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ORIGINAL RESEARCH ARTICLE

Physicochemical Assessment of Two Plant-Derived Rabbit Semen Extenders (Apple and Orange) Compared to an Inorganic Buffer

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ABSTRACT

Semen analysis is vital for evaluating male reproductive status, especially in artificial insemination and fertility management of farm animals. In this study, semen quality collected from twelve New Zealand rabbits (*Oryctolagus cuniculus*) was assessed using three treatments: Treatment one was (semen with 100% tris-egg yolk), which served as the control; treatment two was (semen with 50% tris-egg yolk extender + 50% apple juice), and treatment three was (semen with 50% tris-egg yolk extender + 50% orange juice). Key parameters measured included spermatozoa, progressive motility, Average Path Velocity (APV), Curvilinear Velocity (VCL), Straight-line Velocity (VSL), linearity, livability, straightness, and Amplitude of Lateral Head (ALH). Results showed significant differences ($p<0.05$) at a 1:1 dilution ratio. The orange juice extender yielded the highest values for motility (92.67%), progressive motility (78.67%), and livability (93.92%), which was better the control tris-egg yolk extender, which had lower values (66.00, 48.33 and 92.41% respectively). However, increasing the dilution ratio to 1:2 caused a decline in semen quality across all parameters. Motility and progressive motility dropped to 76.67% and 66.33% with the orange juice extender, and even lower with the tris-egg yolk extender (48.00 and 35.50%). In conclusion, incorporating apple and orange juices into semen extender can enhance spermatozoa motility and overall semen quality in rabbits. Nonetheless, optimizing the dilution ratio is crucial to avoid quality reduction. These findings offer valuable insights for improving male fertility, reproductive efficiency, and productivity in rabbit farming and animal production.

Keywords: Rabbit farming, semen extenders, dilution ratio, spermatozoa quality, fruit juices

INTRODUCTION

Rabbit farming is currently receiving significant attention because of their short gestational cycle, high prolificacy, and easy management. (Juliana *et al*., 2023). Also, its meat is a vital source of animal protein and income for rabbit farmers so, its profitability and productivity depend on the fertility and reproductive efficiency of the male animals. In rabbits, freshly diluted or cooled semen is preferred for insemination (Nagy *et al*., 2002) because semen storage processes such as cryopreservation induce physical and chemical stress in the sperm membrane, resulting in reduced post-thaw spermatozoa survival (Laghouati *et al*., 2023). In artificial insemination, a semen extender (diluent) is a chemical medium used for the preservation, extension and protection of spermatozoa against various shocks during processing, storage and transportation. Interestingly, some factors affect spermatozoa fertility during storage, such as pH fluctuations (buffer capacity), cold shock protection, microbial contamination, osmotic changes, cryo-damages, and energy depletion during metabolism and freezing-thawing procedure (De Ambrogi *et al*., 2006; Raheja *et al*., 2018; Jimoh and Nwachukwu 2022). The gains of artificial insemination have not been optimized by rabbit farms in Nigeria, due to the unavailability of diluents and extenders for its practice partly due to the high cost of importing commercial diluents and extenders (Jimoh and Ayedun, 2020). The propagation of the technical know-how and practice of AI in farms lies in the development of readily available diluents at low production costs. The significant role of diluents used for semen extension determines the success of artificial insemination (Jimoh 2019). The application of natural juices from fruits such as pineapple, watermelon, coconut, and citrus (tangerine and sweet orange) as components of semen extenders has been investigated with chickens and rabbits. (Jimoh and Ayedun, 2020; Jimoh *et al.*, 2020a, 2020b, Jimoh *et al*.,2021). Plant extracts and phytochemicals can be a dependable, secure, and inexpensive treatment for animal fertility problems. Herbs and fruits are essential in antioxidative defense against oxidative damage, potentially protecting cell biological functions. (Shi *et al.*, 2006). There is growing interest in the protective and biological role of natural antioxidants found in herbs, which are options for oxidative damage prevention. (Jimoh *et al.*, 2018). The inclusion of fruit juices from orange (*Citrus sinensis*), cucumber (*Cucumis sativus*) and pineapple (*Ananas comosus*) as constituents of semen extenders has been proven to improve the quality of stored spermatozoa

(Daramola *et al.,* 2016). However, research on how apple and orange juice impact the quality of rabbit semen when mixed with bud extract to protect sperm is scarce, making it difficult to determine the optimal concentration for the extract. Thus, this study seeks to compare the efficacy of two plant-based rabbit semen extender (Apple and Orange) with inorganic buffer.

MATERIALS AND METHODS

Experimental location

Semen collection was carried out at the Rabbitry Unit of the Teaching and Research Farm, University of Ibadan (7º20'N, 3º50'E; 200 - 300 above sea level), while the analyses of semen was carried out at the Animal Physiology and Bioclimatology Laboratory of the Department of Animal Science of the same institution.

Experimental Animals and Management

Twelve (12) mature New Zealand White bucks were used. The bucks were housed in a disinfected standard pen (wire cage). They were fed with commercial pelleted feed and water provided *ad-libitum*.

Experimental layout

The rabbits were allotted to 3 treatments in a completely randomized design. Each treatment was replicated 3 times.

T1 = Treatment one (100% tris-egg yolk extender)
T2 = Treatment two (50% tris-egg yolk extender + 50% Apple juice)
T3 =Treatment Three (50% tris-egg yolk extender + 50% Orange juice)

The statistical model which was a fixed model is as written below:

$$Y_{ijl} = \mu + Bi + e_{ijl}$$

Where Y_{ijl} represents the value of semen characteristics measured in the lth animal;
μ is the overall mean for each character;
Bi is the fixed effect of ith fruit juice extenders at 50% level of inclusion and tris-egg yolk extender as control,
e_{ijl} is the random residual effect

Preparation of extenders

For the control extender, the following was used to prepare the Tris-egg yolk extender

(i) Tris - 2.42g - Primary Extender
(ii) Citric acid - 1.34g
(iii) Glucose - 1.0g
(iv) Penicillin - 0.028g
(v) Glycerol - 10%
(vi) Egg yolk - 10mL

These combinations were added to deionized water to make 100mL and then mixed for proper blending

Fruit extenders

Orange juice extender

Ripe Orange fruit was purchased, washed, and peeled and the fruit pulp blended. The fruit juice was clarified using a juice extractor which was designated as orange juice and kept at 5 ℃. 50mL of the orange juice was then mixed with 50mL of tris-egg yolk extender to give 100mL.

Apple juice extender

100g of Apple was weighed and blended with 50mL of deionized water and strained out juice extracted made up to 100mL with deionized water. 50mL of the apple juice was then mixed with 50mL of tris-egg yolk extender to give 100mL.

Semen collection and evaluation

Semen was collected from the bucks with the use of artificial vagina warmed with water at 40 °C to equilibrate the temperature of the artificial vagina while mimicking the average temperature of the doe's vagina. The experienced bucks were introduced to the does to ensure natural stimulation for ejaculation. The buck's penis was located to guarantee penetration into the artificial vagina for ejaculation. Semen was collected two times a week and only ejaculates with minimum of 0.5 mL volume were used in this study. Gels in ejaculates were removed using micropipette and collected semen was transferred to the laboratory for assessment, processing and storage.

Semen evaluation

Semen evaluation was done using the Computer-Assisted Semen Analysis (CASA). The parameters assessed were:

Curvilinear Velocity (VCL) (μm/s), measured by summing the distance between the spermatozoa head centroid positions frame by frame, divided by the elapsed time.

Straight Line Velocity (VSL) (μm/s), the distance between the first and last points of the spermatozoa track, divided by the elapsed time.

Average Path Velocity (VAP) (μm/s), the average path length, determined by smoothing the spermatozoa head position in a running average (determination of the algorithm which changes among CASA-Mot brands), divided by the elapsed time.

Linearity (LIN) (%), the level of linear progression calculated as the ratio VSL/VCL in percentage.

Straightness (STR) (%), the ratio of VSL/VAP in percentage to measure track compactness;

Wobble (WOB) (%), the oscillation of the actual path about the average path expressed as the ratio of VAP/VCL in percentage;

Amplitude of lateral head (ALH) (μm), the amplitude of the approximate sinusoidal oscillation of the spermatozoa head around the track (can be considered as the maximum or the mean value along the track),

Beat cross frequency (BCF) (Hz), the frequency with which the spermatozoa head crosses the

average path length during acquisition (Soler *et al.*, 2017).

Physicochemical Analysis of the fruit juice

Determination of pH

The pH was determined in ten milliliters of the juice dispensed into a beaker after calibration with phosphate buffer of pH 4.0 and 7.0 (Adubofuor *et al.*, 2010).

Determination of total titrable acidity (TTA).

For the measurement of the titratable acidity the standard method of Talasila *et al.*, (2012) was used. Five grams of concentrated fruit juice was diluted with distilled water (20mL) and filtered using filter paper (Whatman No. 1). The indicator (two drops of phenolphthalein) was added to 20mL of the filtrate and titrated against 0.05 M NaOH. The Total Titratable Acidity was calculated thus: (Eq. (1)).

TA= ¼ (M_{NaOH} x C_{NaOH} x 0.064 _ 100)/V (1)

Where: TA: Titrable acidity; M_{NaOH}: Molarity of NaOH used; V_{NaOH} volume of NaOH used; 0.064: Equivalent weight of citric acid V: volume of juice.

Determination of total solids (TSS).

Total solids content was determined by weighing an empty filter paper and then passing a known weight of juice through a Whatman No. 1 filter paper that retained particle or solids. After drying inside a ventilated oven at 103 ℃ for 2h, the solid left on the filter after evaporation was weighed and used to calculate the TSS (material remaining on the filter after moisture have been evaporated) (AOAC, 2005) as illustrated in Eq. (2).

% Total solids= ¼ (W2 x 100) / W1¼ (100 - % moisture)

Where;
W1: Initial weight;
W2: Dried weight.

Determination of vitamin C

Vitamin C content was determined with the dichlorophenol indophenol (DCP) method of Covenin (AOAC, 2005) with a slight modification.

Titration of DCP with fruit juice.

Standardization process was repeated by replacing ascorbic acid solution with 5mL of juice made up to 10mL with distilled water. After repeating the titration 2 times, the vitamin C content was calculated from standard volume and expressed as mg ascorbic acid/100mL of juice.

Mineral analysis

The method outlined by the Association of Official Analytical Chemists (AOAC 2005) was used to evaluate the samples' proximate analysis: The moisture content was determined gravimetrically, while the AOAC method was used to calculate crude fiber, ash, fat, and protein. (2005). The Association of Official Analytical Method (2005) was used to examine Na, P, Zn, Mg, Ca, Fe, and K.

Table 1 Chemical composition of the orange and apple juice

Extracts	Orange	Apple
Crude Protein (%)	1.05	0.53
Total Sugar (m g / 1 0 0 g)	40.00	20.00
Total Solid (%)	13.70	8.50
pH Measurement	4.83	4.02
Acidity (mg/l)	4.20	3.20
Vit. C Conc. (m g / 1 0 0 g)	35.22	1.76
Vit. E Conc. (m g / 1 0 0 g)	0.14	0.16
Glucose (g / 1 0 0 g)	17.89	20.94
Fructose (g / 1 0 0 g)	9.16	11.58

Table 2 Mineral composition of orange and apple juice (mg/litre)

	Orange	Apple
Na (m g / L)	8.70	5.10
Fe (m g /L)	1.39	1.57
Ca+ (m g / L)	42.40	2.30
Mg (m g / L)	11.74	3.03
Zn (m g / L)	0.44	0.38
P (m g / L)	0.72	0.90
K (m g / L)	13.60	14.40

Table 3: Microbial profile of orange and apple juice

Juice Extracts	Microbial Load (x 10^{-5} c f u / m L)
Orange	0.2 X10-5
Apple	0.4 X10-5

Statistical Analysis

Data obtained were subjected to analysis of variance at $\alpha = 0.05$, the general linear model procedure of SAS (2004), while means were separated using Duncan's multiple range test of the same software.

RESULTS

Semen quality of Buck in tris, apple and orange juice extender at ratio 1:1

Presented in Table 4 is the semen quality of buck in Tris, Apple and Orange juice extender at ratio 1:1. The percentage motility was significantly ($p<0.05$) highest in T3 and in T1. Similarly, Progressive motility of spermatozoa was significantly ($p<0.05$) highest in T3 and lowest in T1. Non-progressive motility of spermatozoa on T1 was significantly ($p<0.05$) higher than in T2 and T3 which were also significantly different ($p< 0.05$). Average path velocity, Straight line velocity and Curvilinear velocity were significantly ($p < 0.05$) highest in T2 and lowest in T3. Amplitude of lateral head of spermatozoa in T2 was significantly ($p<0.05$) higher than spermatozoa on T1 and T3 which were statistically similar ($p<0.05$).

Table 4 Semen quality of buck in Tris, Apple and Orange juice extender at ratio 1:1

Parameters	T1	T2	T3	SEM
Percentage motility, %	66.00^{c}	76.33^{b}	92.67^{a}	3.89
Progressive motility, %	48.33^{c}	66.00^{b}	78.67^{a}	4.40
Nonprogressive motility, %	17.67^{a}	10.33^{c}	14.00^{b}	1.07
Average path velocity (VAP), µm/s	4.56^{b}	9.36^{a}	3.80^{c}	0.87
Curvilinear velocity (VCL), µm/s	5.07^{b}	11.41^{a}	3.65^{c}	1.19
Straight line velocity (VSL), µm/s	2.98^{b}	5.13^{a}	2.77^{c}	0.38
Amplitude of lateral head (ALH), µm	0.24^{b}	0.57^{a}	0.23^{b}	0.06
Beat cross frequency (BCF), Hz	0.38^{c}	1.19^{a}	0.46^{b}	0.13
Linearity, %	67.67^{b}	42.65^{c}	71.89^{a}	4.56
Straightness, %	72.19^{b}	52.19^{c}	75.85^{a}	3.68
Liveability %	92.21^{c}	92.97^{b}	93.92^{a}	0.25
Spermatozoa concentration (x10^{9})	0.18	0.18	0.18	0.00

abc: means in the same row with different superscripts are significantly (P < 0.05).
T1: Semen + Tris-egg yolk extender; T2: Semen + 50% Tris-egg yolk extender + 50% apple juice extender; T3: Semen + 50% Tris-egg yolk extender + 50% orange juice extender; SEM: Standard Error of Mean

Beat cross frequency of spermatozoa in T2 was significantly (p<0.05) higher than that on T1 and T3 which also differed one from another (p< 0.05). Linearity and Straightness of spermatozoa were significantly (p<0.05) higher in T3 and significantly (p<0.05) lowest in T2. Livability of spermatozoa was significantly (p<0.05) highest in T3 and lowest in T1.

Semen quality of buck in tris, apple and orange juice extender at ratio 1:2

Semen quality of buck in Tris, Apple and Orange juice extender at ratio 1:2 is presented in Table 5. The percentage motility was significantly (p<0.05) highest in T3 while the significantly (p<0.05) lowest in T1. Similarly, Progressive motility of spermatozoa was significantly (p< 0.05) highest in T3 and the significantly (p<0.05) lowest values was obtained in T1. Non-progressive motility of spermatozoa on T2 was significantly (p<0.05) higher than spermatozoa on T1 and T3 which were also significantly different (p<0.05). Average path velocity, Straight line velocity and Curvilinear velocity were significantly (p<0.05) higher in T2 and significantly (p<0.05) lower in T3. Amplitude of lateral head of spermatozoa in T2 was significantly (p<0.05) higher than spermatozoa in T1 and T3 which were statistically different (p<0.05). Beat cross-frequency of spermatozoa in T2 was significantly (p<0.05) higher than for spermatozoa in T1 and T3. For linearity, T1 was significantly (p<0.05) higher than other treatments while T3 was significantly (p<0.05) lowest. Straightness of spermatozoa was significantly (p<0.05) highest in T1, while the lowest value was obtained in T2 (p<0.05). The livability of spermatozoa was also significantly (p<0.05) higher in T1 and lower in T3 (p<0.05).

DISCUSSION

Extenders are important in preserving semen because nutrients essential for metabolism are supplied, as well as help in regulating pH and osmolarity of the semen (El-Kelawy *et al*., 2012; Ewuola *et al*., 2017).

Table 5. Semen quality of buck in Tris, Apple and Orange juice extender at ratio 1:2

Parameters	T1	T2	T3	SEM
Percentage motility, %	48.00^{c}	62.33^{b}	76.67^{a}	4.14
Progressive motility, %	35.50^{c}	47.67^{b}	66.33^{a}	4.48
Nonprogressive motility, %	12.50^{b}	14.67^{a}	10.33^{c}	0.63
Average path velocity (VAP), µm/s	5.07^{b}	8.43^{a}	4.00^{c}	0.67
Curvilinear velocity (VCL), µm/s	5.50^{b}	10.23^{a}	4.78^{c}	0.85
Straight line velocity (VSL), µm/s	4.44^{b}	4.64^{a}	2.10^{c}	0.41
Amplitude of lateral head (ALH), µm	0.34^{b}	0.53^{a}	0.25^{c}	0.04
Beat cross frequency (BCF), Hz	0.32^{b}	1.11^{a}	0.33^{b}	0.13
Linearity, %	76.56^{a}	44.98^{b}	34.33^{c}	6.34
Straightness, %	83.67^{a}	53.18^{c}	53.76^{b}	5.03
Liveability %	90.56^{a}	88.95^{b}	82.99^{c}	1.15
Spermatozoa concentration (x10^{9})	0.09	0.09	0.09	0.00

abc: means in the same row with different superscripts are significant (P < 0.05).
T1: Semen + Tris-egg yolk extender; T2: Semen + 50% Tris-egg yolk extender + 50% apple juice extender; T3: Semen + 50% Tris-extender + 50% orange juice extender; SEM: Standard Error Mean.

Meanwhile, diluents for rabbit semen have been reported to enhance sperm quality and improve fertility and prolificacy (Ewuola *et al.,* 2017). Furthermore, male fertility is dependent on spermatozoa quality for effective reproduction, improvement, and expansion. The examination of the reproductive status of male animals through artificial insemination, fertility, and reproductive efficiency improves the qualitative and quantitative evaluation of semen. The study of physical characteristics of semen (color, odor, pH, viscosity, and liquefaction), volume, concentration, morphology, spermatozoa viability, spermatozoa motility, and progression are known as semen analysis (Baker, 2007). Seminal plasma has a limited antioxidant capacity (Jimoh and Ewuola, 2018), the use of diluents having high antioxidant activities are required to maintain the viability and fertilizing capacity of spermatozoa.

Inclusion of semen extenders can protect and maintain spermatozoa cell properties such as morphology, motility, and viability, as well as membrane, acrosomal, and DNA integrity towards fertilisation. Semen extenders regulate semen pH, adenosine triphosphate, anti-cooling and anti-freeze shock, and antioxidant activity, thereby increasing semen quality for fertilisation (Bustani and Baiee, 2021). This suggests that orange and apple juice contain certain nutrients or components that enhance the motility, progressive movement of spermatozoa cells and the velocity of spermatozoa cells. In this experiment, the maintained spermatozoa viability that accompanied supplementation of semen extenders with apple and orange juices revealed that the fruit juices have the ability to maintain motility, which could be due to their excellent source of antioxidants such as vitamins C and E found in these fruits (Djuric and Powell, 2001; Gebhardt and Thomas, 2002; Martin *et al.,* 2002). The results agree with the findings reported by Ball *et al.,* (2001), Reza *et al.,* (2011), and Adikwu and Dio (2013), who found that adding vitamin E or C to preserved semen improved spermatozoa motility.

It has been suggests that orange juice contains components that enhance the motility of semen as noted by Daramola, *et al.* (2016). Similarly, semen with 50% apple juice extender, had the highest values of average path velocity, straight line velocity, and curvilinear velocity compared to other extenders. This suggests that apple juice may contain components that improve spermatozoa velocity at dilution ratio 1:2. Moreover, semen with 50% apple juice extender had higher amplitude of lateral head and beat cross frequency than other extenders, indicating that apple juice may have a positive effect on the quality of the spermatozoa head.

Although, Table 5 showed a similar trend with progressive motility, average path velocity, curvilinear velocity, straight line velocity, and amplitude of lateral head having higher value in semen extender containing 50% apple and orange juice compared to the 100% egg-yolk semen extender. Linearity, livability, straightness and non-progressive motility were lower in values of the semen extender containing 50% apple and orange juice compared to the 100% egg-yolk semen extender. The results of this study therefore agrees with Fenton reaction (O'Flaherty *et al.,* 2003) that antioxidants influence the removal of hydrogen peroxide to produce hydroxyl radicals

Nevertheless, there was a reduction in quality across all parameters measured in Table 4 with dilution ratio 1:2 compared to dilution ratio 1:1. The results revealed that the higher the dilution ratio, the lesser the progressive motility of the spermatozoa in the medium. This finding supports the results of Lahnsteiner *et al.,* (2004) and Sadeghi *et al.,* (2013), who reported that increasing the dilution ratio causes spermatozoa plasma to lose its protective effect, resulting in decreased spermatozoa viability. The highest percentage motility of spermatozoa was also obtained for treatments with a dilution ratio of 1:1, which agrees with the findings of Liu *et al.,* (2006), who got the highest motility percentage at the same dilution ratio. There was also a decrease in the spermatozoa concentration in semen extender with dilution ratio 1:2. The observed decreased in the spermatozoa concentration may be attributed to the dilution factor. The trend was expected since the concentration was expressed per mL of the sample. The higher the rate of dilution, the lesser the spermatozoa in the sample (Kondracki, 2003). This is due to increase in the volume of seminal plasma when the spermatozoa cells in the medium remain constant. It was logical because the dilution ratio is inversely related to the concentration (Kondracki, 2003). Ewuola *et al.,* (2014) reported a similar result in concentration and attributed this to the dilution factor, due to an increase in the volume of seminal plasma when the spermatozoa in the medium remain constant.

CONCLUSION

This study provides baseline information on the physicochemical evaluation of Tris-egg yolk semen extender and the extender containing 50% inclusion level of apple and orange juice with 50% tris-egg yolk extender. It can be said that the fruits (apple and orange) inclusion, had a positive effect in maintaining the spermatozoa quality. The spermatozoa in semen extender with 50% apple and orange juice showed a better response compared to the extender with only tris-egg yolk extender. The semen extender having the 50% orange juice had the best response. The use of orange and apple-

based semen extenders in rabbit breeding programs offers several advantages, and improved semen quality. However, there are also challenges, such as limited shelf-life, variability in composition, risk of microbial contamination, and lack of standardization.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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