humane endpoints & euthanasia

10th Care and Use of Laboratory Animals Course 15/5/2024

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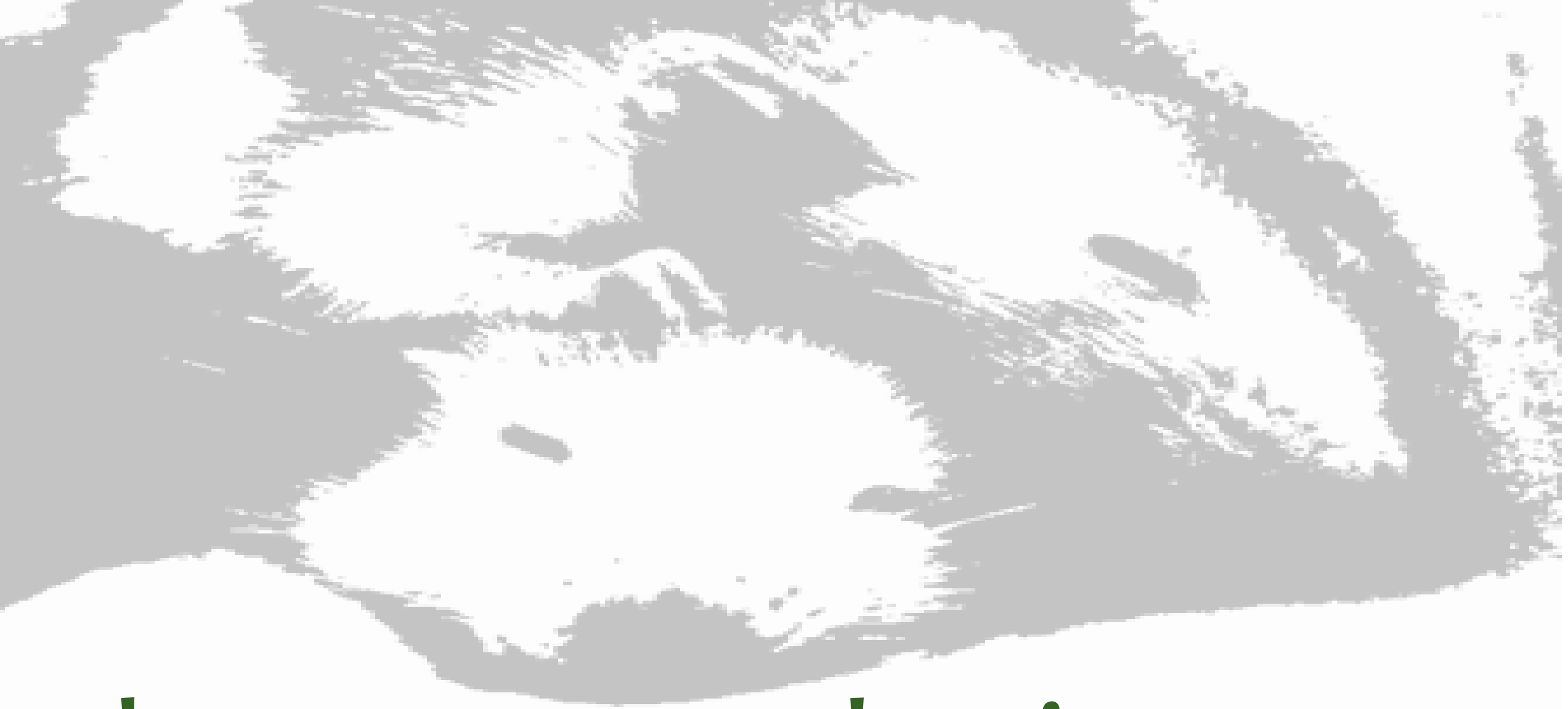
learning outcomes

- Humane Endpoints
  - 1.11. Indicate the circumstances in which animals under the scope of the Directive should be humanely killed or removed from the study to receive veterinary treatment.
  - 5.4. Describe what a humane end point is. Identify criteria to be used to set humane endpoints. Define action to be taken when a humane endpoint is reached and consider possible options for refining methods to finish at an earlier endpoint.
  - 11.15. Identify, assess and minimise all of the welfare costs to animals throughout the animals' lifetime (including adverse effects relating to sourcing, transport, housing, husbandry, handling, procedures and humane killing); Explain and give examples of welfare assessment protocols.
  - 11.16. Define and apply appropriate humane end-points; establish suitable criteria to identify when the humane endpoint has been reached
- Humane methods of killing (Euthanasia)
  - 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.



humane endpoints

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??

- endpoint
  - ➢ end of something
  - ➢ result / event that signifies completion of something
- study endpoint
  - ➢ scientific study aims and objectives are reached
  - ➢ data / samples are taken
- humane
  - ➢ kind, caring esp. for those suffering
- humane endpoint (humane intervention point)
  - ➢ point of intervention for humane reasons

endpoints in project design

study endpoints

- parameters @ time

humane endpoints

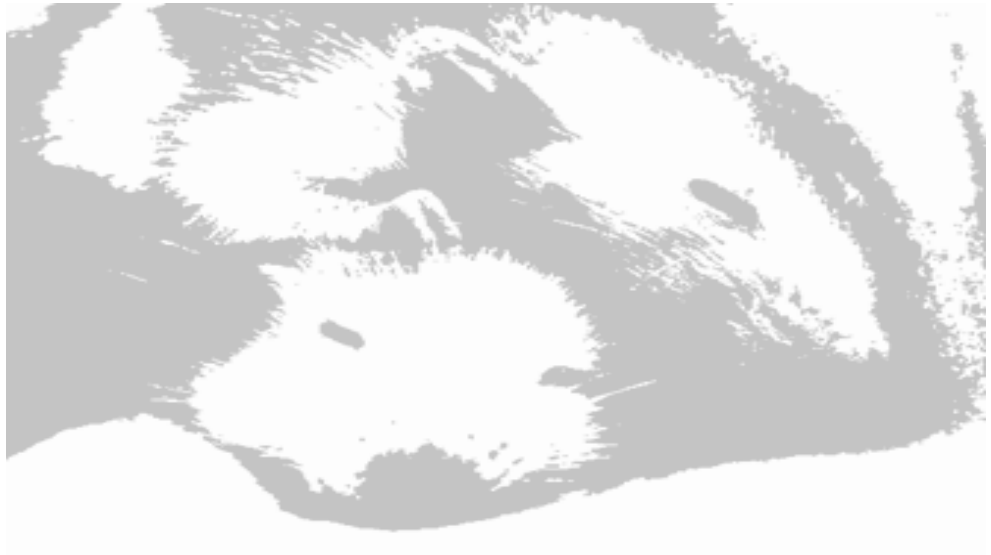
- points of humane intervention

- humane?
- early?
- predetermined
- monitored
- PI, DV, everyone!

humane endpoints

a tool for

refinement & reduction



‘the earliest indicator(s) in an animal experiment of (potential) pain and/or distress that,

can be used to avoid or limit pain and/or distress

by taking actions such as humane killing or terminating or alleviating the pain and distress

within the context of moral justification and scientific endpoints to be met,’

(Hendriksen and Morton, 1999)

indicators

- clinical signs (e.g. tumor formation)
- pathophysiological changes (e.g. hypothermia)
- behavioural changes (e.g. stereotypic behaviour)
- biochemical changes (e.g. ketonury)
- hormonal changes (e.g. prolactin)
- imaging changes (e.g. bioluminescence)

- ? preclinical changes (detectable before the onset of symptoms)

actions

- termination of procedure
- removal from protocol
- modification of experimental design
- refinement
- anesthesia (local, general)
- analgesia (local, general)
- other method(s) for alleviating pain/distress
- treatment
- euthanasia

- (earlier) sample collection?

https://www.humane-endpoints.info/en/why-humane-endpoints

Directive 2010/63/EU

examples

• surgical procedure	• anesthesia and analgesia
• pain	• analgesia
• tumor ulceration	• topical treatment
• tumor size	• euthanasia after set limit is exceeded
• reduction of body weight, and deterioration of body condition	• euthanasia after set limit is exceeded
• detection of preclinical indicator	• experimental endpoint

remember the Directive

- ✓ article 23: personnel must be proficient in the establishment and use of humane endpoints
- ✓ article 37: information on the project's humane endpoints shall be provided in the application for authorization to enable harm-benefit evaluation
- ✓ annex VIII: the nature of the humane endpoints affects the severity classification of the procedure/project
- ✓ article 13: humane endpoints shall be set for each project to prevent unnecessary suffering and apply refinement
- ✓ article 13: early and painless humane endpoints shall be chosen and death as an endpoint must be avoided
- ✓ article 14: in case of expected or observed pain, an appropriate analgesic plan shall exist
- ✓ article 14: when the purpose of the procedure is achieved, animal suffering shall be minimized
- ✓ article 17: when a procedure ends or when further observations cannot be made, appropriate actions shall be taken to relief the animals

Article 13

Choice of methods

1. Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union.

2. In choosing between procedures, those which to the greatest extent meet the following requirements shall be selected:

(a) use the minimum number of animals;

(b) involve animals with the lowest capacity to experience pain, suffering, distress or lasting harm;

(c) cause the least pain, suffering, distress or lasting harm;

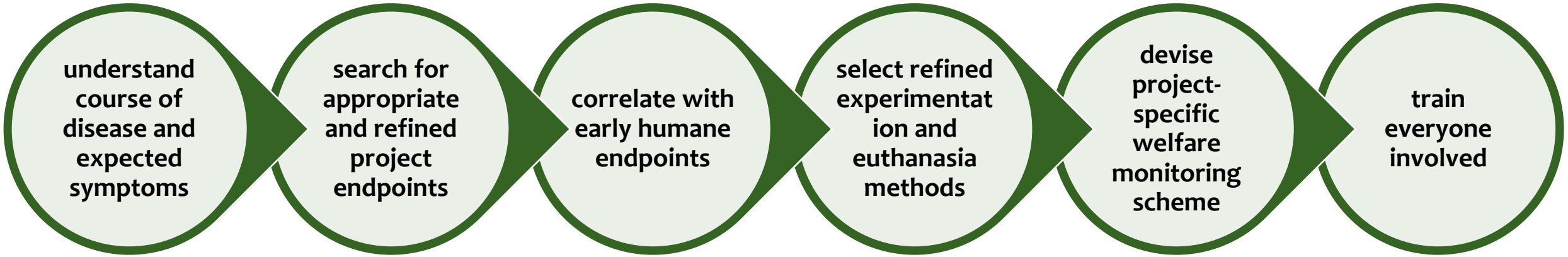
and are most likely to provide satisfactory results.

3. Death as the end-point of a procedure shall be avoided as far as possible and replaced by early and humane end-points. Where death as the end-point is unavoidable, the procedure shall be designed so as to:

(a) result in the deaths of as few animals as possible; and

(b) reduce the duration and intensity of suffering to the animal to the minimum possible and, as far as possible, ensure a painless death.

apply refinement



- ✓ plan actions for all possibilities
  - ✓ both expected and unexpected events!
- ✓ consider quality of measurements/samples
- ✓ consider animal welfare

check!

- are all possibilities considered, both expected and unexpected events?
- is death as endpoint avoided?
- is the earliest stage where scientific objectives are met considered?
- can the objectives be met whilst the animal has these symptoms?
- is the suffering of the animal still justified?
- does keeping the animal have additional value to the results?
- does the situation of the animal have consequences that may interfere with the results?

- quality of measurements/samples
- animal welfare

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early humane endpoints?

healthy

earlier indicators of disease onset

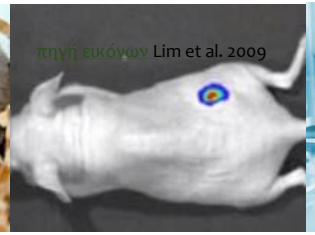
indicators of disease onset

earlier signs of disease

obvious signs of severe disease

dead

know what it looks like !?



e.g. MRI, CT, bioluminescence

e.g. blood glucose, tumor markers

e.g. weight loss, chromodacryrrhea

e.g. cachexia, moribund state

start

experiment timeline

end?

increasing harms

Efficacy of novel pharmaceutical agents on tumour growth

Procedures included in the protocol				
Procedure	**Frequency**	**Duration**	**Person who will carry out the procedure**	**Induced severity**
Maintenance of immunocompromised mice (30 male BALB/c nude mice)	-	3 months	Establishment staff	mild
Subcutaneous injection of tumor cells	once	5 minutes	Jim	mild
Tumor growth	-	2 months	-	moderate
Intraperitoneal injection of pharmaceutical agents or placebo	7 times	7 days	Jim	moderate
Overall assessment of protocol severity: SEVERE?				

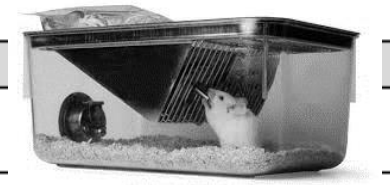
Evaluation of Humane Endpoints	
How often will the animals be monitored?	once a day
Which criteria will define the HE	☒ Weight loss ☐ Loss of mobility ☐ Loss of sensation ☐ Coma status for 24-48hrs after the intervention ☒ Serious disturbance of animal welfare Other:
Who will assess the animals?	Establishment staff.
Attach welfare assessment sheet.	

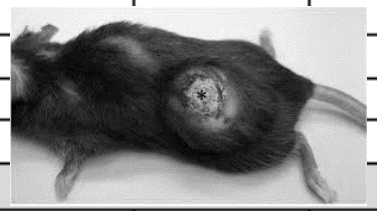
discussion points

- What kind of cages are available? Have you discussed with the staff about the needs and care of immunocompromised animals? Are they familiar with the strain?
- Are you comfortable with the procedures you will perform?
- Are there enough data on the effect on the animals, or we need a pilot experiment.?
- Are you familiar with the cell line? Do you know the effect this tumor will have? What symptoms do you expect?
- Are the side effects of the drug known? What are they?
- How much volume will you inject?
- How is the prospective severity assessed?
- What HE are selected?
  - There are specific guidelines regarding humane endpoints in tumor research.
- How often would you weigh and assess the animals?

Example of a score sheet

Animal no.				
Date	01/06	02/06	03/06	04/06
Appearance				
Body weight				
Coat condition				
Body function				
Dyspnoea and/or tachypnoea				
Food intake				
Environment				
Loose stools or diarrhoea				
Blood in diarrhoea				
Behaviours				
Handling				
Aggression				
Abnormal gait				
Abnormal posture				
Reluctance to move				
Procedure-specific indicators				
Tumour size				
Ulceration of tumour				
Tumour impeding movement				
Total score				
Any other observations				





Examples of clinical scores

Appearance	Score
Bodyweight	
5-10% weight loss	1
11-15 % weight loss	2
16-20% weight loss	3
20% + weight loss	HEP
Coat Condition	
Coat slightly unkempt	1
Slight piloerection	2
Marked piloerection	3
Body Function	
Tachypnoea (fast breathing)	1
Dyspnoea (difficulty breathing)	3
Environment	
Loose stools or diarrhoea	1
Blood in diarrhoea	HEP
Behaviour	
Tense and nervous on handling	1
Markedly distressed on handling, e.g. shaking, vocalizing, aggressive	3
Locomotion	
Slightly abnormal gait/posture	1
Markedly abnormal gait/posture	2

Actions	
Score 1	Review frequency of monitoring
2	Consider supplementary care, *e.g.* extra fluids
4	Consult veterinarian
6	Implement humane endpoint

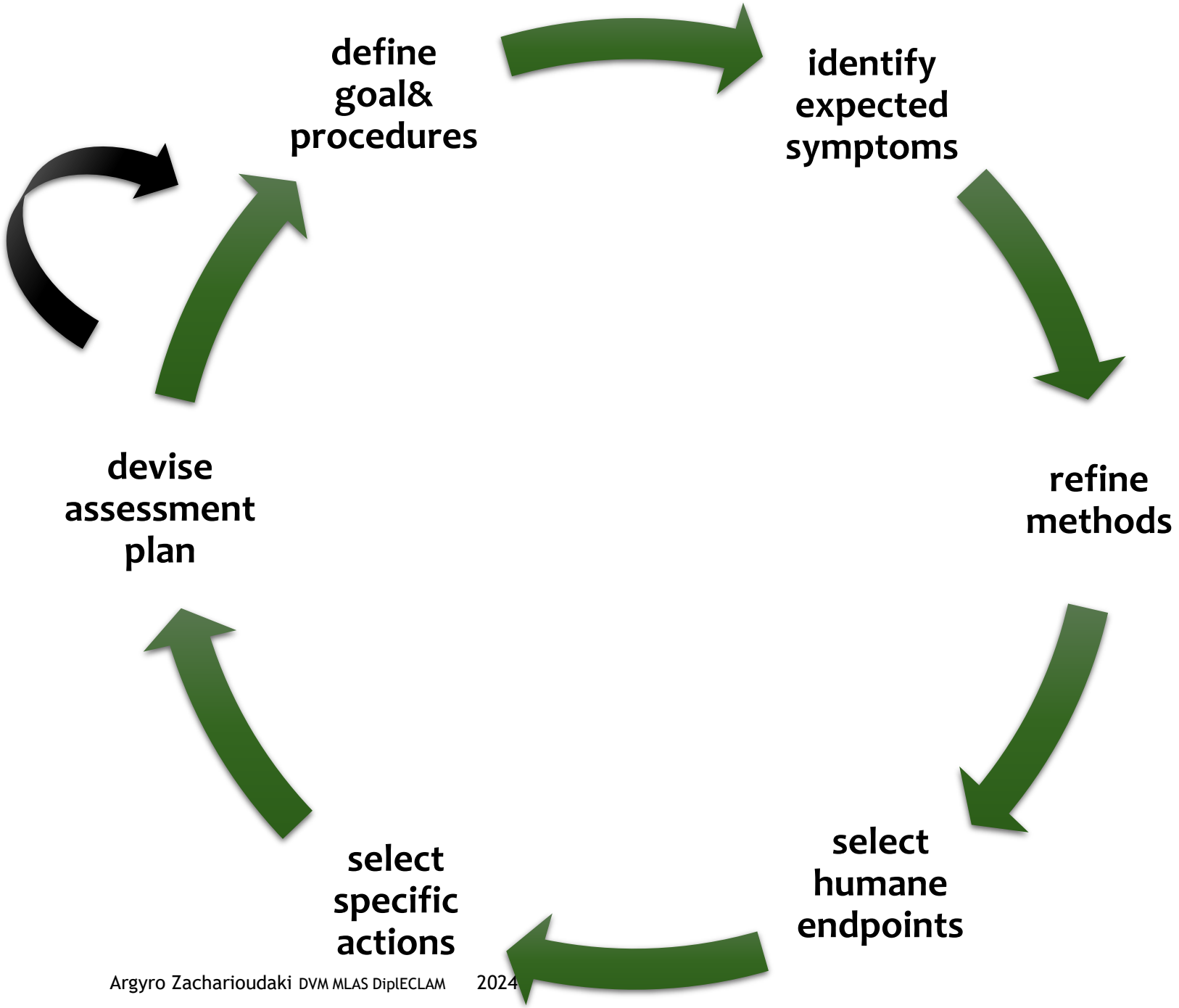
earlier HE
lower severity!

define project-specific symptoms and signs → **select endpoints based on significance** → **assure scientific validity** → **decide on appropriate action** → **devise assessment sheet and inform assessors**

assessment of actual severity

- 3 animals did not develop tumours and were euthanized as unusable for the experiment → mild
- 2 animals developed ulceration at the tumour injection site before treatment started and were euthanased. → moderate
- 10 animals receiving drug B at dose H had tumours that remained relatively small, with no significant BW loss and no clinical signs. → mild
- 7 animals receiving drug B at dose X had a decrease in tumour size, a BW loss of 15% and presence of loose stools, but were kept until the end of the experiment. → moderate
- 3 animals receiving drug B at dose X had a decrease in tumour size, a BW loss of 15%, presence of loose stools, anorexia and were very lethargic; these were humanely killed on day 25. → severe
- 5 Animals receiving drug C at dose Y had a continued increase in tumour size, body weight increased, no clinical signs apart from tumour growth. These animals were euthanised when the tumour size exceeded 1.2 cm. → moderate

on-going evaluation!



decision wheel

humane endpoints
example – gastrointestinal surgery

define goal& procedures

- study of new implant
- enteroanastomosis surgery, 1month observation, euthanasia for sample collection

identify expected symptoms

- surgical site (healing, pain, infection), anastomosis (GI function, failure, peritonitis)

refine methods

- surgical technique, post-op care, analgesia

select humane endpoints

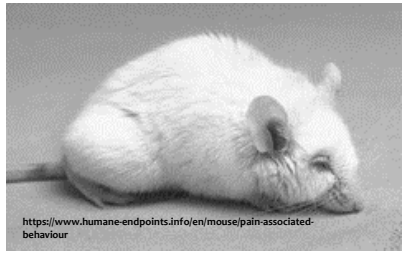
- pain signs: hunched posture, grimace scale
- incision observation: erythema, edema, open wound
- GI function observation: fecal production
- food/water intake
- body weight, BCS
- temperature

select specific actions

- treatment of infection
- palatable, accessible food, prokinetics?
- analgesia
- euthanasia

devise assessment plan

- frequency
- parameters & methods
- training



humane endpoints

example – arthritis research

define goal& procedures

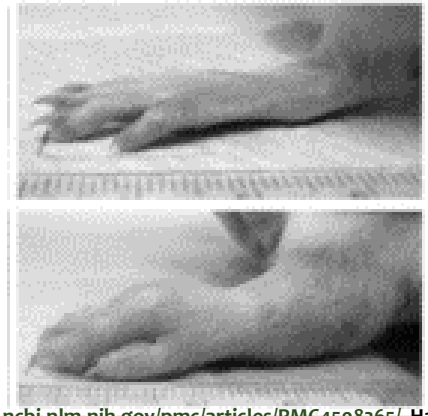
- study of new treatment or pathophysiology
- arthritis induction, other procedures
- duration

identify expected symptoms

- arthritis
- pain
- obstruction of movement & normal behavior

refine methods

- procedures (number of limbs, location of injection etc)
- handling!
- housing



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4508365/ Hawkins et al 2015

Arthritis score	Maximum points—hindpaws	Maximum points—forepaws
1 point for each swollen or red digit	5	4
1 point for each swollen knuckle	5	4
1 point for swollen midfoot	1	1
1 points for a swollen ankle/wrist	1	1
Total (×2)	24	20

select humane endpoints

- pain signs
- arthritis
- movement: paralysis
- food/water intake
- body weight, BCS

select specific actions

- treatment of ulceration
- accessible food & water
- analgesia???
- euthanasia

devise assessment plan

- frequency
- parameters & methods
- training

humane endpoints

example

National Centre for the Replacement Refinement & Reduction of Animals in Research

https://www.nc3rs.org.uk/refined-animal-model-multiple-sclerosis

Consider the HE in each case

A refined animal model of multiple sclerosis

traditional model: Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease that is widely used as a model of multiple sclerosis. It is induced by immunisation with myelin antigens. Studies of EAE in mice involve **paralysis of the hindlimbs** and sometimes forelimbs and, as such, are classified as causing severe suffering. In a typical study, animals are **killed at various time points** to examine disease progression. This necessitates the use of large numbers of mice; eight to 25 per group depending on the experiment. Analysis requires detailed histology which is time consuming. EAE models are, however, limited in that they represent acute central nervous system inflammation, whereas progression of multiple sclerosis has been shown to involve other mechanisms of neurodegeneration which are independent of the immune system.

refined model: With NC3Rs funding, Professor David Baker, the Blizard Institute at Queen Mary, University of London, has developed a new mouse model of multiple sclerosis which uses fewer animals and avoids paralysis, based on **optic neuritis** which is typically an **early symptom** of multiple sclerosis in humans. Two lines of **transgenic** mice have been crossed: one which has T cells which target a protein called myelin oligodendrocyte glycoprotein (leading to demyelination) and the other which expresses cyan fluorescent protein in the retinal ganglion cells. Levels of myelin oligodendrocyte glycoprotein are higher in the optic nerve than in the spinal cord and as a result the mice develop optic neuritis without EAE and the associated paralysis. Optic nerve damage can be tracked by following the expression of cyan fluorescent protein in the retinal ganglion cells. This provides a novel model which allows autoimmunity, neurodegeneration and neuroprotection to be studied and can be used to replace some use of the EAE model. The whole experiment can be performed in one to two weeks rather than the typical three to four weeks with the EAE model and optical damage can be **monitored non-invasively using optical coherence tomography.**

humane endpoints
example

National Centre for the Replacement Refinement & Reduction of Animals in Research

https://www.nc3rs.org.uk/refinement-mouse-model-pulmonary-embolism

Consider the HE in each case

Refinement of a mouse model of pulmonary embolism

traditional model: intravenous administration into conscious mice of a lethal dose of clotting agents such as collagen or thrombin, which causes massive pulmonary embolism and **paralysis or death is observed in 90% of the mice within 15 minutes** - the effects of drugs and genetic modifications are studied by measuring their ability to significantly change the proportion of mice that develop paralysis or die

disadvantages: severity (severe), use for massive pulmonary embolism but not earlier stages, reliance on non-specific clinical signs (paralysis, death)

refined model: With NC3Rs funding, Dr Michael Emerson, Imperial College London, has refined and reduced the use of mice in pulmonary embolism research, providing an in vivo model which better mimics the physiology and biochemistry of the condition in man and models the earlier stages of the disease. Dr Emerson developed a model which allows thrombus formation to be tracked in vivo using **radiolabelled platelets** isolated from the blood of donor mice. The radiolabelled platelets are injected into a recipient mouse, **under terminal anaesthesia**, and a sublethal dose of the clotting agent is administered. Platelet accumulation can then be measured non-invasively using **a spectrometer** connected to a gamma scintillation probe. This is a major refinement which avoids paralysis and death. The model also has a number of other advantages in that it allows measurement of platelet aggregation and disaggregation directly in real-time and takes into account non-platelet factors such as endothelial status and blood flow. Dr Emerson has further evolved the new model by using radiolabelled human platelets. The human platelets are then injected into NOD-SCID mice which lack T and B lymphocytes and are therefore amenable for transplantation studies. The human platelets remain viable and can be tracked in the mouse. The humanised mouse model is currently being validated by studying the anti-thrombotic effect of aspirin administered to human volunteers.

By optimising the procedures for repeat administration of clotting agents, **one mouse can be used several times whilst under terminal anaesthesia**. This has enabled the number of animals used to be reduced from 200 with the traditional model, to 30 per study where mice are used as platelet donors and to 15 per study where human platelets are used.

humane endpoint resources

20.10.2010 EN Official Journal of the European Union L 276/33

DIRECTIVES

DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
of 22 September 2010
on the protection of animals used for scientific purposes
(Text with EEA relevance)

THE EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION,

Having regard to the Treaty on the Functioning of the European Union, and in particular Article 114 thereof,

Having regard to the proposal from the European Commission,

Having regard to the opinion of the European Economic and Social Committee (1),

After consulting the Committee of the Regions,

Acting in accordance with the ordinary legislative procedure (2),

Whereas:

(1) On 24 November 1986 the Council adopted Directive 86/609/EEC (3) in order to eliminate disparities between laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Since the adoption of that Directive, further disparities between Member States have emerged. Certain Member States have adopted national implementing measures that ensure a high level of protection of animals used for scientific purposes, while others only apply the minimum requirements laid down in Directive 86/609/EEC. These disparities are liable to constitute barriers to trade in products and substances the development of which involves experiments on animals. Accordingly, this Directive should provide for more detailed rules in order to reduce such disparities by approximating the rules applicable in that area and to ensure a proper functioning of the internal market.

(2) Animal welfare is a value of the Union that is enshrined in Article 13 of the Treaty on the Functioning of the European Union (TFEU).

(3) On 23 March 1998 the Council adopted Decision 1999/575/EC concerning the conclusion by the Community of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (4). By becoming party to that Convention, the Community acknowledged the importance of the protection and welfare of animals used for scientific purposes at international level.

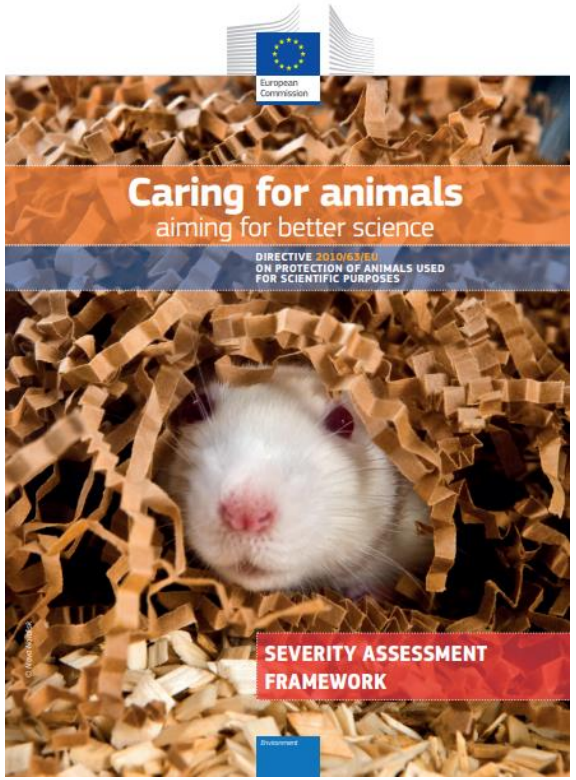
(4) The European Parliament in its resolution of 5 December 2002 on Directive 86/609/EEC called for the Commission to come forward with a proposal for a revision of that Directive with more stringent and transparent measures in the area of animal experimentation.

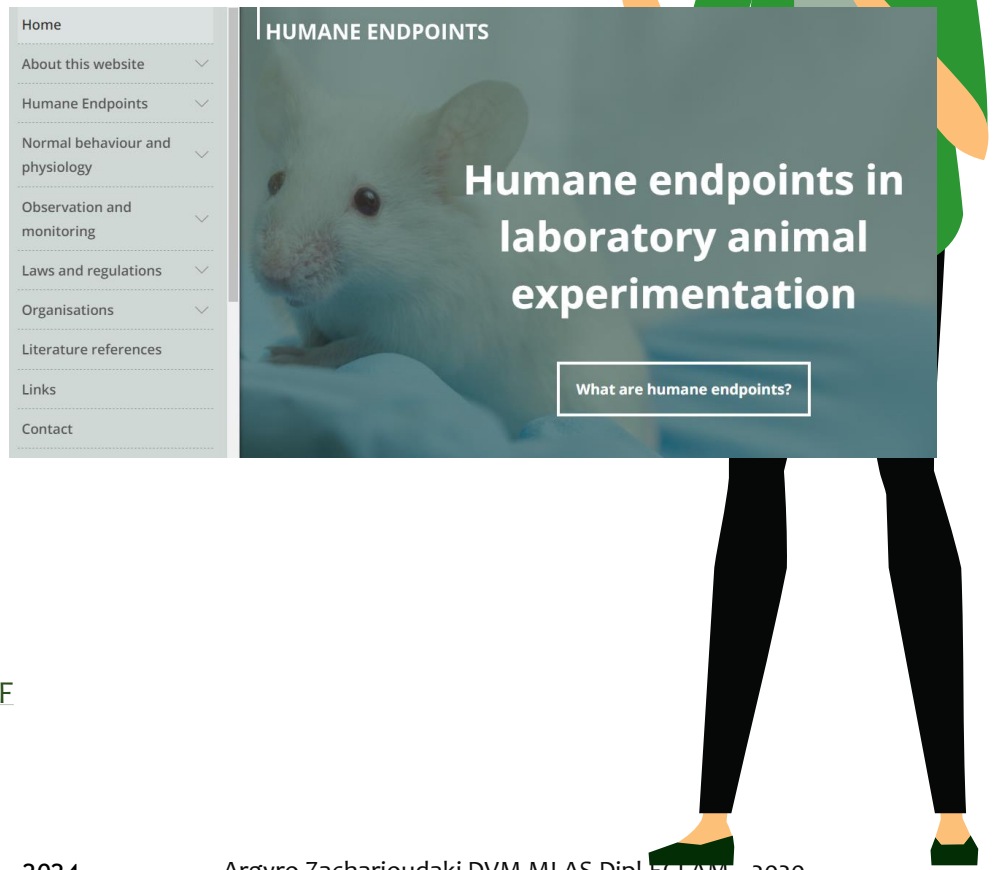
(5) On 15 June 2006, the Fourth Multilateral Consultation of Parties to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes adopted a revised Appendix A to that Convention, which set out guidelines for the accommodation and care of experimental animals. Commission Recommendation 2007/526/EC of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (5) incorporated those guidelines.

(6) New scientific knowledge is available in respect of factors influencing animal welfare as well as the capacity of animals to sense and express pain, suffering, distress and lasting harm. It is therefore necessary to improve the welfare of animals used in scientific procedures by raising the minimum standards for their protection in line with the latest scientific developments.

(7) Attitudes towards animals also depend on national perceptions, and there is a demand in certain Member States to maintain more extensive animal-welfare rules than those agreed upon at the level of the Union. In the interests of the animals, and provided it does not affect the functioning of the internal market, it is appropriate to allow the Member States certain flexibility to maintain national rules aimed at more extensive protection of animals in so far as they are compatible with the TFEU.

(1) OJ C 277, 17.11.2009, p. 51.
(2) Position of the European Parliament of 5 May 2009 (OJ C 212 E, 5.8.2010, p. 170), position of the Council of 13 September 2010 (not yet published in the Official Journal) and position of the European Parliament of 8 September 2010 (not yet published in the Official Journal).
(3) OJ L 358, 18.12.1986, p. 1.
(4) OJ L 222, 24.8.1999, p. 29.
(5) OJ L 197, 30.7.2007, p. 1.





- https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF
- https://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/severity/en.pdf
- https://www.humane-endpoints.info/en

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how?

- search and read

✓make a scoresheet

✓monitor carefully

- think and challenge

✓make a replacement, reduction or refinement!

euthanasia

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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
- 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				(4)	(5)				
Concussion/ percussive blow to the head				(7)	(8)				
Decapitation				(11)	(12)				
Electrical stunning	(13)	(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 used in gradual fill. Not to be used for foetal and neonate rodents.
4. Only to be used for birds under 1 kg. Birds over 250 g shall be sedated.
5. Only to be used for rodents under 1 kg. Rodents over 150 g shall be sedated.
6. Only to be used for rabbits under 1 kg. Rabbits over 150 g shall be sedated.
7. Only to be used for birds under 5 kg.
8. Only to be used for rodents under 1 kg.
9. Only to be used for rabbits under 5 kg.
10. Only to be used on neonates.
11. Only to be used for birds under 250 g.
12. Only to be used 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.

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 / percussive blow

electrical stunning

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
    - 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 10’ after cessation of opercular movement
- physical methods
  - cervical transection or decapitation or concussion followed by pithing
  - maceration

(AVMA Guidelines for the Euthanasia of Animals 2020. Collymore et al 2016)

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

Argyro Zacharioudaki DVM MLAS Dipl.ECLAM

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 e.g. MS222

~~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

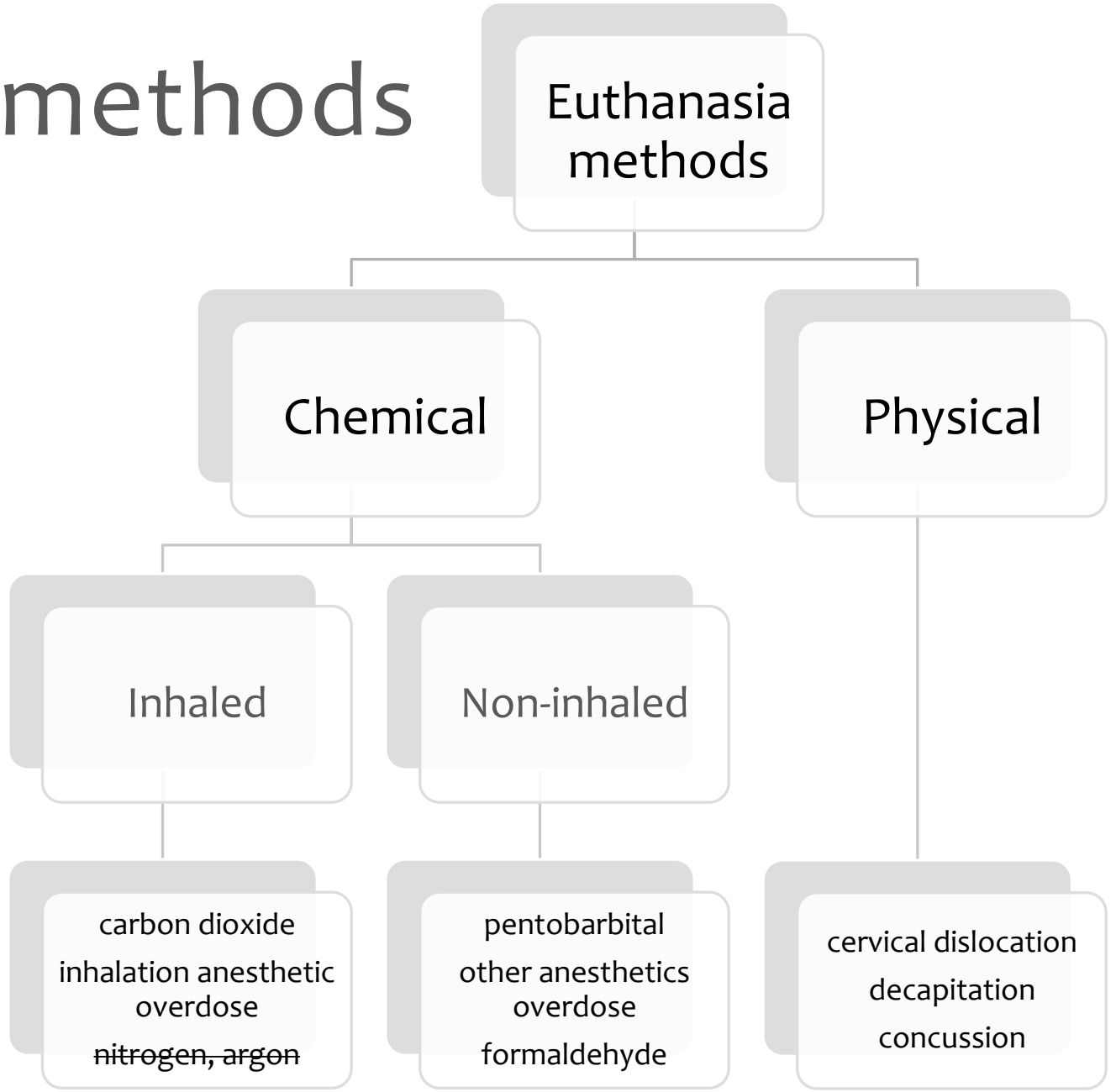
- availability
- prescription?
- good working order and maintenance

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. inert gases (Ar, N2) (? → unacceptable by AVMA Guidelines)
7. other methods under anesthesia
8. 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…

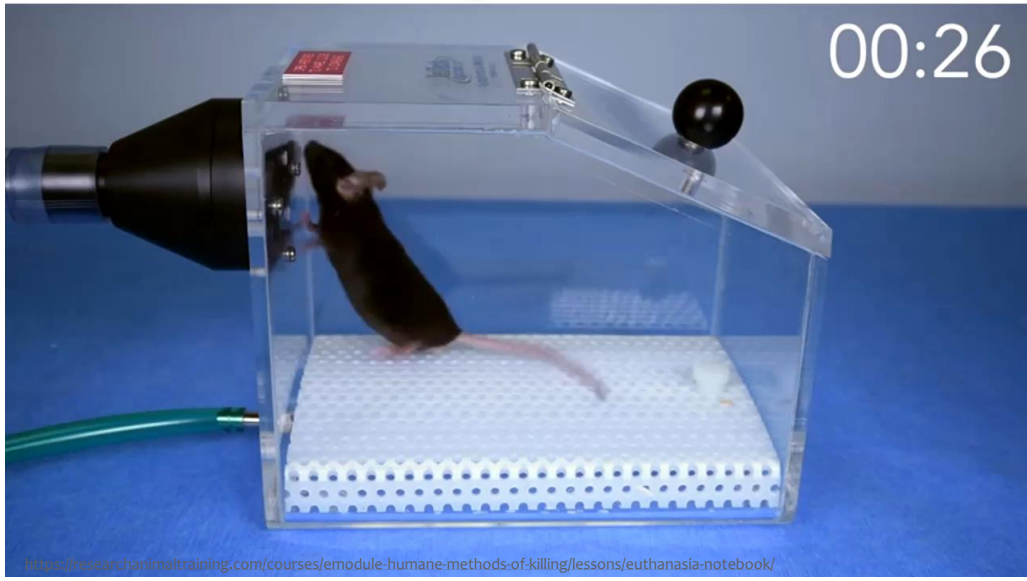
➢ **administered in an induction chamber via a vaporizer**

- 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)

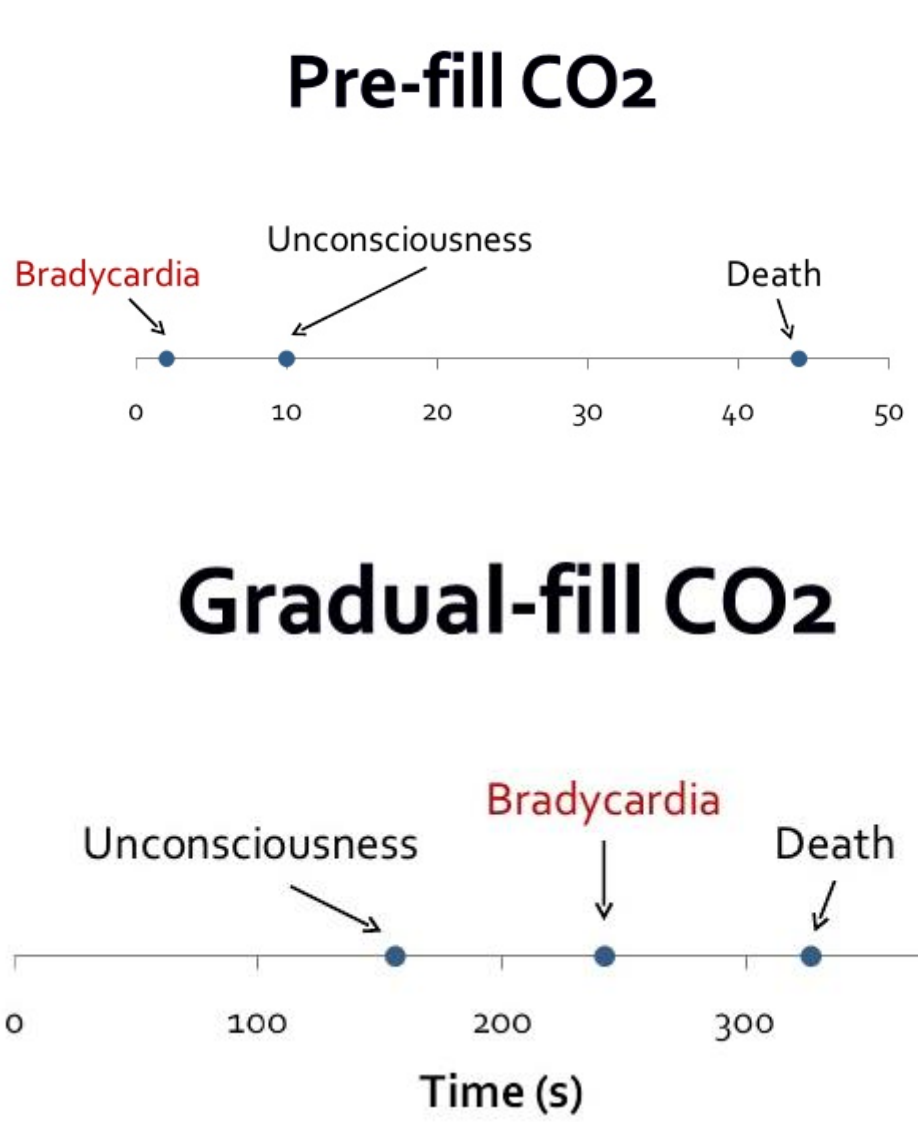
carbon dioxide

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)



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

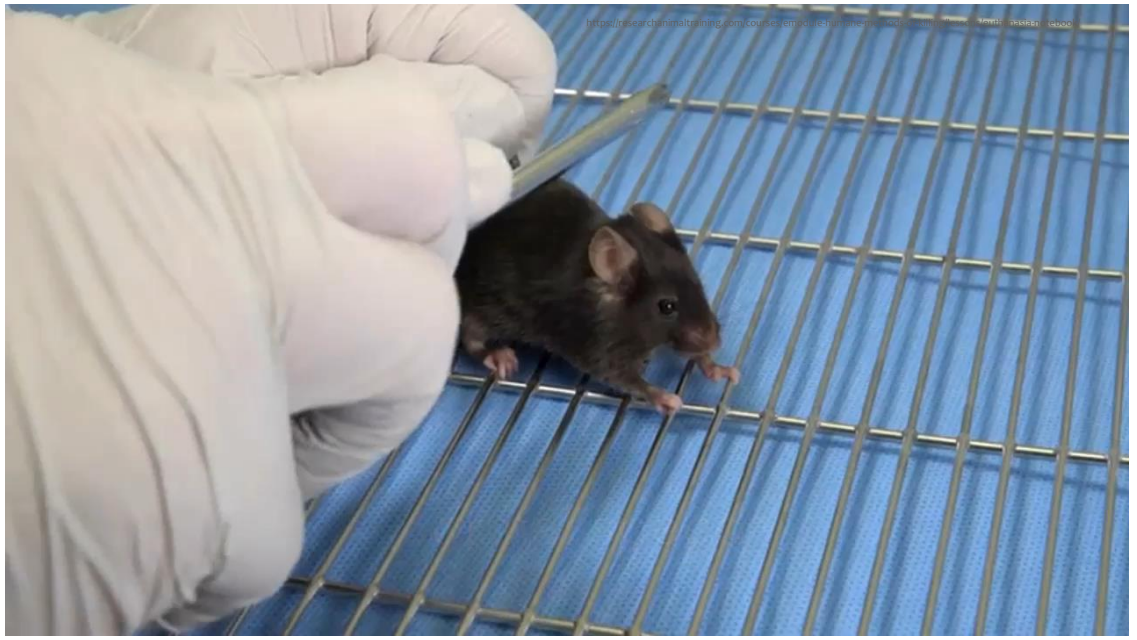
- preferably under sedation or anesthesia

- 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.

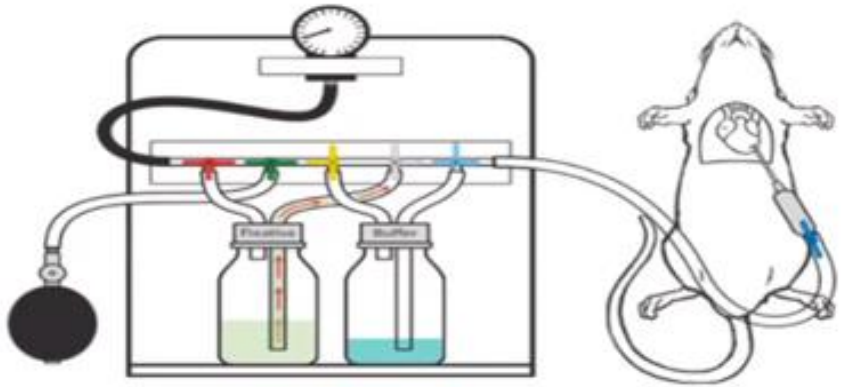
The equipment used to perform decapitation should be maintained in good working order and serviced on a regular basis to ensure sharpness of blades.

(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

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

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.

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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
- 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