Morphology and Morphometric of Frozen-thawed Pasundan Cattle Sperm Incubated in Extreme pH Media

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ABSTRACT

An evaluation of sperm morphology and morphometry is useful to predict sperm quality and in the diagnosis of fertile animals. The purpose of this study is to assess the morphology and morphometry of frozen-thawed Pasundan Cattle sperm, as well as the correlation between the two parameters. The sample was obtained from frozen-thawed Pasundan Cattle sperm incubated in pH 4 as acidic media, pH 7.2-7.4 as a control, and pH 11 as alkali media. After 5 minutes of incubation in media, all samples were returned to pH 7.2-7.4 by adding buffer media and immediately evaluated. The results revealed that the percentage of sperm with normal morphology was significantly lower (p<0.05) in the alkali or acidic groups compared to the control. Furthermore, the width of all sperm areas was significantly different in acidic compared to the control and alkali groups (p<0.05). There was no significant difference in treatments or control groups for the entire head and neck area morphometric (p>0.05). Moreover, the width of the sperm tail area displayed the same pattern as all widths of the sperm area. Furthermore, it can be concluded that extreme acidic and alkali media significantly impact on morphometry and morphology of frozen-thawed Pasundan Cattle sperm.

Keywords: sperm, acid, alkaline, morphology, morphometry, Pasundan Cattle.

1. INTRODUCTION

Pasundan Cattle is a Sundanese icon in West Java, Indonesia, which has superior characteristics. It has a small body size, good reproductive performance, and carcass proportion in rounds 52-55%. It has a distinct reddish coat color, and for bulls, it changes to black during sexual maturity due to the androgen hormone [1]. Pasundan cattle, like other local cattle in Indonesia, is relatively resistant to parasitic disease and adaptable to extreme environments, including poor feed conditions. Despite becoming a Sundanese icon, the population of Pasundan Cattle has been declining year by year, with only around 28,000 remaining in 2015 which spreads along the southern coast of West Java and the buffer zone area along the northern Priangan [2]. Based on these circumstances, it is critical to converse the Pasundan Cattle from extinction [3]. One method of conserving Pasundan Cattle is in-situ conservation by preserving their natural habitat, or ex-situ conservation by preserving the diversity of their habitats.

For ex-situ conservation, using frozen semen for artificial insemination or in vitro embryo production...
may be an important method for increasing the population of the Cattle. Frozen sperm is commercially used all over the world for artificial insemination (AI) or \textit{in vitro} embryo production. Moreover, Pasundan Cattle frozen sperm has been established in Indonesia and is widely used for AI by Indonesian local farmers. Frozen sperm will be exposed to various environmental conditions during the manufacturing process, distribution, and finally thawing for direct use in AI or \textit{in vitro} embryo production. The changes in environmental conditions such as temperature and pH would lead to changes in reactive oxygen species (ROS) levels due to alteration in metabolic activities [4]. Previous research found that external pH has a significant impact on the motility, viability, and capacity of sperm [5] and it is critical for sperm motility [6]. When the sperm is exposed to an environment (media) with low pH or high pH, it may experience a detrimental effect on its cellular and molecular level [7].

There is a lack of information on the effect of extreme pH media on morphology and morphometry of freeze-thaw Pasundan sperm studies during \textit{in vitro} conditions. A more investigation is necessary. Sperm morphology and morphometry are two of the quality parameters used to determine fertility. The purpose of this study is to assess the morphology and morphometry of frozen-thawed Pasundan cattle sperm, as well as the correlation between the two parameters after short time incubation in extreme pH media.

2. MATERIALS AND METHODS

2.1. Semen Sample and Sperm Preparation

The study was conducted with commercial frozen semen samples from 2 Pasundan bulls with the same batch number obtained from Lembang Artificial Insemination Center (Lembang, Indonesia). All the procedures related to this study were approved by the ethics committee of the Faculty of Veterinary Medicine, IPB, Indonesia (No:033/KEH/SKE/VI/2021). Three different solutions with different pH values were prepared by adding 5 M HCL or 5 M NaOH to buffer media (G-MOPS) just before the experiment. pH 4 represented an extreme acidic condition, while pH 11 represented an alkali condition while for the control group G-MOPS with pH 7.2-7.4 was prepared in this study.

In each experiment, frozen sperm were thawed in the water bath at 37°C for 10 seconds, pooled, and then diluted (1:1) with G-MOPS (Vitrolife, Sweden) following centrifugation at 500 g for 5 minutes to remove the cryoprotectant. The supernatant was then discarded, and the pellet was re-suspended with 0.5 mL G-MOPS and divided into three equal aliquots. Each aliquot was then diluted in the specific pH media (treatment). The addition of sperm samples slightly altered the original buffer solution's pH, therefore we prepared buffer such that it always indicates the pH of the final sperm sample buffer mixture. Each sample was incubated for 5 minutes with a specific pH media at room temperature. Then, it was immediately returned to pH 7.2-7.4 by buffer solution addition. The assessment of sperm morphology was performed after 10 minutes of incubation at room temperature.

2.2. Evaluation of Sperm Morphology

Sperm morphology was determined by evaluating the number of normal versus abnormal sperm per 200 sperm in the eosin-nigrosine-stained smear with microscopy at 1000x magnification. A total of 200 sperms were examined for morphological abnormalities based on the previous method [8].

2.3. Morphometric Measurements of Sperm

Morphometric measurements of sperm were performed using the Scanning image analysis system (Computer Scanning Systems) connected with an Olympus IX70 microscope at 6.000 x magnification (100 x oil immersion objective). In this study, the sperm measurements were evaluated using software namely OLYMPUS FLUOVIEW ver. 4.2a. The width area of the sperm head, neck tail, and all of the sperm surface was measured.

2.4. Statistical analysis

All the treatments in this study were performed six times. Data were then analyzed using GraphPad Prism (version 8; GraphPad Software Inc., La Jolla, CA). The Shapiro-Wilk test was used to determine the data normality, and comparison between the group was compared using analysis of variance (ANOVA) followed by post-hoc test (Tukey test). The significance level in this study was set at \( \alpha < 0.05 \).

3. RESULTS

The sperm morphology measurements from respective treatments are shown in Table 1. The number of sperm with abnormal morphology increased threefold in the treatment group versus the control group \((p<0.05)\). In both media at pH 4 and pH 11, the most common abnormality found is abnormal head morphology. Furthermore, when compared to controls, sperm with abnormal head morphology increased threefold in pH 4 and fourfold in pH 11 \((p<0.05)\). When compared to the control, sperm with abnormal mid-piece and tail were not significantly different in any of the group treatments \((p>0.05)\).
Table 1. The percentage of sperm with abnormal morphology after a brief incubation in different pH of media

<table>
<thead>
<tr>
<th>pH media</th>
<th>Sperm with abnormal morphology (%)</th>
<th>Part of sperm with abnormal morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Head</td>
<td>Neck</td>
</tr>
<tr>
<td>4</td>
<td>45.33±2.78a</td>
<td>39.43±1.71a</td>
</tr>
<tr>
<td>7.2-7.4</td>
<td>14.71±0.70b</td>
<td>10.58±1.03b</td>
</tr>
<tr>
<td>11</td>
<td>47.35±4.48a</td>
<td>40.72±3.98a</td>
</tr>
</tbody>
</table>

Data presented as average±SD. The different superscripts in the same column showed a significant difference.

Table 2. Wide surface area of frozen-thawed Pasundan Cattle sperm after a brief incubation in different pH of media.

<table>
<thead>
<tr>
<th>pH media</th>
<th>Sperm wide-area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
</tr>
<tr>
<td>4</td>
<td>112.30±19.18a</td>
</tr>
<tr>
<td>7.2-7.4</td>
<td>102.79±16.81b</td>
</tr>
<tr>
<td>11</td>
<td>116.71±15.33a</td>
</tr>
</tbody>
</table>

Data presented as average±SD. The different superscripts in the same column showed a significant difference.

The parameters for the morphometric characteristics of sperm incubated in extreme acidic, extreme alkali, and control groups are summarized in Table 2. There is none group of treatment which have significantly different for the wide area of the head and the mid-piece compared to control (P>0.05). Interestingly, both groups of treatment have a smaller all surface and tail area compared to control (p<0.05).

4. DISCUSSION

Sperm is considered normal if the shape, size, and proportions of the head, mid-piece, and tail fall within the classification for the given species. Sperm morphology research is regarded as an important component of sperm analysis because it reflects not only motility but also DNA integrity [9, 10]. In artificial reproduction practice, sperm morphology evaluation is closely related to sperm morphometric which has been directly linked to male fertility, fertilization success rate, early embryonic development, and pregnancy rate [11]. The sperm's abnormal morphology is believed to be often multifactorial and caused by both genetic and extrinsic [12, 13].

In our study, we demonstrate that exposing frozen-thawed Pasundan Cattle sperm to extreme acidic or alkaline media for a short period increases the number of sperm with abnormal morphology, particularly in the head area. Furthermore, the morphometric characteristics of sperm reveal a significant reduction in sperm surface area, indicating a strong correlation between sperm head abnormal morphology and the reduction in sperm surface area in frozen-thawed Pasundan Cattle sperm incubated in extremely acidic or alkali media for a short period. The results are consistent with previous research that reported a short exposure of frozen-thawed sperm in different incubation media which can significantly increase sperm with abnormal morphology [8]. The reduction of sperm surface area in bovine may be manifested in sperm with head abnormal morphology [14] that is associated with chromatin structure [15, 16] or DNA fragmentation [17, 18].

Naturally, sperm is very sensitive to acidic and alkali extreme environments. In such conditions, sperm will die within 10 minutes at pH 4 and 11. Furthermore, pH 4 is a natural analog of vaginal pH and functions as a naturally spermicidal or microbicidal agent [19]. Furthermore, previous research found that sperm is susceptible to acid denaturation or heat, so exposing frozen-thawed sperm to extreme acidic media for a short period may cause slight shrinkage, resulting in a reduction in sperm surface area. Incubation pH 4 media-induced sperm DNA fragmentation in human sperm [20], as a result, it increases abnormal sperm morphology. At this point we can say that the acidity of media would impact the sperm surface area, suggested it's related to the change of sperm membrane as the response of different media or environment.

All in all, the results of this study indicate that media incubation with both extreme acid and alkaline would increase the number of sperm with abnormalities in the head area, as well as morphometric. Since sperm heads contain DNA and membrane which are responsible for the fertilization process, the change of both morphology and morphometric of sperm may have a detrimental effect on sperm fertilizing ability.

AUTHORS’ CONTRIBUTIONS

RW, DNP, SP, HM, CS, and AB all contributed to the design, experimented, analyzed the data, and...
interpreted the results. Furthermore, RW and NMDH were responsible for sample collection and statistical analysis. RW and SP were writing and review the manuscript and approved by all the authors.

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