Stiff as a Board: Measuring Rigor Mortis in Zebrafish

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Aim: To determine the rate of rigor mortis using different anaesthetic agents

Introduction

Schedule 1 Killing (S1K) methods require a two step process: a humane method of death, typically an anaesthetic overdose for zebrafish, and a confirmation of death, such as confirmation of rigor mortis. There is widespread variation of anaesthetics used for S1K, and there is debate about refining anaesthesia for zebrafish, as adverse effects are becoming a concern. Anecdotal evidence suggests that different anaesthetics can inhibit or reduce the rate of the onset of rigor mortis, with some taking an hour, whilst others take more than three. We conducted an trial in order to determine the rate of rigor mortis for four different agents.

Rigor Mortis

Rigor mortis (RM) is a stage of death characterised by muscle stiffness. Following death, lactic acid accumulates, decreasing cellular pH[1]. This disrupts glycolysis and in turn adenosine triphosphate (ATP) levels fall, which breaks actin-myosin cross-bridges. Its depletion results in prolonged muscle stiffness (fig. 1). The rate of onset and duration of RM is dependent on many factors: size; temperature; and pre-mortem exhaustion. Studies on anaesthetics used in zebrafish are limited, and the effects on RM are mostly unknown.

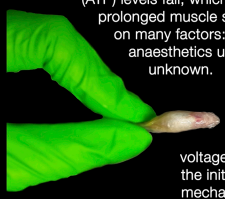

Fig. 1: a zebrafish that is in rigor mortis

Four common anaesthetics are benzocaine, lidocaine, 2-phenoxyethanol (2-PE), and tricaine (MS-222) (fig. 2). Of these, lidocaine, benzocaine and MS-222 all reversibly bind to voltage-dependent sodium channels[2]. This inhibits Na+ uptake which stops the initiation and propagation of action potentials at the site of pain. The mechanism of action for 2-PE is currently unknown in fish.

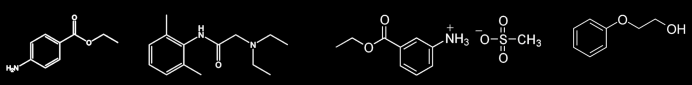

Fig. 2: The chemical structure of each anaesthetic used. Left to right: Benzocaine, (4-Aminobenzoic acid ethyl ester, Ethyl 4-aminobenzoate) chemically similar to MS222. Lidocaine hydrochloride. MS222, (Ethyl 3-aminobenzoate methanesulfonate). 2-Phenoxyethanol.
Attributions: Benzocaine: Mykhal [Public domain]. Tricaine: Edgar181 [Public domain]. 2-Phenoxyethanol: Edgar181 [Public domain]. Lidocaine hydrochloride: Prisonblues at the English language Wikipedia [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0/)]

Methods

A total of 57 hybrid (AB;TupLF) 10 month old adult fish of the same stock were used. The fish were maintained in a recirculating 10 L tank, 28.2 – 28.4°C, pH (7.1-7.3), in a 14:10 hour light/dark cycle and fed on a combination dry food diet. All fish used were of similar size for each respective sex (table 1). The fish were scheduled for euthanisia according to S1K methods, as found in Animals (Scientific Procedures) Act 1986 (ASPA).
The 2-PE dose followed UCL protocol, whilst the other three were based on published recommendations (table 2).

	Average Weight
Males	0.783 g
Females	1.178 g

Table 1: The average weights of each sex. The females weighed more overall.

Anaesthetic	Dosage	Average time of no observed opercular movement	Average time of no observed movement
Benzocaine	0.7 g/L	00:00:26	00:01:01
MS-222	0.5 g/L	00:00:38	00:01:35
2-PE	6 mL/L	00:00:12	00:00:31

Table 2, above: Anaesthetic doses. The the time of no observed movement is the time of assumed death, which occurs after respiration ceases.

Lidocaine Dose	Observation
<500 mg/L	Still swimming >2 minutes
600-700 mg/L	Still swimming >2 minutes Fish exhibited erratic behaviour

Table 3, left: Lidocaine was too adverse; the fish did not loose consciousness quickly enough.

Lidocaine was exlcuded from the trial as it was deemed too adverse to use (table 3); these fish were removed from the trial and euthanised according to UCL protocol. Each anaesthetic was tested in two different post-mortem media: 'removed' into fresh water and 'staying' in dosed water (fig. 2).

Fig.2. Fish immersed into overdose anaesthetic, 10 minutes after observed death transferred to glass petri dish filled with system water (remove) or transferred to dish with same anaesthetic (stay).

For each treatment, three fish were added to the pre-dosed water and left for ten minutes to ensure death. After a knock test to ensure death, individual fish were transferred to labelled petri dishes with the assigned post-mortem media. At 30 minute intervals, each fish was held by the caudal perduncle and measured against a protractor to measure the angle.

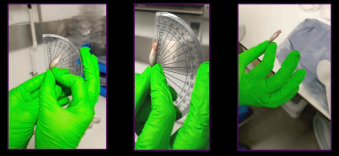

Fig.3. Every 30 minutes, the angle of each fish was measured using a protractor to determine onset/stage of rigor mortis. Most fish reached 0°.

Results

The time points for the measured angles for each treatment were averaged and plotted to compare the rate of RM in each anaesthetic. The most notable difference was 2-PE 'stay', which reached 0° at a faster rate than Benzocaine and MS-222; 2-PE reached 0° at approximately the three hour mark, whereas the other two took more than five hours. The maximum angle was also reached faster in the 2-PE 'remove' media, but at a slower rate.

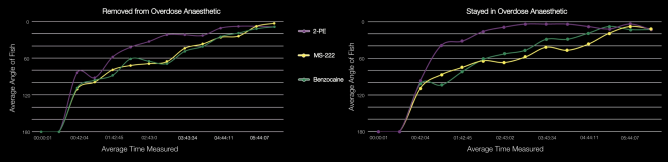

Fig. 4: the different post-mortem media. The fish in 2-PE 'Stay' (right) reached the maximum angle at a faster rate than MS-222 or Benzocaine.

Each anaesthetic was individually plotted to compare the post-mortem media and its effect on the rate of RM.

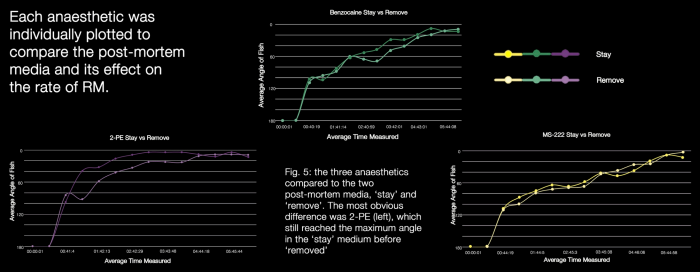

Fig. 5: the three anaesthetics compared to the two post-mortem media, 'stay' and 'remove'. The most obvious difference was 2-PE (left), which still reached the maximum angle in the 'stay' medium before 'removed'

Discssion

It was noted during the trial that the fish euthanised with 2-PE exhibited signs of death sooner than the other anaesthetics by approximately 30 seconds (table 2). Additionally, RM was induced faster with 2-PE compared to the other agents, regardless of post-mortem media (fig. 4). It is unlikely that the small acceleration of death by 2-PE caused such an increase in the onset of RM (fig. 5). Therefore, we must question the possible changes in cellular physiology caused by 2-PE.

When comparing the post-mortem media, it is clear that **2-PE allows for the maximum angle to be achieved first** when the fish stay in the anaesthetic. Benzocaine also appeared to work faster when the fish 'stay' rather than are removed (fig. 5). Surprisingly, MS-222 showed little, if any difference between the media; although they both work similarly at the chemical level, there is a suggestion that there is a larger difference between them than initally assumed at the beginning of the trial.

The dosages may also play a role. Benzocaine and Lidocaine dosages are quantatively small compared to 2-PE (table 2).

Studies on other species suggest possible side effects of 2-PE: raised cortisol and glucose levels pre-mortem; lowered blood O_2; increased CO_2; and reduced pH[3]. Both high levels of cortisol and decreased pH and glucose lower the rate of glycolysis, decreasing ATP, which directly increases the rate of RM.

The potential increased stress that these animals experience during euthanasia should be of great concern. Although the data collected here is focussed on non-protected animals (i.e. dead), a potential application for this information is a **refined** use of S1K anaesthetics for zebrafish. The exclusion of Lidocaine from the trial is a good example: it is assumed that a quick death is the ideal, but for more than two minutes, the fish did not exhibit a desirable anaesthetic depth, unlike the other agents which did so in less than two minutes. Conversely, 2-PE took ~30 seconds, yet there is now a question about cortisol/stress present with this agent. It is possible that we did not wait long enough in our trial to see sufficient effects from Lidocaine for S1K. As a result of this, we need to establish if a **slower, possibly less painful, death** would be more ethical than aiming to achieve the quickest possible induction of death.

Further Work

There are potential variables that were not explored, or explored enough, in this trial, such as strain, dosage, and effect of sex/weight; this will be addressed in a repeat trial. Other factors that affect stress will be constrained further, such as handling and health. We will also attempt to establish if the increased rate of RM with 2-PE is from the agent or the increased dosage. The collected data may aid in the refinement of S1K.

References:
1. Nazir, D. and Magar, N. (1962). *Biochemical Changes in Fish Muscle During Rigor Mortis.*
2. Collymore, C., Tolwani, A., Lieggi, C., & Rasmussen, S. (2014). Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science* 53(2), 198-203.
3. Hedayati A,A , et al (2016) Effects of 2-phenoxyethanol (2-PE) anesthesia on some haematological and biochemical indices of silver carp (Hypophthalmichthys molitrix)

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