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Assessment of clove oil and Benzocaine Anaesthesia on Haematological and Histopathological profile of *Haludaria fasciata*

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Key words: Blood profile, Histology, Melon barb, Stress response, Optimal dose

Abstract

Transportation of live fish is a stressful procedure. Present study assesses the optimal concentration and the degree of effectiveness of clove oil and benzocaine as anaesthetics separately for stress reduced transportation of *Haludaria fasciata*. Study contributes the knowledge on some aspects of stress research include the detection of histological and haematological alteration as a biomarkers for evaluating degree of pollution or toxic effect with the environment of an organism. The effects anaesthetics revealed at some stages (10 min and 24 hr) of experiment. Histology of gills and liver; and haematological parameters included the erythrocyte count (RBC), leukocyte count (Leuko,TC), haemoglobin concentration (Hb), haematocrit (PCV – Packed Cell Volume) as well as mean erythrocyte volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were assessed for determine the effect of clove oil and banzocaine on *H. fasciatus* and compared with nonanaesthetised control group. Both the anaesthetics do not cause to irreversible damage in the test fishes. The effects of benzocaine on fish were comparatively higher than clove oil treatments. The degree of alteration may due to the defensive response with additives in external environment. The obtained result shows that the optimal dose of clove oil (50 µl) and benzocaine (30 µl) anaesthesia are effective, safe and recommended as suitable anaesthetics for the transportation of *H. fasciatus*.

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Introduction

Anesthesia is, by definition, a biological reversible state induced by an external agent, which results in the partial or complete loss of sensation or loss of voluntary neuromotor control, through chemical or nonchemical means (Summerfelt and Smith, 1990). Anesthesia is frequently applied in aquaculture being a valuable tool that helps to minimize fish stress and prevent physical injuries to fish while handling and transportation.However, the type of anaesthetics, the dosage and period of exposure may leads to alterations in blood profile of the fish andcan reflectgill and liver tissues and they are used as stress indicators (Velisek and Svobodova 2004; Bolasina 2006; Hoseini and Ghelichpour 2011; Hoseini *et al*., 2011; Velisek *et al*., 2011). These can provide important information about the internal environment of an organism.

Histological changes observed in a study, widely used as the biomarkers for assessing the health (Meyers and Hendricks, 1985), immune response (Khoshnood *et al*., 2010), degree of pollution and toxicity of contaminants. Histopathological examination also monitors the surrounding environment of an organism. Fish haematology has gained great academic and scientific interest because of its applicability in evaluating the physiological status of fish. Haematological profiles are commonly used for the estimation of anaesthetic effect than other commonly used parameters (Iwama *et al*., 1989; Velisek and Svobodova 2004a,b; Velisek *et al*., 2005a,b, 2006, 2007, 2009, 2011; Kristan *et al*., 2012). Haematological indices are responding quickly to changes with respect to a variety of anaesthetics and their dosage and to the environmental changes (Atkinson and Judd, 1987; Atamanalp and Yanik, 2003; Anver Celik 2004; Velisek *et al*., 2005a,b, 2006, 2007, 2009, 2011; Kristan *et al*., 2012).

In the present study, we estimate the impact of popularly used clove oil and benzocaine anaesthesia on haematological and histological profile and their optimal concentration as anaesthetics during the simulated transportation of an endemic freshwater fish *Haludaria fasciata* (*Puntius fasciatus*). *H. fasciata* popularly known as the Melon barb, occurring in the rivers flowing through the Western Ghats region of India. Histology of gills and liver and haematological parameters were used to assess the effects of anaesthetics.The indices used to evaluate the haematological profile included the erythrocyte count (Red Blood Cell - RBC) (Blaxhall and Daisley, 1973; Atamanap *et al*., 2002), leukocyte count (Total Platelets Count - TC) (Blaxhall and Daisley, 1973; Atamanalp *et al*., 2002), haemoglobin concentration (Hb) (Kocabatmaz and Ekingen, 1984), haematocrit (Packed Cell Volume– PCV) (Schalm *et al*., 1975) as well as mean erythrocyte volume (MCV), mean corpuscular haemoglobin concentration (MCHC), and mean corpuscular haemoglobin (MCH) (Reddy and Bashamohideen, 1989).

Materials and methods

Melon barb; *H. fasciata* (4.2±0.2 cm and 1.3±0.3 g) were collected from Valapattanam River, Kerala, India and their tributaries and acclimatized for captive condition for two weeks and the fishes were placed in glass aquaria (60×30×30 cm). Proper aeration was provided to each aquarium. Fish were fed with commercial pellet feed at twice a day. Unused food and metabolic wastes were discharged through siphon every day. Feeding was stopped 24 h before the experiment (Hicks, 1989).Temperature (27.0±0.5^{o}C), pH (7.0±0.3), dissolved oxygen (6.50±0.5 mgL^{-1}), alkalinity (65.0±6.0 mgL^{-1}), hardness (70.0±4.0 mgL^{-1}) and ammonia (<0.02 mgL^{-1}) were maintained within narrow ranges.

Clove oil (Micro Fine Chemicals, India) and benzocaine (HiMedia Laboratories Pvt. Ltd. Mumbai) were used as anesthesia. Clove oil and benzocaine barely soluble in water, dissolved with 95% ethanol at 1: 10 ratio (clove oil: ethanol) to prepare stock solution containing 100 mg ml/L (Sindhu and Ramachandran, 2013; Yildiz *et al*., 2013); 100 g of benzocaine was dissolved in 1 L of 95% ethanol by following Pramod *et al*. (2010).

Experimental design

The optimum concentration of clove oil (50 µl) and benzocaine (30 µl), were determined in our previous study, transferred separately into the polyethylene bag (Low Density Polyethylene - LDPE) containing 1L water. Ten fish were transferred into the each polyethylene bag. It was then inflated with medical grade oxygen and the top of the bag was tied and made airtight. Each bag was then placed together in a Styrofoam box for thermal insulation for preventing sudden changes of temperature in the transport water. A control group without anaesthetic was also similarly maintained. For haematological analysis, blood was collected by caudal severance (Pirhonen and Schreck, 2002).

Six trials were performed for each parameter using different individual fish.After collecting blood for haematological analysis, gills and liver were dissected for histological study. The factors used to evaluate the hematological profile included hemoglobin (HB), total plate count (TC), erythrocyte (RBC), Mean Corpuscular Volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and packed cell volume (PCV). All analysis was conducted after 10 minutes and 24 hours of the simulated transportation (Velíšek and Svobodová, 2006).

The degree of expression of histological changes in gill and liver tissues and haematological parameters which monitored at 10 min and 24 hr after the simulated transportation of *H. fasciata* (Melon barb).Which distinguished between the control fish group and anesthetic treatments.

Statistical analysis

Statistical analyses were carried out using SPSS 19.0 for Windows. All data were subjected to a one-way analysis of variance (ANOVA) to determine differences in treatments. All data are analysed with Duncan new multiple range test and stated as mean values ± standard deviation (SD). Significant differences were considered at $P<0.05$ levels among the groups.

Results and discussion

Haematological indices

Average number of erythrocyte count (RBC) during the simulated transportation of nonanaesthetised group detected as 0.86±0.01x10^6/mm^3 (after 10 min) and it significantly decreased ($P<0.005$) to 0.82±0.01x10^6/mm^3 after 24 hr (Table 1).

Table 1. Effects of clove oil and benzocaime anesthesia on hematological indices in *H. faciata*.Data are presented as mean ± SD, n=252.

	Control		Clove oil		Benzocaine	
Time	10 m	24 h	10 m	24 h	10 m	24 h
RBC	0.86±0.01^e	0.82±0.01^d	0.59±0.02^a	0.61±0.01^a	0.74±0.02^b	0.77±0.02^c
TC	3.85±0.10^b	4.10±0.23^c	3.40±0.16^a	6.81±0.23^d	3.75±0.18 ^{b}	3.46±0.21^a
HB gm%	7.75±0.24^e	6.98±0.14^b	6.48±0.11^a	6.55±0.18^a	6.46±0.25^a	7.08±0.24^b
PCV %	20.85±0.93^c	18.39±0.68^a	18.60±1.12^{ab}	19.48±1.69^{abc}	20.31±1.24^{bc}	22.52±2.13^d
MCV fl	232.29±2.50^b	227.10±1.22^a	315.21±1.46^e	314.21±1.53^e	271.98±1.63^c	285.60±0.68^d
MCH gm%	89.57±1.96^b	86.80±1.03^a	108.13±1.00^d	106.13±0.93^c	86.88±0.84^a	89.28±1.56^b
MCHC %	38.30±1.29^c	38.23±0.89^c	35.43±0.76^b	33.86±2.57^b	31.46±0.89^a	31.91±1.01^a

Values of each anaesthetic in the same rows with different superscript indicates, they are significantly different ($P<0.05$).

Similar result of armoured cat fish (*Hypostomus paulinus*) to be between 0.66-2.01 x10^{6}mm^6 (Satake *et al*., 1986). It was lower than healthy rainbow trout (*Onchorynchus mykiss*) (Kocabatmaz and Ekinge, 1984; Atmanalp *et al*., 2002) and *Tilapia zilli* (1.8x10^{6}mm^6) Ezzat *et al*. (1974). RBC count was 1.00 x10^{6}mm^6 in *H. punctatus* (Torres *et al*., 1986), and 4.09 x10^{6}mm^6 in *Anabas testudineus* (Santhakumar *et al*., 1999) and higher than cultured *H. regain* 0.69 x10^{6}mm^6 reported by Favaretto *et al*. (1978).

In the study, after 10 min of benzocaine exposure erythrocyte (0.59±0.02x10^6/mm^3) was detected and it was significantly increased after 24 hr (0.74±0.02x10^6/mm^3). At the same time, there was no significance difference detected in clove oil anaesthesia. Compared with control group, clove oil and benzocaine anaesthesia suppressed the erythrocyte count during the exposure time in *H. fascita*.

Erythrocyte count of non anaesthetised *H. fasciata*, increased after 10 min and it return back to normal range after 24 hr, whereas increasing erythrocyte value with increasing time of simulated transportation were observed in clove oil and benzocaine. Similar observation is made Velisek *et al.* (2006) in bull head (*Siluris glanis*). Decreasing erythrocyte count was reported in rainbow trout (Velisek *et al.*, 2005) and brown trout (*Samo trutta*) (Arzu and Atamanalp, 2010) when practised with clove oil.

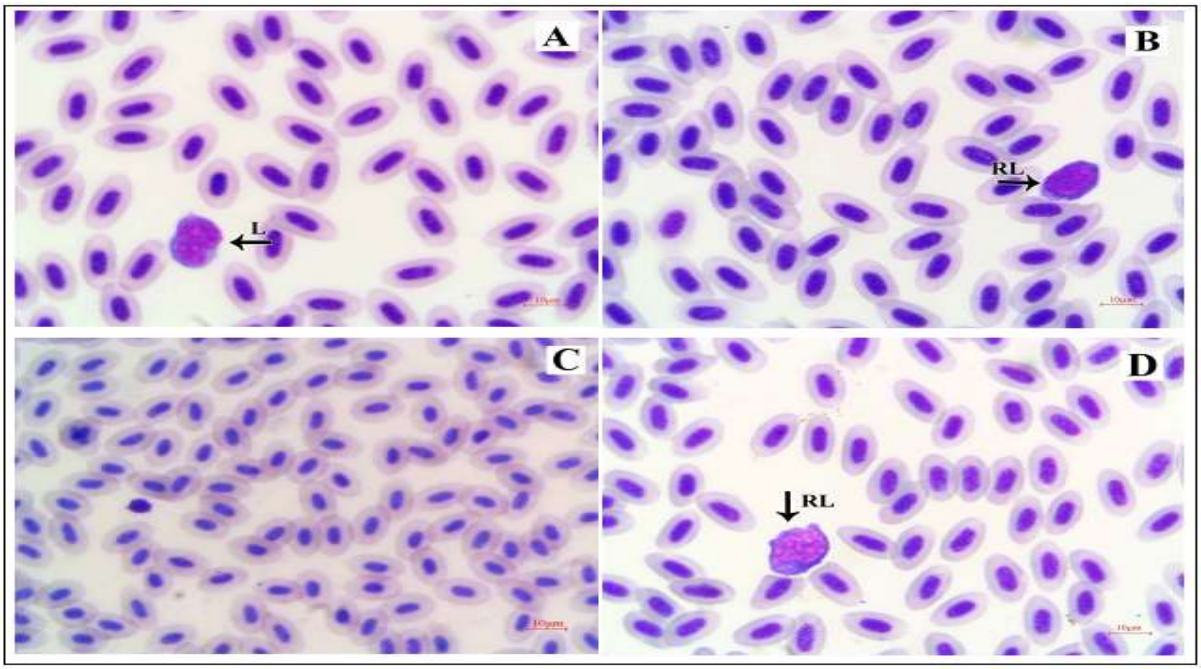

Fig. 1. The morphological studies of *H. fasciata* blood corpuscles after 10 min and 24 hr later in clove oil (A and B) and benzocaine (C and D) anaesthesia. Giemsa stain; 1000x magnification.

Leukocyte count in the control group of *H. fasciata* showed significant increase (P<0.00) with increasing time of exposure. The values were concordant with the results of Kocabatmaz and Ekingen (1984) and Atamanalp *et al.* (2002) in rainbow trout (*O. mykiss*) Arzu and Atamanalp (2010) reported leukocyte count in control group of rainbow trout (4.033±0.30 x10^{3}mm^3) and brown trout (3.86±0.25 x10^{3}mm^3).

Mean leukocyte (TC) count in the blood samples of *H. fasciatus* significantly increased (P< 0.00) with exposure time in clove oil. It was detected right after 10 min of experiment in the clove oil anaesthesia as 3.40±0.16x10^{3}mm^{3}and maximum was 6.81±0.23x10^{3}mm^{3}at 24 h later, whereas, benzocaine anaesthesia reduces the leukocyte production than clove oil and control group. It was observed as3.75±0.18x10^{3}mm^3 after 10 min and it significantly reduces to 3.46±0.21 x10^{3}mm^3. There was no significant difference (P>0.00) detected between the leukocyte value observed after 10 min of control and benzocaine exposure. The value is found similar to the values Kocabatmaz and Ekingen (1984) found for rainbow trout, Arzu and Atamanalp (2010) found in brown trout. Van Vuren and Hattingh (1978) found for common carp (*Cyprinus carpio*) and Das and Mukherjee (2003) for Indian carp (*Labeo rohita*).

Clove oil anaesthesia reduces the haemoglobin countin *H. fasciata*than the simulated transportation of control group. Haemoglobin value detected after 10 min was 6.48±0.11gm% and it was not significantly different with the value (6.55±0.18gm%) detected after 24 hr. Benzocaine anaesthesia leads to increase haemoglobin value from 10 min (6.46±0.25 gm%) of exposure to 24 hr (7.08±0.24 gm%).Similar result was reported by Weinert *et al.* (2015) in Nile tilapia (*Oreochromis niloticus*). Adámek *et al.* (1993) also reported the increase of Hb count in common carp (*C. carpio*) in 2-phenoxyethanol anaesthesia.

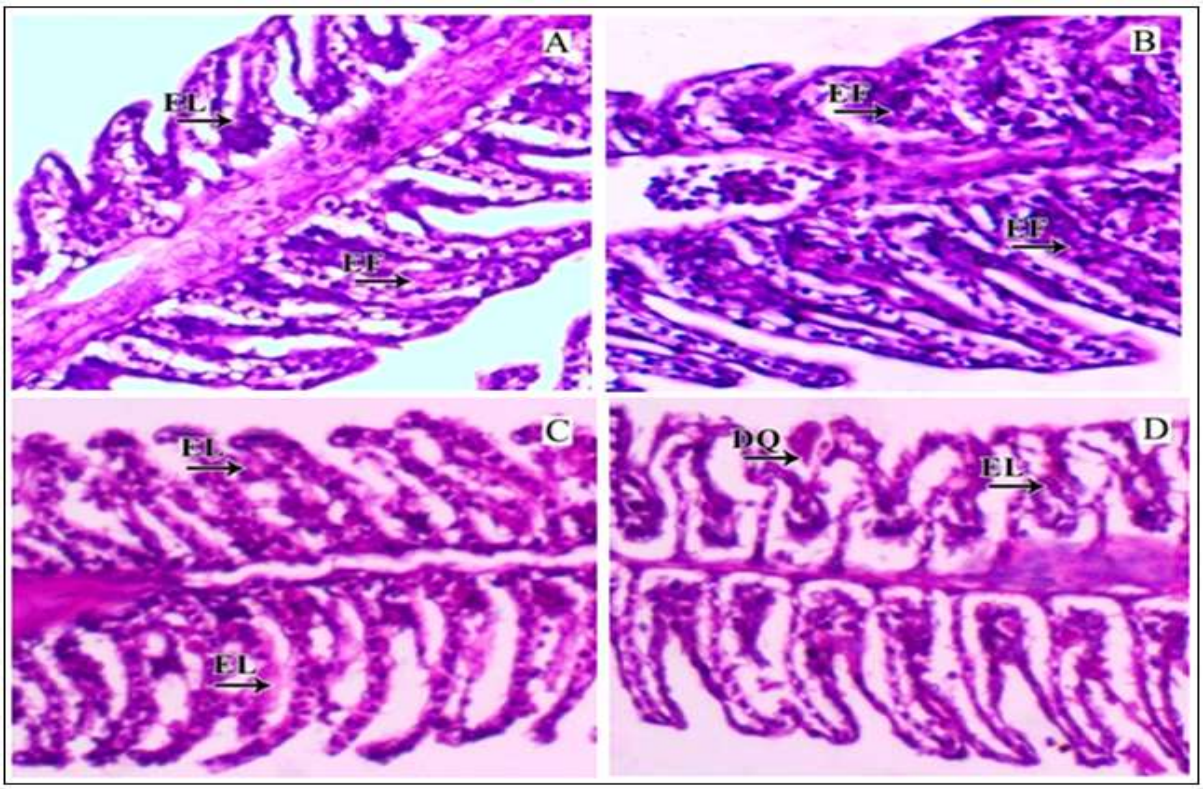

Fig. 2. Sagittal section through the gills of *H. fasciata* after 10 min and 24 hr of exposure on clove oil (A and B) and benzocaine (C and D) H&E stain; 400x magnification.

At the same time, PCV value (19.48±1.69) obtained from clove oil after 24 hr significantly not different from the value obtained from control group (18.39±0.68). These values shows compatibility with the value of Arzu and Atamanalp (2010) found for brown trout (23.0±2.12) and rainbow trout (19.0±2.04) and Kocabatmaz and Ekingen (1984) and Atamanalp *et al.* (2002) reported 28.0±0.08 for healthy trout.

Comparing between the control group of both clove oil and benzocaine showed the increase in the PCV value in *H. fasciatus*. Same result was observed in rainbow trout compare with their control group (Arzu and Atamanalp, 2010).

PCV value observed in clove oil and benzocaine treatments was more or less similar with the result of Arzu and Atamanalp (2010) reported in rainbow trout (19.0±2.04) and brown trout (23.0±2.12) respectively.

Highest MCV values observed in clove oil anaesthesia after 10 m (315.21±1.46 fl) and 24 h later (314.21±1.53 fl). In the study with clove oil; Velisek *et al.*, (2006) detected MCV values 344.05±57.67 μm^3right after the anesthetics and 339.79±56.96 μm^3 after 24 h in bullhead (*S. glanis*).

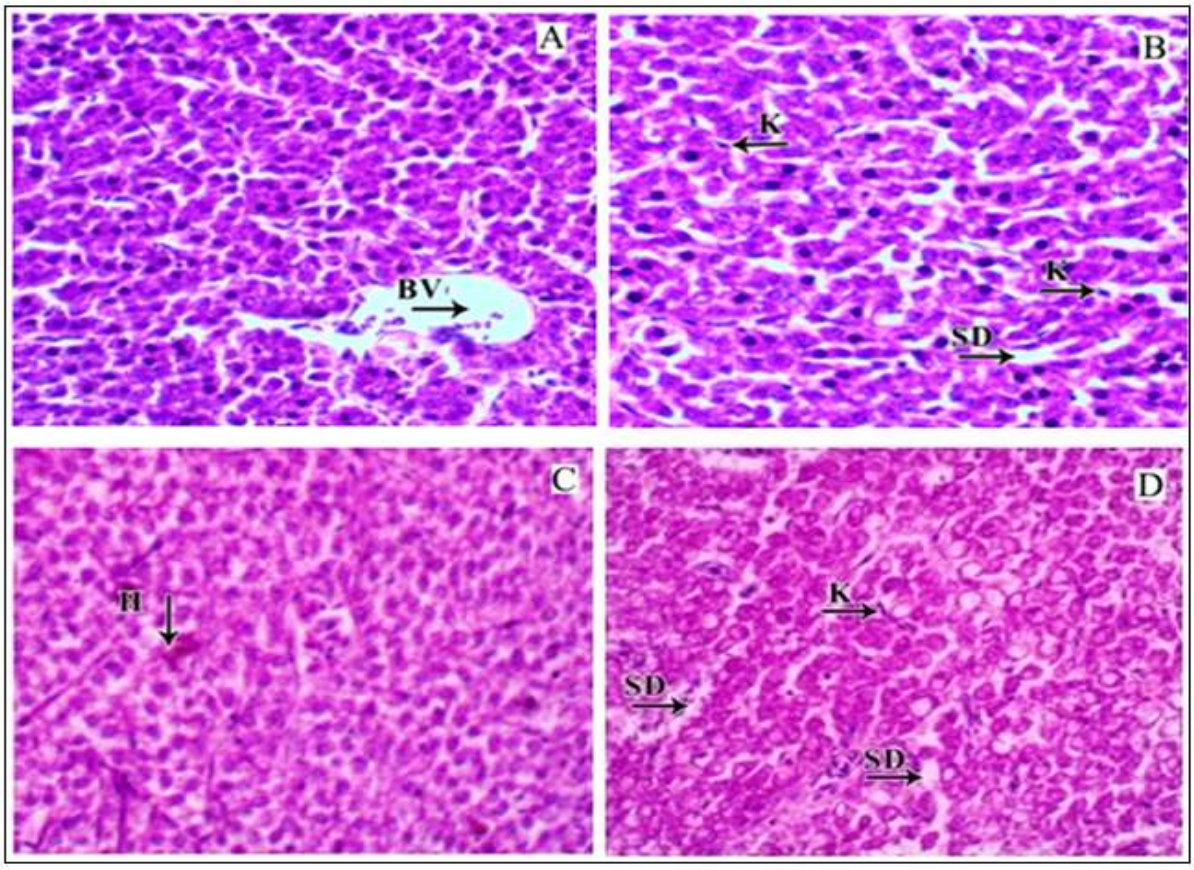

Fig. 3. Section through the liver of melon barbafter 10 min and 24 hr of exposure on clove oil (A and B) and benzocaine (C and D) H&E stain; 400x magnification.

There was no significant difference obtained in the MCV value after 10 min and 24 hr after clove oil exposure. Compared with control group MCV and MCH values are higher in clove oil anaesthesia than benzocaine. It was higher than the values of rainbow trout at 10 min after the application of anesthetics (49.15±11.55 pg) and 24 h later (51.06±9 pg).

When compare with control group, clove oil and benzocaine reduces MCHC values in *H. fasciatus* in both the time periods. There was no significant difference observed between 10 min and 24 hr later in control, clove and benzocaine treatment. Average MCHC value obtained in this study is higher than the values reported by Hasiloglu and Atamanalp (2002) for chub (*Leuciscus cephalus*), Mughal *et al.* (1993) for grass carp (*Ctenopharyngodon idella*), Pages *et al.*, (1995) for bream (*Sparus aurata*), Quentel and Obach (1992) for turbot (*Scophthalmus maximus*), Sandnes *et al.*, (1988) for salmon (*Salmo salar*) and Yamawaki *et al*, (1986) for carp (*C. carpio*). MCH value obtained in *H. fasciatus* is lower than the value observed by Quentell and Obach (1992) for turbot (*S. maximus*).

Morphological studies of blood corpuscles

Morphological studies of blood corpuscles (Fig. 1) of *H. fasciatus* in control and clove oil and benzocaine anaesthesia showed normochromic normocytic cells, no normoblast (Immature blood cells), normal distribution of WBC and adequate lymphocytic platelets where observed. While after 24hr, benzocaine treatment noticed with the presence of reactive lymphocytes (Fig 1 D).

Reactive lymphocyte or immunocyte are antigenitically stimulated lymphocytes. Lymphocytes prevail in the defence mechanisms of fish. Under stress, lymphocytes may present in inflammation (Iwama and Nakanishi, 1996) and may mediate

against different abuses. Lymphocyte is a type of white blood cells which involves in the defence mechanisms in fish. Under stress, lymphocytes may present in inflammation (Iwama and Nakanishi, 1996) and may mediate against different abuses.

Histopathological changes in gills and liver

Histopathological changes have been widely used as biomarkers for evaluating degree of pollution or toxic effect with the habitat of an organism. Limited studies are available on histopathology as biomarker in anaesthetic treatments.

Histopathological studies of *H. fasciata* gill revealed that in control fish, without anesthetics, no histopathological changes were observed. In clove oil treated fish (Fig. 2. A), epithelial lifting (EL) and epithelial fusion (EF) were observed after 10 m of simulated transportation and complete fusion of secondary gill lamellae (EF) were noticed after 24 h exposure (Fig 2. B.).In benzocaine treated fish gill after 10 min (Fig. 2. C) showed epithelial lifting (EL) and after 24 h (Fig. 2. D) of simulated transportation epithelial lifting (EL) associated with desquamation (DQ) of respiratory epithelium were observed.

Epithelial lifting may be due to direct deleterious effect of the anesthetics. Schmidt *et al.* (1999) observed epithelial cell lifting in brown trout, *S. trutta* in diluted sewage plant effluents exposure. This histological alteration reported by Georgieva *et al.*, (2014) in Common Carp (*C. carpio* L.) and Osman (2012) in Nile tilapia, *O. niloticus*. Separation and lifting up of the epithelium might be defense response of the fish (Ramesh, 1994; Van Heerden *et al.*, 2004) in response with surrounding environment. Such cellular damage can increase the water-blood diffusion distance (Ingersoll *et al.*, 1990) and it adversely affect the gas exchange and ionic regulation.

In the present study, other histopathological alterations observed on the gills of test species are desquamation and epithelial fusion. This type of changes occurs as defense mechanism and thus decreases the respiratory area (Poleksic and Mitrovic-Tutundzic, 1994; Van Heerden *et al.*, 2004) in order to increase water-blood diffusion distance. These alteration also found in fish exposed in some other pollutants (Randi *et al.*, 1996; Osman, 2012).

No histopathological changes were noticed in the liver of control fish during the simulated transportation. Mild haemorrhage was observed after 10 m of benzocaine treatment (Fig. 3. C). After 24 h of clove oil and benzocaine treatment (Fig. 3. B & D) the presence of kupffer cells (K) and mild sinusoid dilation (SD) were noticed.

The result of the present investigation on histopathological changes on gills and liver revealed that the effect of clove oil on fish gills were comparatively higher than benzocaine treatment. While, benzocaine anaesthesia moderately affected on the liver of *H. fasciata*.

In liver structure, mild histopathological alteration was observed in test species exposed to all anesthetics, these alterations may due to defensive response with additives in external environment such as anesthetics.

Hepatocytes, the main parenchymal tissue of the liver and the extracellular space includes sinusoids and kupffer cells are seen in the present study. Lateral nucleus of hepatocytes was noticed by Aliakbar *et al.* (2015) in MS-222 treatment. In the present study, sinusoid dilation was the common histological alteration observed in liver tissues of each specimen. Dilation rate may increase with prolonged exposure (Aliakbar *et al.*, 2015).

In a study on the liver of Siberian sturgeons 24 h after MS-222 anesthesia noticed with congestion of sinusoid capillaries (Gomulka *et al.*, 2008). Osman (2012) reported severe sinusoid dilation with other alteration in Nile tilapia *O. niloticus* in a study to assess the impacts of river Nile pollution.

The result of the present investigation on histopathological changes on liver revealed that the effect of benzocaine on fish liver tissues were comparatively higher than clove oil treatments.

Results of the present study suggest that the use of anaesthetics at acceptable dose does not causes irreversible damage in the test fishes and the literatures are also supported the same. All the histopathological alterations such as epithelial lifting, fusion and desquamation in gills and sinusoid dilation in the liver of three test species observed in the study were time and dose dependent. Severe alteration may occur due to prolonged exposure or higher dose and the values determined in the present study suggest that, internal organs and tissues of melon barb are not altered by clove oil and benzocaine anaesthetics dose.

Conclusion

Our present study contributes to increase knowledge on *H. fasciata* analysed with clove oil and benzocaine anaesthesia for proper and stress less transportation. We experienced with the optimal concentration of clove oil and benzocaine does not lead to significant effects on the haematological and histological indices in *H. fasciata*. The optimal concentration of clove oil and benzocaine anaesthesia reduces stress and are safe for transportation of *H. fascita*.

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