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Effect of semen cryopreservation with cucumber (*Cucumis sativus*) fruit juice (CJ) fortified- Extender on milt quality, viability and oxidative enzyme activity of *Clarias gariepinus* (Burchell, 1822)

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Abstract
The present study investigated the quality, viability and oxidative enzyme activity of catfish semen fortified with cucumber fruit juice. The highest milt motility duration time of 66.6 seconds occurred among 20% CJ and was stored for 4 days. The lowest duration time of 50.3 s was recorded among the control group of milt stored for 2 days. The motility rate differed significantly between the control and CJ. The highest rate of 99.21% was recorded in control milt on day 4 compared with the least rate of 81.95% recorded among 15% CJ on day 1. The highest milt fertility rate of 75.35% was recorded in 10% CJ on day 2 compared with the least rate of 56.63% in control on day 1. The least hatchability rate of 64.21% was recorded in control day 3 compared with the highest value of 74.73% in 10% CJ on day 2. The highest milt survival rate of 99.46% was recorded among the control group on day 2, which compared with the least survival rate of 87.66% recorded on day 1 of 10, 15 and 20% CJ. There was a significant difference ($P< 0.05$) between the CAT control and treatments of CJ. The lowest value of 0.25 µmolemm^{-1} mg protein^{-1} recorded in control on day 1 compared with the highest value of 0.34µmolemm^{-1} mg protein^{-1} in 15% CJ on day 4. There was no significant difference in SOD activity between the control and CJ. Recorded values ranged between 0,026-0.56 Umole^{-1}mgprotein^{-1} in 15% on day 2 and 10% day 1 respectively. There was no significant difference in LPO activity between the control and CJ. However, a range of 0.023-0.130 mMoleTbarsmm^{-1} protein^{-1} was recorded on day 1in 10% and control, respectively. 20 and 10% Cucumber fruit juice showed greater values in motility duration and fertility rate than control at respective storage periods for 4 and 2 days in *Clarias gariepinus* semen, and could in combination with DMSO improve its cryopreservation at short periods.

Keywords: Cryopreservation, fish semen, oxidative enzyme activity, motility duration, semen quality

Introduction
Cryopreservation uses low temperatures to preserve structurally intact living cells such as milt of fish in diluents comprising of the extender and cryoprotectant chemicals. The extender increases the volume of the semen and conserves its endogenous energy while the cryoproprotecting chemicals such as dimethyl sulfoxide DMSO limit cell injury resulting from freezing and thawing but more often introduces toxicity concerns [19]. Stock protection from disease, environmental and natural hazards is limited by cryopreservation and could preserve endangered species in perpetuity [2, 1]. It provides easy transport, a suitable supply of sperms in hatcheries and laboratories and availability in selective breeding and gene transfers [18]. Reported that coconut water with suitable salts of potassium, sodium and sugars provides good extender chemical for milt cryopreservation of *Clarias gariepinus*. The effectiveness of cryopreservation success in recent times is the toxicity concerns posed by cryoprotecting chemicals, which have been implicated in oxidative stress of cryopreserved semen. Fish semen generally contains limited antioxidant enzymes and requires nontoxic and environmental friendly natural substances rich in antioxidants such as in fruit juice. Cucumber *Cucumis sativus* fruit juice and seed extracts have been reported to contain high-level antioxidant and antibacterial properties [23], which may be suitable to limit oxidative stress posed by DMSO in milt cryopreservation. There is a scarcity of data on fish milt quality and antioxidant activity and growth of larvae obtained from fertilized eggs by cryopreserved spermatozoa.

The aim of this, the research therefore is to determine the effect of semen Cryopreservation with cucumber fruit juice fortified-Extender on milt quality, viability, anti-oxidative enzyme activity and the growth of *Clarias gariepinus* larvae fertilized by cryopreserved milt. The objectives of this study will be to: [1] Determine the effects of adding cucumber fruit juice to coconut-water extender on cryopreserved milt quality and viability of *Clarias gariepinus* [2]. Compare the anti-oxidative enzymes of cryopreserved fish milt in extender- fortified in cucumber fruit juice with DMSO as control.

Materials and methods

The study area

The study was conducted at the Faculty Research Farm, Fisheries Unit Faculty of Agriculture, Enugu State University of Science and Technology, Agbani, Enugu. This site is located at latitude 074-82.00N; Longitude 068-78E. It has an annual rainfall of 200 mm and daily temperature range from 20 ^{0}C to 35 ^{0}C with an average of 26.7 ^{0}C.

Collection of experimental fish, semen and eggs

Matured hhigh-qualitybrood stock was obtained from a reputable farm in Enugu, Nigeria. The male and female shall be sacrificed and stripped to obtain milt and egg, respectively.

Preparation of extender, cryoprotectant and fruit juice from cucumber

Coconut water obtained from the endosperm of coconut fruit was procured from a local market in Enugu. Similarly, cucumber fruit was obtained from a local market also in the Enugu metropolis.

Diluents (mixture of extender and cryoprotectant) shall be prepared and stored in the refrigerator at 4 ^{0}C for 24hrs before use [18, 20]. 2% of DMSO was prepared and added as a cryoprotectant. Thereafter milt was diluted at 1:20 with diluent and equilibrated for 30 minutes [26] before freezing in a programmable freezer for 4 days. Sperm quality was assessed daily

Sperm quality

Sperm motility and duration were estimated subjectively under the microscope following the method described by [5].

Sperm viability

Cryopreserved sperms straws at the end of freezing, was thawed at 37 ^{0}C for the 30s in a water bath, and cut open from the sealed end to release sperms used to fertilize pooled ripe eggs from gravid brood females. Fertilized eggs were incubated for 30h at the appropriate temperature and pH of water. Fertilization, hatchability and survival rate of larvae were used to assess the viability of the milt.

$$\text{Fertilization rate (\%)} = \frac{\text{Number of egg cells hatched}}{\text{Total number of egg cells counted}} \times \frac{100}{1}$$

$$\text{Hatchability rate (\%)} = \frac{\text{Number of eggs hatched}}{\text{Total number of eggs in a batch}} \times \frac{100}{1}$$

$$\text{Survival rate (\%)} = \frac{\text{Number of hatchling alive to larval stage}}{\text{Total number of hatchlings}} \times \frac{100}{1}$$

Anti-oxidative enzyme activity

The catalase (CAT) in the semen was determined according to the method of [24], which involved H_2O_2 breakdown, and was measured spectrophotometrically at 240 nm. Enzyme activity will be expressed as mini moles of H_2O_2 decomposed min/L mg/L protein.

Superoxide dismutase (SOD) activity was determined using the method of [14], based on the oxidation of epinephrine-adrenochrome transition by the enzymes. Superoxide dismutase activity was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme mg/L of protein required to give 50% inhibition of epinephrine auto-oxidation. Lipid peroxidase (LPO) was determined by estimation of thiobarbituric acid reactive substances (TBARS), according to [22]. TBARS concentration was measured spectrophotometrically at 535 nm at a molar extinction coefficient of 156 nm cm/L. Enzyme activity was expressed in mini moles of TBARS mg/L protein.

Results

The results of milt motility duration, motility rate, fertility rate, hatchability rate, survival and oxidative stress are given in table 1 below.

Table 1: Milt motility duration, motility rate, fertility rate, hatchability rate, survival and oxidative stress using Varying levels of Cucumber Juice for 4 days

Parameters CJ in Days		Mean	Std. Error (±)
Motility duration(sec.)	control day 1	50.8000	.05774
	control day 2	50.3000	.05774
	control day 3	51.2600	.05774
	control day 4	51.6000	.05774
	5% CJ day 1	53.3400	.05774
	5%CJ day 2	52.8033	.05487
	5% CJ day3	53.8067	.05207
	5% day 4	54.5200	.23180
	10%CJ day1	55.8233	.03930
	10% CJ day 2	55.3200	.05774
	10%CJ day 3	56.3800	.05774
	10% day 4	59.3300	.05774
	15% day 1	56.7233	.79386
	15%CJ day2	56.1433	.79386
	15% CJ day 3	57.2100	.81206
	15% CJ day4	64.2433	2.50683
	20% CJ day 1	55.8800	.05774
	20% CJ day2	55.3000	.05774
	20% CJ day 3	56.3000	.05774
	20% CJ day 4	66.6000	.05774
	Total	55.6842	.53275

Motility rate (%)	control day 1	96.4100	.00577
	control day 2	96.9100	.00577
	control day 3	98.4067	.00667
	control day 4	99.2100	.00577
	5% CJ day 1	91.5900	.00577
	5%CJ day 2	92.0700	.00577
	5% CJ day3	93.4900	.00577
	5% day 4	94.2500	.00577
	10%CJ day1	86.5000	.00577
	10% CJ day 2	87.2200	.00577
	10%CJ day 3	88.5700	.00577
	10% day 4	89.2900	.00577
	15% day 1	81.9500	.00577
	15%CJ day2	82.3800	.00577
	15% CJ day 3	83.4100	.00577
	15% CJ day4	84.3300	.00577
	20% CJ day 1	86.4100	.00577
	20% CJ day2	87.2100	.00577
	20% CJ day 3	88.5100	.00577
	20% CJ day 4	89.3400	.33005
	Total	89.8728	.66739
Fertility rate (%)	control day 1	56.6300	.00577
	contrl day 2	68.4667	.03844
	control day 3	64.7000	.00577
	control day 4	59.1000	.00577
	5% CJ day 1	59.4600	.00577
	5%CJ day 2	71.9300	.00577
	5% CJ day3	67.9433	.00882
	5% day 4	62.0600	.00577
	10%CJ day1	62.2900	.00577
	10% CJ day 2	75.3500	.00577
	10%CJ day 3	71.2000	.00577
	10% day 4	65.0100	.00577
	15% day 1	62.1967	.00333
	15%CJ day2	75.3000	.00577
	15% CJ day 3	71.2000	.00577
	15% CJ day4	65.0300	.03512
	20% CJ day 1	62.2000	.00577
	20% CJ day2	75.3000	.00577
	20% CJ day 3	71.2000	.00577
	20% CJ day 4	65.0300	.03512
	Total	66.5798	.72530
hatchability_ Rate (%)	control day 1	57.9700	.00577
	control day 2	67.9400	.00577
	control day 3	64.2100	.00577
	control day 4	59.3700	.00577
	5% CJ day 1	60.8700	.00577
	5%CJ day 2	71.3400	.00577
	5% CJ day3	67.4200	.00577
	5% day 4	62.3400	.00577
	10%CJ day1	63.7700	.00577
	10% CJ day 2	74.7300	.00577
	10%CJ day 3	70.8300	.00577
	10% day 4	65.3100	.00577
	15% day 1	63.7100	.00577
	15%CJ day2	74.7100	.00577
	15% CJ day 3	70.8100	.00577
	15% CJ day4	65.3100	.00577
	20% CJ day 1	63.7100	.00577
	20% CJ day2	74.7100	.00577
	20% CJ day 3	70.8100	.00577
	20% CJ day 4	65.3100	.00577
	Total	66.7590	.64346
Survival rare (%)	control day 1	97.4667	.03333
	control day 2	99.4667	.03333
	control day 3	99.1967	.03333
	control day 4	98.5667	.03333
	5% CJ day 1	92.5967	.03333

	5%CJ day 2	94.4967	.03333
	5% CJ day3	94.2367	.03333
	5% day 4	93.6467	.03333
	10%CJ day1	87.6933	.03333
	10% CJ day 2	89.5267	.03333
	10%CJ day 3	89.2867	.03333
	10% day 4	88.7167	.03333
	15% day 1	87.6667	.03333
	15%CJ day2	89.4667	.03333
	15% CJ day 3	89.2667	.03333
	15% CJ day4	88.6667	.03333
	20% CJ day 1	87.6667	.03333
	20% CJ day2	89.4667	.03333
	20% CJ day 3	89.2667	.03333
	20% CJ day 4	88.6667	.03333
	Total	91.7515	.52402
CAT ($\mu molemm^{-1}mgprotein^{-1}$)	control day 1	.2904	.00007
	control day 2	.2927	.00033
	control day 3	.2947	.00033
	control day 4	.2957	.00033
	5% CJ day 1	.3052	.00032
	5%CJ day 2	.3070	.00032
	5% CJ day3	.3098	.00003
	5% day 4	.3099	.00003
	10%CJ day1	.3194	.00003
	10% CJ day 2	.3213	.00003
	10%CJ day 3	.3235	.00003
	10% day 4	.3246	.00003
	15% day 1	.3339	.00003
	15%CJ day2	.3359	.00003
	15% CJ day 3	.3382	.00003
	15% CJ day4	.3394	.00003
	20% CJ day 1	.3197	.00033
	20% CJ day2	.3217	.00033
	20% CJ day 3	.3237	.00033
	20% CJ day 4	.3249	.00003
	Total	.3166	.00194
SOD ($Umole^{-1}mgprotein^{-1}$)	control day 1	.0513	.00000
	control day 2	.0532	.00000
	control day 3	.0550	.00000
	control day 4	.0560	.00000
	5% CJ day 1	.0538	.00003
	5%CJ day 2	.0553	.00063
	5% CJ day3	.0578	.00003
	5% day 4	.0588	.00003
	10%CJ day1	.5637	.00033
	10% CJ day 2	.0585	.00003
	10%CJ day 3	.0605	.00003
	10% day 4	.0615	.00007
	15% day 1	.0588	.00007
	15%CJ day2	.0266	.01328
	15% CJ day 3	.0633	.00003
	15% CJ day4	.0644	.00003
	20% CJ day 1	.0560	.00000
	20% CJ day2	.0577	.00033
	20% CJ day 3	.0567	.00333
	20% CJ day 4	.0567	.00333
	Total	.0813	.01445
LPO ($mMoleTbarsmm^{-1}protein^{-1}$)	control day 1	.0030	.00000
	control day 2	.0040	.00000
	control day 3	.0070	.00000
	control day 4	.0090	.00000
	5% CJ day 1	.0030	.00058
	5%CJ day 2	.0040	.00058
	5% CJ day3	.0070	.00058
	5% day 4	.0090	.00058
	10%CJ day1	.0023	.00033
	10% CJ day 2	.0033	.00033

	10%CJ day 3	.0073	.00033
	10% day 4	.0133	.00333
	15% day 1	.0030	.00058
	15%CJ day2	.0050	.00058
	15% CJ day 3	.0070	.00058
	15% CJ day4	.0133	.00333
	20% CJ day 1	.0030	.00058
	20% CJ day2	.0040	.00058
	20% CJ day 3	.0070	.00058
	20% CJ day 4	.0090	.00058
	Total	.0062	.00047

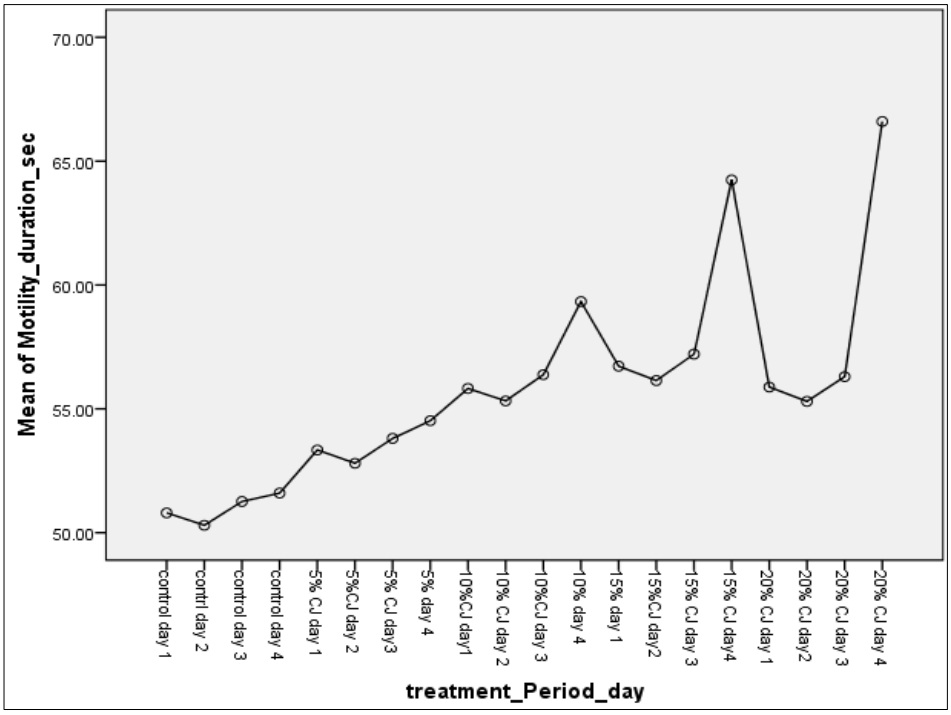

Fig 1: Mean of motility duration

Milt motility duration

The highest milt motility duration time of 66.6 seconds occurred among 20% CJ, stored for 4 days. The lowest duration time of 50.3 s was recorded among the control group of milt stored for 2 days (Figure 1)

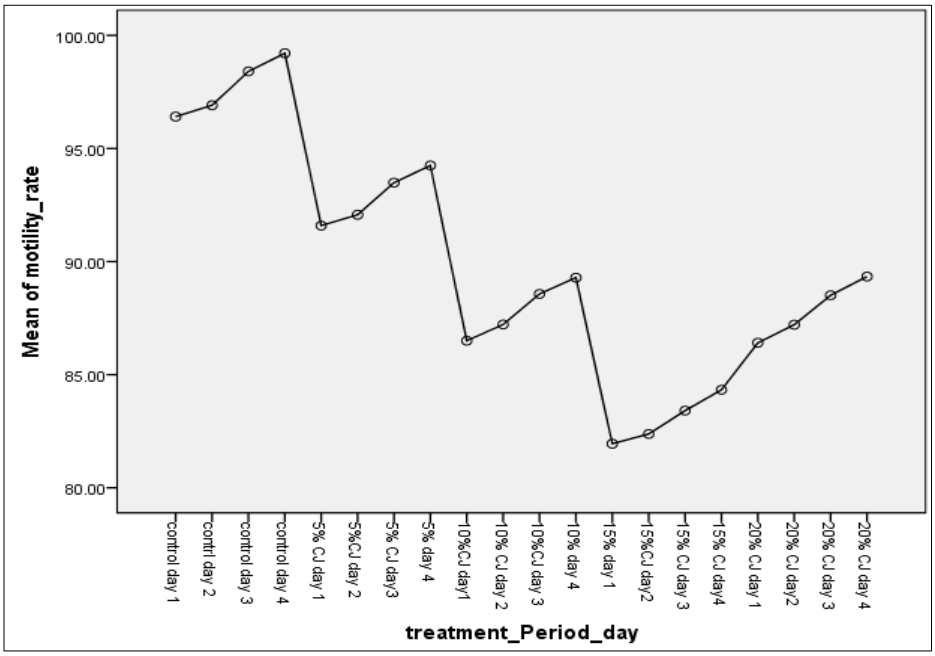

Fig 2: Mean of motility rate

Motility rate

The motility rate differed significantly between the control and CJ. The highest rate of 99.21% was recorded in control milt on day 4 compared with least rate of 81.95% recorded among 15% CJ on day 1 (Fig. 2).

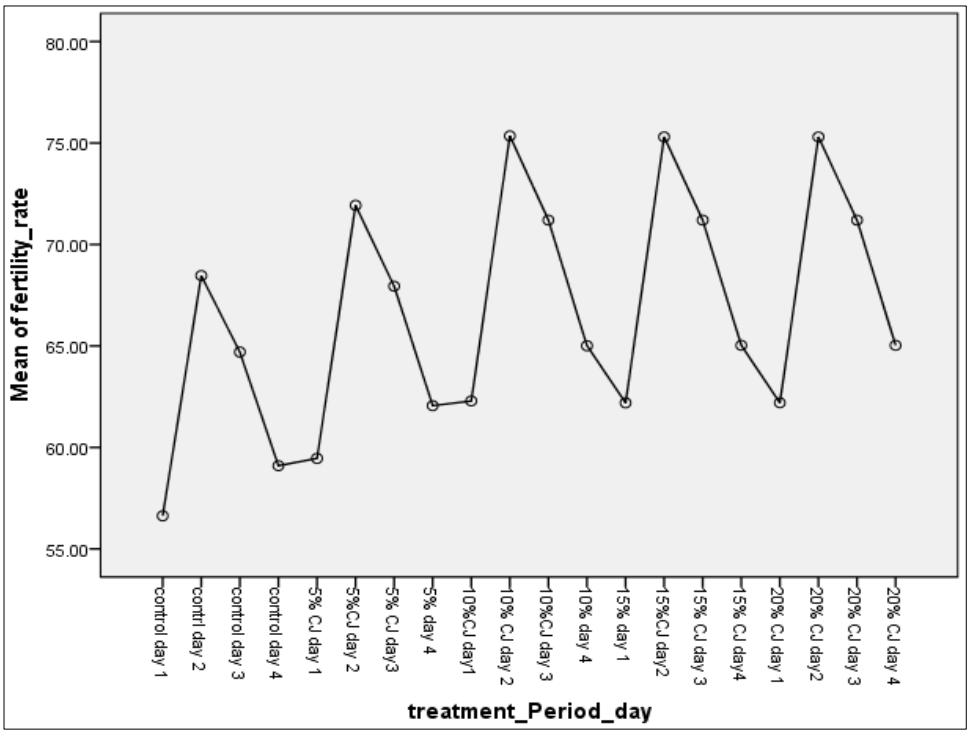

Fig 3: Mean of fertility rate

Fertility rate

The highest milt fertility rate of 75.35% recorded in 10% CJ on day 2 compared with the least rate of 56.63% in control on day 1 (Fig. 3).

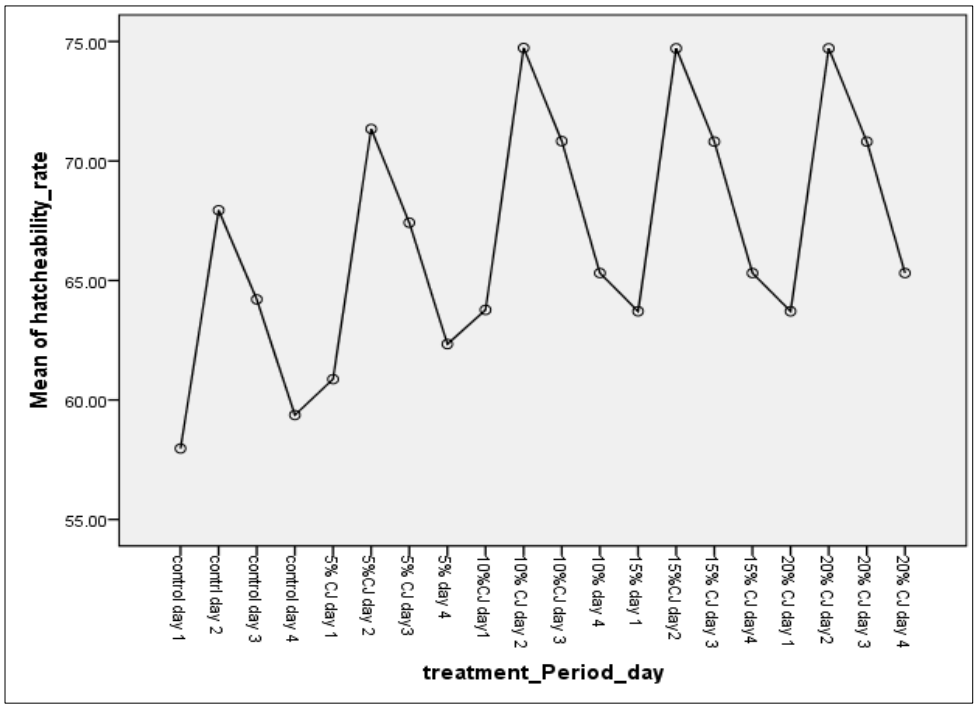

Fig 4: Mean of hatchability rate

Hatchability rate

The least hatchability rate of was 64.21% recorded in control day 3 compared with the highest value of 74.73% in 10% CJ on day 2 (Fig. 4).

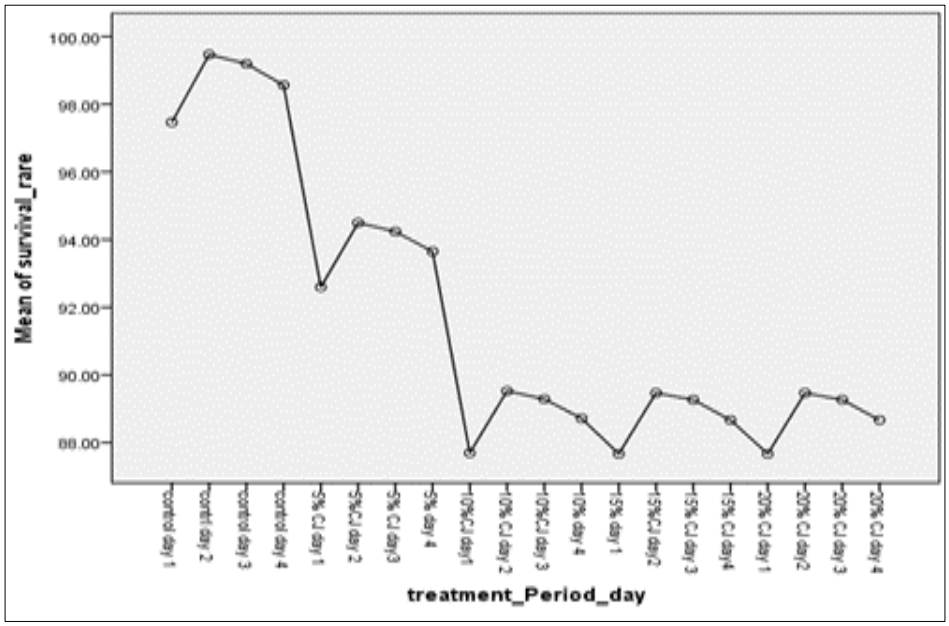

Fig 5: Mean of survival rate

Milt survival rate

The highest milt survival rate of 99.46% was recorded among the control group on day 2 which compared with the least survival rate of 87.66% recorded on day 1 of 10, 15 and 20% CJ (Fig.5).

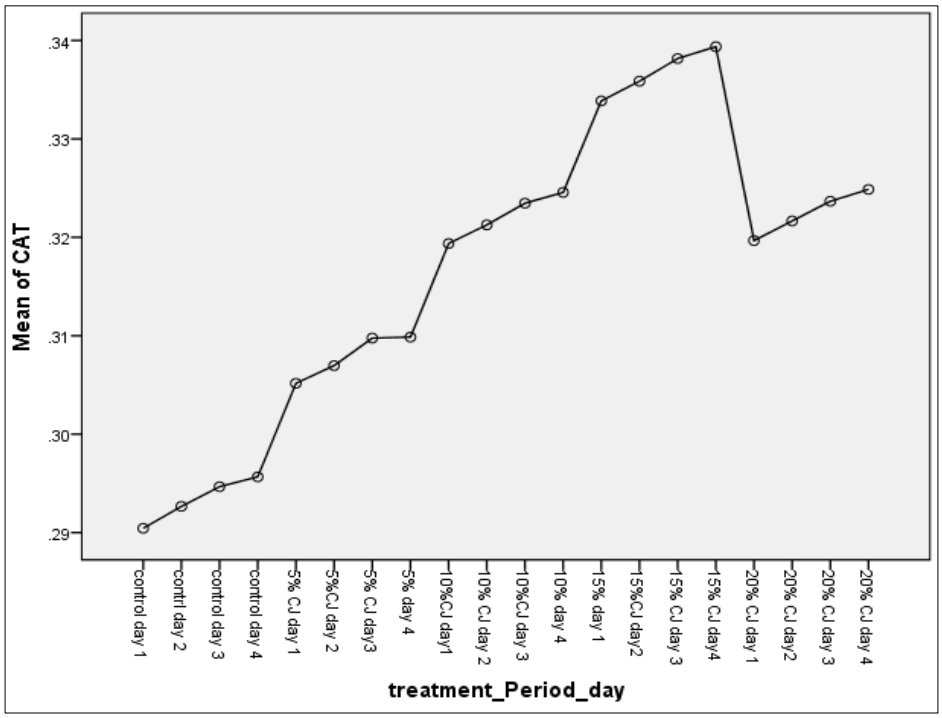

Fig 6: Mean of CAT

Catalase activity

There was a significant difference (P<0.05) between the control and treatments of CJ. The lowest value of 0.25 µmolemm^{-1}mgprotein^{-1} recorded in control on day 1 compared with the highest value of 0.34 µmolemm^{-1}mgprotein^{-1}in 15% CJ on day 4 (Fig. 6).

SOD Activity

There was no significant difference in SOD activity between the control and CJ. Recorded values ranged between 0,026-0.56 Umole^{-1}mgprotein^{-1} in 15% on day 2 and 10% day 1 respectively (Table 1).

LPO

There was no significant difference in LPO activity between the control and CJ. However, a range of 0.023-0.130 mMoleTbarsmm^{-1}protein^{-1} was recorded on day 1in 10% and control respectively (Table 1).

Discussion

The uses of fruit juices as cryopreservants have been reported to be suitable for the cryopreservation of major fish species (Heinstra *et al*., 2005; Horvath and Ubanyi, 2009) [12, 13]. In addition, many cryopreservation studies revealed that fruit juices resulted in higher fertilization and hatching rate

compared to artificial cryopreservants. Van Vuren and Steyn (2017) [25] noted however that the level, type, concentration, temperature, and exposure period of the fruit juice function to evaluate the quality and viability of the milt. Fruit antioxidants include carotenoids, vitamins, phenolic compounds and flavonoids and have proved to function as singlet and triplet oxygen quenchers, free radical scavengers and peroxide decomposers (Anghel *et al*., 2010; Daramola *et al*., 2016;) [6, 9, 10]. Preservation of fish milt has become an uphill task due to the fact that most artificial cryo-protectants in use have oxidative impact on fish milt spermatozoa. Cucumber (*Cucumis sativus*) and orange (*Citrus sinensis*) are fruit-rich natural antioxidants renowned for high concentrations of these vitamins and other antioxidants (Cutis *et al*., 2013; Reda *et al*., 2016; Okiyele *et al* 2019) [8, 21, 19]. Cryopreserved catfish semen fertilizes more number of eggs than natural (Agarwal, 2005; Agarwal, 2011) [4, 5]. Dilution with extenders in cryopreservation of fish semen increases the volume of semen, so that it can be used for multiple inseminate (Agarwal, 2011) [5]. It was noted that in several trials of the experiment, cryopreserved semen resulted in significantly higher fertilization and hatchability percentage than freshly extracted semen. (Kovacs and Urbanyi, 2010; Ezike *et al*., 2019) [14, 11]. The CAT-SOD system of enzyme in the semen may efficiently have removed reactive oxygen species ROS by a trigger from fruit antioxidants (Krzyzosiak *et al*., 2000) [15] produced during storage thus limited the elicitation of LPO. Therefore the use of cucumber fruit juice could advance cryopreservation of fish milt and in particular *Clarias gariepinus* (Adeyemo *et al* 2007; Boryshpolets *et al*., 2011; Liu *et al*., 2015) [3, 7, 16].

Conclusion

Although the control indicated higher milt motility, hatchability and survival rates than CJ. 20 and 10% Cucumber fruit juice showed greater values in motility duration and fertility rate than control at respective storage periods for 4 and 2 days in *Clarias gariepinus* semen, and could in combination with DMSO improve its cryopreservation at short periods.

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