Original Research

Journal of Advanced Veterinary Research
(2025) Volume 15, Issue 3, 289-293

Improving landrace pig sperm motility and kinematics by adding citrate and glucose to coconut water-egg yolk diluent: A CASA study

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ARTICLE INFO

Recieved: 20 January 2025

Accepted: 10 April 2025

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Keywords:

Citrate, Coconut water, Egg yolk, Glucose, Motility and kinematics, Pig sperm

ABSTRACT

Sperm motility and kinematics are considered significant elements that could affect the artificial insemination programs in pigs. The objectives of this experiment were to investigate the impact of citrate and glucose supplementation to coconut water-egg yolk-based diluents on the motility and kinematics of landrace pig sperm. The research material was fresh semen of a landrace pig having total sperm motility greater than 70% and sperm abnormality less than 20%. The semen was diluted with four different diluents: CY (young coconut water-egg yolk, control), CYG (young coconut water-egg yolk with glucose), CYC (young coconut water-egg yolk with citrate), and CYGC (young coconut water-egg yolk with glucose and citrate). Sperm motility and kinematics were assessed at 48 hours of preservation using Computer Assisted Sperm Analysis (CASA), while sperm morphology was assessed under light microscopy. The revealed sperm motility was significantly different (P<0.05) in the experimental treatments. The best result in motility was obtained by CYGC diluent with total motility of 76.86%, progressive motility at 50.35%, and fast motility of 24.76%, while the Sperm abnormalities were no significant difference (P > 0.05). The highest sperm kinematics were also produced by CYGC and were significantly different from CY and CYG for all sperm kinematics parameters (P<0.05), while with the CYC diluent it was significantly different only in several parameters such as DCL, DSL, DAP, BCF, HAC, LIN, and STR (P<0.05). It could be concluded that supplementing the coconut water-egg yolk diluent with glucose and citrate could enhance the motility and kinematics of Landrace pig sperm.

Introduction

Motility and sperm kinematics appear to be the main factors that determine the success of artificial insemination in pigs. For this reason, researchers should keep on looking for methods that might enable sperm motility in samples meant for storage. Few of the semen diluents have been used in the preservation of pig sperm, and one of such is coconut water-egg yolk diluent. Nevertheless, there is an opportunity to develop new optimal formulations with the further increase in the motility and kinematics of sperm at great usage of these diluents. Among the techniques used, there is the use of additives that supplement energy to sperm: glucose (Jian-Hua *et al*., 2016; Hernandez-Aviles *et al*., 2020) and citrate (Susilowati *et al*., 2020), which help to stabilize the pH during storage.

Although diluents that incorporate coconut water and egg yolk have been used in the application of artificial insemination in livestock (Jimoh, 2020; Salim *et al*., 2020; Cabral *et al*., 2023; Soltan *et al*., 2023), some challenges have been identified in the storage of semen quality over extended periods. Reduced sperm quality during storage is observed in relation to the motility and kinematics of sperm, meaning any process of insemination is likely to be affected by the ability of sperm to fertilize an egg. This also caused a decline in sperm quality; the main cause is the inappropriately low energy and buffer concentration in the diluent (Lopez Rodriguez *et al*., 2017; Hussain *et al*., 2018); this may lead to increased oxidative stress and changes in the movement characteristics of sperm (El-Khawagah *et al*., 2020). Therefore, this study will seek to extend the knowledge on this possibility of advancing the capability of the diluent containing citrate and glucose to aid the performance of sperm during the preserved state. Both are considered to act in playing functions for energy of cells and for pH stability (Zhu *et al*., 2022), although the interactions between them affecting Landrace pig sperm quality have not been explored extensively. Therefore, there is a need to establish the impact of citrate and glucose in improving the quality and kinetics of Landrace sperms tested in coconut water-egg yolk-based diluent.

Some earlier research has focused on the utilization of citrate and glucose in the diluents of semen for various livestock breeds, such as cattle and sheep, with an increase observed in the motility of sperm samples (Raheja *et al*., 2018; Bintara *et al*., 2022; Akhter *et al*., 2023). However, research works that are specific to the effects of citrate and glucose in coconut water-egg yolk-based diluents on Landrace pig sperm are still comparatively few. In most of the previous works, only one of the two ingredients was examined in its capacity, but none of the works looked into the combined effects of both in enhancing the quality of sperm (Yeste, 2018; Zhu *et al*., 2022). However, while there are preexisting tests for sperm quality based on routine semen analysis, motility analysis, and other parameters, the sperm kinematic parameters are not thoroughly investigated. These kinematic parameters can give much more detailed data about the capability of sperm to move and interact with eggs. To address this literature gap, the current study intended to employ the Computer Assisted Sperm Analysis (CASA) method (Soler *et al*., 2017), which can give better and more precise sperm kinematic details, and assess the impacts of the combination of citrate and glucose in coconut water-egg yolk-based semen extenders on the semen quality of Landrace pigs.

Therefore, this work presents a new idea to the existing literature on animal reproductive technology on the utilization of the citrate and glucose combination that enhances the sperm quality of the Landrace pigs. It is important to note that the use of these two components together in a diluent prepared from coconut water and egg yolk is an area that has not received much attention in scientific literature. Furthermore, the recent method, the CASA method, enables detailed analysis of sperm kinematic parameters, which helps evaluate sperm quality at a higher level than the usual methods of assessing sperm quality, though it can distinguish changes in sperm kinematics. Therefore, this study also aims at not only enhancing the quality of sperm in semen diluents but also enhancing better and more efficient evaluation systems for the improved productivity of the livestock industry. It is believed that this study will contribute knowledge towards the formulation of better semen diluents for artificial insemination to enhance productivity in the pig farming sector.

The general aim of this research was to assess the impact of citrate and glucose supplementation in the coconut water-egg yolk diluent on the motility and kinematics of Landrace pig sperm.

Materials and methods

Semen diluents preparation

Four different types of diluents were used: young coconut water-egg yolk (CY), young coconut water-egg yolk with glucose (CYG), young coconut water-egg yolk with citrate (CYC), and young coconut water-egg yolk with glucose and citrate (CYGC). The CY diluent was made by the guidelines of Hernawati *et al.* (2024). CY diluent is a basic diluent composed of 80% young coconut water and 20% chicken egg yolk, which are mixed until they are homogenous. CYG diluent was made by adding 1.25 g of glucose (Sigma, G7021-100G) to 100 mL of CY diluent; CYC was made by dissolving 2.9 g of tri-sodium citrate dihydrate (Sigma, S1804-500g) in 100 mL of CY diluent; and CYGC diluent was made by adding 1.25 g of glucose (Sigma, G7021-100G) and 2.9 g of tri-sodium citrate dihydrate (Sigma, S1804-500g) to 100 mL of CY diluent. For every milliliter of these diluents, 1000 IU of streptomycin (Sigma, S1277-5G) and 1000 μg of penicillin (Sigma, P3032-10MU) were added.

Semen collection

Two 2.5-year-old landrace boars with healthy reproductive organs were used for collecting semen. The boars were housed in separate cages and fed 3200 kcal of energy and 16% protein. Semen collection was carried out twice a week using the glove and hand method (Telnoni *et al.*, 2022).

Fresh semen evaluation

Evaluation of fresh semen was consistent with that published by Maside *et al.* (2023). The semen was collected and brought to the lab for both a macroscopic and microscopic assessment. The volume, color, consistency, and pH of semen were all evaluated macroscopically, whereas sperm concentration, motility, viability, and abnormalities were all evaluated under a microscope with a 400x magnification.

Dilution and preservation of semen

After evaluation, fresh semen containing sperm with progressive motility of more than 70% and sperm abnormalities of less than 20% was diluted with one of the following four diluents: CY, CYG, CYC, or CYGC with a ratio of 1 part semen to 3 parts diluent. The diluted semen was then stored at a temperature of 15-20°C (Henning *et al.*, 2022), and sperm motility, abnormalities, and kinematics were analyzed at the 48th hour of preservation.

Analysis of sperm abnormalities

Sperm abnormalities were analyzed using the eosin (Sigma, E4009-5G) - nigrosin (Sigma, 43925-100ML-F) staining method (Kondracki *et al.*, 2017), and observed under a 400x magnification microscope. The proportion of sperm abnormalities was calculated on 200 cells based on the abnormality of both the head and tail of the sperm.

Computer-assisted semen analyses

Sperm motility and kinematics were analyzed using CASA, which is consistent with Fernandez-Novo *et al.* (2021). In brief, the semen samples in each treatment were diluted with the same diluent until the sperm concentration reached 6 x 106 cells per mL. The semen samples were put in a 2 ml test tube with a lid and warmed for two to three minutes at 37°C in a mobile warming unit (minitube). After filling the counting chamber with three microliters of the semen sample, the chamber was placed inside the Androscope (Androscope CASA system 12500/3000, minitube), which had previously been linked to a computer. Following focus, the kinematics and motility of the sperm were examined. At least 8 random fields with a minimum of 1000 sperm/sample were used for analysis. The sperm motility parameters analyzed included total motility, progressive motility, and fast motility, while sperm kinematics included velocity curve line (VCL), velocity straight line (VSL), average path velocity (VAP), distance curve line (DCL), distance straight line (DSL), distance average path (DAP), amplitude of lateral head movement (ALH), beat-cross frequency (BCF), head activity (HAC), linearity (LIN), and straightness (STR).

Experimental design

This experiment used four treatments (diluents), namely, CY, CYG, CYC, and CYGC. Each treatment was repeated 6 times.

Statistical analysis

The data were analyzed using analysis of variance and continued with Duncan’s test using IBM SPSS software version 25.0.

Results

Comparing the four kinds of diluents, the sperm motility of the CYGC diluent proved significantly higher than the three groups (P<0.05) (Table 1). The total sperm motility in the CYGC diluents was 76.86%, progressive motility was 50.35%, and fast motility was 24.76%. For sperm abnormality parameters, diluent had no effect (P > 0.05), where the abnormality rates of the four types of diluents were 6.9–7.9% after 48 hours preservation (Table 1). Therefore, it is safe to assert that the motility of sperm was improved, and normal sperm morphology may be sustained when glucose and citrate were added to young coconut water-egg yolk diluent which is important for fertilization.

Table 1. Sperm motility of Landrace boar after 48-hour preservation in different extenders

Treatments	Total Motility (%)	Progressive Motility (%)	Fast Motility (%)	Abnormality (%)
CY	69.26[b]	27.02[d]	9.81[c]	7.90[a]
CYG	66.64[b]	33.48[c]	9.16[c]	7.70[a]
CYC	68.51[b]	40.01[b]	19.46[b]	7.10[a]
CYGC	76.86[a]	50.35[a]	24.76[a]	6.90[a]

Young coconut water-egg yolk (CY), young coconut water-egg yolk with glucose (CYG), young coconut water-egg yolk with citrate (CYC) and young coconut water-egg yolk with both glucose and citrate (CYGC). Different superscripts in the same column show that the differences between the means are significantly different (P < 0.05).

In the CASA analysis of sperm kinematics, the greatest value was observed with the CYGC in all the sperm kinematics parameters as compared to CY and CYG (P<0.05). Whereas, with CYC diluent was significantly different only in a few parameters like DCL, DSL, DAP, BCF, HAC, LIN, and STR (P<0.05) (Table 2). The curvilinear trajectory velocity (VCL) was 107.74 μm/s, the straight trajectory velocity (VSL) was 35.15 μm/s, and the average trajectory velocity (VAP) was 45.46 μm/s. The total travel distance of curvilinear trajectory (DCL), straight trajectory (DSL), and average position (DAP) were 36.10 μm, 11.01 μm, and 14.68 μm, respectively. This yielded an amplitude of head deviation as represented by the ALH equal to 2.50 μm, as well as a flagellar beat frequency represented by the BCF of 11 Hz. LIN and STR were 0.29 and 0.65 for straightness and path stiffness, respectively, and HAC was 0.39 rad. These results corroborate the beneficial effects of CYGC diluent on sperm velocity, paths, and motilities and, as such, attain higher and more beneficial sperm kinematic quality as compared to other treatments.

Table 2. Kinematics of Landrace boar sperm after 48-hour preservation in various extenders.

Sperm Kinematics	CY	CYG	CYC	CYGC
VCL (µm/s)	64.77±7.5[b]	71.27± 8.0[b]	107.15± 15.6[a]	107.74± 14.5[a]
VSL (µm/s)	17.85± 3.5[b]	18.45± 4.4[b]	31.41± 3.5[a]	35.15± 3.2[a]
VAP (µm/s)	26.18± 2.1[b]	26.34± 2.1[b]	43.29± 4.4[a]	45.46± 1.8[a]
DCL (µm)	21.62± 1.7[d]	24.62± 1.0[c]	31.08± 1.8[b]	36.10± 2.2[a]
DSL (µm)	5.30± 0.4[c]	5.84± 0.5[c]	8.05± 0.7[b]	11.01± 1.6[a]
DAP (µm)	8.32± 0.2[c]	8.71± 0.3[c]	11.93± 0.6[b]	14.68± 0.2[a]
ALH (µm)	1.69± 0.2[b]	1.71± 0.1[b]	2.48± 0.1[a]	2.50± 0.1[a]
BCF (Hz)	6.13±0.7 [c]	8.63± 0.7[b]	8.70± 0.3[b]	11.00± 0.4[a]
HAC (rad)	0.26± 0.0[c]	0.26± 0.0[c]	0.33± 0.0[b]	0.39± 0.0[a]
LIN	0.29± 0.0[a]	0.21± 0.0[b]	0.24± 0.0[b]	0.29± 0.0[a]
STR	0.59± 0.0[ab]	0.52± 0.0[c]	0.58± 0.0[bc]	0.65± 0.1[a]

Young coconut water-egg yolk (CY), young coconut water-egg yolk with glucose (CYG), young coconut water-egg yolk with citrate (CYC) and young coconut water-egg yolk with both glucose and citrate (CYGC). velocity curve line (VCL), velocity straight line (VSL), average path velocity (VAP), distance curve line (DCL), distance straight line (DSL), distance average path (DAP), amplitude of lateral head movement (ALH), beat-cross frequency (BCF), head activity (HAC), linearity (LIN), and straightness (STR). Different superscripts in the same row show that the differences between the means are significantly different ($P < 0.05$).

Discussion

Sperm motility and kinematics improved significantly with the application of CYGC solution compared to the other three diluents. Significant and crucial roles in creating a favorable microenvironment for sperm motility and function are played by the synthesis of these four critical components. Its inherent antioxidants reduce oxidation processes in sperm, and they fulfill the role of electrolyte support in osmotic equilibrium (Halim *et al.*, 2018; Mahayothee *et al.*, 2016). The proteins and phospholipids in the egg yolk (Xiao *et al.*, 2020) provide protection for the sperm plasma membrane. It has also been established that the glucose included in the composition serves as the main source of energy (Lin & Hardie, 2018) for the respiration of sperm, resulting in progressive movement and a high rate of dynamic indicators. Citrate functions that keep the pH of the diluent stable (Balogun *et al.*, 2022) while the enzymes are made fully effective. The synergy of CYGC increases progressive motility and sperm velocity from different aspects using the parameters such as VCL, VSL, and VAP. These advantages also hold the key message that additive mix determines the best route to semen quality.

When there was a comparison between sperm motility and kinematics of CY, CYG, CYC and CYGC, it was observed that the latter gave the best quality in most of the parameters. The only CY treatment had quality that was significantly lower because of the absence of other energy sources (glucose) and pH controllers (citrate) apart from young coconut water and egg yolk. CYG added with glucose improves the progressive motility but removing the citrate buffer might not maintain an optimal pH for the enzymatic activity of spermatozoa. On the other hand, CYC supplemented with citrate and without glucose was unable to supply enough energy for good motility and kinematics of sperm. This experiment supports the hypothesis that the addition of glucose and citrate to the coconut water-egg yolk diluent has a positive effect on sperm motility and kinematics (Amaral, 2022; Setiawan *et al.*, 2023). In turn, this finding contributes to the scientific perspective on improving semen diluents to improve pig fertility.

Currently, citrate has a buffer role, regulating the internal pH of sperm and thus ensuring appropriate enzymatic activity. This is supported by citrate, which is used in the citrate acid cycle (TCA cycle) energy metabolism in sperm cells (Verschueren *et al.*, 2019). Contrary to this, glucose provides energy through glycolysis for the movement of sperm via adenosine triphosphate (ATP) (Schurr, 2017), which is required for sperm movement. That is why CYGC diluents when combined have a synergetic effect, producing relatively higher progressive motility and kinematics in contrast to other treatments. These statements make the idea of fine-tuning substrate availability related to pH and energy necessary to enhance semen quality proposed in the current work more robust, particularly in the context of the artificial insemination of pigs.

It was also found that glucose and citrate both have a positive impact on the motility and kinematics of spermatozoa. Glucose, through the glycolysis pathway, is used as fuel that is converted to pyruvate (Garcia *et al.*, 2018), which enters the Krebs cycle where ATP is synthesized for motility. This metabolic activity aids in the movement of the sperm tail, which is useful in the progressive motility. Citrate, on the other hand, acts as a pH control agent, which ensures that the pH of the diluent does not change (Liu *et al.*, 2016) and causes the membrane of the sperm to be broken. Besides, citrate also participates in sperm energy metabolism as an intermediate of the Kreb's cycle, offering other contributions to energy supply (Verschueren *et al.*, 2019). It has been found that the diluent that is made from glucose and citrate helps support the motility and kinematics of sperms even when stored.

The current research provides a crucial contribution to the development of a coconut water and egg yolk-based solution suitable for semen diluent. The use of coconut water is wise from an ecological and economic standpoint because it is easily accessible and renewable, and its bioactive components include antioxidants and electrolytes (Halim *et al.*, 2018; Rethinam & Krishnakumar, 2022; Shayanthavi *et al.*, 2024). Certain proteins and phospholipids in the egg yolk protect the sperm plasma membrane during the cooling process (Yeste, 2018; Orrego *et al.*, 2019; Selige *et al.*, 2021). Coconut water has been shown to be a natural semen diluent; numerous experiments have demonstrated that it is more effective than water and other common juices (Esguerra *et al.*, 2020; Odrada *et al.*, 2023). This has created a new conversation for the development of more reasonably priced natural semen diluent to be used in the livestock industry.

Coconut water as a base material in semen diluent also has the advantages of cost-effectiveness and renewable resources. Coconut water is a natural product that can be easily sourced from many countries, particularly those that are tactically stationed in the tropics, and is much cheaper than a commercial chemical diluent. This implication is particularly significant for the livestock industry in developing countries where the cost of the commercial diluents may be prohibitive. In general, the use of coconut water and egg yolk-based CYGC diluents can help cut costs of AI production and help the livestock sector become more sustainable.

Coconut water and egg yolk are essential ingredients of semen diluent because of their nutrient composition and other ingredients that enhance sperm movement and longevity (Jimoh, 2020). Its fluid possesses electrolytes including potassium, sodium, magnesium, and calcium helpful in deriving ionic balance and also the osmotic stability in diluent media (Kannangara *et al.*, 2018; Tuyekar *et al.*, 2021). The uptake of glucose and fructose, which are simple carbohydrates, gives sperm additional energy sources, especially when stored. On the other hand, egg yolk contains phospholipid, and lipoprotein consequently has a role in protecting the sperm plasma membrane from oxidative damage. These phospholipids serve as protectants of the sperm membrane primarily from the adverse effects of temperature fluctuations during the preservation process or during storage. When coconut water is mixed with egg yolk, then it proves to be the best combination of physical barrier protection as well as the energy resource for sperm.

The practical relevance of this study is that the CYGC diluent should be incorporated into the pig artificial insemination (AI). CYGC diluent seems to have functional properties that keep the sperm viable with movement and a motility profile that enables extended use of the semen. Since higher sperm kinematics, including VCL, VSL, and VAP, mean a better, faster, and well-targeted movement of the sperm, then AI has better efficiency (Barquero *et al.*, 2021; Waberski *et al.*, 2022; Araya-Zúñiga *et al.*, 2023). This technology may be used in pig farms to enhance reproductive capacity and pregnancy rates. Besides, the use of CYGC diluent can extend sperm longevity while not affecting semen quality, which makes AI services available to remote areas.

This study has limitations; one of those is a lack of information about how long sperm can persist in CYGC diluent before use. CYGC diluent was established as having the better effect on sperm quality among the four diluents, but the overall quality of the diluted sperm decreased with time. Sperm quality may be negatively affected through oxidative stress that reaches out to the sperm membrane as well as damage the sperm mitochondria that are needed in energy production, affecting motility, viability, and kinematics. Other authors report that the extender used for semen preservation at low temperatures over a relatively long period of time can lead to a reduction in the sperm motility and damage to the acrosome membrane (Yeste, 2018). As such, subsequent research is required to determine how long the CYGC diluent can maintain the viability of boar sperm.

Conclusion

The study revealed that treatment with CYGC comprising coconut water-egg yolk-based diluent with glucose and citrate offered the greatest sperm motility and kinematics characteristics of Landrace boar sperm as compared to the other three treatments. After the CYGC treatment, the percentage of total motility was 76.86%, progressive motility was 50.35%, and minimum sperm abnormality rate was 6.91%. In the same manner, sperm kinematics in the CYGC treatment also registered the highest values in the variables of path velocity, progressive velocity, average path velocity, and others, which denote faster, stably, and direct sperm movement.

Acknowledgments

The authors would like to thank the Republic of Indonesia's Ministry of Higher Education, Science, and Technology for helping to fund this study.

Conflict of interest

The authors have no conflict of interest to declare.

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