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Effects of Cellgevity® on the milt quality of catfish, *Clarias gariepinus* extended in sodium citrate during chilled storage

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Abstract

Cellgevity® is a supplement reported to comprise mostly D-Ribose and L-Cysteine enriched glutathione, known to be an effective antioxidant that improves spermatozoa quality. However, its effect on milt characteristics has not been reported. This study, therefore, aimed to evaluate the effects of Cellgevity® on the milt quality of catfish (*Clarias gariepinus)* extended in sodium citrate during chilled storage. Pooled milt sample from three fishes was divided into three groups (T_1, T_2 and T_3). The milt was extended in sodium citrate, and each group in triplicate was supplemented with Cellgevity® at 0 mg (T_1), 125 mg (T_2) and 250 mg (T_3). The spermatozoa motility, concentration, viability and morphology were evaluated on days 0, 1, 2, 3, 4 and 5 of chilled storage. Data were expressed as mean ± standard deviation (SD) and analysed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Mean ± SD spermatozoa motility was significantly ($P < 0.001$) lower in T_2 and T_3 than T_1 before and during the first 3-days storage period. Mean ± (SD) spermatozoa concentration was significantly ($P < 0.001$) higher in T_2 and T_3 than T_1 before and throughout the 5-days storage period. Mean ± SD live spermatozoa were significantly ($P < 0.001$) lower in T_3 than T_1 at day 2 of the storage. Mean ± SD total abnormal spermatozoa did not differ significantly ($P > 0.05$) among the groups before and throughout the 5-days storage period. It was concluded that although supplementation of Cellgevity® at 125 mg and 250 mg in milt of catfish, extended in sodium citrate in chilled storage maintained the sperm cells alive and motile up to four days of the storage. However, it did not improve the milt quality. Hence, it should not be supplemented in sodium citrate extended milt of catfish, *Clarias gariepinus* in chilled storage.

Keywords: Cellgevity®, Chilled storage, C*larias gariepinus,* Milt quality, Sodium citrate

Introduction

African catfish, *Clarias gariepinus* (Burchell, 1822) is a species of catfish originally from Africa and the Middle East commonly called African sharptooth or airbreathing catfish (Teugels, 1996). It was reported

that although the African catfish fertilised externally, however, artificial milt collection is difficult and the volume of milt is very small, hence, males are killed and testes are collected and macerated to obtain the milt (Viveiros *et al.,* 2000). An extender is required to dilute the milt and increase its volume for artificial breeding purposes and storage (Muchlisin, 2005; Ohta *et al.,* 2001). Sodium citrate has been used as milt extender of *Clarias gariepinus* where it enhanced sperm cells survival within the first 24-48 hours post extension (Adeyemo *et al.,* 2007). Chilled storage of milt from various fish species has been studied (Agarwal *et al.,* 2013; Kledmanee *et al.,* 2013; Kowalski *et al.,* 2014; Bernáth *et al.,* 2018; Muthmainnah *et al.,* 2018) in other to enhance fish production, preserve threatened or endangered fish species and to mitigate inbreeding of native species in captivity (Hatipoğlu & Akçay, 2010). Numerous factors including methods of milt collection, sperm motility activation, milt storage, extender solution, etc affect sperm quality and subsequent fertilisation outcome (Beirão *et al.,* 2019). During milt storage, the biological membranes of spermatozoa are mostly affected by reactive oxygen species which ultimately results in their death. Reactive oxygen species are highly reactive in nature and can readily combine with other molecules, directly causing oxidation that can lead to structural and functional changes and result in cellular damage (Agarwal *et al.,* 2005). Various additives which imparts antioxidant properties during semen extension and preservation such as regucalcin, curcumin, sodium pyruvate, glutathione, astaxanthin, virgin coconut oil, epidermal growth factor, coenzyme q10, silymarin, melatonin, etc. have been used (Raheja *et al.,* 2018). Cellgevity® contain the glutathione precursor molecule: riboceine (D-ribose-L-cysteine), alpha lipoic acid, broccoli seed extract, quercertin, milk thistle, vitamin c, turmeric root extract, resveratrol, grape seed extract, selenamethione, cordyceps, black pepper and aloe extract. It is marketed as an antioxidant and its total antioxidant potential has been determined *In vitro* on selected rat liver cytochrome P450 enzyme activity (N'guessan *et al.,* 2018). It is known to be an effective antioxidant that improved spermatozoa quality (Gaucher *et al.,* 2018; Ukwenya *et al.,* 2020). Its effect on milt characteristics has not been reported. Hence, this study aimed to evaluate the effects of Cellgevity® on milt quality of catfish (*Clarias gariepinus)* extended in sodium citrate during chilled storage.

Materials and Methods

Study area

This study was carried out at the Theriogenology Laboratory, Department of Theriogenology and Production, Ahmadu Bello University, Zaria, Nigeria.

Ethical approval

Ethical approval for the use of the fish and milt sample collected were sorted from Animal Care and Use Committee of the Ahmadu Bello University, Zaria, Nigeria, and approval number ABUCAUC/2021/154 was issued.

Testes and milt collection

Three sexually matured male catfish*, C. gariepinus* broodstock of age (15 months), body weight (1,891 ± 32 g) and body length (62.25 ± 0.79 cm) were used for this study. The broodstocks were humanely euthanised and dissected by making a mid-ventral incision between the pectoral fins to about one centimetre to the genital papilla. The testes were located, pulled out and dried using a clean cloth. A small incision was made on each testis using a scalpel blade and holder and the milt was squeezed from the testes into clean transparent test tubes. Milt was quickly checked for gross motility on a score of 0 to 5 based on the wave pattern of spermatozoa motility using light microscope at x10 magnification as describe previously (Cosson *et al.,* 2008; Davida *et al.,* 2015). Only milt whose pre-extension gross spermatozoa motility was ≥ 80% were pooled together for the study (Adeyemo *et al.,* 2007; Müllera *et al.,* 2020).

Preparation of sodium citrate extender and Cellgevity® supplement

Sodium citrate dihydrate (1.45% weight/volume) extender was prepared by dissolving 1.45g of sodium citrate dihydrate in 50ml of double distilled water. Cellgevity® was procured from Max International Company. The capsules were opened and 125mg and 250mg of Cellgevity® powder were weighted using Mettler Toledo® scale and were dissolved in 5ml each of warm double distilled water and allowed to stand for 60 minutes, after which it was centrifuged at 3000rpm for 5 minutes. The supernatant was decanted and used for supplementation.

Experimental design

A complete randomised design was used. The milt sample was assigned to three (3) groups (T_1, T_2 and T_3) in triplicate each and were extended in sodium citrate dihydrate at (1 part milt : 200 parts sodium citrate

dehydrate extender). T_1 served as control while T_2 and T_3 were supplemented with 125 mg and 250 mg Cellgevity®, respectively. All groups were stored at refrigerated temperature (+4°c). Extended and supplemented milt samples of all groups were assessed daily for pH, spermatozoa motility, concentration, viability and morphology immediately before storage and throughout the storage period until all spermatozoa stopped moving. Day-0 was referred to the day before storage. The duration of storage was 5 days.

Determination of milt pH

A drop of sample was transferred using a micropipette on a piece of pH indicator strip. The pH values were read by comparison with standardised pH values.

Determination of spermatozoa motility

Extended milt sample (10µl) was placed on a clean, grease free microscope slide using a micropipette and was activated using 20µl borehole water. This was mixed thoroughly, coversliped and viewed using AmScope Trinocular Compound light Microscope at ×100 magnification. Only forward moving sperm cells were judged motile. The percentage spermatozoa motility was immediately estimated as the percentage of spermatozoa progressively motile (Cosson *et al.,* 2008; Davida *et al.,* 2015).

Determination of spermatozoa concentration

Neubauer haemocytometer chamber was used for determination of sperm concentration by counting spermatozoa. A cover slip was mounted on the counting chamber of the haemocytometer. Extended milt sample was agitated for at least 10 seconds before filling the counting chamber. After agitation an aliquot of 10µl was taken with a diluting pipette to one side of the haemocytometer, then a second aliquot was taken to the other side. Average counts from the two aliquots were calculated). Sperm cells were counted at an objective of x40 using a light microscope. Sperm cells in each 5 small squares were counted according to Kvist & Björndahl (2002). Total number of sperm cells counted per ml of milt was expressed in millions.

Determination of spermatozoa viability

Eosin-Nigrosin stain was used by preparing 25mls each of 0.5% Eosin B and 5% Nigrosin. A 10µl of extended milt sample was mixed with one drop each of the prepared 0.5% Eosin B and 5% Nigrosin on clean, grease free microscope slide. A smear was made on another slide and was examined directly at an objective of x100 under oil immersion. Sperm cells that appeared colourless were classified as 'live' while those that appeared pinkish or redish were classified as 'dead' (Kvist & Björndahl, 2002).

Determination of spermatozoa morphology

Eosin-Nigrosin stained slides were used to study spermatozoa morphology. Spermatozoa abnormalities such as: detached heads, bent tails, free tails, coiled tails etc. (Blawut *et al.,* 2020) were studied. Total abnormal spermatozoa were expressed in percentages.

Statistical Analyses

Data generated from the study were expressed as mean ± SD and represented in percentages, Tables and Figures. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to compare the differences in means between the experimental groups and the control. All tests were performed using GraphPad Prism® Version 5.03 for Windows, GraphPad Software, San Diego California USA, www.grahpad.com. Values of $P < 0.05$ were considered statiscally significant.

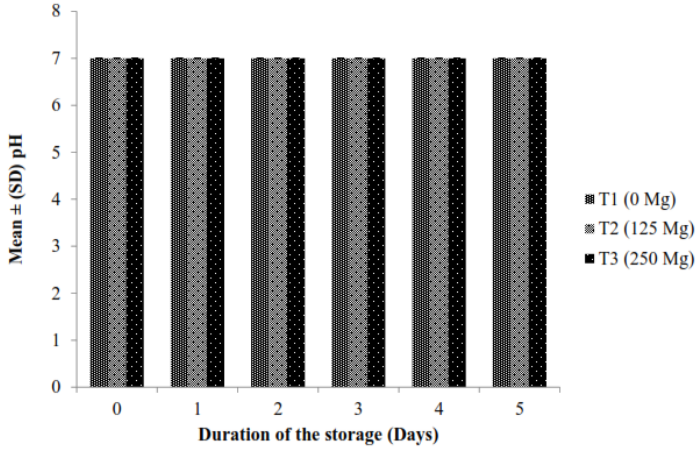

Figure 1: Percentage Mean ± (SD) pH of milt extended in Sodium citrate and supplemented with Cellgevity® before (day-0) and during storage period (days 1-5)

Results

There was no significant difference ($P > 0.05$) in percentage mean ± SD milt pH among the experimental groups. All groups maintained a pH of 7.00 ± 0.00% before and throughout the storage period (Figure 1). There was a progressive decline in the mean ± SD spermatozoa motility from day-0 to day-5 of the storage period in all the groups (Table 1). On day-0 before storage, percentage mean ± SD spermatozoa motility was significantly ($P < 0.001$) lower in T_2 and T_3 than T_1 (Table 1). Likewise from day-1 to day-3 of the storage, percentage mean ± SD spermatozoa motility

was significantly ($P < 0.001$) lower in T_2 and T_3 than T_1 (Table 1). However, on day-4, percentage mean ± SD spermatozoa motility was lower in T_2 and T_3 compared to T_1 although not statistically significant ($P > 0.05$) (Table 1). By day-5, all sperm cells in all the groups where found non motile (Table 1). Mean ± (SD) spermatozoa concentration declined from day-0 to day-5 of the study period in all the groups (Table 2). On day-0, mean ± SD spermatozoa concentration was significantly ($P < 0.01$) higher in T_2 and T_3 than T_1 (Table 2). On day-1, it was significantly ($P < 0.001$) higher in T_2 and T_3 than T_1 (Table 2). On day-2, it was significantly ($P < 0.001$) higher in T_2 as well as significantly ($P < 0.01$) higher in T_3 than T_1 (Table 2). On day-3, it was significantly ($P < 0.001$) higher in T_2 as well as significantly ($P < 0.05$) higher in T_3 than T_1 (Table 2). However, for days 4 and 5, mean ± SD spermatozoa concentration was significantly ($P < 0.001$) higher in T_2 and T_3 than T_1 (Table 2).

There was a progressive decline in the percentage mean ± SD live spermatozoa with a concurrent progressive increase in the mean ± SD dead spermatozoa from day 0 to day 5 of the storage period in all the groups (Table 3). Percentage mean ± SD dead spermatozoa were significantly ($P < 0.01$) lower in T_3 than T_1 on day-0 (Table 3). However, percentage mean ± SD live and dead Spermatozoa were significantly ($P < 0.001$) lower and higher, respectively, in T_3 compared to T_1 on day 2. (Table 3).

Table 1: Percentage Mean ± SD spermatozoa motility of milt extended in Sodium citrate solution and supplemented with Cellgevity® before (day-0) and during storage period (days 1-5)

Storage Period (Days)	T_1 (0 Mg)	T_2 (125 Mg)	T_3 (250 Mg)
0	61.67 ± 2.89^{a}	$25.00 \pm 5.00^{b***}$	$28.33 \pm 7.64^{b***}$
1	23.33 ± 2.89^{a}	$1.67 \pm 2.89^{b***}$	$3.33 \pm 2.89^{b***}$
2	22.67 ± 2.52^{a}	$1.67 \pm 2.89^{b***}$	$3.33 \pm 2.89^{b***}$
3	$23.33 \pm 2.89^{a***}$	$1.67 \pm 2.89^{b***}$	$1.67 \pm 2.89^{b***}$
4	5.00 ± 5.00	1.67 ± 2.89	1.67 ± 2.89
5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[ab]Means with different superscript letters between columns are significantly different at $P < 0.001$ (***)

Table 2: Mean ± SD Spermatozoa concentration ($\times 10^9$ ml^{-1}) of milt extended in sodium citrate and supplemented with cellgevity® before (day-0) and during storage period (days 1-5)

Storage Period (Days)	T_1 (0 Mg)	T_2 (125 Mg)	T_3 (250 Mg)
0	1.39 ± 0.04^{a}	$1.62 \pm 0.03^{b**}$	$1.63 \pm 0.06^{b**}$
1	1.34 ± 0.01^{a}	$1.57 \pm 0.04^{b***}$	$1.48 \pm 0.01^{b***}$
2	1.34 ± 0.02^{a}	$1.50 \pm 0.02^{b***}$	$1.40 \pm 0.02^{b**}$
3	1.36 ± 0.01^{a}	$1.48 \pm 0.01^{b***}$	$1.41 \pm 0.02^{bc*}$
4	0.73 ± 0.03^{a}	$1.52 \pm 0.02^{b***}$	$1.24 \pm 0.04^{b***}$
5	0.43 ± 0.01^{a}	$1.26 \pm 0.02^{b***}$	$1.12 \pm 0.02^{b***}$

[abc]Means with different superscript letters between columns are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)

Table 3: Percentage Mean ± (SD) Live and Dead Spermatozoa of milt extended in Sodium citrate and supplemented with Cellgevity® before (day-0) and during storage period (days 1-5)

Storage Period	T_1 (0 Mg)		T_2 (125 Mg)		T_3 (250 Mg)	
(Days)	Live (%)	Dead (%)	Live (%)	Dead (%)	Live (%)	Dead (%)
0	71.67 ± 2.89	28.33 ± 2.89^{a}	68.33 ± 2.89	31.67 ± 2.89^{a}	80.00 ± 5.00	$20.00 \pm 5.00^{b**}$
1	65.00 ± 5.00	35.00 ± 5.00	66.67 ± 2.89	33.33 ± 2.89	61.67 ± 2.89	37.78 ± 2.55
2	23.33 ± 2.89^{a}	76.67 ± 2.89^{a}	16.67 ± 2.89^{a}	83.33 ± 2.89^{a}	$6.67 \pm 2.89^{b***}$	$93.33 \pm 2.89^{b***}$
3	8.33 ± 2.89	91.67 ± 2.89	11.67 ± 2.89	88.33 ± 2.89	5.00 ± 0.00	95.00 ± 0.00
4	5.00 ± 0.00	95.00 ± 0.00	6.67 ± 2.89	93.33 ± 2.89	5.00 ± 0.00	95.00 ± 0.00
5	3.33 ± 2.89	96.67 ± 2.89	5.00 ± 0.00	95.00 ± 0.00	5.00 ± 0.00	95.00 ± 0.00

[ab]Means with different superscript letters between columns are significantly different at $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

There was a progressive increase in the mean ± SD percentage total abnormal sperm cells from day 0 to day 5 of the storage period in all the groups. Mean ± (SD) percentage total abnormal sperm cells did not differ significantly ($P > 0.05$) among the groups in all the days of the storage (Figure 2).

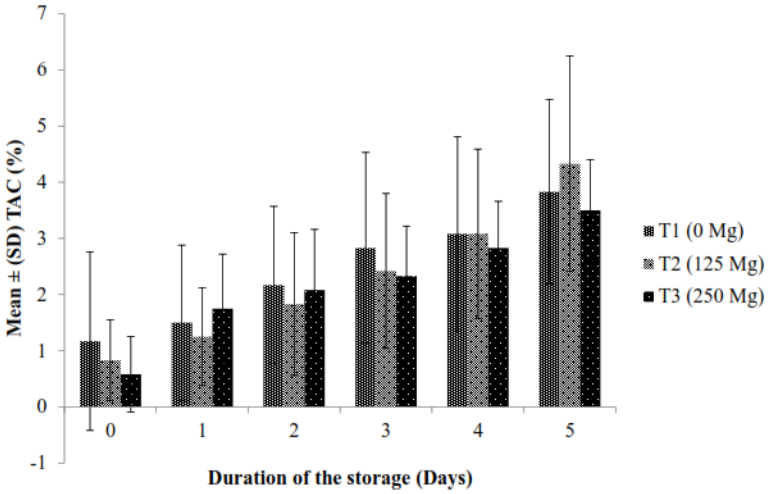

Figure 2: Percentage Mean ± SD total abnormal sperm cells of milt extended in Sodium Citrate and supplemented with Cellgevity® before (day-0) and during storage period (days 1-5)

Discussion

The general pattern of a progressive declined mean spermatozoa motility, spermatozoa concentration and live spermatozoa from before chilled storage through 5-days of the storage is in agreement with the findings recently reported in Carp, *Labeo rohita* (Bibi *et al.,* 2021). The decreased percentage motility of the treated groups in this study is similar to findings by Saroseik *et al*. (2013) that the addition of antioxidants (vitamin C and E, cysteine and glutathione) did not benefit spermatozoa motility during chilled storage of Salmonidae (Arctic char and Rainbow trout) milt. The higher spermatozoa concentrations observed in the treated groups compared to control in this study is similar to findings by Monteiro *et al*. (2017), who reported that spermatozoa concentration was higher in *Prochilodus brevis* semen cryopreserved with vitamins C and E compared to that without supplements. Spermatozoa concentrations values in the treated groups in the present study however was lower than those reported in Carp, *Labeo rohita* (Verma *et al.,* 2009; Khan *et al.,* 2015; Bibi *et al.,* 2021). The progressive decrease in the percentage live spermatozoa in treated groups in this study opposed the highest percentage of sperm viability found in groups treated with L-cysteine (Kledmanee *et al.,* 2013) in Chilled Carp (*Cyprinus carpio*) and findings by Kaeoket *et al.* (2010) in which L-cysteine improved spermatozoa viability by minimising lipid peroxidation of sperm plasma membrane during chilled and frozen storage in pigs. The progressive increase in total abnormal sperm cells in all the treated groups in this study is consistent with the increased abnormal cells observed following a supra-therapeutic dose of Cellgevity® in the study of the toxicological evaluation of therapeutic and supra-therapeutic doses of Cellgevity® on the productive function and biochemical indices in wistar rats (Awodele *et al.,* 2018). Cellgevity® used at 125 mg and 250 mg in the present study might have caused pro-oxidant effect and this may likely be the reason why we noticed reduced percentage mean spermatozoa motility, reduced percentage live spermatozoa and increase percentage mean total abnormal sperm cells throughout the storage period. It was reported that Cellgevity ® at supra-therapeutic doses could have caused pro-oxidant effect in wister rats (Awodele *et al.,* 2018; Ezekiel, 2021).

Generally, in African catfish, untreated testicular semen loses its viability within several hours at 28^{o}C and in just about 2 days at 4^{o}C (Mansour *et al.,* 2002). In the present study, spermatozoa were alive at 4^{o}C up to 5 days in both control and Cellgevity® supplemented groups. This implies that milt extension with sodium citrate helped in preserving the sperm cells. This finding can be used by catfish breeders instead of killing the male broodstock whenever artificial breeding is needed as reported by Viveiros *et al.* (2000) since catfish milt cannot be spawn artificially. However, it was reported that semen of walking catfish (*Clarias macrocephalus*) diluted with calcium-free Hanks' balanced salt solution (Ca-F HBSS) at the ratio 1:1 remained viability at 4^{0}C up to 10 days (Vuthiphandchai *et al.,* 2009). One of the limitations of the present study was that milt were diluted with sodium citrate extender at higher dilution ratio (1:20) in all groups. This might have caused over dilution of antioxidant naturally present in milt plasma. It was reported that for short-term semen storage, the optimal dilution ratio normally ranged from 1:1 to 1:10 in various fish species (Contreras *et al.,* 2019). Semen of Basa catfish (*Pangasius bocourti*) diluted in Ca-F HBSS at ratio 1:1

gave a better preservation of sperm quality during 7 days of chilled storage (Yang *et al.,* 2020).

In conclusion, supplementation of Cellgevity® at 125 mg and 250 mg in sodium citrate extended milt of catfish, *Clarias gariepinus* during chilled storage resulted to reduced percentage mean spermatozoa motility, percentage mean live spermatozoa and percentage mean total abnormal spermatozoa, however, increased percentage mean spermatozoa concentration. We recommend that lower concentrations of Cellgevity® at 125 mg and 250 mg is not suitable as a supplement in sodium citrate extended milt of Catfish, *Clarias gariepinus* in chilled storage.

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Conflict of interest

The authors declare that there is no conflict of interest.

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