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Comparison of the effect of four anaesthetics on haematological profiles, oxidative stress and antioxidant enzymes in barbel (*Barbus barbus*)

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Abstract

OBJECTIVES: Currently, many questions regarding the effect of anaesthetics to fish remain unresolved. Fish species may differ widely in their response to an anaesthetic, the screening of dosages is often necessary. The aim of this study was to compare the effect of tricaine methane sulphonate (MS 222), clove oil, 2-phenoxyethanol and Propiscin on haematological profiles, oxidative stress biomarkers and antioxidant enzymes in barbel (*Barbus barbus*).

DESIGN: The haematological profiles, oxidative stress biomarkers and antioxidant enzymes of barbel were evaluated immediately after a 10 min anaesthesia (MS 222 – 100 mg.L^{-1}, clove oil – 33 mg.L^{-1}, 2-phenoxyethanol – 0.4 mg.L^{-1}, Propiscin – 1.0 mg.L^{-1}), and 24 h after anaesthesia.

RESULTS: The 10 min exposure in the recommended concentrations of tested anaesthetics have no significant effect on haematological profiles, levels of thiobarbituric acid reactive substances, and activity of glutathione reductase of barbel. The activity of superoxide dismutase (SOD) was significantly decreased (p<0.01) in the muscle in all experimental groups. The activity of SOD showed a significant decrease (p<0.01) in the liver 24 h after all anaesthetics; however in the gill the activity of SOD was significantly increased (p<0.01) in Propiscin (10 min). The activity of catalase (CAT) was significantly increased (p<0.05) in the muscle 24 h after all anaesthetics.

CONCLUSIONS: The observed effects on barbel antioxidant systems may be a defence against oxidative damage. The results of this study suggest that the antioxidant systems of barbel are altered by Propiscin anaesthesia, but are slightly affected by MS 222, clove oil, and 2-phenoxyethanol anaesthesia.

ORIGINAL ARTICLE

Abbreviations

ANOVA	- analysis of variance
CAT	- catalase
GR	- glutathione reductase
Hb	- haemoglobin
LPO	- peroxidation of lipids
MCHC	- mean corpuscular haemoglobin concentration
MCH	- mean corpuscular haemoglobin
MCV	- mean corpuscular volume
MRL	- maximum residue levels
MS 222	- tricaine methane sulphonate
PCV	- packed cell volume
RBC	- erythrocyte count
SOD	- superoxide dismutase
TBARS	- thiobarbituric acid reactive substances
WBC	- white blood cell count

INTRODUCTION

The use of anaesthetics is common practice in modern aquaculture. The handling of aquatic animals both in and out of their natural environment almost always involves physical activity. Their characteristic struggling during capture and handling affects physiology and behaviour, and consequently, it is often necessary to immobilise fish before attempting to perform even the simplest task (Ross & Ross 2008). Anaesthetic agents are now used widely, ranging from light sedation that aims to reduce stress during handling and non-invasive procedures to full anaesthesia to avoid inflicting pain during surgery and larger interventions (Ross & Ross 2008; Neiffer & Stamper 2009). The decrease in movement minimizes integument damage, associated osmoregulatory disturbances, and increased susceptibility to pathogens (Ross & Ross 2008). As well as metabolism reduction, resulting in a decreased oxygen demand and the production of less waste (i.e., CO_2 and ammonia) (Hoskonen & Pirhonen 2004; Crosby *et al.* 2006). Activities in the Czech Republic must pursue the Act of Protection the Animals against Maltreating (Kolarova *et al.* 2012).

Many anaesthetics, sedatives and analgesic drugs used in vertebrates reduce stress in fish, decrease handling trauma, minimize movement and physiologic changes in response to nociceptive stimuli. But extrapolating from limited published anaesthetic and sedative data, to all fish species is potentially harmful because of marked anatomic, physiologic, and behavioural variations; instead, a stepwise approach to anesthetizing or sedating unfamiliar species or using unproven drugs for familiar species is advisable (Neiffer & Stamper 2009).

The anaesthetics most commonly used are tricaine methane sulphonate (MS 222), benzocaine, quinaldine sulphate, methomidate, clove oil, and 2-phenoxyethanol (Velisek *et al.* 2011; Kristan *et al.* 2012, 2014). Currently, only MS 222 is licensed for use in food fish in the USA and the European Union. However, compounds such as 2-phenoxyethanol, clove oil, and Propiscin have been evaluated experimentally and are being used in non-food fish and in research (Coyle *et al.* 2004). Their use in food fish remains illegal under EEC Regulation 2377/90, as no maximum residue levels (MRL) have been established.

2-Phenoxyethanol is an opaque, oily liquid. The exact mechanism of its anaesthetic effect in fish has not been reported, but it has been suggested that it involves an expansion of neuronal cell membranes (Burka *et al.* 1997). The recommended concentration for fish anaesthesia is 0.30–0.40 ml.L^{-1} (Kolarova *et al.* 2012). The active substance of Propiscin is etomidate [etomidate (1)-ethyl 1-(α-methylbenzyl) imidazole-5-carbo-xylate]. The recommended concentration for fish is 1.0 ml.L^{-1} water (Kazun & Siwicki 2001). Clove oil is distilled from stems, leaves and flower buds of *Eugenia caryophyllata*, and its active ingredient, eugenol (4-allyl-2-methoxyphenol), makes up to 85% of the oil by weight (Keene *et al.* 1998). The used and recommended concentration for fish is 33 mg.L^{-1} (Kolarova *et al.* 2012). Tricaine methane sulphonate is an isomer of benzocaine with an additional sulphonate radical, making it more soluble but also more acidic in solutions (Congleton 2006). The used concentration for anaesthesia in fish is 100 mg.L^{-1} water (Zarski *et al.* 2012).

Anaesthesia of fish may mitigate against the physiological stress due to handling, but the anaesthetic can itself induce alterations in physiology. The purpose of this study was to compare the effects of MS 222, clove oil, 2-phenoxyethanol, and Propiscin on haematological profile, oxidative stress biomarker and antioxidant enzymes in barbel.

MATERIALS AND METHODS

Anaesthetics and other chemicals

MS 222 was purchased from Sigma-Aldrich Chemicals Ltd. (St. Louis, USA). Clove oil (eugenol concentration 78%) was from the Kulich Company (Hradec Kralove, Czech Republic), and 2-phenoxyethanol from Merck (Hohenbrunn, Germany). Propiscin was supplied by the Division of Fish Pathology and Immunology (Zabieniec, Poland).

Experimental procedure

For assessment of the haematological and blood biochemical profile, 63 barbels (18.41±2.79 g body weight and 114.21±3.76 mm total body length) were used. All fish were starved for 24 h before the experiments. Water temperature was maintained at 20.1–20.8 °C, pH 6.93 and oxygen saturation 8.7 mg.L^{-1} throughout the experimental period. Nine groups of 7 fish each were compared in this study:

1. Control – no anaesthetic, were sampled prior to the treatment of anaesthetized groups.
2. Four groups were sampled immediately after 10 min anaesthesia and designated as: Propiscin (10 min) (1.0 ml.L^{-1}), 2-phenoxyethanol (10 min), (0.4 ml.L^{-1}), clove oil (10 min) (33 mg.L^{-1}) and MS 222 (10 min) (100 mg.L^{-1}).

3. Four groups were sampled 24 h after 10 min anaesthesia and designated as: Propiscin (24 h), 2-phenoxyethanol (24 h), clove oil (24 h) and MS 222 (24 h).

The treatments were duplicated for a total of eighteen groups, each held in 40 L glass aquaria containing freshwater plus the anaesthetic and one control aquarium. Each group was held in a separate aquarium. There were no mortalities during the experiment.

Haematological assays

Blood samples were drawn from *vena caudalis* using a syringe with heparin as anticoagulant (Heparin inj., Leciva, Czech Republic) at a concentration of 5000 IU heparin sodium salt in 1 mL. Erythrocyte count (RBC), packed cell volume (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) and white blood cell count (WBC), were determined by Svobodová *et al.* (2012).

Oxidative stress biomarker and antioxidant enzymes

After blood sampling, fish were killed by severing the spinal cord, and samples of gill, liver, brain, and white muscle were taken for evaluation of oxidative stress. Samples for evaluation of oxidative stress biomarkers and antioxidant enzymes were homogenized and prepared for analysis according to Stara *et al.* (2013).

Indice of oxidative stress

Lipid peroxidation (LPO) measured as thiobarbituric acid reactive substances (TBARS) was estimated spectrophotometrically by Lushchak *et al.* (2005) method.

Antioxidant enzymes

Total superoxide dismutase (SOD) activity was estimated spectrophotometrically by Marklund & Marklund (1974) method. Glutathione reductase (GR) activity was estimated using the Carlberg & Mannervik (1975) method. The catalase (CAT) activity was assayed using the method of Beers & Sizer (1952). Proteins were determined by the method Bradford (1976).

Statistical analysis

Statistical analysis was carried out using Statistica software 12.0 for Windows (StatSoft, Czech Republic). Data were first tested for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, one-way analysis of variance (ANOVA) was employed to reveal significant differences in measured variables among control and experimental groups. When a difference was detected ($p<0.05$), Tukey's multiple comparison test was applied to identify which treatments were significantly different. If the conditions for ANOVA were not satisfied, the non-parametric Kruskal-Wallis test was used.

RESULTS

Haematology profile

A 10 min anaesthesia of MS 222 (100 mg.L^{-1}), clove oil (33 mg.L^{-1}), 2-phenoxyethanol (0.4 mg.L^{-1}) and Propiscin (1.0 mg.L^{-1}) did not have significant effect on haematology parameters (RBC, Hb, PCV, MCV, MCH, MCHC and WBC) in barbel (Table 1).

Oxidative stress indice

Levels of LPO (measured by tissue TBARS level) in the brain, gill, muscle, and liver of all groups are summarized in Figure 1. The test groups were not significantly different from the control group in TBARS levels in tissues of barbel.

Antioxidant enzymes

The activity of SOD was significantly decreased ($p<0.01$) in the muscle in all experimental groups compared to the control group. The activity of SOD in the liver showed a significant decrease ($p<0.01$) in MS 222 (24 h), clove oil (24 h), 2-phenoxyethanol (24 h), and Propiscin (24 h); however in the gill the activity of SOD was significantly increased ($p<0.01$) in Propiscin (10 min) compared to the control group (Figure 2). The activity of GR in the brain, gill, muscle, and liver of all groups are summarized in Figure 3. No test group showed significant differences from the control

Tab. 1. Effect of anaesthetic (MS222, clove oil, 2-phenoxyethanol and Propiscin) on haematology parameters in barbel.

Group	Control	MS 222		Clove oil		2-phenoxyethanol		Propiscin	
Time		(10 min)	(24h)	(10 min)	(24h)	(10 min)	(24h)	(10 min)	(24h)
RBC (T.L^{-1})	1.30±0.32	1.44±0.36	1.26±0.10	1.33±0.20	1.22±0.12	1.35±0.17	1.25±0.15	1.36±0.17	1.28±0.10
Hb (g.L^{-1})	53.19±15.72	68.79±17.05	35.94±7.40	64.19±6.31	41.82±10.62	60.77±5.01	39.39±10.59	67.20±7.26	45.49±13.0
PCV (l.L^{-1})	0.31±0.10	0.43±0.13	0.20±0.04	0.36±0.04	0.22±0.06	0.34±0.06	0.22±0.02	0.38±0.04	0.26±0.04
MCV (fl)	259.73±117.87	325.42±138.31	156.71±38.90	278.09±41.49	185.31±54.41	251.99±44.65	178.88±38.19	279.30±33.20	202.30±47.10
MCH (pg)	46.08±24.29	51.44±18.42	28.71±7.00	49.01±5.58	34.82±10.96	45.86±6.37	32.04±9.73	50.40±10.40	35.56±9.50
MCHC (g.L^{-1})	185.37±54.40	170.66±62.18	192.80±53.60	179.93±34.28	197.18±53.47	186.16±36.79	181.74±48.18	181.10±31.10	188.60±69.10
WBC (G.L^{-1})	34.00±9.91	31.93±7.43	30.57±4.20	31.29±6.88	26.86±6.10	31.57±6.21	33.79±8.00	30.80±4.50	31.14±6.60

All values are mean ± standard deviation.

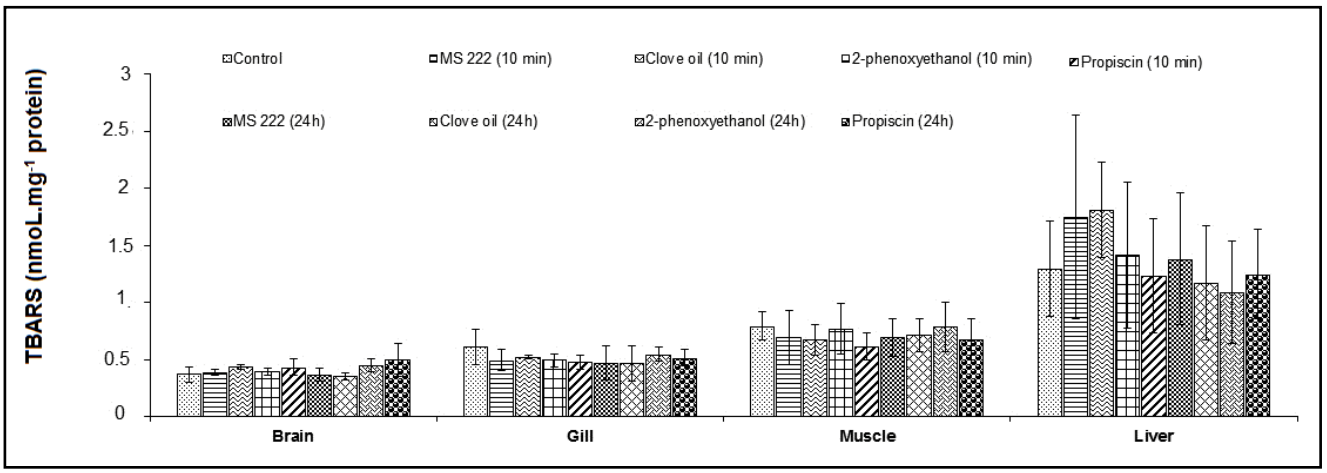

Fig. 1. Effects of MS 222, clove oil, 2-phenoxyethanol and Propiscin anaesthesia on level of thiobarbituric acid reactive substances (TBARS) in barbel tissues. *Significance levels observed are ($p<0.05$) in comparison to the control group. All values are mean ± standard deviation.

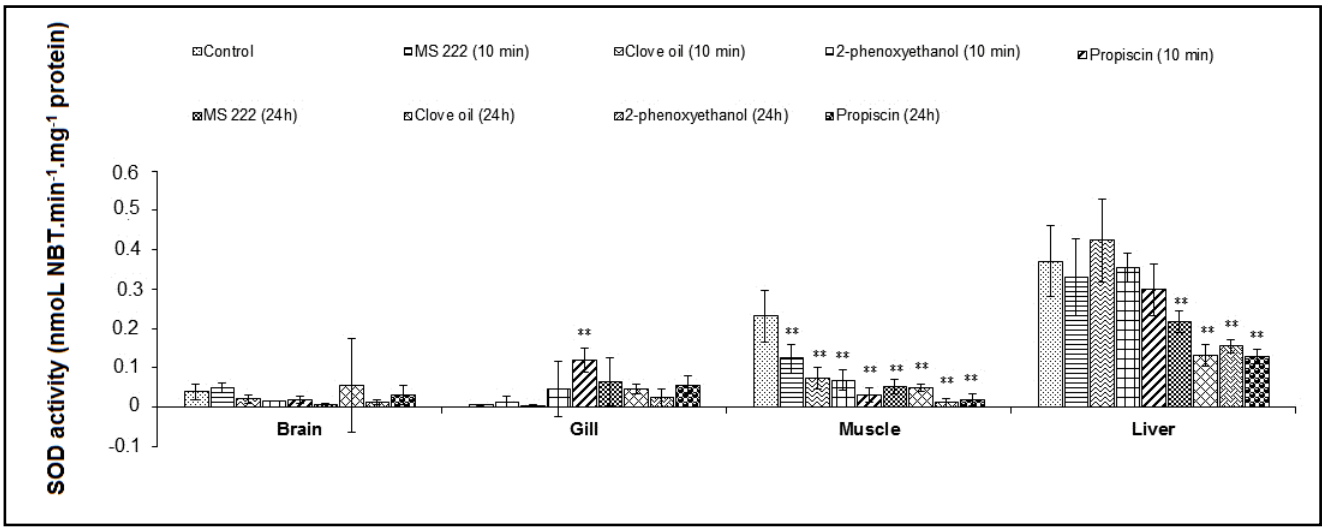

Fig. 2. Effects of MS 222, clove oil, 2-phenoxyethanol, and Propiscin anaesthesia on superoxide dismutase (SOD) activity in barbel tissues. **Significance levels observed are ($p<0.01$) in comparison to the control group. All values are mean ± standard deviation.

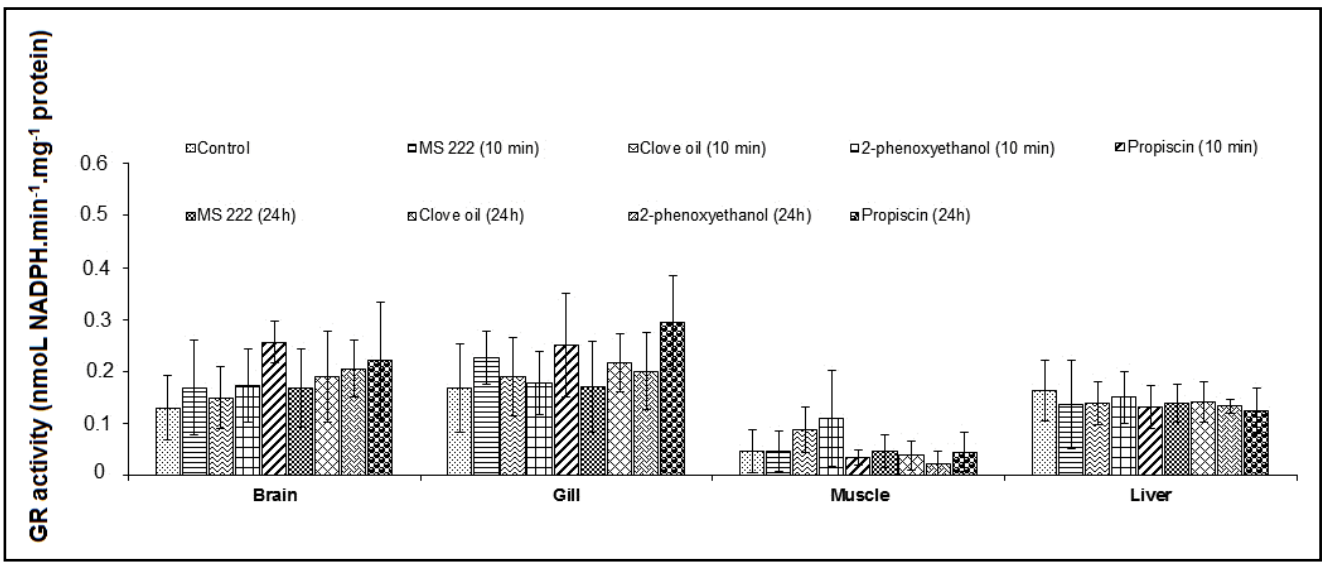

Fig. 3. Effects of MS 222, clove oil, 2-phenoxyethanol, and Propiscin anaesthesia on glutathione reductase (GR) activity in barbel tissues. *Significance levels observed are ($p<0.05$) in comparison to the control group. All values are mean ± standard deviation.

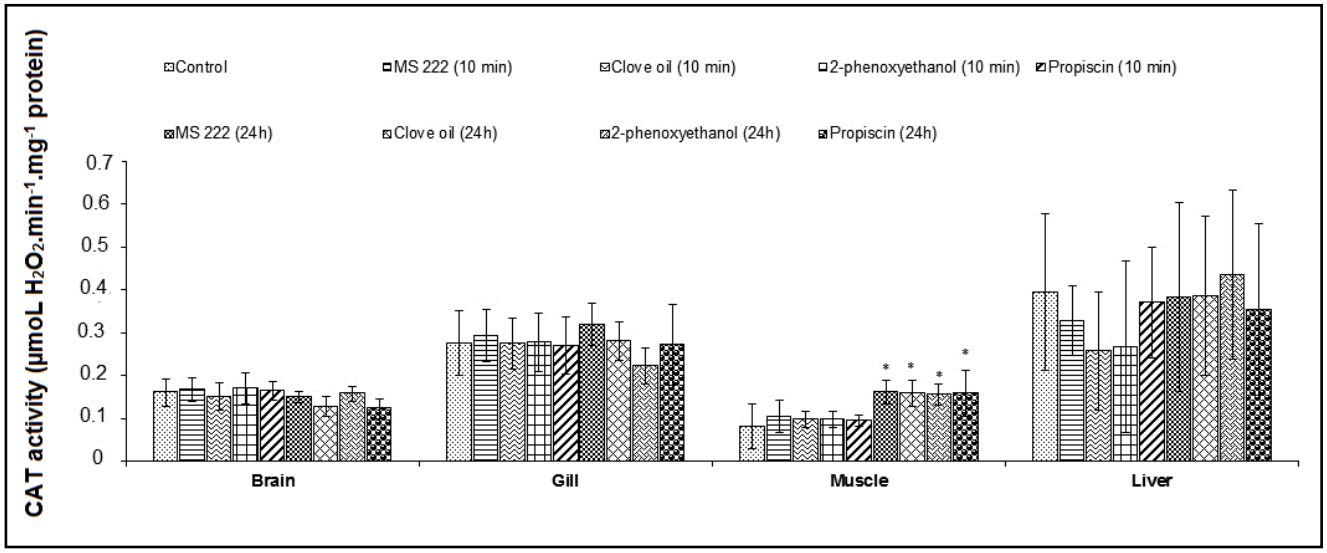

Fig. 4. Effects of MS 222, clove oil, 2-phenoxyethanol, and Propiscin anaesthesia on catalase (CAT) activity in barbel tissues. *Significance levels observed are (p<0.05) in comparison to the control group. All values are mean ± standard deviation.

group in activity of GR in brain, gill, muscle or liver in barbel. The activity of CAT was significantly increased (p<0.05) in the muscle with MS 222 (24 h), clove oil (24 h), 2-phenoxyethanol (24 h), and Propiscin (24 h) compared to the control group (Figure 4). No test group showed significant differences from the control group in activity of CAT in brain, gill, or liver in barbel.

DISCUSSION

The anaesthetics tested in this study were effective as sedatives for routine weighing and measuring procedure of barbel. Haematological profiles are frequently used for evaluating the effect of anaesthetics (Velisek *et al.* 2007; Kristan *et al.* 2012; Lepic *et al.* 2014). To our knowledge, no other data on haematological profiles in barbel anaesthetized with MS 222, Propiscin, 2-phenoxyethanol, or clove oil are available. In our study anaesthesia with MS 222, Propiscin, 2-phenoxyethanol, and clove oil showed no effect on the haematological profile of barbel. Similar results observed in common carp (*Cyprinus carpio* L.) (Velisek *et al.* 2005), European catfish (*Silurus glanis* L.) (Velisek *et al.* 2006) and vimba bream (*Vimba vimba*) (Lepic *et al.* 2014).

In the present study, SOD and CAT activities differed from that of the control group in all tested anaesthetics. The evaluation of antioxidant biomarkers is critical to the investigation of oxidative stress in organisms (Lushchak 2011; Stara *et al.* 2014). SOD and CAT are the major enzymes in eliminating reactive oxygen species, formed during bioactivation of xenobiotics in the tissues (Sk & Bhattacharya 2006) and the induction of SOD and CAT systems provides the first line of defence against reactive oxygen species. Catalase is mainly located in the peroxisomes, and is responsible for the reduction of hydrogen peroxide produced from the metabolism of long-chain fatty acids in peroxisomes (Sheikh *et al.* 1998). Superoxide dismutase is an antioxidant enzyme important in inhibiting oxyradical formation and is used as a biomarker to indicate oxidative stress (Zhang *et al.* 2004). Similar results were observed in a study Velisek *et al.* (2011) with rainbow trout (*Oncorhynchus mykiss*). These authors observed increased reactive oxygen species formation, oxidative damage to lipids and proteins and inhibition of antioxidant capacities in rainbow trout after anaesthesia with MS 222, clove oil, 2-phenoxyethanol and Propiscin. The increase SOD activity may be due to increased production of reactive oxygen species, or SOD activity may be reduced due to direct damage to the structure of proteins exposed to tested anaesthetics and the resulting increase in amounts of hydrogen peroxide. Overall, results indicate disruption of the normal oxidation process, suggesting a failure of antioxidant defence systems represented by SOD and CAT.

On the basis of this experiment, it appears that clove oil and 2-phenoxyethanol was associated with the lowest effects in barbel and therefore would be recommended as an alternative to MS 222. However, the final choice of anaesthetic must take into account legislation, availability, cost-effectiveness, ease of use, and safety for the user and the environment. As clove oil, and 2-phenoxyethanol are not approved for use in food fish, we do not advocate their use in any fish until MRL (EEC Regulation 2377/90) standards are determined and proper licensing is enacted.

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