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The Anesthetic Role of Some Herbal Oils for Zebrafish

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Summary: Anesthetics have been important in ornamental fishes for transport and restriction of behaviors as well as for surgical applications and studies in science. Also, more economical and appropriate anesthetics can be common choice for these stiuations. In the study, a total of two hundred and fifty two zebrafish (forty two females and forty two males in each group) divided in three groups were studied as group A (Anise oil); group T (Thyme oil); group M (Mint oil). Anesthesia intake and lethal dose values were determined by following the anesthetic entry and recovery times. There was no anesthetic effect in group A, while anesthetic effects occured in groups T and M in concentrations 1, 5, 10, 20 and 30 mg/l. Similarity was found in groups T and M in terms of anesthesia entry and recovery times (P>0.05). Nevertheless, no mortality occured in group M. However, 5mg/l tyme oil anesthesia was determined to cause 50% mortality in male fishes (group T). It was also observed that mint oil has an anesthetic effect on zebrafish with increasing doses determined by anesthesia entry and recorvery times (group M, P<0.05). Thereby, mint oil may be an alternative herbal anesthetic agent for zebrafish. However, further studies are neccessary to show the herbal anesthetics effect on ornamental fishes.
Key words: Anise oil, mint oil, thyme oil, zebrafish

Bazı Bitkisel Yağların Zebra Balıklarında Anestezik Rolü

Özet: Süs balıklarında anestezikler, taşıma ve davranışların azaltılmasında olduğu gibi cerrahi müdahale ve bilimsel çalışmalarda da önemlidir. Genellikle daha uygun ve ekonomik anestezikler seçilmektedir. Çalışmada toplam ikiyüz elli iki adet (her grupta kırk iki dişi ve kırk iki erkek) zebra balığı üç gruba ayrıldı; grup A (Anason yağı), grup T (Kekik yağı) ve grup M (Mentol yağı). Anesteziye giriş ve lethal dozlar, anesteziye giriş ve çıkış sürelerine göre değerlendirildi. Çalışma sonunda grup A'da bulunan balıklarda anestezi görülmezken T ve M gruplarında 1, 5, 10, 20 ve 30 mg/l konsantrasyonlarında anestezik etkiler gözlendi. Anesteziye giriş ve çıkış süreleri bakımından grup T ve M'de istatistiksel olarak anlamlı farklılık belirlenmedi (P>0.05). Ayrıca grup M'de hiç ölüm gözlenmedi. Ancak, 5 mg/l kekik yağı anestezisinin erkek balıklarda (grup T) %50 oranında ölüme neden olduğu belirlendi. Mentol yağının ise artan dozlarına bağlı anesteziye giriş ve çıkış süreleri değerlendirilerek anestezik etki oluşturduğu belirlendi (grup M, P<0.05). Bu nedenle mentol yağı, zebra balıklarında alternatif bir bitkisel anestezik madde olarak kullanılabilir. Süs balıklarında bitkisel anesteziklerin etkilerinin belirlenmesi için daha fazla çalışmaya ihtiyaç bulunmaktadır.
Anahtar kelimeler: Anason yağı, mentol yağı, kekik yağı, zebra balığı

Introduction

Anesthetics have been used for reducing stress and protecting from death which are associated with transport, catching, measuring, weighing, vaccination, sampling, photographing and surgical applications of ornimental fishes (Summerfelt and Smith, 1990; Weber et al., 2011). According to the selection criteria of anesthetics, rapid entry and exit time of anesthesia are important as well as the anesthetic agents. Anesthetic agents, which are expected to have no residues in tissues, should be effective in low concentrations, and toxic dose range should be wide. Also an ideal anesthetic agent should be low-cost, easily applicable, and available.

The most known anesthetic for ornamental fishes is Trikain Methane sulphonalfanate-Trikain (MS222) however, it must be used with sodium bicarbonate buffer to balance the water pH (Serezli et al., 2005). Although Food Drug Administration (FDA) limits its usage for human health, it is still used for fish anesthesia. Some researchers reported that MS222 decreases the heart rate and increases the mortality of zebrafish (Huang et al., 2010). Also, Deebani et al., (2019) determined that MS222 may cause vasoconstriction and change the blood parameters. Nevertheless, nowadays, plant derived anesthetics have been preferred due to their several positive effects (Sharif Rohani et al., 2008; Metin et al., 2015). Metin et al. (2015) determined that 200mg/l dose mint oil

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can be used for rainbow trout safely. It was also reported that 3, 5 and 7 ml/l dose of mint oil was found effective on carps (Roohi and Imanpoor, 2015). However, the number of studies on mint oil anesthesia with different species of fishes such as spotted-sorubim, bass and tambaqui is limited (Façanha and Gomes, 2005; Souza et al., 2012; Sanchez et al., 2014). The researchers also indicated that carvone, which is a compund of mint oil, has the anesthetic efficiency. Another plant derived anesthetic agent named thyme oil has been used as a medicinal bandage for human through modern medicinal science (Baydar et al., 2004). Azad et al. (2014) explained that thyme oil has a protective role on immunity after anesthesia, and they also indicated that there is a need for further studies for this purpose. Anise oil known as a sedative, antispasmodic and antibacterial, and also has attracted attention with its essential oil contents in recent years. It was reported that alcoholic extracts and oils of anise may relax the muscles by antagonist action against contraction of different organs (Reiter and Brandt, 1985).

In recent years, Zebrafish has been an important species of ornamental fish used in studies on cancer, toxicology, medicine and genetics because of its anatomical structure, survival rate and easy nutrition (Spitsbergen and Kent, 2003; Hengartner and Horvitz, 1994). In this sense, aquaculture of Zebrafish to provide better quality and more economy has gained importance.

The purpose of this study was to assess the anesthetic effects of anise, thyme and mint oils in zebrafish. We also focused on the appropriate doses of these plant-derived anesthetics for entry and recovery times of anesthesia. There exist no data regarding the use of these oils for anesthesia, although they are used in medicinal studies if limitedly. We hope this study will improve our awareness of the effects of all these anesthetic agents for ornamental fish and scientific studies.

Material and Methods

Ethics statement

All procedures were carried out under ethics licence (Ethichal no: T2018-7) approved by the National Competent Authority for Animal Research in Tekirdag Namik Kemal University.

Animals and housing

The research was carried out in the Tekirdag Namik Kemal University, Faculty of Veterinary Medicine, Fisheries and Diseases Laboratory. Totally two hundred and fifty two zebrafish divided into three groups (n=84; male and female; age, 4 month) were studied as groups; group A (Anise oil); group T (Thyme oil); group M (Mint oil). The animals were acclimatized for seven days in a system tanks of 50 L with water (in 26 to 28 °C water, conductivity, 500 to 600 µS; pH, 8 to 8,2; hardness, 80 ppm; alkalinity, 80 ppm; dissolved oxygen, greater than 8 ppm; ammonia, 0 ppm; nitrate, 10 to 30 ppm; and nitrite, 0 ppm), in a room with a 12:12-h light-dark cycle. Feeding was carried out with an *ad libitum* commercial diet (Bioaqua) twice daily.

Experimental design

The experiment was conducted in 3 L of water in an aquarium with a volume of 5 litres. The anesthetic agents anise oil, thyme oil and mint oil were diluted in 99.8% ethanol, resulting in a concentrated solution 1:9. The experiment was executed with each twelve fishes (six male and six female) based on the 0.1, 0.5, 1, 5, 10, 20 and 30 mg/l concentrations of anesthetic solutions. Within the trial design, three anesthetics groups were studies as two replications (in twenty-one day of quarantine) which was modified from literatures (Chen et al., 2014; Masoumeh and Masoumeh, 2018) The anesthesia stages were analysed in three parts; first entry time of anesthesia, time of anesthesia and exit time from anesthesia. Anesthesia intake and lethal dose values were determined by following the anesthetic entry and exit times. After the induction period, exit times were evaluated, so the animals were removed from anesthesia tank and transferred to another aquarium containing anesthetic free water. Physiological responses such as respiration and behavior were also observed. The different stages of the anesthesia were described according to Cunha (2015) (Table 1). The behavioral patterns was monitored by a digital stopwatch and for 48 hours the animals were put back to an aquarium

Table 1. Behavioral characteristics observed in entry and recovery stages of anesthesia (Cunha, 2015)

Stage	Anesthesia entry	Recovery (exit)
I	Normal reaction to external stimuli Partially decrease of swimming motion	Slight movements in the opercular Slight swimming motion
II	Loss of muscle movement and balance Decreased respiration rate Decreased responses to external stimuli	Mild responses to external stimuli Balance recovery
III	No responses to stimuli Opercular movement absent Total loss of reflexes to external stimuli Immobility at the bottom of aquarium	Normal movement and swimming balance (Full recovery)

(80*35*45 cm) for monitoring mortality with feeding, ventilation and filtration.

Statistical analyses

The results were analyzed with Statistical Package for Social Sciences (SPSS) version 20.0 (Chicago, IL, USA). Data were tested for normality distribution and variance homogeneity assumptions. All the values were grouped, and the means and standard errors were calculated. One-way ANOVA was applied to all parameters of females and males to examine the difference between anesthesia doses. If the difference between groups proved to be significant (P<0.05), differences were evaluated with Tukey's test. On the other hand, in non-homogenous groups, differences between means were analyzed with Kruskal Wallis and following Mann Whitney U test between groups one by one. Also, statistical analysis of either anesthesia entry or recovery times between females and males were determined with Independent samples T-test, which considered P<0.05 to be significant.

Results

The chemical and physical parameters of water were 8.05±0.04 for pH, 26.87±0.16 C for temperature and 8.27±0.18 for oxygen. The difference in body length and weight between males and females is considered to be the natural result of gender although they are peer research material (Table 2).

In the study, neither anesthetic effect nor mortality was occured in group anise oil (group A). Although there was no mortality in group M (mint oil), mortality was occured in fishes which were anesthetized by thyme oil (group T). Also, there is no sedation occured in group T concentrations 0.1 and 0.5 mg/l and in group M concentrations 0.1, 0.5, 1 and 5 mg/l.

When the gender differences were considered, anesthesia entry and recovery times were found to be similar between males and females in group T (Table 2). However, males were found more sensitive than female ones to recovery times. While all the males could not recover from anesthesia in concentration of 10 mg/l of thyme oil, female fishes reached recovery without mortality. Nevertheless, mortality occured in a concentration of 20 mg/l thyme oil with ratio %100 of females (Tables 3-4).

There was no mortality in group M. The anesthesia entry times were found similar in the groups of females and males for concentrations of 10mg/l, 20mg/l and 30mg/l of mint oil (P:0.839, P:0.347, P:0.242, respectively 10mg/l, 20mg/l and 30mg/l concentrations). Also, males were more sensitive than females. Although no difference was found between females and males of anstehesia entry times, the anesthesia recovery times in concentration 20mg/l and 30mg/l were found higher in the males than in the females statistically (Table 5, P:0.04, 20mg/l: 68.50±10.60sec - 215.00±21.63sec; P:0.01, 30mg/l: 162.75±10.44sec - 192.13±24.35sec, respectively the females and males). Furthermore, in the females, anesthesia entry times were found statistically different by increasing concentrations of mint oil (Table 6, P<0.0001, 40.71±3.69sec, 26.88±1.66sec and 20.38±1.08sec, respectively 10mg/l, 20mg/l and 30mg/l). On the other hand, in the female, no differences was found between concentration 10mg/l (65.00±5.67sec) and

Table 2. Anesthesia entry and recovery times (sec) of zebrafish with different concentrations of thyme oil added in aquarium water

		Female (mean±SEM)	Male (mean±SEM)	P values
Weight (g)		0.78±0.05	0.64±0.04	0.60
Total lenght (cm)		3.69±0.08	4.01±0.07	0.19
Anesthesia entry time (sec)	**1 mg/l**	51.14±1.72	36.00±1.63	0.90
	5 mg/l	39.63±2.96	34.13±4.13	0.37
	10 mg/l	27.00±1.73	37.00±1.73	1.00
Anesthesia recovery time (sec)	**1 mg/l**	45.43±1.36	17.86±1.91	0.47
	5 mg/l	139.88±34.47	199.63±41.25	0.51
	10 mg/l	225.63±24.41	-	-

Table 3. Anesthesia entry and recovery times of female zebrafish with different concentrations of thyme oil

Anesthesia Doses	Anesthesia entry time (sec) (mean±SEM)	Anesthesia recovery time (sec) (mean±SEM)
1 mg/l	51.14±1.72[a]	45.43±1.36[a]
5 mg/l	39.63±2.96[b]	139.88±34.47[b]
10 mg/l	27.00±1.73[c]	225.63±24.41[c]
P values	<0.0001	<0.0001

[a,b,c] Means with different superscripts in the same column differ at P<0.05.

Table 4. Anesthesia entry and recovery times of male zebrafish with different concentrations of thyme oil

Anesthesia Doses	Anesthesia entry time (sec) (mean±SEM)	Anesthesia recovery time (sec) (mean±SEM)
1 mg/l	36.00±1.63	17.86±1.91[a]
5 mg/l	34.13±4.13	199.63±41.25[b]
10 mg/l	37.00±1.73	-
P values	0.65	0.001

[a,b] Means with different superscripts in the same column differ at P<0.05.

Table 5. Anesthesia entry and recovery times (sec) of zebrafish with different concentrations of mint oil

		Female (mean±SEM)	Male (mean±SEM)	P values
Weight (g)		0.70±0.04	0.51±0.04	1.49
Total lenght (cm)		3.50±0.11	3.49±0.13	0.45
Anesthesia entry time (sec)	**10 mg/l**	40.71±3.69	40.00±4.23	0.84
	20 mg/l	26.88±1.66	31.00±2.07	0.35
	30 mg/l	20.38±1.08	20.25±1.66	0.24
Anesthesia recovery time (sec)	**10 mg/l**	65.00±5.67	64.29±5.92	0.85
	20 mg/l	68.50±10.60[a]	215.00±21.63[b]	0.04
	30 mg/l	162.75±10.44[a]	192.13±24.35[b]	0.01

[a,b] Means with different superscripts between females and males, differ at P<0.05.

Table 6. Anesthesia entry and recovery times of female zebrafish with different concentrations of mint oil

Anesthesia Doses	Anesthesia entry time (sec) (mean±SEM)	Anesthesia recovery time (sec) (mean±SEM)
10 mg/l	40.71±3.69[a]	65.00±5.67[a]
20 mg/l	26.88±1.66[b]	68.50±10.60[a]
30 mg/l	20.38±1.08[c]	162.75±10.44[b]
P values	<0.0001	0.001

[a,b] Means with different superscripts in the same column differ at P<0.05.

Table 7. Anesthesia entry and recovery times of male zebrafish with different concentrations of mint oil

Anesthesia Doses	Anesthesia entry time (sec) (mean±SEM)	Anesthesia recovery time (sec) (mean±SEM)
10 mg/l	40.00±4.23[a]	64.29±5.92[a]
20 mg/l	31.00±2.07[a]	215.00±21.63[b]
30 mg/l	20.25±1.66[b]	192.13±24.35[b]
P values	0.001	0.001

[a,b] Means with different superscripts in the same column differ at P<0.05.

20mg/l (68.50±10.60sec) for anesthesia recovery time, although 30mg/l (162.75±10.44sec) concentration of mint oil was found significantly different (Table 6, P:0.001). Nevertheless, in the males, only the concentration of 30mg/l mint oil was found significant among anesthesia entry times (Table 7, P:0.001, 10mg/l, 20mg/l and 30mg/l, respectively, 40.00±4.23sec, 31.00±2.07sec and 20.25±1.66sec). Also, anesthesia recovery times in 20mg/l (215.00±21.63sec) and 30mg/l (192.13±24.35sec) of mint oil, statistically different than 10mg/l (64.29±5.92sec) in males (P:0.001).

Discussion and Conclusion

Anesthetic agents can be used for operation, sedation and reducing stress (Neiffer and Stamper 2009; Sisecioglu et al., 2009). Also, to slow down the reflexes and remove consciousness are important for transport as well as for scientific studies of ornamental fishes. In this study, we found that although anise oil has only a sedative effect, both thyme oil and mint

oil have anesthetic efficiency on zebrafish. Besides, in the study, water was blurred due to anise oil, so this situation could not give a choice to use this herbal for either sedation or anesthesia. Nevertheless, it was determined that mortality had a high ratio in concentration 10mg/l of thyme oil anesthesia. Also, males had more sensitivity than females due to death ratio in group thyme oil (group T). However, although 5 mg/l (LD50) and 10 mg/l (LD100) concentration of thyme oil was detected lethal dose for males, mortality in the females occurred in concentrations 10 mg/l (LD50) and 20 mg/l (LD100). Although there was no difference observed in anesthesia entry times in group thyme oil in the males, decreased times were observed in increasing concentrations significantly in females. On the other hand, significant revovery times were observed in increased concentrations in both females (P<0.0001) and males (P:0.001). Females entered anesthesia more quickly with increasing dose of thyme oil. It was stated that the concentration of 10 mg/l noted the highest entry and recovery time from other concentrations (entry times for 1mg/l, 5mg/l and 10mg/l respectively 51.14±1.72sec, 39.63±2.96sec and 27.00±1.73sec – P<0.0001; recovery times for 1mg/l, 5mg/l and 10mg/l respectively 45.43±1.36sec, 139.88±34.47sec and 225.63±24.41sec – P:0.001). On the other hand, although no difference was observed in the males for anesthesia entry times, significant difference (P:0.001) was found in recovery time between concentrations 1mg/l (17.86±1.91sec) and 5 mg/l (199.63±41.25sec). Based on our results, it's thought to that thyme oil could not be a good and safe anesthetic agent for zebrafish. However, in a study with grass carps, it was reported that thyme oil might be a better anesthetic agent (Masoumeh and Masoumeh, 2018). Nevertheless, further studies are neccessary to identify the thyme oil efficiency in fish anesthesia.

There was no mortality in group mint oil (group M) in both males and females. Also, there was no significant difference in anesthesia entry times between females and males. However, recovery times in males were longer than females in concentrations 20mg/l and 30mg/l than 10 mg/l (P:0.01). Metin et al. (2015) reported that mint oil anesthesia in concentration 200mg/l was effective and safe for rainbow trout. Roohi and Imanpoor (2015) detected that 3, 5 and 7ml/l oil mint oil concentrations may be useful for carp. They also indicated that carvone content in mint oil was important during anesthesia procedure. In our study, it could be said that concentrations 10, 20 and 30 mg/l of mint oil may used safely for zebrafish and thereby ornemantal fishes. However, Rezende et al. (2017) observed that mint oil anesthesia should not be recommended for sedation and handling due to their adverse effects on behaviors. On the other hand, Spanghero et al., (2019) reported that 80mg/l peppermint oil has an anesthetic effect on silver catfish. Also, it has been proven as an anesthetic for tropical fishes as sorubim or tambaqui according to some researches (Façanha and Gomes, 2005; Sanchez et al., 2014).

Nowadays, choice of anesthetics in ornemantal fishes depend on cost, avaliability, administration procedure and recovery rates. Also, herbal anesthetic agents have been used as an alternative to chemical ones which are risky for health and economically unfavorable. Especially mint oil may be the most appropriate herbal anesthetic agent. Nevertheless, there is limited study about herbal anesthetics, their usages and also effects on species of ornamental fishes. Thereby it is necessary further studies to identify the herbal anesthetics and their usages for ornamental fishes.

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