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Original Article

Computer aided semen analysis (CASA) to determine the quality and fertility of frozen thawed sumba ongole sperm supplemented with amino acids

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Abstrak

Tujuan: Penelitian ini bertujuan untuk mengetahui kualitas dan kapasitas fertilitas spermatozoa pejantan Sumba Ongole (SO) beku-cair dengan asam amino terpilih yang diuji menggunakan computer assisted semen analyzer (CASA).
Metode: Sebanyak 18 ejakulasi dikumpulkan seminggu sekali dari tiga ekor pejantan Sumba Ongole dan diencerkan dalam pengencer tris-citric-fructose-Egg yolk (TCFY) dan berbagai konsentrasi asam amino terpilih. Spermatozoa pasca thawing diperiksa motilitasnya, pergerakan kepala dan pola geraknya menggunakan teknik CASA. Data dianalisis menggunakan SPSS 24 dengan pengujian ANOVA.
Hasil: Motilitas total (72,56% ±3,20) dan motilitas progresif (71,11%±3,31) penambahan Glutamin 5% berbeda nyata ($p<0,05$) dibandingkan perlakuan lainnya. Penambahan sistein 7mM menghasilkan karakteristik kecepatan yang lebih tinggi secara signifikan ($p<0,05$) pada parameter velocity average path (VAP), velocity curvilinear (VCL), velocity straight line(VSL) serta memiliki nilai distance average path (DAP), distance curve line (DCL), dan distance straight line (DSL) yang lebih tinggi ($p<0,05$).
Kesimpulan: Pengenalan sistem CASA memungkinkan kuantifikasi karakteristik motilitas spermatozoa individu dan CASA dapat digunakan untuk memprediksi kemampuan fertilisasi semen berdasarkan karakteristik kecepatan sperma.

Kata Kunci: CASA; Fertilitas; Kriopreservasi; Spermatozoa; Sumba Ongole

Abstract

Objective: The objective of this research was to investigate the quality and reproductive potential of frozen-thawed Sumba Ongole (SO) bull spermatozoa supplemented with specified amino acids using computer-assisted semen analysis (CASA).
Methods: total of 18 ejaculates were collected once a week from three Sumba Ongole bulls and diluted in tris-citric-fructose-egg yolk (TCFY) extender and various concentrations of selected amino acids. The post-thaw spermatozoa were examined for motility, head behavior, and swimming pattern using CASA technique. Data were analysis using SPSS 24 ebvaluated by ANOVA.
Results: Total motility (72.56%±3.20) and progressive motility (71.11%±3.31) the addition of Glutamine 5% significantly different ($p<0.05$) than other treatments. The addition of 7mM cysteine resulted in significantly ($p<0.05$) in velocity average path (VAP), velocity curve line (VCL), velocity

straight line(VSL) parameters also have higher in distance average path (DAP), distance curve line (DCL), dan distance straight line (DSL) parameters significantly ($p<0,05$).
Conclusions: In conclusion, the introduction of CASA systems enabled quantification of individual spermatozoa motility patterns and CASA colud be used to estimate the fertilizing capacity of sperm based on the characteristics of sperm velocity.

Keywords: CASA; Cryopreservation; Fertility; Spermatozoa; Sumba Ongole

INTRODUCTION

Sumba Ongole (SO) cattle are an indigenous Indonesian breed that has thrived on Sumba Island, East Nusa Tenggara Province. The cattle were declared indigenous to Indonesia by the Indonesian Ministry of Agriculture in a decision numbered 427/Kpts/SR.120/3/2014. SO cattle were Bos indicus breeds that were maintained in Indonesia as beef cattle. In Indonesia, insufficient information on the productivity of SO cattle is available [1]. The ability to retain the quality of spermatozoa throughout the freezing process is a critical aspect of reproductive capability.

During cryopreservation, sperm are subjected to physiological and structural stressors as a consequence of changes in osmotic balance, oxidative stress, and the production of intracellular ice crystals, requiring the use of antioxidants and cryoprotective agents (CPAs) [2]. The male reproductive tract generates a wide variety of antioxidant scavengers capable of shielding spermatozoa against ROS damage. The presence of oxidative stress (OS) and reactive oxygen species (ROS) in the male reproductive tract is highly associated with infertility [3].

Cysteine acts as a direct radical scavenger, increasing intracellular glutathione (GSH) synthesis and protecting proteins, DNA, and membrane lipids, cystine has been proven to protect spermatozoa against cryo-damage [4,5]. Glutathione production is increased in rabbit spermatozoa when glutamine is added to extender solutions by increasing GSH production, glutamine protects the spermatozoa from ROS-induced cryodamage [6]. Previous research discovered that adding 15 mM glycine, 15 mM glutamine, and 5 mM cysteine to bovine bull cryopreservation extender increased its cryoprotecting efficacy and ensured that all DNA spermatozoa were preserved [7].

The evaluation of sperm motility and morphology is regarded as critical parameters in the CASA system. These systems enable the objective evaluation of various cell characteristics (motility, velocity and morphology) [8,9]. The CASA is a new emerging laboratory tool for objectively analyzing semen samples. It enables the assessment of sperm kinematics in ejaculates using single sperm cell measurements [10]. A combination of semen motility characteristics may more accurately predict a bull's reproductive performance than a single test.

Furthermore, [11] reported sperm motility is a major component in evaluating the quality of sperm because it is often recognized as one of the most essential features connected with spermatozoa's capacity to reproduce. The development of CASA systems based on video images allows the determination of individual sperm motility qualities and the assessment of sperm fertility capability utilizing sperm velocity parameters. The purpose of this study was to assess the quality and fertility potential of cryopreserved bull sperm that had been supplemented with selected amino acids using a CASA system.

MATERIALS AND METHODS

Animals

A total of 18 ejaculates, were collected once a week from three Sumba Ongole bulls (5-6 years age) with six replicates. Sumba Ongole bulls were utilized as semen donors at PT. Karya Anugerah Rumpin, a private cattle breeding company in West Java, Indonesia. Every week, sperm was collected using an artificial vagina. Prior to freezing, the volume, concentration, motility, and abnormalities of the sperm were determined. This study used ejaculates that fulfilled the minimal requirement for sperm motility (70%) and sperm morphological normality (80%).

Cryopreservation of spermatozoa

As a control, we used a Tris-citric-acid-fructose egg yolk (TCFY) diluent consisting of 3.09 % Tris (hydroxymethyl) aminomethan, 1.73 % citrate acid, and 1.27 % fructose, with an additional 20% (v/v) egg yolk and 1% (v/v) Penstrep (Pennicilin and streptomycin) antibiotic. The control extender was supplemented with glutamine and glycine at concentrations of 5, 15, and 25 mM, respectively, and cysteine at doses of 3, 5, and 7 mM. Each extender was used to dilute the spermatozoa in a 0.25 mL polyvinyl straw to a concentration of 25 million. Semen were then equilibrated for 2 hours at 4°C in 0.25 mL straw before to analysis. The straws were stacked horizontally on a rack after equilibration and frozen in a vapor 5 cm above liquid nitrogen for 10 minutes. Then it went into liquid nitrogen. [12].

CASA motility assessment

The cryopreserved semen samples were thawed in a water bath at 37°C for 30 seconds and further diluted by adjusting the sperm concentration to $20x10^6$/ml using Tris buffer. CASA analysis was done by using Spermvision™ 3.7.8 Minitube Germany. The prepared semen sample of 3 µl was loaded on the Leja counting chamber (Leja Product, Ltd.) at 37°C. Four fields microscopic were captured at 200X magnification and analyzed (approximately 400 sperm) for each semen sample.

During the analysis, the sperm motion characteristics like sperm motility (Tmot= total motility), Pmot (progressive motility), DAP (Distance average path is time average distance of sperm head along its average path), DCL (Distance curve linear is time average distance of sperm head along its actual curvelinear path), DSL (Distance straight line is time average distance of sperm head along the straight line between its first detected position and its last), VAP (average path velocity is time average velocity of sperm head along its average path), VCL (Velocity curvelinear is a measure of cell vigor in time average velocity of sperm head along its actual curvelinear path), VSL (Velocity straight line is time average velocity of sperm head along the straight line between its first detected position and its last), LIN (Linearity of curve linear path from VSL/ VCL), STR (Straightness is the linearity of average path from VSL/VAP), ALH (Amplitude of lateral head displacement) and BCF (Beat cross frequency is the average rate at which curvilinear path crosses the average path), were studied.

Recovery rate

The percentage of sperm that can be retrieved after thawing frozen sperm is known as the recovery rate, and it must be at least 50% [12]. The recovery rate was calculated by dividing post-thawing spermatozoa motility by fresh semen motility. Recovery rate = (post thawing motility / fresh semen motility) x 100%.

Statistical analysis

The Statistical Product and Service Solution (SPSS) version 24 was used to analyze the data. The Shapiro & Wilk test was used to establish data normality, whereas the Levene test was used to demonstrate data homogeneity. To check whether there were any changes between treatments, the data was evaluated using one-factor variance analysis (ANOVA) and Tukey testing.

RESULTS

Tables 1 and 2 show the macroscopic and microscopic examination of fresh sperm samples. Table 3 and Figure 1 show the effects of adding amino acids (glycine, glutamine, and cysteine) to frozen-thawed Sumba Ongole bull sperm motility and velocity characteristics of TCFY extenders.

Table 1. Macroscopic evaluation of fresh semen samples

Parameters	Value (Mean±SD)
Volume (mL)	6.58 ± 0.37
pH	7.00 ± 0.00
Color	Creamy
Smell	Distinctive
Consistency	Thick milky

In this study fertilizing ability of sperm were estimated based on velocity characteristics (VAP, VCL, VSL) after addition of selected amino acid. Data in Table 3 showed

that among the velocity traits and the variation among the amino acid treatments, cysteine 7mM were significantly ($p<0.05$) higher velocity characteristics (VAP, VCL, VSL) than other. The results are in line with the data shown in Table 4 that cysteine 7mM significantly ($p<0.05$) higher in DAP, DCL, DSL.

Table 2. Microscopic evaluation of fresh semen samples

Parameters	Average ± SD
Concentration (x10^9 cell/mL)	1848.33 ± 49.72
Mass movement (Scooring)	(++)
Motility (%)	74.97 ± 2.89
Viability (%)	71.16 ± 5.10
Plasma membrane integrity (%)	70.42 ± 4.13
Abnormality (%)	7.25 ± 1.32

Data are shown in Table 5 that the greatest recovery rate (RR) value was 5 mM glutamine and the lowest was 25 mM glutamine. One of the effective cryopreservation parameters was the recovery rate (RR) of spermatozoa, which was defined as their ability to recover after freezing.

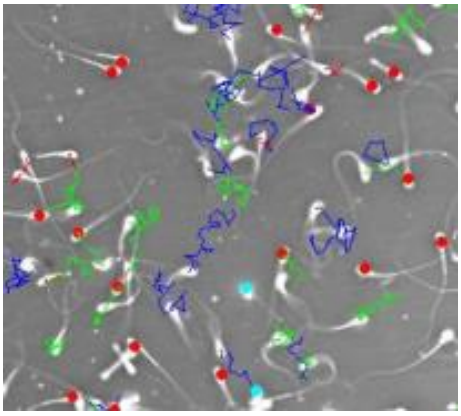

Figure 1. Motion of sumba ongole bull sperm after the addition selected of amino acids; Green line= progressive motility, cyan line = local motility, red line= immotile and blue line= hyperactive

DISCUSSION

According to macroscopic and microscopic examinations, the fresh semen samples utilized in this investigation seemed to be in the normal category and suitable for cryopreservation. Ejaculates with sperm motility of at least 70% and morphologically normal sperm of at least 80% [7].

The activities of sperm processing during cryopreservation induce damage to the sperm membrane, altering sperm physiology and might affecting sperm motility [13]. Reactive oxygen species are formed during the freezing process, which causes sperm cryodamage and impaired fertilization potential. The decreased sperm motility, decreased mitochondrial activity, and plasma membrane, acrosomal motility might all be explained by an increase in reactive oxygen species (ROS) generation [14,15]. Amino acids are seminal plasma components that have been used in different combinations as cryoprotectants for cryopreservation of sperm in several bovine species [16,17].

[18] Reported A number of studies have indicated that the typical microscopic visual approach for determining the motile sperm ratio is erroneous, imprecise, and has limited reproducibility. There is a lack of evidence-based information on the link between bull spermatozoa's motility/velocity properties and their capacity to fertilize. As a result, it is unknown whether these parameters can accurately predict sperm fertility.

Data in Table 3 showed that addition of 5mM glutamine and 5mM when compared to the control, cysteine resulted in significant ($p<0.05$) increase in post-thawing sperm total motility and progressive motility. Furthermore, the addition of more than 5 mM doses of each amino acid significantly increased ($p<0.05$) reduced sperm motility. The distance motility traits of frozen-thawed Sumba Ongole Bull spermatozoa after addition of selected amino acid are shown in Table 4. The data shown in Table 4 that cysteine 7mM is significant ($p<0.05$) higher in DAP, DCL, DSL.

The results are in line with the data shown in Table 4 that cysteine 7mM is significant ($p<0.05$) higher in DAP, DCL, DSL. These data indicated that addition of cysteine 7mM in TCFY extender semen provide faster movement of spermatozoa so that it can be predicted to have higher fertilization ability.

The addition of 7mM cysteine increased VAP, VCL, and VSL significantly ($p<0.05$) when compared to the control (Table 1). When compared to previous research [19], it seems

Table 3. The motility and velocity characteristics of frozen-thawed Sumba Ongole bull sperm after amino acid addition

Treatment		Parameter ± SEM							
		Tmot (%)	Pmot (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	WOB (%)	ALH (μm)	BCF (Hz)
Control		61.51±1.99	59.68±2.18	60.13±8.35	94.37±16.01	47.65±8.40	63.4±2.3	4.09±0.62	26.40±3.56
Glycine	5 mM	60.55±4.26^{a}	59.21±4.67^{a}	62.96±2.28^{a}	117.44±2.28^{a*}	47.33±4.41^{a}	53.0±2.0^{a*}	4.16±0.43^{a}	25.99±3.38^{a}
	15 mM	51.52±7.69^{b*}	49.45±7.67^{b*}	59.25±1.16^{b}	101.45±4.21^{c}	46.97±1.67^{a}	58.0±1.87^{b*}	3.86±0.28^{b}	26.47±1.28^{a}
	25 mM	56.69±8.27^{ab}	54.58±8.35^{ab}	62.83±2.55^{a}	110.01±4.75^{b*}	49.09±1.56^{a}	56.4±1.51^{b*}	4.69±0.26^{c*}	24.67±1.98^{a}
Glutamine	5 mM	72.56±3.20^{a*}	71.11±3.31^{a*}	60.66±1.28^{a}	104.45±2.70^{a}	43.62±0.86^{a}	57.5±1.29^{a*}	5.18±0.16^{a*}	20.94±0.29^{a*}
	15 mM	56.16±8.36^{b}	52.91±8.66^{b}	54.82±2.26^{b}	91.75±2.70^{b}	41.35±1.70^{b*}	59.4±1.51^{a*}	4.07±0.14^{b}	23.91±1.04^{b}
	25 mM	32.38±4.87^{c*}	31.20±5.26^{c*}	58.78±3.71^{a}	90.62±9.95^{b}	48.00±1.48^{c}	64.8±3.49^{b}	2.96±0.50^{c*}	28.12±1.61^{c}
Cysteine	3 mM	58.79±11.46^{a}	56.62±12.11^{a}	56.30±7.88^{a}	91.41±91.41^{a}	43.96±7.73^{a}	61.0±4.00^{a}	4.05±0.49^{a}	22.07±2.63^{a*}
	5 mM	71.03±3.65^{b*}	68.72±3.54^{b*}	53.33±1.79^{a}	86.75±3.43^{a}	41.09±2.22^{a*}	61.0±1.58^{a}	4.99±0.28^{b*}	23.58±1.34^{a*}
	7 mM	56.55±1.31^{a}	54.11±2.19^{a}	65.63±2.46^{b*}	121.72±5.50^{b*}	48.97±2.39^{b}	53.6±1.81^{b*}	4.48±0.33^{a}	24.18±1.30^{a}

Means denoted by different alphabetical superscripts vary significantly within each amino acid ($p<0.05$). Tmot=Total motility; Pmot=Progressive motility; VAP= Velocity Average Path; VCL=Velocity Curvilinear; VSL=Velocity Straight Line; WOB=Wobble; ALH= Amplitude of Lateral Head displacement; BCF= Beat Cross Frequency.

as if the velocity patterns of bull spermatozoa (VAP, VSL, and VCL) are very varied. Several factors can affect sperm motility, these factors include age, the time between ejaculations, the maturity level of sperm, energy storage (ATPase), surface-active chemicals in cell membranes (agglutinins and detergents), viscosity, pH, temperature, the ionic content of seminal plasma, and potentially compounds (Cu, Zn, Mn, and Hg, hormones, kinins, and prostaglandins) that induce or inhibit motility.

In this study, we used TCFY extenders as diluent medium that have high viscosity affected velocity, swimming pattern, sperm head behavior, and so on. The TCFY extender has a high viscosity, which limits spermatozoa movement when compared to PBS and TALP media, which have a lower viscosity.

The fertilizing ability of sperm was estimated in this study using velocity characteristics (VAP, VCL, VSL) after the addition of a selected amino acid. Data in Table 3 showed that among the velocity traits and the variation among the amino acid treatments, cysteine 7mM were significantly ($p<0.05$) higher velocity characteristics (VAP, VCL, VSL) than other. The results are in line with the data shown in Table 4 that cysteine 7mM significant ($p<0.05$) higher in DAP, DCL, DSL. According to [11] velocity average path (VAP) parameter might be utilized to estimate the fertility of frozen-thawed bull sperm. The VCL is linked to the ability of sperm to enter cervical mucus, whereas the VSL is linked to fertility [20]. Total motility, progressive motility, VAP, VSL, and VCL were all shown to correlate positively with in vivo buffalo bull fertility utilizing CASA [21, 22].

Table 4. The distance motility characteristics of frozen-thawed Sumba Ongole bull sperm after amino acid addition

Treatments		Parameters ±SEM		
		DAP (μm/s)	DCL (μm/s)	DSL (μm/s)
Control		26.82±4.05	42.17±7.60	21.29±3.98
Glycine	5 mM	28.32±1.23a	52.97±1.08a*	21.34±2.22a
	15 mM	26.23±0.72b	45.04±2.07b	20.80±0.91a
	25 mM	27.87±1.13b	48.98±1.97c*	21.77±0.93a
Glutamine	5 mM	25.47±0.75a	44.06±1.16a	18.28±0.49a*
	15 mM	23.80±1.05a*	39.99±0.89a	17.99±0.72a*
	25 mM	26.87±1.40b	41.70±4.31a	21.92±0.50b
Cysteine	3 mM	24.53±3.37a	39.88±3.39a	19.19±3.42a
	5 mM	22.93±1.07a*	37.32±1.91a*	17.71±1.09a*
	7 mM	30.20±1.39b*	56.25±3.14b*	22.53±1.36b*

Within each amino acid, means with different alphabetical superscripts are significantly different ($p<0.05$). DAP= Distance average path, DCL=Distance curve linear, DSL= Distance Straight line.

Additionally, [23,24] reported that VAP, VCL, VSL, ALH, and BCF are variables of spermatozoa movement that serve as a sperm fertility indicator and have a high probability of pregnancy rate, because VCL and VSL are proportional to the ability to fertilize, whereas the VAP value has a strong correlation with the pregnancy rate. The findings reported in this research suggested that the addition of cysteine 7mM to TCFY extender sperm results in a rapid motility of spermatozoa, implying a greater potential to fertilize.

Investigations have been conducted to determine the relationship between various motion parameters of bull spermatozoa and oocyte penetration rate, in vitro fertility rate, and field fertility [24]. The CASA approach produces reproducible and extremely accurate data on the kinematics of ejaculates using individual sperm cell measurements. As a result, determining sperm motility using kinetic measures would aid in the evaluation of sperm quality.

Table 5. Recovery rate of frozen-thawed semen samples

Treatments		Recovery Rates (Mean %)
Control group		82.04
Glycine	5 mM	81.72
	15 mM	69.53
	25 mM	76.51
Glutamine	5 mM	97.93
	15 mM	75.79
	25 mM	43.70
Cysteine	3 mM	79.34
	5 mM	95.86
	7 mM	76.32

According to the data in Table 5, the maximum recovery rate (RR) value was 5 mM glutamine and the minimum value was 25 mM glutamine. The recovery rate (RR) of spermatozoa was described as their ability to recover following freezing. 5 mM glutamine had the highest recovery rate (RR) and 25 mM glutamine had the lowest, which did not meet the criteria of a minimum recovery rate of 50%. [20] shown that the percentage of spermatozoa that return after freezing may offer information into the freezing method's efficiency.

CONCLUSION

In conclusion, the introduction of CASA systems enabled the quantification of individual spermatozoa motility patterns and CASA could be used to estimate the fertilizing capacity of sperm based on the characteristics of sperm velocity.

CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript**.**

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