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Original Article

Anesthesia of juvenile Persian sturgeon, *Acipenser persicus*; Borodin 1897, by peppermint, *Mentha piperita*, extract – Anesthetic efficacy, stress response and behavior

Mohammad Mazandarani*[1], Seyyed Morteza Hoseini[2]

[1]Department of Fisheries, Faculty of Fisheries and Environmental Science, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
[2]Inland Waters Aquatics Resources Research Center, Iranian Fisheries Sciences Research Institute, Agricultural Research, Education and Extension Organization, Gorgan, Iran

Abstract: Anesthesia in large animals such as sturgeons is unavoidable, so in this regard it is important to choice a best anesthetic with lowest side effects in fish.in the present study anesthetic efficacy of mentha, *Mentha pipertia*, extract was studied in Persian sturgeon, *Acipenser persicus*, to find if it is a suitable anesthetic for this species. In this regard, the fish were subjected to 300, 500, 750 and 1000 mg L^{-1} mentha extract, or 150 mg L^{-1} clove oil, and behavioral response, stress indices, induction and recovery time were recorded. According to the results, the fish exposed to mentha extract showed more severe exciting movements than those exposed to clove oil. No histopathological effects were recorded in gills and kidneys of the fish after anesthesia with both mentha extract and clove oil. Exposure to either 150 mg L^{-1} clove oil or 750 mg L^{-1} mentha extract for 3 min resulted in the fish serum cortisol change. Result showed a significant increase in serum cortisol at 6 hrs after anesthesia in both mentioned anesthetic. However, in the fish anesthetized by clove oil, serum cortisol level returned to the pre anesthesia value, at 24 hrs post anesthesia. In the fish anesthetized by mentha extract, a further significant increase in serum cortisol level was observed at 24 hrs after anesthesia. However, it returned to the pre anesthesia level at 72 hrs after anesthesia. At all sampling time, serum cortisol levels of the fish anesthetized by mentha extract were significantly higher than those anesthetized by clove oil. Totally it is concluded that, in Persian sturgeon, use of mentha extract as anesthetic results higher stress compared to clove oil, in the other word, it can be used as a good anesthetic agent but clove oil is better.

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Introduction

Anesthetics are commonly used in fish handling, research, surgery, sorting and transportation to minimize stress (Summerfelt and Smith, 1990). Herbal anesthetics have received attention in recent years because of their beneficial effects beside anesthesia, such as antioxidant property (Chaieb et al., 2007; Gressler et al., 2014). The results of the studies on anesthetic properties of different essential oils in fish are variable, as some authors reported efficiency of essential oil to induce anesthesia (Gressler et al., 2014; Toni et al., 2014) and others failed to reach similar conclusion (Benovit et al., 2012). Therefore, it is necessary to examine the anesthetic efficacy of each herbal essential oil in different fish species.

Mentha, *Mentha piperita*, essential oil has antioxidant, antibacterial, antiviral and fungicidal activities (McKay and Blumberg, 2006). The essential oil is mainly (33-60%) comprised of menthol (Clark and Menary, 1981; Sang, 1982; Pittler and Ernst, 1998). Menthol was found to induce anesthesia in some fish species (Façanha and Gomes, 2005; Kasai et al., 2014). The anesthetic property of menthol was suggested to be related, at least in part, to the activation of $GABA_A$ receptors (Kasai et al., 2014).

To have appropriate anesthetics, knowledge on their side effects is necessary. Early diagnosis is difficult because of the lack of clinical signs in the first stage of poisoning and in many cases, paraclinical tests are needed for determining toxicity levels of drugs and chemicals. Histological analysis is a suitable tool to provide acceptable biomarker of toxicity of drugs. There are limited studies on histopathology as paraclinical test in anesthetic agents

*Corresponding author: Mohammad Mazandarani
E-mail address: mazandarani57@gmail.com
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(Padiyoo et al., 2017; Boijink et al., 2017; Velisek et al., 2005; Velisek et al., 2007).

Persian sturgeon, *Acipenser persicus*, is an important fish species inhabiting the Caspian Sea. It is reared by Iranian aquaculturists to produce meat and caviar. So use of anesthesia is unavoidable for handling, catheterization and sorting of this species on farms. At present, clove solution (mixture of powdered clove buds and water), clove oil and MS-222 are used to anesthetize Persian sturgeon. However, there is no data about the anesthetic efficacy of mentha extract in this species. Therefore, it was tested if mentha extract is a suitable anesthetic for Persian sturgeon, compared to clove oil. To determine its suitability, induction time, anesthetic dosage and stress response were compared between mentha extract and clove oil.

Materials and Methods

Fish and maintenance conditions: Persian sturgeons (76.55±2.17 g) were provided from a local farm and 120 fish were used in this experiment. The fish were stocked into two rectangular fiberglass tanks with 1000 L of water equipped with sponge filters. The fish were acclimatized to the experimental conditions for 20 days. During the acclimation period, the fish were fed daily with commercial feed (Biomar Co., France) at 1% of body weight. About 75% of the tanks' water was replaced with fresh water every day.

Preparation of anesthetic solution: Clove oil (purity= 99%) were purchased from Sigma Co. (St Luis, USA), whereas, mentha extract was purchased from Adonis Gol Darou Co. (Tehran, Iran). The mentha extract was obtained by steam-distillation of mentha leaves and contained 50% menthol, according to the product fact sheet. Both clove oil and mentha extract was dissolved in ethanol (1: 5 oil: ethanol) to facilitate solution in anesthetic bath water (Hoseini et al., 2015).

Induction and recovery time record: Ten fish were individually subjected to 300, 500, 750 and 1000 mg L^{-1} mentha extract, or 150 mg L^{-1} clove oil (ten fish per concentration), and induction (stage 5: decreased opercular beat) and recovery times and behavioral responses were recorded. For each concentration, five fish were tested from each 1000 L tanks, therefore, ten data was recorded for induction and recovery in each concentration (n=10). The fish were individually placed into an anesthetic bath (60 L plastic tank). The anesthetized fish were individually placed into fresh water (200 L tank) to recover. Both anesthesia and recovery baths were aerated. The concentration of clove oil was chosen based on a preliminary trial to find the concentration inducing anesthesia (stage 5) within 3 min. Each fish was only once exposed to anesthetic, and was excluded from experiment thereafter. Fresh water was used for induction and recovery baths preparation. The water source has the following characteristics (mean±SD): oxygen 7.12± 0.12 mg L^{-1}, pH=7.77±0.08, temperature= 20.45°C, unionized ammonia=0.001±0.0001 mg L^{-1} and nitrite =0.002±0.0003 mg L^{-1} and total alkalinity=243.8± 8.66 mg L^{-1} $CaCO_3$.

Stress response study: To study the stress response induced by mentha extract and clove oil, a concentration of each anesthetic was chosen to induce anesthesia (stage 5) within 3 min. Stage 5 anesthesia was chosen because fish can easily be handled for blood sampling at this stage. Accordingly, 750 mg L^{-1} mentha extract and 150 mg L^{-1} clove oil was chosen for the stress response study. A total number of 60 fish were randomly distributed into 6 fiberglass tanks filled with 250 L dechlorinated tap water. All tanks were provided by sponge filter and aerators. The fish were allowed to acclimatize to the experimental conditions for 30 days. During the acclimation period, the fish were fed daily with commercial feed (Biomar Co. France) based on 1% of body weight. About 75% of the water was replaced with fresh water every day. Before the start of the study, the fish were fasted for 24 hrs. Then, one fish was sampled from each tank. These samples were considered as pre anesthesia samples. Then, the tanks were assigned into two treatments, each contains 3 tanks. One treatment was exposed to 750 mg L^{-1} mentha extract, and the other was subjected to 150 mg L^{-1} clove oil. Exposure time for both anesthetics was 3 min. Immediately after 3 min, the fish were returned to their tanks to recover. Blood sampling were performed at 6, 24 and 72 hrs

Table 1. Behavioral observation of different anesthesia stages in Persian sturgeon subjected to mentha extract and clove oil.

Stage	Exhibited behavior	
	Mentha extract	Clove oil
0	Normal	Normal
1	Rapid movements, excitation and saltation	Slight excitation
2	Calmness and operculum closure, no swimming	Calmness and no response to tactile touch, normal swimming
3	Spiral and imbalance swimming, normal opercular pulse	Imbalanced swimming, normal opercular pulse
4	Loss of equilibrium, pectoral fin vibrating and irregular opercular pulse	Loss of equilibrium and irregular opercular pulse
5	Decreased opercular beat	Decreased opercular beat
Recovery	Fish regained its equilibrium	Fish regained its equilibrium

after anesthesia. At each sampling time, two fish were sampled from each tank, thus, sample size in all sampling time was six (n=6). The sampled fish was tagged by dorsal fin cutting and returned to their corresponding tank to avoid a change in fish density until 72 hrs and to ensure that the sampled fish were not sampled at the next sampling time. No anesthesia was applied in sampling time. Blood samples were collected by caudal puncture without anticoagulant. Capture and blood collection lasted less than 1.5 min.

Serum preparation and cortisol assay: Immediately after blood collection, the blood samples were allowed to clot at room temperature for 2 hrs. Then, the samples were centrifuged (7000 rpm, 10 min) to obtain the serum. The serums were kept at -20°C until analyses. Serum cortisol was determined by chemiluminescence technique using LIAISON® cortisol assay kit (DiaSorin Inc., USA) according to Hoseini and Hosseini (2010). The procedure was competitive luminometric assay based on a solid phase antigen-linked technique. In this procedure, cortisol is used for the coating of the solid phase (magnetic particles). The tracer consisted of a highly specific monoclonal antibody, which is labeled with an isolumonal derivative.

Histological analysis: For histopathological analysis, tissue samples were taken from gill and middle parts of kidney after anesthesia (before recovery test). Tissues were fixed in 10% buffered formalin and after 24 hours the fixative were renewed. Routine histological methods were followed and totally 5 μm tissue sections prepared (Robert, 2012; Eagderi et al., 2013), the sections were stained using Harris's hematoxylin and eosin (H&E) method and microscopically examined at magnifications of 40 to1,000 (Nikon, Japan). In the present study, fish histological analysis were done for mentha extract and clove oil in 750 mg L^{-1} and 150 mg L^{-1} concentrations, respectively.

Water physico-chemical parameters: Water dissolved oxygen, pH, temperature, unionized ammonia and nitrite was recorded twice a week during the experiment using Portable Physico-Chemical test Kit (Wagtech Projects Ltd, Wagtech Court, Station Road, Thatcham, Berkshire, RG19 4HZ).

Statistical analyses: Normality of the data was confirmed by Shapiro-Wilk test. The mean of induction (n=10) and recovery (n=10) time, and serum cortisol levels (n=6) were compared among the anesthetics' concentrations using one-way ANOVA and Duncan test to find significant difference. $P<0.05$ was considered as significance. Data are presented as mean±SD. All analyses were performed using SPSS v. 16.

Results

Behavioral observations of the fish exposed to mentha extract and clove oil are summarized in Table 1. Five anesthesia stages were identified in the fish anesthetized either by mentha extract or clove oil. Generally, the fish exposed to mentha extract showed more severe exciting movements to anesthetic contact than those exposed to clove oil.

The induction time of the fish exposed to 300 and 500 mg L^{-1} mentha extract was similar, being significantly longer than 750 and 1000 mg L^{-1} mentha extract and 150 mg L^{-1} clove oil (Fig. 1). Also, the recovery time of the fish exposed to 300 and 500 mg

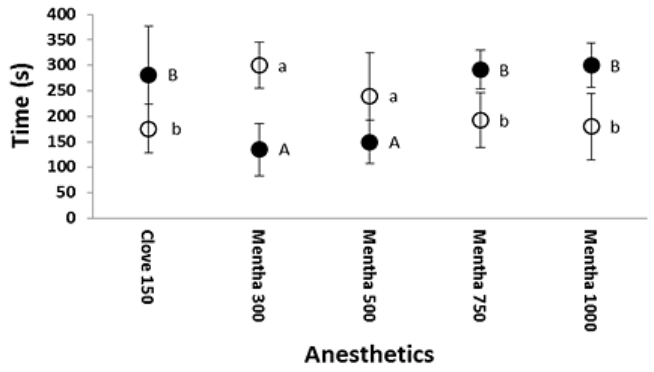

Figure 1. Induction (open circles) and recovery (solid circles) time of Persian sturgeon anesthetized by clove oil or mentha extract. Different lowercase letters show significant difference among the induction time, whereas, different uppercase letters show significant difference among the recovery time.

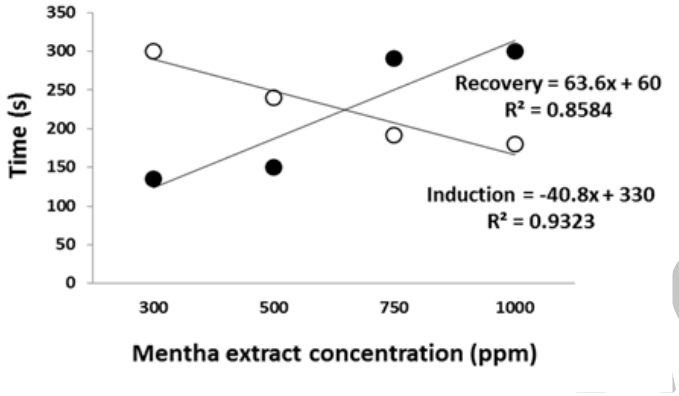

Figure 2. Correlation between induction or recovery time and mentha extract concentrations.

L^{-1} mentha extract was similar, being significantly shorter than 750 and 1000 mg L^{-1} mentha extract and 150 mg L^{-1} clove oil (Fig. 1). Increase in mentha extract concentration resulted in a significant decrease in induction time, and an increase in recovery time (Fig. 2).

Anesthesia with both anesthetics resulted in a significant increase in serum cortisol at 6 hrs after anesthesia (Fig. 3). However, in the fish anesthetized by clove oil, serum cortisol returned to the pre anesthesia value, at 24 and 72 hrs after stress. In the fish anesthetized with mentha extract, a further significant increase in serum cortisol was observed at 24 hrs after anesthesia, however, the cortisol returned to the pre anesthesia level at 72 hrs after anesthesia. In histological analysis, no pathological signs were observed in the fish anesthetized with both clove oil

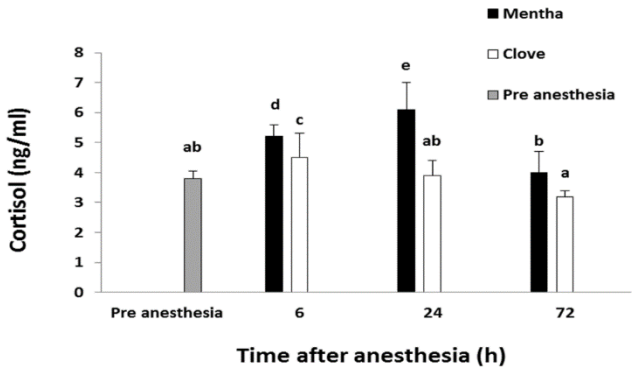

Figure 3. Serum cortisol levels of Persian sturgeons subjected to 750 mg L^{-1} mentha extract or 150 mg L^{-1} clove oil. Different letters above the bars show significant difference among the treatments.

and mentha extract (Fig. 4).

Discussion

Anesthesia of fish by essential oils can offer various health benefits such as antioxidant and antimicrobial effects (Chaieb et al., 2007; Gressler et al., 2014). The most well-known herbal essential oil to anesthetize fish is clove oil. Its anesthetic efficacy was shown in many fish species (Soto and Burhanuddin, 1995; Roubach et al., 2005; Hoseini et al., 2015) and Persian sturgeon (Bagheri and Imanpour, 2011; Vali et al., 2016). Mentha extract has frequently been less studied as an anesthetic in fish, and in this regard there are no data for its application in Persian sturgeon. Mentha extract caused more severe exciting movements than clove oil. Early excitation due to anesthetic exposure of fish have been reported (Hoseini et al., 2010), which may be due to sensation of chemical compound by the olfactory and gustatory systems. The more severe reactions to mentha extract exposure compared to clove oil may be as a result of activation of cold nociceptors (Wei and Seid, 1983; Kasai et al., 2014). Also, behavioral response at stage 2 anesthesia (operculum closure) seems to be related to gill cold nociceptor activation, which forced the fish to prevent gill exposure to mentha extract. Kasai et al. (2014) reported rapid movements in fish anesthetized by menthol, which could be due to cold nociceptor activation.

In the present study, 150 mg L^{-1} clove oil induced anesthesia stage 5 after about 173 s, which is longer

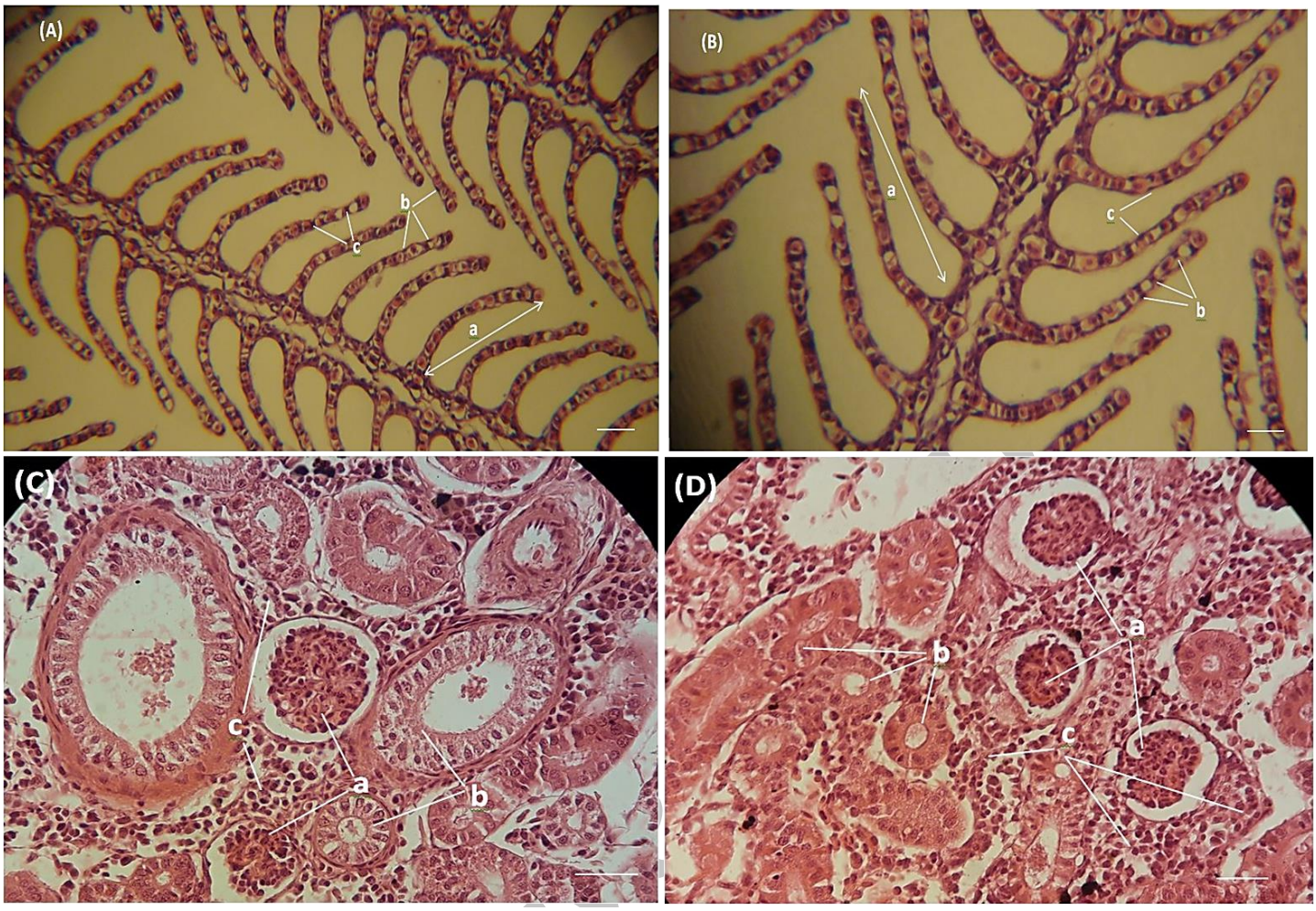

Figure 4. Histological analysis of gill and kidney of Persian sturgeon after deep anesthesia with eugenol and mentha extract (H&E). (A) normal gill structure in fish anesthetized with eugenol, secondary lamella (a), pillar cell (b), epithelial cell of secondary lamella (c), (bar=60 µm), (B) normal gill structure in fish anesthetized with mentha extract, secondary lamella (a), pillar cell (b), epithelial cell of secondary lamella (c), (bar=100 µm), (C) normal kidney structure in fish anesthetized with eugenol, kidney glomerulus (a), tubules (b), interstitial tissue (c), (bar=100 µm) and (D): normal kidney structure in fish anesthetized with mentha extract, kidney glomerulus (a), tubules (b), interstitial tissue (c), (bar=100 µm).

than the time reported by Hoseini et al. (2015) for 2-20 g iridescent shark, *Pangasius hypophthalmus* (at 25°C) and Waterstrat (1999) for 19 g channel catfish, *Ictalurus punctatus* (at 23°C). The reason for longer induction time obtained in the present study compared to those reports may be due to the higher fish weight and lower water temperature in the present study compared to the previous ones. Increase in fish weight affects induction time. For example, Hoseini et al. (2015) found a decrease, and Stehly and Gingerich (1999) found no change in induction time due to fish weight increase. Park et al. (2009) showed that temperature elevation significantly decreased induction and recovery time of clove oil in rock bream, *Oplegnathus fasciatus*.

There is little information about the induction and recovery time of mentha extract in fish. Façanha and Gomes (2005) found 105-358 s induction time for 250-50 mg L^{-1} menthol in tambaqui, *Colossoma macropomum*, which is lower than the present concentrations. Kasai et al. (2014) reported induction times of 50-300 s for 156-31 mg L^{-1} menthol in Japanese medaka, *Oryzias latipes*, goldfish, *Carassius auratus* and zebrafish, *Danio rerio*. It is suggested that mentha extract is not as efficacious as clove oil for Persian sturgeon. The reason for the difference between the results of the present study and those conducted by Façanha and Gomes (2005) and Kasai et al. (2014) is not exactly demonstrated. Beside the environmental factors, which affect anesthetic

efficacy, species-specific traits are important factors affecting anesthetic efficacy. In the case of mentha extract, the present study suggests a potential pain generation in Persian sturgeon. As suggested previously (Wei and Seid, 1983; Kasai et al., 2014), menthol, the major component of mentha extract, activates cold nociceptors and generates a pain sensation. Also, Kasai et al. (2014) reported that rapid movement and pain sensation is induced by high menthol concentration (469 mg L^{-1}), but not low ones (78.1-312.5 mg L^{-1}). Rapid movement and opercular closure may be evidence of pain generation in Persian sturgeon, in this study. In the present study, also, it was observed that the recovered Persian sturgeons were inactive for 0.5-6 hrs, particularly at the high mentha extract concentrations. They remained inactive in the tanks and initiated swimming, only after a tactile touch.

There are many studies showing stress response as a result of anesthesia in fishes (Auperin et al., 1997; Holloway et al., 2002; Park et al., 2009; Hoseini et al., 2010). Cortisol is the best indicator of stress in fish. The present results, generally, showed that mentha extract induced a greater stress response than clove oil. Also, cortisol remained elevated until 24 hrs after anesthesia. The results of serum cortisol trend of different fish species after anesthesia are variable. Park et al. (2008) showed no change in serum cortisol in kelp grouper, *Epinephelus bruneus* 1-6 hrs after anesthesia with clove oil, but a significant increase at 12-48 hrs. On the other hand, Park et al. (2009) found a significant increase in serum cortisol of rock bream, *O. fasciatus* 0-12 hrs after anesthesia with clove oil, which returned to normal level 24-72 hrs after anesthesia. There is no study monitoring serum cortisol in fish anesthetized by mentha extract. Although no histopathologic effects was recorded in anesthetized fish with 750 mg L^{-1} peppermint extract, the higher cortisol response, longer period of cortisol elevation and behavioral responses (fish with minimum activity) suggest that the Persian sturgeons anesthetized by mentha extract experienced more stress in compared to use of clove oil at dose of 150 mg L^{-1} in this fish. However, further studies are necessary to find the reason for these results.

Histological analysis is an applied method to diagnose tissue damage in medication toxicity (Roberts, 2012). There are limited studies about histological effects of anesthetic agents in fish; for example anesthesia with 2-phenoxyethanol in sheatfish, *Silurus glanis* L. (Velisek et al., 2007), clove oil in common carp, *Cyprinus carpio* (Velisek et al., 2005), Benzocaine in *Haludaria fasciata* (Padiyoo et al., 2017) and benzocaine and eugenol in *Colossoma macropomum* (Boijink et al., 2017) led to capillary ectasia of gill filaments with no pathologic effects on other tissues.

In conclusion, although mentha extract can be used for anesthesia in Persian sturgeon, yet it is not as suitable as clove oil, because of lower efficacy and higher stress induction.

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چکیده فارسی

ارزیابی بیهوشی تاسماهی ایرانی (*Acipenser persicus* Borodin, 1897) جوان با کمک عصاره نعنا (*Mentha piperita*): اثرات داروی بیهوشی، پاسخ‌های استرسی و رفتاری

محمد مازندرانی[1]*، سید مرتضی حسینی[2]

[1] گروه تکثیر و پرورش آبزیان، دانشکده شیلات و محیط زیست، دانشگاه علوم کشاورزی و منابع طبیعی گرگان، گرگان، ایران.
[2]سازمان تحقیقات، آموزش و ترویج کشاورزی، موسسه تحقیقات علوم شیلاتی ایران، مرکز تحقیقات ذخایر آبزیان آبهای داخلی، گرگان، ایران.

چکیده:

استفاده از بیهوشی در حیوانات بزرگ از جمله ماهیان خاویاری امری اجتناب ناپذیر است. بنابراین در این راستا انتخاب بهترین ماده بیهوش کننده با کمترین عوارض جانبی از اهمیت بالایی برخوردار است. در این بررسی اثرات بیهوش کنندگی عصاره نعنا (*Mentha pipertia*) در تاسماهی ایرانی و این‌که آیا این ماده می‌تواند داروی بیهوشی مناسبی در این گونه باشد یا نه؟ مورد بررسی قرار گرفته است . به این منظور ماهیان با دوزهای ۳۰۰، ۵۰۰، ۷۵۰ و ۱۰۰۰ میلی‌گرم/لیتر عصاره نعنا و ۱۵۰ میلی‌گرم در لیتر روغن میخک مورد مواجهه واقع شده و پاسخ‌های رفتاری، شاخص‌های استرسی القاء و بازگشت از بیهوشی در ماهیان ثبت و بررسی گردید. بر اساس نتایج ماهیان بیهوش شده با عصاره نعنا در مقایسه با عصاره میخک علایم هیجانی بیشتری از خود نشان دادند. در این بررسی هیچ آسیب بافتی در آبشش و کلیه ماهیانی که با عصاره نعنا و اسانس میخک بیهوش شدند ثبت نگردید. مواجهه با عصاره نعنا ۷۵۰ میلی‌گرم/ لیتر و روغن میخک ۱۵۰ میلی‌گرم /لیتر منجر به افزایش کورتیزول سرم خون در ۶ ساعت پس از مواجهه گردید. در ماهیان بیهوش شده با روغن میخک ۲۴ ساعت پس از بیهوشی کورتیزول سرم به سطح قبلی پیش از بیهوشی برگشت. اما در ماهیان بیهوش شده با عصاره نعنا ۲۴ ساعت پس از بیهوشی میزان کورتیزول سرم به‌طور معنی‌داری بالاتر از زمان پیش از بیهوشی ثبت گردید، در این گروه نیز میزان کورتیزول پس از ۷۲ ساعت به سطح پیش از بیهوشی برگشت. در این بررسی در تمام زمان-های نمونه برداری سطح کوتیزول سرم به‌طور معنی‌داری در ماهیان بیهوش شده با عصاره نعنا بیشتر از ماهیان بیهوش شده با روغن میخک بوده است. نتیجه‌گیری این بررسی حاکی از این امر بوده است که استفاده از عصاره نعنا در مقایسه با روغن میخک به عنوان بیهوش کننده در تاسماهی ایرانی استرس بالاتری ایجاد می‌کند به عبارت دیگر این ترکیب می‌تواند به‌عنوان یک بیهوش کننده در این ماهی مورد استفاده قرار گیرد اما روغن میخک عملکرد بهتری دارد.

کلمات کلیدی: بیهوشی، تاسماهی ایرانی، *Mentha piperita*، استرس، کورتیزول.