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Changes in semen pH and microbial load of post-mortem preserved *Cyprinus carpio* suspended in Dimethylacetamide

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Abstract: **Though post-mortem preserved sperms were shown to be useful in developing restoration procedures, there is a limitation in use due to their quality. Cryoprotectants currently under use are known to play a key role in retaining sperm quality but their impact on sperms under post-mortem is yet to be explored. Sperm motility is considered as the one of the most important quality to attain fertilization success. Chemical quality and microbial growth of stored semen decides the sperm quality. Hence the present study is carried out to study the changes in semen pH and microbial load in *Cyprinus carpio* post – mortem preserved directly or in suspension with Dimethylacetamide (DMA). This study showed that no significant change occurs in semen pH upon post – mortem preservation without / with DMA for upto 90 days compared to the control group maintained at room temperature. Further at PMP temperatures microbial growth was found to be kept significantly low indicating that preservation procedures including DMA utility did not induce any microbial contamination.**

Keywords: C. carpio, **Post-Mortem Preservation, Dimethylacetamide, Semen pH, Microbial growth.**

I. INTRODUCTION

Cryopreservation is used to have high survival of gametes without affecting their efficacy and potency. However due to limitations associated with existing cryopreservation techniques, alternative and more practicable sperm storage techniques are under exploration. Ability of 'cadaveric sperm' of fish in fertilizing or activating eggs demonstrated by Koteeswaran and Pandian (2002) lead to the launch of simple and widely practicable method of using Post-mortem preserved sperm at -20^{o}C substituting cryopreserved sperms. Though Post-Mortem Preservation (PMP) of fish sperms was observed to be useful in earlier studies (Kiran Kumar and Pandian, 2003; Dietrich *et al.*, 2005 and Umaa Rani *et al.*, 2016), limitations related to viability and motility of sperms were reported to be the setbacks. On the other hand *Hemigrammus caudovittatus* males preserved at -20ºC and suspended in 100% glycerol was found to show improved sperm survival for even upto 60 of preservation compared to those stored without glycerol (David and Pandian *et al.*, 2006).

It has been demonstrated that semen pH is the most important factor in determining the motility of sperm and its increase to certain range lead to increased cAMP which in turn activates sperm motility (Cejko *et al.*, 2013). Hamner as early as in 1970 reported that sperms of mammals exhibit good survival at pH 7.0 and considered it as optimum level since lower pH levels resulted in progressive decrease in sperm motility and metabolisim. Likewise the pH of fish milt was also found to affect the maturation process and motility of spermatozoa (Billard *et al*., 1995; Liley *et al*., 2002).

Among Cyprinids extracellular as well as intracellular pH along with the ionic composition of the seminal plasma was shown to influence the initiation and duration of sperm motility (Marian *et al.*, 1992). Increased concentration of solutes and precipitation of salts at the supersaturation level were found to result in pH changes of frozen food materials (Powrie, 1984).

On the other hand microbial contamination of semen was found to result in decreased motility due to adherence of microbes to spermatozoa (Diemer *et al.*, 2003). Compared to all other microbes, bacteria play a major role in the health and occurrence of diseases in different of teleostean fish. High bacterial load in semen was found to cause harmful effects on the spermatozoa due to their toxins and metabolic end products (Bindra *et al.*, 1994). Rapid drop in sperm quality and morphological alterations in the sperm cells manifested in terms of increased sperm abnormalities were found to be associated with microbial contamination (Diemer *et al*., 1996). Fish semen mostly get contaminated by bacteria during collection and storage process eventually minimizing sperm quality and life span (Bielanski, 2007). Limited studies were made so far related to assessment of bacterial load in fish semen. In view of the above and the prerequisite for appropriate monitoring of semen quality it is important to estimate the bacterial load.

Hence the present study is carried out to study the changes in semen pH and microbial load in post – mortem preserved *Cyprinus carpio*.

II. MATERIALS AND METHODS

Procurement and Maintenance of test species:

Adult male ***C. carpio*** weighing about 80 gm obtained from local freshwater aquafarms of Tirupati, Andhra Pradesh were maintained at 1 fish / 2 L of filtered tap water in the laboratory at a water temperature of 28 ± 1° C. They were fed three times a day on commercial pelleted diet along with recharging of at least 1/4th of water every day. Animal handling and conduction of the experiments were done as per the ethical norms (Ref. No 1677/PO/Re/S/2012/CPCSEA/43/ IAEC-06/05/16).

a) Post-mortem preservation of *C. carpio* and collection of semen

Five healthy male ***C. carpio*** each were anaesthetized using 0.2% clove oil and stored in zip-lock bags at -20°C either directly (PMP: Experimental group –I) or by suspending in absolute DMA (Hi-media) (PMP with DMA: Experimental group –II) for 30/ 60/ 90 days. The group maintained parallely at room temperature act as controls. Semen of anaesthetized fresh male *C. carpio* was collected through stripping by applying gentle pressure from either side of the belly in the direction of the anal opening avoiding contamination with urine, blood or feces. Semen was collected into an eppendorf tube using a micropipette for further use.

b) Determination of Semen pH and Microbial load

pH level of semen of fresh *C. carpio* / PMP group / PMP + DMA group at 30/60/90 days was determined using Systronics pH meter. Bacterial load in the semen samples was assessed following standard plate count method (Shukla, 2011). 100 µl of semen samples of control / each of the experimental groups was diluted with 900 µl of autoclaved 0.9% NaCl under laminar air flow and vortexed. 0.1 ml of the above sample was spotted in a sterilized petriplate followed by the addition of 20 ml of agar media. The plates were rotated gently on the working surface for even spreading, kept aside for solidification and incubated for 48 h in inverted position at 37ºC in a bacteriological incubator. The colonies developed were counted and the total microbial load was expressed as CFU /ml of semen.

Comparisons were made among the control and the experimental groups through Two – Way ANOVA ($p<0.01$).

III. RESULTS AND DISCUSSION

Semen pH:

Semen of *C. carpio* maintained at room temperature was found to be viscous and creamy white in colour with a pH of 7.9 ± 0.3 (Table.1). While pH of semen of ***C. carpio***, upon direct PMP for 30 / 60 / 90 days was found to be 8.0 ± 0.2/ 8.2 ± 0.2/ 8.2 ± 0.3 respectively, the pH of the counterparts of PMP with DMA group was 7.9 ± 0.3/ 8.0 ± 0.2/ 8.1 ± 0.2 respectively showing no significant change (Table 1). These values were found to be within the normal physiological range of 7.7–8.2 as observed by Cjezko (2013).

Changes in pH of several storage products such as milk, beef, chicken meat, fish, green beans, cauliflower, peas, and apples were observed to be the resultant of ice formation during freezing (Van den Berg, 1968). Further pH of stored beef was found to rise from 5.6 to 5.9 upon freezing at -10°C which has been attributed to the salt composition and buffering capacity. Samples like milk and haddock, possessing large amounts of sodium and calcium phosphates were found to attain decreased pH during first two weeks of freezing at -10°C because of the precipitation of these alkaline salts. Several

reports supported that diffusion of CO_2 to tissues associated with the formation of carbonic acid may lead to significant drop in pH (Banks *et al.*, 1980; Statham 1984; Debevere and Boskou 1996 and Ruiz-Capillas and Moral 2001).

Cassens and Newbold, (1967) stated that the temperature at which the muscle is stored post-mortem has to have only a small effect on the ultimate pH. Since the post-mortem drop in pH is the consequence of the production of lactic acid from glycogen, the extent of the pH fall may depend on the amount of glycogen present. But fish generally contain very little glycogen (around 0.3%), and hence the production of lactic acid which is mainly responsible for causing changes in pH was found to be very much restricted (Jezek & Buchtova, 2007). Further, since the test species in the present study were preserved at -20^{o}C immediately after anaesthetizing there is no scope for rigor mortis.

When meat is held above 0^{o}C, an increase in pH of the meat occurs as a consequence of aging (Wierbicki *et al.*, 1954; Bouton *et al.*, 1958), which is involved with protein hydrolysis, resulting in the production of different amino acids (Gasperlin *et al.*, 2001; Farouk, Wieliczko & Merts, 2003 and Jayasooriya *et al.*, 2007). On the other hand, Muela *et al.*, (2015) observed decreased pH values in lamb after 1, 9, 15 and 21 months of frozen storage compared to fresh meat. Recently Holman *et al.*, (2017) also reported that ultimate pH of beef increases from 5.4 - 5.7 while stored under chilled-only condition for 5 weeks, but decreased to 5.4 - 5.5 in frozen only storage condition for up to 24 weeks.

Sperm motility and metabolic rate are generally correlated with semen pH. Donoghue *et al.*, (2000) demonstrated higher metabolic rate in the semen of chicken stored *in vitro* with higher pH. But at lower temperatures, where sperm motility or metabolic processes are theoretically lower, a rise in pH may not occur (Johnson *et al.*, 2000; Paulenz *et al.*, 2000). Further the addition of suitable cryoprotective agents that can control the osmolality and pH of cryopreservation medium were demonstrated to minimize cell damage and control the freezing rate (Van den Berg & Soliman, 1969). Hence maintenance of semen pH in post-mortem preserved groups with the addition of DMA on par with the control group provides wider and assured scope for its usage.

Table 1. pH of semen of Post- Mortem Preserved (PMP; @ -20ºC) *C. carpio* without / with DMA for 30 / 60 / 90 Days compared to the '0' Day control.

Duration of PMP (Days)	Control	PMP Without DMA	PMP With DMA
0	7.9 ± 0.2 [a]	_	_
30	_	8.0 ± 0.2 [a]	7.9 ± 0.3 [a]
60	_	8.2 ± 0.2 [a]	8.0 ± 0.2 [a]
90	_	8.2 ± 0.3 [a]	8.1 ± 0.2 [a]

Values are mean ±S.D of 5 individual observations.

Values similarly marked are not significantly different ($p< 0.01$) from each other.

Semen Microbial load

Table.2 and Figure1 show the semen microbial load of control *C. carpio* and those post-mortem preserved without DMA or with DMA for 30 / 60 / 90 . Fresh semen was found to contain 1.16 ± 1.02 x10^3 CFU/ml of microbial load, while post-mortem preserved group without DMA for 30 / 60 / 90 showed 3.8 ± 0.40 / 3.3 ± 0.34 / 3.2 ± 0.36 x10^2 CFU/ml respectively and those preserved with DMA for the same time periods showed 3.7 ± 0.37 / 3.4 ± 0.42 / 3.2 ± 0.39 x10^2 CFU/ml respectively. Significantly low percentages in group without DMA/with DMA 67.2, 71.5, 68.1/ 70.6, 72.4, 72.4 for 30 / 60 / 90 respectively indicates that preservation procedures including DMA utility did not induce any microbial contamination.

Microbial spread plate count was found to increase in chilled meat stored at 4°C, decreased in frozen meat at -10°C with increase in storage time (Kraft *et al.*, 1979). But Perez (2004) demonstrated stoppage of microbial growth at -12°C and total inhibition of cellular metabolism in tissues stored below -18°C. Bacterial load of semen is likely to be an

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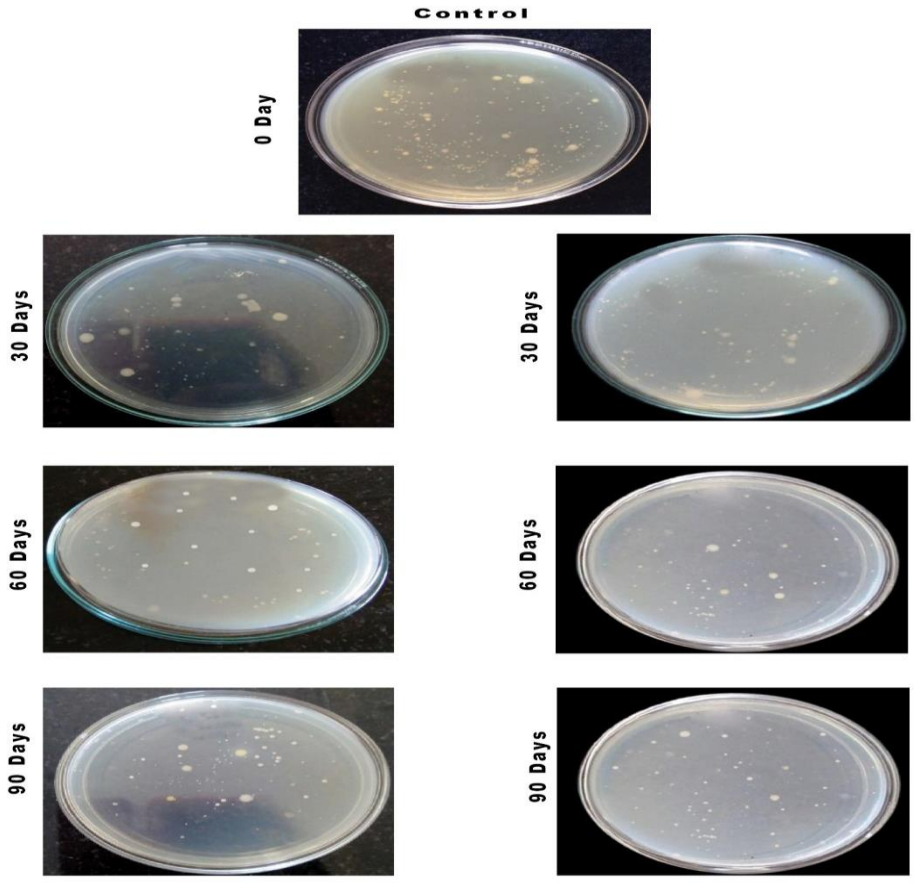
important component during qualitative analysis because it incites the production of macrophages and polymorphonuclear granulocytes as the first line of defence which in turn leads to the production of reactive oxygen species that are detrimental for sperm motility and viability (Morrell, 2006). Though most of the bacteria are declared as commensals, there are potential pathogens which can contaminate the semen upon its collection (Aurich and Spergser, 2007). Dave & Ghaly (2011) observed 60% mortality in microbes along with gradual propagation of the rest at frozen temperature.

Further bacteria are known to compete for nutrients and for oxygen with sperm cells showing spermicidal effect (Rodeheaver, 1997). Contamination of *Staphylococcus* spp., *Escherichia coli*, *Streptococcus* spp. in bovine semen (Corona and Cherchi, 2009) and *Pseudomonas, Streptococcus, Pantoae, Aeromonas, Klebsiella* and non-spore forming rod bacteria being main constituents of channel catfish were reported to reduce motility and sperm viability (Jenkins and Tiersch, 1997). Further *Pseudomonads* were identified as one of the most frequently occuring bacteria in sperms or eggs of fish upon preservation and found to result in their reduced preservation storage capacity (Jenkins and Tiersch, 1997; Holcomb *et al.,* 2005). Presence of 1.0×10^4CFU mL−1 of bacteria was observed in diluted semen of *Piracanjuba*, *Brycon orbignyanus* (Viveiros *et al.,* 2010). *Aeromonas* genera was reported to dominate Gram-negative rods appearing in the stored semen of silver barb (Boonthai *et al.,* 2016).

Cold room stored sperms are commonly used in fish farming to fertilize the eggs. Since low temperatures result in reduced metabolism, sperms stored in appropriate sperm extenders at around 4ºC can be maintained for a few days without significant changes in their quality (Kime *et al.,* 1996) but the onset of aerobic conditions may affect the sperm quality upon prolonged storage. Halting of bacterial proliferation and keeping them sufficiently low in horse meat was demonstrated recently by Coombs *et al.,* (2017) through freezing.

Figure 2. Bacterial colonies developed from semen of Post-Mortem Preserved (PMP; @ -20ºC) *C. carpio* without / with DMA for 30 / 60/90 days compared to '0' Day control.



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Duration of PMP (Days)	Control	PMP Without DMA	PMP With DMA
0	$1.16 \pm 0.09 \times 10^{3}$ **a**	_	_
30	_	$3.8 \pm 0.37 \times 10^{2}$ **b** (- 67.2)	$3.7 \pm 0.30 \times 10^{2}$ **bc** (- 68.1)
60	_	$3.3 \pm 0.24 \times 10^{2}$ **d** (- 71.5)	$3.4 \pm 0.30 \times 10^{2}$ **cd** (-70.6)
90	_	$3.2 \pm 0.16 \times 10^{2}$ **d** (- 72.4)	$3.2 \pm 0.23 \times 10^{2}$ **d** (- 72.4)

Values are mean ±S.D of 5 individual observations.

Values similarly marked are not significantly different ($p < 0.01$) from each other.

Values in parentheses are percent change from that of control.

IV. CONCLUSION

Our observations further demonstrate that post-mortem preservation of cadaveric *C. carpio* at -20ºC without or with the addition of DMA can be chosen for long term semen preservation without considerable change in pH along with keeping of microbial proliferation under control.

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