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Evaluation the performance of anaesthetic effects of Tobacco and Ketamine on Grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844)

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Abstract

The present study aimed to determine the optimal concentration of three anaesthetics, name MS222, ketamine, and tobacco extract, in grass carp juveniles (107.78±22.11 g body weight and 226.74±13.06 mm total body length). Treatments were as follows: 100 mg L^{-1} of MS222 (control group), 5.5, 8, 11, 18.5, and 22 mg L^{-1} of ketamine, and 10, 13.33, 20, 26.27, and 40 mg L^{-1} of tobacco extract. In addition, changes in blood and biochemical parameters were measured at different concentrations. Considering the anaesthesia induction and recovery durations times, the optimal concentration for anaesthesia of studied substances was obtained as follows: 100 mg L^{-1} of MS222 (139.00±20.00 seconds), 18.5 mg L^{-1} of ketamine (140.10±20.20 seconds), and 40 mg L^{-1} of tobacco extract (174.00±20.09 seconds). Based on the results, the shortest recovery time from anaesthesia was observed at 26.27 mg L^{-1} of tobacco extract (870.00±5.3 seconds) and 8 mg L^{-1} of ketamine (967.00±5.5 seconds), respectively. The results indicated that the anaesthesia induction and recovery duration was dependent on the concentration of anaesthetics. Moreover, significant differences were observed between the three anaesthetics in terms of blood parameters. The study findings also revealed a significant difference between treatments in biochemical parameters (cortisol, glucose, and lactate).

Keywords: Anaesthetics, Blood, Grass Carp, *Ctenopharyngodon idella*, MS_{222}, Ketamine, Tobacco

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Introduction

In the modern aquaculture, handling practices like transportation, sizing, grading, weighing, stripping and blood collection are stressful for fish. To reduce the effects of stress, some chemical substances and anaesthetics are routinely utilized for fish before handling (Ross and Ross, 1999; Wagner *et al*., 2003).

Anaesthetic agents are potentially used for light sedation and non-invasive procedures to fulfil anaesthesia in order to avoid inflicting pain during surgery and interventions (Ross and Ross, 2008; Neiffer and Stamper, 2009). Many anaesthetics agents have been used in aquaculture practices, including tricaine methanesulfonate (MS-222), clove oil, xylocaine, benzocaine, 2-phenoxyethanol, quinaldine, quinaldine sulphate, and metomidate (Gutierrez and Herrera, 1995; Massee *et al*., 1995; Ortuno *et al*., 2002; Small, 2003; Kiessling *et al*., 2009).

However, some parameters such as efficacy, cost effectiveness, availability and safety have been considered for a valuable agent. Although, ketamine and tobacco extracts have been experimentally have been applied in researchs (Di Marco *et al*., 2011; Mohammadi and Khara, 2015; Dinesh *et al*., 2017) but, the use of these agents and their impacts on physiological parameters remain unexplored, since no maximum residue levels and species-specific dosage have been determined. At the present, only MS-222 is approved as the most widely used anaesthetics chemical in fish (Topic Popovic *et al*., 2012; Chambel *et al*., 2015).

The evaluation of fish health following induced anaesthesia is linked to study of blood biochemical properties. It has been reported that, anaesthetics may potentially alter blood biochemistry in many fish species (Iwama *et al*., 1989). The stress-induced alterations of post-anaesthesia in blood chemistry indices occur within seconds or minutes after fish are treated (Mazeaud and Mazeaud, 1981; Gingerich and Drottar, 1989). Therefore, precautions must be taken into an account to ensure that handling procedures do not change the indices of interest. These changes are attributed to occur in plasma hormones, energy metabolism and electrolytes balance that enable biologists to use aforementioned parameters for evaluation of fish stress responses and health status (Wagner and Congleton, 2004).

Some studies have been found that MS-222 or clove oil affect blood biochemical parameters, including glucose (Davis and Griffin, 2004), cortisol (Wagner *et al*., 2003), total protein (Cataldi *et al*., 1998) and amino acid (Morales *et al*., 1990). These previous studies mainly focused on conventional anaesthetic agents, while little attention was considered to the physiological effects plant based or other anaesthetic agents. Hence, understanding of the new anaesthetic agents effects have a great practical application in aquaculture. In the current study, we tested haematological profiles and blood biochemical

parameters to determine the potential stress responses of two anaesthetics; ketamine and tobacco extract to compare with MS-222 as conventionally approved anaesthetic on juvenile grass carp. Therefore, the objectives of this study were to determine the appropriate anaesthetic concentrations of ketamine and tobacco compared to MS-222 as a potential substitute anaesthetics and the effect of anaesthetics on blood and biochemical parameters to identify the best anaesthetic for Grass Carp juveniles, particularly regard to reducing stress.

Materials and methods

Fish

165 pieces of Grass Carp juveniles (107.78±22.11 g body weight and 226.74±13.06 mm total body length) were provided from local hatchery near to experiment place. The study was conducted at the Lahijan Branch of Islamic Azad University (Iran). Before beginning of the experiment, fish were initially acclimatized for 10 days in one circular fiberglass tank (1500 L) in a flow-through water system. Water temperature of the holding tanks was maintained at 18-21 °C throughout the experiment. Fish were not fed for 24 h before anaesthesia treatments and blood sampling (Ross and Ross, 2008).

Anaesthesia and experimental procedure

MS-222 was purchased from Sigma-Aldrich Chemicals Ltd (USA, Saint Louis). Ketamine was supplied by Rotexdemica Company (Dresden, Germany) and tobacco extract was provided from Tobacco Company in Rasht (Iran). Before using, the clove oil was dissolved in 96% ethanol (1: 9 v⁄v) and MS-222 in water to prepare a stock solution (Chambel *et al*., 2015). Preliminary trials of ethanol used in each trial did not have any noticeable effects on the fish. The applied concentrations of tobacco extract were calculated as 50% 24 h LC_{50} (24 h LC_{50} of tobacco extract on Grass Carp obtained from preliminary test). Thus, 100 mg of tobacco extract were measured and mixed with 1 L of water to prepare stock solution of 100 mg L^{-1} concentration (Agokei and Adebisi, 2010; Dinesh *et al*., 2017). Ketamine was dissolved in 1 L of water to give desired concentrations (Di Marco *et al*., 2011).

In this study, 11 groups of 15 Grass carp (MS-222: 1 Treatment, 15 pieces, tobacco: 5 Treatments, 75 pieces, ketamine: 5 Treatments, 75 pieces) each were compared in this study:

Control: based on the previous studies, the exact concentrations of MS-222 was served as control treatment to compare effectiveness of other anaesthetics as alternative for anaesthetizing Grass carp (Topic Popovic *et al*., 2012; Chambel *et al*., 2015).

Two groups with blood sampling immediately after anaesthesia in each concentration of tobacco and following recovery time courses.

Two groups blood sampling immediately after anaesthesia in each concentration of ketamine and following recovery time courses.

Three replicates for each treatment were designed for each group, each

held in 30 L glass aquaria (38cm length×27cm width×47cm height) containing freshwater plus the anaesthetic agents. Induction time of anaesthesia was recorded from adding the anaesthetic agent to the water till no opercula movements, decreased respiratory rate and weak response to strong tactile stimuli (Phase II) have been observed.

After anaesthesia, to evaluate the haematological and blood biochemistry parameters, samples were taken from caudal severance and divided into two portions. Half of each blood sample was immediately transferred to heparinized tubes for haematological examination and the rest was put in non-heparinized tubes for serum analysis. For biochemical assays, blood samples were immediately centrifuged (3000 g for 10 min) at room temperature and then the serum was separated and stored at -20°C until analysis. Haematological parameters, including erythrocyte count (RBC), haemoglobin value (Hb, g dl^{-1}), haematocrit value (Hct, %), leukocyte count (WBC), mean corpuscular volume (MCV, fl), mean corpuscular haemoglobin (MCH, pg), and mean corpuscular haemoglobin concentration (MCHC, %) have been measured by using standard procedure (Harikrishnan *et al.*, 2003).

Differential leukocyte counts (neutrophil, lymphocyte, monocyte and eosinophil) were determined by Giemsa staining method of blood smears using a light microscope. The smears obtained from heparinized sample were first air-dried, fixed in 96 % ethanol for 30 min and stained in Giemsa to determine differential leukocyte counts (Klontz, 1994).

Radioimmunoassay (RIA) was used for measurement of cortisol. An assay kit of cortisol was purchased from Immunotech Company, France. Commercial assay kit was contained two necessary reagents, i.e., antibody and tracer (labelled antigen). Aliquots containing antibody and tracer were transferred to assay tubes (polystyrene tube). Tracer and antibody were added in a further 100 µl of assay buffer, and the tubes were left to incubate overnight at 4 °C. The content of polystyrene tubes (antibody, tracer, and serum sample) was discharged completely and then polystyrene tubes applied to a Gamma counter (Wallac, Finland) for measurement of absorption spectrum. Subsequently hormone concentration was calculated using related standard curve. The concentration of glucose and lactate were measured spectrophotometrically using kits supplied by Pars Azmun Diagnostics, Tehran, Iran.

Statistical analysis

A Kruskal–Wallis test was used to determine the difference between the induction and recovery times of different concentrations of the each anaesthetic agent (Differences among means of blood indices were analyzed by one-way ANOVA followed by Duncan's new multiple range tests). Differences were considered significant at $p<0.05$. Data were analyzed using SPSS statistical package (SPSS,

Chicago IL, USA) and expressed as mean±standard error of the mean (SE).

Results

Significant differences (p<0.05) in the induction times of anaesthesia and recovery times were observed at different concentrations of the anaesthetic agents. Induction time of anaesthesia was shortest and longest at MS-222 and 5.5 mg L^{-1} of ketamine, respectively (Table 1). Furthermore, the shortest and longest recovery times were observed in tobacco extract and ketamine at concentrations of 26 mg L^{-1} and 18.5 mg L^{-1}, respectively (Table 2). Haematological parameters of Grass carp including WBC, RBC, Ht and Hb were significantly influenced by anaesthetic agents in induction and recovery times (Tables 2 and 3). There were dose-dependent variations in WBC, RBC, Ht and Hb levels in induction times among different concentrations anaesthetic agents (Table 2). In induction times of anaesthesia and recovery times, significant differences were observed in WBC, RBC, Hb, Ht and MCV values among different concentrations of anaesthetic agents. The highest levels of RBC, WBC and Ht in induction times were observed in tobacco at concentrations of 10, 13 and 40 mg L^{-1} (Table 2). Whereas, in recovery times, the highest levels of RBC and Hb values were obtained in ketamine treatment at concentrations of 5.5 mg L^{-1} (Table 3).

Moreover, in induction and recovery times of Grass carp significant changes in differential leukocyte counts (lymphocyte, neutrophil and monocyte) among different concentrations of anaesthetic agents were detected (Tables 4 and 5).

In the induction and recovery times, serum cortisol concentration was considerably higher in tobacco extract and ketamine at concentrations of 10 mg L^{-1} and 22 mg L^{-1}, respectively (Fig. 1). A similar result was also seen in the serum glucose concentration (Fig. 2). Lactate concentration in the induction and recovery times was significantly higher in ketamine at concentrations of 5.5 mg L^{-1} and 22 mg L^{-1}, respectively (Fig. 3).

Table 1: Induction and recovery times (s) of juvenile grass carp anesthetized with MS-222 (control), Ketamine and Tobacco as anaesthetic agents.

Anesthetics concentration	Induction anesthesia time (second)	Recovery time (second)
MS 222 (100 mg L^{-1})	139 ± 20.1	4100 ± 5.3
Ketamine (5.5 mg L^{-1})	289 ± 10	2112 ± 20
Ketamine (8 mg L^{-1})	243± 15.1	967 ± 5.5
Ketamine (11 mg L^{-1})	243± 20	2595 ± 30
Ketamine (18.5 mg L^{-1})	140 ± 10.1	4776 ± 20.2
Ketamine (22 mg L^{-1})	144 ± 6	4353 ± 16
Tobacco (10 mg L^{-1})	175 ± 10.2	1500 ± 7.5
Tobacco (13 mg L^{-1})	175± 10.1	1521± 15.3
Tobacco (20 mg L^{-1})	174± 10.4	1498± 30
Tobacco (26 mg L^{-1})	175± 10.5	870± 5.3
Tobacco (40 mg L^{-1})	174± 15	1600± 20.9

Table 2: Effects of MS-222 (control), Ketamine and Tobacco extract as anaesthetic agents on haematocrit values of juvenile grass carp following anaesthesia.

Anaesthetics	Concentrations	WBC ($\times10^3$ mm^{-3})	RBC ($\times10^6$ mm^{-3})	Hb (gr dl^{-1})	Ht (%)	MCV (µm^{-3})	MCH (pg)	MCHC (%)
MS 222	(100 mg L^{-1})	4100 ± 100 ^{f}	184 ± 45.9 ^{b}	8.5 ± 0.60 ^{a}	35.6 ± 2.50 ^{a}	187 ± 6.2 ^{b}	46 ± 1	24 ± 1.5
Ketamine	(5.5 mg L^{-1})	4500 ± 500 ^{de}	185 ± 44.9 ^{b}	8.5 ± 0.50 ^{a}	35 ± 1 ^{a}	189 ± 1 ^{ab}	45 ± 2	23 ± 1.1
Ketamine	(8 mg L^{-1})	4816 ± 200 ^{cd}	155 ± 85.1 ^{g}	7.1 ± 0.10 ^{bc}	29 ± 1 ^{cd}	186 ±0.57 ^{b}	46 ± 1	24 ± 1
Ketamine	(11 mg L^{-1})	4850 ± 200 ^{cd}	164 ± 31.8 ^{f}	7 ± 0.0 ^{bc}	27 ± 0.0 ^{de}	181 ± 1.7 ^{cd}	46 ± 1	25 ± 2
Ketamine	(18.5 mg L^{-1})	4700 ± 200 ^{cd}	173 ± 75.3 ^{d}	6.9 ± 0.10 ^{cd}	26 ± 1.5 ^{ef}	179 ± 00 ^{d}	45 ± 2.5	23 ± 0.57
Ketamine	(22 mg L^{-1})	5200 ± 200 ^{c}	173 ± 65.2 ^{d}	6.8 ± 0.10 ^{cd}	26 ± 0.57 ^{ef}	177 ± 00 ^{df}	45 ± 2.5	25 ± 2
Tobacco	(10 mg L^{-1})	6000 ± 200 ^{b}	150 ± 43.5 ^{h}	6.4 ± 0.25 ^{d}	26.4 ± 0.11 ^{d}	173 ± 2.8 ^{f}	42 ± 2.3	24 ± 0.57
Tobacco	(13 mg L^{-1})	4093 ± 310 ^{g}	155 ± 35 ^{g}	6.9 ± 0.15 ^{bc}	26.9 ± 0.25 ^{bc}	186 ± 2.5 ^{b}	46 ± 1	24 ± 0.57
Tobacco	(20 mg L^{-1})	8306 ± 390 ^{a}	149 ± 81.7 ^{i}	6.9 ± 0.20 ^{cd}	28.3 ± 0.57 ^{cde}	188 ± 1.5 ^{bc}	46 ± 0.57	25 ± 0.00
Tobacco	(26 mg L^{-1})	5816 ± 275 ^{b}	189 ± 35 ^{a}	8.4 ± 0.05 ^{a}	35.3 ± 0.67 ^{a}	185 ± 1 ^{a}	44± 0.57	24 ± 0.57
Tobacco	(40 mg L^{-1})	4713 ± 280 ^{cd}	160 ± 39.7 ^{f}	7.9 ± 0.05 ^{b}	30.6 ± 2.5 ^{bc}	193 ± 2.6 ^{a}	45 ± 0.57	23 ± 00

Table 3: Effects of MS-222 (control), Ketamine and Tobacco as anaesthetic agents on haematocrit values of juvenile grass carp following recovery.

Anaesthetics	Concentrations	WBC ($\times10^3$ mm^{-3})	RBC ($\times10^6$ mm^{-3})	Hb (gr dl^{-1})	Ht (%)	MCV (µm^{-3})	MCH (pg)	MCHC (%)
MS 222	(100 mg L^{-1})	4100 ± 100 ^{f}	184 ± 45.9 ^{b}	8.5 ± 0.60 ^{a}	35.6 ± 2.50 ^{a}	187 ± 6.2 ^{b}	46 ± 1	24 ± 1.5
Ketamine	(5.5 mg L^{-1})	4500 ± 500 ^{de}	185 ± 44.9 ^{b}	8.5 ± 0.50 ^{a}	35 ± 1 ^{a}	189 ± 1 ^{ab}	45 ± 2	23 ± 1.1
Ketamine	(8 mg L^{-1})	4816 ± 200 ^{cd}	155 ± 85.1 ^{g}	7.1 ± 0.10 ^{bc}	29 ± 1 ^{cd}	186 ± 0.57^{b}	46 ± 1	24 ± 1
Ketamine	(11 mg L^{-1})	4850 ± 200 ^{cd}	164 ± 31.8 ^{f}	7 ± 0.0 ^{bc}	27 ± 0.0 ^{de}	181 ± 1.7 ^{cd}	46 ± 1	25 ± 2
Ketamine	(18.5 mg L^{-1})	4700 ± 200 ^{cd}	173 ± 75.3 ^{d}	6.9 ± 0.10 ^{cd}	26 ± 1.5 ^{ef}	179 ± 00 ^{d}	45 ± 2.5	23 ± 0.57
Ketamine	(22 mg L^{-1})	5200 ± 200 ^{c}	173 ± 65.2 ^{d}	6.8 ± 0.10 ^{cd}	26 ± 0.57 ^{ef}	177 ± 00 ^{df}	45 ± 2.5	25 ± 2
Tobacco	(10 mg L^{-1})	6000 ± 200 ^{b}	150 ± 43.5 ^{h}	6.4 ± 0.25 ^{d}	26.4 ± 0.11 ^{d}	173 ± 2.8 ^{f}	42 ± 2.3	24 ± 0.57
Tobacco	(13 mg L^{-1})	4093 ± 310 ^{g}	155 ± 35 ^{g}	6.9 ± 0.15 ^{bc}	26.9 ± 0.25 ^{bc}	186 ± 2.5 ^{b}	46 ± 1	24 ± 0.57
Tobacco	(20 mg L^{-1})	8306 ± 390 ^{a}	149 ± 81.7 ^{i}	6.9 ± 0.20 ^{cd}	28.3 ± 0.57 ^{cde}	188 ± 1.5 ^{bc}	46 ± 0.57	25 ± 0.00
Tobacco	(26 mg L^{-1})	5816 ± 275 ^{b}	189 ± 35 ^{a}	8.4 ± 0.05 ^{a}	35.3 ± 0.67 ^{a}	185 ± 1 ^{a}	44± 0.57	24 ± 0.57
Tobacco	(40 mg L^{-1})	4713 ± 280 ^{cd}	160 ± 39.7 ^{f}	7.9 ± 0.05 ^{b}	30.6 ± 2.5 ^{bc}	193 ± 2.6 ^{a}	45 ± 0.57	23 ± 00

Table 4: Effects of MS-222 (control), Ketamine and Tobacco as anaesthetic agents on differential leukocyte counts of juvenile grass carp following anaesthesia.

Anaesthetics	Concentrations	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Monocyte (%)
MS 222	(100 mg L^{-1})	74 ± 2^{bcdef}	20.6 ± 1.1^{cd}	0.33 ± 0.57	5 ± 1^{ab}
Ketamine	(5.5 mg L^{-1})	74 ± 1^{bcdef}	21.3 ± 0.57^{cd}	0.33 ± 0.57	4.3 ± 0.57^{bc}
Ketamine	(8 mg L^{-1})	75 ± 1^{bcdef}	22 ± 1^{bc}	0.33 ± 0.57	2.3 ± 0.57^{abc}
Ketamine	(11 mg L^{-1})	72 ± 1.5^{bcde}	23 ± 0.57^{ab}	0.33 ± 0.57	3.6 ± 0.57^{de}
Ketamine	(18.5 mg L^{-1})	71 ± 0.57^{efg}	24 ± 1^{a}	0.33 ± 0.57	4 ± 1^{abcd}
Ketamine	(22 mg L^{-1})	76 ± 0.57^{fg}	20 ± 1^{cd}	0.33 ± 0.57	3 ± 0^{abcd}
Tobacco	(10 mg L^{-1})	71 ± 1^{b}	24 ± 0.57^{a}	0 ± 0^{d}	4.6 ± 0.57^{abc}
Tobacco	(13 mg L^{-1})	73 ± 2.3^{f}	21.3 ± 1.5^{cd}	0 ± 0^{bc}	5 ± 1^{ab}
Tobacco	(20 mg L^{-1})	76 ± 1.5^{cdefg}	19.6 ± 1.5^{d}	0.66 ± 0.57	3.3 ± 0.57^{bcd}
Tobacco	(26 mg L^{-1})	75 ± 0.57^{bcd}	19.3 ± 0.57^{d}	0.66 ± 0.57	4.6 ± 1.4^{abc}
Tobacco	(40 mg L^{-1})	80 ± 2^{a}	17.3 ± 2^{e}	0.33 ± 0.57	1.6 ± 0.57^{e}

Table 5: Effects of MS-222 (control), Ketamine and Tobacco as anaesthetic agents on differential leukocyte counts of juvenile grass carp following recovery.

Anaesthetics	Concentrations	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Monocyte (%)
MS 222	(100 mg L^{-1})	73 ± 1.5^{b}	21 ± 1^{ef}	0.33 ± 0.57	5 ± 1.7^{bc}
Ketamine	(5.5 mg L^{-1})	76 ± 0.57^{a}	18.3 ± 0.57^{cd}	0.66 ± 0.57	4.3 ± 0.57^{b}
Ketamine	(8 mg L^{-1})	70 ± 0.57^{cd}	23.6 ± 0.57^{a}	1.3 ± 0.57	4.6 ± 0.57^{bc}
Ketamine	(11 mg L^{-1})	67 ± 1^{e}	28 ± 0^{a}	1 ± 1	4.6 ± 0.57^{bc}
Ketamine	(18.5 mg L^{-1})	67 ± 0.57^{e}	27 ± 0.57^{bc}	0.66 ± 1.1	5 ± 0^{bc}
Ketamine	(22 mg L^{-1})	68 ± 1^{de}	24 ± 1^{ab}	1.33 ± 0.57	6 ± 1^{a}
Tobacco	(10 mg L^{-1})	66 ± 2^{e}	26 ± 0.57^{de}	0.66 ± 0.57	6 ± 1^{a}
Tobacco	(13 mg L^{-1})	72 ± 2.5^{bc}	22 ± 0.57^{a}	1.33 ± 0.27	4.3 ± 0.57^{b}
Tobacco	(20 mg L^{-1})	66 ± 1.5^{e}	28 ± 2^{a}	0.66 ± 0.57	4.6 ± 0.57^{bc}
Tobacco	(26 mg L^{-1})	72 ± 2^{bc}	22 ± 1^{cde}	0 ± 0	4.6 ± 0.57^{bc}
Tobacco	(40 mg L^{-1})	72 ± 2.5^{bc}	22 ± 2.5^{de}	0.66 ± 0.57	4.3 ± 0.57^{b}

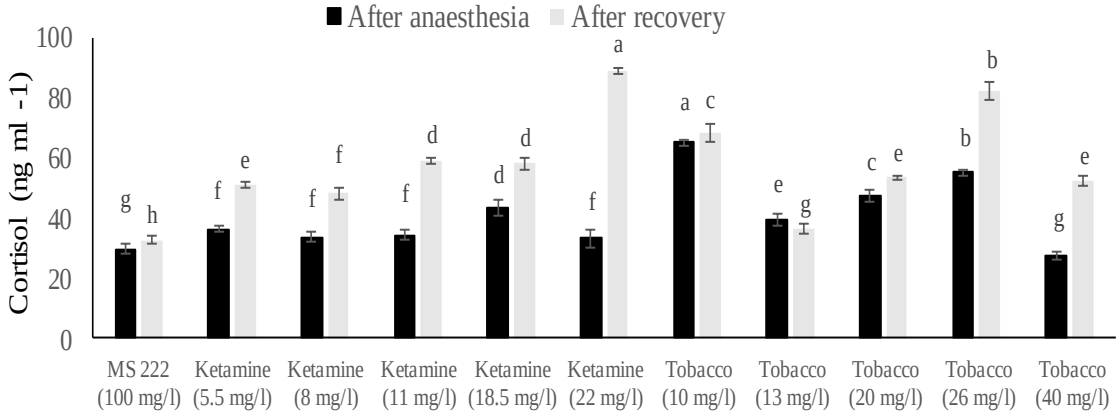

Figure 1: Changes in serum cortisol concentration of juvenile grass carp following anaesthesia with MS-222 (control), ketamine and tobacco and recovery time. Data are expressed as Mean±SD; n=10 fish per group. Different letters indicate differences among groups at *p*<0.05.

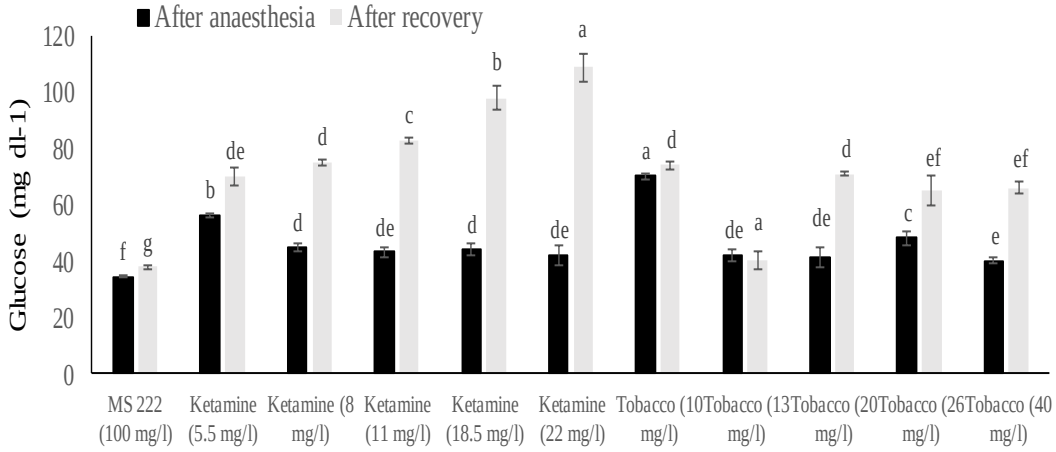

Figure 2: Changes in serum glucose concentration of juvenile grass carp following anaesthesia with MS-222 (control), ketamine and tobacco and recovery time. Data are expressed as Mean±SD; n=10 fish per group. Different letters indicate differences among groups at *p*<0.05.

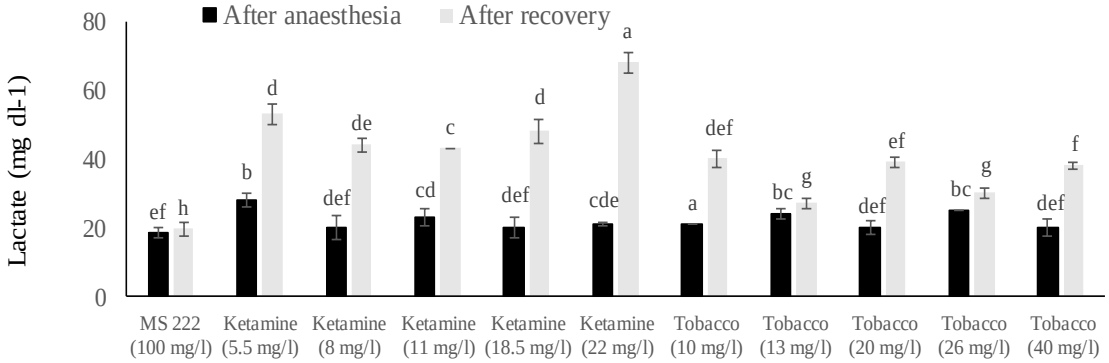

Figure 3: Changes in serum lactate concentration of juvenile grass carp following anaesthesia with MS-222 (control), ketamine and tobacco and recovery time. Data are expressed as Mean±SD; n=10 fish per group. Different letters indicate differences among groups at *p*<0.05.

Discussion

Applications of anaesthetic agents are common practice to handle fish stress and prevents physical injuries during all aquaculture practices (Iversen *et al*., 2003). Despite utilization of various anaesthetics agents in modern aquaculture, the knowledge of comparative effects of different suitable dosage for handling operations is still limited (Di Marco *et al*., 1999; Fleming *et al*., 2003; Gomulka *et al*., 2008). Therefore, it is important to determine the appropriate anaesthetic concentrations and exposure the time to minimize the stress (Feng *et al*., 2011). It has been recommended, the adequate induction time of an anaesthetic for fish should be below 15 min, with a recovery time of lower than 5 min (Marking and Meyer, 1985). However, Cardenas *et al*.(2016), have had been pointed that the anaesthetic efficacy of anaesthetic agents is obviously species-specific, size-dependent, and affected by water temperature.

The anaesthetic concentrations used in this study were safe and effective in reducing anesthetizing induction time in fish. The lowest anaesthetic induction time in each of the MS_{222} test materials with a time of 139.00±20.00 seconds, followed by a concentration of 18.5 mg L^{-1} of ketamine with a time of 140.120 20.20 sec, and 40 mg L^{-1} tobacco with a time of 17.09±20.09 seconds.

Anaesthetic dosages used in this study were safe and effective in order to rapid induction of anaesthesia in grass carp with MS-222 followed by ketamine with the lowest concentration. A similar result on sturgeon hybrid *Acipenser naccarii*×*Acipenser baerii* was reported in concentration of 150 mg L^{-1} MS-222 (Di Marco *et al*., 2011). The results of the present study demonstrated that the most effective anaesthetic that induce anaesthesia with the lowest recovery time were MS- 222, 5.5 mg L^{-1} and 26 mg mg L^{-1} of tobacco for Grass carp. Similarly, MS-222 efficacy for induction (≤3min) and recovery (≤5min) times were reported at the same dose of the present study (100 mg L^{-1}) in several fish (Hseu *et al*., 1998; Bystriansky *et al*., 2006; Ibarra-Zatarain *et al*., 2011). Conversely, the appropriate ranges of ketamine and tobacco extract concentrations in fish are rarely studied. However, it has been reported that the effects of ketamine and tobacco extract in terms of acquiring optimal concentration in induction and recovery times for rainbow trout was varied (Mohammadi and Khara, 2015). This discrepancy is likely due to the differences in experimental protocols or fish species studied. In line with this, Williams *et al.* (1988) have noted that there were interspecies differences in the successful dosage of anaesthetic. In this study, the minimum return time of anesthesia in Amor fish was 26.67 mg L^{-1} of tobacco with 870.00±3.5 g followed by a concentration of 8 mg L^{-1} of ketamine with 967±5.5 seconds. In the present study, anaesthetic induction time significantly decreased with anesthetic concentration increase.

The reaction of fish exposed to excess of the recommended dose of any anaesthetic will be accompanied with an obvious stress response. For

instance, Molinero and Gonzalez (1995) reported that gilthead sea bream *Sparus aurata* exposed to MS-222 and 2-phenoxyethanol at a dose exceeding 25 mg L^{-1} and 0.075 mg L^{-1} respectively represented a stress response.

In this regard, blood chemistry parameters may help to provide some indicators to determine the optimal concentration range for anaesthesia and decrease stress (Czesny *et al*., 2003; Feng *et al*., 2011). Generally, cortisol and glucose levels are the main biomarkers of physiological stress that have been used as indicators (Wagner *et al*., 2002; Gesto *et al*., 2015). Blood erythrocytes were also used as an indicator of anaesthetic stress (Gontijo *et al*., 2003). However, almost no study has reported that other blood variables could be used as indicators of anaesthetic stress for fish. In the present study, significant changes in plasma parameters including cortisol, glucose, and lactate levels in induction and recovery times were dose-dependent. However, the literature suggests that both glucose and lactate levels are typically correlated with an increase in cortisol (Wendelaar Bonga, 1997; Barton, 2002), as it was occurred in the present study. An increase in plasma glucose after only a few minutes of anaesthesia was observed in juvenile salmonids exposed to a lethal dose of MS-222. However, earlier studies reported that plasma glucose was stable for 12-15 minutes in salmonids exposed to 80-100 mg L^{-1} neutralized or un-neutralized MS-222 (Houston *et al*., 1971; Nieminen *et al*., 1982; Congleton, 2006). On the other hand, elevation of plasma glucose have been reported for several species of marine fish exposed to 80-100 mg L^{-1} MS-222 (Bourne, 1984; Thomas and Robertson, 1991). It is not known whether the increase of plasma glucose observed in the present study was related to the examined species, or to the high concentration of anaesthetics used. Our results for lactate level are in accordance with the reports of other authors, who reported that lactate concentration increased significantly in all anaesthetized fish as a metabolic response to anaesthesia (Di Marco *et al*., 2011; Cardenas *et al*., 2016). It has been shown that, plasma lactate level could be dependent upon secondary effects of anaesthesia, related with oxygen insufficiency for cell aerobic metabolism (Iversen *et al*., 2003). Although, longer periods of anaesthesia or even delayed sampling points are the main necessary factors for existing of variations in this plasma metabolite.

Differential leukocyte counts are important characteristics of the health status of the fish and in many cases they are also used to evaluate the effect of drugs and anaesthetics on fish (Gomulka *et al*., 2008; Witeska *et al*., 2015). Very few data on the effects of anaesthetics on thrombocyte counts in fish are available. In the present study, juveniles of Grass carp subjected to different anaesthetics agents have been showed significant changes in lymphocyte and neutrophil counts. In line with our findings, Witeska *et al*. (2015) had reported an increase in neutrophil and monocyte of Grass carp

under handling anaesthesia with 2-phenoxyethanol or etomidate.

Several studies have been shown that control of the stress responses in fish is dependent on the type of anaesthetic, the concentration used and exposure time (Strange and Schreck, 1978; Barton and Peter, 1982; Davidson *et al.*, 2000). In the present research, the appropriate anaesthetic concentrations for juvenile of Grass Carp were just relative values, which should be concluded based on blood biochemical parameters, anaesthetic time, recovery time, exposure time, and so on. According to the present study, juvenile of Grass Carp were physiologically affected by MS-222 as same as tobacco, indicating that tobacco extract is an appropriate alternative to MS-222. Since, tobacco extract is inexpensive, easily obtained, and non-toxic to fish and human (Dinesh *et al*., 2017), it can be potentially used as a proper anaesthetic for fish.

In general, data analysis showed that the physiological effects were higher with tobacco anaesthetics than ketamine and MS222. The results of current and previous studies had indicated the importance of considering all the physiological potential or other anaesthetic effects of other substances used in aquaculture and fishery studies. It is clear that various anaesthetics can have a definite effect on the fish's blood-type indices. Therefore, when choosing an anaesthetic agents for fish whose anaesthetic effects on them are unknown, a careful examination should be made, especially when changes in the blood-counting index are used as the decisive indicator in a study.

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