Applying new formulated herbal anesthetic comparing to tricaine methanesulfonate (MS-222) in beluga (*Huso huso*)

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Abstract

Applying anaesthetic agent is recommended in aquaculture during fish handling specially tagging. However, it should be noted that anesthesia itself is likely to induce stressful condition due to its improper and high concentration. By means of nanoemulsions, drug delivery improves which in turn contributes to fewer amounts of pharmaceutical agents' usage. In this research, nanoemulsions of clove oil (NCL) and lemonbeebrush (NLB) were used to develop the anesthetic efficacy and reduce the concentration needed for anesthesia in *Huso huso* (mean weight: 1115 ± 242.25 g) as an endangered species. Based on the results, anesthesia induction time (s) of stage 3 & 4 was significantly decreased in NCL and NLB when compared to MS-222 ($p<0.05$). NCL treated beluga were induced stage 5 anesthesia much quicker than NLB and MS-222. In contrary, recovery time did not show significant difference among treatments ($p>0.05$). Among measured biochemical factors, lactate had the highest value in NCL group during anesthesia ($p<0.05$). In contrast, lactate level had highest level in NLB, 24h post anesthesia ($p<0.05$). NLB and NCL produced higher non-specific immune (Lysozyme and ACH50) enhancements at 0 and 24h post anesthesia. Furthermore, results showed that MS-222 induced increased serum cortisol levels ($p<0.05$). Cortisol and glucose were not depressed by NLB and NCL, suggesting that these anesthetics are safer anesthetic for *Huso huso* as it does not cause immune depression in anesthetized fish.

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Introduction

Anesthesia is a reversible state of insensibility of cells, tissues and organisms induced by chemical or nonchemical means (Summerfelt & Smith 1990). In contrary, recovery is the time to return to a normal state from surgical anesthesia (Needham, 1988). There are many available chemical anesthetics for fishery research and aquaculture practices in market; including tricaine methanesulfonate (MS-222), xylocaine, benzocaine, 2-phenoxyethanol, quinaldine, quinaldine sulphate, and metomidate (Feng, Liu, Zhuang, Zhang & Duan 2011). MS-222; white crystalline chemical powder is one of the popular anesthetics which is easily dissolved in water. However, after an anaesthesia treatment, a 21-day withdrawal period is required before human can safely consume the fish (Pirhonen & Schreck 2003).

In recent years, herbal medicines are widely under consideration by researchers in order to decrease the use of chemicals and their probable side effects. Clove oil has been popular topical anaesthetic since ancient times to help with toothache, headaches and joint pains. Clove is relatively inexpensive (Ross & Ross 2008) and is more potent than other anaesthetics used in fish. In addition, it has a long history as a local anaesthetic for humans (Woody, Nelson & Ramstad 2002; Soto & Burhanuddin 1995). The plant *Aloysia triphylla* (L'Herit) Britton "a natural shrub in South America" was introduced to Europe in the late seventeenth century (Carnat, Carnat, Fraisse & Lamaison 1999). The essential oil of this plant is effective anesthetic which stimulates oxidative protection and mitigate stress in the silver catfish, *Rhamdia quelen* (Gressler, Riffel, Parodi, Saccol, Koakoski, Costa, Pavanato, Heinzmann, Caron, Schmidt, Liesuy, Barcellos & Baldisserotto 2014) and in sub adults of the shrimp *Litopenaeus vannamei* (Parodi, Cunha, Heldwein, Souza, Martins, Garcia, Wasielesky, Monserrat, Schmidt, Caron, Heinzmann& Baldisserotto 2012). However, due to immiscible feature of essential oils in water they are less bioavailable.Meanwhile, nanotechnology will probably play important roles via contributions to some of medical area.Indeed, by applying nanoemulsion as heterogeneous, transparent and stable solutions of oil with droplet size of less than 100 nm, essential oils solubility enhances, which consequently increases anesthetic efficacy as well (Guglielmini, 2008).

Numerous authors have remarked the supreme characteristics of anaesthetic agents typically when considering as chemical. According to Marking and Meyer (1985), a suitable anesthetic is the one which can induce anesthesia within 3 min or less and allow recovery within 5 min or less. Although, the anesthetic agents are practical means for reducing or minimizing handling impact in fish but treatment with immoderate anesthesia and long term exposure time are very stressful to the fish (Iwama, McGeer & Pawluk 1989).

Beluga (*Huso huso*), one of the most important species of sturgeon in the Caspian Sea, is considered as endangered species by the IUCN (Sturgeon Specialist Group 1996). This

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species is a commercially important source of caviar and meat. Stressful conditions such as breeding practices, restocking programs and research experiments might affect this species survival therefore using anesthetic agents could be helpful. However, besides consideration to ideal anesthetic agents in this valuable species, assessing the effect of these substances on fish physiological statue seems crucial. Evaluation of blood cells, immune responses, blood biochemistry and hormones are useful indicators of the physiological or sub lethal stress response in fishes to endogenous or exogenous alterations (Cataldi, Marco, Mandich & Cataudella 1998). In this regard, the raise in plasma cortisol and glucose indices is a usual indicator of stressful conditions (Wendelaar Bonga 1997).

In order to examine the efficacy of new formulation of nano clove oil and nano *Aloysia triphylla* as well as MS-222 as an anesthetic, we conducted an experiment to investigate whether above anesthetic agents suppress the normal plasma biochemical, hematological and immunological values in beluga.

Materials and Methods

Fish preparation and experimental conditions

The experiments conducted on 40 hatchery reared beluga (average weight: 1115 ± 242.25 g; Total length 64.22 ± 3.39 cm) at the Institute of Aquaculture research of Gharehsoo for Sturgeon, Golestan, Iran. The average values for water used in the both acclimation and experiments were pH 7.36 ± 0.50, dissolved oxygen 7.30 ± 0.06 mg L^{-1}, temperature at 22.70 ± 0.2 ^{o}C, EC 865 ± 1.00 and total hardness 340 ± 2.35 mg L^{-1} as CaCO3. Fish were kept under natural photoperiodic conditions at the research Centre and were fasted for 24 h prior to experiment.

Anesthetic

Preparation and characterization of clove oil and lemon beebrush nanoemulsion were by mixing the separately prepared oily and water phases through spontaneous emulsification method with major modification based on Ebrahimi (2014). Then essential oils were added drop wise to nanoemulsion as final nanoemulsion composition containing clove oil at 8 mg mL^{-1} and lemonbeebrush at 8mg mL^{-1} in the aqueous phase. The average size of droplet (z-average size) as well as distribution size of the obtained nanoemulsions were measured using Zetasizer instrument (PCS, Nano ZEN 3000 Malvern Instruments Corp., U.K.). Afterwards, the prepared oil nanoemulsions were stored at 4°C to determine their physicochemical stability.

Anesthesia Preparation and Experiment

Examining anesthetic effects on fish was the first step. A pilot study was conducted then the characteristics of inducting different stages of anesthesia as well as recovery was defined according to Table 1. The concentrations at 25 mg L^{-1} of our nano treatments (Nano clove oil (NCL), Nano lemonbeebrush (NLB) were chosen. similar concentration (25 mg L^{-1}) of MS-222 (tricaine methanesulfonate; Sigma Aldrich Co., St. Louis, USA) was used to compare anesthetic effect with the nanoemolsions we used.

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All test fish were placed individually in anaesthetic baths supplied with aeration. Each anesthetic treatments (NCL, NLB and MS-222) had 10 fish in it. The times to achieve sedation as well as light and deep anesthesia were also recorded. After reaching the onset of deep anesthesia, fish were immediately removed from the tank and then transferred to a 300 L, well-oxygenated 'Recovery' tank (without anesthesia agent) and maintained at 22°C fully recovered. The fish behavior was observed and times to recover active swimming were recorded during the recovery period. The recovered fish were left in the recovery aquarium for approximately 3 days. Any abnormal behavior or mortalities were recorded during this 14-day recovery period.

Table1. Stages of anesthesia and recovery from anesthesia in fish quoted from Keene, Noakes, Moccia & Soto (1998)

Stage	Behavior in anesthesia stages	Behavior in recovery stages
1	Normal reactions to external stimuli; normal opercular rate and muscle tone	Decrease in opercular movement
2	Light sedation, slight loss of reactivity to external visual and tactile stimuli; minor decrease in opercular equilibrium normal	Partial recovery of equilibrium; partial recovery of swimming motion
3	Deep sedation, Total loss of reactivity to external stimuli excluding 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

Hematological parameters

Blood sample were taken from anesthetized fish and immediately transferred to non-heparinized and heparinized 1.5-mL tubes. Obtained serum and plasma samples were stored in -80°C until further analysis. After bleeding, fish were taken to a recovery tank. Moreover, blood sampling was repeated 24h post anesthesia for some of biochemical analysis. Samples were also taken from control group (no exposure to anesthetic agent). Red blood cells (RBC: 10^6 mm^{-3}) and white blood cell (WBC: 10^3 mm^{-3}) populations were counted manually by haemocytometry, using Neubauer haemocytometer. Haemoglobin concentration (Hb: g dL^{-1}) was measured using spectrophotometer at 540 nm with cyanmethemoglobin method. Haematocrit (Hct: %) was measured using standard heparinized capillary tubes with microcentrifuge method, (75 mm at 10000 rpm for 10 min). The derived erythrocytes of mean corpuscular volume (MCV: Fl), mean corpuscular haemoglobin (MCH: pg) and mean corpuscular haemoglobin concentration (MCHC: %) were calculated according to the following formula:

MCH = Hb / RBC; MCHC = (Hb × 10) / Hct; MCV = (PCV × 1000) / RBC

Biochemical Parameters

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Alanine aminotrans-ferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined in serum using a commercially available kit (Parsazmoon, Co) according to the manufacturer's instructions. Plasma cortisol levels were determined by radioimmunoassay using the Diaplas company kit (Grutter & Pankhurst 2000). Glucose levels were measured using standard spectrophotometric assays (using Parsazmoon kit) and urea by Parsazmoon® kit. Lactate was determined by spectrophotometer using commercial kits (Parsazmoon, Co) (Chaney & Merbach 1962).

Immunological parameters

Lysozyme activity was measured with the turbidimetric method described by Sahoo, Meher, Mahapatra, Saha, Jana & Reddy (2004) with slight modification. Suspension of 175 µL lyophilized *Micrococcus lysodeikticus* (Sigma M 3770) (0.2 mg mL^{-1} as the substrate in 0.1M sodium acetate buffer adjusted to pH = 7.4) added to 25 ul of each serum samples in a 96-well flat-bottom plate. Initial and final OD was taken at 450 nm at 24°C. The final OD was taken 30 min after incubation. Serum lysozyme values were expressed as µg ml^{-1} equivalent to HEWL activity.

Alternative complement pathway activity was assayed according to Matsuyama, Tanaka, Nakao and Yano (1988). After washing Sheep red blood cells with Alsever's solution the blood were stored at 4 ^{o}C.

The buffers used in this experiment were: 10 EGTA-Mg-GVB, veronal-buffered saline containing 10 mM ethylene glycol bis (ß-amino ethyl ether)-N, N, N, N-tetra acetic acid, 10 mM MgCl2 and 0.1% gelatin (µ=0.15, pH = 7.8); 10 mM EDTA-GVB: Veronal-buffered saline containing 10 mM ethylene diamine tetra acetic acid and 0.1% gelatine (µ= 0.15, pH = 7.5). Briefly, 0.5 ml of serially 8-fold diluted *Huso huso* serum in EGTA-Mg-GVB was placed in a set of test tubes then 0.2 ml of sheep red blood cells suspension (2×10^6 cells ml^{-1}) was added. This mixture was incubated at 15 °C for 90 min then the hemolytic reaction was stopped by adding 2.8 ml cold 10 mM EDTA-GVB buffer. The value y (percentage haemolysis 10^{-2}) of supernatant was read at 414 nm. The ACH 50 (units ml^{-1}) and the reciprocal dilution giving 50% haemolysis (y $(1-y)^{-1}$) = 1 were read from the log log graph.

Statistical analysis

The mean ± standard deviation (SD) was calculated for each group by using SPSS 16 statistical software. One-way analysis of variance (ANOVA) with Bonferony range test performed to detect significant differences between means when $p<0.05$.

Results

Mean induction time of sedation, light and deep anesthesia as well as recovery time are shown in Table 2. Induction time (s) of stage 3 was significantly decreased in NCL (88 ± 18) and NLB (84 ± 9) when compared to MS-222 (186 ± 16) ($p<0.05$) as well as decrease in reaching stage 4 ($p<0.05$). Moreover, NCL (186.48 ± 26.79a) treated sturgeons were induced stage 5 anesthesia much quicker than NLB (331 ± 23) and MS-222 (758 ± 10).

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Recovery time did not show significant difference among treatments ($p>0.05$). All anesthetised fish were alive after 72 h post-treatment monitor.

Table 2. Mean ± SD of reaching three anesthesia stages (3, 4&5 ofanesthesia) with NLB (nano lemonbeebrush). NCL (nano clove oil) and MS-222and recovery time (sec) in beluga

Stages (s)	NLB	NCL	MS-222
Sedation	84 ± 9^{b}	88 ± 18^{b}	186 ± 16.2^{a}
Light anesthesia	135 ± 15^{b}	115 ± 14.9^{b}	421 ± 26^{a}
Deep Anesthesia	331 ± 23^{b}	186 ± 26^{c}	758 ± 10^{a}
Recovery	191 ± 7^{a}	198 ± 19^{a}	235 ± 24^{a}

Data in same row with different superscripts are significantly different ($p<0.05$), N=50.

Effects of different anesthetic agents on the hematological indices of beluga are shown in Table 3. The hematological parameters (RBC, PCV, Hb, MCV, MCH, MCHC and WBC) were at comparable levels in all treatments ($p>0.05$).

Table 3. Effects NLB (nano lemonbeebrush) NCL (nano clove oil) and MS-222 on hematological indices of blood in beluga during anesthesia

	Control	NLB	NCL	MS-222
Hb (g dL^{-1})	1.15 ± 0.10^{a}	0.57 ± 0.00^{a}	0.63 ± 0.08^{a}	1.80 ± 1.03^{a}
MCV(μ^3)	778.67 ± 193.80^{a}	943.00 ± 481.36^{a}	944.50 ± 367.10^{a}	662.77 ± 69.46^{a}
MCH (pg)	27.76 ± 3.20^{a}	32.21 ± 6.60^{a}	40.28 ± 9.04^{a}	56.45 ± 36.78^{a}
MCHC(g dL^{-1})	5.52 ± 1.93^{a}	2.85 ± 0.44^{a}	2.25 ± 0.37^{a}	8.06 ± 5.37^{a}
PCV (%)	28.32 ± 5.68^{a}	27.06 ± 1.43^{a}	31.07 ± 4.02^{a}	35.49 ± 4.71^{a}
RBC($10^6 mm^{-3}$)	0.43 ± 0.07^{a}	0.35 ± 0.05^{a}	0.44 ± 0.04^{a}	0.57 ± 0.07^{a}
WBC($10^3 mm^{-3}$)	2.86 ± 0.40^{a}	2.890 ± 0.18^{a}	3.89 ± 0.46^{a}	2.90 ± 0.41^{a}

Data are presented as mean ± SD; Data in same row with different superscripts are significantly different ($p<0.05$), N=10.

The effects of anesthetic agents on serum biochemical indices of beluga are summarized in Table 4.Glucose level was significantly lower in NLB and NCL compared to other groups during anesthesia (0h) ($p<0.05$). Moreover, Glucose level was higher in NLB at 24h post anesthesia compared to other groups ($p<0.05$).Lactate had the highest value in NCL group ($p<0.05$) during anesthesia. In contrary, lactate level had its highest level 24h post anesthesia comparing to other treatments ($p<0.05$). MS-222 increased cortisol level during anesthesia comparing to the other groups ($p<0.05$). The remaining biochemical parameters (ALT, AST, ALP and Urea values) had no significant differences among all treatments ($p>0.05$).

Table 5 represents innate immune responses in different anesthesia treatments and non anesthetised beluga. Increase in lysozyme activity was significant in NLB and NCL treatment ($p<0.05$) in comparison with the MS-222 and control group during anesthesia as well as at 24h post anesthesia. In addition, alternative complement activity (ACH50) reached its significant level during anesthesia and 24h post anesthesia in all treatments in comparison with control.

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Table 4. Effects NLB (nano lemonbeebrush), NCL (nano clove oil) and MS-222 on biochemical indices of blood in beluga during anesthesia and 24h post anesthesia

	Time	NLB	NCL	MS-222	Control
Glucose (mg dL^{-1})	0h	37.00 ± 2.64^{b}	51.33 ± 6.93^{b}	78.33 ± 11.28^{a}	62.33 ± 0.66^{a}
	24h	88.33 ± 25.20^{a}	50.66 ± 1.66^{ab}	78.00 ± 8.08^{b}	40.66 ± 5.36^{b}
Urea (mg dL^{-1})	0h	2.33 ± 0.33	3.00 ± 0.00	2.33 ± 0.33	2.00 ± 0.00
	24h	2.66 ± 0.33	1.66 ± 0.66	2.66 ± 0.33	2.66 ± 0.88
AST(IU L^{-1})	0h	210.33 ± 21.34	184.00 ± 19.08	255.33 ± 18.94	180.00 ± 113.57
	24h	291.67 ± 42.38	191.00 ± 24.58	201.67 ± 5.78	212.33 ± 16.74
ALT (IU L^{-1})	0h	2.66 ± 0.33	2.33 ± 0.88	2.00 ± 0.57	2.66 ± 0.88
	24h	2.00 ± 0.57	3.00 ± 0.57	1.66 ± 0.33	2.00 ± 0.00
ALP(IU L^{-1})	0h	308.33 ± 45.40	347.00 ± 62.74	345.00 ± 47.24	360.33 ± 10.52
	24h	307.00 ± 53.26	349.67 ± 46.18	323.33 ± 19.22	421.00 ± 12.53
Lactate (mg dL^{-1})	0h	26.66 ± 2.18^{b}	33.00 ± 7.00^{a}	20.33 ± 2.60^{b}	14.33 ± 1.66^{b}
	24h	29.33 ± 2.90^{a}	6.33 ± 0.88^{b}	9.33 ± 1.85^{b}	11.66 ± 2.33^{b}
Cortisol (ng mL^{-1})	0h	3.90 ± 1.30^{b}	5.96 ± 0.24^{b}	8.26 ± 0.08^{a}	4.83 ± 1.88^{b}
	24h	3.20 ± 0.11^{a}	5.86 ± 2.04^{a}	4.06 ± 0.85^{a}	3.23 ± 0.14^{a}

Data are presented as mean ± SD; Data in same row with different superscripts are significantly different ($p<0.05$), N=10.

Table 5. Effects NLB (nano lemonbeebrush) NCL (nano clove oil) and MS-222 on immunological indices of blood in beluga during anesthesia and 24h post anesthesia

	Time	NLB	NCL	MS-222	Control
Lysozyme (µg mL^{-1})	0h	35.60 ± 5.07^{a}	28.08 ± 1.74^{b}	20.70 ± 1.03^{c}	15.50 ± 0.80^{c}
	24h	34.04 ± 2.25^{a}	28.55 ± 2.39^{a}	19.07 ± 0.90^{b}	16.01 ± 0.16^{b}
ACH50 (Unit mL^{-1})	0h	94.42 ± 1.82^{a}	88.67 ± 0.92^{a}	74.53 ± 0.91^{b}	17.68 ± 2.75^{c}
	24h	83.70 ± 0.72^{a}	74.48 ± 1.03^{b}	47.14 ± 1.64^{c}	17.59 ± 0.48^{d}

Data are presented as mean ± SD; Data in same row with different superscripts are significantly different ($p<0.05$), N=10.

Discussion

The present study demonstrates that the nanoemulsions of clove oil and lemon beebrush have significant anesthetic effect in beluga. Additionally, these new formulated herbal agents could induce anesthesia much quicker than MS-222. According to keen *et al* (1998), an effective anesthetic induce within 3min with a recovery at most 10 min. Clove oil has been shown to be safe for humans (Miller, Swanson, Phillips, Flethcer, Liem & Miller 1989) and the U.S. Food and Drug Administration has classified it as a generally considered as safe (GRAS) compound (Summerfelt & smith, 1990). Because of its organic substance, clove oil does not require any withdrawal period in contrast to some anaesthetics like MS-222 (Ross & Ross 2008). According to present research, NCL induced anesthesia in 3min, which shows the best induction time. Moreover, the recovery time was less than 4 min in beluga.

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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 organisms in water. For instance, a clove oil / ethanol mixture can reduce the growth and increase the mortality of the branching coral, *Pocillopora verrucosa* (Mulochau & Durville 2004). In addition, Frisch, Ulstrup and Hobbs (2007) reported that use of 100 ml clove oil-ethanol mixture (10% clove oil) bleached and incurred tissue mortality in *P. damicornis* colonies. Other researches show that ethanol as a solvent is likely to increase bleaching, but not disturb growth rate comparing to clove oil dissolved in seawater (Boyer, White, Stier & Osenberg 2009).

By applying new formulation for delivery of clove oil we could produce an extremely soluble and clear solution with highly safe materials which can reach to deep anesthesia with lower amount. Nanoparticles are best potential drug delivery methods, since these tiny particles are easier to be taken up by the cells, while larger particles end up being removed from the body. Several drugs have the problem of poor water solubility. For instance, Propofol is also insoluble anesthetic agent in water and therefore is instead formulated as a nano size emulsion in soybean oil and egg phospholipid, with a mean globule size in the range of 300 nm (Park, Park, Chi, Kil & Lee 2003).

Shaluei, Hedayati, Jahanbakhshi & Baghfalaki (2012) compared different concentration of 2phenoxy ethanol in juvenile beluga and at concentrations of 0.7 and 0.9 mL L^{-1}, all the fish were anaesthetized within 3 min of exposure. Recommended maximum clove oil concentration for induction time (stage 4) during the short time anesthesia is equal to 100 mgL^{-1} (73.5 mg L^{-1} eugenol) with 9-14 min time for stage 3 of recovery (Mohammadi Arani, Shahbaz & Safari 2013) which is quadruple higher than used NCL concentration. 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 (Rotllant, Balm, Perez-Sanchez, Wendelaar-Bonga & Tort 2001; Skjervold, Fjaera & Oestby 1999). Thaylise, Parodi, Cunha, Becker, Zeppenfeld, Martins, Koakoski, Barcellos, Heinzmann & Baldisserotto (2014) recommended the optimum concentration of lemon beebrush essential oil which gives the best response considering time to anesthesia (5.35 min) would be 200 ul L^{-1}. In addition, Persian sturgeon *A. persicus* exposed to a 200-mgL^{-1} solution of clove oil had induction times ranging from 2.3 to 2.8 min and recovery times ranging from 2.4 to 3.0 min post sedation (Imanpoor, Bagheri & Hedayeti 2010). Above data, obviously demonstrates the improvement of anesthetic effects of lemonbeebrush and clove oil in new formulation developed by present study.

Fish are routinely anesthetized during stressful handlings (Shi, Li, Zhuang, Nie & Long 2006), but inappropriate anaesthetic dosage or exposure time probably induce stress which affect the blood biochemical parameters.

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Mostly, cortisol has been used as a common indicator of different stressors for fishes (Wagner, Arndt & Hilton 2002; Czesny, Rinchard, Garcia & Dabrowski 2003). In accordance with present research, Feng *et al* (2011) showed physiological parameters in juvenile Siberian sturgeons were less affected by clove oil than by MS-222, suggesting that clove oil is a good alternative to MS-222. In addition, Wagner, Singer and McKinley (2003) found that 1 h exposure to MS-222 significantly elevated cortisol levels in rainbow trouts signifying MS-222 as less effective anesthetic compared to clove oil in reducing stress. In contrast, Thyalise *et al*. (2013) reported that *Aloysia triphylla* essential oil (lemon beebrush) increases whole-body cortisol levels in the albino strain of silver catfish.

Decrease in glucose plasma levels reflect inhibition of catecholamines and glucocorticoids release from adrenal tissues in fish (Iwama *et al*. 1989) which means stressful conditions have been harnessed during anesthesia. Our findings in current research, was somewhat in agreement with Johnson, Trushenski and Bowker (2016), results in Pallid sturgeon exposed to60 mg mL^{-1} eugenol.In contrary,normal plasma cortisol and glucose level increased in Persian sturgeon exposed to 200-400 mg L^{-1} clove oil (Bagheri & Imanpoor 2011). Applying respectively clove oil and MS-222 in Persian sturgeon and Adriatic sturgeon *A. naccarii* as a sedative agents affected mean glucose levels ranged from 41 to 61 mg dL^{-1} (Cataldi *et al*. 1998; Imanpoor *et al*. 2010).

Hoseini, Hosseini & Jafar Nodeh (2011), reported Beluga exposure to low concentrations of clove solution would result in lactate increase which is in accordance with our findings in NCL treatment. Increase in lactate refers to circumstances that tissue oxygen delivery is inadequate. The inadequate oxygen supply slows mitochondrial metabolism and pyruvate is converted to lactate (and NADH to NAD+). This situation is known as anaerobic metabolism and results in a small net ATP production: two moles of ATP per mole of glucose (Kruse & Carlson 1987). 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 & Ross 2008). It is assumed that, quicker anesthesia induction by new formula leads to sudden drop of blood oxygen, which conduces to increase in lactate.

Our results showed that different anesthetic upsets some blood parameters. Total and differential leukocyte counts are factors, which might change during exposure to stressful conditions (Wedemeyer, Barton & McLeay 1990). Previous studies have confirmed that stress impacts on total and differential leukocyte count are in accordance with our results. WBC count change in *Huso huso* following exposure to clove solution reported by Hoseini & Gelichpour (2012). Moreover, previous works on anesthesia showed no significant change in WBC and differential leukocyte count after clove oil exposure (Mohammadizarejabad, Bastami, Sudagar & Pourali Motlagh 2009) (Velisek, Svobodova & Piackova 2005a; Velisek, Svobodova, Piackova, Groch & Nepejchalova 2005b). Despite of some reports inconsistent

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to our findings, Velisek *et al*. (2005a, b) report is in agreement with present findings. They observed no significant effects of clove oil exposure on hematological parameters in common carp, *Cyprinus carpio* (L.) and rainbow trout, *Oncorhynchus mykiss* (walbaum). The increase in Hct and RBC reported by some researcher during exposure to anesthetics is due to the effect of anesthetic concentration, fish species and water parameter which indeed cause stressful condition for fish. Stressful environment impacts stress hormone release such as cathecolamines which are primary stress response contributing erythrocytes to swell and spleen to release new erythrocyte to blood (Wendelaar Bonga 1997). The absence of significant difference in these parameters indicates that exposure to the anaesthetics is not an important stimulus to activate the sympathetic nervous system, in which the end point is the release of catecholamines into blood from the chromaffin tissue (Weber, Pe´rez-Maceira, Peleteiro, Garcı´a-Martı´n & Aldegunde 2011).

Complement and lysozyme activities are major immune parameters to be used in fish stress studies (Ortuno, Esteban & Meseguer 2002). According to our research, it was clearly demonstrated that these non-specific immune parameters get activated following anaesthesia thus enhance disease resistance properties of the *Huso huso*. The best effect was recorded in fish anaesthetised with NLB followed by NCL and MS-222. Immunosupresive effects of anesthesia has been formerly described in fish (Ortuño *et al*. 2002). Indeed, the is not sufficient information available on the relationship between anaesthetics and the fish immune system. Based on Azad, Al-Yaqout & Al-Roumi (2014) investigation on anesthetic effect of thyme oil , clove oil, Aqui S and quinaldine, these anesthetics elevated lysozyme activity in blue- fin bream (*Sparidentax hasta*) and yellow fin bream (*Acanthopagrus latus*) at 20, 40 and 60 mg L^{-1} concentrations.

The results of our preliminary study suggested that using clove oil and lemonbeebrush nanoemulsions are safe and very effective products for reducing the influence of poly etiological stress in beluga.

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Conflict of interests

The authors declare that there is no conflict of interest.

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استفاده از ماده بی هوشی گیاهی نوین در مقایسه با تریکایین متان سولفانات (MS-222) در فیل ماهی

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چکیده

استفاده از داروی بی هوشی در هنگام دستکاری ماهی به ویژه در مواقع نشانه‌گذاری توصیه می‌شود. هرچند باید توجه داشت که استفاده از غلظت نامناسب بی هوشی به نوبه خود می‌تواند موجب افزایش استرس گردد. به کارگیری نانوامولسیون‌ها در فرمولاسیون دارویی می‌تواند با بهبود دارورسانی مؤثر منجر به استفاده از غلظت کمتر دارو شود. در پژوهش حاضر، تأثیر بی‌هوشی نانوامولسیون اسانس میخک و نانوامولسیون اسانس به لیمو به منظور افزایش کارایی ماده بی‌هوشی با اعمال کمترین غلظت بر روی فیل ماهی با میانگین وزنی (۲۴۲/۲۵ ± ۱۱۱۵) گرم مورد بررسی قرار گرفت. نتایج به دست آمده نشان‌دهنده کاهش معنی‌دار مراحل ۳ و ۴ بی هوشی در نانو امولسیون‌های میخک و به لیمو در مقایسه با تریکایین متان سولفانات بود ($p<0.05$). رسیدن به مرحله ۵ بی‌هوشی در فیل ماهیان بی‌هوش شده با نانوامولسیون میخک به طور معنی‌داری سریع تر از فیل ماهیان بی‌هوش شده با نانو امولسیون به لیمو و داروی شیمیایی تریکایین متان سولفانات بود ($p<0.05$). در مقابل، مدت زمان بازگشت از بی‌هوشی در هیچکدام از تیمارهای بی هوشی تفاوت معنی‌داری نشان نداد ($p>0.05$). در میان فراسنجه های خونی اندازه‌گیری شده، مقدار لاکتات در ماهیان بی‌هوش شده با نانوامولسیون میخک در مقایسه با گروه‌های دیگر افزایش معنی‌داری داشت ($p<0.05$). در حالیکه بیشترین مقدار لاکتات در فیل ماهی بی‌هوش شده با نانوامولسیون به لیمو در ۲۴ ساعت بعد از بی هوشی دیده شد ($p<0.05$). مقادیر پارمترهای ایمنی ذاتی (لیزوزیم و کمپلمان) در ماهیان بی هوش شده با نانوامولسیون میخک و به لیمو در هر زمان بی هوشی (۰) و ۲۴ ساعت بعد از بی‌هوشی بیشتر بود ($p<0.05$). بعلاوه عدم افزایش مقادیر کورتیزول و گلوکز در فیل ماهیان بی‌هوش شده با نانوامولسیون های میخک و به لیمو و همچنین بهبود فاکتورهای ایمنی می تواند نشان‌دهنده کم خطر بودن نانوامولسیون میخک و به لیمو در فیل ماهی می‌باشد.

کلمات کلیدی: *Aloysia triphylla*، *Eugenia caryophyllata*، ایمنی ذاتی، بی‌هوشی، فیل ماهی

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