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The Viabilities of Freeze-Thaw Pasundan-Bull Sperms After a Short-Term Exposure to Media with Different pHs

R. Widyastuti[a,b], N. M. D. Haq[a], D. N. Pristihadi[a], Wahyudin[a], H. Maheshwari[a], C. Sumantri[c], & A. Boediono[a,*]

[a]Department of Anatomy, Physiology, and Pharmacology, Faculty of Veterinary Medicine, IPB University
Jalan Agatis, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia
[b]Department of Animal Production, Animal Husbandry Faculty, Universitas Padjadjaran
Jalan Raya Bandung-Sumedang Km. 21, West Java, Indonesia
[c]Department of Animal Production, Faculty of Animals Science, IPB University
Jalan Agatis, Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia
*Corresponding author: ab@apps.ipb.ac.id
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ABSTRACT

External pH is crucial in preserving sperm viability and ensuring fertilization during in vitro conditions. The purpose of this study was to determine the maximum pH value that can be tolerated by frozen-thawed Pasundan bull sperms and the effect on sperm quality. Around 250x10^6 sperms/mL of frozen-thawed Pasundan bull sperms were divided into ten equal aliquots, and each was diluted in the medium within a particular pH value. HCL or NaOH was added to the buffer media to create ten different solutions with varying pH values of 3, 4, 5, 6 as acidic, 7.2-7.4 as a control, and 8, 9, 10, 11, and 12 as alkaline. Furthermore, the samples were incubated for 5 minutes at room temperature within a particular pH medium before being immediately supplemented with a buffered medium to achieve a pH of 7.2-7.4. After 10 minutes of incubation at room temperature, all parameters were assessed. The results showed that sperm motility, viability, normal morphology, and acrosome intactness in sperms incubated in the acidic or alkaline media were significantly lower compared to control ($p<0.05$, respectively). Interestingly, the sperm still had a good tolerance to pHs 6 and 8. This tolerance was evidenced by all the parameters of sperms that were not sharply decreased compared to the control group. The significant loss of motility occurred at pHs 3 and 12. It could be concluded that frozen-thawed Pasundan bull sperms are still tolerable in pHs 4-11, but the sperm quality degrades as the acidity or alkaline level increases.

Keywords: acidic; alkaline; Pasundan bull; sperm

INTRODUCTION

Pasundan Cattle is one of the local genetic resources in West Java, Indonesia, with a specific aspect that needs to be developed and conserved. The most superior characteristic of the cattle is that they can reproduce effectively in extreme environmental conditions and with low-quality feed (Sutarno & Setyawan, 2015). Additionally, Pasundan cattle are more resistant to parasitic diseases than the other breeds (Sumaryadi *et al.*, 2021). Pasundan Cattle farming is still traditionally carried out by local farmers along the North Priangan region and the south coast of West Java, Indonesia (Sulasmi *et al.*, 2017) . One of the efforts to improve the population and genetic value of this breed of cattle is the implementation of reproductive biotechnology such as oestrus synchronization (OS), artificial insemination (AI), sperm cryopreservation, sperm sexing, and embryo transfer (ET). Moreover, sperm cryopreservation, sperm sexing, and ET will be effective and beneficial when are applied to the beef cattle fattening or breeding industry (Hall & Glaze, 2014). Sperm sexing increases the likelihood of having offspring of a specific sex to increase production efficiency. For example, females are preferred in dairy cattle, whereas males are preferred in the beef cattle industry.

The Pasundan bull has good quality sperms in their ejaculates. Previous research found that the progressive motility of the bull's fresh sperms is around 80%-84% and declined by approximately 33.27% after freeze-thawing (Baharun *et al.*, 2017). The Pasundan bull sperm concentration ranges between 725.20x10^6 and 856.60x10^6 mL-1 and can produce frozen sperm up to 167 straws per ejaculate, equal to 13,400 doses over a year (Santoso *et al.*, 2021). Therefore, it proves that the production of frozen sperm or sperm with a specific sex chromosome through sperm sexing has a high probability of success.

Several sperm sexing methods have been developed based on the specific characteristics of X or Y bearing-chromosomes sperms. To date, methods for sperm

sexing that have been developed are percoll gradient (Noguchi *et al*., 2014), albumin separation (Hadi, 2019), swim-up (Arias *et al*., 2017), and flow cytometry (Mir & Kumar, 2012). These methods rely on the differences in sperm morphology characteristics, deoxyribose nucleate acid (DNA) contents, protein macromolecules, weight, size, specific gravity, the electric charge on the surface, immunological properties, and the sperm swimming ability (Bhalakiya *et al*., 2018). Among them, flow cytometry gives the highest accuracy, greater than 90%. Still, this method is less effective due to resulting low sperm fertility and requires expensive equipment and has a complicated process (Yadav *et al*., 2018). Moreover, a previous study reported that many sperm sexing methods still give inconsistent results (Naniwa *et al*., 2019). Therefore, there is still a wide opportunity to develop a sperm sexing method that considers natural and physiological X and Y sperms. Later the method also needs to be easily applied and preserve sperm quality for further fertilization.

Scientists believed that X and Y sperm could be selected based on different migration rates in acid and alkali media. This was considering the nature of the vagina, which has moderate pH in a range 3.8 to 4, which may play a role in the selection of X- or Y- chromosome bearing sperm, thus affecting the sex of the offspring (Nakano *et al*., 2015). A previous study shows that Y sperm cannot survive for a long time when exposed to acidic media, high temperatures, or environments with high reactive oxygen species (ROS) content (Chen *et al*., 2014b; Zhou *et al*., 2015), meanwhile X sperm is on the contrary with Y sperm (Oyeyipo *et al*., 2017). Then it could be inferred that media acidity allows separating X and Y sperm.

There is no information about the range of pH value tolerable by frozen-thawed sperms of Pasundan bull and their impacts on sperm quality. It is necessary to conduct preliminary studies to provide sufficient data of medium incubation with limited pH values that still support the function of frozen-thawed sperms of Pasundan bull. Therefore, the study aims to determine the pH range that Pasundan bull sperm can tolerate and how that affects the quality of the sperm. The survival of sperms in both mediums will become valuable information in implementing good media to maintain the quality of frozen-thawed sperms of Pasundan bull. Furthermore, understanding the limited pH value media that frozen-thawed sperms of Pasundan bull can tolerate could aid in developing sperm sexing methods based on sperm survival in alkaline or acidic media.

MATERIALS AND METHODS

Semen Sample and Sperm Preparation

The study was conducted with commercial frozen semen samples from 2 Pasundan bulls with a similar batch from Lembang Artificial Insemination Center (Lembang, West Java, Indonesia). All the procedures related to this study were approved by the ethics committee of the Faculty of Veterinary Medicine, IPB University, Indonesia (No:033/KEH/SKE/VI/2021). For each experiment, 10 of 0.25 mL straws (approximately 250×10^6/mL) were thawed in a 37 °C water bath for 10 seconds before being mixed into the sperm pool. This pool was then diluted (1:1) with G-MOPS (Vitrolife, Swedeen) and centrifuged for 5 min at 500 g to remove the sperm dilution media and cryoprotectant. The suspension was also diluted in 0.5 mL G-MOPS and divided into equal aliquots after removing the supernatant.

Experimental Design

G-MOPS buffer solution (Vitrolife, Sweden) was used in this study. The addition of HCL or NaOH to buffer solution just before the start of the experiment created ten different solutions with different pH values. The pH values 3, 4, 5, and 6 represented acidic conditions, while 8, 9, 10, 11, and 12 represented alkaline conditions. The G-MOPS, which have a pH of 7.2-7.4, represents a control.

Each sample aliquot was diluted in G-MOPS with a specific pH with a final concentration of around 25×10^6 sperm/mL. Because the addition of sperm samples altered the pH of the original buffer solution slightly, a buffer that consistently indicates the pH of the final mixture of sperm sample and buffer was created. Furthermore, each sample was incubated for 5 minutes at room temperature with a specific pH media. The pH was immediately returned to pH 7.2-7.4 by adding a buffer solution. After a 10-minute incubation at room temperature, the sperm motility, viability, acrosome intact, and morphology were evaluated.

Sperm Analysis

Sperm motility. The percentage of motile sperms in each sample was determined visually by viewing 10 μL of a drop of sperm suspension in an object-glass and coverslip under a 400x magnification upright microscope (Olympus CX21, Japan). Two hundred sperm cells were counted and classified as motile or immotile. Furthermore, motility was graded on a scale of 0 to 100% (Vasan, 2011).

Sperm viability. To ascertain the sperm viability, a drop of sperm was mixed with 2% eosin solution in the ratio of 1:1 and spread between the object-glass and the coverslip. After the lay down of the mixture, the preparation was air-dried and then observed under a 400× magnification microscope (Olympus CX21, Japan). In total, 200 sperms were evaluated, and viable sperms were characterized by their pale color, whereas nonviable sperms were characterized by red color in their head area. The proportion of viable sperm to total observed sperm was expressed as a percentage (%) (Mbaye *et al*., 2019).

Evaluation of the acrosomal status. The staining solution contained 2% (w/v) Fast Green FCF and 0.8% (w/v) Eosin B in a glutamate-based extender. Twenty microliters of sperm suspension were mixed with 10 μl of stain on one end of a slide. The smears were then dried at room temperature immediately after preparation and

examined under a microscope (Olympus B.X.21, Japan). Finally, 200 sperms were classified as stained or unstained during a differential count based on the degree of eosinophilia (Almadaly *et al*., 2012).

Sperm morphology. Sperm morphology was determined by counting the number of ordinary versus abnormal sperm per 200 sperms in the eosin-nigrosin stained smear under 1000× magnification. Moreover, the evaluation of sperm morphology followed previous research (Widyastuti *et al*., 2020).

Data Analysis

Data from the six replications were obtained. GraphPad Prism was used to analyze the data (version 8; GraphPad Software Inc., La Jolla, CA). Furthermore, the Shapiro-Wilk test was used to determine the normality of the value distribution. A one-way factorial analysis of variance (ANOVA) was performed on the data, followed by multiple comparisons using a posthoc test (Tukey test). The significance level was set at $p<0.05$. Bivariate associations between the level of acidity in the incubation media, sperm motility and viability, acrosome intactness, and sperm morphology were evaluated using Pearson's correlation coefficient.

RESULTS

Overall, when frozen-thawed Pasundan bull semen was incubated for a short period in acidic or alkaline media, the percentage of sperm motility and viability decreased significantly in all treatment groups compared to the control ($p<0.05$). At pHs 8 and 6, sperms remained tolerable to changes in the alkaline and acidic environments. Furthermore, when comparing acidic and alkaline mediums, sperms incubated in the alkaline medium at pH 8 had significantly higher motility than those incubated in the acidic medium at pH 6 ($p<0.05$, 54.94% vs 40.89%). A drastic decrease in sperm motility percentage occurred between pHs 5 and 9, approximately 50%. At pHs 3 and 12, the percentage of post-incubation sperm motility declined compared to the control group, falling from about 60% to less than 3% (Figure 1).

The decrease in sperm viability percentage seemed to be less severe than the decrease in motility. Surprisingly, after incubation on alkaline media, the percentage of sperm viability at pHs 9, 10, and 11 was around 40%. Meanwhile, at pH 3, the viability significantly dropped by more than four folds compared to the control group (from 87.7% to about 16.6 %) (Figure 2).

Determining acrosomal morphology concerning sperm viability is critical in assessing the acrosome reactions involved in fertilization and sperm cell death. The percentage of intact acrosomes had a significant influence on acrosomal intactness. Additionally, it was observed that there was a significant decrease in acrosome intactness compared to acidic media. The results also showed that the percentage of intact acrosome significantly decreased in all treatment groups compared to control ($p<0.05$) (Figure 3).

There was a significant difference in the percentage of sperm with abnormal morphology after incubation in the acidic and alkaline media. In general, the percentage of sperm with abnormal morphology increased with the degree of acidity and alkaline (Figure 4). In the acidic media of pHs 5 and 4, the percentage of sperm with normal morphology decreased around 1.5 folds compared to the control group and statistically differed significantly ($p<0.05$). This study found that the highest percentage of sperm with abnormal morphology was found in mediums with pHs of 3 and 12. Furthermore, the sperm abnormal morphology was dominated by sperm head abnormal morphology than the neck and tail abnormalities. Head and neck abnormal morphologies significantly increased in all treatment groups compared to control ($p<0.05$). While the abnormal neck morphology significantly increased in pHs 4, 10, 11, and 12 ($p<0.05$) (Table 1).

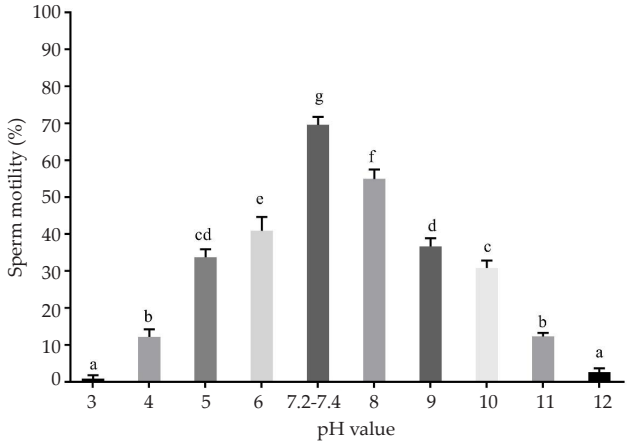

Figure 1. The percentage of motility in freeze-thawed Pasundan bull sperm after a short period of incubation at media with different pH values. The data are presented in average, bars with the same letter above, and superscripts with the same letter were not significantly different ($p<0.05$).

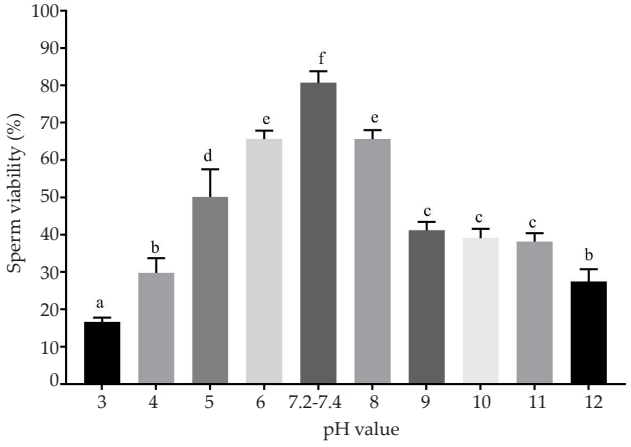

Figure 2. The percentage of viability in freeze-thawed Pasundan bull sperm after a short period of incubation at media with different pH values. The data are presented in average, bars with the same letter above, and superscripts with the same letter were not significantly different ($p<0.05$).

Through bivariate analyses, a statistically significant positive correlation was found between the acidity of the incubation media, sperm motility, the percentage of viable sperm, acrosome intactness, and the percentage of sperms with normal morphology. Interestingly, statistically significant negative correlations were seen between the alkaline incubation media and sperm motility and between the percentage of sperm viability, intact acrosome, and sperm with normal morphology. Pearson's correlation coefficients between the level of acidity of the incubation media and the sperm parameters in this study varied between 0.933 and 0.975. Meanwhile, for the alkaline media, it varied between -0.986 and -0.877 (Table 2).

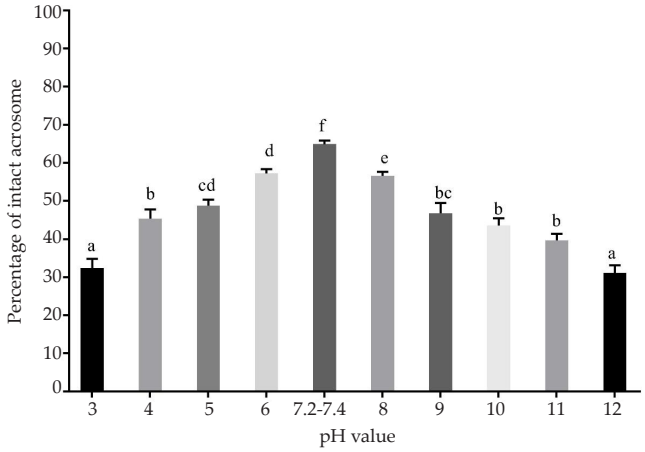

Figure 3. The percentage of intact acrosomes in frozen-thawed Pasundan bull sperm after a short incubation in different pH media. The data are presented in average, bars with the same letter above, and superscripts with the same letter were not significantly different (p<0.05).

DISCUSSION

Sperm is one of the valuable genetic resources produced in the male gonad. After being released, it is exposed to an extracellular environment with fluctuating ion concentrations, pH, temperature, and other physiochemical variables that could impair metabolism (Sullivan & Saez, 2013). Sperm pH is an essential factor in maintaining normal functions (Chen *et al.*, 2014a). This study found that exposing frozen-thawed sperms of Pasundan bulls to acidic or alkaline solutions for a short time significantly reduced sperm motility, viability, and morphology, including acrosome intactness.

Motility is an essential factor in determining sperm quality, fertilizing ability, and it is directly affected by pH (Zhou *et al.*, 2015). This study showed that the acidity or alkalinity of the media used to frozen-thawed the sperms of Pasundan bull affected its motility. The sperm motility at pH 8 was not significantly reduced and is comparable to the motility at pH 7. These findings are similar to the results of a previous study, which discovered that ram epididymal sperm are activated when diluted in an alkaline medium with a pH of 7 to 8 (Mahdi *et al.*, 2019). Meanwhile, alkaline pH of 8 had a minor effect on ram epididymal sperm after a long incubation period. However, the ideal pH for sperm incubation media would be around 7.2 (Swain, 2012). This study showed the decreased sperm motility after incubation in pHs 5, 4, and 3 following previous findings that bovine sperm tolerated a decrease in medium pH to 6. However, a lower pH was spermicidal and caused an immediate cessation of motility (Suthutvoravut & Kamyarat, 2016). The kinematic study of bull sperm showed higher values for sperm motility characteristics at pHs 7 and 7.5, while at pH values lower than 6.5 and

Table 1. The percentage of sperm with abnormal morphology

pH media	Total sperm abnormal morphology (%)	Part of sperm		
		Head abnormality (%)	Neck abnormality (%)	Tail abnormality (%)
3	50.85±2.54^{a}	42.29±2.44^{ae}	5.33±2.24^{ab}	3.23±1.42^{ab}
4	42.13±2.05^{bc}	39.53±1.55^{abe}	3.12±1.31^{a}	2.79±0.99^{ab}
5	40.54±2.01^{c}	35.03±2.63^{b}	2.61±1.19^{a}	2.89±0.92^{ab}
6	26.19±2.94^{d}	20.31±2.81^{c}	3.25±2.28^{a}	2.63±1.20^{ab}
7.2-7.4	14.81±0.67^{e}	10.52±0.92^{d}	2.81±1.49^{a}	1.48±0.52^{a}
8	29.67±4.13^{d}	24.30±4.13^{c}	3.00±1.20^{a}	2.16±0.74^{ab}
9	38.55±1.43^{bc}	30.40±1.37^{b}	4.72±1.03^{a}	3.42±0.51^{b}
10	47.22±3.20^{ab}	38.10±3.74^{ab}	6.30±1.35^{b}	2.81±0.76^{ab}
11	48.01±4.32^{a}	41.18±3.73^{ae}	4.71±1.32^{ab}	2.13±0.74^{ab}
12	51.85±2.52^{a}	44.06±2.43^{e}	5.04±0.73^{ab}	2.74±1.03^{ab}

Note: The data on sperm with abnormal morphology presented in average and the column with the same letter were not significantly different (p<0.05).

Table 2. Bivariate Pearson's correlation coefficient between the parameters of sperm motility, viability, acrosome intactness, including sperm with normal morphology in the cohorts of acidic and alkali incubation media

Sperm parameters	Acidic media (pH 3-6)		Alkali media (pH 8-12)	
	r	p value	r	p value
Motility	0.974	p<0.05	-0.986	p<0.05
Viability	0.975	p<0.05	-0.877	p<0.05
Acrosom intact	0.956	p<0.05	-0.960	p<0.05
Normal morphology	0.933	p<0.05	-0.892	p<0.05

Note: r= correlation coefficient.

higher than 8, there was suboptimal sperm motility (Contri *et al.*, 2013).

The previous research reported that the alkaline pH resulted in immobilization of bull sperm through a significant reduction in their mitochondrial activities, while acidic media reduced the sperm motility by causing membrane damage (Contri *et al.*, 2013). Mitochondria have a main role in sperm motility regulation because they provide adenosine triphosphate (ATP) to support sperm function and synthesis through glycolysis and oxidative phosphorylation (OXPHOS) pathways (Ferramosca & Zara, 2014; Tourmente *et al.*, 2015).

Our results showed that the reduction in the viability of frozen-thawed sperms of Pasundan bull after a short time incubation in acidic and alkaline media does not appear to be as dramatic as that of sperm motility. Therefore, the condition indicated that immotile sperm may still be alive and capable of fertilization. Previous research reported that the increased level of acidity induced sperm membrane damage (Contri *et al.*, 2013). The disruption of membrane permeability has a direct impact on sperm viability because it disrupts the transport membrane process, particularly the calcium pump by ATP-dependent sodium/calcium and the voltage-dependent calcium channel, both of which are essential for sperm metabolism and fertility (Said *et al.*, 2010; Shukla *et al.*, 2012; Kwon *et al.*, 2013).

The results showed that intact acrosomes significantly decreased after incubation in the media with the highest and lowest acidities. These results agreed with the previous study, which found that high pH could induce acrosome reaction by increasing the Ca^{2+} trapping capacity of mitochondria, and in this manner, stimulating Ca^{2+} uptake into the sperm (Nowicka-Bauer & Szymczak-Cendlak, 2021).

Another parameter that was influenced by the short incubation of the frozen-thawed Pasundan bull sperm in the acidic and alkaline media is sperm morphology. Sperm morphological abnormalities have long been linked to male infertility and sterility, making their evaluation a critical component of sperm quality analysis (Enciso *et al.*, 2011). In Artificial Reproductive Technology (ART) practice, sperm morphology significantly impacts fertilization success, early embryonic development, and pregnancy rate (Berger *et al.*, 2011). The decrease in sperm with normal morphology after short-term incubation in acidic and alkaline media in this study could be attributed to acrosomal damage or loss or over condensation of the nuclear chromatin. Previous research found that a brief incubation in extremely acidic or alkaline media increased the percentage of abnormal head shape and acrosome looseness (Widyastuti *et al.*, 2020).

The strong correlation between the pH of the incubation medium and the percentage of sperm motility indicates that the increased level of acidity or level of alkalinity of the media may inhibit sperm motility. The changes in external pH during incubation may induce an increase in ROS production that harms ATP production, resulting in the disturbance of sperm motility (Zhu *et al.*, 2019). Furthermore, the increased ROS production may affect sperm viability through BAX gene activation. BAX is a pro-apoptosis gene that plays an essential role in cell death (Liu *et al.*, 2015). Its activation may disturb sperm membrane permeability, causing a significant decrease in sperm viability in an acidic or alkaline medium after a short incubation period. The disruption of membrane permeability is likely to disrupt the transport of Ca^{2+} ions, which triggers acrosomal reactions. The loss of intact acrosome causes an increase in primary morphological abnormalities in the sperm head. Overall, this study discovered that short incubation in an acidic or alkaline medium negatively impacted the sperm structure and function.

This study should be remembered that frozen-thawed sperm was used as a sample. Therefore, the damage could be induced by incubation in acidic or alkaline media and due to the freezing and thawing processes. During this procedure, sperms were exposed to several potential risks, including plasma membrane damage (Pini *et al.*, 2018), acrosome loss (Khalil *et al.*, 2018), and a change in the integrity of its chromatin (Mukhopadhyay *et al.*, 2011). In practice, even though it reduced the motility and viability of frozen-thawed sperms of Pasundan bull after a short period of incubation, it was still adequate to fertilize oocytes using various techniques. When the sperm was incubated at pHs 5-6 and 8-10, it still had around 40% motile sperms, making it suitable for conventional IUI and IVF. Moreover, the sperm incubated at pHs 3, 4, 11, and 12 experienced a reduction in motility to below 15%, making them only suitable for in vitro embryo production using the intracytoplasmic sperm injection (ICSI) strategy. According to Salamone *et al.* (2017), the method requires only a single viable sperm to be mechanically inserted into the cytoplasm of oocytes using micromanipulators attached to inverted microscopes.

Furthermore, the results showed that the media is vital for maintaining the sperm during assisted reproduction practice. This vital role of media is because it could create a new ambient environment for the preservation of sperm from a superior bull, humans for infertility treatment, and endangered species for in-situ conservation. The knowledge about the limit of pH value of media that the sperms can tolerate becomes essential information to improve the method of simple sperm sexing based on sperm survival in both mediums. It needs further investigation about the effect of incubation at extreme pH media in the enrichment of sperms bearing X or Y sex chromosome in Pasundan bull.

CONCLUSION

The range of pH values the Pasundan bull's frozen-thawed sperms can tolerate is between 4 and 11. Incubation in acidic or alkaline media reduces sperm quality as measured by a decrease in the percentage of motility, membrane integrity, intact acrosomes, and an increase in the percentage of sperm with abnormal morphology, depending on the level of acidity or alkalinity.

CONFLICT OF INTEREST

Cece Sumantri and Arief Boediono serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors declared that there was no conflict of interest in the publication of this paper.

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