

**Research Article****Coconut Water and OPTIXcell™ for Alpine Goat Semen Extension: A Comparative Evaluation of Post-Extension Semen Parameters with and without Seminal Plasma**

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Article History: Received: June 13, 2018 Revised: October 10, 2018 Accepted: October 13, 2018**ABSTRACT**

The detrimental effect of reactions of seminal plasma in goat semen with common components of extenders is known and has been the point of focus on developing appropriate extender for goat semen. The current study compared post-extension semen viability parameters of a laboratory generated, coconut water-egg yolk-based extender (COC), against similar parameters of semen extended in a commercial extender - OPTIXcell™ (OPT), to approve COC for extending goat semen for further processing with and without seminal plasma. Semen was collected from four mature Alpine goats serially, three times, three days apart. Each ejaculate was split into two and one part had seminal plasma removed while the other part had intact seminal plasma. Respective portions were further split into two for extension with each of the extenders. Semen viability parameters were then compared within two hours of extension. Semen evaluation parameters included individual progressive motility and percentage live spermatozoa.

The results showed that individual progressive motility of the spermatozoa significantly reduced in presence and absence of seminal plasma following extension with each extender ($p < 0.05$). Similarly, the percentage of live spermatozoa reduced significantly in samples where seminal plasma was removed ($p < 0.05$). However, both extenders maintained the percentage of live spermatozoa at similar level to pre-extension when seminal plasma was intact ($p > 0.05$). This implied that the process of removal of seminal plasma was one of the factors responsible for death of spermatozoa. The observed reductions in viability parameters were within values acceptable for further processing of the semen using the Kenyan standards of bull semen evaluation. Semen samples extended with each of the extenders with and without seminal plasma were therefore approved for further processing. The laboratory generated, coconut water-egg yolk-based extender as constituted for purposes of this study, was thereby approved for extending goat semen for further processing.

Key words: Coconut water-egg yolk-based extender, OPTIXcell™, Post-extension evaluation, Goat semen**INTRODUCTION**

Semen extension using extenders is a critical step in semen processing for preservation. Extenders are used to increase the volume of semen from one ejaculate, which can then be packaged in several breeding doses for artificial insemination (AI).

In any semen processing enterprise, survivability of spermatozoa following extension is a critical determinant for continuity of the whole process. The ability of spermatozoa to maintain post-extension viability depends on both the semen composition and characteristics and the biochemical composition of the extender. It is known that semen extension interferes with cellular mitochondrial respiration of the spermatozoa (Kaya *et al.*, 2014). This

affects the vigour of semen motility. The chemical balance of the extender may also affect the integrity of the plasma membrane (Agarwal *et al.*, 2005). Spermatozoa with faulty cell membranes can easily be detected through differential staining. Cells that show a breach in the membrane integrity are usually considered dead when establishing the live dead ratio. In establishment of appropriate extender, most laboratories test the ability of extenders to maintain post-extension semen viability to meet acceptable level before passing a given extender for a given animal species.

In the livestock industry, most studies to establish the ideal extender dwelt on bull semen (Baracaldo *et al.*, 2007; Rehman *et al.*, 2013). The same extenders have been adopted for use in other species including goats but

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with variable results (Purdy, 2006). Most of such extenders contain egg yolk and skimmed milk (Purdy, 2006). In the goat, there is interactivity between seminal plasma and milk or egg yolk that is deleterious to the sperm, a situation not observed with bovine seminal plasma (Purdy, 2006). The effect resulting from interaction of seminal plasma of the goat and egg yolk was first demonstrated by Roy, in late 1950s. He found that spermatozoa maintained motility in egg yolk when seminal plasma was removed unlike when whole ejaculate was used. In addition, he noted that egg yolk coagulated and it was determined that an enzyme of bulbourethral gland origin was responsible for the coagulation, thus called Egg Yolk Coagulating Enzyme (EYCE). A similar interactivity was observed between milk and goat semen by Nunes and colleagues in the early 80s and the enzyme responsible, also from the bulbourethral gland, was a 50-60 kDa glycoprotein lipase called SBUIII (Pellicer-Rubio *et al.*, 1997). These effects elicited several researches on goat semen to verify the extent of the effect of the interaction and if such reactions affect post extension viability.

In the 1980s, many researchers agreed with the interactive effects of seminal plasma with both milk and egg yolk. Later, some researchers found no difference in quality of semen extended in presence and absence of seminal plasma (Peterson *et al.*, 2007; Jiménez-Rabadán *et al.*, 2012). As a corrective measure to the largely known effect, researchers have resorted to alternative extenders that do not have either milk or egg yolk. Coconut water (Sule *et al.*, 2007) and soybean (Vidal *et al.*, 2013) have been some of such alternatives. Though high concentration of coconut water has been associated with preventing free motility of spermatozoa, it has given good viability values especially for semen stored at room temperature (Sule *et al.*, 2007). Detrimental effects over frozen-thawed sperm viability however, have been recorded with ram semen when coconut water was used (Santillana and Garcia 2000; Gutierrez *et al.*, 2006).

This study compared the post-extension qualities of buck semen extended with a non-egg-yolk based commercial extender OPTIXcell™ (OPT) versus a laboratory constituted coconut water based extender (COC) which contained egg yolk. It also compared such qualities with and without removal of seminal plasma in each extender. Assessment of viability was made within two hours following extension in order to establish if the extended semen would be taken through further semen processing for storage.

MATERIALS AND METHODS

Study design

This was a longitudinal study where semen samples collected serially (three times) from same goats were extended with or without seminal plasma and observed at a point in time for post-extension viability of spermatozoa. The effect two extenders, coconut egg yolk-based extender (COC) and commercially available extender - OPTIXcell™ (OPT), on post extension viability of spermatozoa was evaluated in presence and absence of seminal plasma. Viability parameters included individual progressive motility and percentage live spermatozoa.

The ability of coconut water-egg-yolk-based extender (COC) to maintain semen viability following extension was compared against a commercially available semen extender (OPT) for acceptability for further processing using the Kenyan standards. Two parallel experiments were done on split ejaculates, one with semen containing the seminal plasma and another with semen whose seminal plasma had been removed via centrifugation and aspiration of supernatant. Both experiments were run simultaneously utilizing each half aliquot of semen collected from the same buck on the same day. Three serial collections of ejaculates were obtained from every experimental buck three days apart except buck5 from which only second trial of ejaculate collection was successful.

Buck recruitment and care

Five mature (2-4 years old, weighing between 40-56kg live body weight), Alpine bucks were randomly selected out of 10 bucks in a buck stud located at the Animal Health and Industry Training Institute (AHITI)-Ndomba - Kenya. The 10 bucks were housed in individual pens without following any order. The bucks were numbered from one to ten and random numbers were generated from a scientific calculator (FX-82MS- CASIO) to select five bucks.

The bucks were already familiar with the artificial vagina method of semen collection. They were re-evaluated for breeding soundness based on standard procedures (Mushtaq *et al.*, 2007). They were then transported by vehicle to the University of Nairobi (UoN) Animal unit at the Department of Clinical Studies. They were treated for external and internal parasites using 1% ivermectin (supermec® Bimeda Ltd. Nairobi, Kenya) injected subcutaneously at 0.2mg/kg body weight. At the animal unit, the bucks were kept in pairs in cubicles except one buck (buck 1), which was kept on its own. Within the cubicles, the bucks were tethered far from each other to avoid fights. The goats were allowed *ad libitum* access to good quality grass hay and clean water. In addition, each goat was given 1kg of a commercially available concentrate mixture (dairy meal; Pembe Millers Ltd, Kenya) daily divided in two meals.

Daily health check was done on all the goats. This included daily visual clinical examination, auscultation of various body regions as well as recording their body vital parameters each morning.

The bucks were allowed two weeks of acclimatization, after which two semen collections were obtained from them to re-affirm their maintenance of quality semen production in the new environment. To augment this, twice-weekly testicular palpation and ultrasonography was performed on them during which one of the bucks (buck 2) was withdrawn from the study after he developed orchitis thus leaving four bucks for the experiments.

Semen collection and initial evaluation

This study was conducted in the month of February - April 2015. From the third week, semen was collected from each buck using the standard artificial vagina (AV) technique (Jiménez-Rabadán *et al.*, 2012). Collection was done after every three days as from 0900hrs on each

collection day, to obtain three ejaculates per buck for the experiments. In brief, the AV had an insulated outer casing (15cmx5.5cm) and an inner liner made of thin rubber. In assembling the AV, the liner was extended at least 2-3cm beyond the end of the outer casing, folded back, and secured with rubber bands to form a watertight jacket. The jacket was then filled with water (at 50°C) through a tap on the side of the AV, to about two-thirds to achieve the recommended 45°C inside the AV. The end of the AV through which the penis would be introduced was lubricated with a non-spermicidal gel (#xB640, L'aigle Cedex, France). At the other end of the AV, a plastic cone with a calibrated glass tube was fixed and covered with a leather material.

The prepuce of the buck was wiped with a clean paper towel to reduce contaminants at the prepuce. A teaser doe was immobilized in a standing position from her head end by an assistant exposing her rear end. The assistant held firmly a rope tied to the neck of the doe with the right hand. The left hand was used to secure the head between the hand and the waist of the assistant. This ensured minimal movement of the doe. The operator crouched at the right of the doe and held a fully assembled AV with the right hand along her flank with the open end facing downwards at an angle of 45° towards the male held on a leash by another person and led toward the female. The male was allowed two false mounts but finally allowed to mount the doe. The penis was directed by holding the prepuccial skin with the left hand to the open end of the AV. An upward forward thrust with an upward jerking of the head by the buck was an indication that ejaculation may have occurred. The buck was then allowed to dismount. The graduated tube (covered in a leather covering) containing the ejaculate was separated from the cone and capped with aluminum foil then labeled with the buck's identity. The tube was removed from the covering and immediately placed in a water bath at 37°C. Only one ejaculate was obtained from each individual buck on a given day of semen collection. Once collected, routine evaluation for colour, volume, consistency and admixtures was done (Shamsuddin *et al.*, 2000).

Semen was also evaluated for mass activity on a scale of + to ++++ (Mushtaq *et al.*, 2007). Mass activity was determined by placing a drop of raw semen on a warm slide on a heated microscope stage at 37°C and examined at x10 objective lens. If only individual cells were observed moving then a single plus sign (+) would be assigned. Islands of individual cells plus slow waves would be represented by (++) , vigorous mass activity was indicated by (+++) and turbulent mass activity was assigned (++++) . In this study, numerical value scaling of 1-4 was awarded in the same order for purposes of statistical analysis.

Progressive forward motility (Burcuet *et al.*, 2009; Soe Win Nianget *et al.*, 2011; Gupta *et al.*, 2012) and membrane integrity, using eosin-nigrosin staining technique (Kulaksizet *et al.*, 2013) were also evaluated. To assess progressive forward motility, a drop of semen was mixed with a drop of warm 0.9% saline (Claris Otsuka Private Limited- village-Vasana- Chacharwadi India) on a warm microscope slide to achieve a 1/1 v/v extension. The drop was covered with a cover slip and motility determined through visual observation of 8-10 separate fields under a

warm-stage light microscope at x400 (oil emulsion lens) by one experienced person throughout this study. Percentage individual progressive motility was subjectively determined by counting ten spermatozoa and indicating how many of them moved across each microscopic field and the totals averaged to determine the percentage of motility score. This was repeated for all bucks on each day of semen collection. Values obtained, (mean±SD) formed the baseline progressive motility for the ejaculates from each buck for the day of semen collection.

Plasma membrane integrity was evaluated using the method described by Gupta *et al.*, (2012) with minimal modifications. A drop of semen was placed on a warm glass-slide at 37°C side by side with warm eosin-nigrosin stain and the two allowed to mix for 15-60 seconds by gently tilting the slide back and forth. A thin smear was then prepared, air dried and observed under microscope at x400 to determine live/dead ratio. Nigrosin provided a dark background while eosin stained the cytoplasm of the spermatozoa pink if the plasma membrane was broken. Sperm cells showing partial or complete colorization were considered non-viable or dead (Kulaksizet *et al.*, 2013). Cells that did not pick stain were considered viable. A minimum of two hundred sperm cells were counted from several microscope fields. All cells on the last field were counted even if the 200 cells target had been reached. Viable spermatozoa were counted against non-viable ones and percentage live (viable, non-stained) cells determined.

Ejaculate concentration was determined using a spectrophotometric machine (Acucell™-IMV technologies), as per the directions provided (www.imv-technologies.com), after a pre-test showed that such concentrations of the experimental buck semen fell within the normal range for goats. Three thousand nine hundred and sixty micro-liters (3960µL) of 10% sterile sodium chloride solution at 37°C (water bath), was transferred into a sterile cuvette using a micropipette. Forty micro-liters (40µL) of semen was then added to the saline to top up the volume to 4000µL and by gentle mixing, the spermatozoa allowed to distribute evenly in it. The cuvette was then immediately inserted into its slot in the spectrophotometer and ejaculate volume fed into the machine. The machine was then able to display the total sperm content of the ejaculate in billions per milliliter. The machine was set for an extension rate of 40million spermatozoa per milliliter for purposes of this study. The machine was then able to generate the volume of extender to be added to the ejaculate.

Semen extender preparation

Two extenders were used in the current study; coconut water based preparation (COC) and a commercially available extender called OPTIXcell™ (OPT).

Coconut water extender

Coconut fruits were bought from the market, brought into the laboratory, and selected. Selection was based on presence of coconut water determined by shaking the fruit. Greener fruits were selected because they were considered fresh. The fruits were washed with clean water, rinsed, and dried with disposable paper towel. They

were routinely opened to access the water in them. The coconut water was obtained by aspiration using sterile rubber capped pipette from each fruit in separate 100mL beakers. Each sample was taken through organoleptic evaluation by one staff from the Kenya Animal Genetic Resource Centre (K.A.G.R.C) who had the experience of relating taste to coconut water quality. A sample was accepted for use if it had a strong sour taste. Selected coconut water samples were then pooled together and used for preparing the final extender.

To obtain egg yolk, fresh non-fertilized chicken eggs (less than seven days old) were obtained from the poultry unit at the Animal Production Department, University of Nairobi on every extender preparation day. The eggs were wiped using paper towels to clean off particulate dirt. The surfaces of the eggs were further wiped with a cloth towel dampened in distilled water and allowed to dry. The eggshell was carefully broken across the longitudinal axis of the eggs. The content of the eggs was gently emptied onto a sterile absorbent paper placed over a sterile paper foil, care taken not to break the egg yolk membrane. The albumen was removed by gently rolling the contents from each egg from one absorbent paper to the other. The egg yolk membrane was then carefully punctured and the egg yolk drained into a sterile beaker while ensuring the membrane was held back in the paper.

The antimicrobials used in this study included sulphamylamide (1.2g) (LobaChemie-India), streptomycin (0.54g) (CSPC Group pharmaceuticals-China) and penicillin (0.24g) (CSPC Group pharmaceuticals-China). Penicillin, streptomycin, and Sodium citrate (Manigate agencies) were weighed and put in one flask while sulphamylamide (1.2g) was weighed and placed in a different flask.

Twentyeight milliliters (28mL) of egg yolk were measured into a third flask. One hundred milliliters (100mL) of double distilled water were added to the flask containing sulphamylamide and another 100mL of the water added to the flask containing the other mixture. Each of the flasks was warmed in a larger container containing hot water and shaken continuously to allow the solutes to dissolve. The contents of the flasks were then mixed in one flask and allowed to cool to room temperature, by placing the flask in water at room temperature and the temperature of its content monitored. Separately, coconut water was boiled for 15minutes, sieved, and allowed to cool to room temperature by placing the flask in water at room temperature as well. Sieving was done by placing sterile double folded gauze in a glass funnel and the coconut water transferred into a different beaker via a funnel. Sixty-eight (68mL) of the coconut water was then added to the flask with the dissolved solutes. Twenty-eight milliliters (28mL) egg yolk was then added to the mixture. The mixture was mixed by gently tilting the flask back and forth. Double distilled water was then added to the mixture at room temperature to top it up to 400mL. The temperature of the mixture was raised to 37°C by placing the flask in water bath at the same temperature while monitoring the temperature. The pH was read using a pH meter (Hanna instruments) and adjusted to between 7.3-7.5 using 10% sodium hydroxide (Manigate agencies) at 37°C. Fresh

extender was made on each day of semen collection and the extender used within six hours of preparation.

OPTIXcell™ extender

For purposes of this study, OPTIXcell™ was identified as OPT. It was a commercially available extender made without any animal protein as indicated by the manufacturers (IMV Technologies – www.imv-technologies.com). It was prepared for use as per the manufacturer's instructions. One hundred and fifty milliliters (150mL) of the extender was reconstituted by mixing 50mL of OPT with twice the amount (100mL) of double-distilled de-ionized water at 37°C. To achieve this, the capped 50ml bottle of OPT was placed in water bath for 10 minutes. One hundred (100mL) of double distilled water was put in a flask and the flask placed in the same water bath at 37°C for 10 minutes to equilibrate their temperatures. The bottle of OPT was removed from water bath, wiped dry using a paper towel and its content poured into the flask containing distilled water and the bottle rinsed with the same solution. The fresh extender was kept in the same water bath and used within six hours.

Sample aliquots

Each ejaculate was divided into two equal aliquots using micropipette; one for experiments with seminal plasma (A) and the other for experiments without seminal plasma (B). Each of the aliquots (A and B) was divided further into two aliquots for extension with two extenders, COC and OPT. The aliquots were kept in centrifuge tubes placed in water bath at 37°C throughout the processing.

Removal of seminal plasma

Volumes of aliquots B were measured using micropipette and recorded. The aliquots were added to 5mL of 0.9% of a commercially available normal saline (Clarix Otsuka Private Ltd - India) in capped centrifuge tubes at 37°C. Seminal plasma separated from the cells through centrifugation at 3000 revolutions per minute (rpm) for 10mins at 37°C and the supernatant carefully aspirated using a glass pipette with a rubber bulb at one end.

The pellet remaining at the bottom of the centrifuge tube was reconstituted to the original volume of aliquot B using normal saline at 37°C. The centrifuge tube was then gently tilted back and forth until the entire pellet was re-suspended then placed again in water bath ready for addition of extender.

Semen viability evaluations

Parameters for semen viability used in this study included individual progressive motility herein referred to as motility, and percentage live spermatozoa. Evaluation was done within two hours following extension by modification of methods described by Gupta *et al.*, (2012) and Kulaksiz *et al.*, (2013). Motility was assessed by placing a drop of semen on a warm microscope slide at 37°C for 15 seconds prior to visual observations of microscopic fields. Percentage motile spermatozoa were estimated by visual examination of the average number of spermatozoa that swam across 8-10 observation fields. Percentage of live spermatozoa was determined as a ratio of live spermatozoa of all cells counted for each sample.

Data analysis

Data were coded for analysis with Statistical Package for Social Sciences (SPSS) version 20.0. (SPSS Inc.-Chicago). The median and range of semen parameters before treatments were displayed. The values of semen parameters obtained initially after collection of semen served as the reference for comparison with post-treatment values obtained within two hours following treatment. Pre- and post-treatment median values were compared to determine effect of extension using General linear Model (GLM).

Median values for motility and percentage live spermatozoa were compared before and after semen samples were extended with specific extenders, with and without removal of seminal plasma, using T- test statistic within GLM analysis. The results of these analyses were as presented in the Table 1.

RESULTS

Individual progressive motility of the spermatozoa significantly reduced in presence and absence of seminal plasma following extension with each extender ($P < 0.05$) (Table 1). Similarly, the percentage of live spermatozoa reduced significantly in samples where seminal plasma was removed ($P < 0.05$). However, both extenders maintained the percentage of live spermatozoa at similar level to pre-extension irrespective of seminal plasma status ($P > 0.05$) with intact seminal plasma. This implied that the process of removal of seminal plasma was one of the factors responsible for death of spermatozoa.

DISCUSSION

The current study investigated the effect of two extenders (COC and OPT) and seminal plasma on post-extension viability (motility; percentage live spermatozoa) of buck (goat) semen. Assessment of viability was done within two hours following extension to establish if the newly constituted coconut water extender (COC) would sustain viability of spermatozoa of the goat that would allow further processing, alongside a commercial extender. The study was also aimed at establishing effect of seminal plasma on the viability of spermatozoa within two hours after extension.

The results from this study showed that the two extenders lowered individual progressive motility of goat spermatozoa. The results also showed that the observed increase in percentage of dead spermatozoa (decreased percentage in live spermatozoa) could be attributed to the effects associated with removal of seminal plasma. The post-extension viability values however allowed the

extended semen with and without seminal plasma for further processing.

This study routinely examined such pre-treatment ejaculate parameters from the experimental bucks. The values of volume and individual motility (Sathe and Shipley 2014), concentration (Mushtaq *et al.*, 2007), percentage live spermatozoa (Sule *et al.*, 2007), mass activity (Olayemi *et al.*, 2011; Sathe and Shipley, 2014), and colour (Mushtaq *et al.*, 2007), were within acceptable ranges. There was a significant buck effect on mass activity. This could be traced to relatively large fluctuation in mass activity values of buck 1 and 4 on different days of semen collection, which could have been due to individual difference in degree of response to similar sexual stimulation of the bucks (Mushtaq *et al.*, 2007).

Post-extension viability of semen is known to differ between extenders probably based on difference in their composition and species of animal from which semen is collected (Sathe and Shipley, 2014). The two extenders used in the current study maintained post-extension mean motility and percentage live spermatozoa above the acceptable levels of 70% with and without seminal plasma, as analyzed within two hours following extension without significant differences in percentage motility. This implied that semen with and without seminal plasma extended in each of the extenders could immediately be used for insemination in the goat or would further be processed for storage. There was however a significant reduction in motility within this time limit, a finding that was also reported by Sule *et al.*, (2007) in West African Dwarf goats.

Immediate effect of extenders on sperm viability has been elaborately studied and is known to affect motility and percentage of live spermatozoa. Extenders interfere with mitochondrial energy metabolism pathways, through poor control in production of Reactive Oxygen Species (ROS) (Kaya *et al.*, 2014). The effect is a reduction in motility vigour (Kaya *et al.*, 2014). The degree of reduction in motility is therefore depended on the level of ROS produced. Increased ROS production has been established as a modulator of sperm cell capacitation, (Argarwal *et al.*, 2005) a process that interferes with the integrity of sperm plasma membrane compromising their viability (Kaya *et al.*, 2014). Yet, such effects should not compromise viability of spermatozoa to level that would deter further processing.

The ability of any extender to support short-term survival and motility of spermatozoa is pegged on many factors that include its micronutrient composition and biochemical composition of the extender. The laboratory generated coconut-water-egg yolk based extender (COC) recorded very promising results as an extender for further

Table 1: Comparison of significance of pre- and post-extension of median of evaluation parameters

Parameter	Treatment	N	Median Percentage	Minimum percentage	Maximumpercentage	P value
Motility	Pre-extension	10	90	85	100	
	Post-extension (ACOC)	10	80	70	85	0.0
	Post-extension (BCOC)	10	80	70	85	0.0
	Post-extension (AOPT)	10	80	70	90	0.004
	Post-extension (BOPT)	10	80	80	85	0.0
% live cells	Pre-extension	10	97.6	95.1	100	
	Post-extension (ACOC)	10	96.7	94.7	100	0.09
	Post-extension (BCOC)	10	93.1	84.2	98.1	0.001
	Post-extension (AOPT)	10	97.3	93.1	100	0.22
	Post-extension (BOPT)	10	94.3	87	98.1	0.005

goat semen processing compared to the commercial extender OPT.

This study concluded that the laboratory generated coconut water-based extender was equally appropriate in extending goat semen for immediate use or for further processing compared to the commercially available extender, OPTIXcell™. The researchers strongly believed that the composition of the laboratory-generated extender COC as constituted for purposes of this study was appropriate for extension of goat semen.

It is recommended that further research be done especially in Kenya to commercialize coconut production for purposes of extracting coconut water for goat semen production. Further research to establish viability of such semen under different storage modalities and subsequent conception success is necessary as well.

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