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# Effect of the addition of soursop juice (*Annona muricata L.,* 1753) to egg yolk citrate extender on the quality of bull semen

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## Abstract

The present study was carried out to investigate the effect of the addition of soursop (*Annona muricata L.,* 1753) juice to the citrate-egg yolk extender (EYC) on the quality of cryopreserved bull semen. After pre-evaluation, the semen was distributed in five extenders T1, T2, T3, T4 and T5 containing respectively 0% soursop juice (SJ) (T1), 25% SJ (T2), 50% SJ (T3), 75% SJ (T4), 100% SJ (T5) and microscopic evaluation was performed daily to assess plasma membrane integrity (PMI), progressive motility of spermatozoa (MP) and sperm pH until all sperm were dead. The results indicate a significant increase in spermatozoa PMI and MP ($p$<0.05) in the case of treatment with 50% SJ (T3). Spermatozoa showed a motility of 50.78% 9 days later in the citrate-egg yolk extender containing 50% SJ (T3), whereas at day 9, spermatozoa in the other extenders had a progressive motility of less than 50%. Similar results were obtained for PMI with 50.78% of spermatozoa having an intact plasma membrane 9 days later in the citrate-egg yolk extender containing 50% SJ whereas the PMI of cells in the other stents was less than 50%. However, the pH of the extender containing 50% SJ was closest to 7 during the experimental period. The results of this experiment suggest that the addition of 50% soursop juice (T3) to the citrate-egg yolk extender could be adopted as an acceptable alternative to traditional egg yolk-based extenders for the cryopreservation of bull semen.

**Keywords:** Cryopreservation; Extenders; Soursop juice; Spermatozoa

## 1. Introduction

In modern cattle breeding, where artificial insemination (AI) is the most widely used tool to facilitate the large-scale use and distribution of semen from genetically superior bulls, cryopreservation is an essential component. The effects of cryopreservation on sperm function and fertility have been extensively studied in many species, particularly in bulls. Since the inception of bovine semen cryopreservation, egg yolk-based extender has been used to protect sperm from the adverse effects of cooling and freezing. In recent years, there has been a demand for alternatives to conventional commercial extender, as the risk of introducing exotic diseases when transporting egg yolk-based products has been recognized. Egg yolk can also interfere with sperm evaluation and the presence of particles in the extender can reduce fertility [1].

Soursop (*Annona muricata L.,* 1753) belongs to the *Annonaceae* family found in all tropical regions. Soursop is astringent, cholagogic and promotes digestion [2]. It also has a number of properties, including being an antioxidant [3]. Antioxidant nutrients are important for limiting damaging oxidative reactions in cells, which can predispose to the

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development of major clinical conditions such as oxidative stress disorders [4]. The fruit is also rich in B vitamins, potassium, fructose, vitamin C and certain enzymes such as catalase and peroxidase [2]. Several biological activities and the general mechanisms underlying the effects of *Annona muricata L.,* 1753 have been tested both in vitro and in vivo. *Annona muricata L.,* 1753 contains chemicals such as acetogenins (annomuricins and annonacin), alkaloids (coreximine and reticuline), flavonoids (quercetin) and vitamins, which are predicted to be responsible for the biological activity of *Annona muricata L.,* 1753 [5].

However, few data are available in the literature on the antioxidant activity of soursop juice to counter the deleterious effects of lipid peroxidation that occurs naturally during in vitro storage of bull semen. This study sought to contribute to the improvement of livestock productivity by increasing the duration of storage of refrigerated semen destined for artificial insemination. More specifically, it evaluated the addition of soursop juice to citrate-egg yolk extender as an acceptable alternative to traditional egg yolk-based extenders for the cryopreservation of bull semen.

## 2. Materials and methods

### 2.1. Description of the study site

This study was carried out on a collective agricultural farm: the Babete livestock breeders' cooperative with a board of directors, located in the locality of Mbouda, in the West Cameroon region. It is one of the largest farms in the region, practicing an intensive breeding system with a herd of more than 25 cattle, oriented exclusively towards milk production.

### 2.2. Biological material and management

#### *2.2.1. Semen collection*

Semen was collected from four healthy, fertile *Holstein* bulls aged between 4 and 7 years. Semen was collected twice weekly for 4 weeks. The bulls were reared under standard feeding and management conditions. Semen was collected using an artificial vagina preheated to 42 °C. The percentage of progressive motility of each sample was determined using a phase contrast microscope at 200x magnification. Ejaculates with greater than 75% motility were used for the present study [6]. Collected semen was immediately transported to the laboratory for evaluation, processing and extension. Each treatment was carried out in eight replicates, giving a 4 x 8 factorial design.

#### *2.2.2. Preparation of soursop juice (Annona muricata L.,* 1753*)*

A ripe, healthy soursop (*Annona muricata L.,* 1753) weighing 1 kg, purchased from a supermarket, was washed under running water and sterilized with alcohol. Using a sterilized knife, the soursop fruit was dissected and seeded using a clean sterile spoon, the flesh was removed, cut and crushed with the spoon in a dish and the juice was extracted. The extracted juice was collected in centrifuge tubes and centrifuged at 3000 rpm for 15 minutes. The supernatant from the first centrifugation was centrifuged again at 3000 rpm for 15 minutes. The supernatant from the second centrifugation was collected in test tubes. The volume and pH of the soursop juice were measured and recorded, and the juice was stored in a refrigerator at 5 °C until used in the experiment.

#### *2.2.3. Preparation of the soursop juice-based extender*

The extenders used were prepared on the basis of citrate-egg yolk extenders with the addition of soursop juice at 3 levels in accordance with FAO guidelines [7] as shown in Table 1.

**Table 1** Soursop juice-based extender

| Extender | Soursop Juice (%) | Citrate (g) | Penicillin (g) | Streptomycin (g) | Egg yolk (ml) | Soursop Juice (ml) | Distilled $H_2O$ (ml) | Total (ml) |
|---|---|---|---|---|---|---|---|---|
| T1 | 0 | 0.294 | 0.0075 | 0.0125 | 2.5 | 0.000 | 10 | 12.814 |
| T2 | 25 | 0.294 | 0.0075 | 0.0125 | 1.875 | 0.625 | 10 | 12.814 |
| T3 | 50 | 0.294 | 0.0075 | 0.0125 | 1.25 | 1.25 | 10 | 12.814 |
| T4 | 75 | 0.294 | 0.0075 | 0.0125 | 0.625 | 1.875 | 10 | 12.814 |
| T5 | 100 | 0.294 | 0.0075 | 0.0125 | 0.000 | 2.5 | 10 | 12.814 |

The eggs used for these experiments were laying hen eggs bought from a local supermarket and stored in a refrigerator. The various salts, citrate salt, penicillin and streptomycin sulphate were weighed using a sensitive electronic balance and dissolved in distilled water as indicated in Table 1 above. After homogenization of the diluents, the pH was adjusted to 7.4 using an electronic pH meter. The solutions were then placed in a water bath at 34.5 °C pending semen collection.

### 2.3. Post-extension evaluation of the semen

Extended semen was stored at 5 °C. Refrigerated semen samples during storage were further evaluated for progressive mobility of spermatozoa, plasma membrane integrity and sperm pH.

The percentage of progressive motility of spermatozoa in each sperm sample (10 µL) was determined using a phase contrast microscope equipped with a hot stage set at 37 °C. Plasma membrane integrity was assessed using the hypoosmotic swelling test as described by Correa et al and Barszcz et al [8,9]. The sperm pH of each solution was assessed using an electronic pH meter and values were recorded daily. Semen parameters were assessed daily until all spermatozoa were dead.

### 2.4. Statistical analysis

Mean percentages of progressive motility of spermatozoa, plasma membrane integrity and sperm pH were assessed using STATGRAPHICS Centurion 16.1. A one-way ANOVA was used to test for differences between factors. An angular transformation of the data was performed to make them more uniform. Fisher's LSD test was used to separate treatment means. Significance levels were considered to be $p<0.05$.

## 3. Results

### 3.1. Effects of soursop juice-based extender on progressive mobility of spermatozoa, plasma membrane integrity and sperm pH

The results of adding soursop juice (SJ) (*Annona muricata L.,* 1753) to the citrate-egg yolk extender on bull sperm quality are summarized in Table 2 below.

**Table 2** Effects of soursop juice-based extender on progressive mobility of spermatozoa, plasma membrane integrity and sperm pH

| Percentage of soursop juice added | Means (%) | | |
|---|---|---|---|
| | **PM** | **PMI** | **pH** |
| T1: 0% SJ | 40.21±2,55ab | 39.80±0.88ab | 6.78±0.01a |
| T2: 25% SJ | 41.53±2,55ab | 41.59±0.88ab | 6.89±0.01a |
| T3: 50% SJ | 43.65±2,55a | 43.70±0.88a | 6.97±0.01a |
| T4: 75% SJ | 37.18±2,55b | 37.30±0.88b | 6.96±0.01a |
| T5: 100% SJ | 21.59±2,55c | 21.74±0.91c | 6.80±0.01a |

SJ: Soursop Juice; (PM): progressive motility; PMI: plasma membrane integrity; a, b, c: least square means with the same superscript in the same column do not significantly differ from each other at ($p<0.05$).

Looking at Table 2, we can see that there was no significant difference between the pH levels in the five extenders throughout the storage period. However, we did notice that T3 maintained a pH closest to the adjusted pH (7.4) compared to the other four extenders. The control extender T1 was furthest from the adjusted pH value.

Progressive motility (PM) averages of spermatozoa were highest in T3 containing 50% SJ and lowest in T5 containing 100% SJ. However, there was no significant difference between T1, T2 and T3. On the other hand, significant differences ($p<0.05$) were observed between T3 and T4 as well as between T3 and T5. We also noted that the PM of the T5 extensor differed statistically ($p<0.05$) from the other four extenders. The same observations were made for plasma membrane integrity (PMI) of spermatozoa during storage time with T3 recording the highest value while T5 recorded the lowest mean value.

### 3.2. Effects of the soursop juice-based extender on progressive motility of spermatozoa as a function of storage time

Figure 1 shows the evolution of the percentage of progressively motile spermatozoa in the different extenders as a function of storage time. Figure 1 shows that, for all five extenders, progressive motility of spermatozoa decreased with time from the day of dilution until the end of the experiment when all cells were dead. We also observed that T1, T2 and T3 extenders showed the same trend from the first day of dilution until day 7 when progressive motility was still above 50%. On the other hand, the extender containing 50% egg yolk and 50% soursop juice gave better results with 50.78% motile sperm 9 days after the start of storage.

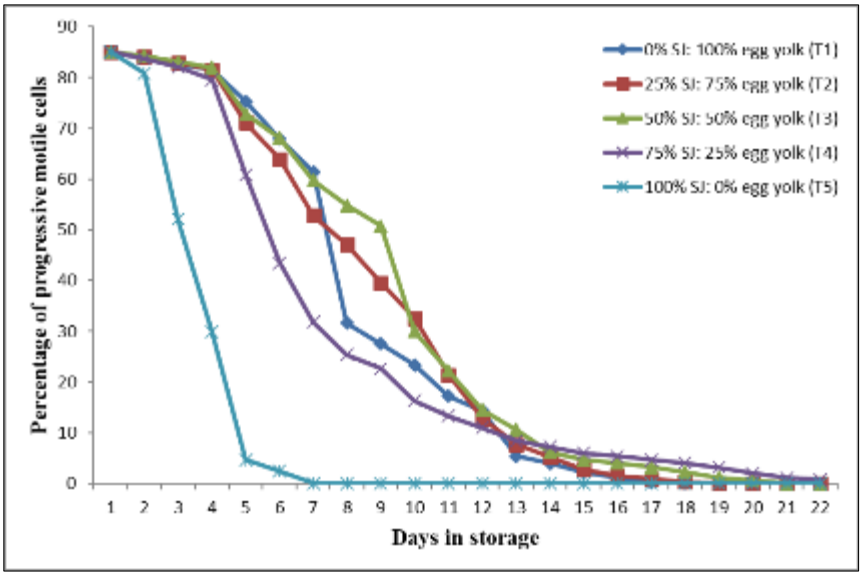


**Figure 1** Evolution of the percentage of progressive motility cells in the different extenders (T) as a function of storage time

### 3.3. Effects of soursop juice-based extender on the evolution of the spermatozoa plasma membrane integrity as a function of storage time

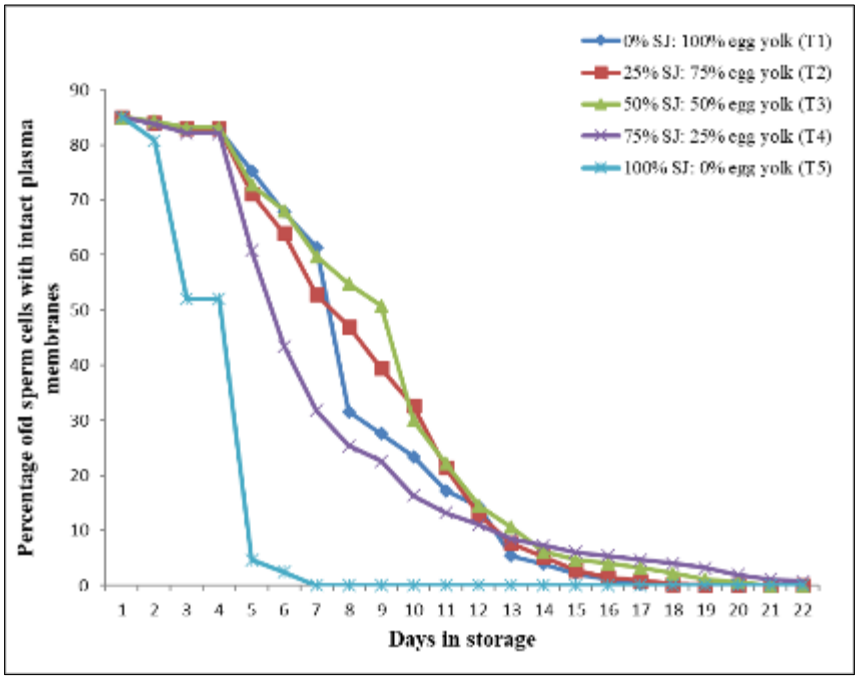


**Figure 2** Evolution of the percentage of cells with intact membranes in different extenders (T) as a function of storage time

Figure 2 showing the effects of the soursop juice-based extender on the evolution of plasma membrane integrity of spermatozoa as a function of storage time revealed that the percentage of spermatozoa with an intact plasma membrane decreased progressively with the number of days of storage for all treatments. This PMI fell rapidly in T5 from day 1 to 0% at day 7. As in the case of progressive motility, the results showed that up to 50.78% of spermatozoa still had an intact plasma membrane 9 days after storage in T3.

### 3.4. Effects of the soursop juice-based extender on changes in pH as a function of storage time

The evolution of the pH of the different extenders as a function of storage time is shown in Figure 3. According to this figure, the pH values seem to decrease progressively with increasing storage time. All extenders apparently maintained a similar pH trend throughout the storage period. However, pH levels fluctuated over the days of storage, with extender 3 (T3) containing 50% egg yolk and 50% SJ (soursop juice) having the best pH at 7 still 13 days after storage.

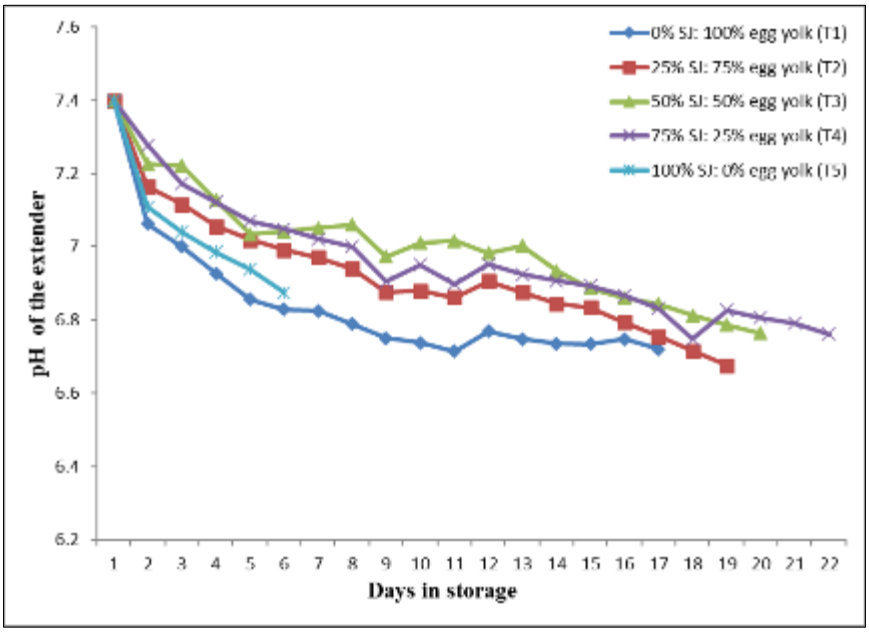


**Figure 3** Evolution of the pH of different extenders (T) as a function of storage time

## 4. Discussion

Egg yolk citrate extenders have been widely used for the dilution and preservation of chilled bull semen. However, with the emphasis on biosecurity issues and disease control during semen transfers, egg yolk extenders have become suspect of facilitating disease transmission. *Escherichia coli, Staphylococcus sp., Streptococcus sp., Pseudomonas sp., Haemophilus sp., Salmonella sp., Avian influenza, Campylobacter sp., Listeria sp.* and *Mycoplasma sp.* can be transmitted through egg yolk [1]. In addition to the risk of disease transmission, the particles present in egg yolk cause problems for seed evaluation and quality control [10,11].

The results of this study indicated that there is a significant difference between extenders with respect to progressive motility and membrane integrity. The egg yolk extender containing 50% soursop juice performed best for progressive motility of spermatozoa, plasma membrane integrity and sperm pH. The performance obtained with extender T3 compared with the extender containing 100% egg yolk (T1) on the one hand, and the other extenders (T2, T4 and T5) on the other, could be attributed to the antioxidant properties of soursop juice, which is known to be rich in vitamin C. Similar observations were obtained by Ekaluo et al [12], who showed that soursop fruit extract could attenuate caffeine-induced toxicity on testicular and epididymal weight, motility of spermatozoa, number of spermatozoa and the abnormality of the head of spermatozoa. Ekaluo et al [12] suggested that the attenuating effect of soursop fruit extract could be attributed to its rich vitamin C content. Vitamin C acts as an electron donator, to neutralize free radicals that are generated from normal metabolic activity in addition to environmental challenges. This ability to donate electrons allows for the reduction of oxidative stress from ascorbate free radicals [13]. In a study performed by Mittal et al. [14] supplementation of 5 mM vitamin C to pooled bull ejaculates significantly improved seminal characteristics and significantly decreased the number of observed abnormal sperm as compared to the control group measurements. Enzymes such as catalase and peroxidase have been detected in soursop pulp [5]. The presence of catalase and

peroxidase in soursop fruit, which like vitamin C are potent antioxidants, may be responsible for the increase in progressive motility and plasma membrane integrity of spermatozoa observed with T2, T3 and T4 extenders.

The present study revealed that T3 recorded the best pH, close to the adjusted pH. This pH value remained around 7 until 13 days after the start of storage. Thus, soursop juice may have contributed to maintaining a more stable pH during cold storage. As spermatozoa are highly polarized cells with a compartmentalized distribution of lipids and proteins in their plasma membrane, changes in membrane lipids and phospholipids in heat shock-sensitive spermatozoa are responsible for the drop in pH and concomitant decrease in sperm motility and plasma membrane integrity during storage [15].

On the other hand, Hydrogen peroxide ($H_2O_2$), Nitric oxide (NO), and superoxide anion ($O_2^-$) in semen have positive effects on intracellular signaling, sperm capacitation, and acrosome reactions [16]. Although at the appropriate levels of these molecules play a significant role in sperm physiology, namely capacitation and acrosome reaction, they are detrimental to sperm function at high concentrations due to toxicity.

Since soursop juice contains antioxidants, their antioxidant properties scavenge hydrogen peroxide ($H_2O_2$) generated by spermatozoa, preventing lipid peroxidation of polyunsaturated fatty acids and thus the production of reactive oxygen species that appear to be the main process of pH variation, sperm membrane deterioration and motility [17,18].

In addition, the catalase present in soursop juice is capable of reacting directly with reactive oxygen species and reducing peroxides ($H_2O_2$) to water and oxygen. This reaction attenuates the production of free radicals, reducing toxicity and pH variations in stored sperm, protecting spermatozoa from oxidative stress and preserving sperm parameters. The significant difference in plasma membrane integrity and motility observed between the egg yolk citrate extender containing 50% soursop juice and the other extenders could be due to the soursop's low fatty acid content [19].

## 5. Conclusion

According to the results of the present study, the addition of 50% soursop juice to egg yolk citrate extender reduces the negative effect of cooling on progressive motility of spermatozoa, plasma membrane integrity and pH of bull semen maintained at 5 °C. This is because soursop is an antioxidant agent, rich in B vitamins, potassium, fructose, vitamin C and certain enzymes such as catalase and peroxidase. Cryopreservation of bull semen could therefore be maximized by combining adequate levels of a source of unsaturated fatty acids such as egg yolk and soursop juice. Therefore, substituting 50% soursop juice for egg yolk in the egg yolk citrate extender for bull semen can be used to improve progressive motility of spermatozoa, plasma membrane integrity and pH of bull semen and thus improve fertility and fertilization capacity of spermatozoa.

## Compliance with ethical standards

### *Acknowledgments*

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### *Disclosure of conflict of interest*

The authors declare that there is no conflict of interest regarding the publication of this document

### *Statement of ethical approval*

This study is accordance to ethical guidelines of the Institute of Agricultural Research for Development (IRAD), Cameroon.

### *Statement of informed consent*

Informed consent was obtained from all individual participants included in the study.

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