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Motility and Oxidative Stress of Cryopreserved Fish Milt using Juices of Orange, Cucumber and Pineapple as Cryoprotectants

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***Abstract*— The present research assessed the use of fruit juice as cryopreservants of fish milt with the intent of obtaining a semen preservation protocol that can serve as a means of making fingerlings available to fish farmers all year round. The motility values recorded in Pineapple (44.30, 45.83, 45.83, 46.03) and Orange (44.83, 44.86, 45.00, 45.43) ($p < 0.05$) on day 1, 2, 3 4 were significantly higher compared to control. Significant differences did not exist ($p > 0.05$) between cucumber and orange compared to control in all the treatment days, but significant differences ($p < 0.05$) was shown to be higher in COP (0.017 – .018) and Pa (0.029, .031, .04) on days, 1, 3, 2 and 3, 1, 4 respectively for COP and Pa. Catalase activity was significantly elevated ($P < 0.05$) in treatments compared to the control (0.0256 – 0.0303). The highest value of CAT was recorded to be 0.298 in COP on day 4, while the least value of 0.18 recorded in COP in day 1. Superoxide dismutase (SOD) was significantly higher ($p < 0.05$) in all treatments compared to the control (0.293 – 0.347). The highest value of (0.084) was recorded in COP on day 4 while the least value was recorded in Cu (0.513) on day 1. Significant differences did not exist ($p > 0.05$) between cucumber and orange compared to control in all the treatment days for LPO, but significant differences ($p < 0.05$) was shown to be higher in COP (.0167 – .0183) and Pa (.0290, .0310, .0400) for LPO on days, 1, 3, 2 and 3, 1, 4 respectively for COP and Pa. Therefore the use of orange and cucumber fruit juice which did not elicit lipid peroxidation may be used with the control to improve cryopreservation of C.gariepinus semen.**

***Index Terms*— cryopreservation, oxidative stress, fruit juice, cryoprotectants, extenders.**

I. INTRODUCTION

Cryopreservation is a process whereby biological materials such as cells and tissues are preserved by cooling to very low temperatures, usually at -196°C (the temperature of liquid nitrogen), yet remain viable after later warming to temperatures above 0°C. Cryopreservation addresses the need to preserve semen from the fish such that it could be used for subsequent artificial insemination over an extended period of time. Artificial insemination with preserved semen is a viable option for genetic upgrading of fish. It makes possible almost indefinite storage of the desirable gene pools and ensures the availability of cryopreserved semen for artificial insemination/breeding, (Babiak *et al.*,2008). The first work done on freezing fish semen was done in1953 by Blaxter, who made it possible to cross different populations of fish species which spawn at different times of year (Suquet *et al.* 2000; Liu *et al.* 2006). Semen preservation protocols have been determined experimentally with more than 200 species of fish (Billard and Zhang 2001; Billard et al., 20004; Gwo 2011). Cryopreservation of African cat fish semen normally presents better results for motility rate and fertilizing capacity after thawing in species like *Clarias gariepinus*, with mean embryo survival of 80%, than in freshwater species like Atlantic salmon, which present embryo survival of around 65% with cryopreserved semen (Suquet et al., 2000; Gwo, 2011). These results are probably related with the fact that spermatozoa from marine species are adapted to high osmotic pressure (Cuevas-Uribe et al. 2011; Cuevas-Uribe et al. 2013). In recent years, due to the rapid development of aquaculture and the conservation problems facing some fish species, cryopreservation has played an important role in freezing gametes to protect fish of high economic and biological value. Among catfish like the African and European catfish (*Clarias gariepinus*, *Silurus glanis*) and carps like the common (*Cyprinus carpio*) and silver carp (Hypophthalmichthys molitrix) (Suquet et al., 2000). Extenders and cryoprotectants are important and play a vital role in cryopreservation. Muchilsin et al. (2009) noted that an extender is a medium used to dilute sperm and to get a larger quantity of diluted sperm for artificial induced breeding purposes while a cryoprotectant is a material which is added to an extended sperm dilution to protect the sperm from cold and heat shock and cryo toxicity during cryopreservation. Irrespective of the species, fish semen requires dilution before it has to be cryopreserved. Extenders used for diluting the fish semen are generally designed to be compatible with the physio-chemical composition of seminal fluid of the candidate species, (Muchlisin et al., 2009). The African catfish (*Clarias gariepinus*) is widely cultured in Nigeria. This species of fish is one of the best aquaculture species under culture (FAO, 1994). It is known for its resistance to handling stress, ability to tolerate a wide range of environmental parameters, wide food range, high fecundity, fast growth, disease resistance, high stocking densities under culture condition, and high meat quality (Kryzyzosiak *et al.*, 2000; Reza *et al.*, 2011). Therefore, there is need to preserve semen from this breed such that it could be used for subsequent artificial insemination over an extended period of time. Artificial insemination with preserved semen is a viable option for genetic upgrading of this breed. The aim of the present study was to determine motility and oxidative stress of cryopreserved fish milt using juices of orange, cucumber and pineapple as cryoprotectants

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II. Materials and Methods

This research was carried out in Prof. Nwamba Research and Teaching Farm, Mbaukwu Street along Old airport road in Emene, Enugu State. This location lies between latitude of $6^0$30'E and Longitude of 6.500^0N – 7.500^0E.The average day time temperature was 26^oC and the night time temperature was 20^oC with a mean elevation of 450m above sea level and an annual rainfall of 1800mm to 2100mm. Diluents (mixture of Extender and Cryoprotectant) was prepared in the first day of the research and stored in the refrigerator at 4^0C for 24hrs before use according to (Argwal *et al* 2004). The fruit-juice was prepared following standard methods Agrawal (2011) with some modifications: fresh cucumber, pineapple and orange was washed thoroughly using distilled water. The fruits were first peeled (pineapple and orange) and thereafter cut into pieces and the seeds removed (cucumber and orange) and then the fruit were blended for five minutes separately, placed in a sieve and pressure was applied manually to squeeze the juice out from the blended fruits. The juices collected from each fruit were put in separate plastic test tubes and centrifuged. The supernatant fluid obtained were decanted into a clean beaker and used immediately for the experiment. 2% of Dimethyl Sulphide (DMSO) was prepared and used as Cryoprotectant. 2% Dimethyl sulphide was prepared by addition to 98ml of distilled water to 2ml of DMSO. About 100ml of distilled water was heated to boiling point and allowed to cool under running tap water. The preparation of 2.9% weight/volume (w/v) of sodium citrate was then made by using 2 x 2.9g of sodium citrate. This amount was dissolved in 200ml flat bottom flask. This solution was then shaken together and allowed to cool for some few minutes.

Four beakers were washed and rinsed with saline solution, then with a 5ml syringe, 2ml of diluent was collected and poured into the four beakers each, subsequently each cryovials containing 0.5ml catfish semen was gradually emptied into each beaker, to avoid osmolarity and pH shock. 0.5ml of semen was diluted with 2ml of diluent which is equivalent to the ratio 1:4 (Rana 1995). The beaker containing the diluted semen was shaken to obtain even dilutions before storage. The method used was Agarwal, (2011), Semen was extracted by dissecting male broodstocks from the abdomen towards the pointed papilla, using a new sharp razor blade the testis was obtained and the semen was obtained by dissecting the testis. The male broodstock was first weighed and the weight was 800g. The male broodstock was cleaned with towel before dissection. The testis was washed with saline solution to remove bloodstains, and mucos to avoid contamination. The testis was opened into a cryovial to obtain 0.5ml of catfish semen which afterward dilution followed. The stored semen in cryovial (Syringe) was laid in the Freezer, before then the freezer was allowed to freeze up to -30^0C before the semen was kept. The semen was stored in the freezer at -30^0C for 4days (Billard, et al., 2004) but at the end of each day, each tube containing the cryopreserved semen was thawed (Akcay et al., 2004; Alvarez et al., 2008). Ampoules/vials/visotube method was used in storing semen for freezing (Akcay et al., 2004). The diluted semen was stored with syringe (visotube). This was done for the four beakers containing the diluted semen, therefore four syringes were used to store the semen for freezing. The volume of semen in each syringe was 2.5ml (2ml of diluents and 0.5ml of catfish semen). After the storage, the semen were allowed to stay for equilibration before freezing. The semen was kept in the fridge at 4^0C for equilibration. The period of this equilibrum is termed "equilibrium time, which is the time allowed for facilitating the penetration of cryoprotectant into the cells for effective protection during freezing. (Grout and Morris 1987). The semen was allowed to equilibrant for 45mins with the time of gradual addition of diliuent to semen and storage taken into account. Equilibrium time was not longer than 45 – 60mins to maximize later recovery. (Agarwal 2007). The most appropriate equilibrium time is 45mins for DMSO (Sanni, 2008).

A. Thawing and artificial fertilization with cryopreserved semen

The semen was thawed in a water bath at 57^0C for 10secs. But prior to the time of removal from the freezer, hot water was boiled to be used for the thawing (warming). The hot water was used for thawing at 57^oC for 10secs (Rana 1995; Alvarez et al., 2008). In several fresh water fish species, thawing is done at 30 – 80^0C in a water bath. The tube containing the cryopreserved semen were layed in the water bath for 10secs. Afterwards, a dropper was used to collect a drop of the semen for Analysis under the microscope to determine the viability of the sperm cells which include the sperm swimming velocity (motility), motility percentage and motility duration.

B. Experimental design

During the cryopreservation period, the cryopreserved milt was monitored every six (6) hours to check the motility and viability, after which the oxidative stress was checked at the end of the experiment. The experiment was arranged in a completely randomized design (CRD) with five (5) treatment and each was replicated three (3) times. The experiment lasted for 4 days.

C. Oxidative Stress Parameters

Fish milt were weighed and homogenized in 0.1M potassium phosphate at 15000g, 4^0C for 20minutes. The supernatant was collected for biochemical parameter of the antoxidant enzymes and lipid peroxidation.The catalase (CAT) in the blood was determined according to the method of Takahara et al. (1960) which involved H_2 O_2 breakdown, and was measured spectrophotometrically at 240 nm. Enzyme activity was expressed as nanomoles of H_2 O_2 decomposed min/L mg/L protein. Superoxide dismutase (SOD) activity were determined using the method of Misra and Fridovich (1972), based on the oxidation of epinephrine- adenochrome transition by the enzymes. Superoxide dismutase activity was assed spectrophotometrically at 420 nm and expressed as the amount of enzyme mg/L of protein required to give 50% inhibition of epinephrine auto-oxidation. Glutathione peroxidase (GPX) activities was assayed according to Paglia and Valentine (1967) which was based on the oxidation of gluthathione in the presence of NaN_3.

Determination of Lipid Peroxidation (LPO)

Lipid peroxidase LPOX in the liver tissue was determined by estimation of thiobarbituric acid reactive substancs (TBARS), according to Sharma and Krishna-Murti (1968).TBARS concentration was measured spectrophotometrically at 535

nm at molar estinction coefficient of 156 Nm cm/L in mMole/TBARS/ mg protein.

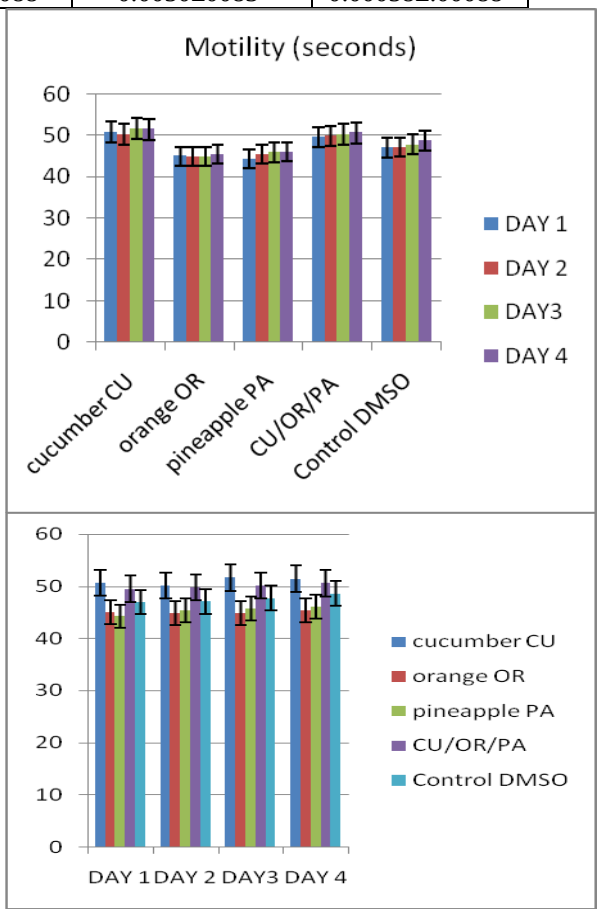
D. Statistical Analysis

Data was collected using Statistical Package for Social Sciences (SPSS) computer package, version 20.0 and subjected to one-way analysis of variance (ANOVA). Mean differences was separated using the Duncan multiple range test ($P < 0.05$).

3. Results

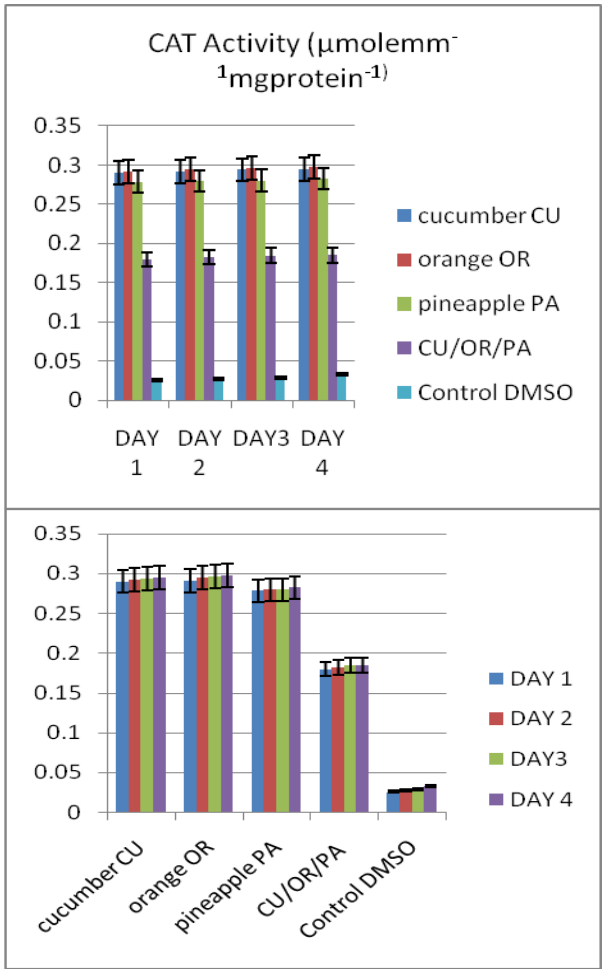
Table 1: Motility and oxidative stress of cryopreserved fish milt for 4 days

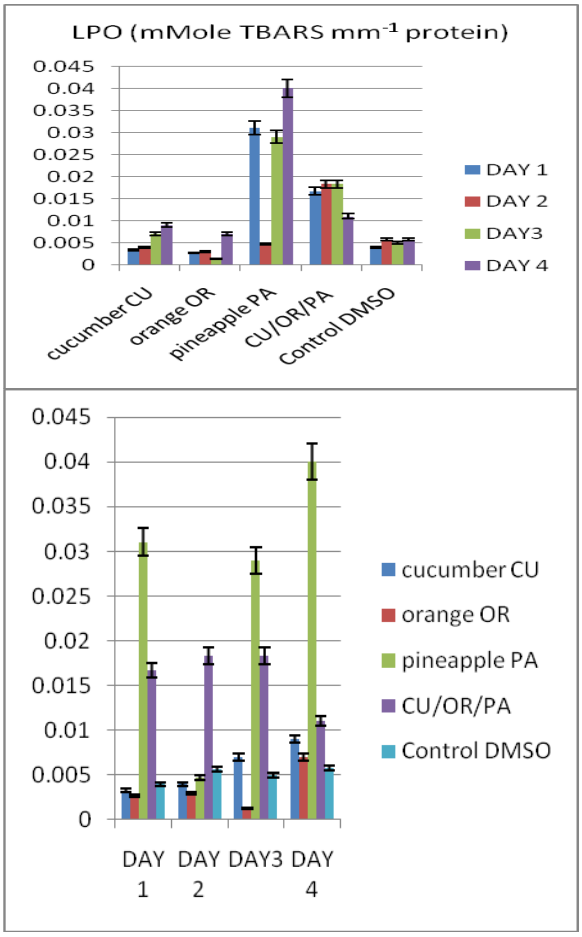
Fruits	Day 1	Day 2	Day 3	Day 4
Motility				
Cucumber	50.7000^{i}	50.1667^{h}	51.6667^{j}	51.4667^{j}
Orange	45.0000^{b}	44.8333^{b}	44.8667^{b}	45.4333^{c}
Pineapple	44.3000^{a}	45.4333^{c}	45.8333^{d}	46.0333^{d}
Cop	49.5333^{h}	49.8333^{h}	50.1333^{h}	50.6000^{i}
Ctrl	46.9667^{e}	47.1333^{e}	47.7333^{f}	48.6333^{g}
Superoxide Dismutase (SOD)				
Cucumber	$0.0513 \pm .00033^{d}$	$0.0537 \pm .00088^{d}$	$0.0550 \pm .00058^{d}$	$0.0560 \pm .00058^{e}$
Orange	$0.0517 \pm .00167^{d}$	$0.0520 \pm .00058^{d}$	$0.0533 \pm .00088^{d}$	$0.0543 \pm .00088^{d}$
Pineapple	$0.0780 \pm .00058^{f}$	$0.0783 \pm .00067^{f}$	$0.0813 \pm .00088^{g}$	$0.0813 \pm .00088^{g}$
Cop	$0.0807 \pm .00033^{g}$	0.0827 ± 00088^{g}	$0.0813 \pm .00088^{g}$	$0.0840 \pm .00058^{b}$
Ctrl	$0.0293 \pm .00033^{c}$	0.0320 ± 00058^{c}	0.0333 ± 00088^{c}	0.0347 ± 00120^{c}
Catalase Activity				
Cucumber	$0.2903 \pm .00088^{i}$	$0.2920 \pm .00058^{j}$	$0.2940 \pm .00058^{j}$	0.2950 ± 00058^{j}
Orange	$0.2913 \pm .00145^{i}$	$0.2947 \pm .00088^{i}$	$0.2960 \pm .00100^{j}$	$0.2980 \pm .00058^{j}$
Pineapple	$0.2787 \pm .00088^{f}$	$0.2797 \pm .00088^{g}$	$0.2800 \pm .00100^{g}$	$0.2827 \pm .00100^{g}$
Cop	$0.1800 \pm .00058^{d}$	$0.1823 \pm .00186^{d}$	$0.1847 \pm .00088^{e}$	$0.1850 \pm .00058^{e}$
Ctrl	$0.0260 \pm .00058^{a}$	0.0270 ± 00058^{b}	$0.0290 \pm .00058^{c}$	$0.0303 \pm .00088^{c}$
Lipid Peroxidation (LPO)				
Cucumber	$0.0033 \pm .00088^{a}$	0.0040 ± 00058^{a}	$0.0070 \pm .00058^{a}$	$0.0090 \pm .00058^{a}$
Orange	$0.0027 \pm .00088^{a}$	$0.0030 \pm .00115^{a}$	$0.0013 \pm .00033^{b}$	$0.0070 \pm .00115^{a}$
Pineapple	$0.0310 \pm .00058^{d}$	$0.0047 \pm .00120^{d}$	$0.0290 \pm .00058^{d}$	$0.0400 \pm .00603^{e}$
Cop	$0.0167 \pm .00088^{c}$	$0.0183 \pm .00088^{c}$	$0.0183 \pm .00033^{c}$	$0.0110 \pm .00058^{b}$
Ctrl	$0.0040 \pm .00058^{a}$	$0.0057 \pm .00088^{a}$	0.0050 ± 0083^{a}	$0.00058 \pm .00088^{b}$

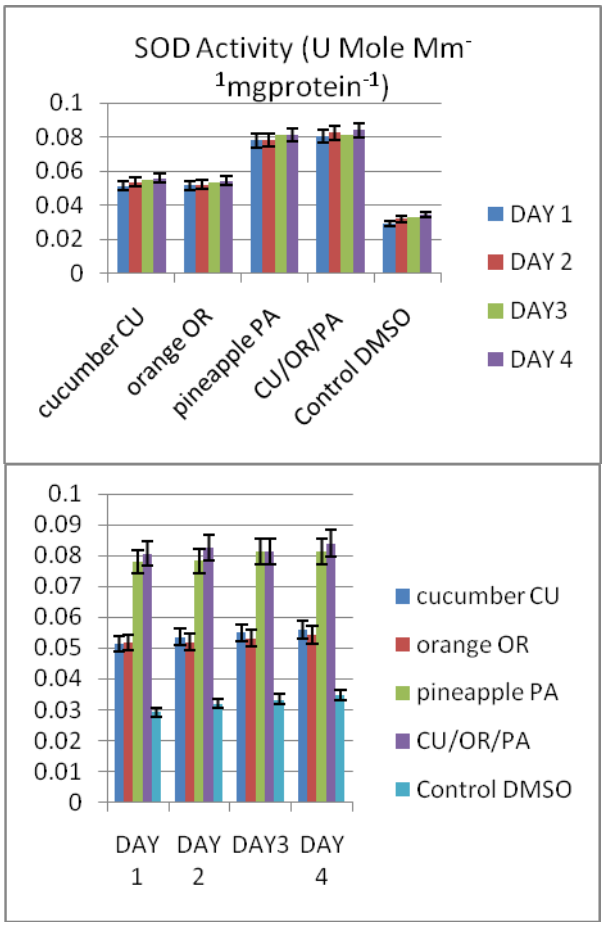


Sperm motility and oxidative stress

The motility values recorded in Pineple (44.3000, 45.8333, 45.8333, 46.0333) and Orange (44.8333, 44.8667, 45.000, 45.4333) ($p < 0.05$) on day 1, 2, 3 4 were significantly lower compared to control and cucumber on day 1, 2, 3, 4 respectively. On the other hand superoxide dismutase (SOD) was significantly higher ($p < 0.05$) in all treatments compared to the control (0.293 – 0.347). The highest value of (0.084) was recorded in COP on the day 4 while the least value was recorded in Cu (0.513) on day 1. Catalase Activity was significantly elevated ($P < 0.05$) in treatments compared to the control (0.0256 – 0.0303). The highest value of CAT was recorded to be 0.298 in COP on day 4, while the least value of 0.18 was recorded in COP in day 1. Significant differences did not exist ($p > 0.05$) between cucumber and orange compared to control all the treatment days, but significant differences ($p < 0.05$) was shown to be higher in COP (.0167 – .0183) and Pa (.0290, .0310, .0400) on days, 1, 3, 2 and 3, 1, 4 respectively for COP and Pa.







III. Discussion

The extenders inhibits the activation of spermatozoa and thereby preserves the vitality and viability of sperm by conserving the energy (Park and Chapman, 2005) and also function as a medium for cryoprotectant - the chemicals that minimize the cryo-injuries to the cells due to the formation of intracellular ice crystals during cryopreservation time of semen freezing and thawing. Determining the cryoprotectant type and its right concentration in extender medium is an important step of developing cryopreservation protocol for a species, (Gwo et al., 2005; Muchlisin et al., 2009). 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). In addition, many cryopreservation studies revealed that fruit juices resulted in higher fertilization and hatching rate compared to artificial cryopreservants. Level of toxicity of a cryopreservants depends on the type, concentration, temperature, and exposure period, (Van Vuren and Steyn, 2017). The survival of sperm after collection in seminal plasma for longer periods during preservation at low temperatures requires dilution with appropriate extender, in order to maintain viability of spermatozoa. Advantage of cryopreserving catfish semen is well established. It is not only a useful management tool but also offer several benefits such as stock protection (Cabrita et al., 2005) from being totally eliminated due to sudden outbreak of disease, natural disaster, over exploitation and stable supply of sperm for optimal utilization in hatcheries; improvement in selective breeding (Bakhach, 2009) whereby stock can be maintained more

economically, effectively and experimental material for advanced studies such as gene transfer (Bakhach, 2009).

Fruits are good sources of natural antioxidants, containing many different antioxidant components (FAO,

2000; Liu et al., 2015). These 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). Preservation of fish milt has become an uphill task as cryoprotectants used has an oxidative effect on the spermatozoa. Oxidative is imbalance between oxygen reacting species in cells and tissues (ROS) and anti-oxidant. DMSO and other cyroprotectants used have been reported to act as chemo-preventive agents against oxidative damage (Kiwon et al., 2003; Ondei et al., 2009). Cucumber (*Cucumis sativus*), pineapple (*Ananas comosus*) and orange (*Citrus sinensis*) are fruit-rich natural antioxidants renowned for their high concentrations of these vitamins and other antioxidants (Cutler *et al*., 2008). Cryopreserved catfish semen fertilizes more number of eggs than natural (Agarwal, 2005; Agarwal, 2011). Dilution with extenders in cryopreservation of fish semen increases the volume of semen, so that it can be used for multiple inseminate (Agarwal, 2011). It was noted that in several trials of experiment, cryopreserved semen resulted in significantly higher fertilization and hatchability percentage than freshly extracted semen. (Kovacs and Urbanyi, 2010). A number of different protocols are advocated in literature for the preservation of fish semen but most of them are concerned with the salmonids, tilapia, and carp (Martınez-Paramo et al., 2009).

Catalase activity CAT in semen enriched with fruit juice increased significantly compared to control, and ranged from the highest value in orange and cucumber juice- enriched semen followed by pineapple contained semen > semen with a mix of all the 3 juice compared to the control. The trend was witnessed throughout the duration of the study. Similarly, dismutase superoxide SOD increased significantly among semen enhanced with fruit juice compared to control and ranged from the highest level in semen contained with pineapple and mixed juice than cucumber and orange compared to control, and followed the same trend throughout the experimental period. On the other hand, lipid peroxidation was shown to be highest in semen contained with pineapple than mixed juice enriched semen followed by cucumber and control and was least in orange enriched semen. The foregoing gave an indication that juices of orange and cucumber enhanced sufficient CAT and SOD levels in the cryopreserved semen. The CAT-SOD system of enzyme in the two fruit juice efficiently removed reactive oxygen species ROS (Krzyzosiak et al., 2000) produced due to the presence of DMSO and thus limited the elicitation of LPO. Therefore the use of orange and cucumber fruit juice could advance (Boryshpolets et al., 2011; Liu et al., 2015) cryopreservation of *C.gariepinus* semen.

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