American Journal of Animal and Veterinary Sciences

Original Research Paper

In vitro Kinematics Semen Tolerance to Oxidative Stress

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Article history
Received: 22-05-2024
Revised: 13-06-2024
Accepted: 22-06-2024

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Abstract: Interpretation of semen kinematic parameters remain as a point of discussion in predicting semen quality following processes like cryopreservation. During cryopreservation, oxidative stress remains a challenge in promoting post-thaw semen quality. Comparison of livestock semen exposed to oxidative stress, in vitro, highlight various aspects of sperm cell movement, like kinematics, to predict their qualities. Sperm cell kinematics includes the measurement of the distance between each head point for a given sperm cell during the acquisition period. Regrettably, the semen kinematic parameters are often reported with the limited interpretation. The apparent difficulty in interpreting these kinematic parameters can be interpreted clearly by looking at semen kinematics from various livestock species under similar conditions. Induced oxidative stressed conditions in cockerel, buck and boar semen present such an opportunity to investigate and interpret the significance of semen kinematic parameters and also check if any of the investigated livestock species can be used to set a threshold for semen quality. In this study, we are demonstrating that cockerel, buck and boar semen under induced oxidative stress present an opportunity to interpret the kinematic parameters associated with their semen quality. Such kinematic include curvilinear velocity, straight line velocity, average path velocities, linearity, straightness and wobble. All these kinematic parameters are readily available following computer aided sperm analyzer semen analysis. Three species, namely, buck boar and cockerel semen were collected. The semen was then exposed to oxidative stress inducing agent, hydrogen peroxide, for 3 h. The semen kinetic parameters were then compared. Our data seem to indicate that buck semen kinematics could be used as predictors of livestock semen quality under oxidative stress, at least for livestock species investigated.

Keywords: Kinematic Parameters, Livestock Species, Oxidative Stress, Semen

Introduction

There are several factors to consider when predicting livestock semen fertility. Such factor include total motility, progressive motility, rapid motility, viability and morphology (Guzick *et al*., 2001; Fernández-López *et al*., 2022). However, semen kinematic parameters can also be useful in predicting the fertilizing ability of spermatozoa, despite being continuously ignored or insufficiently discussed as to their relevance during semen fertility analysis (Fernández-López *et al*., 2022; Barbas *et al*., 2018). Obviously, the semen kinematic patterns form the basis for motility evaluations done following Computer Aided Sperm Class Analyzer (CASA) analysis. A large volume of data is obtained following CASA which requires multivariate statistical analysis for accuracy of data interpretation. Furthermore, for accuracy of assessment of these data, a number of factors needs to be taken into consideration. Such factors include the software used, the capture field, the recording time and the frame rate, all of which must be adjusted and standardized prior to analysis. At species specific CASA settings of assessment, the kinematic parameters can be used to predict fertility of livestock semen.

While the species-specific setting can be used to assess their semen kinematics using CASA, the differences in the sensitivity and accuracy could indicate negligible differences overall for various species (Finelli *et al*., 2021). Additionally, the behavior of the semen kinematic parameters under similar conditions can be used to indicate how the inherent molecular properties of the sperm cells influence their response to stimulus (Barbas *et al*., 2018).

During cryopreservation, sperm cells show high lipid peroxidation as measured using Malondialdehyde (MDA) as an indicator (Tsikas, 2017). This is due to the high generation of free radicals that interact with membrane lipids resulting in membrane lipid peroxidation (Len *et al*., 2019). For affected spermatozoa the semen kinematics changes, accompanied by compromised semen quality. Interestingly, various livestock species show variable tolerance level to these free radicals and different degrees of compromised semen qualities under similar conditions (Pilane *et al*., 2016; 2019). These observations are due to the inherent properties of the sperm cell make-up per livestock species. For example, boar semen has been reported to contain high levels of polyunsaturated fatty acids which renders them vulnerable to induced oxidative stress, hence the exaggerated lipid peroxidation levels (Am-In *et al*., 2011).

Previous studies have also demonstrated that the first line of defense against oxidative stress in spermatozoa includes the cell membrane (Brzezińska-Ślebodzińska *et al*., 1995; Al-Omar *et al.*, 2004). Once the cell membrane is compromised, the sperm cell is then doomed to lose its ability to move and fertilize, accompanied by compromised kinematic parameters (Pilane and Mapeka, 2021). Individual motile spermatozoa can be defined by six kinematic parameters, among others, such as, curvilinear velocity [VCL], straight-line velocity [VSL], average path velocity [VAP], Linearity [LIN], straightness [STR] and wobble (WOB). The curvilinear velocity (VCL) is the time-average velocity of the sperm head along its actual path, measured in µm/sec. A compromised sperm cell motility will have a low VCL (Pathak *et al*., 2019). On the other hand, the time-average velocity of the sperm head that is projected along a straight line (VSL) will also be low in motility compromised sperm cell. In fact, the average path velocity (VAP), linearity (LIN), straightness (STR) and the degree of oscillation (WOB), will all drop in a motility-compromised sperm cells. For example, in boars, the total motility decreases by 40% following 3 h of oxidative stress as compared to 20% in cockerels and 10% in buck semen and thus will expect to see corresponding compromised kinematic parameters (Am-In *et al*., 2011; Brzezińska-Ślebodzińska *et al*., 1995; Pilane and Mapeka, 2021).

Ironically, the sperm cell's kinematic parameters from various livestock species are affected differently in a species-dependent manner, as elucidated. Thus, a comparative semen kinematic study to investigate the tolerance to oxidative stress *in vitro* will be helpful to provide a clear interpretation in this regard. In this study, we expose the semen from various livestock species, namely, buck, boar and cockerel to oxidative stress under comparable assessment conditions and compare the behavior of their VCL, VSL, VAP, LIN, STR and WOB kinematic parameters.

Materials and Methods

Study Area

The study was approved and carried out according to the guidelines of the Agricultural Research Council ethics committee. The study was conducted at the Agricultural Research Council (ARC) Irene at the Germplasm Conservation and Reproductive Biotechnology laboratory. The area is located at 25° 53′ 59.6″ South latitude and 28° 12′ 51.6″ East longitude in Pretoria, South Africa.

Semen Collection and Processing

All procedures were approved by the animal ethics committee of the ARC in the presence of a local veterinarian. Four superior South African indigenous bucks were stratified based on age (2 years) and weight (25-45 kg). The South African indigenous unimproved bucks were in good health condition throughout the duration of the study (APIEC2011/38). For boar, three exotic Large White boars were used for this study at the ages of 2-3 years of age with ethical clearance numbers APIEC/13/002 and APIEC 10/01. Semen was collected from five Venda cockerels using the abdominal massage technique with an ethical clearance number APIEC/08/06. For all the livestock species, water was given at ad libitum basis throughout the duration of the study. After collection, the semen samples were placed into the thermo-flask at 37°C and transported to the laboratory.

Treatments

For semen treatment, H_2O_2 stock solution was prepared in pre-warmed BO-Wash and kept at 4°C until use. During the experiment, semen at equal concentration and volume was treated with pre-warmed H_2O_2 stock to make 0, 5, 50 and 200 µM concentrations. The treated cells were then incubated at 37°C for 3 h in a humidified 5% CO_2 and 95% atmospheric air incubator (Sanyo, Japan). After 3 h the cells were evaluated for semen kinematic parameters and viability.

Viability Assay

For cell viability, SYBR-14 and PI Live/Dead kit were used and the cells were treated according to the manufacturer's recommendation (Invitrogen, Molecular probes, USA). Briefly, 50 µL of semen was diluted with pre-warmed BO-Wash to 1 mL and 5 µL of a 50 times diluted SYBR-14 was added to the cells followed by incubation at 37°C for 10 min. After 10 min, 5 µL of Propidium Iodide was added to the cells followed by incubation for an additional 10 min. After 10 min, 5 µL of cells were immediately placed on a pre-warmed glass slide and observed under a fluorescent microscope (Olympus, model BX51). The percent cell viability was determined by counting the number of green cells out of

a hundred cells in a field. The data was represented in a table as percent viability versus H_2O_2 concentrations.

Semen Kinematic Parameters

The semen kinematic parameters were assessed for all livestock species following CASA analysis. Six semen kinematics namely VCL, VSL, VAP were used to measure individual sperm cell velocities, while STR, LIN and WOB, were used to measure the directional movement of sperm cells. Indicated values represent the average of five replicates per species.

Statistical Analysis

Data was analyzed using ANOVA for treated versus untreated samples. Comparison among species was analyzed using the multivariate analysis of variance (ANOVA). All analyses were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). Significance was set at $p<0.05$.

Results

Sperm cells exposed to oxidative stress become leaky at the membranes due to the damage induced by ROS. This can be observed when such cells are stained with various dyes to show membrane permeability. The SYBR-14/PI viability staining has been used for a while now and it has become popular because of the ease of distinguish between the viable and the non-viable cells irrespective of the species used. The viable sperm cells become green following the staining as their membranes remain intact while the non-viable cells become red due to the presence of the Propidium Iodide (PI) within the stain itself, as shown for Cockerel, Boar and Buck semen (Fig. 1. A-C, respectively). The SYBR-14 dye was readily permeable to the cells irrespective of the cell's membrane integrity, while PI was selective and only enters "leaky" cells.

The viability analysis is shown in Fig. (2), where semen from the three species exposed to H_2O_2 for 3 h show varying tolerance to induced oxidative stress.

Figures (3-5), show the semen motility for cockerel, Boar and Buck, respectively. Analysis of these data revealed that the projection of the movement of cockerel semen is not influenced by H_2O_2 treatments as seen by minimal changes is rapidly and progressively moving sperm cells.

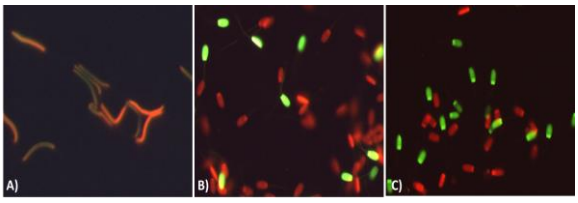

Fig. 1: (A) Cockerel semen stained with SYBR-14/PI, (B) Buck semen stained with SYBR-14/PI and (C) Boar semen stained with SYBR-14/PI at 60X magnification

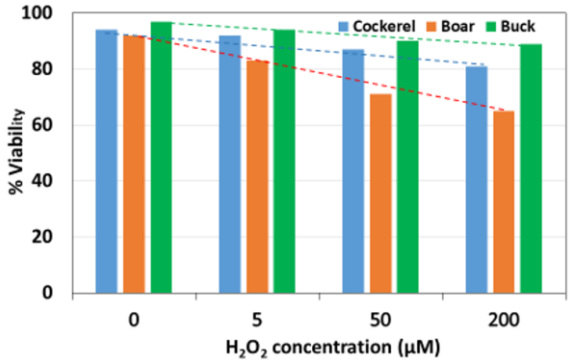

Fig. 2: The percent viability of cockerel (blue), boar (orange) and buck (green) semen following treatment with 0, 5, 50 and 200 µM H_2O_2 for 3 h at $p<0.05$. The dotted lines indicate the slope of corresponding sample

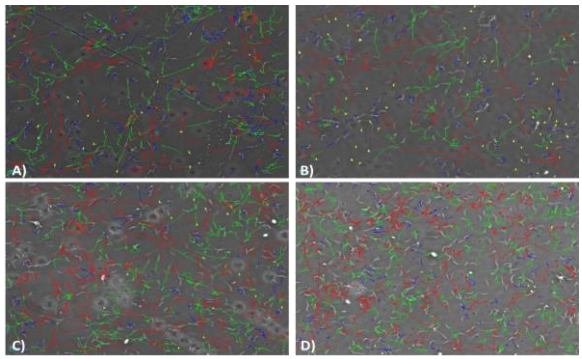

Fig. 3: Motility of cockerel semen as analyzed using CASA after 3 h where the RAP (red), progressive motility (green), Non-progressive motility (blue) and static (yellow) sperm cells are shown. The untreated control (A) and 5µM H_2O_2 treated cells (B), 50 µM H_2O_2 treated sperm cells (C) and 200 µM H_2O_2 treated sperm cells are shown

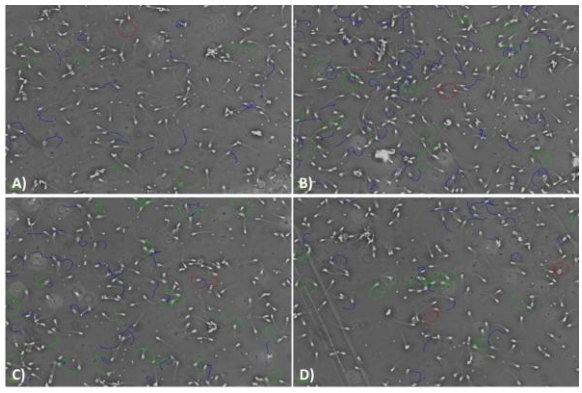

Fig. 4: Motility of boar semen as analyzed using CASA after 3 h where the RAP (red), progressive motility (green), Non-progressive motility (blue) and static (yellow) sperm cells are shown. The untreated control (A) and 5 µM H_2O_2 treated cells (B), 50 µM H_2O_2 treated sperm cells (C) and 200 µM H_2O_2 treated sperm cells are shown

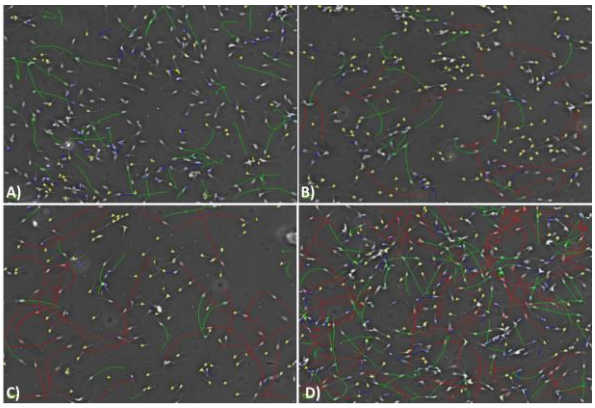

Fig. 5: Motility of buck semen as analyzed using CASA after 3 h where the RAP (red), progressive motility (green), Non-progressive motility (blue) and static (yellow) sperm cells are shown. The untreated control (A) and 5 µM H_2O_2 treated cells (B), 50 µM H_2O_2 treated sperm cells (C) and 200 µM H_2O_2 treated sperm cells are shown

Also, boar semen revealed no changes overall from the untreated control semen with no rapidly moving sperm cells. While the Buck semen revealed an absolute activation of the cells accompanied by large projections as shown by rapidly moving and progressively moving sperm cells. Moreover, the number of rapidly moving sperm cells increases as the H_2O_2 concentrations were increased.

As for the semen kinematics, analysis of raw cockerel semen and untreated controls preserved for 3 h revealed that there was no significant differences between the raw cockerel semen VCL values and the untreated controls after three hrs. However, VSL, LIN, STR and WOB were significantly increased as compared to untreated raw cockerel semen values, while VAP showed an non-signicant increase (Table 1).

For Boar semen, there was a significant decrease in VCL only, while the rest of the kinematic parameters showed non-significant drop in such values (Table 2.).

For Buck semen, only LIN showed a significant decrease in untreated controls after three hours as compared to VCL, VSL, VAP, STR and WOB (Table 3.).

In Buck semen, the kinematic parameters VCL, VSL, VAP and LIN, all showed significant increases following 3 h of 5 µM H_2O_2 induced oxidative stress (Table 4.).

At 50 µM H_2O_2 induced oxidative stress, there were no significant differences among all three species of their semen VCL, VSL, VAP, LIN, STR and WOB (Table 5.), while only STR was marginally significantly increased in buck semen under 200µM H_2O_2 induced oxidative stress for 3 h as compared to Cockerel and Boar semen kinematic parameters (Table 6.).

Table 1: The kinematic parameters of raw Cockerel semen and untreated controls after 3 h ($p<0.05$)

Kinematic parameters	Raw cockerel semen	Untreated cockerel semen control
VCL	67.13±11.85	65.10±4.0
VSL	23.40±04.24	31.10±3.6
VAP	39.83±07.85	45.50±3.9
LIN	34.30±00.74	47.60±3.5
STR	58.20±00.88	68.06±3.1
WOB	59.11±01.64	69.80±2.1

Table 2: The kinematic parameters of raw boar semen and untreated controls after 3 h ($p<0.05$)

Kinematic parameters	5 µm H_2O_2 treated cockerel semen	5 µm H_2O_2 treated boar semen	5 µm H_2O_2 treated buck semen
VCL	56.5±13.5(a)	59.10±02.01(a)	86.60±17.7(b)
VSL	27.3±11.4(a)	27.67±06.90(a)	60.50±23.5(b)
VAP	38.6±12.9(a)	47.83±03.86(a)	75.80±20.5(b)
LIN	46.7±09.37(a)	47.00±12.20(a)	67.20±13.1(b)
STR	68.9±05.84(a)	57.43±11.30(a)	77.25±09.75(a)
WOB	67.1±07.50(a)	80.96±05.63(a)	86.30±06.12(b)

Table 3: The kinematic parameters of raw Buck semen and untreated controls after 3 h ($p<0.05$)

Kinematic parameters	200 µm H_2O_2 treated cockerel semen	200 µm H_2O_2 treated boar semen	200 µm H_2O_2 treated buck semen
VCL	68.1±8.70(a)	66.70±5.76(a)	78.60±9.30(a)
VSL	28.9±0.98(a)	24.83±7.1(a)	52.70±0.70(a)
VAP	44.5±2.98(a)	49.3±12.81(a)	66.40±4.65(a)
LIN	43.3±6.45(a)	36.6±80.07(a)	68.30±9.13(a)
STR	65.4±5.50(a)	50.0±10.74(a)	79.80±6.70(a)
WOB	65.8±4.29(a)	72.7±13.89(a)	85.05±4.15(a)

Table 4: The kinematic parameters of cockerel, boar, and buck semen following induced oxidative stress at 5 µm H_2O_2 after 3 h ($p<0.05$)

Kinematic parameters	Raw boar semen	Untreated boar control
VCL	66.20±3.69(a)	46.03±15.00(b)
VSL	28.40±2.52(a)	24.27±12.00(a)
VAP	48.27±2.89(a)	36.00±16.60(a)
LIN	58.67±2.81(a)	50.50±07.89(a)
STR	73.30±7.72(a)	67.23±04.57(a)
WOB	77.95±4.95(a)	79.40±04.94(a)

Table 5: The kinematic parameters of cockerel, boar, and buck semen following induced oxidative stress at 50 µm H_2O_2 after 3 h ($p<0.05$)

Kinematic parameters	Raw buck semen	Untreated buck control
VCL	70.30±1.56(a)	65.70±29.50(a)
VSL	30.83±4.32(a)	37.70±28.40(a)
VAP	44.00±7.21(a)	49.30±32.50(a)
LIN	64.60±5.32(a)	24.80±02.86(b)
STR	67.73±5.28(a)	73.20±08.87(a)
WOB	63.50±0.50(a)	68.43±14.60(a)

Table 6: The kinematic parameters of Cockerel, Boar, and Buck semen following induced oxidative stress at 50 µm H_2O_2 after 3 h ($p<0.05$)

Kinematic parameters	50 µm H_2O_2 treated cockerel semen	50 µm H_2O_2 treated boar semen	50 µm H_2O_2 treated buck semen
VCL	59.7±14.40(a)	61.57±04.70(a)	75.05±4.20(a)
VSL	27.1±07.40(a)	26.70±07.58(a)	49.3±11.40(a)
VAP	40.8±10.50(a)	46.63±11.60(a)	63.6±17.80(a)
LIN	45.2±06.00(a)	43.50±12.90(a)	65.0±11.40(a)
STR	70.6±05.70(a)	57.20±10.70(a)	77.75±8.55(a)
WOB	68.1±04.90(a)	75.20±14.90(a)	83.55±5.65(a)

Discussion

The interest to interpret and understand the significance of the semen kinematic parameters is shown in this study (Aghazarian *et al*., 2021). Cockerel, Buck and Boar semen respond differently to various conditions which affect their kinematic parameters. Raw cockerel semen and the 3 h untreated controls show varying kinematics whereas the untreated control shows increases in linearity, straightness, straight line velocity and wobble. This data indicates that cockerel raw and the 3 h untreated semen do not lose their kinematics (Jimoh *et al.*, 2021). Interestingly, after 3 h of induced oxidative stress at 5µM H_2O_2, the kinematic parameters are increased even further (Pilane and Mapeka, 2021). Moreover, no further increases were observed at 50 and 200 µM H_2O_2 of induced oxidative stress for the same period indicating that cockerel semen kinematics are resistant to oxidative stress effects after three hrs. Others have shown that high levels of ROS, higher than the antioxidant capacity of a sperm cell, can lead to a poor quality sperm cell (Leão *et al*., 2021). The interpretation gathered from this observation indicates that low amounts of ROS are harmless for cockerel semen for only a short period of time.

Furthermore, raw boar semen and their 3 h of untreated controls show a significant decrease only in VCL. All of the other kinematic parameters showed numerical decreases in VSL, VAP, LIN and STR. Interestingly, numerical but insignificant increases were observed for VCL at increasing induced oxidative stress. Accompanying these, was the numerical decreases in VSL, VAP, LIN, STR and WOB at increasing concentrations of induced oxidative stress. The implication here is that semen quality is lost during oxidative stress.

Another livestock species, namely buck, show interesting observations. Buck semen showed an initial increase for VCL, VSL and VAP. All of these are velocity parameters indicating overall motility and improved semen quality (Pilane *et al.*, 2019). Also, buck semen showed a decrease in only the LIN after 3 h in untreated control semen. Under oxidative stress induced at 5 µM H_2O_2 for 3 h, buck semen showed a significant recovery of linearity to raw semen values, while all other kinematic parameters remained unchanged. Surprisingly, there were no further changes in all other kinematic parameters at 50 and 200 µM H_2O_2 of induced oxidative stress.

A comparison of the kinematic parameter changes was also investigated after 3 h of induced oxidative stress. Our data showed that at 5 µM of induced oxidative stress for 3 h, buck semen had a superior VCL, VSL, VAP, LIN and WOB accompanied by a moderate increase in STR as compared to cockerel and boar semen. A further investigation of 50 and 200 µM H_2O_2 induced oxidative stress showed that only STR was improved albeit non-significantly, especially at 50 µM H_2O_2 induced oxidative stress for 3 h.

In cockerel semen, few if any studies seem to emphasize their vulnerability to oxidative stress (Tesfay *et al.*, 2020). Our data revealed that while all other kinematic parameters namely, VCL, VSL, VAP and WOB are numerically higher in buck semen, followed by cockerel semen and lastly boar semen, only STR and LIN seem to change in the order of decrease. That is, cockerel semen becomes numerically higher for LIN and STR after buck semen at all H_2O_2-induced levels of oxidative stress. Overall, this data appears to suggest that the robustness and resistance of buck semen to induced oxidative stress is due to its ability to maintain its semen STR in addition to its velocity parameters.

Conclusion

Livestock semen kinematic parameters can be used to distinguish semen with supreme qualities by investigating their responses following stimuli like oxidative stress. Thus far, buck semen seems to show superior movement as compared to cockerel or boar semen following oxidative stress stimuli. Buck semen can maintain STR and LIN accompanied by VAP, VSL and VCL. As such, our observation seems to suggest that buck semen kinematics, can be used, under oxidative stress conditions, as a baseline for predicting other livestock semen tolerance to oxidative stress and cryoinjury, although further investigation is required to confirm this in other livestock species.

Acknowledgment

The author wish to acknowledge the Agricultural Research Council (ARC) and National Research Foundation (NRF) for providing necessary facilities and financial support.

Funding Information

Grants from the National Research Foundation (NRF), Agricultural Research Council (ARC) and Department of Agriculture, Land Reform and Rural Development (DALRRD) funded the study.

Ethics

The ethical clearance were obtained from the ARC-animal ethics committee with the ethical clearance numbers APIEC2011/38 for bucks, APIEC/13/002 and APIEC 10/01, for large white boars and APIEC08/06 for cockerels.

Conflict of Interest

The authors declare no conflicts of interest.

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