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Anesthetic Effect of Clove Oil Loaded on Lecithin based Nano Emulsions in Gold Fish, *Carassius auratus*

[1]Hosna Gholipourkanani, [1]Iraj Gholinasab-Omran, [2]Pouneh Ebrahimi and [1]Hojatollah Jafaryan

[1]Department of Fisheries, Faculty of Agriculture, Gonbad Kavous University, Gonbad Kavous, Iran
[2]Department of Chemistry, Faculty of Basic Sciences, Golestan University, P.O. Box 155, Post Code 4913815759, Gorgan, Iran

Corresponding Author: Hosna Gholipourkanani, Department of Fisheries, Faculty of Agriculture, Gonbad Kavous University, Gonbad Kavous, Iran Tel: +989111159791 Fax: +981722224060

ABSTRACT

The anesthetic effects of clove-oil nano essence and clove oil loaded nano emulsions were studied in the gold fish, *Carassius auratus*. Acute lethality and the effects of exposures to various dosages of nano clove oil were measured. The estimated 24 h LC_{50} for nano clove oil was 31.28 ppm. Times to induction and recovery from anesthesia were measured and compared with clove oil under similar conditions. Nano clove oil at 50 ppm induced anesthesia faster when compared to clove oil. The recovery times for fish exposed to nano clove oil were generally shorter compared to similar concentrations of clove oil. In contrast, serum urea level in nano clove oil experiment increased significantly when compared to clove oil treated gold fish.

Key words: Nano emulsions, clove oil, gold fish, anesthesia

INTRODUCTION

Biological state with the partial or complete loss of sensation or loss of voluntary neuromotor control induced by chemical or nonchemical means is called anesthesia (Summerfelt and Moyle, 1990). Anesthetics induce a calming effect followed by loss of equilibrium, mobility and consciousness. Thus, anaesthesia is a way to minimize fish hyper motility during handling and transport and probable injuries which may reduce susceptibility to pathogens and infection (Woody *et al*., 2002; Matin *et al*., 2009) and abolish pain in fish (Summerfelt and Moyle, 1990). Anaesthetics are also used in aquaculture activities during artificial spawning, weighing, tagging, grading, blood sampling, surgery and surgical procedures (Matin *et al*., 2009). Nowadays, there are strict control in chemicals used in aquaculture, particularly with regard to their safety and efficacy (Taylor and Roberts, 1999). A number of considerations are important when choosing an anesthetic such as the type of anesthetic, dosage and the time it can affect the physiology of the fish, cost, availability and ease of use, toxicity to fish, humans and the environment. The choice may also depend on the nature of the experiment and species of fish are the important points (Ortuno *et al*., 2002; Soto and Burhanuddin, 1995; Akbulut *et al*., 2011). When choosing an anaesthetic such as efficacy, cost, availability and ease of use as well as toxicity to fish, humans and the environment (Soto and Burhanuddin, 1995; Akbulut *et al*., 2011) and the choice may also depend on the nature of the experiment and species of fish (Summerfelt and Moyle, 1990; Munday and Wilson, 1997).

Clove oil (natural product obtained by distillation of the flowers, stems and leaves of the clove tree *Syzygium aromaticum*) is considered to be a potential fish anaesthetic (Woody *et al*., 2002) and varies in its composition, eugenol, the active ingredient of clove oil, inhibits the synthesis of prostaglandin H (PHS) which accounts for the analgesic effect of clove oil (Thompson and Eling, 1989; Pongprayoon *et al*., 1991). Clove oil is generally believed to have few health concerns and is used in many countries as a topical anesthetic for humans (Soto and Burhanuddin, 1995) and fish (Ross and Ross, 2009).

Many researchers use alcohols or detergents as dissolving agents because clove oil does not easily emulsify with water without vigorous agitation (Munday and Wilson, 1997). Thus, even if clove oil is not harmful, the dissolving agent itself or the combination of chemicals, may have harmful effects on live organisms. More importantly, some field researchers do not use ethanol or detergents due to expense or limited access in remote field locations.

The term nanoemulsion is derived from the prefix "Nano" meaning one-billionth of something and "Emulsion" that is a heterogeneous system, consisting of at least one immiscible liquid, intimately dispersed in another in the form of droplets with diameters that in general exceed 0.1 mm (Guglielmini, 2008). Novel Nano Emulsifier System has been demonstrated to be an innovative system in the field of emulsions with nano size droplets. Using nanoparticles can overcome poor solubility problem of the compounds. The oil/water nanodispersions are a group of preparations in which the poorly soluble essence could be dissolved.

The aim of this study was to disperse clove oil essence by loading on two nano emulsifiers in order to increase its anesthetic effects in lower doses and decrease the needed time to surgery anesthesia and recovery time.

MATERIAL AND METHODS

Experimental fish: Approximately 100 sexually immature, gold fish were used, with an average weight of 8±2 g (Mean±SD) and a mean fork length of 100±20 mm. Dechlorinated tap water was used for all of the experiments. The fish population was distributed equally among two 150 L holding tanks, each maintained at 22°C with well aeration. The fish were maintained on a lighting regime representative of the local natural environment (12 L:12 D) and fed twice daily. Tanks were siphoned once every second day and approximately 2 L of water was exchanged during each cleaning.

Preparation and characterization of clove oil nanoemulsion: Nanoemulsions were prepared by mixing the separately prepared oily and water phases through spontaneous emulsification method (Ebrahimi and Salmanpour, 2014; Ebrahimi *et al*., 2013). The experiments were performed with various combinations of the values of three variables: the contents of tween-80 and lecithin in the aqueous phase (%, w/w) and the sonication time. Spontaneous emulsification consists of injecting an aqueous phase into an organic phase (ethanol) containing components of the oil core (egg lecithin, tween-80) under moderate magnetic stirring (15 min) conditions. Then, the mixture of nanoemulsion was homogenized by using an ultrasonic bath (Bondelin Sonorex equipment) for a 10 min. The organic solvent was completely removed by evaporation under reduced pressure at approximately 40°C. Then clove essential oil was added drop wise to nanoemulsion as final nanoemulsion composition containing clove oil at 8 mg mL^{-1} in the aqueous phase.

The average droplet size (z-average size) and size distribution of the obtained nanoemulsions were determined by photon correlation spectroscopy using a Zetasizer instrument (PCS, Nano ZEN 3000 Malvern Instruments Corp., U.K.) after dilution in water at 25°C under a fixed angle of 90° in disposable polystyrene cuvettes. In addition, the prepared oil nanodispersions were stored at 4°C to determine their physicochemical stability. The size distribution was represented by the Poly

Dispersity Index (PDI) values. The PDI is a measure for the width of the distribution. It is a measure for the width of the distribution ranging from 0 (monodispersed) to 0.5 (relatively broad distribution).

Acute toxicity of nano clove oil (LC_{50}): Acute toxicity of nano clove oil was ascertained by the OECD 203 "Fish, acute toxicity test" for the 24 h LC_{50} trials. At first Experimental fish (n = 60) were exposed to concentrations 3.125, 6.25, 12.5, 25 and 50, mg L^{-1} of clove oil loaded on nano emulsifiers dissolved in dechlorinated tap water and controls were placed in dechlorinated tap water with no tested substance added in five plastic basin filled to a volume of 10 L. Twelve gold fish were randomly used for each concentration and for the control group in 2 replicate. The fish and its behavior, water temperature, pH and oxygen saturation were monitored throughout the tests at individual concentrations and in the control aquarium. The total mortalities, behaviors, temperature and oxygen saturation were recorded every hour for the first 12 h of the experiment, every 3 h for the next 12 h. Fish were considered dead when there were no opercular beats observed for 15 continuously monitored min. Mean lethal concentration at 24 h LC_{50} also 24 h LC1and 24 h LC_{99} was calculated from mortality rates over the period of 24 h by the EPA probit analysis program version 1.5 software.

Onset and recovery from anesthesia: The observations of stages 5-anesthesia onset were made using nano clove oil and clove oil under the same experimental conditions. Three liter experimental aquarium was maintained at a temperature of 22°C with oxygen saturation greater than 85%. Gold fish (n = 60) were randomly distributed into the experimental tank at the treatment concentrations of either 50 ppm clove oil and 50 ppm of nano clove oil . Four replicates of 5 fish were used for each anesthetic concentration treatment of nano clove oil and clove oil.

The times to achieve sedation, narcosis, light and surgery anesthesia were also recorded. Once an individual fish had reached the onset of stage 5 anesthesia, a dip net was used to immediately remove it from the tank. The fish was then transferred to a 20 L, well-oxygenated 'Recovery' tank (i.e., no anesthesia present) maintained at 22°C and observed until it fully recovered. During this recovery period, the fish behavior was observed and times to recover opercular movement, complete equilibrium and active swimming were recorded.

Once a fish had been used for a treatment, it was left in the recovery aquarium for approximately 3 days prior to being transferred back to a 150 L recovery holding tank for the remainder of a 14-day observational recovery period. Any abnormal behavior or mortalities were recorded during this 14-day recovery period. Anesthesia and recovery stages are presented in Table 1.

Table 1: Stages of anesthesia in fish Keene *et al*. (1998) , modified from McFarland (1959), Jolly *et al*. (1972) and stages of recovery from anesthesia in fish Keene *et al*. (1998) modified from Hikasa *et al*. (1986)

Stages	Behavior in anesthesia stages	Behavior in recovery stages
1	Normal reacts to external stimuli, opercular rate and muscle tone normal	Decreased opercular movement
2	Light sedation slight loss of reactivity to external visual and tactile stimuli, opercular rate slightly decreased, equilibrium normal	Partial recovery of equilibrium, partial recovery of swimming motion
3	Deep sedation total loss of reactivity to external stimuli except very strong pressure	Total recovery of equilibrium
4	Slight decrease in opercular rate, equilibrium normal partial loss partial loss of muscle tone, increased opercular rate, reacts of equilibrium only to strong tactile and vibrational stimuli	Reappearance of avoidance swimming motion, reaction to external stimuli, behavioral response still stolid
5	Total loss of muscle tone and equilibrium; slow but regular of equilibrium opercular rate, loss of spinal reflexes	Swimming, rarely striking head firmly to sides or against bank of the tank
6	Loss of reflex total loss of reactivity, opercular movements slow and reactivity irregular, heart rate very slow, loss of all reflexes	Total behavioral recovery; normal swimming

Blood sample collection, hematological and biochemical assay: Separate group of 10 Gold fish obtained from experimental groups were used to determine blood parameters. Thus, the fish was blood sampled from caudal vein by sterile syringe.

Serum samples obtained by centrifugation of blood at 3000 g for 15 min were stored at -20°C until analysis. Total Protein (TP) (Henry, 1974), Albumin (Alb) (Fazlolahzadeh *et al*., 2011), cholesterol (CHOL) (Zlatkis *et al*., 1953) and Urea (Chaney and Marbach, 1962) by Parsazmoon® kit. Globulin content was calculated by subtracting albumin values from serum total protein.

Statistical analysis: The means and standard deviation of the parameters were estimated and the data analysis was done through running t- student test using SPSS16 software. The p values less than 0.05 were considered as significant.

RESULTS

During the Acute toxicity of nano clove oil the 24 h LC_{50} tests, the mean water temperature was 22°C, pH was 7.5 and water oxygen levels were 75-85% saturation. Based on tests of acute toxicity to gold fish, the 24 h lethal concentrations of nano clove oil were determined (24 h LC_{50} 31.28 mg L^{-1}, 24 h LC_1 10.31 mg L^{-1} and 24 h LC_{99} 94.89 mg L^{-1}). The first three concentrations (3.12, 6.25 and 12.5 ppm) survived the 24 h trial with 0% mortality. In concentration of 25 there was 2 mortality and in 50 ppm all of the fish were dead after 16 h of exposure.

Figure 1 shows the intensity vs. droplet size for the studied solutions. The obtained result shows that nano-droplets are below 100 nm.

The results of induction time in each anesthesia stage is shown in Table 2. The times to induce sedation, narcosis, light and surgery anesthesia in gold fishes were significantly quicker in nano clove oil group compared with clove oil group (p<0.05).

The needed time for 3 recovery stages are shown in Table 3. All of the recovery times were noticeably decreased in nano clove oil experiment (p<0.05).

Table 2: Induction times for gold fish anesthetized with 50 ppm clove oil and nano clove oil

	Stages			
Treatments	Sedation	Narcosis	Light anesthesia	Surgery anesthesia
Nano clove oil	0.83±0.41*	1.62±0.56*	2.35±0.8*	3.11±0.9*
Clove oil	1.86±0.71	4.17±1.72	6.72±2.92	9.63±3.47

Results are expressed as the Mean±SE, N: 20 for each anesthetic,*p<0.05

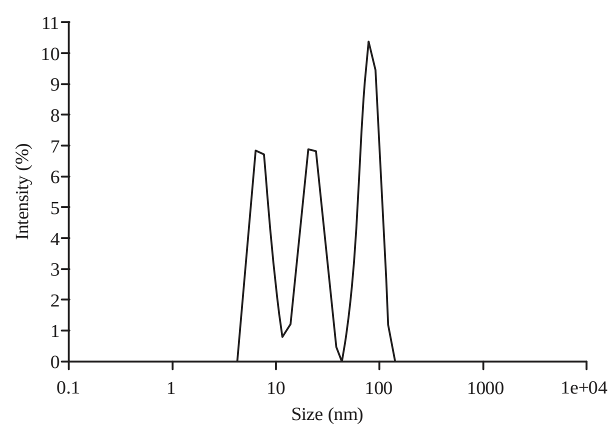

Fig. 1: Intensity vs. droplet size, in a nanoemulsion solution containing of clove essential oil, lecithin and tween-80 after 10 min sonication time

PCO2 showed more increase in fish of nano clove oil group ($p<0.05$). In contrary, there was significant decrease in sodium and potassium in nano clove oil group ($p<0.05$) (Fig. 2 and 3). The values of biochemical parameters are showed in Table 4. The levels of total protein, glucose,

Table 3: Recovery time for gold fish anesthetized with 50 ppm clove oil and nano clove oil

	Recovery stage		
Treatments	Opercular movement	Complete equilibrium	Active swimming
Nano clove oil	1.83±0.86*	6.85±2.04*	10.56±2.17*
Clove oil	3.09±1.28	8.21±3.01	12.27±3.81

Results are expressed as the Mean±SE, N = 20 for each anesthetic *p<0.05

Table 4: Biochemical parameters of gold fish in clove oil and nano clove oil anesthetic group

	Biochemical parameters						
Treatments	Urea mg (dL^{-1})	Glucose (mg dL^{-1})	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	Cholesterol (mg dL^{-1})	pH
Nano clove oil	12.35±0.87*	58.49±5.41	2.68±0.22	1.49±0.08	1.18±0.28	105.71±7.49	7.30
Clove oil	2.82±0.17	35.14±5.21	3.28±0.33	1.74±0.13	1.54±0.35	83.53±6.63	7.32

Values are Mean±SE values within the same column, with *are significantly different ($p<0.05$, n = 10)

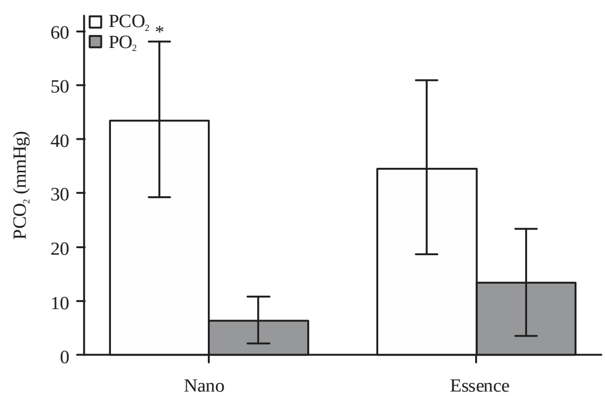

Fig. 2: Mean±SE of PCO_2 for arterial blood samples obtained during anesthesia in gold fish exposed to nano clove oil (nano) and clove oil (essence) at 50 ppm concentration. (*) values between nano and essence during anesthesia differ significantly ($p<0.05$)

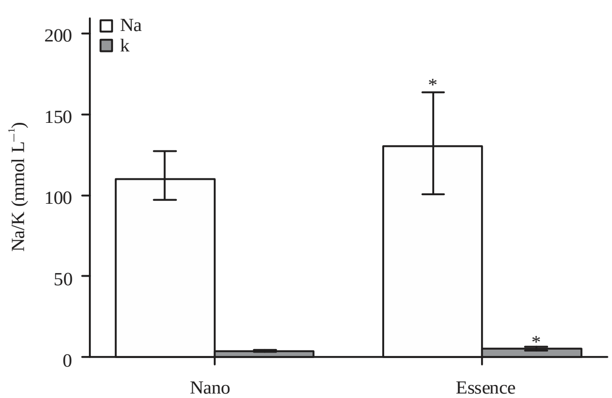

Fig. 3: Mean±SE of Sodium and potassium for arterial blood samples obtained during anesthesia in gold fish exposed to nano clove oil (nano) and clove oil (essence) at 50 ppm concentration. (*) values between nano and essence during anesthesia differ significantly ($p<0.05$)

cholesterol, albumin and globulin in blood of fish treated in both anesthetic group were not significantly different ($p>0.05$). There were significant increases ($p<0.05$) in the levels of urea in the nano clove oil anesthetic group compared with clove oil group.

DISCUSSION

In part of this study acute toxicity of nano clove oil to *Carassius auratus* is investigated from the point of view of nano clove oil use as an anaesthetic, with anaesthetizing baths.

Some anesthetics, such as quinaldine, MS-222, 2-phenoxyethanol and benzocaine are limited in their use because of concerns about their health effects on humans or fish (Lewis *et al*., 1985; Clark, 1990; Brown, 1993). However, Clove oil has been shown to be safe for humans (Miller *et al*., 1989) and the U.S. Food and Drug Administration has classified it as a generally considered as safe (GRAS) compound (Summerfelt and Moyle, 1990). It also, used as organic substance which does not require any withdrawal period which are rapidly emitted from blood and tissues (Fischer *et al*., 1990) in contrast to some anaesthetics like MS-222 (Ross and Ross, 2009). Therefore, clove oil seems to be more appropriate for use in commercial aquaculture situations, where anaesthetics may be used in large quantities by unskilled laborers and released in natural water bodies. On the other hand, clove oil is not easily dispersible in water, it should be first mixed with ethanol to make a stock solution of 1:10 or 1:9 (eugenol:ethanol) prior to use, to assist with emulsification, which can be harmful for fish and other organisms in water. Also for clove oil dispersing the water temperature should be more than 15°C which is not optimal for cold water fishes. We tried to apply nano technology for delivery of clove oil that is poorly water soluble with highly safe materials.

The optimal clove oil dosage to induce anaesthesia in *carassius arratus* varies between 15.5-100 ppm and is 10-30 mg L^{-1} for handling (Endo *et al*., 1972). Therefore, in present study, we used 50 ppm dosage for anesthesia.

Regarding no abnormalities such as apparent changes (color change), behaviors changes (ataxia) and tachy ventilation in fish behaviors monitoring at 14 days after anesthesia, clove oil loaded on lecithin based nano emulsion is safe for gold fish.

The efficacy of an anesthetic is dependent on several factors such as temperature (Stehly and Gingerich, 1999), size which seems to be positively correlated with anesthetic efficacy (Taylor and Roberts, 1999) and species (Stehly and Gingerich, 1999).

Clove oil has shown to immobilize fish effectively at low dosage. Rainbow trout within 1.8-0.6 min at concentrations of 20-100 ppm (Keene *et al*., 1998), white sturgeon (16 gm in weight) exposed to 25 ppm of clove oil required less than 3 min for the induction time (Taylor and Roberts 1999), Juvenile chinook salmon (*O. thsawytscha*) (7.0°C, weight 40 g) reached anesthesia or loss of reactivity after 2 min at a 20 ppm clove oil (Cho and Heath, 2000) and the 25 ppm of clove oil anesthetized juvenile mullet *Valamugil cunnesius* (9 g in weight) in less than a minute (Durville and Collet, 2001).

The present results showed that, the induction time of surgery anesthesia in nano treatment is approximately three times quicker than clove oil treatment. The shorter the exposure time to the anaesthetic bath, the smaller the amount of anaesthetic absorbed by the body and the faster its removal from the blood and recovery of the fish. once placed in clear water (Skjervold *et al*., 1999). Marking and Mayer (1985) recommend that the optimal anesthetic is the one which can induce anesthesia in 3 min and its recovery time is 5 min. Additionally, Gilderhus and Marking (1987) stated that the criteria of efficacy of fish anesthetic are induction time or total loss of equilibrium in 3 min or less, recovery of normal swimming in 10 min or less and no mortality of fish following 15 min of exposure. Furthermore, Brown (1993) added that in order for a chemical to be a good anesthetic agent it should have smooth induction and recovery.

Regarding recovery time, gold fish exposed to 50 ppm of nano clove oil demonstrated recovery within 10.56 min. It indicates that anesthetization with nano clove oil required shorter recovery time than clove oil (12.27 min), specially starting opercular movement was about 2 times faster in fish anesthetized with nano clove oil.

Munday and Wilson (1997) noted that recovery time after anesthesia with clove oil was two to three times longer than recovery from quinaldine, benzocaine, MS 222 and 2-phenoxyethanol. In addition, the recovery times for rainbow trout exposed to clove oil were six to ten times longer than in those exposed to similar concentrations of MS 222. According to Sladky *et al.* (2001), the longer of recovery time is caused by the increased duration of exposure or the physical properties of clove oil. It seems that, coating of anatomic structure, particularly gill epithelial by oily clove oil or eugenol, lead to prolonged exposure, potentially for sustained anesthetic effects. Indeed, the efficacy of anesthetic depends on its solubility in lipids which make the anesthetic drug to be permeable into cell wall of the gills (Ross and Ross, 2009). According the present results regarding the induction and recovery time of anesthesia, applying nano emulsions could improve clove oil permeability.

Biochemical profiles of blood can provide important information about internal environment of the organism. In our experiment with the gold fish serum urea increased significantly during anesthesia with nano clove oil. The elevation in the urea level in nano clove oil experiment may be due to gill dysfunctions as the urea excreted mainly through the gills (Murray and Harper, 1990) also this finding may be attributed to adverse injury of the kidney.

Fish in nano clove oil group had an increase in PCO2 of mixed arterial blood when anaesthetized in comparing with the fish in clove oil group. Along with hypoxia and hypercapnia, blood pH decreased, which is consistent with respiratory acidosis (Sladky *et al.*, 2001).

Significant increase in blood sodium and potassium concentrations were observed when fish were exposed to clove oil essence. This could be attributed to retention of sodium or potassium by the gills to compensate for loss of hydrogen ions (Heisler, 1993), muscle contractions causing movement of sodium or potassium ions out of myocytes into plasma, release of catecholamines into the circulation, which stimulated Na^+-H^+ exchange in RBC to maintain constant pH or artifactual increases caused by RBC lysis during the collection of blood samples.

In the study reported here, found 100% clove oil loaded on lecithin based nano emulsions to be an effective agent for immobilization of gold fish, characterized by rapid induction and shorter recovery times, compared with clove oil. This nano emulsion does appear to have promise as an effective and safe anesthetic for use on gold fish. However, further studies are needed to be conducted regarding more physiological effects such as tissue. Since, the information about anesthetic nano based emulsions are lacking, the further work is required to determine standard procedure of applying this new formulated anesthetic in different fish species intended for human consumption and in different water temperature. It is imperative that the efficacy and safety of novel immobilization chemicals for use in fish are systematically, qualitatively and quantitatively evaluated.

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