This document has been developed by The Australian National University's (ANU) Research Ethics Office. It has been endorsed by the ANU Animal Ethics Committee (AEC). It is designed to provide guidance regarding current best practice to institutional animal users and carers on the care and use of animals for scientific purposes. It has been prepared in consultation with the Australian code for the care and use of animals for scientific purposes 8th edition 2013.

Document_022_Guideline_Euthanasia of Laboratory Rodents V2.0

Background

The purpose of this document is to provide clear directives as to the appropriate methods of euthanasia of rodents in a laboratory setting. The term euthanasia has been used throughout this document and is considered synonymous with "humane killing" as defined by the Code. .

Methods of euthanasia for laboratory rodents must have prior approval from the ANU Animal Ethics Committee (AEC). In biomedical research the method of euthanasia must take into account any potential impacts on research quality and the ability to collect meaningful specimens from the animal(s). All personnel working independently with rodents must be able to act immediately to competently euthanise any animal found in distress.

As per the Code:

2.4.22 *Investigators must use humane procedures for killing an animal that are appropriate to the species and circumstances*

2.5.8 Animal carers must use humane procedures for killing an animal that are appropriate to the species and circumstances (

3.3.45: *"The method and procedures used for killing an animal must be humane and:*

- *i) avoid pain or distress and produce rapid loss of consciousness until death occurs*
- *ii) be compatible with the purpose and aims of the project or activity*
- *iii) be appropriate to the species, age, developmental stage and health of the animal*
- *iv) require minimum restraint of the animal*
- *v) be reliable, reproducible and irreversible*
- *vi) ensure the animals are killed in a quiet, clean environment away from other animals*
- *vii) ensure that death is established before disposal of the carcass, foetuses, embryos and fertilised eggs.*

3.3.46 - Dependent offspring of animals to be killed must be cared for or humanely killed.

4.7 Institutions must ensure that humane killing of animals is not demonstrated to, or carried out by, primary or secondary level students unless it is required:

(i) to achieve an educational outcome in science as specified in the relevant curriculum or competency requirement, or

(ii) as part of veterinary clinical management of an animal, under the direction of a veterinarian

The method of euthanasia must be undertaken in accordance with current best practice.

Definitions

Humane killing: the act of inducing death using a method appropriate to the species that results in a rapid loss of consciousness without recovery and minimum pain and/or distress to the animal. Code: The NHMRC's *Australian code for the care and use of animals for scientific purposes 8th Edition 2013.*

Unexpected adverse event: An event that may have a negative impact on the wellbeing of any animals and was not foreshadowed in the approved project or activity.

General Information and Considerations

ANU Training Requirements

It is a requirement that all personnel working independently in a laboratory environment with rodents must be trained and deemed competent in euthanasia. Personnel working with mice must be competent, at a minimum, in cervical dislocation and where applicable, the use of carbon dioxide for euthanasia. Personnel working with rats must be competent in the approved technique for euthanasia as listed on the protocol and carbon dioxide euthanasia.

Training Prerequisites

Prior to being trained in methods of euthanasia, personnel must have completed appropriate training in the handling and restraint of the relevant species.

For certain methods, additional prior training may be required e.g. injection procedures, anaesthesia.

Risks and Work Health and Safety Considerations

You must consult your local standard operating procedures for a full list of work health and safety considerations.

The AEC strongly recommends that personnel work in a 'buddy system' for euthanasia, particularly when working with a large number of animals due to the potential impacts on mental wellbeing and the effects of compassion fatigue.

You should also ensure that you are familiar with carbon dioxide monitoring practices in your laboratory.

Environmental Considerations

Rodents can experience significant stress if they can hear other animals being euthanased.

The movement to a laboratory for euthanasia and handling prior to euthanasia may create additional distress.

The environment should be kept quiet to reduce stimulation. The area should be cleaned between animals to remove any olfactory stimulation. Animals should be kept out of view of euthanised animals or the area where the euthanasia is to take place. It is most humane to remove the animals to be humanely culled away from the animal holding area where possible.

Euthanasia Requirements – All Methods

Rodents should not be euthanased in close proximity to live rodents. A separate space, away from the home cage, should be utilised when performing cervical dislocation of animals.

Where utilising carbon dioxide for euthanasia it is preferable to use the animal's home cage where possible as this reduces stress for the animal.

Always ensure that you have selected the correct animals and that you are using an appropriate method of euthanasia for any samples that you need to collect, and that the method you are using is approved in your ethics protocol.

Records, including the number of animals euthanised and the reason for euthanasia, must be kept for all animals that are euthanised.

Cervical Dislocation

Cervical dislocation is not permitted in rats over 150 grams in weight.

Cervical dislocation must be undertaken by the application of pressure to the cervical neck region and must not be performed by the extension of the head and tail. The tail must only be held for restraint purposes and must not be pulled back as part of the euthanasia procedure as it can result in paralysis without death. The vertebrae must be completely separated to make sure that there has been dislocation of the atlanto-occipital joint and destruction of the brain stem.

It is recommended that no more than 35 animals be cervically dislocated in a single session by a single staff member. This is to avoid fatigue in the fingers that may reduce the effectiveness of the procedure and risk animal welfare. This may vary between facilities and each should assess the risk as it applies to their situation.

The use of instruments to perform cervical dislocation is not considered acceptable practice at ANU and is not authorised in any situation. The use of instruments can reduce the ability for the individual to feel that the cervical dislocation has been complete and this may lead to poor welfare outcomes due to prolonged time to death or paralysis without death. The ANU recognise that other institutions may allow the use of instruments however the AEC have made a decision not to allow this based on significant discussion with experienced individuals.

Carbon Dioxide (CO^2)

The introduction of carbon dioxide must be a gradual introduction and pre-charging of chambers must not be undertaken. Significant research has proven that the use of high flow rates of carbon dioxide or the use of pre-charged chambers increased the stress and can cause pain and suffering to animals. Concentration >70% or prefilling a chamber will cause rapid death, however it will also cause pain for 10-15 seconds before death, irritating the nasal, ophthalmic and respiratory tract, causing distress and discomfort. If concentrations above 30% need to be used for any reason, the animals need to be anaesthetised before being introduced.

A flow meter to control the introduction of carbon dioxide into the chamber must be used regardless of whether the carbon dioxide is introduced from an in room tank, piped in gas or using a more complex euthanasia chamber.

The chamber must be allowed to vent out residual carbon dioxide between rounds of euthanasia. This can most efficiently be achieved by turning small chambers upside down as CO^2 is heavier than air.

Asphyxiation by carbon dioxide must not be used in neonatal animals (<10 days of age) due to their resistance to hypoxia.

Acceptable flow rates of carbon dioxide in euthanasia should displace between 30%-70% of the chamber or cage volume per minute.

Animals must remain in the euthanasia chamber/cage for at least three minutes beyond visible cessation of breathing/gasping.

Confirmation of death after removal from the chamber must be performed.

Decapitation

Decapitation is used for the euthanasia of neonatal mice (<10 days of age) and neonatal rats (<5 days of age) and embryonic forms post mid-gestation.

Scissors are suitable for neonatal mice less than 10 days of age. Scissors must be kept sharp and able to perform decapitation rapidly. The use of a guillotine is required for larger mice and rats over 5 days of age and should include sedation or anaesthesia prior where applicable. Guillotines must be kept clean and sharp. The use of restraint aids (e.g. DecapiCones) should be considered when performing decapitation, to reduce handling distress, minimize risk of personnel injury, and to improve positioning of the animal.

Any use of decapitation in animals greater than the above age restrictions must be clearly specified, justified and the procedure detailed in an approved animal ethics protocol.

Overdose with Barbiturates

The use of barbiturates for euthanasia must be detailed and justified in an approved animal ethics protocol. In Australia, pentobarbitone is the most commonly used barbiturate drug, also known as pentobarbital.

Only those individuals competent in the relevant injection method are approved to undertake euthanasia with barbiturates.

The use of IV injection of barbiturates is recommended over the use of IP injections, however the committee acknowledge the technical challenge with this procedure in small rodents. The use of barbiturates using intra-peritoneal injection can cause pain due to the pH of the barbiturate substance and irritation of the abdominal cavity. For this reason it is a requirement that animals are anaesthetised with a different compound prior to the use of barbiturate. An accepted method is the use of isoflurane inhalational anaesthesia prior to IP injection with pentobarbitone.

Barbiturates can also cause physiological changes in the animal that may greatly impact samples collected and this must be considered.

The dose rate for pentobarbitone (325mg/ml) is 1ml/2kg (equivalent of 160mg/kg). For an average sized mouse of 30g the effective dose is 0.015ml. It is recommended that pentobarbitone is diluted to ensure accurate dosing due to the low volumes. When using barbiturates the dose per animal

must be recorded, and the barbiturate stock must be kept secure in accordance with ANU WHS regulations.

Euthanasia of Pregnant Females

The above methods are appropriate for euthanasia of pregnant female rodents. It is not necessary to remove embryos or foetuses for secondary euthanasia after the dam is deceased. Embryos are unconscious in utero during pregnancy, so euthanasia of the dam is considered sufficient to euthanise foetuses if they remain in the uterus.

However, if embryos/foetuses need to be removed from the uterus for independent tissue collection or experimentation, then they should be euthanised using methods acceptable for neonates, e.g decapitation or injection with barbiturates.

Other Methods of Euthanasia

Exsanguination can be used as a method of euthanasia as long as it is undertaken under anaesthesia and is followed by a supplementary approved method of euthanasia.

The use of rapid cooling (not freezing) followed by immersion in cold fixative without decapitation is suitable for embryos, foetuses and neonates up to 2 days of age. This method should only be used if other methods are not a viable option.

Monitoring Intervention and Reporting

Indicators of Death

It is essential that all personnel undertaking euthanasia are competent in recognising the indicators of death in rodents, which are:

- No movement or breathing
- Dilated pupils, glassy eyes
- Loss of corneal reflex – this reflex can be checked by touching the eye of the animal. If there is any movement of the eye or the animal blinks, it is not dead.
- Absence of a heartbeat – although difficult to detect in small rodents
- Absence of a pedal reflex – this can be checked by squeezing the foot pad of the animal. The animal will not react.
- Cyanosis – loss of pink colour can be noted around the mucous membranes and in the limbs with these areas turning blue.

  *Note that some of these indicators are also absent under heavy anaesthesia and therefore where chemical euthanasia is utilised or the animal is anaesthetised prior to anaesthesia secondary methods are required to confirm euthanasia. More than one sign of death should be observed to confirm death or a secondary method undertaken.

Recording of Animal Usage

All animal usage must be recorded by the investigator responsible for the animal. This may be on a database provided by the animal facility or in a laboratory record system/laboratory book. This

information must be made available to the animal ethics committee or their representatives at any time.

Unexpected Adverse Events (UAE)

If an animal is found in pain or distress due to an unexpected event all effort must be made to contact the University Veterinarians as soon as possible. The requirements of the University's UAE procedure must be followed.

Minimum Requirements

- All personnel with access to animal laboratories and working **independently** with rodents must be able to demonstrate competency in cervical dislocation.
- Euthanased animals must be checked for indicators of death immediately after euthanasia.
- The method of euthanasia to be used must be included in the approved animal ethics protocol.
- A flow meter must be used to control the entry flow rate of carbon dioxide into a euthanasia chamber.
- Individuals must be knowledgeable in the use of local area equipment (e.g. euthanasia chambers) and any specific requirements for its operation prior to undertaking euthanasia procedures.
- When using barbiturates the dose per animal must be recorded, and the barbiturate stock must be kept secure in accordance with ANU WHS regulations.

Some methods of euthanasia are not acceptable under any circumstances in the laboratory environment and these include (but may not be limited to); ether, hydrogen cyanide, carbon monoxide, nitrogen, chloroform, decompression, rapid freezing.

References and Resources

AVMA. AVMA guidelines for the euthanasia of animals 2020

NHMRC. Australian code for the care and use of animals for scientific purposes 8th Edition 2013 (Section 4.4.3)

NHMRC. Best practice methodology in the use of animals for scientific purposes 2017 (Part III Humane killing and euthanasia)

ANU Procedure for Managing & Reporting Unexpected Adverse Events

UIOWA (2020) Vertebrate Animal Research: Euthanasia (Guidelines)

Vaughan, L. (2019) Flinders University safe work method statement: methods of humane euthanasia in mice Version 7