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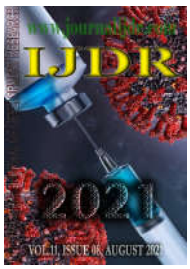

RESEARCH ARTICLE OPEN ACCESS

POWDERED COCONUT WATER (PCW-108) AS ROOSTER SEMEN EXTENDER

Carlos Tadeu Bandeira de Lavor[1]*, Thalita Evangelista Bandeira[1], Marcone Sampaio Oliveira[2] Thales Sampaio do Nascimento[3] and José Ferreira Nunes[1]

[1]Postgraduate Program in Biotechnology of the Northeast Network of Biotechnology, State University of Ceará, Silas Munguba Avenue, 1700, Itaperi Campus, CEP: 60740-903, Fortaleza-Ce, Brazil
[2]Postgraduate Program in Biotechnology of the Faculty of Veterinary Science of the State University of Ceará, Silas Munguba Avenue, 1700, Itaperi Campus, CEP: 60740-903, Fortaleza-Ce, Brazil
[3]Graduate of the Faculty of Veterinary Science University Center FAMETRO Conselheiro Estelita Street, 500, CEP: 60010-260, Fortaleza – Ce, Brasil

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***Corresponding author:**
Carlos Tadeu Bandeira de Lavor

ABSTRACT

Powdered coconut water was used to preserve the integrity of the rooster's sperm cell for long periods and improve the quality of the ejaculated semen. The composition of the powder is natural, obtained from coconut water, presenting osmolarity around 500 mOsm / kg H2O and pH around 4.5 to 5.0, which were corrected for pH 7.5-8.5 and osmolarity 350-400 mOsm / kg H2O similar to seminal plasma. The filtrate is homogenized and pulverized, with high solubility. The in vitro analysis protocol of semen with the addition of powdered coconut water as a storage medium was carried out using 1:1 volumes, with fresh semen and stored for 1, 24, 48, 72 and for 96 hours at 5°C Immediately after semen collection, the sample volume was evaluated, with a mean of 316.00 ± 68.79, ranging from 150 to 450 µL, semen pH of 8.41 ± 0.28, mean osmolarity of 365, 42 ± 27.48 mOsm / kg H2O, mean fresh semen motility of 88.93 ± 5.06% and mean progressive motility of 4.46 ± 0.35. Sperm and their motility were analyzed for up to 96 hours at 5°C, showing a significant decrease from $p < 0.05$. The morphological structure and integrity of the sperm membrane, classified as intact (89.03%) or dead (10.97%), were examined.

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INTRODUCTION

The method of abdominal massage and pressure in the cloaca region, (Burrows and Quinn, 1937) was developed to collect semen from roosters. With the semen collection technique, the rapid handling and transport of this semen to the females allowed the flexibility of the artificial insemination and allowed the development of efficient procedures to preserve the semen of the birds in in vitro conditions for a few hours (Rutz et al., 2007). To address management challenges, it is the use of assisted reproduction techniques, including the preservation of sperm along with artificial insemination. The growing importance of artificial insemination in poultry reproduction has led researchers to become interested in developing the appropriate conditions for storage of cooled (short term) and frozen (long term) (Lake, 1960; Van Wambeke, 1967; Lake and Ravie, 1981; Lukaszewicz, 2002; Lukaszewicz et al., 2004).

From the first synthetic extender (Lake, 1960), experiments on the ideal extender composition for poultry semen storage are still in progress. Since the biochemical composition of seminal plasma was described for Lake (1966) and Cherms (1967), the extenders were based on this composition. Glutamic acid, the most important component of seminal bird plasma, has become a standard component of extenders (Lake and Mc Indoe, 1959). Many buffered salt solutions are available and can be used as rooster semen extender (Sexton and Fewlass, 1978), both commercial products and diluents developed by individual researchers. These diluents have different compositions, sometimes with special additives such as skim milk, egg albumin, gentamicin sulfate (Van Wambeke, 1967), caproic acid (Lake and Ravie, 1981), catalase and vitamin E Moghbeli et al., 2016a; Moghbeli et al., 2016b, or even antibiotics (Harris, 1963). They are evaluated by the time they conserve stored semen, with different compositions (Siudzinska and Lukaszewicz, 2008), by the time they conserve stored semen, with variation in the dilution rate (Hudson et al., 2016), by the ability to preserve spermatozoa in cryopreservation protocols (Nabi et al., 2016). Ensuring adequate pH and osmotic

pressure (Latif et al., 2005) of the suspension is of great importance to sustain the viability of spermatozoa. The list of extenders, protectives, and different protocols used to improve the semen performance of various bird species is expanded by trying to optimize the production of animal protein for human consumption and also to optimize the preservation of the diversity of still existing species. Among these extenders is added coconut water, a natural product, which according to Vigliar et al. (2006) found results for electrolytes and glucose concentrations and osmolarity of coconut water with 6 months of maturity stage, sodium (3 mEq/L), potassium (98 mEq/L), chloride (48.7 mEq/L), calcium (6 mmol/L), magnesium (9.4 mmol/L), glucose (2.9 g/L) and osmolarity (422.9 mOsmol/L), which transformed into powdered material, while maintaining the fundamental properties of in natura liquid with stability and longevity, can be used in biotechnological processes, replacing imported, chemically constituted and priced products high levels (Moreira-Neto et al., 2009). The objective of this work was to use in vitro PCW-108 as a means of preservation of poultry semen, in order to preserve for a long-term, the characteristics of sperm cell integrity and improve the quality of semen ejaculated from roosters.

MATERIAL AND METHODS

Local: This study was conducted in accordance with ethical standards and approved by the Ethics Committee (N°-09144395-4) of the State University of Ceará, at the Itaperi campus, Fortaleza, Ceará, in an experimental shed with individual wire cages measuring 60x60x80 cm, height, depth and width respectively.

Birds: Thirty male breeding cocks (*Gallus gallus*) of the Agro-ross strain were used. The experimental age of the roosters was between 29th to 40th week (12 experimental weeks). All roosters were fed balanced ration with 2700 kcal.kg-1, 13% protein, 1% calcium and 0.45% phosphorus. The roosters received light stimulus only from sunlight which in the region of the experiment was equivalent to 12-13 hours per day.

Coletion of semen: Males were previously conditioned to the production (release ejaculate) of sperm by mechanical action. The experimental collection was performed once a week. The rooster was removed from the cage, and placed with his chest propped on a platform attached to a chair, where the operator stood. The rooster was massaged in the dorsal and abdominal region with cranio-caudal movements and then gentle pressure was applied with the thumb and index finger of the operator's left hand, close to the cloaca of the bird, thus exposing the phallus and the drop of semen on this. The drop of semen was then mechanically collected by the operator by aspiration. For this purpose, a hermetically sealed harvesting system was used that had a tip at one end, directed towards the roostres phallus, which came in contact with the semen on the phallus of the bird that was sucked until the harvest deposit. At the other end was a hose, which led down to the operator's mouth, which sucked air from the inside of the collection tube, causing a vacuum, which caused the semen to be transported into it. Five rooster semen were collected forming a pool of ejaculates to avoid the male effect, as the mixture was well homogenized.

Extender: The composition of PCW-108 is natural, obtained from coconut water from fruits with six months of maturation, selected for their physicochemical properties and presence of carbohydrates, amino acids, minerals, vitamins, amino acids, osmolarity around 500 mOsm / kg H_2O and pH around 4.5 to 5.0. To obtain PCW-108, the pH is increased to 7.5-8.5 and the osmolarity is reduced to 350-400 mOsm/kg of H_2O, similar to the indices of the seminal rooster plasma. The water is filtered, homogenized and subjected to dehydration by heat treatment, the sample is dried and transformed into a powder, free from liquid, with high solubility. The product maintains all the chemical characteristics of fresh coconuts. For use in the experiment the powder is rehydrated with distilled water.

Evaluation of semen quality: The protocol for in vitro analysis of semen with addition of PCW-108 as a storage medium was performed using semen and PCW-108 (v:v): fresh; stored for 1, 24, 48, 72 and for 96 hours at 5 °C. Samples of ejaculates of all the above items were analyzed by macro and microscopic aspects such as coloring, volume, pH, osmolarity and concentration obtained from the 5 roosters pool, being six collections with five replications totaling 30 pools; sperm motility; progressive motility and; sperm morphology obtained by smears on slides stained with eosin-nigrosin according to the method IV established by Lukaszewicz et al., (2008) and adapted for pH and osmolarity of the test rooster semen. From 6 pools were made 6 slides (replicates) and 100 cells (5 x 6 x 100) were counted on each slide.

RESULTS

Immediately after semen collection, the volume of the samples was evaluated, establishing the mean value, ranging from 150 to 450 µL. The pH of the pure semen was analyzed, presenting an mean value of 8.41 ± 0.28. Osmolarity presented an mean of 365.42 ± 27.48 mOsm/kg. The mean spermatic motility of fresh semen diluted in PCW-108 was 88.93 ± 5.06% and the progressive motility with a mean of 4.46 ± 0.35 (Table 01).

Table 1: Mean of the volume, pH, osmolarity, sperm concentration, sperm motility (ME%) and progressive motility (MP 0-5) of fresh rooster ejaculates (*Gallus gallus*)

Item	Mean ± Sd	CV%
Volume of ejaculate (µL)	316,00 ± 68,79	21,77
pH of pure semen	8,41 ± 0,28	3,37
Osmolarity pure semen (mOsm / kg H_2O)	365,42 ± 27,48	7,52
Spermatic concentration (x10^9 sptz / mL)	5,642 ± 1,63	29.01
Sperm motility (%)	88,93 ± 5,06	5,69
Progressive motility (0-5)	4,46 ± 0,35	7,87

Table 2. Mean of motile sperm motility (SM%) and progressive motility (PM 0-5) of rooster semen diluted in PCW-108, volume 1:1, fresh and stocked cooled at 5 °C for 1, 24, 48, 72 and 96 hours

Blocos	Sperm motility (%)	Progressive motility (0-5)
Fresh	88,44 ±4,53^a	4,31 ±0,25^a
1h cooled	79,44 ±5,27^a	2,93 ±0,72^{bc}
24h cooled	60,55 ±11,84^b	3,18 ±0,65^b
48h cooled	45,55 ±5,83^c	2,18 ±0,65^{cd}
72h cooled	40,55 ±3,90^c	2,31 ±0,45^{cd}
96h cooled	37,22 ±10,34^c	2,06 ±0,56^d

Letters a-d indicate statistical difference between the data $p < 0.05$.

Table 3. Incidence (%) of the normal and defective appearance of the morphological structure of spermatozoa of diluted roosters in PCW-108 at room temperature

Morfology		%	Mean	± Sd
•	Espermatozoa intact	89,03	89,03	9,81
Head defect				
•	Curved head	0,57	2,43	1,90
•	Macrocephaly	0,13	1,33	0,58
•	Acephaly	0,20	2,00	1,00
Tail defect				
Intermediate piece				
•	Disjointed spermatozoa	0,67	2,86	1,35
Main piece				
•	Tail cut	0,23	1,75	0,96
•	Tail rolled	0,17	2,50	0,71
Multiple defects				
•	Cytoplasmatic drop	7,50	9,38	8,92
•	Acrossomic drop	1,47	2,32	1,25
Total of uncorrected		10,97	10,97	-

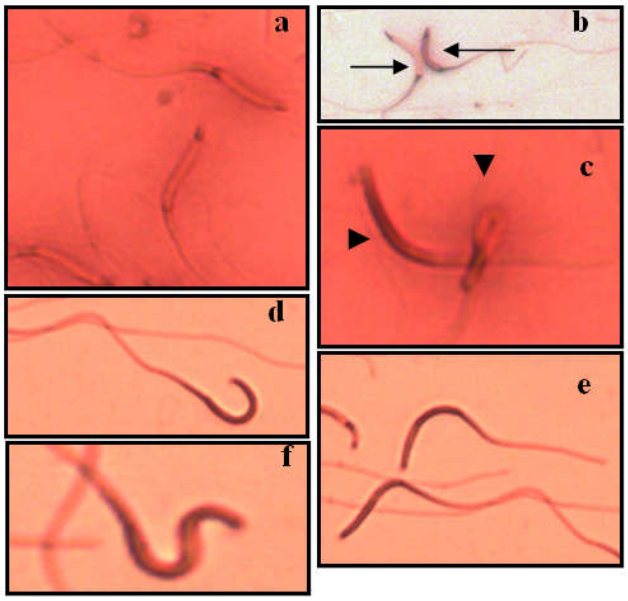

Figure 01. Optical micrograph illustrating the morphology of the spermatozoon of the rooster (*Gallus gallus*). (a) morphologically normal (live), (b) →live sperm and ←dead sperm, (c) dead ► and head bent ▼, (d) hooked head, (e) head curved beyond usual, (f) S-shaped head

The motile and progressive motility of the semen stored for up to 96 hours at a temperature of 5 °C was also analyzed, according to the data presented in Table 02, showing that there was a statistically significant ($p<0.05$) decrease in motility and progressive motility, but showing that 48h up to 96h there was no statistical difference in the parameters studied. The evaluation of the morphological structure shown in (Figure 01) and the integrity of the spermatozoa membrane was examined, being the spermatozoa classified as intact (89,03%) or uncorrected (10.97%) differentiating them by the related defects: Head defect (bent head, macrocephaly, acephaly). Tail defect (intermediate part: disjointed spermatozoon; main part: tail cut, tail curled). Multiple defects (cytoplasmic droplet, acrossomic drop) as present in Table 03.

DISCUSSION

The seminal volume standard of roosters ranges from 100-900 µL and 200-1500 µL, found in works by Etches (1996) and Blesbois and Brillard (2005) respectively, as well as in the work of Mal'tsev and Dymkov (2008) who found in two Russian lines of rooster medium volume of 340-430 µL and 530-700 µL and work comparing fresh semen collected from roosters of different breeds volume of ejaculates ranging 240-520 µL (Siudzinska and Lukaszewicz, 2008). Adeoye et al. (2017), studying the main effects of race and age on semen volume found the mean semen volume was 520 ± 50 µL. The volume found in the present study was consistent with studies by other authors. According to Correia and Correia (1985) the semen of roosters presents concentration of hydrogen ions (pH) between 6.3 and 8.4; and also the mean of 7.27 according to Parker et al. (1942); mean of 7.71 samples of undiluted semen according to Parker and Mc Daniel (2006); Blanco et al. (2000) found an mean pH of 6.85; Zheng et al. (2007) found an mean pH of 7.02±0.06 and 6.83±0.06 in normal roosters and dwarf roosters respectively and 6.96±0.15; 7.05±0.18; 7.33±0.21 and 7.21±0.24 from four roosters breeds (Siudzinska and Lukaszewicz, 2008). Comparing sêmen collected from four roosters breeds Siudzinska and Lukaszewicz (2008) found osmotic pressure (mOsmol/kg) 326±10.3 (green-legged partridge); 327±3.37 (blak minorca); 311±15.2 (White crested black polish); 323±8.17 (italian partridge). The osmotic pressure (325 mOsm / L) is stable in fresh seminal plasma of roosters, reports Sauveur, (1982), but there is variability between ejaculates and / or between roosters; Latif et al. (2005) showed the pH effect 7.4 on the motility spermatozoa stored for 48 hours at 5 °C, keeping the osmotic pressure constant at 375 mOsm and concluded that an experimental extender with 375 mOsm of pressure was satisfactory and improved the fertilizing capacity of rooster spermatozoa. Adeoye et al. (2017), found the mean concentration and sperm count (6.42×10^9 mL-1 and 3.49×10^9-1 respectively); sperm motility had an average of 70.76±2.53%; sperm morphology (mean 76.46±1.91); percentage of dead, live sperm and sperm viability had a mean of 18.78±2.37, 81.89±1.90 and 75.51±1.93 respectively, data that are similar to the data in the present study. The dilution of rooster sêmen, reports Clarke (1982), increases sperm motility. This increase is possible due to increased vailability of nutrients, such as BPSE fructose (Kamar and Risik, 1972) and glucose (Parker and Mc Daniel, 2006). With Herrera et al (2017), sperm motility in fresh semen was higher ($P<0.05$) when BPSE medium (63.7±2.3) was used compared to Lake medium (68.4±1.9), lower data than those found in this study. For Herrera et al (2017), there was no significant difference in the amount of live sperm (BPSE 98.0 ± 0.2) and (Lake 96.0 ± 1.0), but it was higher $P<0.05$), with statistical difference, comparing Lake (94.6 ± 1.4) and BPSE (98.6±0.1) for normal morphology and BPSE abnormalities (1.2±0.13) Lake (5.2±0.5), presenting values higher than those of this study classified as intact (89.03%) or dead (10.97%), the morphological data of sperm were also observed by Najafi et al., (2019), with the aim of evaluating the effect of supplementing semen extender with resveratrol, which is the most prevalent non-flavonoid polyphenol contained in almost all grapes, which has an antioxidant effect, diluted the semen in two stages with Beltsville freezing diluent and found a percentage of live sperm, 61.0% and dead, 25.5%.

In stored turkeys, semen in vitro, Douard et al., (2005) found decreased motility when stored for up to 48 hours at 4 °C. Penfold et al. (2001) analyzed the effect of fresh duck semen (*Anas acuta*), undiluted and diluted in commercial extender at two temperatures (4 and 24 ºC) and found the best motility and progressive motility rates in semen diluted at 4 ºC. Holm and Wishart (1998), using 5 extensors, adjusting the concentration to 1 billion spermatozoa/mL and the pH between 5 and 9 analyzed the motility of rooster, turkey and quail semen at temperatures of 30 ºC and 40 ºC and concluded that pH alkalinization increases sperm motility in these three species. Wishart and Wilson (1999), studying the inhibition of sperm motility by temperature dependence in *Gallus gallus*, *Meleagris gallopavo*, *Anas platyrhynchos*, *Coturnix japonica* and *Chlamydotis undulata undulata* found the increase of motility in the presence of NaCl (30 ºC) and in the presence of 5 mM calcium (40 °C), except in quail that showed inhibition by calcium. For sperm preservation, Froman (2015) concluded that the high percentage of mobile spermatozoa was more important than the type of rooster extender chosen or the type of storage. The type of semen diluent and the dilution rate, influence sperm motility and metabolism immediately after dilution and have shown that sperm motility not only differs due to the type of diluent, but that the dilution factor also affects the rate of movement of the sperm (Parker and Mc Daniel, 2006). When the semen is diluted excessively, a dilution effect occurs and the sperm motility is altered (Mann, 1964).

Numerous studies are carried out using diluents to try to extend, preserve and protect the semen of birds using a variety of diluents, and in these experiments the percentage of live, dead spermatozoa and the most recurrent defects caused by the addition of these substances. The quality of semen is an important factor that affects fertility. There is a need to select males based on the fertilization capacity of the semen (Mc Daniel et al., 1995; Donoghue, 1999; Mellor, 2001). The fertilization of eggs, in a chronological sequence of ovulation, in the female poultry depends on the ability of this female to store viable cells in the sperm glands and to supply the infundibulum with morphologically normal spermatozoa, which are able to migrate in the oviduct to the region where these glands (Bakst et al., 1994). The fertilization capacity of sperm depends on several parameters, mainly sperm motility and the ability of sperm to undergo an acrosome reaction (Mocé et al 2010, Ahammad et al 2013), which makes in vitro evaluations necessary. For stocked semen, Clarke et al., (1982) reported that sperm motility in semen of undiluted and

diluted roosters is lower when stored at 41 °C, close to the body temperature of the chicken. This contrasts with semen stored at 25, 15 or 5 °C. The authors also revealed that sperm motility is not affected by dilution with storage temperature of 15 and 5 °C. Dumpala et al, (2006) showed that as the percentage of dead spermatozoa grows in undiluted semen mainly at 41 ºC during storage, the number of live spermatozoa and sperm motility interacting with light decreases, resulting in a reduction in the readings performed (Howarth, 1981) when compared with pure and undiluted semen. Morphological percentage of duck's spermatozoa (*Anas acuta*) diluted in BPSE, kept at 4 ºC, at 24 ºC and found lower rates of normal cells (Penfold et al., 2001), than those of this work. Lukaszewicz et al, (2008) evaluated the effectiveness of sperm morphology of four rooster strains using four different smear staining methods to facilitate the daily practice of sperm analysis prior to artificial insemination. These authors used pure semen added to dyes and obtained good results (% normal morphology), but not better than the results of this experiment that adapted one of the four methods of the cited authors, modifying only the use of semen, which instead of pure, was diluted in PCW-108. Malecki and Martin, (2000); Du Plessis et al., (2010) emphasize the need for analysis of sperm morphology in birds (works in commercial productions of *Dromaius novaehollandiae*) using simple but reliable techniques. Diluent osmolarity is associated with morphological changes in the sperm structure and variations in semen can occur during semen cooling. The ideal range for bird spermatozoa is between 250 and 460 mOsm/Kg H_2O and the temperature should be kept at 5-7 °C (Bakst, 1990; Etches, 1996).

The effect of diluent and dilution rates on motile spermatozoa of guinea fowl for short periods of storage time, observed that the magnitude of the decrease in seminal motility with increasing dilution ratio can be attributed to the efficacy of the diluent in maintaining motile spermatozoa (Hudson et al., 2016). The superiority of the BPSE extender over IMV is evident in the maintenance of guinea fowl sperm regardless of the dilution rate and shelf life. Sperm motility and viability gradually decrease after collection (Rahman et al. 2018). The delay in the inclusion of the extenders in the semen can cause loss of fertilizing capacity of the rooster sperm, but the numbers of the studied parameters decrease with increasing time. This is supported by the findings of Hudson et al. (2016) who reported that the storage period at 5 °C has a significant influence on sperm motility in guinea fowl, as well as in the results of this study that demonstrated that coconut water at 4 °C keeps sperm alive for long periods of time, which facilitates the management of artificial insemination with the maintenance of life and sperm activity. Comparing the Nabi and Beltsville diluents for rooster semen cryopreservation, they adjusted, before and after including glycerol and lecithin in the diluent, osmotic pressure and pH to 310 and 340 Osmol / kg, 7.4 and 7.5, respectively (Nabi et al., 2016) They found a significant number of motile and progressive sperm from the Nabi extender compared to the Beltsville extender. The inclusion of soy lecithin in the Nabi extender can replace the loss of phospholipids in the sperm membrane, which, as far as it is concerned, improves the integrity of the sperm membrane and increases the motility (more than 60%) and viability (more than 50%) of the spermatozoa after the freezing process and concluded that the extender is an adequate cryopreservation means for the semen of the rooster, which can lead to the maintenance of the quality of the semen of the rooster after freezing and thawing. Like these authors, this study also adjusted the osmotic pressure and pH (350-400 mOsm/kg and 7.5-8.5 respectively) of PCW-108 by adding it to rooster semen and found improvements in sperm motility stored up to 96 hours at 5 °C. The effect of rooster semen extenders and storage time on the sperm morphology of four broiler strains, found the number of dead spermatozoa in semen collected from examined breeds and stored did not vary with the evaluated extender used, however there were significant differences in the number of sperm with deformities (Siudzinska and Lukaszewicz, 2008). By using the of nigrosine-eosin staining technique and evaluation of morphological forms of sperm, it was possible to disclose that the largest group of spermatozoa with abnormalities (mainly curved laps) occurred when semen was diluted with the Lake extender.

Other methods of evaluating sperm that qualify cells only as live and dead perhaps would not have revealed that extensors had varied effects on sperm morphology. PCW-108, as semen extender at room temperature (v:v - semen:PCW-108), analyzed by the nigrosin and eosin staining technique, showed to be able to preserve membrane integrity in most spermatozoa, however in the group of dead spermatozoa there are varied deformities in the principal piece, midpiece and head, besides other defects, like cytoplasmic droplet and acrosomal droplet, suggesting that these membranes rupture abruptly.

CONCLUSION

The PCW-108 added to the still fresh rooster semen and after an hour of cold showed good rates of motility and progressive motility. Normal sperm morphology presented good percentage index, showing that the sperm plasma membrane was preserved after the extender addition.

Conflicts of interest: None of the authors have any conflict of interest to declare.

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