Impact of Extender pH on Motility of Bovine Sexed Semen by M-Zlex Process

ผลกระทบของความเป็นกรด-ด่างของสารละลายเจือจางน้ำเชื้อต่อการเคลื่อนที่ของน้ำเชื้อโคที่ผ่านการแยกเพศด้วยกระบวนการ M-Zlex

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ห้องปฏิบัติการนวัตกรรมปศุสัตว์และชีวหมุนเวียน ภาควิชาสัตวศาสตร์และสัตว์น้ำ คณะเกษตรศาสตร์ มหาวิทยาลัยเชียงใหม่ จ.เชียงใหม่ 50200

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บทคัดย่อ การเคลื่อนที่ของอสุจิเป็นตัวบ่งชี้ที่มีประสิทธิภาพของภาวะเจริญพันธุ์ โดยปัจจัยแวดล้อม เช่น ค่าความเป็นกรด-ด่าง ความเข้มข้นของออสโมติก และอุณหภูมิ สามารถส่งผลต่อการทำงานและการเคลื่อนที่ของอสุจิได้ การศึกษานี้จึงมีจุดประสงค์เพื่อประเมินผลของค่าความเป็นกรด-ด่างของสารละลายเจือจางน้ำเชื้อที่แตกต่างกัน ต่อการเคลื่อนที่ของน้ำเชื้อแยกเพศโค โดยในการทดลอง ได้แบ่งตัวอย่างน้ำเชื้อออกเป็นสองกลุ่ม ได้แก่ น้ำเชื้อปกติ ซึ่งถูกเจือจางด้วยสารละลายเจือจางน้ำเชื้อที่มีค่าความเป็นกรด-ด่างแตกต่างกัน ได้แก่ 6.2, 6.6, 7.0, 7.4 และ 7.8 และน้ำเชื้อที่ผ่านกระบวนการแยกเพศด้วยเทคนิค M-Zlex โดยใช้สารละลายเจือจางน้ำเชื้อที่มีค่าความเป็นกรด-ด่างแตกต่างกัน ได้แก่ 6.2, 6.6, 7.0, 7.4 และ 7.8 ในกระบวนการแยกเพศและเจือจาง โดยอัตราส่วนของอสุจิเอ็กซ์ ถูกประเมินด้วยเครื่อง imaging flow cytometer และการวิเคราะห์การเคลื่อนที่ของอสุจิในกลุ่มน้ำเชื้อหลังการเจือจาง และหลังการแช่แข็งถูกประเมินด้วยเครื่อง CASA ผลการทดลอง พบว่า ค่าเฉลี่ยของอสุจิเอ็กซ์ ในกลุ่มน้ำเชื้อปกติ อยู่ที่ 50.06 เปอร์เซ็นต์ ในขณะที่ กลุ่มน้ำเชื้อแยกเพศ M-Zlex สูงถึง 73.89 เปอร์เซ็นต์ นอกจากนี้ ในน้ำเชื้อหลังการเจือจาง น้ำเชื้อปกติมีการเคลื่อนที่สูงสุดที่ค่าความเป็นกรด-ด่าง 6.6 และ 7.0 ในขณะที่ น้ำเชื้อแยกเพศ M-Zlex มีการเคลื่อนที่สูงสุดที่ค่าความเป็นกรด-ด่าง 6.6 เท่านั้น ในทางตรงกันข้าม ในน้ำเชื้อหลังการละลาย น้ำเชื้อปกติ มีการเคลื่อนที่สูงที่ค่าความเป็นกรด-ด่าง 6.6, 7.0 และ 7.4 ในขณะที่ สารละลายเจือจางน้ำเชื้อที่มีค่าความเป็นกรด-ด่าง 6.6 และ 7.0 เหมาะสมต่อการเคลื่อนที่ของน้ำเชื้อแยกเพศ M-Zlex มากที่สุด เนื่องจาก สารละลายเจือจางน้ำเชื้อที่เป็นกรดอ่อนสามารถกระตุ้นการเคลื่อนที่ของอสุจิเอ็กซ์ได้ นอกจากนี้ ค่าความเข้มข้นของออสโมติกของสารละลายเจือจางน้ำเชื้อในการศึกษานี้ อยู่ในช่วงที่เหมาะสมต่อการเคลื่อนที่ของอสุจิ

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Abstract: Sperm motility is an effective indicator of sperm fertility, and environmental factors such as pH, osmolarity, and temperature can modify the function and motility of sperm. In this study, we evaluated the effect of different extenders pH values on the motility of bovine sexed semen. Semen samples were divided into two parts: conventional semen (CONV) diluted with different extenders pH 6.2, 6.6, 7.0, 7.4, and 7.8 and sexed semen by M-Zlex process (M-Zlex) that used different extenders pH 6.2, 6.6, 7.0, 7.4, and 7.8 during the sexing process. An imaging flow cytometer was used to evaluate X-sperm ratio, and CASA was used to analyze the motility of sperm after dilution (AD) and post-thaw semen (PT). The results showed an average of X-sperm for 50.06% in CONV, while M-Zlex reached up to 73.89%. Moreover, CONV exhibited the highest motility at pH 6.6 and 7.0 during the AD, stage while M-Zlex was the most motile at only pH 6.6. The motility in PT of CONV was high in pH 6.6, 7.0, and 7.4, whereas pH 6.6 and 7.0 are suitable for M-Zlex because weakly acidic solutions have an effect on stimulating X-sperm motility. Furthermore, the extenders osmolarity was in an appropriate range for sperm motility.

Keywords: Extender, osmolarity, pH, sexed semen, sperm motility

Introduction

Assisted reproductive technologies (ARTs), particularly sperm sexing, have developed the cattle production industry by allowing targeted production of offspring with desired sex, significantly enhancing breeding efficiency and economic gains (Orsolini *et al.*, 2021; Yadav *et al.*, 2017). Sexed semen is fresh semen in which the proportion of X-chromosome-bearing sperm (X-sperm) and Y-chromosome-bearing sperm (Y-sperm) has been altered from the normal ratio (50:50%) using any sperm sexing procedure to produce a specific gender (Thongkham *et al.*, 2021). Moreover, the immunological sexing approach with antibodies offers low-cost, high-volume production. A previous study demonstrated that the X-enriched fractions from sperm sexing using magnetic-activated cell sorting and scFv antibodies specific to Y-sperm (M-Zlex) contained a high proportion of X-sperm, up to 80%. (Paitoon *et al.*, 2024; Sringarm *et al.*, 2022). For this reason, sexed semen contains a significantly higher proportion of X-sperm than conventional semen.

Sperm motility is an important component in determining semen quality and fertilization potential (Bonato *et al.*, 2012). Environmental factors, including pH, osmolarity, and temperature, can modify the function and motility of sperm (Contri *et al.*, 2013). In mammals, the X- and Y-sperm have different motility activities under different pH conditions (He *et al.*, 2023a). Several studies have shown that pH affects sperm performance differently across species. In rabbits, sperm motility was significantly reduced under acidic conditions without affecting the offspring sex ratio (Muehleis and Long, 1976). Meanwhile, in hamsters, mating later in estrus when vaginal pH is higher results in smaller litters and more male offspring, suggesting that elevated pH may favor Y-sperm fertilization by affecting motility or longevity (Pratt *et al.*, 1987). In humans, incubation in acidic pH increasing the X:Y ratio from 1:1 to about 1.64:1, suggesting that low pH reduces Y-sperm viability

(Oyeyipo *et al*., 2017). Furthermore, in dairy goats, semen diluted at different pH levels significantly influenced the enrichment of X-and Y-sperm. At pH 6.2, the proportion of X- sperm in the upper sperm layer reached 67.24%, resulting in 66.67% female offspring, whereas at pH 7.4, the X-sperm proportion dropped to 30.45%, with 29.73% female offspring. This indicates that slightly acidic conditions enhance the motility and fertilization capacity of X- sperm, whereas alkaline conditions favor Y-sperm (He *et al*., 2021).

Therefore, this study aimed to evaluate the effect of different extender pH levels on the motility of bovine sexed semen (X- enriched fractions) and to identify the pH range that is appropriate for use with the M-Zlex sexed semen.

Materials and Methods

Animal and semen sample

Semen samples were collected using an artificial vagina from sexually mature tropical Holstein Friesian bulls housed individually at the Livestock Semen Production Centre, Inthanon Royal Project (RAGIACUC006/2564). Only fresh semen samples that were used in the experiment had a sperm concentration of greater than 650 × 10^6 cells/mL and total motility of more than 80%.

Preparation of different pH extender

The extender composition of this study was tris (3.025% w/v), citric acid (1.7% w/v), D-fructose (0.2% w/v), egg yolk (20.0% v/v), glycerol (8.0% v/v), gentamicin (0.3 mg/mL), and penicillin (1000 IU/mL). To evaluate the impact of extender pH on sperm motility, extenders with different pH of 6.2, 6.6, 7.0, 7.4, and 7.8 were prepared using tris to increase pH and citric acid to lower pH. The pH of each extender was measured using a calibrated pH meter (Metrohm AG, Herisau, Switzerland) at room temperature.

Measurement of different pH extender osmolarity by osmometer

After preparation, each extender with different pH was measured for osmolarity. Osmometer (Osmomat 3000-basic, Gonotec®, Berlin, Germany) was calibrated using 300 and 850 mOsmol/kg of standard solution before measurement. Then, 50 µL of extender was dropped into the tubes and measured with an osmometer (5 replicates/treatment).

Production of bovine sexed semen by M-Zlex process

Semen samples were separated into two groups. The first group was used to produce conventional semen (CONV). Fresh semen was further divided into five parts and diluted to a final concentration of 8 × 10^7 cells/mL with five extenders at pH 6.2, 6.6, 7.0, 7.4, and 7.8. The second groups were further divided into five parts to produce sexed semen by M- Zlex process (M-Zlex). In the sexing process, fresh semen was dissolved in five different extender pH (6.2, 6.6, 7.0, 7.4, and 7.8) to 1.0 × 10^9 cells/mL. Then, 100 mg of PY-microbeads produced following Sringarm *et al*. (2022) was added in five dissolved semen and incubated for 15 min at 37 °C. After incubation, the PY-microbeads were trapped in the bottom of the tube by resting on a strong neodymium magnet for 5 min at room temperature. The unbound PY - microbeads were removed and placed in new tubes, which contained a high concentration of X-sperm and were called M-Zlex sexed semen. After that, diluted to a final concentration of 8 × 10^7 cells/mL using extenders based on the pH used in sexing. Each different extender pH of CONV and M-Zlex semen was separated into two parts: the first was incubated after dilution for 30 min at 37 °C, and the second was frozen and stored in liquid nitrogen (196 °C) for 24 h before sperm motility evaluation. A diagram of the steps involved in the production of conventional and M-Zlex semen in this study is shown in Figure 1.

Evaluation of X-sperm percentage in bovine sexed semen by imaging flow cytometer

The percentage of the X-sperm in each treatment from CONV and M-Zlex semen was determined, according to Thongkham *et al.* (2024). Sperm samples were diluted to 1 × 10^6 cells/mL in DPBS and mixed with 1.2 µL of Hoechst 33342 (50 µg/mL). Then, incubated for 10 min at 37 °C in the dark before analysis using imaging flow cytometer (FlowSight, Seattle, WA, USA). The Hoechst 33342 stain was excited by a 15 mW 405-nm laser. Histograms were created to determine the fluorescence of X-sperm, which differs from Y-sperm in that its density is higher.

Evaluation of sperm motility in bovine sexed semen by CASA

The motility of sperm after dilution (AD) and post-thaw (PT) was evaluated using computer-assisted sperm analysis, or CASA (AndroVision software, Minitube of America-MOFA®, Verona, WI, USA). Before analysis, 5.5 µL of semen aliquots were dropped on prewarmed slides and covered with a coverslip (Hamilton 2X-CEL®, MA, USA) and then subjected to rapid CASA. The CASA system was operated at 30 frames per second, with at least 1,000 sperm tracked per sample and a minimum motility threshold of 20%. The analysis parameters were measured in this study: total sperm motility (TM) and progressive sperm motility (PM).

Statistical analysis

The percentages of X-sperm, total sperm motility, and progressive sperm motility in CONV and M-Zlex semen were analyzed by one-way ANOVA using the statistical software program

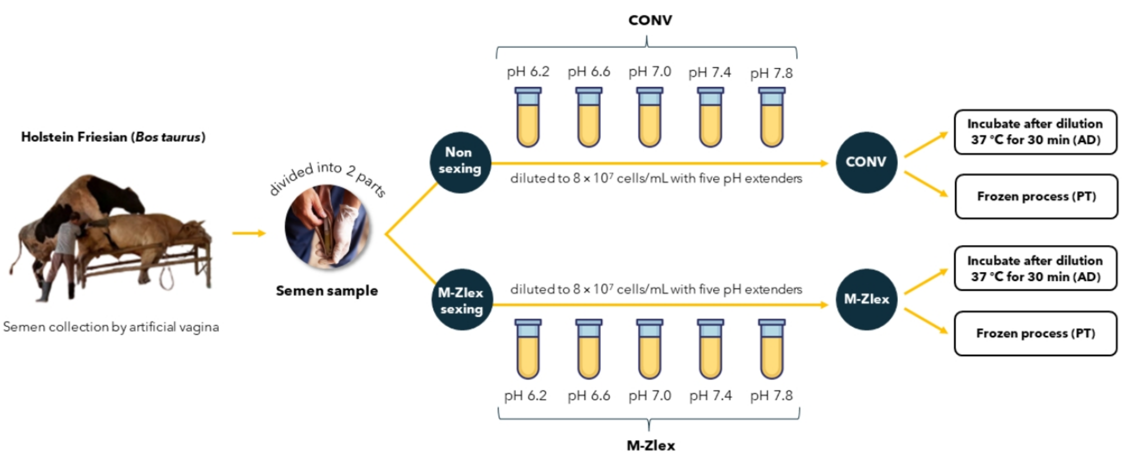

Figure 1. A diagram of the steps involved in the production of conventional and M-Zlex semen using five extenders

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Duncan's new multiple range test was used to compare the mean between individual treatments when the *P*-value was less than 0.05.

Results

Osmolarity of extender with different pH

Each treatment was measured for osmolarity after preparation. Five extenders with different pH have an osmolarity of 300-335 mOsmol/kg. The osmolarity of each treatment is shown in Table 1.

X-sperm ratio in bovine sexed semen

The patterns of X-and Y-sperm were observed with Hoechst 33342, and sperm frequency histograms created after Hoechst 33342 staining exhibit distinct fluorescence peaks for X-and Y-sperm in the CONV and M-Zlex groups, as shown in Figure 2. The percentage of X-sperm in different extender pH of CONV and M-Zlex semen is shown in Figure 3. A significant difference in the percentage of X-sperm was observed between CONV and M-Zlex ($P < 0.05$). The range of CONV was between 48.96-51.34%, while M-Zlex was up to 73.22-74.36%.

Table 1. The osmolarity of extenders with different pH (*n* = 5 replicates/treatment)

Treatment	pH 6.2	pH 6.6	pH 7.0	pH 7.4	pH 7.8
Osmolarity (mOsmol/kg)	328.60 ± 3.21	302.20 ± 1.92	310.80 ± 4.09	320.00 ± 2.34	333.80 ± 6.22

Values are presented as mean ± standard deviation

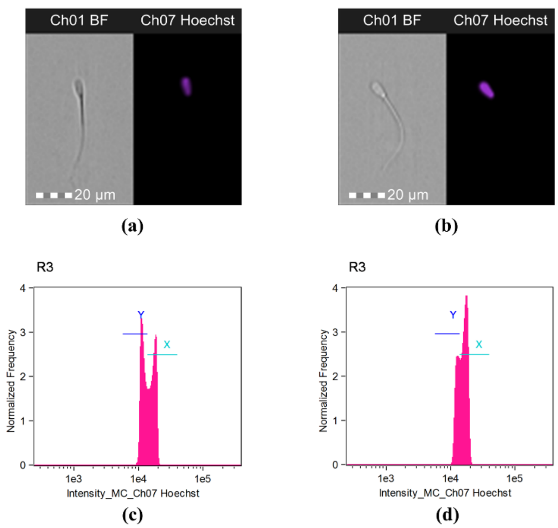

Figure 2. Discrimination of the X- and Y-sperm ratios in post-thaw between CONV and M-Zlex semen. (a) Patterns of Y-sperm were observed with Hoechst 33342; (b) Patterns of X-sperm were observed with Hoechst 33342; (c) Sperm frequency histograms exhibit distinct fluorescence peaks for X- and Y-sperm in CONV groups; (d) Sperm frequency histograms exhibit distinct fluorescence peaks for X- and Y-sperm in M-Zlex groups

Sperm motility in bovine sexed semen

All data on sperm motility of CONV and M-Zlex semen were obtained at various pH levels and examined them with CASA at two time periods: after dilution (AD) and post-thaw (PT). In this study, the results for total sperm motility (TM) and progressive sperm motility (PT) in AD are shown in Figure 4. After dilution, TM and PM of CONV in pH 6.6 and 7.0 were significantly higher than other pH levels, but only pH 6.6 of M-Zlex was the highest (P < 0.05). Additionally, the TM and PM of sperm diluted by extender with different pH in PT are shown in Figure 5. In post-thaw, pH 6.6, 7.0, and 7.4 of CONV had more TM and PM than other pH levels. However, pH 6.6 and 7.0 provided the most TM and PM of M-Zlex compared to other treatments (P < 0.05).

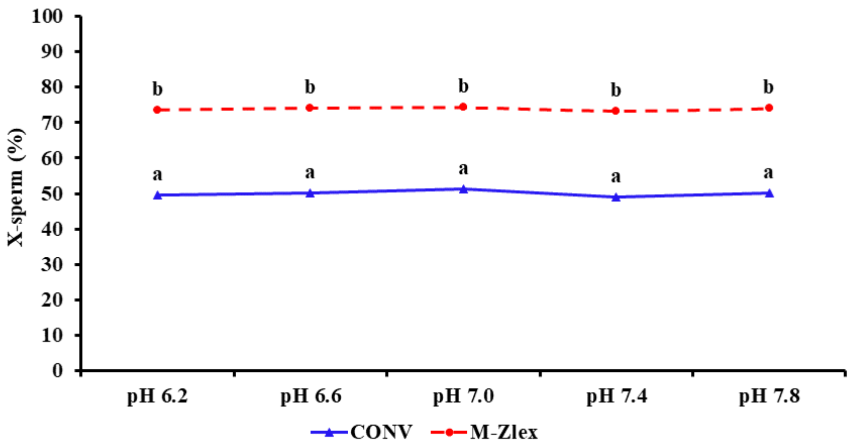

Figure 3. The percentage of X-sperm in CONV and M-Zlex semen

[a, b] indicates a significant difference in each treatment (P < 0.05)

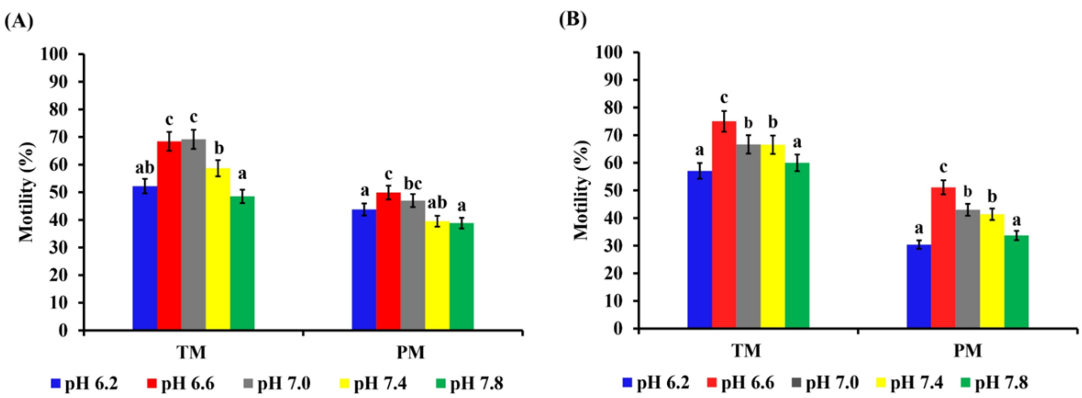

Figure 4. The total motility and progressive motility of sperm diluted by extender with different pH after dilution (AD); (A) CONV; (B) M-Zlex

Different letters indicate a statistically significant difference in each treatment (P < 0.05)

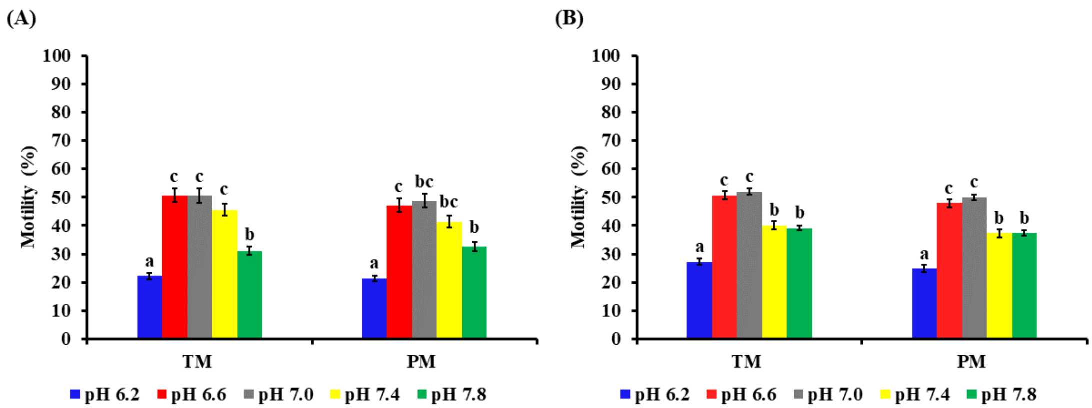

Figure 5. The total motility and progressive motility of sperm diluted by extender with different pH in post-thaw (PT); (A) CONV; (B) M-Zlex

Different letters indicate a statistically significant difference in each treatment (*P* < 0.05)

Discussion

After maturation, sperm become completely dependent on their environment because they are no longer able to grow, divide, repair, biosynthesize, or perform any metabolic functions (Contri *et al*., 2013). For this reason, different osmolarity and pH of the extender can affect the quality in terms of motility of CONV and M-Zlex semen differently due to different proportions of X-/Y-sperm.

Our findings show that all extenders had an osmolarity range of 300-335 mOsm/kg, which is appropriate for bovine sperm motility. This is consistent with a previous study by Liu et al. (1998), who reported that bovine sperm motility was greatest when extender osmolarity ranged between 270 and 340 mOsm/kg. Based on these findings, osmolarity alone did not significantly affect sperm motility and did not exert any synergistic or interactive effects with extender pH in influencing motility outcomes. Therefore, osmolarity was confirmed as not a contributing factor to sperm motility in this study.

Extracellular pH (pHe) has a direct impact on the pH of the sperm cell's cytoplasm, or intracellular pH (pHi), which is directly associated with sperm function. Mammalian sperm under different pH extenders shows differences in terms of the DNA damage, sperm motility, and mitochondrial activity (He *et al*., 2023a). In particular, mitochondrial activity for ATP production was significantly positively correlated with sperm motility (Contri *et al*., 2013). In this study, we discovered that the various pH levels of extenders clearly affected sperm motility and that the CONV and M-Zlex semen with different X-/Y-sperm ratios had slightly varied suitable pH values for the greatest sperm motility. In post-thaw, conventional semen has excellent motility in slightly acidic to slightly basic solutions (pH 6.6-7.4), whereas M-Zlex semen with a higher X-sperm content has high motility in weakly acidic to neutral conditions (pH 6.6-7.0). Furthermore, the influence of pH on sperm motility becomes increasingly clear when fresh semen is continuously exposed to that different acid or base environment (after dilution, incubate for 30 minutes). The M-Zlex semen exhibits the highest sperm motility at slightly acidic (pH 6.6). Conversely, CONV semen exhibits the highest sperm motility between pH 6.6 and 7.0. In addition, strongly acidic (pH < 6.2) or highly alkaline (pH > 7.8) extenders frequently cause reduced sperm motility in both CONV and M-Zlex semen, indicating an undesirable environment for sperm motility. The extender pH has an impact on sperm motility because it changes mitochondrial activity.

Previous studies have shown that different environmental pH can affect mitochondrial activity of sperm. Contri *et al*. (2013) found that high mitochondrial activity led to high sperm motility; for conventional semen, solutions with pH 7.0-7.5 had the highest mitochondrial activity and sperm motility. This is similar to our findings, which showed that CONV semen in post-thaw had the greatest motility at pH 6.6-7.4 and pH 6.6-7.0 after incubation for 30 min. In addition, the researchers investigated the association between pH and sperm sex ratio by incubating sperm in solutions with varying pH values to distinguish between X- and Y-sperm. Several studies have demonstrated that in acidic solutions (pH 6.2), the upper sperm has a greater proportion of X-sperm, whereas the upper sperm contains a higher percentage of Y-sperm in alkaline solutions (pH 7.4). This is one of the fundamental concepts of swim-up sperm sexing, with the upper layer containing 67.24% of X-sperm at pH 6.2 and 69.53% of Y-sperm at pH 7.4 (He *et al*., 2023b, 2021). This difference in the amount of swim forward (upward) of X- and Y-sperm indicates an optimal pH environment or may promote sperm motility. Consistent with the current study, M-Zlex semen (rich in X-sperm) proved the greatest motility in the pH 6.6 extender after incubation for 30 min. The motility of X- and Y-sperm differs in an acidic-alkaline environment due to changing ATP levels. In weakly acidic conditions, X-sperm mitochondria function better than in alkaline conditions, in contrast to Y-sperm (He *et al*., 2023b). This differential mitochondrial activity contributes to improved motility of M-Zlex semen (enriched with X-sperm) in slightly acidic extenders, as enhanced mitochondrial function increases ATP production specifically in X-sperm. Conversely, mitochondrial activity in Y-sperm is reduced under the same conditions, resulting in lower motility. Meanwhile, CONV semen containing a roughly equal proportion of X- and Y-sperm exhibits balanced mitochondrial activity across mildly acidic to mildly alkaline pH ranges, leading to overall motility that is less influenced by pH variations. Consequently, the optimal pH range for maintaining sperm motility is broader in CONV semen than in M-Zlex semen, reflecting the complementary mitochondrial responses of X- and Y-sperm to pH fluctuations. Moreover, M-Zlex semen motility decreases at pH levels above 7.4 due to impaired mitochondrial function in X-sperm, which reduces ATP production necessary for movement. Therefore, using a slightly acidic extender (pH 6.6–7.0) is beneficial for promoting the motility of post-thaw sexed semen enriched with X-sperm.

Conclusion

The pH of the semen extender influences the motility of both conventional and sexed sperm. A pH range of 6.6 - 7.4 is generally optimal for conventional semen motility. However, for M-Zlex sexed semen, which is enriched with X-sperm, motility is enhanced within a slightly acidic range of pH 6.6-7.0. This finding suggests that X-sperm exhibit improved motility under weakly acidic conditions. Therefore, adjusting the extender's pH to 6.6-7.0 presents a viable approach for optimizing the production of sex-sorted semen enriched with X-sperm.

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