UNIVERSITI PUTRA MALAYSIA

PATHOLOGICAL CHANGES IN GILLS, LIVER AND KIDNEY OF GOLDFISH (*Carassius auratus*) WHEN EXPOSED TO CLOVE OIL USING THE FISH ANAESTHESIA DELIVERY SYSTEM

HANISAH NORIDN

PATHOLOGICAL CHANGES IN GILLS, LIVER AND KIDNEY OF GOLDFISH (*Carassius auratus*) WHEN EXPOSED TO CLOVE OIL USING THE FISH ANAESTHESIA DELIVERY SYSTEM

HANISAH NORIDN

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

MARCH 2018

CERTIFICATION

It is hereby certified that we have read this project entitled "Pathological Changes in Gills, Liver and Kidney of Goldfish (*Carassius auratus*) When Exposed to Clove Oil using the Fish Anaesthesia Delivery System", by Hanisah Nordin and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999-Project.

DR. MAZLINA MAZLAN
DVM (UPM), MSc. (UPM), PhD. (UPM)
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

DR. MOHD FUAD BIN MATORI
DVM (UPM)
Veterinary Officer
Aquatic Animal Health Unit
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

PROF. DR. MOHD HAIR BEJO

DVM (UPM), PhD. (LIVERPOOL)

Professor

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

DEDICATIONS

This project paper is dedicated to

My family

My friends

ACKNOWLEDGEMENTS

First and foremost, my sincere gratitude and appreciation for my project supervisor, Dr. Mazlina Mazlan for her patience, guidance, knowledge and support throughout this entire project.

In addition, I would also like to thank my co-supervisors, Dr. Mohd Fuad Matori and Prof. Dr. Mohd Hair Bejo for sharing their experiences and thoughts for the improvement of this project.

Also, not forgetting the staff and post-graduate students of the Veterinary Histopathology Laboratory and Animal Health Unit of the Faculty of Veterinary Medicine, including Madam Latifah, Madam Jamilah, Mr. Azmi and Mr. Zainal, whom have guided and assisted me with this project.

My heartfelt gratitude towards Nabila Farahin Ishak, my FYP mate without whom I would have gone through a major breakdown and also to my wonderful friends Adeline, Syahirah, and Crystal for all their support and for their presence in my life.

I would very much like to extend my deepest gratitude and appreciation towards Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia and the Dr's that have kick started this project and those who have helped me in making this project a success.

Lastly, I thank my family, for all their love and support throughout my lifetime.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

G1	Group 1
G2	Group 2
G3	Group 3
G4	Group 4
MS-222	Tricaine methanesulfonate
%	Percent
=	Equal
<	Less than
>	More than
-	Negative
+	Plus
++	Two plus
+++	Three plus
AUP	Animal Utilisation Protocol
cm	Centimetre
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
H&E	Haematoxylin & Eosin
IACUC	Institutional Animal Care and Use Committee
L	Liter
mg	Milligram
mg/L	Milligram/Liter
mL	Milliliter
Ppt	Parts-per-trillion

SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
TBL	Total body length

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek

PERUBAHAN PATOLOGIK PADA INSANG, HATI DAN GINJAL IKAN EMAS (*Carassius auratus*) BERIKUTAN PENDEDAHAN KEPADA MINYAK CENGKIH PADA DURASI BERBEZA DENGAN MENGGUNAKAN SISTEM PENGHANTARAN ANESTESIA IKAN

Oleh

Hanisah Nordin

2018

Penyelia: Dr. Mazlina bint Mazlan

Penyelia bersama:

Dr. Mohd Fuad Matori

Prof. Dr. Mohd. Hair Bejo

Perubahan patologi pada insang, hati dan ginjal ikan Emas (*Carassius auratus*) berikutan pendedahan kepada minyak cengkih dengan menggunakan sistem penghantaran anestesia ikan telah dikaji. Dalam kajian ini, tempoh pengendalian semasa pembedahan dalam ikan Emas adalah 5 minit (G1), 15 minit (G2) dan 30 minit

(G3) menggunakan minyak cengkih sebagai anestesia dengan kepekatan sebanyak 50 mg/L manakala ikan Emas dalam kumpulan 4 (G4) bertindak sebagai kumpulan kawalan. Sampel insang, ginjal dan hati telah diambil daripada semua kumpulan untuk penilaian secara makroskopik dan mikroskopik. Hasilnya menunjukkan bahawa walaupun tidak ada perubahan kasar yang diperhatikan, terdapat perubahan secara histopatologi pada insang iaitu pembengkakan sel epitelium, pengangkatan sel epitelium, edema dan pendilatan saluran darah pada ikan dalam G1, G2 dan G3 dengan skor lesi tertinggi diperhatikan dalam G3. Di samping itu, keputusan menunjukkan bahawa minyak cengkih tidak mempunyai kesan berbahaya terhadap buah pinggang dan hati secara makroskopik mahupun mikroskopik. Kesimpulannya, kajian ini mengesahkan bahawa tempoh pendedahan kepada minyak cengkih selama 5 minit dan 15 minit adalah lebih selamat dalam ikan Emas kerana kurang kesan lesi yang ditunjukkan oleh ikan khususnya pada insang dan sistem penghantaran anestesia ikan menggunakan minyak cengkih menyokong anestesia optimum untuk menjalankan prosedur pembedahan dalam ikan Emas. Oleh itu, kajian ini mencadangkan bahawa tempoh pendedahan kepada minyak cengkih selama 5 minit dan 15 minit adalah lebih selamat kerana tidak memberi kesan patologikal pada insang. Sebagai kesimpulan, hasil kajian ini menunjukkan minyak cengkih boleh digunakan sebagai anestesia untuk pembedahan yang berkaitan dengan ikan Emas dengan menggunakan sistem penghantaran anestesia ikan.

Kata kunci: minyak cengkih, ikan Emas (*Carassius auratus*), anestesia, perubahan patologi, insang, hati, buah pinggang, sistem penghantaran anestesia ikan

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999-Project

PATHOLOGICAL CHANGES IN GILLS, LIVER AND KIDNEY OF GOLDFISH (*Carassius auratus*) WHEN EXPOSED TO CLOVE OIL USING THE FISH ANAESTHESIA DELIVERY SYSTEM

By

Hanisah Nordin

2018

Supervisor: Dr Mazlina Mazlan

Co-supervisors:

Dr. Mohd Fuad Matori

Prof. Dr. Mohd. Hair Bejo

The pathological changes in gills, liver and kidney of Goldfish (*Carassius auratus*) following exposure to clove oil using the fish anaesthesia delivery system were studied. In this study, the duration of maintenance stage during surgery in Goldfish were 5 minutes (G1), 15 minutes (G2) and 30 minutes (G3) using 50 mg/L clove oil while the Goldfish in group 4 (G4) served as the control. The gills, kidney and liver were sampled from all groups for macroscopic and microscopic evaluation. The

results revealed that although there was no gross changes observed, histopathological alterations of gills characterised by epithelial swelling, epithelial lifting, oedema and dilatation of blood vessels were present in fish in G1, G2 and G3 with the highest lesion scoring was observed in G3. In addition, results showed that clove oil has no harmful effect towards kidney and liver both macroscopically and microscopically. In conclusion, this study verified that the duration of exposure to clove oil for 5 minutes and 15 minutes are relatively safer in Goldfish as less injurious effects were observed in gills and the fish anaesthesia delivery system using clove oil can provide optimum anaesthesia to conduct surgical procedures in Goldfish. Thus, this study suggests that the duration of exposure to clove oil for 5 minutes and 15 minutes is relatively safer as there were less pathological effects on the gills. In conclusion, the result from this study revealed that clove oil can be used as anaesthesia for surgery related to Goldfish by using the fish anaesthesia delivery system.

Keywords: Clove oil, Goldfish (*Carassius auratus*), anaesthesia, pathological changes, gills, liver, kidney, fish anaesthesia delivery system

1.0 INTRODUCTION

General anaesthesia techniques are widely used in aquatic animals for many purposes including for a variety of major and minor surgery, in fisheries research and aquaculture. Clove oil is one of the anaesthetic agent for general anaesthesia in fish.

Clove oil is a dark-brown liquid, a distillate of flowers, stalks and leaves of the clove tree *Eugenia aromatica* (Soto & Burhanuddin, 1995). The major constituent (70 to 90 percent by weight) is the oil eugenol, but clove oil contains a wide range of other compounds that impart its characteristic odour and flavour (Coyle *et al.*, 2004). Similarly, acccording to Isaacs (1983), clove oil is distilled from stems, leaves and flower buds of *Eugenia caryophyllata* and its active ingredient, eugenol (4-allyl-2-methoxyphenol) makes up 70 to 90% by weight of clove oil. Clove oil also contains eugenol acetate and kariofilen.

The main advantages of clove oil lies in its low cost and its relative safety to both fish and humans (Keene *et al.*, 1998). It is necessary to provide both anaesthesia and analgesia in fish as the interest in ornamental fish medicine is increasing. Goldfish (*Carassius auratus*) is a common and popular pet nowadays. As Goldfish has a long life span, the pet owner will develop sentimental feeling towards their fish. They will be keen enough to proceed with any procedures if the fish develop diseases. Therefore, surgical procedures in Goldfish is not rare anymore. This study can be one of the references to establish the use of clove oil as anaesthesia via the fish anaesthesia delivery system for Goldfish surgery. Even though, a lot of literature exist about the anaesthetic usage on food fish species, not much information seems to be available on ornamental fish aquaculture (Bystriansky *et al.*, 2006).

Other than that, anaesthesia is important for surgery out of water for extended periods of time. Brown (1987) reported that currently, two methods are available for maintaining fish under general anaesthesia. Lewbart & Harm (1999) also stated that for short procedures, anaesthesia is generally achieved by immersing the fish in MS-222 bath. Immersion anaesthesia with alfaxalone can be used to produce an adequate plane of surgical anaesthesia in a Goldfish (Fernandez-Parra *et al*., 2017).

For longer procedures, continuous anaesthesia delivery can be maintained by pumping anaesthetic-containing water over the gills. Complex recirculation systems which pass an anaesthetic solution over the gills have been constructed and reported by Ross & Ross (1983). An attempt to study the recirculation systems was done by Brown (1987) by using MS-222 on Catfish (*Ictaturus punctatus*). Clove oil recirculation systems has never been attempted and thus, in this study, this method will be attempted. There has been one attempt to study the effects of tricaine as an anaesthetic on Goldfish (*Carassius auratus)* at the different salinities and concentrations, performed by Küçük *et al.* (2016). From their result, MS-222 + salt combination is carried out quickly for Goldfish anaesthesia. It is recommended that 200 mg/L of MS-222 at 12 ppt can be used in Goldfish aquaculture practice. Nevertheless, there is still lack of study in *Carassius auratus* when exposed to clove oil. Thus, this study was conducted to investigate the effect of clove oil at different duration of maintenance stage during surgery in *Carassius auratus*.

This study was undertaken to fulfil the following objectives:

1) To investigate the effect of anaesthetic clove oil in *Carassius auratus* when exposed at different duration by using fish anaesthesia delivering system.

2) To investigate the gross morphological lesion and histological changes on gills, livers and kidneys on *Carassius auratus* upon the exposure of clove oil at different duration by using fish anaesthesia delivery system.

2.0 LITERATURE REVIEW

2.1 Anaesthesia in aquaculture

The use of anaesthesia is extremely crucial in aquaculture and fisheries research to ease in different handling procedures. For instance, tagging, weighing or any veterinary treatment (Kazuṅ & Siwicki, 2001). The main purpose of anaesthesia is to reduce the stressful impacts and discomfort during handling.

2.2 Stages of anaesthesia

Anaesthesia can be divided into induction, maintenance and recovery. Coyle *et al.* (2004) stated that it can be further divided into several stages which are sedation, anaesthesia, surgical anaesthesia and death. The stage of anaesthesia will depend on the dose and the length of exposure with the anaesthetic agent (Table 1).

Table 1: The different stages of anaesthesia in fish.

Stages of anaesthesia	Condition	Behaviour/Response
I	Sedation	Motion & breathing reduced
II	Anaesthesia	Partial loss of equilibrium Reactive to touch stimuli
III	Surgical anaesthesia	Total loss of equilibrium No reaction to touch stimuli
IV	Death	Breathing & heart beat stop Overdose-eventual death

Adopted from Coyle *et al.,* (2004)

Coyle *et al.* (2004) also declared that it is important to maintain desired degree of anaesthesia once it is reached for some time. There will be difficulty in maintaining a uniform depth of anaesthesia as the drug dose and exposure time are cumulative and it will accumulate in the brain and muscle even after blood levels have achieved equilibrium. Reducing the dosage can help in maintaining the desired level of anaesthesia. During this maintenance period, the fish must be closely monitored to observe for change in breathing rate that may indicate over-exposure of the anaesthetic agent.

Other than that, Coyle *et al.* (2004) also described about the recovery stage. Recovery stage is important to return the fish to a normal state and it may vary depending on the type of anaesthetic agent administered. During this stage, great care must be taken in order to prevent mortality and minimize stress. Increasing the flow of anaesthetic-free water over the gills can be done if the fish fails to recover. This may help in accelerating and normalizing the heartbeat. Other than that, gently passing water over the gills and moving the fish backwards and forwards in the recovery bath will absolutely increase gill blood flow and subsequently eliminate the drug rapidly. Table 2 shows the different stages of recovery in fish.

Table 2: The different stages of recovery in fish

Stages of Recovery	Condition	Behaviour/Response
I	Sedation	Motion & breathing reduced
II	Anaesthesia	Partial loss of equilibrium Reactive to touch stimuli
III	Surgical anaesthesia	Total loss of equilibrium No reaction to touch stimuli

Adopted from Iwama *et al.,* (1989)

2.3 Factors affecting anaesthesia

The efficacy of anaesthesia in fish will depend on many factors which are the biological and environmental factors.

Rate of anaesthetic drugs varies among fish species as it depends on the gill area to body weight ratio (Coyle *et al.*, 2004). Different metabolic rates in between fish will also affect the rate of chemical absorption and induction of anaesthesia.

2.4 Clove oil as an anaesthetic agent

The safety range and efficacy of the clove oil absolutely varies and will depend on the species and size of the fish apart from the different concentration and purity of the clove oil itself. Therefore, a pilot study is really important before clove oil can be used in a large volume for any procedure. Clove oil can be used in fish as long as the fish will not be used in human or animal food. This is mainly because clove oil is prohibited for any fish that will be used for consumption by the Canada or United

States and only Japanese can use 10% of eugenol for anaesthetic purposes legally (Hikasa *et al.,* n.d.).

The main benefit of the clove oil would be the inexpensiveness and practically easy to be administered. Clove oil is easy to work with and can be obtained relatively easily (Coyle *et al.,* 2004). Other than that, clove oil is generally recognized as a safe (GRAS) substance by the FDA due to its noncarcinogenic and nonmutagenic properties as described by Maura *et al.,* (1989). Following oral administration, eugenol can be absorbed and metabolized rapidly with 24 hours due to its complete excretion via urine with no evidence of illness effects to the fish (Fischer *et al.,* 1990).

2.5 Methods of maintaining general anaesthesia

There are two methods of maintaining general anaesthesia for fish. Firstly, immersion in the anaesthetic solution can be done for the fish. Brown & Richards (1979) stated that the fish will be operated on air after being removed from the solution or the fish will be placed where the gills can stay in the anaesthetic solution while the body can be raised out for examination. Another method to maintain general anaesthesia would be through a complex recirculation system that can pass the anaesthetic solution through the gills (Ross & Ross, 1983) and deliver them rapidly to the central nervous system.

3.0 MATERIALS AND METHODS

3.1 Experimental Fish

A sum of 20 Goldfish with same total body length (TBL) was bought at a local fish store located in Sungai Buloh, Selangor. The fish were acclimatized for 1 week in a tank prior to trial, in aerated water with same water parameters and water quality. The fish were fed with same feeding regime using the same commercial feed. The water supply to the laboratory comes from a main supply which was passed through a dechlorination unit and the water was changed daily. The feeding were fasted for 24 hours prior to the experiment.

3.2 Recirculating Delivery System

The recirculating anaesthesia delivery system that was assembled for this study is as illustrated in Appendix A. Basically, 2 aquariums were placed where one of the aquarium is at the bottom while another one is at least 40 cm above the top of the other. A tube approximately 0.5 cm in diameter was led from the bottom of the upper aquarium, where it is held down by lead weights, clipped at the aquarium with clothespin and inserted into the mouth of the fish. The fish was placed on a fenestrated tray over the lower aquarium. The water was removed manually by using a beaker to allow the anaesthetic solution to be returned to the upper aquarium. Negative pressure was created in the 0.5 cm diameter tubing. The fish was laid in between the sponges and gently secured in position by wetting the sponges with water.

3.3 Preparation of clove oil stock solution

The clove oil was dissolved in water by shaking it vigorously in a bottle. This is due to the clove oil property that disallow it to dissolve with water naturally. For induction, 100 mg/L of clove oil was added to the induction tank and it was then thoroughly mixed with the water. A total of 0.7 mL of clove oil is then mixed with 6 L of water. Initially, the clove oil was shook vigorously with 375 mL of water in a bottle for 30 seconds to dissolve the clove oil.

For maintenance, 50 mg/L clove oil was calculated and mixed thoroughly with about 375 mL of dechlorinated water in a bottle. Therefore, approximately 2.4 mL of clove oil was added into 40 L of water.

3.4 Experimental design

From a total of 20 fish, the fish were randomly divided equally into 4 groups which are groups 1, 2, 3 and 4. Each group had 5 individual fish. The treated groups which comprised of fish in groups 1 (G1), 2 (G2) and 3 (G3) were exposed to clove oil and subjected to surgery simulation for 5, 15 and 30 minutes respectively. Group 4 (G4) served as the control. The fish in G4 were kept inside an aerated tank that is free from clove oil or any anaesthetic agent.

The fish from G1 was individually transferred from the acclimation tank into the induction tank by using a net. After the fish has been induced, the fish was then ready for surgical simulation procedure. This solution was used for induction for 5 minutes and it was changed for 10 minutes and 15 minutes.

The fish was maintained with a mixture of oxygen and 50 mg/L clove oil by using fish anaesthesia delivery system which is a recirculating delivery system. A total of 2.4 mL of clove oil was mixed in 40 L of water. The same method just like during induction was used to dissolve the clove oil in the water. The recirculating delivery system enabled both the delivery of anaesthetic water from a reservoir to the gills and the recycling of the effluent manually.

Once the fish was quiescent, it was positioned on a fenestrated board lower than the anaesthetic water tank. This was done to provide positive pressure from the anaesthetic water tank to the fish where the anaesthetic water will be continuously flowed from this tank, into the animals' mouth and pass over the gills.

Another tank was put under the fenestrated board to allow the effluent to be recycled back to the fish by submersible pump.

The fish were left under anaesthesia to mimic simulated surgery for 5, 15 and 30 minutes. After each of the stipulated time interval has been reached, the fish were sacrificed via cervical pithing. The same procedure was repeated on all the fish in G2 and G3.

3.5 Histological and morphological observation

Samples such as the gills, kidneys and livers were taken and prepared for histological observation following the routine standard histological technique. Briefly, all of the samples were immediately fixed in Davidson's fixative for 24 hours (Appendix B) and then rinsed with 10% ethanol for 30 minutes. After that, the samples were fixed with 10% ethanol, drained and processed in tissue processor. Next, the samples were embedded in paraffin. Samples were sectioned at 4 μm before being stained by using haematoxylin & eosin (H&E). Any pathological changes involving the gills, kidneys and livers were observed histologically under light microscope**.**

Pathological changes in the sampled organs were scored accordingly. Angsirijinda (2011) stated that the severity of alterations were determined by comparing with control sections. The degree of histopathological lesions were scored from -, +, ++ to +++ based on the percentage of the total fields with histological alterations found per total observed in the five samples for each group, whereby - = no tissue damage in any field of the slides, + = mild histopathological lesions that involved <25% of the field, ++ = moderate lesions that involved >75% of the field and +++ = severe lesions that involved all fields of the slides when compared to the control sections.

3.6 Statistical analysis

Statistical analysis between groups were examined by using SSPS (version 20) with Kruskal Wallis and proceeded with Mann Whitney U test if there was any significant difference in between groups. The data are presented as mean ± SD. Level of significance established in all tests was $p<0.05$. The experimental protocol approved by the UPM Institutional Animal Care and Use Committee with AUP number of UPM/IACUC/AUP-U027/2018 dated 8th January 2018.

4.0 RESULTS

The behaviour and response of each individual fish during anaesthesia was observed and staged according to the criteria stated in Table 1. Once there was total loss of muscle tone, the fish was immediately placed on the fenestrated tray. Anaesthesia was initiated as soon as the tube was placed into the fish's mouth. The anaesthesia took generally 3 minutes to be induced in all of the fish.

4.1 Gross lesion

The gross lesions for all organs for each time intervals were observed in all fish following necropsy and sampling. This study revealed that no lesion was observed in any of the organs sampled from all the fish from all of the groups.

4.2 Histopathological lesion

There was no lesion observed in kidney and liver samples of all the fish in all of the groups. However, microscopic changes were noted such as epithelial lifting, epithelial swelling, oedema and dilatation of blood vessel in the gills of all the fish in the treated groups (G1, G2 and G3). These lesions were observed as shown in Figure 1.

Statistical analysis of the histopathological lesions from the Kruskal-Wallis test revealed that there were significant differences ($p<0.05$) between G1, G2, G3 and G4 (control) for all lesions involving the gills. Table 3 shows the results from the statistical analysis.

Table 3: Effect of clove oil on lesion scoring of gills (Kruskal-Wallis Test)

Test Statistics[a,b]				
	Epithelial cell swelling	**Epithelial cell lifting**	**Dilatation of blood vessel**	**Oedema**
Chi-Square	**14.095**	**16.294**	**16.811**	**15.606**
Df	**3**	**3**	**3**	**3**
Asymp. Sig.	**.003**	**.001**	**.001**	**.001**
a. Kruskal Wallis Test				
b. Grouping Variable: Group				

*Significant level at *$p<0.05$* (Kruskal-Wallis Test)

After that, further test showed significant difference ($p<0.05$) between G4 and all the other groups for all lesions. Table 4 shows that the longer the duration of exposure to clove oil for maintenance stage, the higher the mean lesion score for all lesions. Group 3 displayed the highest mean lesion scores for all lesions when compared to Group 1 and Group 2. Group 1 and Group 2 had mild to moderate histopathological changes in gills for epithelial cell swelling, epithelial cell lifting, oedema and dilatation of blood vessel while Group 3 had moderate to severe lesions for all lesions when observed under the microscope.

All the organs in group 4 appeared as normal cells and there was no lesion observed.

Table 4: Histopathological lesion scores (mean±SD) of gills in groups treated with clove oil (Group 1, Group 2 & Group 3) and control group (Group 4)

Organs	**Lesion type**	**Mean Lesion Score ± SD**			
		Group 1	**Group 2**	**Group 3**	**Group 4**
Gills	**Epithelial cell swelling**	**1.40 ± 0.55^{a}**	**2.20 ± $0.84^{a,b}$**	**2.60 ± 0.89^{b}**	**0.00 ± 0.00^{c}**
	Epithelial lifting	**1.40 ± 0.54^{a}**	**2.20 ± 0.45^{b}**	**2.80 ± 0.45^{b}**	**0.00 ± 0.00^{c}**
	Dilatation of blood vessel	**1.20 ± 0.45^{a}**	**1.40 ± 0.55^{b}**	**2.20 ± 0.45^{c}**	**0.00 ± 0.00^{d}**
	Odema	**1.20 ± 0.45^{a}**	**1.60 ± $0.55^{a,b}$**	**2.40 ± 0.55^{b}**	**0.00 ± 0.00^{c}**

*Each value is the mean ± SD of 5 fish from each group. ^{Ab}Different superscript indicate significant difference ($p < 0.05$).

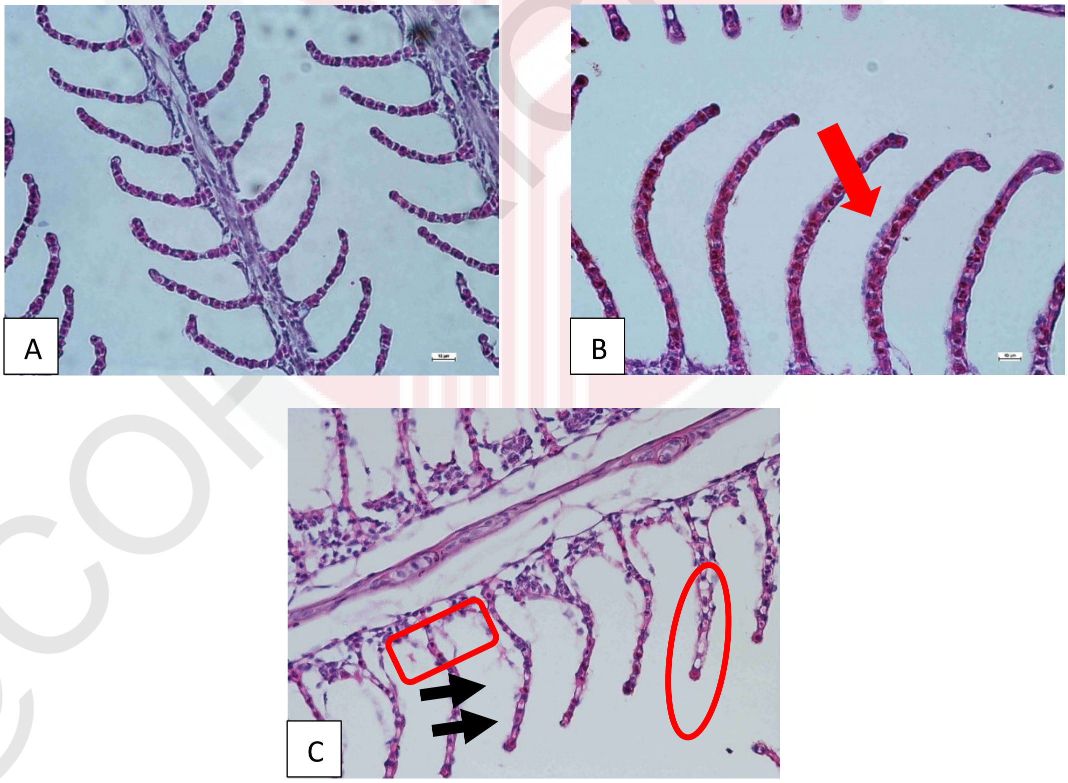


Figure 1: Photomicrograph of gills of *Carassius auratus* when exposed to clove oil (A) The control gills (B-C); The treated groups showed histological changes; (B) epithelial cell swelling (red arrow) (C) epithelial cell lifting (black arrow); oedema (red square); dilatation of blood vessel (red circle). Scale Bar: 10 micron; H&E

5.0 DISCUSSION

Velisek (2005) reported that upon exposure to clove oil for 10 minutes, the histological changes of common carp that can only be found was in the gills which developed capillary ectasia of gills filaments without histopathological changes observed in kidney and liver. Similarly, it was revealed that throughout this experiment, there were presence of histological changes on the gills but no alterations can be detected in the liver and kidney of all the Goldfish (*Carassius auratus*) upon the exposure of clove oil at different time duration by using the fish anaesthesia delivery system.

Anaesthesia of fish is a necessary technique in handling and in any operative procedures that will require any surgical intervention. Green (1979) as well as Ross & Ross (1983) reported that they are many reviews on how to administer the anaesthetic agents to the fish. Brown (1987) stated that the main benefit of the recirculation anaesthesia system is that many surgical techniques can be done over long periods of time despite having difficulty in maintaining a constant depth of anaesthesia and trouble in controlling oxygenation. In this study, we managed to use the clove oil recirculation anaesthesia system successfully. The system has proven to be an extremely useful, economic and reliable long-term recirculation system.

Javahery *et al.* (2012) reported that the use of clove oil as fish anaesthetic would be inexpensive, easy to handle apart from the fact that clove oil can be obtained relatively easily and is claimed to be safe. Throughout this experiment, we found that clove oil can be used effectively for maintenance stage for Goldfish.

Grush *et al.* (2004) stated the need for the clove oil to be prepared by dissolving 1 part of clove oil to 10 parts of ethanol. In this study we shook the clove oil vigorously with 375 mL of water in a bottle. This step was done to ease the use of clove oil and to cease if not to minimise the use of ethanol.

The main reason for choosing the time intervals of 5, 15 and 30 minutes as duration for maintaining the general anaesthesia will depend on the type of procedures that shall be carried out on the fish. The short time interval of 5 minutes is suitable for any short and simple procedures such as routine physical examination while a duration of 15 minutes is appropriate for any procedures that will involve simple but lengthier procedure than others such as tumour removal whereas the 30 minutes of time interval is preferred for any extensive surgery such as laparotomy.

CONCLUSION

In conclusion, fish anaesthesia delivery system using clove oil at the concentration of 50 mg/L as maintenance dose can provide optimum anaesthesia to conduct surgical procedures in goldfish and the duration of exposure to clove oil for 5 minutes and 15 minutes is relatively safer in Goldfish as less injurious effects were observed in gills.

RECOMMENDATIONS

Recommendations for future studies in this area will include the study of haematological parameters of *Carassius auratus* in different time of exposure for maintaining general anaesthesia. Further study of safety range for using clove oil as maintenance in between 15 to 30 minutes can also be done. Further research about the effect of clove oil exposed to Goldfish at different concentration could also be investigated.

Other recommendations that can be done for this study would be to raise the fish by ourselves. This must be done to make sure that only healthy fish will be used throughout this study. Furthermore, it is better to change the anaesthetic solution for every groups in different time of exposure for maintaining the general anaesthesia in order to get more accurate result for the study.

REFERENCES

Abdolazizi, S., Ghaderi, E., Naghdi, N. and Kamangar, B.B. (2011). Effects of clove oil as an anaesthetic on some hematological parameters of *Carassius auratus. Journal of Aquaculture Research & Development,* 2(1), 108.

Angsirijinda, W. (2011). Sedative efficacy of clove oil in Siamese fighting fish *Betta splendens* Regan, 1910 (Doctoral dissertation, Chulalongkorn University).

Bauquier, S., Greenwood, J. and Whittem, T. (2013). Evaluation of the sedative and anaesthetic effects of five different concentrations of alfaxalone in goldfish, *Carassius auratus*. *Aquaculture,* 396-399, pp.119-123.

Bell, T.A. and Lightner, D.V. (1988). *A handbook of normal penaeid shrimp histology,* (No. 595.3843 B4).

Brown, L.A. (1987). Recirculation anaesthesia for laboratory fish. *Laboratory animals,* 21(3), 210-215.

Brown, L.A. (1988). Anaesthesia in fish. *Veterinary Clinic North America Small Animal Practi*ce, 18(2), 317-330.

Bystriansky, J.S., LeBlanc, P.J. and Ballantyne, J.S. (2006). Anaestetization of arctic charr Salvelinus alpinus (L.) with tricaine methanesulfonate or 2-phenoxyethanol for immediate blood sampling. *Journal of Fish Biology,* 69:613-621.

Coyle, S. D., Durborow, R. M., & Tidwell, J. H. (2004). *Anesthetics in aquaculture* (No. 3900). Publication 3900, Stoneville, Mississippi: Southern Regional Aquaculture Center.

Fernández-Parra, R., Donelly, T.M., Pignon, C, Noirault, A. and Zilberstein, L. (2017). Immersıon Anaesthesia with Alfaxalone in a Goldfısh (*Carassius auratus*), *Journal of Exotic Pet Medicine,* 26(4), 276-282

Grush, J., Noakes, D.L.G. and Moccia, R.D. (2004). The efficacy of clove oil as an Anesthetic for the Zebrafish, *Danio rerio* (Hamilton), *Zebrafish*, 1:46-53.

Hajek, G., Kłyszejko, B. and Dziaman, R. (2006). The anaesthetic effect of clove oil on common carp, *Cyprinus carpio* L. *Acta Ichthyologica et Piscatoria,* 36(2), 93-97.

Hikasa, Y., Takase, K., Ogasawara, T. and Ogasawara, S. (1986). Anesthesia and recovery with tricaine methanesulfonate, eugenol and thiopental sodium in the carp, *Cyprinus carpio*. *Japanese Journal of Veterinary Science,* 48:341–351.

Husen, M. A., & Sharma, S. (2015). Anaesthetics efficacy of MS-222, Benzoak vet, AQUI-S and clove oil on Common Carp (Cyprinus carpio) fry. *International Journal of Research in Fisheries and Aquaculture,* 5, 104-114.

Isaacs, G. (1983). Permanent local anaesthesia and anhidrosis after clove oil spillage. *The Lancet,* 321(8329), 882.

Iversen, M., Finstad, B., McKinley, R. S., & Eliassen, R. A. (2003). The efficacy of metomidate, clove oil, Aqui-S™ and Benzoak® as anaesthetics in Atlantic salmon (Salmo salar L.) smolts, and their potential stress-reducing capacity. *Aquaculture,* 221(1-4), 549-566.

Javahery, S., Nekoubin, H. & Moradlu, A.H. (2012). Effect of anaesthesia with clove oil in fish. *Fish Physiology and Biochemistry*, 38(6), 1545-1552.

Jiraungkoorskul, W., Upatham, E. S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S., & Pokethitiyook, P. (2003). Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*). *Environmental toxicology,* 18(4), 260-267.

Kazuṅ, K. and Siwicki, A.K. (2001). Propiscin - a safe new anaesthetic for fish. *Archives of Polish Fisheries*, 9, 183 – 190.

Keene, J.L., Noakes, D.L.G., Moccia, RD. and Soto, C.G. (1998). The efficacy of clove oil as an anaesthetic for rainbow trout, (*Oncorhynchus mykiss Walbaum*). *Aquatic Research,* 29, 89-101.

Küçük, S., & Çoban, D. (2016). Effects of Tricaine as an Anaesthetics on Goldfish, Carassius auratus (Linnaeus 1758) at Different Salinities and Concentrations. *Turkish Journal of Fisheries and Aquatic Sciences,* 16(3), 605-610.

Lewbart, G.A. and Harms, C. (1999). Building a fish anaesthesia delivery system. *Exotic Doctor of Veterinary Medicine*, 1(2), 25-28.

Maura, A., Pino, A., & Ricci, R. (1989). Negative evidence in vivo of DNA-damaging, mutagenic and chromosomal effects of eugenol. *Mutation Research Letters,* 227(2), 125-129.

Neiffer, D.L. and Stamper, M.A. (2009). Fish sedation, anaesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *Institute for Laboratory Animal Research Journal,* 50(4), 343-360.

Ross, L.G. and Ross, B. (1983). Principles and practice of fish anaesthesia. *Association of Veterinary Anaesthetists of Great Britain and Ireland,* 11, 154-189.

Soto, C.G. and Burhanuddin, S. (1995). Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*), *Aquaculture,* 136, 149-152.

Summerfelt, R. C., Smith, L. S., Schreck, C. B., & Moyle, P. B. (1990). Methods for fish biology. *Anaesthesia, surgery and related techniques. American Fisheries Society, Bethesda,* 213-272.

US Food and Drug Administration. (1998). Scientific Literature Review of Eugenol and Related substances in Flavor Usage. Vol. 1, Accession No. PB 283-501. National Technical Information Service, U.S. Department of Commerce, Washington, D.C.

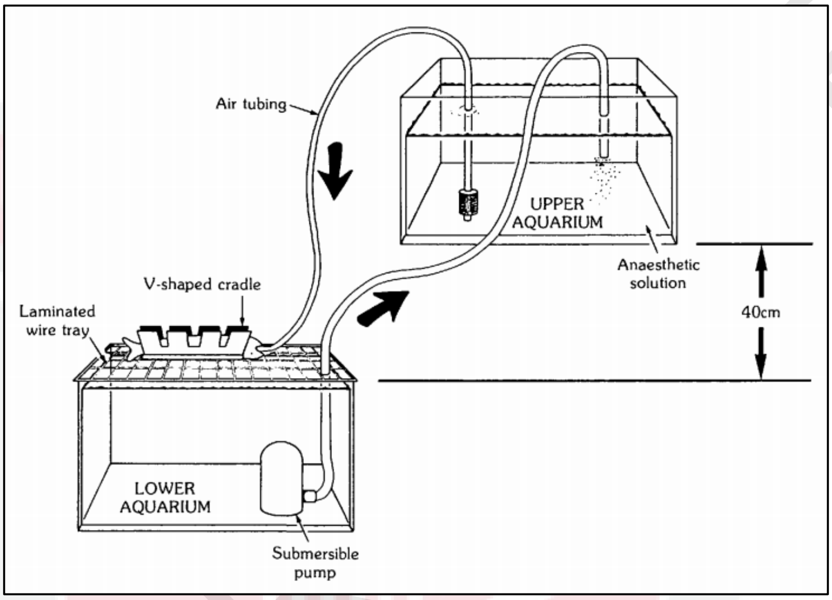
Velisek, J., Svobodova, Z., Piackova, V., Groch, L. and Nepejchalova, L. (2005). Effects of clove oil anaesthesia on common carp (*Cyprinus carpio L*.). *Veterinary Medicine,* 50(6), 269-275.

Velmurugan, B., Selvanayagam, M., Cengiz, E.I. and Unlu, B. (2007). Histopathology of lambda-cyhalothrin on tissues (gill, kidney, liver and intestine) of *Cirrhinus mrigala*. *Environmental Toxicology and Pharmacology.* 24(3), 286-291.

APPENDICES

APPENDIX A

Recirculation anaesthesia for laboratory fish



Recirculation anaesthesia for laboratory fish. (Adopted from Brown, 1987)

Photograph of recirculation anaesthesia delivery system for Goldfish surgery

APPENDIX B

Davidson's Fixative Preparation:

Bell & Lightner (1988) stated that, Davidson's fixative was prepared by mixing:

- 33 mL of 95% ethanol
- 22 mL formalin
- 11.5 mL of glacial acetic acid
- 33.5 mL of distilled water

Tissue Fixation:

The tissues are fixed in Davidson's fixative overnight at room temperature. After that, the tissues are rinsed in 70% ethanol for one hour. Then, the tissues are stored in a new 70% ethanol that can last at least for a few weeks.

APPENDIX C

Histological Procedure (Tissue Processing)

Tissue was prepared by performing the following outlined steps:

1. Fixation
2. Dehydration
3. Clearing
4. Infiltration
5. Embedding
6. Sectioning with microtome
7. Mounting section on glass microslide
8. Staining
9. Coverslipping

The tissue were placed in the specimen cartridges and put into the beaker of histokinette to perform the first four steps automatically.

1. Fixation: The fixative used which is the 10% neutral buffered formalin stops the metabolic processes of the tissues, preventing autolysis. It preserves cytologic and histologic elements and preserves their actual form. It also hardens and gives consistency to soft tissues and mordants the tissue for differential staining. It should prevent dehydration and bacterial growth.

2. Dehydration: Since paraffin is not miscible with water, the water present in the tissue must be removed. Removal of water must be done gradually to minimize shrinking and distortion. Dehydration is accomplished by immersing the fixed tissues into gradually ascending strength of alcohol. (80%, 85%, 90%, 100%, 100%, 100%).

3. Clearing: The function of chloroform as clearing agent is twofold. Alcohol and paraffin are not miscible; therefore, alcohol must be removed and replaced with a solution, which is miscible with paraffin. On the other hand, alcohols have a tendency to cause tissue to become white and opaque. When these alcohol treated tissues are stained, the component parts will not be differentiated. Thus it must be removed.

4. Infiltration: After clearing is complete, the tissue is immersed in melted paraffin (68˚C). The paraffin replaces the clearing agent and the tissue then is infiltrated. It is necessary to heat paraffin for it to liquefy. Therefore, the tissue should not be left in the paraffin longer than necessary. Tissue can be distorted badly by overheating.

5. Embedding: This is a process whereby the tissue is encapsulated and included in a paraffin mould to facilitate sectioning on a microtome. The mould is filled with hot liquid paraffin and the infiltrated tissue is placed so that the bottom of the mould touching the copper plate.

6. Sectioning: Tissue embedded in paraffin is sectioned on the rotary microtome. It is usual to trim the block of paraffin as close to the tissue as possible prior to sectioning. This procedure removes excess paraffin and will keep the knife sharp longer. This section cut as thin as 4 µm. If the knife is sharp and angled correctly, a ribbon will form. This ribbon represents the consecutive sections of the tissue in the paraffin block.

7. 'Fishing' tissue sections: The fishing of tissue is facilitated by placing the ribbon on the surface in such a manner as to cause stretching of the ribbon. This action will remove all wrinkles that have been caused by sectioning. The slide is placed in a dry air incubator set at temperature just below the melting point of the paraffin (40 to

45˚C) for 45 minutes. The heat causes the albumin to coagulate thereby, forming an adhesive bond between the tissue and slide.

8. Staining with H&E: This is a routine stain with both nuclear (Haematoxylin) and cytoplasmic (Eosin) staining. Haematoxylin gives out the basophilic or bluish colouring while Eosin will stain the cytoplasm red in colour (eosinophilic).

9. Coverslipping: Stained tissue must be preserved so that it can be studied microscopically and filed for future reference.