0.5 volts and 1.0 volts) and time (5 minutes and 10 minutes) on the motility and viability of spermatozoa.

Table 3 indicates that treatment number 18 at 0.5 volts for 10 minutes (V1T2) obtained the best or highest motility percentage of 43% and the lowest motility of 20% was in treatment number 8 at 1.0 volts for 5 minutes (V2T1). The best or highest percentage of viable spermatozoa (viability) was obtained at 0.5 volts for 10 minutes (V1T2) at 78%, and the lowest occurred at 1 volt for 5 minutes (V2T1) at 51%.

Data Analysis of the Effect of Voltage and Time on Spermatozoa Motility and Viability

Based on the normality test, data were obtained that were normally distributed ($P > 0.05$) for both variables, namely the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode. Data that is normally distributed is further tested to determine the homogeneity of the data. The homogeneity of the data ($P > 0.05$) was obtained for the motility and viability of X spermatozoa at the anode motility and viability of Y spermatozoa at the cathode.

Data that were normally distributed and homogeneous were subjected to parametric statistical tests with One Way Anova analysis to determine the effect of the treatment of voltage factor and time factor on motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode. (see table 4 and table 5). To analyze the interaction of voltage factor and time factor on the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode, the data were analyzed by 2 factor multivariate analysis (table 6).

The results of statistical tests using One Way Anova analysis (table 4) showed that the voltage had a very significant effect on the motility and viability of Y spermatozoa at the cathode and the viability of X spermatozoa at the anode ($P<0.01$). The results of the analysis of the effect of voltage on the motility of X spermatozoa at the anode had no significant effect ($P > 0.05$).

Overall, the effect of voltage on the motility and viability of X spermatozoa at the anode and Y

spermatozoa at the cathode showed significant results. Figure 1 (chart A, B, C, D) shows that the lower the voltage at the anode and cathode within certain limits, the higher the motility and viability of the spermatozoa.

Tabel 3. Spermatozoa Y at the cathode

No.	Spermatozoa Y at Cathode		
	Treatment	Motility (%)*)	Viability (%)*)
1.	V1T1	30	65
2.	V1T1	39	74
3.	V1T1	25	68
4.	V1T1	39	73
5.	V1T1	30	72
6.	V1T1	37	69
7.	V2T1	31	53
8.	V2T1	20	56
9.	V2T1	28	51
10.	V2T1	23	53
11.	V2T1	25	55
12.	V2T1	22	57
13.	V1T1	42	78
14.	V1T1	35	70
15.	V1T1	40	68
16.	V1T1	40	75
17.	V1T2	41	72
18.	V1T2	43	76
19.	V2T2	30	67
20.	V2T2	35	61
21.	V2T2	39	54
22.	V2T2	35	72
23.	V2T2	38	70
24.	V2T2	31	66
Related		33.25	65.63
Minimum		20	51
Maksimum		43	78

*) Source: Primary Data.
Information:
V1 = 0.5 volt; V2 = 1.0 volt; T1 = 5 minute; T2 = 10 minute

Table 4. One-way ANOVA analysis of the effect of voltage on X spermatozoa in the anode and Y spermatozoa at the cathode.

				Sum of Squares	df	Mean Square	F	Sig.
Motilities Anoda	Between Groups	(Combined)		150.000	1	150.000	3.536	.073
		Linear Term	Contrast	150.000	1	150.000	3.536	.073
	Within Groups			933.333	22	42.424		
	Total			1083.333	23			
Viabilities Anoda	Between Groups	(Combined)		352.667	1	352.667	29.260	.000
		Linear Term	Contrast	352.667	1	352.667	29.260	.000
	Within Groups			265.167	22	12.053		
	Total			617.833	23			
Motilities Katoda	Between Groups	(Combined)		294.000	1	294.000	8.224	.009
		Linear Term	Contrast	294.000	1	294.000	8.224	.009
	Within Groups			786.500	22	35.750		
	Total			1080.500	23			
Viabilities Katoda	Between Groups	(Combined)		876.042	1	876.042	25.643	.000
		Linear Term	Contrast	876.042	1	876.042	25.643	.000
	Within Groups			751.583	22	34.163		
	Total			1627.625	23			

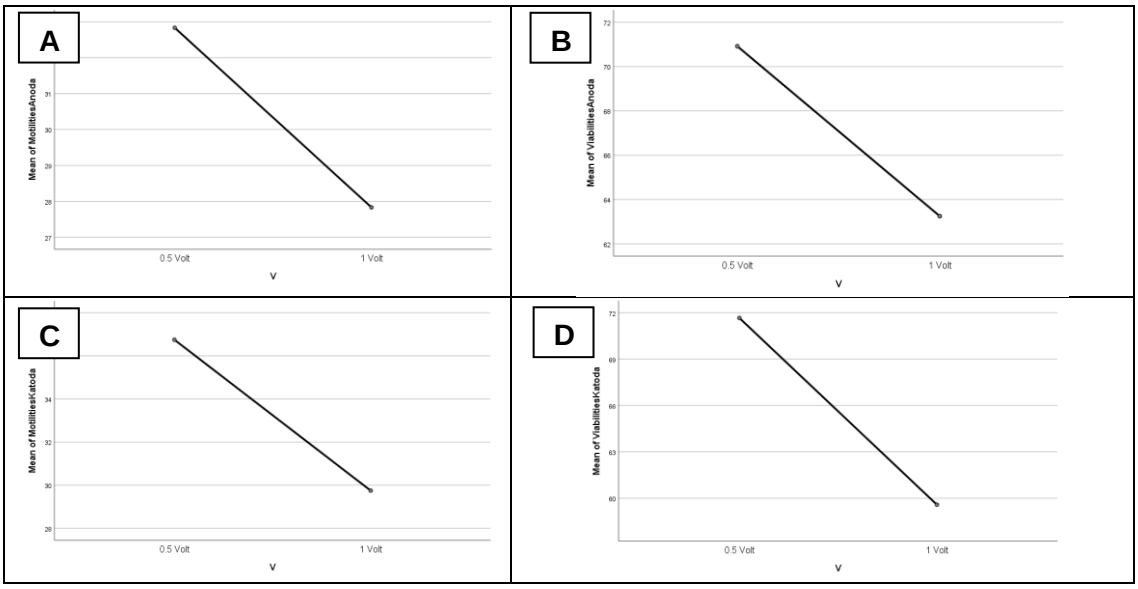

Figure 1. Effect of voltage on X spermatozoa at the anode and Y spermatozoa at the cathode

Table 5. One-way ANOVA analysis of the effect of time on X spermatozoa In the anode and Y spermatozoa at the cathode

				Sum of Squares	df	Mean Square	F	Sig.
Motilities Anoda	Between Groups	(Combined)		280.167	1	280.167	7.674	.011
		Linear Term	Contrast	280.167	1	280.167	7.674	.011
	Within Groups			803.167	22	36.508		
	Total			1083.333	23			
Viabilities Anoda	Between Groups	(Combined)		4.167	1	4.167	.149	.703
		Linear Term	Contrast	4.167	1	4.167	.149	.703
	Within Groups			613.667	22	27.894		
	Total			617.833	23			
Motilities Katoda	Between Groups	(Combined)		416.667	1	416.667	13.809	.001
		Linear Term	Contrast	416.667	1	416.667	13.809	.001
	Within Groups			663.833	22	30.174		
	Total			1080.500	23			
Viabilities Katoda	Between Groups	(Combined)		287.042	1	287.042	4.711	.041
		Linear Term	Contrast	287.042	1	287.042	4.711	.041
	Within Groups			1340.583	22	60.936		
	Total			1627.625	23			

The analysis using the One Way Anova statistical test (table 5) showed that the time treatment showed varying results, both for X spermatozoa at the anode and Y spermatozoa at the cathode. Time treatment had a significant effect on the motility of X spermatozoa at the anode ($P < 0.05$) and a highly significant effect on the motility of Y spermatozoa at the cathode ($P < 0.01$). The time treatment on the viability of X spermatozoa at the anode did not show significant results ($P > 0.05$), and the viability of X spermatozoa at the cathode showed significant values ($P < 0.05$).

Results of spermatozoa X at the anode and Y spermatozoa at the cathode (figure 2, charts A and C) show that the longer the time used, the higher the percentage of motility. Figure 2 shows that the shorter the time used to a certain extent, the higher the percentage of X spermatozoa viability at the anode. On the other hand, in graph D, the longer the time given to a certain time limit, the higher the percentage of viability of Y spermatozoa at the anode.

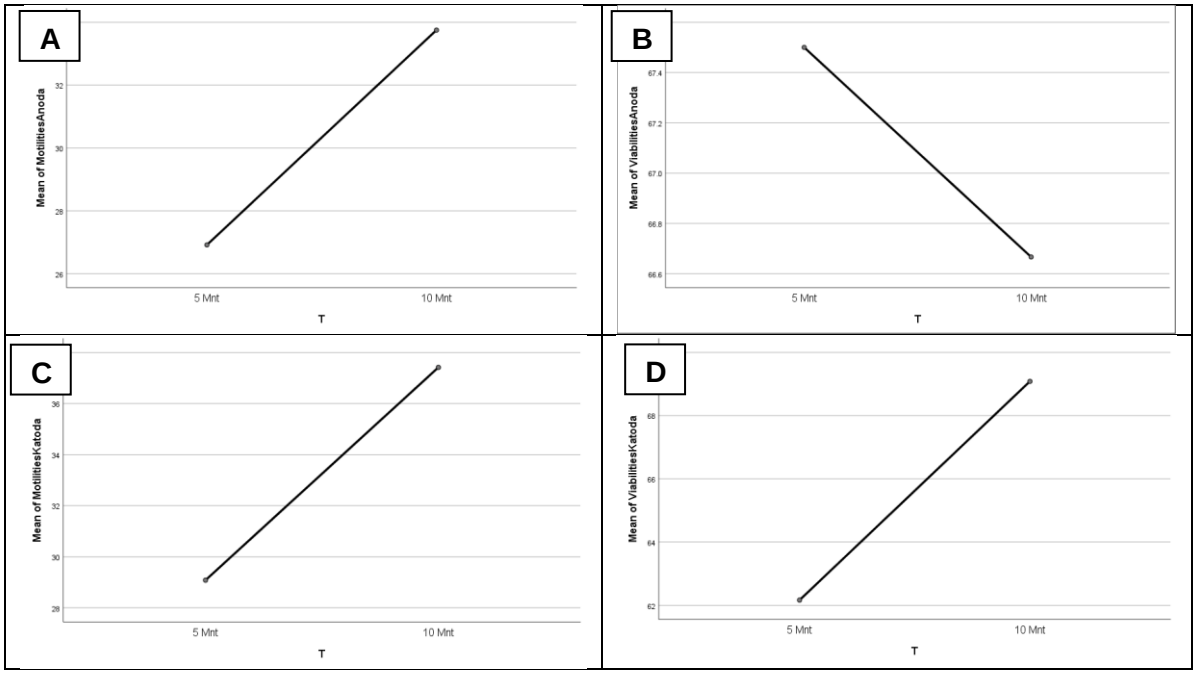

Figure 2. Effect of time on X spermatozoa at the anode and Y spermatozoa at the cathode

Table 6. Multivariate Test Analysis of the effect of the interaction of voltage and time against X spermatozoa at the anode and Y spermatozoa at the cathode

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.999	3519.525[b]	4.000	17.000	.000
	Wilks' Lambda	.001	3519.525[b]	4.000	17.000	.000
	Hotelling's Trace	828.124	3519.525[b]	4.000	17.000	.000
	Roy's Largest Root	828.124	3519.525[b]	4.000	17.000	.000
V (Voltage)	Pillai's Trace	.816	18.855[b]	4.000	17.000	.000
	Wilks' Lambda	.184	18.855[b]	4.000	17.000	.000
	Hotelling's Trace	4.437	18.855[b]	4.000	17.000	.000
	Roy's Largest Root	4.437	18.855[b]	4.000	17.000	.000
T (Time)	Pillai's Trace	.664	8.396[b]	4.000	17.000	.001
	Wilks' Lambda	.336	8.396[b]	4.000	17.000	.001
	Hotelling's Trace	1.975	8.396[b]	4.000	17.000	.001
	Roy's Largest Root	1.975	8.396[b]	4.000	17.000	.001
V * T	Pillai's Trace	.318	1.978[b]	4.000	17.000	.144
	Wilks' Lambda	.682	1.978[b]	4.000	17.000	.144
	Hotelling's Trace	.465	1.978[b]	4.000	17.000	.144
	Roy's Largest Root	.465	1.978[b]	4.000	17.000	.144

a. Design: Intercept + V + T + V * T
b. The statistic is an upper bound on F that yields a lower bound on the significance level
c. Exact statistic

Table 6 shows that the analysis of the effect of the voltage factor on the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode showed very significant results. Likewise, the analysis of the effect of the time factor on the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode showed significant results. ($P < 0.01$)

The results of the analysis of the interaction of voltage and time on the motility and viability of spermatozoa Y at the cathode and X at the anode were analyzed using 2 multivariate factor analyses. The analysis showed a less significant effect on the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode ($P > 0.05$) (Table 6).

Spermatozoa Motility and Viability Data Analysis.

The results of observing motility with a light microscope at 400x magnification showed that at the cathode, the average percentage of motility (44%) was slightly better than the average motility of spermatozoa at the anode (43%).

Viability observation data with 400x and 1000x magnification obtained data that at the anode (Table 2) the average viability of X spermatozoa was obtained which was higher (67.08%) compared to the average viability of Y spermatozoa at the cathode (table 3) of 65.63%.

Table 2 shows that at the anode, the average percentage of X spermatozoa motility ranges from 20% to 44%. The highest percentage (44%) was obtained in the V1T2 treatment (0.5 volts for 10 minutes), and the lowest (20%) was obtained in the V2T1 treatment (1.0 volts for 5 minutes). At the cathode (Table 3), the average motility percentage of Y spermatozoa ranged from 20% to 43%. The highest (43%) was obtained in the V1T2 treatment (0.5 volts for 10 minutes). The lowest (20%) was obtained in the V2T1 treatment (1.0 volts for 5 minutes).

Table 2 shows that the percentage of viability ranges from 58% to 76%. The highest viability (76%) was obtained in 0.5-volt treatment for 5 minutes (V1T1). The lowest (58%) was obtained by treating 1.0 volts for 5 minutes (V2T1). At the cathode, the viability percentage ranges from 51% to 78%. The highest (78%) was obtained by treating 0.5 volts for 10 minutes and the lowest (51%) was obtained by treating 1.0 volts for 5 minutes (V2T1).

Spermatozoa motility is influenced by several things, including time of examination after ejaculation, the time between ejaculations, temperature, ionic composition, electromagnetic radiation, the orientation of spermatozoa, viscosity, pH, osmotic pressure, immunological aspects as well as the presence of motility stimulation and inhibition factors [6].

The speed of moving spermatozoa decreases along with the time after ejaculation. The speed of spermatozoa decreased to 31 microns per second after 1 hour, and after 5 hours decreased to 12 microns per second [6). Increased motility is also affected by an increase in temperature. There is no movement at 5-10 °C, and the movement speed increases until the temperature is 40-44 °C. The increase in motility is almost linear at 5-35 °C. Sudden cold temperatures can cause a decrease in ATP content so that motility can be lost. When the temperature is cooled slowly, and a cryoprotective agent such as glycerol is added, the spermatozoa can survive up to –196 °C for a long time. In frozen storage, viability and motility decline after 1 year [6].

Spermatozoa motility will be better if the amount of non-electrolyte substances exceeds the electrolyte content. Spermatozoa tend to be permeable to inorganic ions, and these ions greatly affect the bioelectric status of the spermatozoa membrane, which will affect membrane permeability. Potassium ions will affect the membrane potential, while sodium ions have little effect on the membrane. Adding calcium ions will reverse the membrane potential from –10mV to +10mV. The distribution of ions across the membrane depends on the metabolic energy [6].

Ultraviolet rays and X-rays can affect spermatozoa. X-ray radiation at 32000 R affects the motility of spermatozoa. Even a much lower dose emitted during spermatogenesis can cause genetic damage [6]. Changes in hydrogen ion concentration greatly affect the motility of spermatozoa. At pH 3-4 the motility will decrease, and the motility of spermatozoa will increase with increasing pH and reach optimal pH between 7-8, while partial motility can be maintained at pH between 5-10 [6].

The motility of spermatozoa should be examined after 20 minutes from ejaculation but not more than 60 minutes because with increasing time, the movement of spermatozoa will worsen, and the pH and smell can change [7]. Spermatozoa motility under field microscopy, which was observed carefully and systematically, was divided into four categories (graded), namely: a. Move very fast, go straight ahead; b. Movement less fast/slow, forward straight ahead; c. Move on the spot; d. Silent/not moving.

Spermatozoa viability is the ability of spermatozoa to survive after experiencing liquefaction and is an important factor in determining the quality of a man's spermatozoa. The higher the viability of the spermatozoa, the higher the chance for fertilization to occur during copulation, both naturally and artificially [8].

When motility is reported to be less than 5% to 10%, viability testing is recommended because very low motility can result from dead sperm or necrospermia. The most common viability assessment involves staining with Eosin Y, followed by the blue-black stain of Nigrosin. Live sperm with intact cell membranes will not pick up the dye and remain unstained. This test will differentiate necrospermia from immotile sperm secondary to structural flagellar defects such as in Kartagener's syndrome and primary ciliary dyskinesia. [8].

Viability of spermatozoa in cattle (Balinese cattle), when compared with the percentage of viability of fresh spermatozoa (95%), the percentage of viability of spermatozoa after liquefaction has passed tends to decrease. It is caused by the reduced nutrient content in the diluent and the longer time, so the percentage of live spermatozoa produced also decreases. It is the opinion of Utomo and Sumaryati [9] that the length of storage time greatly affects the quality of spermatozoa. The longer the storage time, the more

nutrients contained in the diluent will decrease. According to Salisbury [10], spermatozoa quality during storage decreased due to cold shock, changes in pH, and reduced nutrients contained in the diluent.

The results of sperm analysis, including viability, can be negatively affected by external factors such as the temperature at which the sample was left before the start of the analysis and the time that elapses between ejaculation and the start of the test. This information should be recorded in the results report before making necessary judgments.

Analysis of the Effect of Voltage and Time

Statistical test results (table 6) show that voltage and time have a very significant effect on the motility and viability of X spermatozoa at the anode and Y spermatozoa at the cathode with a significance value of 0.000 ($P < 0.01$) on the value of Pillai`s, Wilks Lambda, Hotelling`s and Roy`s ($P < 0.05$). The interaction of voltage and time factors on the separation of X and Y spermatozoa did not significantly affect the motility and viability of spermatozoa, with a significance value of 0.114 ($P > 0.05$).

Musanip [4] stated that the voltage treatment and the length of separation time at the anode and cathode greatly influenced the results of the separation of X spermatozoa and Y spermatozoa. It was also reported that X spermatozoa tended to be negatively charged (-), and Y spermatozoa tended to be positively charged (+). In accordance with this statement, spermatozoa containing the dominant X chromosome are on the positive current (anode), and spermatozoa containing the dominant Y chromosome are on the negative current (cathode).

Based on that finding, the motility and viability of the separation results are closely related to the ionic charge of the cell membrane found in spermatozoa. So that an electric current with a certain time can affect the spermatozoa membrane and even damage this cell membrane. Therefore, treatment with electric current, both anode, and cathode, cannot be given for too long.

A cell membrane covers spermatozoa from head to tail, which has a very complex arrangement both in molecular composition and functionally. The spermatozoa membrane of each region has a special function. The membrane on the head plays a role in the capacitation process, the acrosome reaction, and then plays a role in the penetration of the zona pellucida in the fertilization process. The membrane on the back of the acrosome (post acrosomal region) makes first contact and becomes one with the Oolema ovum in the fertilization process. In comparison, the membrane on the tail obtains a substrate for spermatozoa energy and transmits waves of movement [12].

There are several ways to transport materials (substances) through the membrane, namely: free diffusion and conditional/facilitated diffusion, active transport (symport and antiport), endocytosis, and exocytosis [11]. The highest number or yield is the best-expected motility and viability. On the viability of X spermatozoa at the anode, the effect of time gave opposite results to the viability at the cathode; namely, the longer the treatment time, the lower the viability results.

Factors causing the low percentage values of motility and viability at both the anode and cathode are mainly due to the high or low voltage supplied. The data above shows that the higher the voltage, the lower the value of the motility and viability variables. Conversely, the motility and viability variables will be higher if the lower applied voltage is. Another reason for the low percentage of motility and viability is the length of treatment time. It also greatly affects the visual acuity (concentration) of spermatozoa. The longer the treatment time, the more visual field of spermatozoa will be higher. Still, the variable percentage of motility and viability will decrease along with the changes in the cell membrane of spermatozoa.

The ion pump in the spermatozoa cell membrane is certainly influenced by the voltage and treatment time. So the interaction of these two factors had no significant effect on the motility and viability of spermatozoa.

Anode and Cathode Analysis

The basic principle of this separation is that the X and Y spermatozoa have opposite electrical charges. This technique uses two metal plates, namely copper (Cu) as the anode and zinc (Zn) as the cathode, where both positive and negative charges flow simultaneously. The results of this separation obtained a significant increase at each pole. The Y spermatozoa at the cathode averaged 83.91%. In comparison, the X spermatozoa in the anode averaged 81.19% [4].

Based on the characteristics and differences between X spermatozoa and Y spermatozoa, various techniques have been developed by researchers to obtain the separation of spermatozoa with higher accuracy. To date, various techniques have been developed to exploit some of these differences to separate spermatozoa carrying X and Y chromosomes, including:

Electrophoretic technique

Engelman et al. in Puji Sari [1] used a free-flow electrophoresis system and obtained a significant increase in Y spermatozoa in the anode. At the same time, the X spermatozoa in the cathode decreased.

Flow cytometry

A technique developed using a Flow cytometric cell sorting tool, which can separate X

and Y spermatozoa based on differences in DNA content. The principle of this technique for separating X and Y spermatozoa is that the difference in DNA content is measured using a fluorescent DNA binding stain. The sorting results were then tested using the FISH (Fluorescent In Situ Hybridisation) technique using an alpha satellite probe DNA.

Separation with this technique succeeded in obtaining the viability of X and Y sperm which have been sorted and can last several hours in good conditions. Thus, this flow cytometry technique can be used for in vitro fertilization or intra oviductal insemination, the desire to have a boy or a girl, and the detection or prevention of X-strand disease. About 82% of the sorted spermatozoa provide a probe hybridization signal with a Y chromosome, while the rest signal hybridization with the X chromosome.

Cephadex column

Cefadex column is a non-equilibrium sedimentation technique (which is based on sedimentation rate) in the form of spermatozoa filtration through a cefadex column containing cefadex gel grains/seeds with a diameter of 40-120 µm. Spermatozoa are taken from a certain fraction of the filtrate. Separation using filtration, it was found that X spermatozoa moved down on the basis of their own specific gravity and motility, while Y was still held by the cephalex gel particles, so they could not go down. Another cause is the attraction of negatively charged Y spermatozoa with positively charged cefadex. Thus positively charged X spermatozoa will be released, while Y spermatozoa with a negative electrical charge will be attracted [17]. In humans, the success of this technique reaches 70-80% [14]. Filtration with cefadex resulted in 70% spermatozoa X in the filtrate [13]. The results of the research by Mahaputra [15] showed that by separating sheep spermatozoa with a G-200 cefadex column, they succeeded in obtaining X and Y spermatozoa with a ratio of 81.3% and 18.7%. The results of research by Susilawati et al. [16] the results of the filtration of cattle spermatozoa using cefadex G-200 showed that the proportion of X spermatozoa was the same as that of the female calf that was born was 83.1%.

Bovine serum albumin (BSA).

According to Ericson et al. [17], who reported the separation of human X spermatozoa and Y spermatozoa using a column containing bovine serum albumin, the separation of Y spermatozoa was up to 85%. The research was based on the premise that Y spermatozoa have a greater ability to penetrate the surface between solutions with a discontinuous gradient than isolated medium. In research conducted by Tjokronegoro and Herman [18], separating X and Y spermatozoa using the "human serum albumin gradient" yielded an average percentage of Y sperm of 72.18%.

Swim up and side migration techniques

Swim-up is a preparation procedure that allows motile spermatozoa to migrate to the surface of fresh media [19]. Parrish et al. [20] modified this technique from human medicine for use in in vitro fertilization in cattle. Morphological differences and biological activity of Y spermatozoa are lighter and slimmer than spermatozoa with X chromosome, so it is possible that their migration power will be faster on the surface or sideways migration.

Based on the differences in movement characteristics between spermatozoa with X and Y chromosomes, a swim-up and side migration method were developed to separate spermatozoa [21]. Madura cattle preparation with Swim up or a combination of Swim up with Side migration can significantly change the proportion of spermatozoa with Y chromosome, which is more than spermatozoa with X chromosome [15]. In detail, the results of Yuliani's research [21], it was found that the ratio of spermatozoa with the Y chromosome in Bali cattle based on chromosome analysis was 4:73, 3:14, 2:56, 6:55, and 1:18, respectively for 30-minute swim up, 45 minutes, 60 minutes, combined Swim up with Aside migration and control. This data shows that the highest ratio of X and Y spermatozoa is in the combination of Swim up with Side migration.

H-Y antigen technique

The H-Y antigen technique is an attempt to separate spermatozoa based on the presence of a specific male antigen, namely the H-Y antigen. To identify Y spermatozoa containing this antigen, H-Y antibodies competed with fluorescently labeled antibodies. The success of this technique does not exceed 80-90% [22].

This technique is based on the theory that on the Y chromosome, there is a gene known as the M gene (male-determining gene). The presence of the M gene will induce the indifference of the medulla gonad to form testicular tissue. Besides that, the presence of the M gene can control the formation of cell surface proteins (surface antigens) known as HY antigens (Histocompatibility Y). The presence of these antigens will trigger the gonads to form an orderly structure of the testicular tissue. It is known that the M gene is located in the pseudoautosomal region of the Y chromosome (SRY). If there is no HY antigen, it is not formed or there is an effect on the HY protein receptor, causing testicular failure to occur.

Percoll Column

Separation of X and Y sperm using another density technique was carried out by Kaneko et al. [23]. This study used Percoll and Ficoll Paque density gradient centrifugation (12-step Discontinuous Percoll Gradient) to increase the number of spermatozoa carrying the X chromosome. Sperm that had been passed through

the Percoll gradient was then labeled with FISH (Fluorescence In situ Hybridization) using a specific chromosomal DNA probe. The average ratio of X and Y spermatozoa is 49: 48%. Research conducted by Suhana et al. [24] stated that with increasing Percoll density, the number of X spermatozoa would also increase, and conversely, the number of Y sperm would decrease.

To increase the effectiveness of harvesting X spermatozoa with a Percoll column with a number of different gradients with different densities, Schwering (1991) separated bovine spermatozoa by fractionating 2 times through 10 Percoll gradients with densities ranging from 1,034-1,068 g/ml.

In human and animal FIV, percoll has been used to obtain motile spermatozoa with a recovery of about 50% (discontinuous Percoll gradient 55 and 90%, density 1,082-1,117 g/ml) [25].

CONCLUSION

The best motility of spermatozoa X separated at the anode was obtained in the 0.5-volt treatment for 10 minutes with an average yield of 35.5%. The best viability was obtained in the 0.5-volt treatment for 5 minutes with an average value of 73.17%. The best motility of Y spermatozoa from the cathode separation was obtained at 0.5-volt treatment for 10 minutes with an average value of 40.17%, and the best viability was obtained at 0.5-volt treatment for 10 minutes with an average value of 73.17%. The voltage and time treatments significantly affected the motility and viability of X and Y spermatozoa resulting from in vitro cathode-anode separation ($P < 0.01$). The interaction of voltage and time treatment did not significantly affect the motility and viability of X and Y spermatozoa due to in vitro cathode-anode separation technique ($P > 0.05$).

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