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Comparison of Electroanesthesia with Chemical Anesthesia (MS222 and Clove Oil) in Rainbow Trout (*Oncorhynchus mykiss*) using Plasma Cortisol and Glucose Responses as Physiological Stress Indicators

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Abstract: This study investigates Alternating Current (AC) electroanesthesia of rainbow trout (*Oncorhynchus mykiss*) in comparison with MS222 and clove oil, using plasma cortisol and glucose concentrations as stress assessment indicators. A microcontroller-based apparatus was designed and constructed to allow a programmable voltage-time Pulse-Width Modulated (PWM) electrical wave application through 19×20 cm submersible electrodes for 91sec in a 33 cm long tank to induce loss of equilibrium and immobility with recovery after 52±27 sec. Recovery after 660±102 sec was observed in MS222-anesthetized fish (after induction for 720±72 sec) and a recovery time of 546±102 sec was observed in clove oil-anesthetized fish (after induction for 144±42 sec) both are significantly longer recovery times in comparison with electroanesthesia ($p<0.001$). Using direct enzyme-linked immunosorbant assay (ELISA) for cortisol and enzymatic colorimetric assay for glucose assessments at 0, 1, 6, 12 h after each anesthesia, the anesthetics indicated similar trend of cortisol responses during 12 h of investigation. The dilatory trend of glucose changes and response derived from anesthetics and electricity and its surge at 6 h after anesthesia ($p<0.05$) confirmed glucose as a second order indicator of stress responses. Electroanesthesia is a fast, economic, eco-friendly and safe anesthetic method provides desirable trout immobility for aquaculture activities.

Key words: Electroanesthesia, stress, MS222, clove oil, cortisol, rainbow trout

INTRODUCTION

Fish anesthetics (physical/chemical) are valuable tools that help to reduce fish struggling and physical damages due to stressful rearing and propagation activities (e.g., handling, sorting, spawning, transportation, vaccination, injection etc.). A number of parameters such as expense, availability, ease

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of use and human safety, have to be considered prior to the choice of an anesthetic. Appropriate induction and recovery times of the anesthesia and level of anesthetic-induced stress responses also require consideration. Although, chemical anesthetics like tricaine methane sulphonate (MS222 is the most-frequently used and ouly FDA approved anesthetic for edible fish in U.S.) and clove oil (eugenol) and derivatives (generally considered as safe by FDA; GRAS) are used to alleviate negative consequences of stress responses (Summerfelt and Lynwood, 1990) but there is some evidence of undesirable and stressful consequences after chemical anesthetic application. For example MS222, Metoimdate and AQUI-S reduce the contractile efficacy of ventricular myocardium of Chinook salmon (*Oncorhynchus tschawytsha*) (Hill *et al*., 2002).

MS222 alters brain currents needed to extract an action potential of supramedullary/dorsal neurons of Cunner (*Tautogolabrus adspersus*) (Arnolds *et al*., 2002) . Also MS222 and eugenol are not considered to be completely suppressive of stress-induced cortisol and glucose responses in rainbow trout (Wagner *et al*., 2002). Palić *et al*. (2006) believe that MS222 and eugenol do not prevent the cortisol response of stress and MS222 is not capable of preventing the degranulation of neutrophil primary granules as a stress indicator in fish. In toad *Bufo marinus* an immense disturbance of hematological and cardiorespiratory parameters accures after anesthesia with benzocain (Andersen and Wang, 2002). Kiessling *et al*. (2009) described that both MS222 and isoeugenol caused a marked increase in plasma cortisol at the end of exposure and believe that different anesthetics provoke physiological stress on their own. Although, the eugenol derivatives are relatively less expensive than MS222, but extraction of a standard pharmaceutical form from clove plant needs laboratory facilities and investments which may not always be available.

The cost beneficial alternative which eliminates the problems of chemical residues, withdrawal periods and ecological pollution and is assumed to supply short induction and recovery time and immobility for aquaculture activities, is electroanesthesia which is considered to have fewer effects on plasma and tissue electrolyte changes in comparison with MS222 (Jenning and Looney, 1998).

The development of an apparatus to investigate fish electroanesthesia was defined as the first objective using evaluation of endocrine anesthetic-induced stress indicators (plasma cortisol and glucose) in comparison with current chemical anesthetics (MS-222 and clove oil), introduced as second objective of this study.

MATERIALS AND METHODS

Fish

Three-hundred rainbow trout (fasted for 24 h) weighing 60±15 g (Mean±SD), were purchased two weeks before the experiment (Parvaresheh Ghezelala Jajrood Co., Tehran, Iran) and transported to experimental tanks (stocking density = 3 kg m^{-3}) at the Faculty of Veterinary Medicine, University of Tehran, supplied with 15±2°C aerated flowing well water. Water quality parameters (pH = 7.8, Electrical conductivity; EC = 870 μs cm^{-1}, Dissolved oxygen; DO = 8.3 mg L^{-1} and Hardness = 300 mg L^{-1} as $CaCO_3$) were recorded using a Multi-340i detector (WTW, Germany).

Feeding started 24 h after arrival with the same batch of food used at the original rearing farm (Chinneh Co., Iran). Experimental procedures started after 14 days of acclimation on December 2007 and final raw data were elicited nntil April 2008.

Experimental Design

The experiment was performed for three treatments of anesthesia; electricity, MS-222, clove oil and control group. The experiment was triply repeated for each treatment and control (n = 48). The control group fish were transferred (in nets) from the acclimation tank to a recovery tank located at the Ichthyology Lab. Fish intended as treatment designated animals were transported (in nets) to an anesthetic bath supplied with air stones. After treatment these fish were placed in recovery

tanks under similar conditions to the control group. Blood samples were taken from all animals (treatment and control) through peduncle vessels (Wagner *et al*., 2002) at 0, 1, 6 and 12 h after placement in recovery tanks.

Electroanesthesia

A prototype of an alternating current (AC) electroanesthesia apparatus was designed and constructed based on Chiba *et al*. (2006) study and Ross *et al*. (2008). This unit was equipped by microcontroller system to allow an automatic voltage-time adjustments of the anesthesia steps instead of manual adjustments (Chiba *et al*., 2006). In order to adjust and calibrate the apparatus, a number of pilot experiments were performed on rainbow trout, common carp (*Cyprinus carpio*) and gold fish (*Carassius auratus*) to select the optimal voltage wave form and time of insult for the minimum vigorous movements of fish during anesthesia. The sinusoidal PWM electrical waves were found to have the least effect on behavior in rainbow trout allowing the authors to devise a pattern of voltage-time application. Groups of 4 fish were transported to the anesthesia tank (19×20×33 cm) and placed between two submersible stainless steel plate electrodes (19×20 cm) which inserted in a distance 33 cm from each other. The PWM voltage waves form was applied to electrodes for 91 sec with the defined pattern of voltage-time variations.

Chemical Anesthesia

The fish were immersed in 100 mg L^{-1} of Tricaine methane sulphonate (Ross *et al*., 2008) (MS-222, Finquel®; Argent TR2905, Redmond, WA, USA) and 100 mg L^{-1} of clove oil (Soltani *et al*., 2002) (Zardband Co., Tehran, Iran) up to stage 3 of anesthesia (surgical anesthesia) (Ross *et al*., 2008). Then the fish were immediately transported to recovery tanks and time of recovery was noted.

Analytical Procedures

Cortisol

Blood samples collected in 1.5 mL tubes containing Heparin (5000 IU) were centrifuged for 12 min (1500 r min^{-1}) at laboratory room temperature to separate the plasma which was preserved at-18°C.

Although, radio immunoassay (RIA) is the most accepted method for quantitative detection of cortisol in fish (Patiño *et al*., 1987; Small and Davis, 2007) the problems of handling radio isotopes are immense due to short half-life and human health risk. Therefore, in the present study we applied direct enzyme-linked immunosorbant assay (ELISA) which has been validated by Sink *et al*. (2008) for non salmonid species.

An ELISA kit based on the competitive link between cortisol and related monoclonal antibody was purchased (RE 52061, Lot: 43K028-2, 43K107, IBL, Hamburg, Germany). Since, the kit was fabricated for human samples it was necessary to validate its use for fish samples prior to analysis. Validation was performed by filling the first 8 wells of the ELISA plate with 20 µL of 0, 2.5, 5, 10, 20, 50, 100, 400 and 800 ng mL^{-1} of standard solution (Sink *et al*., 2008). The following four wells were filled with 20 µL of a plasma sample from a highly-stressed rainbow trout (kept out of water for 2 min), assumed to contain a high amount of cortisol diluted in 1/4, 1/3, 1/2, 1 proportions. The next eight wells contained 20 µL of undiluted plasma from another 8 highly stressed fish. According to the kit instructions, the parallelism and linearity in trend of optic absorbance of cortisol between serial-diluted standard solutions of the kit and highly-stressed fish plasma, indicated the kit reliability for use as quantitative assays of cortisol in rainbow trout. Subsequent cortisol assay of treatments and control samples were analyzed according to booklet instructions.

Glucose

An enzymatic-colorimetric kit (1500017, Pars Azmoon Co., Karaj, Iran) was applied for single-spot detection of glucose (Adetunji *et al*., 2008). This method allows the oxygen released by

glucose (catalyzed by glucose oxidase) to react with phenol and 4-amino pyridine (catalyzed by peroxidase) to produce Kinonimin which is photometric-detectable and its quantity is indicative of glucose volume.

Statistical Analysis

A student t-test was used to define level of differences of induction and recovery derived from each method of anesthesia. Based on statistics used by Wagner *et al*. (2002) the confidence level of 95% was used for analysis performed by SPSS software version 16 and the Kolmogorov-Smirnov test and examination of box plots and histograms were used for preliminary assessment of the normal distribution. Also, the Pearson correlation coefficient was applied to investigate presence of correlation between cortisol and glucose changes.

RESULTS

Fish were anesthetized after 720±72 sec (Mean±SD, n = 30) of induction with MS222, after 144±42 sec of induction with clove oil (n = 30) and after 84±33 sec (n = 24) of induction of immobility using the apparatus. Recovery times for anesthetics were 660±102 sec for MS-222, 546±102 sec for clove oil and 52±27 sec for electroanesthesia (n = 13). Significant differences were observed between anesthetics in induction and recovery times ($p<0.001$, Table 1).

No significant differences of plasma cortisol concentrations were observed among three anesthetic treatments and among anesthetic treatments and control group at 0, 1, 6 and 12 h after anesthesia (Fig. 1). Plasma cortisol concentrations of anesthetized and non anesthetized fish increased at 1 h after anesthesia so that the highest concentration belonged to clove oil and MS222, respectively however

Table 1: Duration of induction and recovery times (Mean±SD) for electroanesthesia (n = 24, n = 13 respectively), clove oil and MS222 (n = 30)

Anesthetic	Induction time (sec)	Recovery time (sec)
Electroanesthesia	84±33a	52±27d
Clove oil	144±42b	546±102e
MS222	720±72c	660±102f

Values in each column with different letters are significantly different ($p<0.001$)

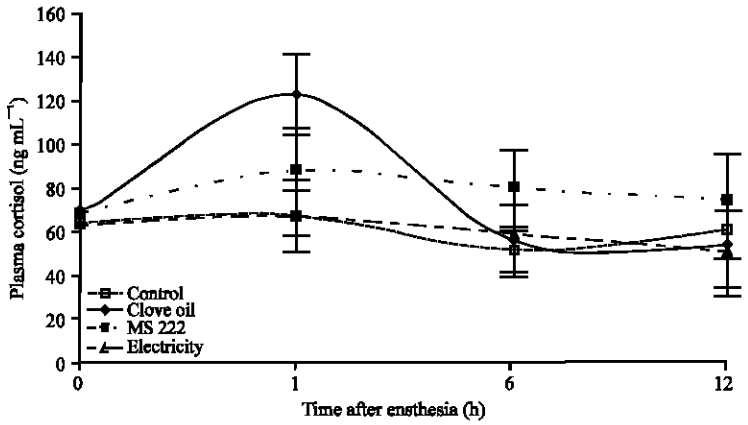

Fig. 1: Cortisol responses of rainbow trout (Mean±SE) to clove oil, MS222 and electroanesthesia during 12 h after anesthesia. No significant difference is observed among groups in trend of cortisol changes

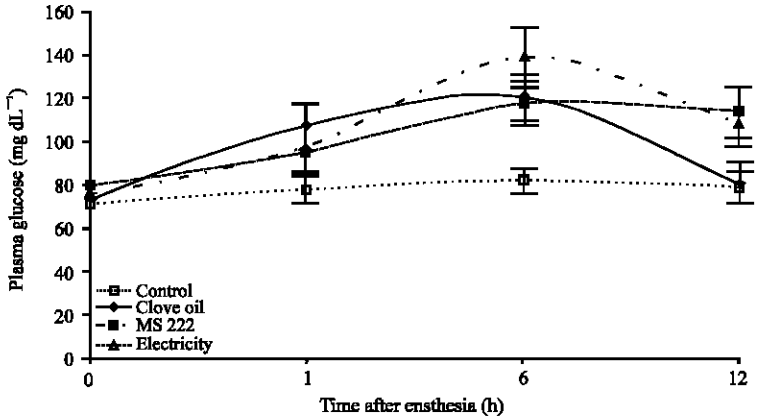

Fig. 2: Glucose responses of rainbow trout (Mean±SE) to clove oil, MS222 and electroanesthesia during 12 h after anesthesia. At 6 h plasma glucose of control group is significantly different from other treatments

after 6 h of anesthesia cortisol concentrations of all groups indicated a decrease that in clove oil, electroanesthetized and control fish was to level lower than the time of anesthesia (0 h) (Fig.1). This decrement was observed for anesthetized groups until 12 h of anesthesia while level of cortisol in control group started to increase after 6 h nevertheless at 12 h was still lower than time of anesthesia (Fig. 1).

Plasma glucose concentrations of anesthetized groups were not different from each other at all sampling times and from control group at 0, 1 and 12 h after anesthesia while they were significantly higher than control group after 6h of anesthesia ($p<0.05$) (Fig. 2). A notable increment of plasma glucose concentrations of anesthetized fish from 0 to 6 h after anesthesia and its recurrence to the level higher than time of anesthesia (0 h) was observed afterwards. The recurrence was notable in electricity and clove oil anesthetized fish (Fig. 2).

DISCUSSION

During normal aquaculture activities some unavoidably stressful procedures have to be performed. Therefore it has become necessary to introduce a number of practical approaches in an attempt to alleviate the detrimental consequences of stress in fish (Barton and Iwama, 1991). One such method applied to reduce the risk of procedural stress is anesthesia, however the application of anesthetics is known to be stressful in itself (Barton and Barton, 1982; Wagner *et al*., 2002; Kiessling *et al*., 2009). For some routinely performed activities e.g., sorting, weighing, injections etc. anesthesia with shorter induction and recovery times are more appropriate. This characteristic is achieved by electro anesthesia in our study with the shorter induction (84 sec) and recovery (52 sec) times in comparison with other treatments. The resulting stress responses of anesthesia can be categorized as endocrine, metabolic and whole animal responses (Barton, 2002). It is of primary importance that reliable indicators are defined in order to evaluate anesthetics in relation to their stress responses. Plasma cortisol and glucose have been introduced as stress indicator in fish (Pickering, 1981; Pickering and Pottinger, 1989; Ortuño *et al*., 2002) and a number of anesthetic-induced increases in plasma cortisol have been observed in several species i.e. sea bream, red drum, rainbow trout and channel catfish (Robertson *et al*., 1998; Barton and Barton, 1982; Tort *et al*., 2002; Small, 2003).

In Chinook and Atlantic salmons, MS222 and eugenol derivatives provoke similar post-recovery cortisol and glucose responses. Cho and Heath (2000) and Kiessling *et al*. (2009) concluded that the

level of nervous depression achieved by anesthetics does not necessarily mitigate certain physiological stress responses.

In Channel catfish when MS222 was compared to metomidate by Small (2003), it caused an eightfold higher concentration of plasma cortisol than metomidate and when it was used at the required dose for anesthesia of Japanese eel, MS222 raised the cortisol level more than 2-phenoxyethanol (Chiba *et al*., 2006). This study showed that the anesthetic-induced cortisol responses did not differ among anesthetics in comparison with control. The electro-anesthetized trout showed no significant difference of cortisol concentration from chemical anesthetics which is a similar observation to that found in electro-immobilized Gold fish (*Carassius auratus*) in comparison with MS222-anesthetized fish (Singley and Chavin, 1975). This similarity in stress responses among electroanesthesia and two chemical anesthetics of our study on one hand and fast induction (84 sec) and recovery time (52 sec) of electroanesthesia on the other hand, are criteria of a suitable anesthetic (Son *et al*., 2001) which was previously confirmed in stripped bass surgery and electroanesthesia was considered as an alternative to MS222 that eliminates risk of chemical overdose-caused death (Jenning and Looney, 1998). Increment of cortisol concentrations in treatment and control groups at 1 h which can be caused by anesthesia, netting and handling and its decrement from 1 to 6 h after anesthesia is in agreement with Wagner *et al.* (2002) findings although this decrement in our anesthetized fish continued to the level lower than time of anesthesia (0 h) in clove oil and electro anesthetized fish until hour 12. The decrement to this level is in contrast to findings of Park *et al.* (2008) in clove oil anesthetized kelp grouper and is representative of species differences in stress responses and suggests efficacy of electroanesthesia besides chemical anesthetics to recover fish from stress caused plasma cortisol increment and its probable long term negative consequenses.

Continuation of decreasing trend of cortisol responses in anesthetized fish from hour 6 to 12 in this study is in contrast to increasing cortisol concentrations in Aqui-s® and MS222 anesthetized trout brood stock and similar to stress responses of CO_2 anesthetized fish performed by Wagner *et al*., (2002). This decreasing trend from 6 h after anesthesia approves alleviating role of anesthetics and electroanesthesia against physiological consequences of stressful handlings nettings and other activities. Increment of cortisol in control group since 6 h after anesthesia in spite of its decrement in anesthetized groups after 6h, implies that anesthesia speeded up recovery from handling and netting stresses.

Lack of significant difference of cortisol response among electro anesthetized fish and other treatments and control is in contrast with Barton and Dwyer (1997) study in juvenile bull trout (*Salvelinus confluentus*) suggesting different physiological responses in different species.

During 12 h of investigation, plasma glucose fluctuations of anesthetized fish followed a similar trend and did not return to 0 h level. Although electro anesthesia and MS222 induced faster increase of glucose concentration than other groups during first 6 h, after this time glucose response of electrical and clove oil anesthetized fish indicated a faster recurrence to normal level than MS222 anesthetized fish. The trend in plasma glucose fluctuations and its magnitude 6 h after anesthesia with electricity and two anesthetics which is in accordance with Wagner *et al.* (2002) findings in Aqui-s® anesthetized trout and observed correlation ($p = 0.00007$ and $r = 0.27$) between cortisol and glucose concentrations in electro anesthetized and control group of this study could be indicative of the influence of cortisol magnitude 1 h after anesthesia and confirm the categorization of cortisol and glucose responses by Barton (2002) as indicators of first and second phase of stress responses, respectively.

It is concluded that in comparison with current chemical anesthetics (MS222, clove oil), electroanesthesia with fast induction and rapid recovery times offers an inexpensive, safe and eco friendly anesthetic alternative which can be useful in aquaculture activities need a kind of brief immobility to do something. This method facilitates rearing activities for more fish per time unit while reducing residues in fish and the environment. The fish electro anesthesia apparatus allows adjustable anesthesia for different sizes and types of fish improving farm application and enhancing physiological

studies. The anesthetics used in this study suggest no different impact on stress responses of plasma glucose and cortisol and no controlling effect on stress responses but precipitating influence on recovery from stress responses. In order to improve fish welfare, future neurophysiologic studies can provide advantageous information on level and duration of unconsciousness, gained by application of this apparatus.

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