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Anesthetic and Physiological Effects of Clove oil and Lidocaine-HCl on the Grass Puffer, *Takifugu niphobles*

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Abstract : The aim of this study was to determine the physiological response and the applicable concentration ranges of anesthetic clove oil and anesthetic lidocaine-HCl, and to investigate the synergistic effect of a mixture of these two anesthetics on the grass puffer (*Takifugu niphobles*). The anesthesia times decreased and the recovery times increased with increasing concentrations of clove oil and lidocaine-HCl. Applicable concentration ranges for long-term transportation requiring more than 1 hour were 2 ppm for clove oil and 50 ppm for lidocaine-HCl. With mixtures of the two anesthetics, the anesthesia time decreased as the admixture concentration of clove oil and lidocaine-HCl increased. Anesthesia times of experimental groups with the combined anesthetics were shorter than those with the same concentrations of clove oil or lidocaine-HCl alone. Plasma cortisol concentrations were highest at 6 hours in all experimental groups anesthetized with the mixture of clove oil and lidocaine-HCl, while all groups with clove oil or lidocaine-HCl alone had the highest plasma cortisol concentrations at 12 hours. Plasma glucose concentrations were highest at 12 hours in experimental groups anesthetized with the mixture of clove oil and lidocaine-HCl, while groups with clove oil or lidocaine-HCl alone had the highest plasma glucose at 24 hours. The results of this study provide basic information about anesthetics and the synergistic effect of mixtures of anesthetics in this fish species. This information should be useful for aquaculturists who require methods for safe and easy fish handling, and for transporters who require that minimal stress is imposed on fish during transport.

Key words : clove oil, grass puffer, lidocaine-HCl, synergy effect, *Takifugu niphobles*

1. Introduction

The grass puffer, *Takifugu niphobles*, is a teleost fish with a wide distribution in Korea, Japan and the Northwest Pacific Ocean. This species is one of around 24 puffer fish species in the Tetraodontine genus *Takifugu*. Grass puffer belongs to the family Tetradontidae and exhibits a lunar-synchronized spawning rhythm: spawning occurs on a beach several hours before high tide and only during several days around the new moon and full moon

from April to August (Honma et al. 1980; Yamahira 2004). Since spawning occurs at the same place every year and every two weeks (spring tide) during a spawning season (Nozaki et al. 1976), we are aware of the time and place of spawning, and thus can obtain spawning fish as well as non-spawning fish that subsist in the southern coastal area of Korea. Recently, Masaomi et al. (2013) reported that gonadal development and fertility of triploid grass puffer is induced by cold shock treatment, and researchers took note of the potential possibility of breeding this species. Breeding puffer fish species in captivity involves handling the captive fish broodstocks

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removing the teeth of broodstocks, and the collection of gametes both from males and females for use in *in vitro* fertilization (Gallego et al. 2013). The aquaculturist has to handle grass puffers for artificial spawning and breeding, and can be bitten by the teeth of captive fish. Handling grass puffers is difficult because of toxicity, bites and inflation of captive fish. Anesthesia is necessary for easy and safe handling of the grass puffers and for developing aquaculture technology.

Anesthetics are used in fisheries primarily to immobilize animals so they can be handled more quickly and less stressfully (Summerfelt and Smith 1990). Anesthesia facilitates the following operations: weigh and measure; mark and tag; study fish physiology and behavior; perform surgery; collect fish with scuba and in tidal pools; photography; preparation for live shipment and transport; manually spawn; inject vaccines and antibiotics; and collect blood and other tissues (Park et al. 1998a, 1998b). An ideal anesthetic will have considerations for toxicity (safety to handlers and to fish), efficacy, price, regulations for use, and the purpose for using an anesthetic. In addition to these considerations, an ideal anesthetic agent should meet the following criteria: (1) anesthesia time within 3 min and recovery time within 5 min; (2) no effect on the physiology and the movement of the handled fish; (3) excreted from the fish thereby eliminating a requirement for a withdrawal period; (4) no side effects and no accumulative effects of repetitive anesthetizing; and (5) cost-effective (Park et al. 2003). Traditionally, chemicals such as urethane, ether and chloroform were used to anesthetize fish. However, these substances are now restricted because they all contain carcinogens (Hasler and Meyer 1942). Currently, tricaine methanesulfonate (MS-222) is widely used to anesthetize fish, including fish for food, although it is considered mildly toxic as an edible anesthetic according to the US Food and Drug Administration (FDA). The FDA requires that fish treated with MS-222 undergo an obligatory 21-day withdrawal period (Summerfelt and Smith 1990).

The active ingredient of clove oil is eugenol [4-allyl-2-methoxyphenol]. It is an inexpensive and safe anesthetic that has been used as a substitute for such traditionally employed anesthetics as MS-222 (Seol et al. 2007). Compared with MS-222, clove oil is characterized by a faster induction of anesthetic effects, a prolonged recovery time, and a narrower range of safe doses in at least three teleost species (Hikasa et al. 1986; Keene et al. 1998; Sladky et al. 2001). Also, clove oil is considered to be safe, inexpensive, and non-toxic to the environment, and

does not require a withdrawal period such as is required with other anesthetic chemicals (Park et al. 2008). The anesthetic compound, lidocaine-HCl [2-(diethylamino)-N-(2, 6-dimethylphenyl) acetamide hydrochloride], is a white, water soluble powder which was first administered to fish by Carrasco et al. (1984). Lidocaine-HCl has been safely used in the dentistry industry, and has been proven as a safe substitute for use on some freshwater and marine fishes in Korea (Park et al. 1998a, 1998b; Hur et al. 2005). A number of studies have investigated its effectiveness, economic viability, reusability, toxicity and side-effects when used as a fish anesthetic (Summerfelt and Smith 1990; Park et al. 1998a, 1998b).

No research has been performed to test the anesthetic effect of a clove oil and lidocaine-HCl mixture and the possible synergistic effect of these two anesthetics combined, despite the importance of anesthesia as an essential technique for handling experimental fish. Thus, the aim of this study is to determine the physiological response and the applicable concentration ranges of anesthetic clove oil and anesthetic lidocaine-HCl, and the possible synergistic effects of mixtures of the two anesthetics in grass puffer.

2. Materials and Methods

On 12 October 2015, 4,000 grass puffers, *Takifugu niphobles* were obtained by fishing from the dock of the Korea Maritime and Ocean University (KMOU), Korea. The fish were reared and bred in the aquarium of the Fishery Genetics and Breeding Sciences Laboratory of the KMOU, Korea. Grass puffers used in the experiment were measured using a vernier caliper (Mitutoyo, Japan) and an electronic balance (Shimadzu, Japan). Mean body lengths and body weights of the fish used in this study were 10.2 ± 2.21 cm ($n = 50$) and 160.3 ± 37.6 g ($n = 50$), respectively. The fish were reared in five 400 L glass tubes containing seawater with a total water salinity of 32 ppt, and having a circulation pump, an aeration system and a temperature control system. Water temperature was maintained at $20 \pm 1^\circ\text{C}$ using a heater (OKE-6422H, IMTech, Korea). Dissolved oxygen was maintained at 6.5 ± 0.3 ppm. The values of salinity and dissolved oxygen were checked by a refractometer (OxyGuard International, Denmark). The systems used for anesthesia and recovery were 10 L plastic, rectangular parallel-piped tubes with identical conditions as the breeding tubes of water composition, salinity, temperature, and dissolved oxygen.

Ninety fish were randomly selected to investigate the anesthetic effects of clove oil (containing 85% eugenol;

Table 1. Stages of anesthesia and recovery in a clove oil and lidocaine-HCl efficacy test performed on the grass puffer, *Takifugu niphobles* (modified from stage 1 to stage 22 of Summerfelt and Smith (1990) and Woolsey et al. (2004))

Stage	Characteristic behavior
Anesthesia	
A1	Normal swimming; operculum movement and normal general movement
A2	Swimming speed slowed; rolling from side to side
A3	Partial loss of equilibrium; swimming erratically
A4	Complete loss of equilibrium; swimming perfectly inside-out; pectoral fin, pelvic fin and dorsal fin movement stop
A5	Little sedation; anal fin and tail fin movement stop
A6	Perfect sedation; only operculum movement
A7	Operculum movement ceased
Recovery	
R1	Resume operculum movement
R2	Preferential movement of pectoral fin and tail fin
R3	Dorsal fin, pelvic fin, and anal fin movement
R4	Swimming perfectly inside-out
R5	Swimming erratically; redressing the balance
R6	Normal swimming; responsiveness to visual stimuli

Sigma, USA), lidocaine-HCl (HongSung Chemical, Korea), and mixtures of the two anesthetics. All fish were fasted for 24 hours before the study. For anesthesia and long-term transportation experiments, one fish was randomly selected from the breeding tube using a net and transferred to an anesthesia tube. After fish were anesthetized in the anesthesia tube, they were immediately moved to a recovery tube. The anesthetic effect decision-based table (Table 1) was modified from data reported by Summerfelt and Smith (1990) and Woolsey et al. (2004). Anesthesia time was defined as the time duration from when fish were placed in the anesthesia water to the time of stage A6 state of Table 1, in which fish were perfectly sedate but with only a minimum opercular movement. Recovery time was defined as the time duration from when the fish were placed in recovery water to the time of stage R6 state of Table 1, in which normal swimming and responsiveness to visual stimulation resumed.

The anesthetic effect of clove oil was investigated at six concentrations: sham control, 10, 20, 30, 40 and 50 ppm. The stock solution of clove oil was dissolved in 95% methanol (Sigma, USA) at a ratio of 1:10. Sham control was dissolved 5 mL of 95% methanol, and maintained 500 ppm concentration of 95% methanol. The anesthetic

effect of lidocaine-HCl was examined at seven concentrations: sham control, 500, 600, 700, 800, 900 and 1,000 ppm. To neutralize the lidocaine-HCl solution and to amplify its effect, NaHCO₃ (Sigma, USA) was included in all lidocaine-HCl solutions and the sham control at a total concentration of 1,000 ppm (Carrasco et al. 1984; Park et al. 1998b, 2009). The synergistic anesthetic effect of a clove oil and lidocaine-HCl combination was investigated by mixing the two anesthetics in ten concentrations: sham control, 30 + 800, 30 + 900, 30 + 1,000, 40 + 800, 40 + 900, 40 + 1,000, 50 + 800, 50 + 900 and 50 + 1,000 ppm mixtures of clove oil and lidocaine-HCl, respectively. Each group was prepared with 1,000 ppm NaHCO₃. Sham control was dissolved 5 mL of 95% methanol and 10 g of NaHCO₃, and concentrations of methanol and NaHCO₃ were maintained 500 ppm and 1,000 ppm, respectively. The effects of long-term transportation in clove oil and lidocaine-HCl were investigated at the following anesthetic concentrations: sham control, 2, 4, 6, 8 and 10 ppm for clove oil; and sham control, 50, 100, 150, 200, 250 and 300 ppm for lidocaine-HCl.

The physiological responses were determined from blood analyses of plasma cortisol and glucose at set time intervals. Blood samples were collected from five randomly selected fish at 0 (pre), 1, 6, 12, 24 and 48 hours post anesthesia. Fish used in blood analyses were not involved in the anesthetic effect experiments. Blood was collected from the caudal vasculature using a disposable syringe (3 mL, Sung Shim Medical Co., Ltd, Bucheon, Korea) containing heparin sodium (Shin Poong Pharm Co., Ltd, Ansan, Korea). Blood was extracted within a 1 minute period to minimize handling stress, and allowed to sit for 10 minutes at room temperature prior to centrifugation (Centrifuge Micro 17R, Hanil Science Industrial Co., Ltd, Incheon, Korea) for 10 minutes at 20,000 g. The collected plasma was transferred to another 1.5 mL microtube and stored at -70°C (CLN-50UW Nihon Freezer, Nihon Co., Japan) prior to analysis. The plasma cortisol concentration was measured using the 1470 WIZARD Automatic Gamma Counter (Cobra, Packard Co., Ramsey, MN, USA) after the antigen antibody response was derived using Coat-A-count TKCO Cortisol RIA Kit (DPC, Los Angeles, CA, USA) according to the method of Donaldson (1981). The plasma glucose concentration was analyzed according to the method of Raabo and Terkildsen (Raabo and Terkildsen 1960; Kit 510, Sigma, St Louis, MO, USA), in which production of H₂O₂ by glucose oxidase in the presence of

o-dianisidine is measured as an absorbance increase at 450 nm.

One- and two-way analyses of variance (ANOVA) were used to test the significance ($P < 0.05$) of concentration effects and the synergistic effects of clove oil, lidocaine-HCl and combinations of the two anesthetics. The differences among groups were analyzed by ANOVA using the SPSS statistics package (SPSS 9.0, SPSS Inc., Chicago, IL, USA) and multiple comparisons were performed using Duncan's multiple range test.

3. Results

No fish died due to the stress of anesthesia during the experiments with clove oil and lidocaine-HCl, or their combinations. Table 2 lists the parameters associated with the anesthesia and recovery times of the different anesthetic concentrations. Anesthesia time was significantly affected by the clove oil concentration, and decreased linearly with increasing clove oil concentrations ($P < 0.05$). Recovery time was also significantly affected by the clove oil concentration ($P < 0.05$), increasing as the clove oil concentration was increased. Similar to the anesthetic effect of clove oil, the anesthesia time decreased linearly with increasing concentrations of lidocaine-HCl ($P < 0.05$). Recovery time was also significantly affected by the lidocaine-HCl concentration ($P < 0.05$), increasing as the concentration of lidocaine-HCl increased ($P < 0.05$). There

were four applicable concentration ranges of clove oil excluding 10 ppm, and five applicable concentration ranges of lidocaine-HCl, excluding 500 ppm.

The ratios of recovery times to anesthesia times are also given in Table 2. In terms of trends, the ratios of recovery time to anesthesia time in clove oil and lidocaine-HCl anesthesia groups gradually increased as the concentrations of each anesthetic increased ($P < 0.05$). The ratios in the 30, 40 and 50 ppm clove oil anesthesia group were higher than those in all the lidocaine-HCl anesthesia groups. Comparing all of the clove oil and lidocaine-HCl anesthesia groups, the ratio of recovery time to anesthesia time was lowest in the 10 ppm clove oil anesthesia group, and was the highest in the 50 ppm clove oil anesthesia group.

Plasma cortisol concentrations in the clove oil anesthesia groups were not significantly different over the range of anesthetic concentrations. The mean plasma cortisol level was 1.1 ± 0.15 $\mu\text{g/dL}$ prior to the anesthesia experiment (Fig. 1a). The plasma cortisol concentrations of the 10 ppm and 50 ppm clove oil anesthesia groups increased from 15.0 ± 1.51 $\mu\text{g/dL}$ and 16.2 ± 1.65 $\mu\text{g/dL}$ at 1 hour after anesthesia to 30.1 ± 1.56 $\mu\text{g/dL}$ and 31.0 ± 1.59 $\mu\text{g/dL}$ at 12 hours ($P < 0.05$). After 48 hours the plasma cortisol concentrations of these anesthesia groups were recovered to 5.2 ± 1.54 $\mu\text{g/dL}$ and 5.2 ± 1.81 $\mu\text{g/dL}$, but were higher than those of the pre-anesthesia group ($P < 0.05$). The plasma cortisol concentrations in lidocaine-HCl anesthesia groups were not significantly different

Table 2. Effects of clove oil and lidocaine-HCl on anesthesia in grass puffer, *Takifugu niphobles**

Anesthetic	Dose (mgL ⁻¹)	Time (sec)		Time ratio (Recovery/exposure)
		Exposure	Recovery	
Clove oil	Sham control	-	-	-
	10	845 ± 29.7 ^a	112 ± 25.1 ^a	0.1 ± 0.02 ^a
	20	134 ± 16.1 ^b	172 ± 19.3 ^b	1.3 ± 0.14 ^b
	30	91 ± 15.7 ^c	189 ± 21.7 ^c	2.1 ± 0.20 ^c
	40	85 ± 13.6 ^d	194 ± 19.3 ^d	2.3 ± 0.25 ^d
	50	72 ± 11.4 ^e	242 ± 14.9 ^e	3.4 ± 0.22 ^e
Lidocaine-HCl	Sham control	1271 ± 80.3 ^a	111 ± 50.1 ^a	0.1 ± 0.07 ^a
	500	181 ± 22.9 ^b	74 ± 16.1 ^b	0.4 ± 0.03 ^a
	600	88 ± 11.6 ^c	79 ± 9.4 ^b	0.9 ± 0.09 ^b
	700	84 ± 12.5 ^d	91 ± 13.5 ^c	1.1 ± 0.15 ^c
	800	78 ± 10.0 ^e	93 ± 12.8 ^c	1.2 ± 0.12 ^c
	900	76 ± 13.0 ^e	109 ± 12.3 ^d	1.4 ± 0.13 ^c
	1,000	68 ± 6.7 ^f	124 ± 15.1 ^e	1.8 ± 0.16 ^d

*Each value is mean ± standard deviation of triplicate experiments ($n = 90$). Values in the same column not sharing common superscripts are significantly different ($P < 0.05$). The sham control group's concentrations of clove oil and lidocaine-HCl were maintained 500 ppm methanol and 1,000 ppm NaHCO₃, respectively

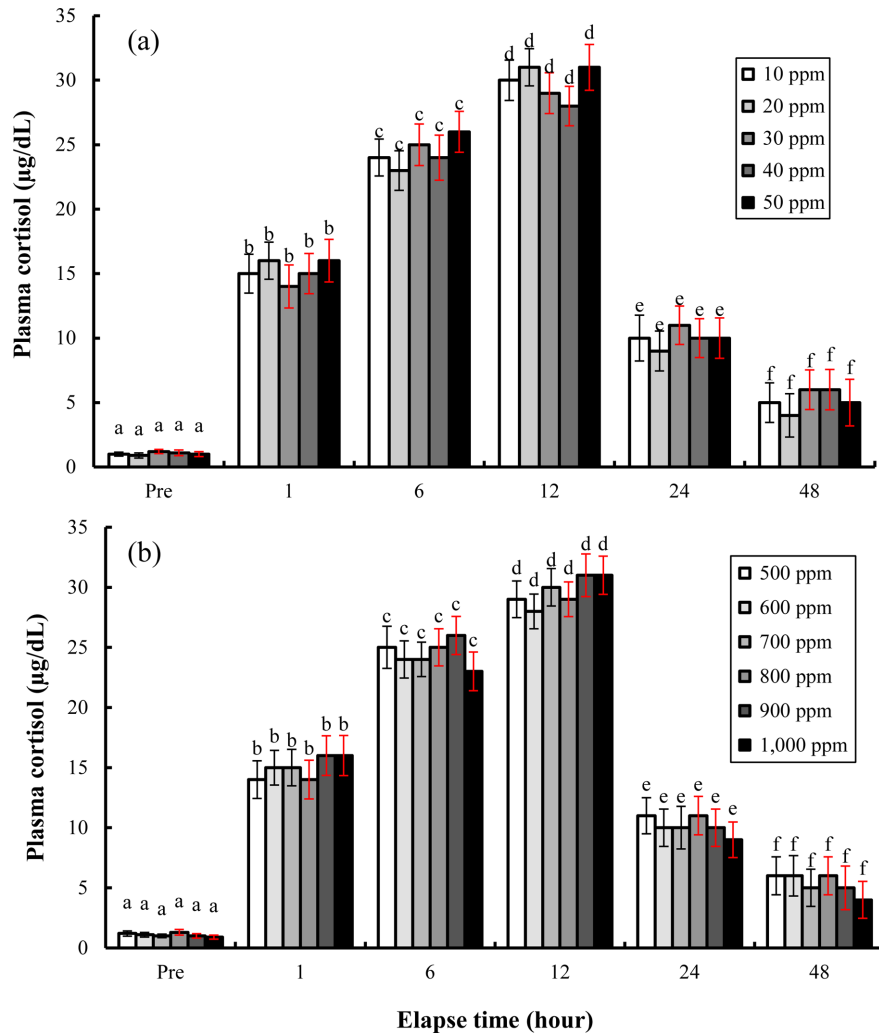


Fig. 1. Variations in plasma cortisol concentrations in the blood plasma of the grass puffer, *Takifugu niphobles*, during 48 hours of anesthesia with (a) clove oil and (b) lidocaine-HCl anesthetics. Vertical and error bars are means \pm SE of triplicate experiments ($n=90$). Different letters on the bars indicate statistical significance between pre and experimental groups ($P < 0.05$). The 'Pre' group means experimental group before anesthesia. Actually $n=90$ for each experiment because the means and SE were calculated separately for each group

over the range of anesthetic concentrations, similar to the trend with clove oil anesthetic. Mean plasma cortisol concentration levels were 1.2 ± 0.22 $\mu\text{g/dL}$ before the anesthesia experiment (Fig. 1b; $P < 0.05$). Plasma cortisol concentrations of the 500 ppm and 1,000 ppm lidocaine-HCl anesthesia groups increased from 14.6 ± 1.56 $\mu\text{g/dL}$ and 16.7 ± 1.67 $\mu\text{g/dL}$ at 1 hour after anesthesia to 29.4 ± 1.50 $\mu\text{g/dL}$ and 31.1 ± 1.58 $\mu\text{g/dL}$ at 12 hours ($P < 0.05$). Plasma cortisol concentrations of the 500 ppm and 1,000 ppm lidocaine-HCl anesthesia groups at 48 hours were decreased to 6.0 ± 1.57 $\mu\text{g/dL}$ and 4.0 ± 1.54 $\mu\text{g/dL}$, but higher than those of the pre-anesthesia group ($P > 0.05$).

Plasma glucose concentrations in clove oil anesthesia

groups were not significantly different over the range of clove oil concentrations. Plasma glucose concentration was 5.0 ± 0.2 mg/dL before the anesthesia experiment (Fig. 2a). Mean plasma glucose concentration level increased from 10.0 ± 1.44 mg/dL at 1 hour to 32.5 ± 1.55 mg/dL at 24 hours for the 10 ppm clove oil anesthesia group ($P < 0.05$). After 48 hours, the plasma glucose concentration of the 10 ppm clove oil anesthesia group was recovered to 8.2 ± 1.68 mg/dL, the same level as that of the pre-anesthesia group ($P < 0.05$). The plasma glucose concentrations in lidocaine-HCl anesthesia groups were not significantly different over the range of lidocaine-HCl concentrations, similar to the result with the clove oil anesthetic. Plasma glucose concentration was 5.0 ± 0.16

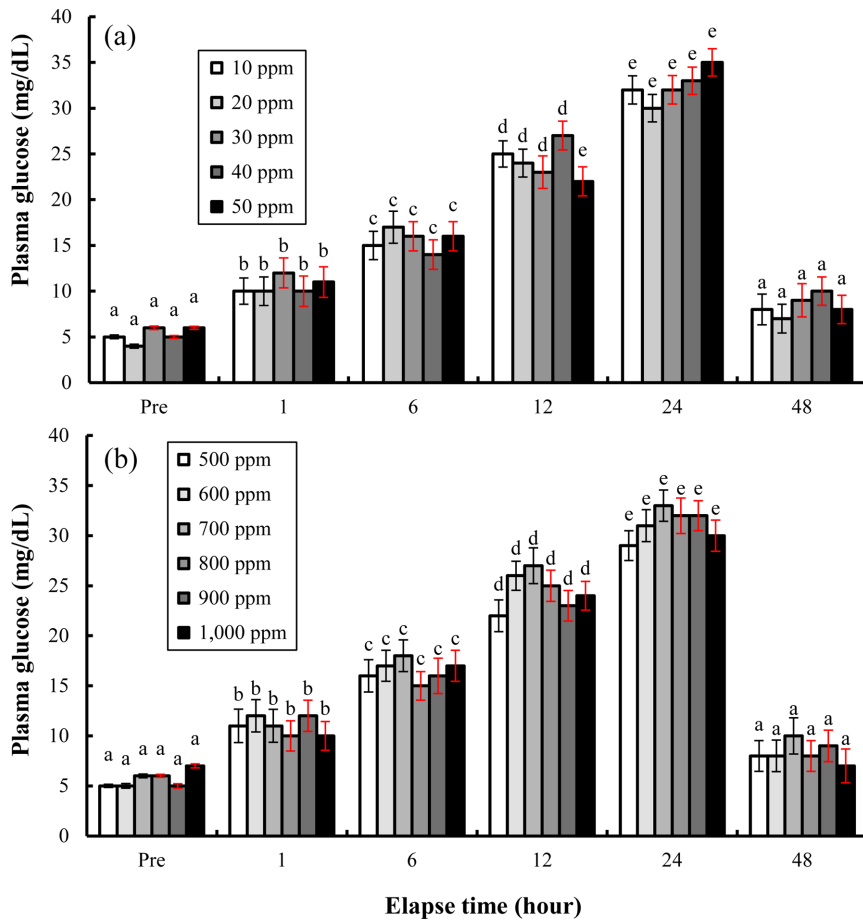


Fig. 2. Variations in the plasma glucose concentrations in the blood plasma of the grass puffer, *Takifugu niphobles*, during 48 hours of anesthesia with (a) clove oil and (b) lidocaine-HCl anesthetics. Vertical and error bars are means \pm SE of triplicate experiments ($n=90$). Different letters on the bars indicate statistical significance between pre and experimental groups ($P < 0.05$). The 'Pre' group means experimental group before anesthesia. Actually $n=90$ for each experiment because the means and SE were calculated separately for each group

mg/dL before the anesthesia experiment (Fig. 2b). Mean plasma glucose concentration level of the 500 ppm lidocaine-HCl anesthesia group increased from 11.2 ± 1.67 mg/dL at 1 hour to 29.0 ± 1.49 mg/dL at 24 hours ($P < 0.05$). After 48 hours, the plasma glucose concentration this group was recovered to 8.1 ± 1.54 mg/dL, similar to that of the pre-anesthesia group ($P < 0.05$).

During long-term transportation of fish anesthetized with the two anesthetics, no fish died due to anesthesia stress. Table 3 contains the parameters associated with the anesthesia and recovery times at each concentration of clove oil and lidocaine-HCl. Anesthesia and recovery times with clove oil were significantly affected by clove oil concentrations, and decreased drastically as the concentration of clove oil increased ($P < 0.05$). Similarly, anesthesia and recovery times with lidocaine-HCl were significantly affected by lidocaine-HCl concentrations,

and decreased drastically as the concentration of lidocaine-HCl increased ($P < 0.05$). Contrast the tendency of anesthetic effect's result (Table 2), recovery time was decreased by increasing concentration of clove oil and lidocaine-HCl. Anesthesia times and recovery times were the longest with 2 ppm clove oil and 50 ppm lidocaine-HCl. In conclusion, the optimum anesthetic concentrations for long-term transportation requiring more than 1 hour were 2 ppm for clove oil and 50 ppm for lidocaine-HCl.

No fish died due to anesthesia stress during the experiments to evaluate synergistic effects of the two anesthetics combined. Table 4 lists the parameters associated with the anesthesia recovery times of groups anesthetized with clove oil + lidocaine-HCl mixtures at each concentration of the mixture components. Anesthesia time was significantly affected by the mixture concentration, and decreased linearly as the mixture concentration increased

Table 3. Effects of clove oil and lidocaine-HCl on long-term transportation of grass puffer, *Takifugu niphobles**

Anesthetic	Dose (mgL ⁻¹)	Time (min)		Time ratio
		Exposure	Recovery	(Recovery/exposure)
Clove oil	Sham control	-	-	-
	2	342 ± 29.7 ^a	62 ± 2.1 ^a	0.18 ± 0.04
	4	41 ± 16.1 ^b	23 ± 1.3 ^a	0.56 ± 0.09
	6	39 ± 15.7 ^b	21 ± 2.7 ^b	0.53 ± 0.08
	8	20 ± 3.6 ^c	10 ± 1.3 ^c	0.5 ± 0.05
	10	14 ± 0.4 ^d	2 ± 0.2 ^d	0.1 ± 0.04
Lidocaine-HCl	Sham control	-	-	-
	50	378 ± 22.9 ^a	54 ± 4.1 ^a	0.14 ± 0.03
	100	81 ± 11.6 ^b	32 ± 3.4 ^b	0.39 ± 0.10
	150	65 ± 12.5 ^c	31 ± 3.5 ^b	0.47 ± 0.08
	200	38 ± 10.0 ^d	23 ± 2.8 ^c	0.60 ± 0.06
	250	16 ± 3.0 ^e	10 ± 2.3 ^d	0.63 ± 0.03
	300	8 ± 0.7 ^f	7 ± 1.1 ^e	0.8 ± 0.15

*Stages of anesthesia and recovery in each group were A6 and R6, respectively. Each value is mean ± standard deviation of triplicate experiments ($n = 30$). The sham control group's concentrations of clove oil and lidocaine-HCl were maintained 300 ppm methanol and 1,000 ppm NaHCO₃, respectively

Table 4. Effects of combination of two anesthetics, clove oil and lidocaine-HCl, on grass puffer, *Takifugu niphobles*

Clove oil (mgL ⁻¹)	Lidocaine-HCl (mgL ⁻¹)	Time (sec) [*]		Time ratio
		Exposure	Recovery	(Recovery/exposure)
Sham control		1281 ± 100.4 ^a	113 ± 54.7 ^a	0.1 ± 0.08 ^a
30	800	68 ± 11.8 ^b	171 ± 23.5 ^b	2.5 ± 0.77 ^b
	900	63 ± 10.4 ^b	224 ± 19.3 ^c	3.6 ± 0.81 ^c
	1,000	57 ± 14.5 ^c	258 ± 15.8 ^d	4.5 ± 0.91 ^d
40	800	65 ± 9.3 ^b	203 ± 21.8 ^b	3.1 ± 0.90 ^c
	900	56 ± 10.1 ^c	276 ± 28.1 ^c	4.9 ± 0.89 ^d
	1,000	47 ± 9.4 ^d	334 ± 32.7 ^d	7.1 ± 0.88 ^e
50	800	63 ± 11.7 ^b	254 ± 24.9 ^b	4.0 ± 0.79 ^c
	900	54 ± 10.2 ^c	293 ± 30.1 ^c	5.4 ± 0.81 ^d
	1,000	41 ± 7.9 ^d	338 ± 33.1 ^d	8.2 ± 0.85 ^e

*Each value is mean ± standard deviation of triplicate experiments ($n = 90$). Values in the same column not sharing common superscripts are significantly different ($P < 0.05$). Sham control was dissolved mixture of 5 mL 95% methanol and 10 g NaHCO₃

($P < 0.05$). As the concentrations of clove oil + lidocaine-HCl increased, the anesthesia time decreased ($P < 0.05$) and the recovery times increased ($P < 0.05$). Anesthesia times of the mixture were shorter than those of the same clove oil concentrations alone, and were also shorter than those of the same lidocaine-HCl concentrations alone (Tables 2 and 4). Recovery times of the mixture were slower than those with the same concentrations of individual anesthetics. Anesthesia and recovery times of the mixture satisfied the requirements for an anesthesia time within 3 minutes and a recovery time within 5

minutes. Anesthesia and recovery times of the sham control group were significantly different from those of the lidocaine-HCl and mixture anesthetics. The ratios of recovery time to anesthesia time for the mixed anesthetics are shown in Table 4. This ratio drastically increased as the concentration of each anesthetic in the mixture increased ($P < 0.05$). Furthermore, the ratios of recovery time to anesthesia time in the mixture anesthesia groups were higher than those in the groups with clove oil or lidocaine-alone (Tables 2 and 4).

There was no significant concentration effect of the

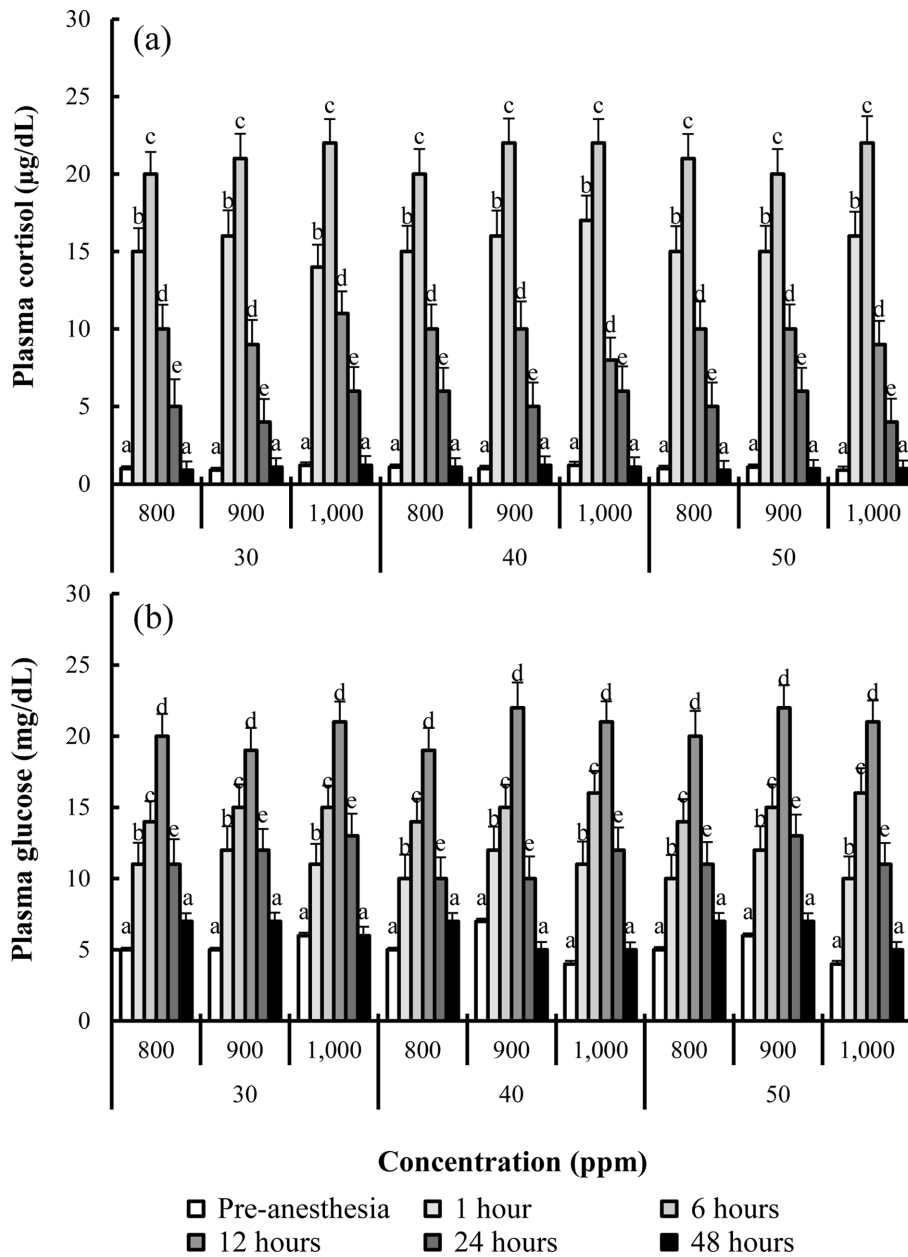


Fig. 3. Variations in the (a) plasma cortisol and (b) plasma glucose concentrations in the blood plasma of the grass puffer, *Takifugu niphobles*, 48 hours after anesthesia with the mixture of clove oil (30, 40 and 50 ppm) and lidocaine-HCl (800, 900 and 1000 ppm) anesthetics. Vertical and error bars are means \pm SE of triplicate experiments ($n=90$). Different letters on the bars indicate statistical significance between pre and experimental groups ($P < 0.05$). The 'Pre' group means experimental group before anesthesia. Actually $n=90$ for each experiment because the means and SE were calculated separately for each group

combined anesthetics on plasma cortisol levels. The mean plasma cortisol concentration level was 1.0 ± 0.2 $\mu\text{g/dL}$ in the pre-anesthesia group (Fig. 3a). Plasma cortisol concentrations of the 30 + 800 ppm and 50 + 1,000 ppm anesthesia mixtures increased from 14.2 ± 1.66 $\mu\text{g/dL}$ and 15.8 ± 1.56 $\mu\text{g/dL}$ at 1 hour after anesthesia to 24.0 ± 1.43 $\mu\text{g/dL}$ and 23.0 ± 1.75 $\mu\text{g/dL}$ at 6 hours ($P < 0.05$).

After 48 hrs, the plasma cortisol concentrations of the 30 + 800 ppm and 50 + 1,000 ppm mixtures recovered to 0.9 ± 0.55 $\mu\text{g/dL}$ and 1.0 ± 0.52 $\mu\text{g/dL}$, similar levels as those of the pre-anesthesia group ($P < 0.05$). Plasma glucose concentrations in the mixed anesthetic groups were not significantly different among anesthetic concentrations. Plasma glucose concentration was 5.2 ± 0.15 mg/dL in

the pre-anesthesia group (Fig. 3b). The mean plasma glucose concentration levels of the 30 + 800 ppm and 50 + 1,000 ppm mixtures increased from 11.0 ± 1.51 mg/dL and 10.1 ± 1.56 mg/dL at 1 hour to 22.5 ± 1.77 mg/dL and 25.5 ± 1.52 mg/dL at 12 hours ($P < 0.05$). After 48 hours, the plasma glucose concentrations of the 30 + 800 ppm and 50 + 1,000 ppm mixtures recovered to 7.1 ± 0.56 mg/dL and 5.3 ± 0.55 mg/dL, similar levels as those of the pre-anesthesia group ($P < 0.05$).

Plasma cortisol concentrations of the anesthetic mixtures were highest at 6 hours in all experimental groups, and those of clove oil or lidocaine-HCl alone were highest at 12 hours in all experimental groups. Plasma glucose concentrations of the anesthetic mixtures were highest at 12 hours in all experimental groups, and those of clove oil or lidocaine-HCl alone were highest at 24 hours in all experimental groups. The stress responses of the mixed anesthetics groups recovered perfectly at 48 hours, and the velocity of the stress response in the mixed anesthetics group was faster than that in the clove oil anesthesia group and the lidocaine-HCl anesthesia group.

4. Discussion

The anesthesia time is the time required for an animal to reach the anesthesia criterion for that species, and the recovery time is the time required for the animal to recover its vitality completely (Summerfelt and Smith 1990). This study indicates that clove oil and lidocaine-HCl are both effective anesthetics for anesthesia and long-term transportation of grass puffer, *Takifugu niphobles*. To identify the anesthetic concentration that met the efficacy criteria for anesthesia within 3 minutes, recovery in 10 minutes and no mortality during experiment, this study assessed a range of clove oil concentrations from 10 to 50 ppm and a range of lidocaine-HCl concentrations from 500 to 1,000 ppm (Gilderhus and Marking 1987; Son et al. 2001; Park et al. 2003).

Our study obviously showed that the higher the concentration of anesthetic, the shorter the anesthesia time. The anesthesia times obtained in this study for grass puffer anesthetized with clove oil are similar to those reported elsewhere for sockeye salmon, *Oncorhynchus nerka*, rock bream, *Oplegnthus fasciatus*, and kelp grouper, *Epinephelus bruneus* with clove oil and greenling, *Hexagrammos otakii* and winter flounder, *Pleuronectes americanus* with lidocaine-HCl (Woody et al. 2002; Park et al. 2003, 2004, 2008, 2009). The dose response of marine medaka to clove oil and lidocaine-HCl followed a

negative exponential curve, with increasing doses resulting in a decreasing time to stage A6 anesthesia. At each concentration, the higher the water temperature, the shorter the anesthesia time. The relationship between water temperature and anesthesia time showed a negative exponential curve, with increasing water temperature resulting in a decreasing anesthesia time. This observation of the dependency of anesthetic effect on water temperature is identical with that of other species. European sea bass, *Dicentrarchus labrax* and gilthead sea bream, *Sparus aurata* anesthetized by clove oil have significantly longer anesthesia and recovery times at lower temperatures (ANOVA, $P < 0.001$) (Constantinos et al. 2005). Also, kelp grouper and greenling showed similar trends for clove oil and lidocaine-HCl, respectively (Park et al. 2003, 2008; Constantinos et al. 2005).

When the ratio of the recovery time to the anesthesia time is greater than 1, the recovery time is longer than the anesthesia time (Park et al. 2009). For the clove oil and lidocaine-HCl anesthetics, the ratio of all groups excluding 10 ppm clove oil, 500 ppm lidocaine-HCl and 600 ppm lidocaine-HCl anesthesia groups were greater than 1, and for each group, the ratio increased as the anesthesia concentration was increased ($P < 0.05$). In particular, all ratios of the groups with clove oil + lidocaine-HCl were greater than 2, and they increased as the clove oil and lidocaine-HCl concentrations were increased ($P < 0.05$). That is, the anesthesia time was shortened as the anesthesia concentration increased, but the increase in recovery time was relatively higher. A similar result was reported for rock bream which showed that the increment of clove oil concentration corresponded to the increment in the ratio of the recovery time to the anesthesia time (Park et al. 2009). For lidocaine-HCl, all the ratios of recovery time to anesthetic time were above 1 which means that the recovery time is longer than the anesthesia time for lidocaine-HCl (Park et al. 2009).

The trends in plasma cortisol and plasma glucose concentrations of grass puffer observed in these experiments are symptomatic of stress reactions. Plasma cortisol and plasma glucose are recognized as useful indicators of stress in fish (Schreck 1982; Park et al. 2008). Plasma cortisol and glucose levels in red drum, *Sciaenops ocellatus*, simultaneously exposed to MS-222 and Quinaldine anesthetic, were reported to be elevated (Massee et al. 1995). Barton and Iwama (1991) stated that "Usually, phenomenon that plasma cortisol concentration of fishes rises by stress is first order reaction, phenomenon that plasma glucose concentration rises is result of second-

order first order reaction by hormone rise reaction by stress.” This trend has been reported in the gray mullet, *Mugil cephalus* and kelp grouper (Chang and Hur 1999; Park et al. 2008). Das et al. (2004) suggested that the increased use of glucose for increased cell metabolism during early anesthetic exposure may overwhelm the increase in blood glucose, even though glycogenolysis would have increased during this period (Martinez-Alvarez et al. 2002). However, because of dysfunctional cell metabolism the lower use of glucose later in the exposure period (after 48 hours) resulted in an increase in blood glucose levels. Our results showed that the plasma cortisol level increases faster than the glucose concentration. This result was similar to a study carried out by Chang and Hur (1999) and Park et al. (2008).

The synergy of clove oil and lidocaine-HCl in combination was evaluated by analyzing the anesthetic effect of mixtures of the two anesthetics. The mixture had a shorter anesthesia time and a more rapid recovery of stress response than each anesthetic alone. No previous studies have analyzed the synergy of clove oil and lidocaine-HCl by mixing the two anesthetics. As mentioned by Park et al. (2011), the anesthesia times are significantly affected by water temperature and the clove oil or lidocaine-HCl concentration, and decrease proportionally as the clove oil or lidocaine-HCl concentration or water temperature increases. The results of Park et al. (2011) suggest that the anesthetic effects of clove oil are similar to those of lidocaine-HCl. Our results with grass puffer suggest that the anesthetic effects of clove oil are similar to those of lidocaine-HCl, and that mixtures of clove oil and lidocaine-HCl are more effective than clove oil and lidocaine-HCl alone. This study provides basic information about grass puffer’s anesthesia and the synergistic effects of anesthetic mixtures. The results should be useful for aquaculturists who require safe and easy handling of fish and for transporters who require that the stress on fish is minimal during transport.

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