Global Veterinaria 12 (3): 292-298, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.12.03.8236

# Effect of Egg Yolk Concentration in Semen Extender, pH Adjustment of Extender and Semen Cooling Methods on Bovine Semen Characteristics

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**Abstract:** This work aimed to improve cattle production through Artificial Insemination using refrigerated semen. The effect of egg yolk concentrations (10, 15, 20, 25 and 30%) in semen extender, pH adjustments of the extender and semen cooling methods [from 35 to 5°C using a water bath (WB), 35 to 5°C without the WB, 35 to 20°C then to 5°C with WB and 35 to 20°C then to 5°C without WB in the refrigerator respectively] on the characteristics of chilled bovine semen were evaluated. Daily, the post-thaw live cells concentration (LCC), individual (IM) and progressive motilities (PM) were registered and grouped into 3 preservation periods; from day 1 to 4, day 5 to 8 and day 9 to 12. Results showed that whatever the preservation period, the IM, PM and LCC at 10% egg yolk were significantly higher ($p<0.05$) than those in other egg yolk concentrations. The LCC at 1 to 4 days was significantly higher ($p<0.05$) when semen was cooled from 35 to 5°C in water bath (23.58±04.81x$10^6$/ml), compared to all other cooling methods. From 9 to 12 days, the IM (15.42±06.90%), PM (05.83±05.57%) and LCC (01.83±01.47 x$10^6$/mL) when cooled from 35 to 20°C then to 5°C in WB were significantly higher. The PM and LCC in the extender with unadjusted pH were significantly higher from day 5 to 8 (44.17±15.93% and 18.75±03.68 x$10^6$/ml respectively), as well as from 9 to 12 days (12.00±11.83% and 04.90±02.92 x$10^6$/ml respectively) compared to those of adjusted pH. In conclusion, sperm lifespan is prolonged for up to 8 days in an extender with unadjusted pH, containing 10% egg yolk and cooled through the water bath.

**Key words:** Semen Quality · Egg Yolk · Cooling Methods · Semen pH · Semen Extender

## INTRODUCTION

The preservation of mammalian sperm is a complex process that involves many factors in order to obtain good results. The most commonly used extenders for liquid or frozen semen have egg yolk as a basic ingredient [1]. Egg yolk provides an excellent protection for mammalian spermatozoa in resisting against cold shock [2,3] and the lipid-phase transition effect [3]. Egg yolk is added in bull semen extenders as energy source as it provides the sperm cells with lecithin, proteins, lipoproteins and similar complexes [4]. The development of buffers to combine with egg yolk and a reduction in its concentration to 20% (v/v) in mammalian semen extenders improved sperm survivability [5].

Many studies have indicated the possibility of the use and commercialization of bovine semen in liquid form under refrigeration [6]. The main advantages related to the use of liquid-stored semen are a higher viability than cryopreserved semen, the possibility of using reduced insemination doses, the optimization of high-merit bulls, the lower cost of storage and the practicality for use in artificial insemination [7, 8].

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The success of artificial insemination depends greatly on the development of satisfactory semen extenders which performs the same function as the seminal plasma. The seminal plasma secreted from the accessory reproductive organs serves as a transport medium for the spermatozoa [9, 10]. It also provides an environment with adequate pH for optimum metabolic activities. The plasma serves as a buffered nutrient medium which suspends and maintains the fertility of spermatozoa [11].

Plasma buffers are often not present in sufficient quantities to prevent a reduction in semen pH when in storage; therefore, a good semen extender should have buffering capacity [12, 13]. Bovine cauda epididymal sperm were shown to be inhibited, in vitro, in a pH-dependent manner by a quiescence factor present in cauda epididymal fluid; the pH of neat semen collected any time from 5 mm to 6h postmortem from bovine epididymides is 5.8 [13]. At this acidic pH, an epididymal quiescence factor(s) immobilizes bovine cauda epididymal sperm. Acott and Carr [14] have presented evidence that low sperm pH is responsible for the in vitro inhibition of motility by bull cauda epididymal fluid.

Chilled semen as an alternative to frozen semen is faced with the challenge of short viability period of about four days [15-17]. If improved upon, chilled semen can be used on a wider scope and in enclave regions, which may take a couple of days to access. As main objective therefore, this study sought to contribute in improving cattle production by increasing the preservation duration of chilled semen destined for artificial insemination. More specifically it tested the effect of five levels of egg yolk incorporation in citrate egg yolk extender on the characteristics of chilled bovine semen; compared the viability of spermatozoa at an adjusted pH of 7.4 and an unadjusted pH during preservation;tested the effect of semen cooling methods on its properties.

## MATERIALS AND METHODS

**Bulls:** Semen was collected from four healthy bulls. The bulls were aged 5-5.5 years, weighed 485-550 Kg and the scrotal circumference was 50-60 cm. The breed of the bulls was Holstein x White Fulani. Each received a daily ration of 4.0 kg protein-based concentrate (cottonseed) cake and grazed freely in a paddock that was composed principally of *Pennisetum clandestinum* and *Brachiaria ruziziensis*. They were regularly vaccinated against bluetongue, Brucellosis, Contagious bovine pleuropneumonia, foot and mouth disease and mycoplasma. Bulls were regularly dewormed and controlled for ectoparasites.

**Extenders Preparation:** All extenders were prepared in the laboratory using reagent-grade chemicals purchased from Sigma. Briefly, in each of the replicates of this experiment, 20 ml of each extender was prepared by introducing in a graduated tube, double distilled water, trisodium citrate dihydrate salt, antibiotics (Penicillin G and streptomycin) and egg yolk.

The composition of each extender is reported in Table 1. The content of each of these cylinders was properly mixed and the pH adjusted to 7.4 using buffers at pH 6.865 or 9.180. These prepared extenders were then put in the water bath at a temperature of 35°C

**Semen Collection and Evaluation:** Semen was collected by artificial vagina as described by Williams [20]. The bulls were allowed at least one to two false mounts before collection. The sheath was lightly grasped and deviated into the artificial vagina. Immediately after collection, the collecting tube was detached from the artificial vagina, taken to laboratory and placed in a beaker containing lukewarm water (37°C) until evaluation and processing. Semen samples were collected three times per week in each bull.

Sperm motility was assessed independently by two well-trained evaluators, using a phase-contrast microscope (Laborlux-11, Germany) at a 400 x magnification and equipped with a heated stage (37°C). After a 200-fold dilution, the percentage of progressively motile spermatozoa was assessed in two drops of semen from each sample in three different fields resulting in a total of 12 observations for each semen sample. Means of the recorded observations were used for further analysis.

Table 1: Composition of extenders

| Egg yolk volume (ml) | Citrate buffer solution (ml) | Penicillin (g) | Streptomycin (g) | Egg yolk concentration (%) |
|---|---|---|---|---|
| 2 | 18 | 0.005 | 0.010 | 10 |
| 3 | 17 | 0.005 | 0.010 | 15 |
| 4 | 16 | 0.005 | 0.010 | 20 |
| 5 | 15 | 0.005 | 0.010 | 25 |
| 6 | 14 | 0.005 | 0.010 | 30 |

With respect to the live cell concentration, an eosin-nigrosin stain was used and equal volumes of the stain and semen (50il) were mixed at 36°C and incubated for 30 seconds. A 20 fold dilution of this semen was then prepared with tap water, properly mixed, mounted on the haemocytometer then the live cells (light in color) were counted and noted. The spermatozoa were counted in five large squares; four at corners and one in the centre of 25 large squares. The number of heads in the large squares was recorded. The concentration of sperm per millilitre of semen was calculated by multiplying the total number in large squares and expressed as million per millilitre.

**Experimental Design:**

- The first experiment tested the effect of five levels of hen egg yolk concentration (10, 15, 20, 25 and 30%) in citrate-egg yolk extender on chilled bovine semen characteristics. Each collected semen was diluted at 35 °C with extender in order to provide approximately 30 x $10^6$ spermatozoa/mL for each treatment of this experiment. The samples in the 5 tubes were then corked, put in plastic cups containing warm water from the water bath into the refrigerator at 5°C. On a daily basis, 75il of each sample was pipetted into 5 other empty tubes in the water bath from which the post-thaw individual and progressive motility and live cell concentration readings were gotten after incubating for about 3 minutes. This procedure of monitoring was carried out until all the cells in each sample die (point of zero motility).
- The second experiment tested the effect of semen cooling methods on its post-thaw properties. The extender was prepared at 35°C with a concentration of 10% hen egg yolk (results in the first experiment showed that extender with 10% hen egg yolk was the best). Semen was then collected in three replicates, split into four representing the four treatments (cooling methods), then diluted with a split fraction of the extender. The four treatments (T) were the following:

**T1:** Cooling from 35°C to 5°C using a water bath in the refrigerator;

**T2:** Cooling directly in the refrigerator from 35°C to 5°C without water bath;

**T3:** Cooling from 35°C to 20°C then from 20°C to 5°C with a water bath in the refrigerator;

**T4:** Cooling from 35°C to 20 then from 20°C to 5°C without the water bath.

Daily readings of the individual mobility, progressive mobility and live cells concentration for each treatment was gotten using the microscope as in experiment 1.

3-In the third experiment, the extender solution was prepared at a concentration of 10% egg yolk and a volume of 40 ml from which 5ml was deducted to constitute the solution of unadjusted pH while 35ml was the solution of pH adjusted to 7.4. Semen was then collected and diluted in these volumes at a cell concentration of 30 million spermatozoa /ml. On daily basis, the IM, the PM and the LCC were noted from these solutions and equally, 4ml was removed from the 35ml solution before adjustment of pH to 7.4.

The third experiment equally had three replicates and the preparation of the artificial vagina was done in a similar manner to the first and the second experiments. Each replicate had a total of 40ml of extender being prepared at 10% egg yolk volume. Here, 36ml of double distilled water was measured in a graduated cylinder and 1.058g of Trisodium citrate dihydrate salt was dissolved in it. Also, 0.005g of penicillin and 0.010g of streptomycin sulphate salt were added into the citrate buffer solution, mixing thoroughly for proper dissolution. Four milliliters (4ml) of egg yolk was then mixed with this solution to compose the 40 ml of extender and its pH noted. From this prepared solution, 5 ml was removed and put in a test tube in the water bath. This constituted the treatment of unadjusted pH. The pH of the remaining 35ml was then adjusted to 7.4 using the buffer solutions. It was then immersed in the water bath at 35°C awaiting extension. On daily basis, the IM, the PM and the LCC were noted from these solutions and equally, 4ml was removed from the 35ml solution before adjustment of pH to 7.4.

**Statistical Analyses:** The effect of treatments was analyzed using the General Linear Model (GLM) procedure of SPSS 19.0. Data transformation to log(x+1) was done for percentages before analyses [19]. The data of each treatment was grouped into three (3) preservation periods of 4 days each from 1 to 4 days, 5 to 8 days and 9 to 12 days before analyses so as to monitor and interpret the evolution during preservation. A p value of $< 0.05$ was considered statistically significant.

## RESULTS

**Effect of Egg Yolk Concentration in the Extender and Preservation Duration on Individual Motility, Progressive Motility and Live Cell Concentration:** It appear from table 2 that during the 1 to 4 days preservation period, the individual and the progressive motility of the sperm cells as well as the live cell concentration generally decreased as the egg yolk concentration in the extender increased, even though no significant differences (p>0.05) were recorded.

From 5 to 8 days of preservation, these parameters equally decreased with increasing concentrations of egg yolk in the extender. Statistical analyses revealed significant differences (p<0.05) only between the individual motility of the sperm cells in the extender containing 10% egg yolk as compared to those containing 25% and 30% egg yolk in the extender.

From 9 to 12 days of preservation, the individual and progressive motility as well as the live cell concentration generally decreased with increasing egg yolk concentration in the extender. Statistical analyses showed significant differences (p<0.05) between the individual motility of the sperm cells in the extender containing 10% compared to that containing 30% egg yolk.

The progressive motility of the sperm cells at 10% egg yolk was significantly higher (p<0.05) when compared to those of all the other levels of egg yolk concentration in the extender. Also, significant differences were recorded between the live cell concentration in the extender containing 10 and 15% egg yolk when compared to those in the extenders containing 20%, 25% and 30% egg yolk.

A highly positive and significant correlation (r=0.919, P<0.05) was recorded between the individual and progressive motility while the live cell concentration was negatively but not significantly correlated with the individual (r=-0.275, P>0.05) as well as with the progressive motility (r=-0.264, P>0.05).

**Effect of Semen Cooling Method and Preservation Duration on Individual Motility, Progressive Motility and Live Cell Concentration:** As shown in Table 3, from 1 to 4 days of preservation, the individual motility as well as the progressive motility was not significantly affected (p>0.05) by the cooling method, though highest motility values were registered when cooling from 35°C to 5°C in the water bath, while least values were recorded when cooling from 35°C to 5°C without the water bath. The live cell concentration when cooled from 35°C to 5°C in the water bath was significantly higher (p<0.05) than those of all the other cooling methods tested;

From 5 to 8 days of preservation, no significant difference (p>0.05) was recorded between the individual motility, the progressive motility as well as the live cell concentrations of the semen samples whatever the cooling method;

From 9 to 12 days of preservation, the individual motility of the sperm cells as well as the live cell concentrations in the semen cooled from 35°C to 20°C then to 5°C in water bath was significantly higher (p<0.05) than those cooled through the other methods tested. The progressive motility of the sperm cells was significantly higher (p>0.05) when cooling from 35 through 20°C to 5 °C in water bath compared to other cooling methods.

Table 2: Effects of Egg yolk concentration in the extender on semen characteristics

| | Preservation period (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1-4 | | | 5-8 | | | 9-12 | | |
| Egg yolk concentration (%) | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) |
| 10 | 84.00±11.19a | 83.75±13.75a | 35.00±18.69a | 49.25±21.04b | 41.50±18.72a | 19.30±11.08a | 23.50±21.10b | 18.25±17.57c | 04.65±04.77b |
| 15 | 83.75±09.30a | 82.25±12.61a | 29.80±15.60a | 45.75±19.95ab | 36.25±18.63a | 15.30±09.98a | 19.25±18.08ab | 12.75±14.09a | 02.45±02.98b |
| 20 | 80.00±13.57a | 80.50±14.77a | 27.15±11.44a | 40.75±16.80ab | 33.50±18.29a | 14.15±11.25a | 14.00±15.44ab | 08.75±12.34b | 01.90±02.86a |
| 25 | 77.25±14.46a | 76.25±16.93a | 26.15±12.12a | 33.00±17.35a | 29.75±15.85a | 12.30±09.20a | 09.75±12.08ab | 06.00±08.97b | 01.40±02.62a |
| 30 | 75.50±16.85a | 73.75±20.38a | 28.95±17.79a | 31.00±15.78a | 26.75±14.89a | 12.60±10.95a | 06.50±09.05a | 04.25±06.93b | 01.10±01.97a |

Table 3: Effects of cooling methods on semen characteristics

| | Preservation period (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1-4 | | | 5-8 | | | 9-12 | | |
| cooling methods | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) |
| 35 to 5°C in WB | 82.08±11.77a | 75.42±14.53a | 23.58±04.81a | 40.41±18.64a | 31.67±18.01a | 11.00±04.99a | 03.75±05.28a | 01.25±02.26a | 00.67±00.98a |
| 35 to 5°C no WB | 75.42±16.44a | 70.42±18.64a | 19.75±03.72b | 36.67±16.00a | 28.75±12.81a | 08.25±03.93a | 06.67±04.92ab | 00.83±01.95a | 00.50±00.80a |
| 35 to 20°C then 5°C in WB | 79.58±13.89a | 74.58±13.90a | 17.67±04.01b | 41.25±12.99a | 30.00±12.61a | 08.92±03.23a | 15.42±06.90b | 05.83±05.57b | 01.83±01.47b |
| 35 to 20°C then 5°C no WB | 80.42±14.37a | 75.00±17.32a | 18.17±04.49b | 36.25±11.89a | 26.67±09.13a | 07.83±03.01a | 04.58±04.98a | 00.83±01.95a | 00.67±00.98a |

Table 4: Effects of pH adjustment on semen characteristics

| | Preservation period (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1-4 | | | 5-8 | | | 9-12 | | |
| H adjustment | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) | IM (%) | PM (%) | LCC (millions/ml) |
| Unadjusted | 82.50±13.06a | 76.25±17.34a | 31.75±05.62a | 52.50±13.73a | 44.17±15.93b | 18.75±03.68b | 23.00±13.58c | 12.00±11.83b | 04.90±02.92b |
| Adjusted on day 1 | 81.67±14.67a | 77.50±16.99 a | 35.67±09.52a | 35.83±15.50a | 27.92±15.73ab | 14.83±06.98ab | 05.00±04.71b | 01.00±02.11a | 01.10±01.45a |
| Adjusted on day 1 and 2 | 79.58±16.85a | 73.75±20.01a | 35.25±09.71a | 30.42±16.44a | 23.75±15.24ab | 12.75±06.84ab | 03.00±03.50a | 00.50±01.58a | 00.80±01.03a |
| Adjusted on day 1 to 3 | 80.42±15.44a | 71.82±20.89a | 35.58±09.38a | 28.75±18.11a | 21.25±15.97ab | 12.75±07.56ab | 00.50±01.58a | 00.00±00.00a | 00.10±00.32a |
| Adjusted on day 1to 4 | 80.42±15.44a | 24.58±16.02a | 02.00±02.58a | 73.33±20.60 a | 17.92±14.38a | 00.50±01.58a | 35.58±09.38a | 11.00±06.9a | 00.30±00.48 a |
| Adjusted on day 1 to 5 | 80.42±15.44a | 27.08±17.38a | 01.50±02.42a | 73.33±20.60a | 20.83±15.50a | 00.50±01.58a | 35.58±09.38a | 09.92±07.23a | 00.20±00.63a |
| Adjusted on day 1 to 6 | 80.42±15.44a | 29.17±16.07a | 02.00±03.50a | 73.33±20.60a | 21.25±15.39a | 00.50±01.58a | 35.58±09.38a | 11.00±06.61a | 00.10±00.32a |

**Effect of pH Adjustment and Preservation Duration on Individual Motility, Progressive Motility and Live Cell Concentration:** When comparing the various levels of pH adjustment (Table 4), from 1 to 4 days of preservation, no significant differences ($p>0.05$) in individual motility of the sperm cells were recorded between the various pH adjustments as well as with the progressive motility and the live cell concentration.

From 5 to 8 days of preservation, no significant differences ($p>0.05$) were recorded between the individual motility of the sperm cells of the various pH adjustments. The progressive motility of the sperm cells in the extender with unadjusted pH as well as its live cell concentration were significantly higher ($p<0.05$) than those of the extenders adjusted from day 1 to 4, day 1 to 5 and day 1 to 6;

From 9 to 12 days of preservation, the individual motility of the sperm cells in the extender with unadjusted pH was significantly higher ($p<0.05$) than those of all the other levels of pH adjustment. The progressive motility of the sperm cells in the extender with unadjusted pH as well as its live cell concentration was significantly higher ($p<0.05$) than all the other levels of adjusted pH.

## DISCUSSION

The results reveal that as the concentration of egg yolk in the extender increases, there is a decrease in the quality of the semen expressed through a decrease in the various response variables (individual motility, progressive motility and live cell concentration) with preservation time. Bispo *et al.* [21] also observed that low egg yolk concentration (2.5%) in buck semen gave superior fertility results compared to high concentration (20%). On the other hand, Arhangari *et al.* [22] working with ram semen, found no significant effect on these parameters with extenders containing 10, 15 and 20% egg yolk. This decrease in the semen quality might be because as this egg yolk concentration increases, the extender becomes more and more viscous and the sperm cells therein tend to lose more energy in swimming than in less viscous samples thus, their life span is greatly reduced. It is therefore evident that the solution with least egg yolk concentration which is the treatment having 10% egg yolk gives the best results with respect to the parameters studied as it has the longest preservation duration (up to 12 days), confirming previous results obtained in collared peccary by Alves *et al.* [23] that semen samples having 10 and 20% egg yolk concentration is good for semen preservation. Based on the fact that semen samples can be used when motility is above 30% [24], the semen samples in the present experiment at 10% egg yolk can be used for up to 8 days, which are better than those expressed by Campbell *et al.* [16], stating that such samples can be used for up to 4 days. Similarly, Gil *et al.* [25] showed that there was no positive effect increasing egg yolk above 10% on the post-thaw motility, membrane integrity and induction of sperm capacitation when they incorporated 5%, 10%, 15% and 20% egg yolk in the extenders.

Generally, egg yolk in semen preservation provides the nutrients the cells need and also prevents the efflux of cholesterol and phospholipids as it attaches firmly to the cell membrane [26]. It also acts as a cryoprotectant [27].

The results show that both treatments involving preservation through the water bath are better than those without the water bath. This is because the semen characteristics in the former are slightly higher than those of the latter and too, their preservation durations are slightly longer. It is likely due to the fact that cooling without the water bath provokes a slight cold shock, which in this case is expressed through a slight decrease in these parameters of the semen samples [28].

The seminal plasma is composed of a number of organic and inorganic compounds. The major organic ion found in seminal plasma is bicarbonate, produced by the vesicular glands and it functions as a buffering agent, regulating pH changes of semen [39-31]. From the results, it is better not to adjust the pH of the extender at all to obtain better results with regards to the motility and live

cell concentration because the quality of the semen decreases as the pH of the extender is being adjusted to 7.4 using buffer solutions. This is because as the level of adjustment intensifies, the quality of the semen drops progressively as expressed through a drop in the post-thaw parameters. This can be accounted for in that as the buffer solution are being incorporated in the extender, they tend to cause a disequilibrium in the ionic composition of the sperm cells as their cell membranes are highly permeable [32], permitting the influx of these solutions in the sperm cells. This therefore causes a reduction in their life spans, for this disequilibrium is a shock to the cells whose susceptibility is expressed through a reduction in motility with time [33].

## CONCLUSION

- Egg yolk incorporation in bovine semen extender at a 10% concentration yields comparative better results up to 8 days in citrate buffer solution under refrigeration conditions.
- The use of the water bath in semen cooling yields better comparative results to those cooled without the water bath.
- Adjusting the pH of semen during storage to 7.4 has no positive effect on its properties.

**Communication Strategy and Impact:** Based on these results, the productivity of cattle can be improved upon (for both milk and meat production) through A.I using chilled semen collected from highly productive bulls. Through organized extension services, farmers can be made aware of chilled semen usage and local A.I stations created where they can get this semen.

## ACKNOWLEDGEMENTS

The author thanks the head of the Institute of Agricultural Research for Development (IRAD), Bambui Regional Center who gave them the opportunity to work in his Bovine Artificial Insemination Unit.

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