euthanasia

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Humane methods of killing (Euthanasia)
Learning Outcomes

- 6.1.1. Describe the principles of humane killing (e.g. what constitutes ‘a good death’)
- 6.1.2. Describe the different methods by which the relevant animals are allowed to be killed, the influence different methods can have on scientific outcomes, and how to select the most appropriate method.
- 6.1.3. Explain why someone competent to kill animals should be available at all times (whether care staff or person carrying out procedures)
- 6.2.1. Proficiently and humanely carry out euthanasia using appropriate techniques on relevant species of laboratory animals
- 6.2.2. Demonstrate how death is confirmed and how cadavers should be processed or otherwise disposed of.

what is euthanasia…

- euthanasia = eu (good) + thanatos (death)

(Greek etymology)

- ending life in a way that minimizes pain and distress

(AVMA Guidelines for the Euthanasia of Animals)

- the act of humanely killing animals by methods that induce rapid unconsciousness and death without pain or distress

(Guide for the Care and Use of Laboratory Animals)

- killing of animals (humane)

(Directive 2010/63/EU)

"The Thinker" by Leonard Filgate

when is euthanasia performed?

- ✓ for the use of organs or tissues [Art.3-1]
- ✓ as a procedure, within the framework of a project [Art.12-2]
  - the specific methods of killing shall be presented in the application for project authorization and approved [Art.37-1-c, Annex VI]
- ✓ when the humane endpoints are met [Art.13-3]
  - the humane endpoints and actions to be taken shall be presented in the application for project authorization and approved [Art.37-1-c, Annex VI]
- ✓ as soon as the purpose of the procedure has been achieved, as one appropriate action to minimise the suffering of the animal [Art.14-5, 17-2]
  - this includes unexpected suffering, distress, pain which cannot be treated and will result in the exclusion of the animal from the project, according to veterinary advice [Art. 19, 25]
- ✓ for excess or old animals in breeding establishments

Directive 2010/63 EU

Article 17

End of the procedure

1. A procedure shall be deemed to end when no further observations are to be made for that procedure or, as regards new genetically modified animal lines, when the progeny are no longer observed or expected to experience pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle.

2. At the end of a procedure, a decision to keep an animal alive shall be taken by a veterinarian or by another competent person. An animal shall be killed when it is likely to remain in moderate or severe pain, suffering, distress or lasting harm.

3. Where an animal is to be kept alive, it shall receive care and accommodation appropriate to its state of health.

euthanasia and welfare assessment plans

- Animals shall be monitored according to a welfare assessment plan specifically set for each project, based on the humane endpoints that are selected.
- Frequency of monitoring depends on expected severity and symptoms.
- All animals shall be checked at least daily.
- A person competent to perform euthanasia shall be present at all times, in order to alleviate unnecessary pain and suffering and collect samples if appropriate.

ANNEX III

3. Care of animals

REQUIREMENTS FOR ESTABLISHMENTS AND FOR THE CARE AND ACCOMMODATION OF ANIMALS

3.1. Health

(a) Establishments shall have a strategy in place to ensure that a health status of the animals is maintained that safeguards animal welfare and meets scientific requirements. This strategy shall include regular health monitoring, a microbiological surveillance programme and plans for dealing with health breakdowns and shall define health parameters and procedures for the introduction of new animals.

(b) Animals shall be checked at least daily by a competent person. These checks shall ensure that all sick or injured animals are identified and appropriate action is taken.

Article 33

Care and accommodation

1. Member States shall, as far as the care and accommodation of animals is concerned, ensure that:

(a) all animals are provided with accommodation, an environment, food, water and care which are appropriate to their health and well-being;

(b) any restrictions on the extent to which an animal can satisfy its physiological and ethological needs are kept to a minimum;

(c) the environmental conditions in which animals are bred, kept or used are checked daily;

(d) arrangements are made to ensure that any defect or avoidable pain, suffering, distress or lasting harm discovered is eliminated as quickly as possible; and

(e) animals are transported under appropriate conditions.

euthanasia legal aspects
in brief

Matters regarding the euthanasia of laboratory animals are regulated by the legislation. Directive 2010/63/EU dictates:

- selecting refined methods that cause minimum pain, suffering and distress
- selecting acceptable methods for each species and provides a brief guideline for such methods
- training personnel until competence in the relevant methods is achieved
- keeping records of euthanasia procedures, numbers and associated health and welfare issues
- stating and justifiying the selected euthanasia methods in the application for project authorization and taking those into account for the severity classification and harm-benefit evaluation of projects

Performing euthanasia is most of the times inevitable in order to permit sample collection after the end of projects, or to alleviate unnecessary suffering after certain humane endpoints are reached. The euthanasia methods shall be chosen balancing animal welfare, project goals and personnel preferences - as emotional aspects may play a significant part in this case. A person competent to perform euthanasia shall be present at all times, in order to alleviate unnecessary pain and suffering and collect samples if appropriate.

evaluating euthanasia methods

impact on animal welfare

- ability to induce loss of consciousness and death with a minimum of pain and distress
- time required to induce loss of consciousness
- reliability
- irreversibility
- compatibility with species, age, and health status
- ability to maintain equipment in proper working order

impact on research goals

- compatibility with intended animal use and purpose
- compatibility with subsequent evaluation, examination, or use of tissue

impact on people & environment

- legal requirements
- safety of personnel
- documented emotional effect on observers or operators
- drug availability and human abuse potential
- safety for predators or scavengers should the animal's remains be consumed
- environmental impacts of the method or disposition of the animal's remains

(AVMA Guidelines for the Euthanasia of Animals 2020)

selecting euthanasia methods

animal welfare

- impact of method on animal welfare
- refinement obligation
- competence of personnel

personnel

- competence
- safety
- emotions

project

- impact of method on samples and results
  - stress
  - method/drug
  - parameters measured

?

euthanasia methods

Directive 2010/63/EU

- acceptable methods summarized in Annex IV
- amended by Directive 2024/1262
- other methods:
  - in unconscious animals or
  - after justification accepted at project evaluation

AVMA guidelines

- acceptable
  consistently produce humane death
- acceptable with conditions
  produce humane death
  when conditions are met
- unacceptable
  inhumane or dangerous for humans
- adjunctive methods
  used in conjunction with others

+ confirmation of death !

ΥΑ 2416-83725 2016
Τροποποίηση Παραρτήματος IV του ΠΔ 56

Animals-remarks/ methods	Fish	Amphibians	Reptiles	Birds	Rodents	Rabbits	Dogs, cats, ferrets and foxes	Large mammals	Non-human primates
Anaesthetic overdose	(1)	(1)	(1)	(1)	(1)				
Captive bolt			(2)						
Carbon dioxide					(3)				
Cervical dislocation					(5)				
Concussion/ percussive blow to the head				(7)					
Decapitation									
Electrical stunning	(13)	(13)							
Inert gases (Ar, N_2)									
Shooting with a free bullet with appropriate rifles, guns and ammunition			(15)						

Requirements

1. Shall, where appropriate, be used with prior sedation.
2. Only to be used on large reptiles.
3. Only to be [illegible] gradual fill. Not to be used for foetal and neonate rodents.
4. Only [illegible]irds under 1 kg. Birds over 250 g shall be sedated.
5. [illegible]d for rodents under 1 kg. Rodents over 150 g shall be sedated.
6. [illegible]be used for rabbits under 1 kg. Rabbits over 150 g shall be sedated.
7. [illegible]y to be used for birds under 5 kg.
8. [illegible]y to be used for rodents under 1 kg.
9. [illegible]e used for rabbits under 5 kg.
10. [illegible]d on neonates.
11. Only [illegible] birds under 250 g.
12. Only to be [illegible] if other methods are not possible.
13. Specialised equipment required.
14. Only to be used on pigs.
15. Only to be used in field conditions by experienced marksmen.
16. Only to be used in field conditions by experienced marksmen when other methods are not possible.

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14. Only to be used on pigs.
15. Only to be used in field conditions by experienced marksmen.
16. Only to be used in field conditions by experienced marksmen when other methods are not possible.
17. To be used only for zebrafish (*Danio rerio*) ≥ 16 days post fertilisation (dpf) and with a maximum body length of 5cm. Temperature of hypothermic shock shall be ≤ 4 °C and the temperature difference from holding temperature shall be ≥ 20 °C. Fish shall not have direct contact with ice. Minimum exposure time shall be 5 minutes.'.

'Animals-remarks/methods	Fish	Amphibians	Reptiles	Birds	Rodents	Rabbits	Dogs, cats, ferrets and foxes	Large mammals	Non-human primates	Cephalo
Anaesthetic overdose	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	
Captive bolt			(2)							
Carbon dioxide					(3)					
Cervical dislocation				(4)	(5)	(6)				
Concussion/percussive blow to the head				(7)	(8)	(9)	(10)			
Decapitation				(11)	(12)					
Electrical stunning	(13)	(13)		(13)		(13)	(13)	(13)		
Inert gases (Ar, N_2)								(14)		
Shooting with a free bullet with appropriate rifles, guns and ammunition			(15)				(16)	(15)		
Hypothermic shock	(17)'									

Directive 2010/63/EU, Annex IV &
Directive 2024/1262/EU

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zebrafish euthanasia

- quiet, non-stimulatory environment
- reduced light intensity e.g. dark container
- tank water quality e.g. water from the home tank
- euthanasia water quality: monitor conditions and anesthetic concentration when euthanizing large populations
- appropriate equipment e.g. nets, gloves
- trained personnel

Acceptable for zebrafish by Directive 2010/63/EU

anesthetic overdose e.g. MS222

concussion / head percussive blow

electrical stunning

hypothermic shock

Acceptable by AVMA Euthanasia Guidelines 2020

anesthetic overdose

physical methods

hypothermic shock

(AVMA Guidelines for the Euthanasia of Animals 2020)

some zebrafish euthanasia methods

adult zebrafish

- immersion in anesthetic solutions
  - buffered tricaine methanesulfonate (MS222) 250-500mg/l
    - aversive…
  - lidocaine 400mg/l
  - leave for at least 10' after cessation of opercular movement
- hypothermic shock
  - rapid chilling at 2-4°C
    - why? because zebrafish are tropical and this is a lethal temperature
    - death in 10-20 seconds
    - only zebrafish > 16 dpf & < 5cm length
    - temperature <4°C with >20°C difference from home tank
    - water probe thermometer
    - no direct contact with ice – form a depression in the ice slurry to expose all fish surface to cold water
    - leave for at least 5' – rec. 10' after cessation of opercular movement
- physical methods
  - cervical transection or decapitation or concussion followed by pithing
  - maceration

embryos <3dpf

- immersion in anesthetic solutions or rapid chilling MUST be followed by adjunctive method such as immersion in 500mg/ml calcium hypochlorite

fry 4-7 dpf

- leave for at least 20' in anesthetic/ice after loss of opercular movement
- may need higher anesthetic concentration

(Directive 2024/1262, AVMA Guidelines for the Euthanasia of Animals 2020. Collymore et al 2016)

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zebrafish euthanasia in brief

select euthanasia method

balance research goals, sample quality, OHS, animal welfare

Acceptable for zebrafish by Directive 2010/63/EU

anesthetic overdose

hypothermic shock

concussion / percussive blow

electrical stunning

confirm death

use combination of criteria

cessation of opercular movement for 30'

no vestibulo-ocular reflex

no heartbeat *

flaccidity → rigor mortis

use secondary methods

to ensure death

exsanguination

removal of organs (heart, lungs, brain)

destruction of brain

cervical dislocation

dispose of cadaver

establishment SOP

bag, label, freeze → incinerate

record

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mouse & rat euthanasia in brief

select euthanasia method

balance research goals, sample quality, OHS, animal welfare

Acceptable for rodents by Directive 2010/63/EU

anesthetic overdose e.g. pentobarbital

carbon dioxide with gradual fill (not for fetuses/neonates)

cervical dislocation, under sedation for >150gr (not for >1kg)

concussion (not for >1kg)

decapitation, only if other methods are not possible

other methods under anesthesia

exemptions approved by project evaluation committee

confirm death

use combination of criteria

no breath

no pulse/heartbeat

no pain reflexes

gray mucous membranes

± rigor mortis

use secondary methods

to ensure death

exsanguination

removal of organs (heart, lungs, brain)

pneumothorax (open chest cavity)

destruction of brain

cervical dislocation

dispose of cadaver

establishment SOP

bag, label, freeze → incinerate

record

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considerations to minimize distress

transport

- stress of transport vs other factors?

handling

- animals accustomed to handling?

established groups and scents

- preferably no disruption of compatible groups
- preferably in familiar smell of home cage

vocalizations, feromones, odors during euthanasia

- always in other location
- no other animals in the room or within hearing/smelling distance

(AVMA Guidelines for the Euthanasia of Animals 2020)

prerequisites

competence of personnel

- training and experience in Module 6
- animal handling, transport, recognition of pain and distress, euthanasia methods, confirmation of death and equipment handling

equipment & consumables

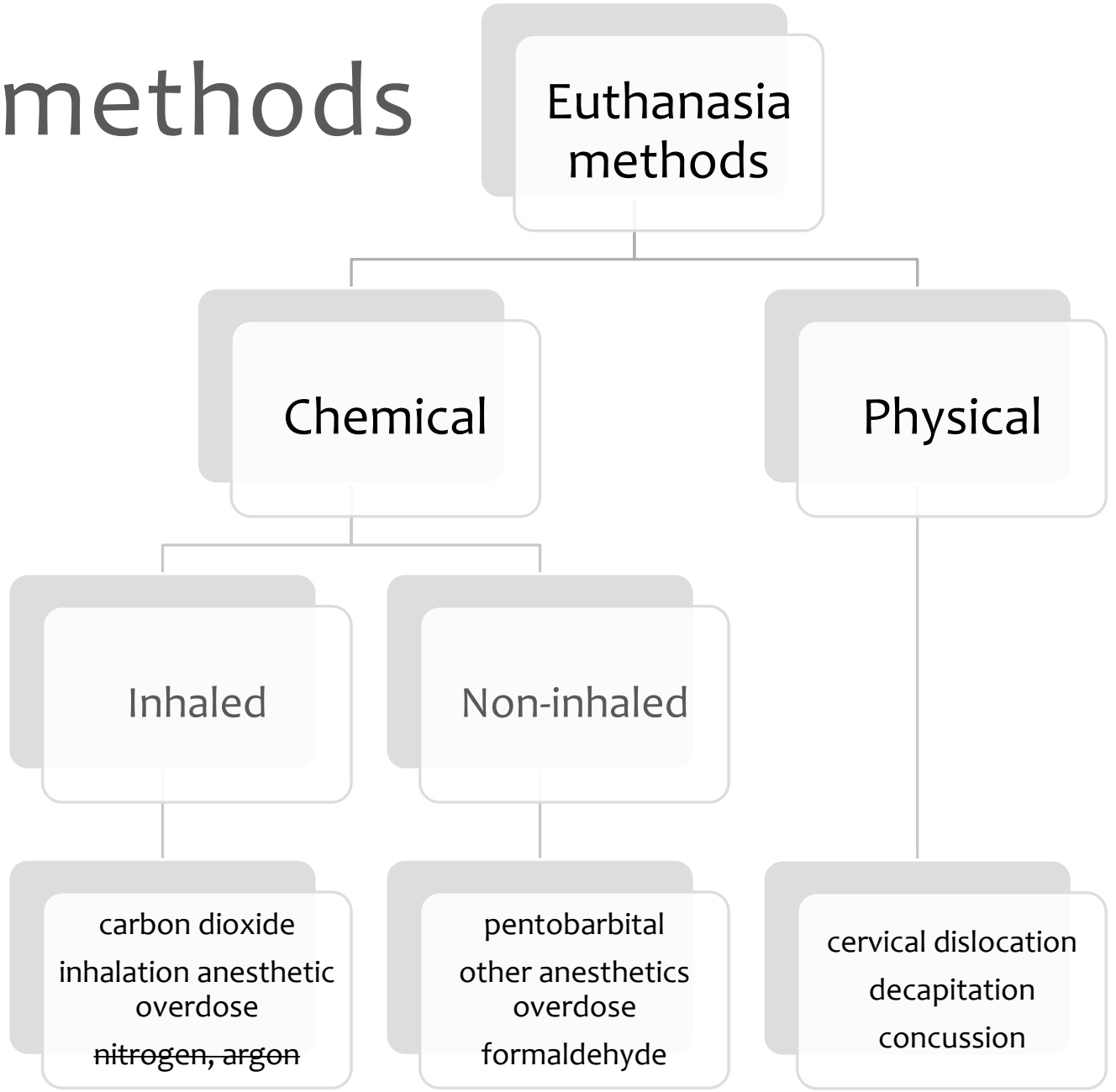
- availability
- prescription?
- good working order and maintenance

space set-up

- PPE, safety SOPs relevant to method
- location
- no other animals in the room or within hearing/smelling distance
- clean and quiet

(AVMA Guidelines for the Euthanasia of Animals 2020)

euthanasia methods



(AVMA Guidelines for the Euthanasia of Animals 2020)

rodent euthanasia methods

Acceptable for rodents by Directive 2010/63/EU

1. anesthetic overdose, preferably preceded by sedation
2. carbon dioxide with gradual fill (not for fetuses/neonates)
3. cervical dislocation, under sedation for >150gr (not for >1kg)
4. concussion (not for >1kg)
5. decapitation, only if other methods are not possible
6. other methods under anesthesia
7. exemptions approved by project evaluation committee

mechanisms of euthanasia

1. direct depression of neurons necessary for life
2. hypoxia
3. physical disruption of brain activity

(AVMA Guidelines for the Euthanasia of Animals 2020)

comparison of euthanasia methods

humane?? assess:

- stress
- perception of pain
- loss of consciousness
- time to death

Type of Evidence
Behavioural indicators of stress, e.g., aversion
Behavioural indicators of pain, e.g., attention to eyes or injection sites
Positive indicators that suffering is minimal or absent
EEG data
Success rate
Known properties of agents, e.g., pH, mechanism of action
Experience of animal before euthanasia process
Time to cease moving
Time to death
EMG and other activity data

Adverse Effect for the Animal	Potential Sources of Suffering or Factors to Consider	Evidence
Pain	Injection Physicochemical properties of agent (e.g., unbuffered PBS, H_2CO_3 on mucous membranes) Muscle spasms/seizures Pain from decapitation	Behavioural; physical reactions, vocalisation, attention to site(s) of pain Active EEG Data on duration, incidence, severity of spasms/seizures, observations or **electromyogram** (EMG)
Aversion to inhaled agents	Molecular structure of agent Concentration Flow rate Whether other agents used to induce anaesthesia or as additives Highly species and strain specific	Behavioural; physical reactions (e.g., escape), conditioned place preference/avoidance Information on properties of agent, e.g., pH NOT time to recumbency or to cease movement
Suffering between administration and death	Dyspnoea Pain from injury with physical methods Anxiety, fear Inability to escape from aversive agent Other unpleasant effects of inhaled agents	Behavioural (physical reactions, vocalisation, defecation) Active EEG Respiration rate and depth Corticosterone NOT time to recumbency or to cease movement

https://www.mdpi.com/2076-2615/6/9/50

(AVMA Guidelines for the Euthanasia of Animals 2020, Newcacstle 2nd Consensus Meeting)

comparison of euthanasia methods

Method	Time to loss of consciousness	Time to death
Cervical Dislocation	< 10 - 15 seconds	< 10 - 15 seconds
Exposure to Carbon Dioxide (20-30% chamber volume per min)	1 - 3 minutes	5 - 10 minutes
Overdose of Anaesthetic by intravenous injection	< 5 seconds	< 10 seconds
Overdose of Anaesthetic by intraperitoneal injection	1 - 3 minutes	4 - 10 minutes
Overdose of Anaesthetic by inhalation (at normal concentration for anaesthetic induction)	1 - 3 minutes	20 - 30 minutes
Concussion by striking the cranium	< 0.1 seconds	< 5 seconds

(references, Valentine et al, 201211, Cartner et al, 200712, Schoell et al, 2009, Boivin et al, 201613, Hickman et al, 2016, Kongara et al, 201314)

http://cbctraining.ncl.ac.uk/eM-EU6/story_html5.html

comparison of euthanasia methods

Welfare Concerns for Rodent Euthanasia	CO_2	Isoflurane	Barbiturate(IP)	Decapitation	Cervical Dislocation
Distress from required restraint			++	++	++
Social stress in chambers prior to euthanasia method	++	++			
Exposure to other animals' alarm calls or pheromones	+	+	+	+	+
Pain experienced from time method is applied to unconsciousness	++		++	++	++
Persistent cortical activity after method has been applied				+	+
Distress and aversion from time method is applied to unconsciousness	++	++			
Likelihood of operator error occurring			+	++	++
Consequences of operator errors				++	++
Potential for reversal and recovery	++	++	++		+

Animal Welfare Science and Euthanasia: Empirical Studies

++ = high level of concern; + = possible

injectable anesthetic overdose

- acceptable method (Directive & AVMA)
- euthanasia dose = usually 3+ times the anesthetic dose
- barbiturates iv or ip
  - most common: **pentobarbital**
  - administer in concentration <200 mg/ml
  - dose: 120-200 mg/kg
  - + formulation for veterinary euthanasia, colored to avoid accidents, long shelf-life, rapid action, minimal discomfort
  - - reported pain when administered ip, prescribed drug, cost, residues in tissues
    - may be used with same amount of lidocaine (local anesthetic) when administered ip
    - local anesthetics take a while to take effect and also cause pain ip…
- ketamine combinations
  - ketamine combination with xylazine, medetomidine, diazepam etc.
- loss of consciousness and death
  - iv within 20-30 sec
  - ip within 5-10 min

(AVMA Guidelines for the Euthanasia of Animals 2020, Directive 2010/63/EU)

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inhalation anesthetic overdose

- **acceptable** (Directive) – **acceptable with conditions** (AVMA)
- **isoflurane > halothane > sevoflurane > others**
  - - time to death 20'+, cost, equipment, OHS, may be aversive, pungent odor, possibility of struggling and apnea
    - learned aversion – better experience in 1st exposure…
  - + useful when handling is not possible
  - NOT ether → ocupational health and safety issues, animal welfare issues: irritating to eyes and respiratory system, pungent odor, stress…

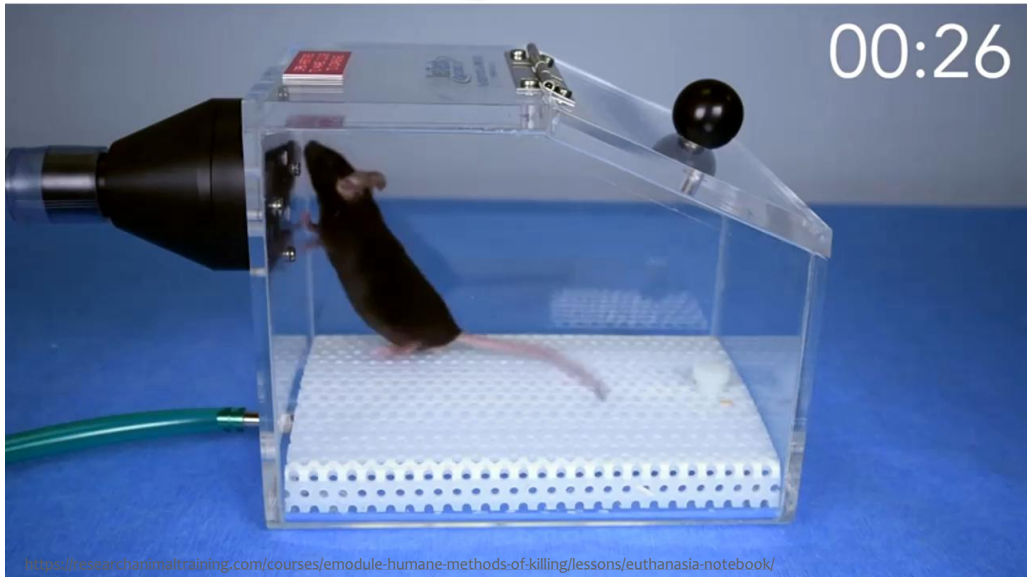
➢ **administered in an induction chamber via a vaporizer**

- begin with 2-3% → continue to maximum → leave for at least 2 min after death → confirm!
- don't pre-fill the chamber, anesthetic is aversive

➢ **administered via open-drop technique in a jar**

- **not recommended**: occupational health and safety issues and unstable dosing
- if you have to… handle under hood, dose: >1 ml/lt/animal in cotton pad, ensure there is no direct contact of the animal with the anesthetic by using mesh

(AVMA Guidelines for the Euthanasia of Animals 2020)



carbon dioxide CO_2

- **acceptable with conditions** (Directive & AVMA)
  - **only with graduall fill**
  - produce tissue acidosis and loss of consciousness followed by death by respiratory and cardiac failure
  - preferably in the home cage (adjust lid or place home cage in chamber)
  - use compressed CO2 from a gas cylinder controlled by a regulator and flow meter
  - flow rate to displace 30-70% of the chamber volume per minute – fill rate of 20% of the chamber volume per minute
    - W x D x H (cm) = VOLUME (L) x (15 – 30%) = FLOW LPM
  - flow maintained for 2’ after observation of death
  - don’t pre-fill chamber, empty and clean chamber between uses
    - exposure to high concentration is painfull!

(AVMA Guidelines for the Euthanasia of Animals 2020)

carbon dioxide

disadvantages

- CO_2 is heavier than air, so incomplete filling of the chamber can induce some animals to avoid exposure by climbing or jumping
- may be distressful to some animals due to irritation of the mucous membranes of the respiratory tract and stimulation of respiratory centers in the brain
- not applicable to neonates!
- special equipment required, OHS

advantages

- inexpensive, non-flammable, non explosive, minimal OHS hazard, available
- no chemical residues into tissues, no cell distortion
- rapid loss of consciousness and anesthesia
- no handling and restraint (preferably the animal's home cage)
- requires little training, and it saves time since many animals can be euthanized at once

(AVMA Guidelines for the Euthanasia of Animals 2020)

carbon dioxide

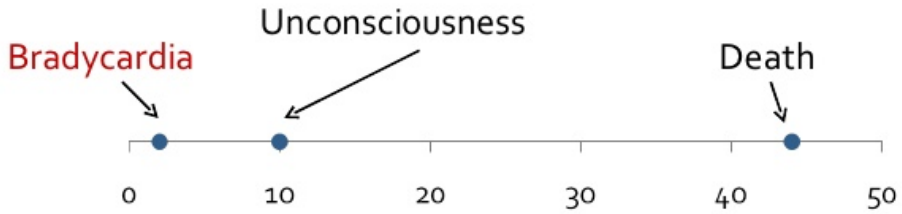
carbon dioxide has the potential to cause distress in animals via 3 different mechanisms:

1. pain due to formation of carbonic acid on respiratory and ocular membranes
   - humans report discomfort beginning at 30% concentration
   - gradual displacement methods are less likely to produce pain prior to unconsciousness
2. production of so-called air hunger and a feeling of breathlessness
3. direct stimulation of ion channels within the amygdala associated with the fear response

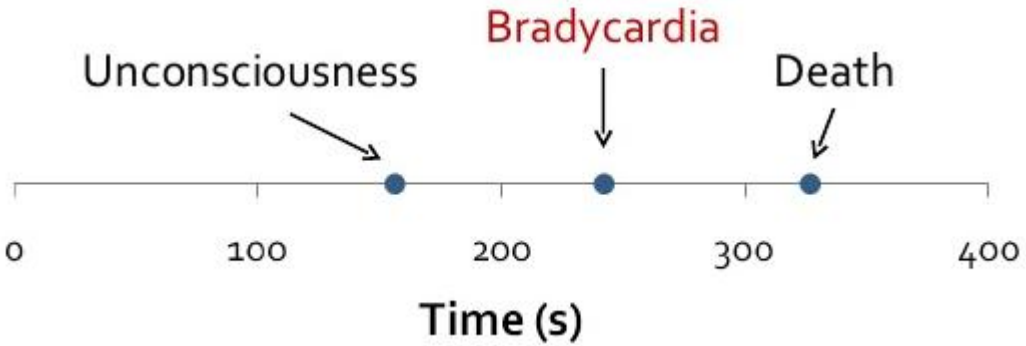
substantial species and strain differences are reported

(AVMA Guidelines for the Euthanasia of Animals 2020)

Pre-fill CO_2



Gradual-fill CO_2



Golledge H, Roughan J, Niel L, Richardson C, WrightWilliamson S and Flecknell P. Carbon dioxide euthanasia in rats – behavioural and autonomic system responses to exposure. In: SECAL–ESLAV 2005 International Congress, Elche, Spain, 5–7 October 2005.

physical methods

- + rapid, cheap, no chemical residues
- - emotional cost, OHS, training
- - require animal handling
- - risk of unsuccessful application
- - some tissues may be damaged

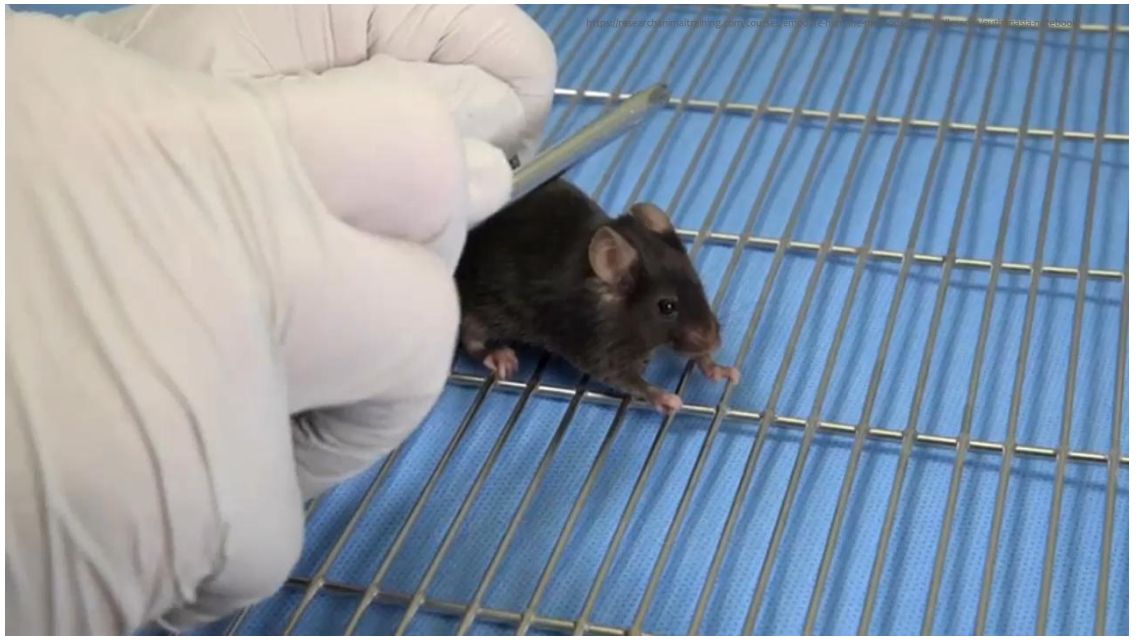
- preferably under sedation or anesthesia

- cervical dislocation
  - under sedation for 150gr - 1 kg
  - only for < 1 kg
- concussion
  - only for <1kg
- decapitation
  - acceptable only if justified that other methods are not possible

(AVMA Guidelines for the Euthanasia of Animals 2020, Directive 2010/63/EU)

cervical dislocation

- only for < 1kg, under sedation for 150 gr - 1 kg
- doesn't require specialized equipment but must be conducted rapidly and effectively
- extensive damage and disruption to cervical spinal cord and brainstem by dislocating and compressing the cervical vertebrae
- rapid loss of cortical function: 5-10 sec



Perform the procedure on a flat surface or surface where the animal can grip (e.g., the wire bar grid of the cage).

Hold the base of the tail with one hand and allow the animal to stand in a normal position.

With the other hand, the thumb and index finger are placed on either side of the neck at the base of the skull. Alternatively, a narrow, blunt instrument such as the dull edge of a scissor blade, acrylic ruler or cage card holder can be used.

To accomplish the cervical dislocation, quickly push down and forward with the hand or the object pressed at the base of the skull while pulling backward with the hand holding the base of the tail.

Note: A 2-4 mm space should be palpable at the base of the skull, between the occipital condyles and the first cervical vertebra or within the upper third of the neck.

https://www.mcgill.ca/research/files/research/301-_rodent_euthanasia_-_jan_2020.pdf

concussion

- only for <1kg
- doesn't require specialized equipment but must be conducted rapidly and effectively
- extensive damage to the brain resulting in very rapid loss of consciousness
- hold firmly by the tail and hindquarters and swing downward rapidly and forcibly so that the head strikes a hard surface, usually the edge of a benchtop
- ensure death using a secondary method

(AVMA Guidelines for the Euthanasia of Animals 2020, Directive 2010/63/EU)

decapitation

- acceptable only if justified that other methods are not possible
- requires equipment: guillotine and decapicones
- guillotine must be cleaned before each use, blades must be maintained to be sharp
- head is completely severed from body at the atlanto-occipital joint
- rapid loss of cortical function: 5-30 sec

Guillotines that are designed to accomplish decapitation in adult rodents in a uniformly instantaneous manner are commercially available.

The use of plastic cones to restrain animals is recommended as it reduces distress from handling, minimizes the chance of injury to personnel, and improves positioning of the animal in the guillotine.

Guillotines are not commercially available for neonatal rodents, but sharp blades (e.g. scissors) can be used for this purpose.

Consider using strong and sharp scissors, .e.g., surgical scissors or kitchen shears, for decapitation of adult mice to reduce the risk of injury to personnel.

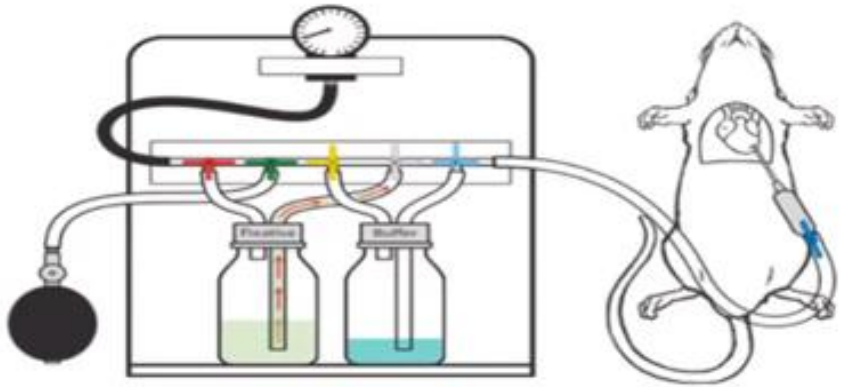
The equipment used to perform decapitation should be maintained in good working order and serviced on a regular basis to ensure sharpness of blades.

(AVMA Guidelines for the Euthanasia of Animals 2020, Directive 2010/63/EU)

methods under anesthesia

other common methods that can be used **under general anesthesia :**

- intravenous or intracardiac administration of potassium chloride ~100 mg/kg to induce cardiac arrest
- formaldehyde perfusion
- secondary methods
  - exsanguination
  - dissection of major organ(s)
  - bilateral thoracotomy
  - decapitation
  - cervical dislocation



https://www.jove.com/v/3564/whole-animal-perfusion-fixation-for-rodents

(AVMA Guidelines for the Euthanasia of Animals 2020, Directive 2010/63/EU)

any other method…

…requires justification by the research group in the application

…and authorization of exemption by the project evaluation committee!

(Directive 2010/63/EU)

euthanasia of fetuses and neonates

Fetuses

pain perception?

- mammalian fetuses are unconscious *in utero* due to a combination of factors, such as low oxygen tension and hormonal influences in the uterus suppressing consciousness - low arterial oxygen concentrations may limit higher cortical processing that would mediate fetal arousal and awareness
- by the 3rd trimester of gestation (>15d), the neural tube has developed into a functional brain, and the likelihood that a fetus may perceive pain should be considered
- no definitive evidence indicates that prenatal rodents perceive pain, but reflexive behavior observed in fetal animals correlates with adult responses to painful stimuli

Neonates

pain perception?

- rat and mouse pups are born neurologically immature when compared with humans, and their afferent pain pathways are not well developed until after postnatal day 5 to 7, with cortical development occurring later

(AVMA Guidelines for the Euthanasia of Animals 2020) (Report of ACLAM Taskforce on Rodent Euthanasia)

euthanasia of fetuses and neonates

Methods

- euthanasia of the dam
  - rat and mouse fetuses are unconscious in utero and hypoxia does not evoke a response → it is unnecessary to remove fetuses for euthanasia after the dam is euthanized…?
  - CO_2 followed by cervical dislocation
  - pentobarbital
- removal of the fetus before 15d of gestation (non viable)
- pentobarbital or anesthetic overdose ip
- hypothermia (<6d) followed by a secondary method
- decapitation with sharp scissors
- cervical dislocation by pinching and disrupting the spinal cord in the high cervical region
- rapid freezing in liquid nitrogen only under anesthesia!
- perfusion under anesthesia
- NOT CO_2 – unacceptable method for neonates!
  - > 10 days of age, pups may first be anesthetized with CO_2 or injectable anesthetic and then must have a physical method performed

(AVMA Guidelines for the Euthanasia of Animals 2020) (Report of ACLAM Taskforce on Rodent Euthanasia)

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euthanasia of neonates

- Neonatal rodents are resistant to hypoxia.
- Methods which do not induce hypoxia, are preferable

	Minimum time in 100% CO_2	
AGE	**MICE** (*Pritchett et al. 2005*)	**RATS** (*Pritchett-Corning 2009*)
Non-haired pups 0-6 days	60 minutes	40 minutes
Haired pups, eyes closed 7-13 days	20 minutes	20 minutes
Haired pups, eyes open, preweaning 14-20 days	10 minutes	10 minutes
Weanlings and adults 21+ days	5 minutes	5 minutes

confirmation of death

Directive 2010/63/EU, Annex IV &

Directive 2024/1262/EU

- ✓ confirmation of permanent cessation of the circulation
- ✓ desctruction of the brain
- ✓ dislocation of the neck
- ✓ exsanguination
- ✓ confirmation of *rigor mortis* onset
- ✓ appropriate for the species

AVMA Guidelines

- ✓ combination of criteria
  - apnea may give the false impression of death
- ✓ lack of pulse, breathing, corneal reflex, toe-pinch reflex, blink reflex
- ✓ graying of mucous membranes
- ✓ *rigor mortis*

confirm death

using combination of criteria

→

no breath

no pulse/heartbeat

no pain reflexes

gray mucous membranes

± rigor mortis

→

use secondary methods

to ensure death

→

exsanguination

removal of organs (heart, lungs, brain)

pneumothorax (open chest cavity)

destruction of brain

cervical dislocation

cadaver disposal

legal regulations

for hazardous waste

- animal remains
- chemical residues

  e.g. prevention of secondary poisoning from pentobarbital
- sharps

- ✓ labelling
- ✓ safe storage
- ✓ freeze or refrigerate
- ✓ incinerate
- ✓ records

(AVMA Guidelines for the Euthanasia of Animals 2020)

biological effects of euthanasia methods

- "The selection of specific agents and methods for euthanasia will depend on the species involved and the objectives of the protocol." *Guide for the Care and Use of Laboratory Animals*
- The euthanasia technique should minimally impact the welfare of the animal and the handler and must support collection of reproducible scientific data
- The specific impact of any euthanasia method on scientific results may require case-by-case validation
- may affect
  - blood biochemistry
  - tissue and organ samples
  - due to stress, hypoxia, acidosis or physical damage

(Report of ACLAM Taskforce on Rodent Euthanasia)

biological effects of euthanasia methods

Table 1. Biologic effects of decapitation[3,5,16,49,56,60,66]

Effect	Mechanism
Increase in plasma sodium Increase in plasma potassium Increase in GABA concentrations (brain) Increase in Alanine (brain) Increase in plasma ascorbic acid (30-40% > resting state)	Hemolysis
Increase in blood catecholamine levels	Continued postmortem neurochemical alterations
Increased plasma calcium, magnesium No change in vasoactive intestinal peptides (brain) No change in neuropeptide Y (brain) Alteration in rat heart mitochondria function	
Increase in serum corticosterone	Stress stimulus → mobilization from tissues to blood; generalized metabolic response secondary to sympathoadrenal response some handling related stimulation. Possible handling stress

(Report of ACLAM Taskforce on Rodent Euthanasia)

biological effects of euthanasia methods

Table 2. Effects of physical and pharmacological euthanasia methods

Method	Physiologic effect
Methoxyflurane and decapitation[10]	Increase in prostacyclin (vasodilator that inhibits platelet aggregation) Vascular contractility suppressed Decreased vascular contractility
Ether and decapitation, or decapitation alone[50]	No statistical difference in prolactin levels or LH/FSH secretory properties of cultured anterior pituitary cells
Ether and decapitation[74]	No change in estrogen receptors/progesterone receptors in rat uteri
Ketamine and decapitation[50,74]	No change in estrogen receptors/progesterone receptors in rat uteri
Pentobarbital and decapitation[4]	Increase in acetylcholine release in the brain
Halothane and decapitation[21]	Increase in plasma ascorbic acid Increase in plasma catecholamines

(Report of ACLAM Taskforce on Rodent Euthanasia)

biological effects of euthanasia methods

Table 3. Effects on reproductive hormones: The following combinations may be unsuitable for studies of serum androgens

Decapitation in combination with agents listed below[49, 71]	Male rats								Mechanism: direct effect on testes	
	Immature				Mature				Circulating Androstenedione	
	LH	FSH	Prolactin	Testosterone	LH	FSH	Prolactin	Testosterone	Castrated	Intact
Xylazine	–	–		↓	–	–	↑	↓		↓ or –
Biotal	–	–			–	–		↓		↓ or –
Thiopental	–	–			–	–		↓		↓ or –
Pentobarbital	–	–		↓	–	–	↑	↓		↓ or –
Ketamine	↓	↓		↓	–	–		↓	↑	↓ or –
Halothane	↓	↓		↓	–	–		↓		↓ or –
Ether (tested on castrated rats)	↑	↑	↑	↓	–	–		↓		↓ or –

↓ = decreased ↑ = increased – = no change.

(Report of ACLAM Taskforce on Rodent Euthanasia)

Table 4. Biologic effects of euthanasia induced by pharmacologic and/or physical methods

Method of euthanasia	Effect	Mechanism
Injectable Pentobarbital[5,53,61] [a,b]	Decreased muscular contractility in isolated muscle preps Decreased GI smooth muscle contractility when given orally or intravenously; not seen in intraperitoneal route Intraperitoneal administration causes increased colonic contractility in response to acetylcholine Decreased spontaneous and drug induced vascular smooth muscle contractility Decreased catecholamine levels	Decreased calcium transport
	Increased partial pressure of CO_2 in arterial blood Increased serum activity renin Increased plasma aldosterone Splenic enlargement	
	Increased plasma glucose and insulin Increased liver glycogen	Increased CO_2 in arterial blood may change blood pH, which then changes metabolic indices
	Decreased plasma triglycerides	
	Increase in plasma insulin	Increased glucose production or decreased glucose clearance
Cervical dislocation/ cervical fracture[32, 68, 72]	Decreased coronary flow; decreased contractile function in isolated perfused heart preparations	Possible decreased sensitivity of B-adrenergic receptors secondary to cervical fracture
	Normal lymphocyte proliferation	
	High levels of serotonin in lung	Entrapment of platelets in pulmonary capillaries
	Increase in granulocyte and macrophage colony forming cell counts in murine bone marrow cultures	Apparent alteration of marrow stem cell pool
CO_2[8,52,69]	100% CO_2: decreased mean corpuscular hemoglobin (NP)[d] Increased total leukocytes and granulocytes (P)[e] Decreased liver glycogen, pyruvate, ATP No change in platelet counts	CO_2 causes acidosis that affects RBC parameters
Isoflurane[8]	No change in liver glycogen	

(Report of ACLAM Taskforce on Rodent Euthanasia)

biological effects of euthanasia methods

Table 6. Gross/histopathology changes[1,24,25,33,64]

Ether	Decapitation	CO_2[a]	Methoxyflurane	Pentobarbital	Physical Methods (DC, CD)	Methods Listed in this Chart
Lung: interstitial edema, marked alveolar emphysema	Lung: emphysema, hemorrhage, blood in alveolar spaces	Lung: congestion, hemorrhage, emphysema, atelectasis; Cardiac muscle: variable degenerative changes (influenced by time of exposure to CO_2 causing acidosis, hypoxia) $CO_2 + O_2$ Lung: severe edema and hemorrhage, extravasation to alveoli Cardiac muscle: variable degenerative changes (influenced by time of exposure to CO_2 causing acidosis, hypoxia), capillary bleeding causing marked extravasation of blood	Lung: congestion Spleen: splenomegaly	Lung: emphysema congestion Spleen: emphysema, congestion GI serosa: emphysema, congestion Cardiac muscle: Acute degenerative lesions Kidney cortex: circulatory changes Other: Peritoneal congestion, sanguinous fluid in abdominal cavity	Lung: emphysema, bleeding Neck/Brain: local tissue trauma	No change in sperm motion

NOTE: DC (decapitation), CD (cervical dislocation), CO_2, Intracardiac pentobarbital more suitable for histology of abdominal viscera.
[a]produces changes in hemodynamics—capillary contraction, followed by dilation of capillaries and veins (except lung vessels); depresses cerebral cortex, stimulates chemoreceptors; extravasation to alveoli: Not seen in all rodent species.

(Report of ACLAM Taskforce on Rodent Euthanasia)

biological effects of euthanasia methods

Table 7. Additional Factors that Influence the Outcome of Euthanasia[6,7,18,22,38,56]

1. Handling: May cause sympathoadrenal discharge, which affects plasma glucose, progesterone plasma catecholamines. Habituating the animals to handling may mitigate this effect.
2. Environmental stimuli (for example, noise) can increase plasma corticosterone concentrations.
3. Sequence: The order of euthanasia for rats housed in pairs produced significant differences in plasma tryptophan and unesterified fatty acids, plasma corticosterone, plasma protein lactate levels, substance P, cholecystokinin, somatostatin.

(Report of ACLAM Taskforce on Rodent Euthanasia)

euthanasia resources

- Directive 2010/63/EU http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF
- Directive 2024/1216/EU https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L_20240126 2
- Report of the ACLAM Task Force on Rodent Euthanasia, 2006 https://www.aaalac.org/pub/?id=DA493B29-D28D-9B8A-3E64-142F58D51546
- AVMA Guidelines for the Euthanasia of animals, 2020 https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

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