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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

EFFECTS OF ANESTHETIC SUBSTANCES ON SOME ANTIOXIDAN ENZYME ACTIVITIES OF TROUTS

Arzu Uçar[1], Muhammed Atamanalp[1], Murat Cankaya[2*], Hasan Özdemir[3]

[1]Ataturk University, Fisheries Faculty, Erzurum, 25240, Turkey

[2]Erzincan University, Science and Art Faculty, Department of Biology, Erzincan, Turkey

[3]Atatürk University, Science Faculty, Department of Chemistry, 25240 Erzurum, Turkey

Abstract: In this study where the effects of naturel (clove oil) and synthetic (2-phenoxyethanol) anesthetic substances were investigated, rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta fario*) were used as fish material. In the blood samples taken from the fishes exposed to the doses of both compounds for 28 days, enzyme activity values were inspected. When enzyme activity values are considered, although the effect of anesthetic substance application on glucose 6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase enzymes have been found to be important ($p<0.05$), the type and interaction have been found unimportant ($p>0.05$). The effect of type on the glutathione reductase enzyme activity values is found to be important ($p<0.05$), whereas catalase enzyme is not effected by application, type and interaction. Clove oil showed inhibition effects on glutathione reductase but 2-phenoxyethanol did not. 2-phenoxyethanol did not exhibit inhibition on catalase enzyme from brown trout.

Keywords: Clove oil, phenoxyethanol, enzymes

Özet:

Anestezik Maddelerin Alabalıklarda Bazı Antioksidan Enzim Aktivitelerinin Üzerine Etkileri

Bu çalışmada naturel (karanfil yağı) ve sentetik (2-fenoksietanol) anestezik maddelerin etkileri araştırıldı. Gökkuşağı alabalığı (Oncorhynchus mykiss) ve kahverengi alabalık (Salmo trutta Dere) kullanılmıştır. 28 gün boyunca her iki bileşik belli dozlarda balıklar üzerinde uygulandı ve balıklardan alınan kan örneklerinde, enzim aktivitesi değerleri incelendi. Enzim aktivitesi değerleri dikkate alındığında, glukoz 6-fosfat dehidrogenaz ve 6-fosfor glukonat dehidrogenaz enzimleri üzerine anestezik madde uygulamasının etkisinin önemli olduğu tespit edilmiş ($p<0.05$) olmasına rağmen tipi ve etkileşim önemsiz bulunmuştur ($p>0.05$). Glutatyon redüktaz enzim aktivitesi değerleri üzerine etkisi önemli bulunmuştur ($p<0.05$), ancak katalaz enzimi uygulamasında her hangi bir etki yapmamıştır. Karanfil yağı glutatyon redüktaz üzerindeki inhibisyon etkileri gösterdi ancak 2-fenoksietanol her hangi bir etki göstermedi. 2-fenoksietanol, kahverengi alabalıkta uygulandığında katalaz enzimi üzerinde inhibisyon gözlenmemiştir.

Anahtar Kelimeler: Karanfil yağı, 2-fenoksietanol, Enzim

* **Correspondence to:** **Murat ÇANKAYA**, Erzincan University, Science and Art Faculty, Department of Biology, Erzincan, TURKEY

Tel: (+90 446) 2243032 **Fax:** (+90 446) 2243016

E-mail: mcankaya@erzincan.edu.tr

Introduction

Anesthesia is a process which is used before performing an operation on a living organism, to prevent pain caused by the operation, to slow down the metabolism rate by decreasing and removing the sensation and consciousness and to slow down or stop the reflex reactions of the organism (Çetinkaya, 1991; Sisecioğlu et al., 2009). Enzymes are the biological catalysts which are synthesized by living cells and speed up the chemical reactions in the living metabolism and bring 100% product efficiency without producing any by-products (Lehninger, 2005). They are synthesized inside the cell and are used in accordance with their purposes. But, in some pathological cases, in the intercellular fluid or blood plasma, the enzyme level increases. It has been informed that the reason for this can be either the increase of enzyme synthesis, or the increase in cell membrane permeability or it can be because of the decomposition of cell, in other words cell necrosis (Lehninger, 2005).

The active substance of 2-phenoxyethanol is ethylene glycol monophenyl ether. Its summary formula is $C_8H_{10}O_2$, the molar weight 138.17 gl^{-1}, density 1.107-1.108 $g \cdot dm^{-3}$, peroxide content less than 0.005% and the boiling temperature is 245°C. The anesthetic is slightly soluble in water (26.7 gl^{-1}) at 25°C but readily soluble in ethanol. The anesthetic affects fish through the skin and gills.

Main advantages of clove oil can be pointed out as; its regulating attribute is limited, its excreting time from the body is short, it is rather safe for human and animals, it can be provided easily and it is inexpensive. It almost meets every measure that should be considered during the selection of anesthetics (Marking et al., 1985; Keene et al., 1998**).**

Besides this, the most important disadvantages of clove oil can be pointed out as follows; its application for a long time would affect the oxygen consumption, it causes color changes in perch, brown trout and atlantic salmon; it causes equilibrium loss for fishes when there is small concentration increases at different carriage conditions in large carrying tanks and it is hard to control the concentration (Hoskonen et al., 2004), the long time of exposure during carriage causes increase of ammonia level. It is mixed with solvents as alcohol as it is not dissolved in water and when it should be used the least dosage that produces anesthesia should be used (Kaiser et al., 2002).

Antioxidant defense system protects the cell from the oxidative damage of free radicals or other reactive molecules (Fridovich, 1976). Antioxidants synthesized in the body may also be taken with diet from the outside. Antioxidant defense systems in living organisms are divided into two main groups. These are produced in metabolism (endogenous) and outside of the diet (exogenous) antioxidant systems (Gulcin, 2002). Endogenous antioxidant system consists of antioxidant enzymes, phospholipase and damaged molecules repellent proteases. Endogenous antioxidants in the structure of the enzyme remove oxidative damage (Fridovich, 1976). Therefore in this defense system, antioxidant enzymes are very important (Gulcin, 2002). Glutathione reductase, catalase and peroxidases are very important antioxidant enzymes. Catalase (CAT) (EC 1.11.1.6) is a characteristic enzyme that is abundant in protein structure. This enzyme is widely present in animals, plants and microorganisms. Also, it plays an important role in removal of toxic hydrogen peroxide from the cells (De Duve, 1983).

Catalase makes the conversion of hydrogen peroxide to water as a one way reaction (Kunce et al., 1986; Havir et al., 1987; Keha et al., 2007) and includes each group as prosthetic group (Kar et al., 1976; Yamaguchi et al., 1984). Glutathione reductase (GR) (EC 1.8.1.7) enzyme catalyzes the electron transfer between low or high molecular weight disulphate substrates and reduced pyridine nucleotides. One of the most important targets of glutathione reductase enzyme that is known is to keep the ratio of GSH (Reduced glutathione)/GSSG (Oxidized glutathione) in the cell medium (Keha et al., 2007). In clinical chemistry, glutathione reductase is used in determination of hepatic disorders and cancer, nutrition, measurement of riboflavin deficiency and determination of some genetic deficiencies (Beutler, 1969). 6-Phospho glyconate dehydrogenase (6PGD) (EC 1.1.1.44) is responsible from very important molecules in living organisms NADPH and ribose 5-phosphate and any defect related with the enzyme would bring many conditions that would be nega-

tive for them. Deficiency of 6PGD negatively affects glucose metabolism in the cell. Because of its vital importance, 6PGD has to be purified from several types and its structural and kinetic characteristics should be determined (Tandogan et al., 2003). Glucose 6-phosphate dehydrogenase (G6PD) (E.C. 1.1.1.49) is one of the most important enzymes of pentose phosphate pathway. The reaction catalyzed by this enzyme is the first reaction of pentose phosphate pathway and constitutes the control point. It is present in almost every animal tissue, plant, yeast and microorganisms (Anderson et al., 1974; Bergmeyer et al., 1983; Lehninger, 2005). The main physiological function of these two enzymes are to produce NADPH, which is essential for reductive biosynthesis and nucleic acid synthesis and protects the cell against oxidants by producing reduced glutathione (GSH). Therefore these two enzymes are similar to antioxidant enzymes (Kuo et al., 2000; Bianchi et al., 2001). The studies that are performed to understand the physiological responses of fishes against the stress elements (stock density, temperature, oxygen etc.) that they are exposed to, their disease conditions, several drugs, heavy metals and pollutants as pesticides are mostly based on hematological, biochemical and histopathological methods (Pickering et al., 1987; Asztalos et al., 1990; Sohlberg et al., 1990; Mazur et al., 1993; Shakoori et al., 1994). However, none of the used methods alone are satisfactory for understanding the physiological responses of fishes.

This study is performed in order to build up the required basis to accurately determine the physiological responses in *in vivo* medium by measuring activity of some enzymes (GR, G6PD, 6PGD, CAT) from the blood samples taken after the application of anesthetic substances (clove oil and phenoxyethanol) to rainbow trout and brown trout. It is aimed to demonstrate that clove oil, which is an organic anesthetic, has no deficiency when compared with its synthetic equivalents and it has no negative effect on the important fish species in inland water fish farming.

Materials and Methods

Experimental groups of 48 fish with an average weight of 180 ±25 g, reared in well water with a constant temperature of 8.5°C at our farm, located at the Research and Extension Center in Atatürk University, was transferred to the Central Laboratory in the Aquarium Fish Rearing Facility and was exposed to a anesthesia dose of 0.5 ppm of clove oil and 0.2 ppm of 2-phenoxyethanol for 4 weeks in circular fiberglass tanks 780 lt in volume (100 cm diameter and 100 cm depth) under natural lighting conditions with a constant flow (1.5-l min^{-1}) of aerated dechlorinated tap water at 9-11°C and with no recirculation. The dissolved oxygen and pH levels and total water hardness were 8-9 ppm, 7.8 and 102 mg in $CaCO_3$, respectively. The tanks were aerated with an air pump.

Eight fish were placed into each of 6 tanks, for testing the clove oil, 2-phenoxyethanol and the other as the control. At the end of each day of exposure, 8 fish from the control tank, 8 fish from the clove oil treatment tank and 8 fish from the 2-phenoxyethanol tank (Ahmad et al., 1995; Shakoori et al., 1996) were taken out and their blood was subjected to hematological analysis (Shakoori et al., 1996; Santhakumar et al., 1999; Atamanalp et al., 2002).

Clove oil, and 2-phenoxyethanol application of anesthetic agent's forms:

Completely insoluble in water bath, clove oil used in the study of anesthesia in 95% ethanol, diluted to 1:10 ratio. Anesthesia; 0.5 ml liter tank was created by adding the anesthetic bath. 2-phenoxyethanol was diluted 1:10 with ethanol bath and 0.5 ml / l was prepared by adding phenoxyethanol. Both anesthetic baths were applied for 1 minute and the fish were stored in clean water tanks. And recovery phases of anesthesia were recorded by monitoring the process.

Measurement of Enzyme Activities

Measurement of G6PD and 6PGD Enzyme Activity:

Glucose 6-phosphate dehydrogenase (G6PD) activity was determined by monitoring NADPH production at 340 nm and 25°C. The assay mixture contained 10 mM magnesium chloride, 0.2 mM $NADP^{+,}$ and 0.6 mM G6PD in 100 mM Tris-hydrochlorid buffer solution at pH 8.0. Assays were carried out in triplicate and the activities were followed up for 60 s. One unit of activity (U) is defined as the amount of enzyme required to reduce 1 µmol/min of $NADP^+$ under the assay conditions (Beutler, 1971).

Measurement of Glutathione Reductase Enzyme Activity:

Glutathione reductase enzyme activity was measured by Beutler's method. One enzyme unit is defined as the oxidation of 1 mmol NADPH per

min under the assay condition (25°C, pH 8.0) (Beutler, 1971).

Measurement of Catalase Enzyme Activity:

The catalase activity was measured by the Aebi method. In this method, 20 ml enzyme solution was added to the 1 ml 10mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 25^0C for 1 min. Initial reaction rate was measured from the decrease in absorbance at 240 nm (Aebi, 1984).

Statistical analysis

The results are presented as means ± SD. Differences between parameters were analyzed by one-way analysis of variance (ANOVA), and significant means were subjected to a multiple comparison test (Duncan) at the a = 0.05 level (Hayran et al., 1995).

Results and Discussion

The variance analysis table for mean values of G6PD enzyme is given in table 1, mean G6PD values according to types are given in table 2 and G6PD values of groups are given in Figure 1. In the control group G6PD enzyme value was found as 2.170±1.477 for brown trout and 1.848±0.113 for rainbow trout. When phenoxyethanol was applied, for brown trout this value was determined as 6.430±0.227, and for rainbow trout 6.591±1.491. For clove oil group, the mentioned parameter was found as 6.591±3.410 for brown trout and 6.109±2.955 for rainbow trout.

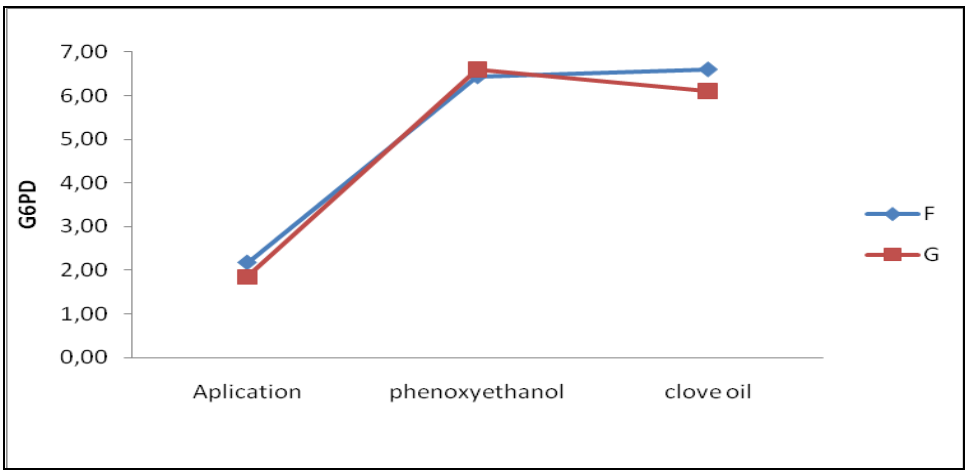
Variance analysis table for 6PGD enzyme mean values is given in table 2. 6PGD enzyme value of the control group was found as 2.049±1.193 for brown trout and 2.049±1.534 for rainbow trout. This parameter was found as 4.702±2.557 for brown trout and 6.511±1.705 for rainbow trout from the application groups for which phenoxyethanol was applied. In clove oil group, for brown trout 6PGD enzyme value was found as 4.702±2.557 and for rainbow trout it is found as 6.993±1.705.

Mean GR and CAT enzymes values are given in table 3- table 4 were observed as 0.602±0.284 for brown trout, 1.004±0.852 for rainbow trout. This parameter was found as 0.602±0.284 for brown trout and 1.004±0.284 for rainbow trout from the application groups for which phenoxyethanol was applied. In clove oil group, for brown trout GR enzyme value was found as 0.401±0.260 and for rainbow trout it was found as 1.808±0.852.

CAT enzyme value of the control group was found as 0.605±0.02 EU/g for brown trout and 0.566±0.159 EU/g for rainbow trout. In the application groups, phenoxyethanol application gave 0.618±0.055 EU/g value for brown trout and 0.154±0.203 EU/g value for rainbow trout. In clove oil group for brown trout was 0.377±0.306 EU/g and for rainbow trout was 0.222±0.145 EU/g.

The importance of G6PD and 6PGD in metabolism has been well known for many years. GSH is used by antioxidant defense mechanisms and its production requires NADPH to be synthesized in the pentose phosphate metabolic pathway in which G6PD and 6PGD participate. For this reason, G6PD and 6PGD are considered as antioxidant enzymes (Reiter et al., 1997).

Figure 1. Glucose 6-Phosphate Dehydrogenase value according to groups (F: *Salmo trutta fario*) (G: *Oncorhynchus mykiss*)



According to the results given in Table 1, both application groups, both application groups have high G6PD enzyme values. Direct measurement of stress may be performed by the measurement of secreted hormones. But this type of approach is fairly expensive. Instead, it is possible to have information about the encountered stress level by (secondary) determination of physiological changes happened in blood and tissues after the stress response. Determination of enzymes in serum which indicate organ or tissue damage, glucose level in blood (indirect indicator of hormonal activity), and CI amount in blood as the ion regulation indicator can be used in measurement of stress effect for different places and purposes (Ogut, 2005). G6PD activity changes depending on nutrition, hormones and especially NADPH concentration. Ozmen et al., 2004 inspected the effects of recirculation system on antioxidant enzymes of rainbow trout and when G6PD enzyme is compared with control group the result were found very important. In the application groups, a significant increase was observed in G6PD enzyme activity as it stops the enzyme synthesis.

6-phosphogluconate dehydrogenase is the third enzyme of the pentose phosphate pathway which converts 6-phosphogluconate compound to D-ribulose 5-phosphate in the presence of $NADP^+$. The importance of the enzyme is because of its reduction of $NADP^+$ to NADPH. In many microorganisms it takes charge as glucose and gluconate catabolic enzyme (Yoshida et al., 1997).The enzyme is important in keeping the equilibrium between glycolytic and pentose phosphate pathway of glucose 6-phosphate (Delmar et al., 1986). As a result of phenoxyethanol and clove oil application, it is determined that the values for brown trout and rainbow trout are found to be close to each other and higher than the values determined for control group. When statistically examined the difference between application, species and application x species interaction is found to be insignificant.

The results of this in vivo experiment indicate that an increase in the activities of G6PD and 6PGD enzymes in the rainbow trout, brown trout were induced in the presence of clove oil, 2-phenoxyethanol. These increased enzyme activity levels ultimately served to protect fish from oxidative stress by increasing the level of GSH because the regeneration of GSH from glutathione disulfide (GSSG) depends on the presence of glutathione reductase (GR) and NADPH in the environment; high activity of the NADPH-generating enzymes, G6PD and 6PGD, leads to an increase in GSH levels (O'Brien et al., 2001).

Catalese and glutathione reductase enzymes directly effects radicals. When statistically examined the effect of application, species and application x species interaction on GR is found to be insignificant. The effects of recirculation system on antioxidant enzymes of rainbow trout and when GR enzyme is compared with control group the result were found very important (Ozmen et al., 2004).

Table 1. Mean Glucose 6-Phosphate Dehydrogenase values according to the type of applied anesthetic substance and multiple comparison test results

Group	Type	G6PD (IU)
Control Group	*Salmo trutta fario*	2.170 ± 1.477^{b}
	Oncorhynchus mykiss	1.848 ± 0.113^{b}
Phenoxyethanol	*Salmo trutta fario*	6.430 ± 0.227^{a}
	Oncorhynchus mykiss	6.591 ± 1.591^{a}
Clove oil	*Salmo trutta fario*	6.591 ± 3.410^{a}
	Oncorhynchus mykiss	6.109 ± 2.955^{a}

Means in columns with different superscripts are significantly different at*p<0.05 important **p<0.01 very important

Table 2. 6-Phospho Gluconate Dehydrogenase results according to the type of applied anesthetic substance

Group	Type	6PGD (IU)
Control Group	*Salmo trutta fario*	2.049±1.193 [a]
	Oncorhynchus mykiss	2.049±1.534 [a]
Phenoxyethanol	*Salmo trutta fario*	4.702±2.557 [a]
	Oncorhynchus mykiss	6.511±1.705 [a]
Clove oil	*Salmo trutta fario*	4.702±2.557 [a]
	Oncorhynchus mykiss	6.993±1.705 [a]

Means in columns with different superscripts are significantly different at*p<0.05 important **p<0.01 very important

Table 3. Mean Glutathione reductase values according to the type of applied anesthetic substance

Group	Type	GR(IU)
Control Group	*Salmo trutta fario*	0.602±0.284 [a]
	Oncorhynchus mykiss	1.004±0.852 [a]
Phenoxyethanol	*Salmo trutta fario*	0.602±0.284 [a]
	Oncorhynchus mykiss	1.004±0.284 [a]
Clove oil	*Salmo trutta fario*	0.401±0.000 [a]
	Oncorhynchus mykiss	1.808±0.852 [a]

Means in columns with different superscripts are significantly different at*p<0.05 important **p<0.01 very important

Table 4. Mean Catalase values according to the type of applied anesthetic substance

Group	Type	Catalase(IU)
Control Group	*Salmo trutta fario*	0.605±0.002 [a]
	Oncorhynchus mykiss	0.566±0.159 [a]
Phenoxyethanol	*Salmo trutta fario*	0.618±0.055 [a]
	Oncorhynchus mykiss	0.154±0.203 [a]
Clove oil	*Salmo trutta fario*	0.377±0.306 [a]
	Oncorhynchus mykiss	0.222±0.145 [a]

Means in columns with different superscripts are significantly different at*p<0.05 important **p<0.01 very important

Compared with control group for catalase enzyme, the values obtained by application of phenoxyethanol with brown trout are close to normal, but lower in rainbow trout. It has been found that the catalase enzyme value obtained after clove oil application is less than control group for brown trout and rainbow trout. Catalase value has not been affected by application, species and application x species interaction. In sea bream it was reported that organic pollutants increase the catalase activity (Uner et al., 2001; Sayeed et al., 2003). This case shows parallelism with the result of the study. But the same chemical has decreased catalase activity in *Channa punctatus*. It is reported that the seasons are effective in catalase activity (Ronisz et al., 1999).

As a result, the necessity of using anesthetics and sedatives in many stages of farming and the advantages it provides make the usage of anesthetics inevitable. But using anesthetics is an application that needs experience and great care. The anesthetic should be not only chosen and used according to the aim, but also its effects on human health should be known very well. Currently, there is only one anesthetic which is ap-

proved by FDA. This is MS-222. Recently, an anesthetic called as aqui-s has been approved by FDA and is used in several countries freely. However, these fishes can be submitted to human consumption 21 days after using anesthetics (Serezli et al., 2003).

Anesthetics provide important ease in many fish handling applications and clove oil is considered as a good choice against synthetic anesthetics.

Conclusion

In this study it was determined that when the natural and synthetic anesthetic substances were used in recommended dosages, they had no negative effects on the blood biochemistry, hematology and enzyme activities of both fish species. In accordance with these results, in fish farming activities it was determined that clove oil is equivalent to 2-phenoxyethanol substance in every respect, practically have ease of use and economically have more advantages. Nowadays, the organic agricultural products gain importance and the clove oil which is a natural compound will come into prominence in fish farming.

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