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A study of different euthanasia techniques in guinea pigs, rats, and mice. Animal response and postmortem findings

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Introduction

It is of prime concern that methods for killing animals fullfil the definition of euthanasia – induction of unconsciousness and death with a minimum of pain and distress.

Not only aspects of animal welfare, but also safety of personnel and the scientific purpose must be considered when selecting an appropriate method for killing laboratory animals. When working in the fields of health monitoring and animal experimentation we repeatedly observed the fact that the responses of animals to different killing methods showed a wide variation. Histological findings also varied with the killing method.

In spite of the fact that the importance of a careful choice of killing method is now being more widely recognized definition of the appropriate method for each specific requirement is still not sufficiently understood. The purpose of the present study was to relate the effect of four available killing methods to animal responses and postmortem findings.

Material and methods

Animals

Twentyfour adult animals of each species were used, six animals (3 + 3) for each killing method.

All animals were clinically healthy but only one colony was microbiologically monitored. All animals were killed on the day of delivery.

Guinea pigs: Non barrier reared Dunkin-Hartley with body weights ranging from 505 to 800 g. (Males 505–755 g, females 560–800 g). The animals were delivered by a local breeder.

Rats: Outbred barrier reared Sprague Dawley with body weights ranging from 175–180 g (ALAB Laboratorietjänst AB, Sollentuna, Sweden). The rats were health monitored and found positive for *Pasteurella pneumotropica,* Coronavirus and *Syphacia sp.*

Mice: Non barrier reared NMRI from an outbred stock, with body weights ranging from 18–22 g. (National Veterinary Institute, Uppsala, Sweden).

Killing methods

Three of the killing methods used were chosen among common laboratory techniques recommended from animal welfare point of view (*AVMA* 1978, 1986, *CCAC* 1984, *UFAW* 1987). The CO_2 + O_2 induction method was included as a supposedly less distressing alternative to pure CO_2 (*MacArthur* 1978, *Iwarsson et al.* 1985b, *AVMA* 1986).

Stunning (guinea pigs) was performed by a blow to the back of the neck.

Decapitation (rats) was performed by means of a standard small rodent guillotine.

Cervical dislocation (mice) was accomplished by the standard procedure using a pencil.

Pentobarbital sodium (Mebumal vet, 60 mg/ml, ACO, Sweden) was administered i.p. in all species at a dose of 150 mg/kg body weight. For injections in mice the commercial 60 mg/ml solution was diluted with saline to a concentration of 30 mg/ml.

Carbon dioxide (CO_2). The animals were exposed to pure CO_2 in a specially constructed, airtight, 10-litre perspex box, originally described as an induction chamber of an

Table 1. Response of guinea pigs to different killing methods.

		METHOD OF EUTHANASIA			
		STUNNING n = 6	PENTOBARBITAL i.p. n = 6	CO_2 n = 6	CO_2 + INITIAL O_2 n = 6
Response until unconscious		-	Drowsiness Decreased respiratory rate	Moderate uneasiness Drowsiness Moderately laboured breathing Urination Defecation	Mild uneasiness Drowsiness Urination Defecation
Handling/restraint stress		Short	Apparent*	Minimal	Minimal
Induction of unconsciousness	Time	Instant	x = 210 sec (130-368)	x = 30 sec (22-40)	x = 29 sec (20-35)
	Stress response	-	Mild	Mild	Mild
Observations after induction of unconsciousness		Fits Muscle fasciculations	Gradually decreased respiratory rate - gasping-respiratory arrest	Tachypnoea during 50 sec, followed by forced, laboured breathing. Shallow breathing just before death.	Initial shallow tachypnoea followed by dyspnoea with intermittent very deep breaths every 2-4 sec during > 4 min. Shallow breathing just before death.
Time for respiratory arrest		μ 10 sec	x = 490 sec (364-730)	x = 165 sec (100-180)	x = 360 sec (290-390)

* Compared to other methods of handling used in this study
x = mean value; range within brackets.

inhalational anaesthesia system for small laboratory animals (*Iwarsson et al.* 1985a). The box has one inlet for CO_2 and a T-piece outlet equipped with an open 0.5 litre reservoir on one limb and the other connected to a vacuum ejector for scavenging excess gases. The device has been used in daily routine work at the National Veterinary Institute since 1984. The 10 litre box was precharged for 5 min at a gas flow rate of 10 litre/min before introduction of the first animal and for 1 min between animals. After visible breathing had ceased the animal was left in the box for another 2 minutes.

Carbon dioxide plus oxygen (CO_2 + O_2). A mixture of CO_2 (80 %) and O_2 (20 %) was used during a 60 sec. exposure period followed by pure CO_2 as described above. The same technical device was used.

The gases used were of medical care grade (AGA, Gas AB, Lidingö, Sweden).

All animals were treated the same way before killing, and they were introduced into the laboratory one at a time. All devices and equipment were thoroughly cleaned between animals.

Recording of animal response

The response of each animal to the killing procedure was recorded by the same person (Tables 1–3). To standardize the procedure, one experienced person performed all animal handling, including the restraint and the killing, with special emphasis on creating a calm experimental situation. For non physical methods unconsciousness was defined as loss of the rightening reflex.

Clinical death was considered to have been achieved at the time of respiratory arrest. Time was recorded by means of a stopwatch.

Post-mortem examination

The animals were subjected to necropsy. Tissue samples from lung, brain, heart, kidney, liver, muscle, spleen and intestine (jejunum) were fixed in a 10 % buffered formaldehyde solution, embedded in paraffin, cut in 5 µm sections, and stained with haematoxylin-eosin.

Table 2. Response of rats to different killing methods.

		METHOD OF EUTHANASIA			
		GUILLOTINE n = 6	PENTOBARBITAL i.p. n = 6	CO_2 n = 6	CO_2 + INITIAL O_2 n = 6
Response until unconscious		-	Drowsiness, ataxia, crawling movements	Initially explorative, followed by "Freezing". Moderate uneasiness. Tachypnoea Urination Defecation	Initially explorative, followed by mild uneasiness. Mildly laboured breathing.
Handling/restraint stress		Short	Apparent*	Minimal	Minimal
Induction of unconsciousness	Time	Instant	x = 152 sec (105-195)	x = 13 sec (10-18)	x = 27 sec (15-40)
	Stress response	-	Moderate	Mild-moderate	Mild
Observations after induction of unconsciousness		Tonic and clonic fits Muscle fasciculation	-	Intermittent severe laboured breathing. Pale mucous membranes. Convulsive breathing just before death.	Hypopnoea with successively increasing shallow irregular breathing
Time for respiratory arrest		Instant	x = 676 sec (510-815)	x = 116 sec (90-135)	x = 196 sec (180-240)

* Compared to other methods of handling used in this study
x = mean value; range within brackets.

Results

Animal response

Tables 1–3 summarize the animal responses to the different killing techniques used. The presentation includes the times for induction of unconsciousness and respiratory arrest, the animal reaction before and after induction of unconsciousness as well as subjective judgements of the stress response to handling/restraint and to the induction procedure per se.

Physical methods

Stunning in guinea pigs, decapitation using a guillotine in rats and cervical dislocation in mice, all resulted in an apparently instant loss of consciousness.

Although emotionally displeasing, the animal distress was judged to be minimal, if at all, and limited to the handling and restraint necessary for fixation immediately before killing.

In guinea pigs, respiration was observed up to 10 seconds after stunning and occasional heart beats were recorded for up to 3 minutes.

Pentobarbital sodium i/p

Intraperitoneal injection of pentobarbital sodium at 150 mg/kg body weight induced unconsciousness smoothly within 2–3 minutes in all species, following a faster but otherwise normal pattern of an ip barbiturate narcosis. In guinea pigs the induction time varied between 2–6 minutes.

For two reasons the handling/restraint stress in guinea pigs and rats was judged to be greater as compared to the other methods used. The injection, including the whole procedure from being picked up one by one, grasped, and injected imposed the longest handling time and induced a light to moderate, but short, flight behaviour immediately after being set free in the perspex box. It is possible, however, that a somewhat prolonged induction, with ataxic paddling movements and increasingly laboured breathing may cause distress and fear in the animals.

Carbon dioxide

In all three species, exposure to pure carbon dioxide rapidly caused unconsciousness, soon followed by death. The handling stress

Table 3. Response of mice to different killing methods.

		METHOD OF EUTHANASIA			
		CERVICAL DISLOCATION n = 6	PENTOBARBITAL i.p. n = 6	CO_2 n = 6	CO_2 + INITIAL O_2 n = 6
Response until unconscious		-	Drowsiness Decreased respiratory rate.	Initial exploration, moderate hyperpnoea. Body stiffness and excitement before sudden loss of rightening reflex. Urination. Defecation	Explorative. Mild hyperpnoea. Mild shivering.
Handling/restraint stress		Short	Minimal	Minimal	Minimal
Induction of unconsciousness	Time	Instant	x = 80 sec (45-120)	x = 8 sec (6-10)	x = 13 sec (7-15)
	Stress response	-	Mild	Mild	Mild
Observations after induction of unconsciousness		Weak muscle fasciculations.	Gradually decreased respiratory rate, gasping, respiratory arrest.	Initial laboured breathing followed by dyspnoea with shallow, intermittent breaths. Convulsions.	Shallow, jerky breathing with gradually decreased respiratory rate.
Time for respiratory arrest		Instant	x = 482 sec ** (315-720)	x = 35 sec (32-38)	x = 130 sec (85-215)

**: Regular breathing stopped at x = 370 (270-495) sec.
x = mean value; range within brackets.

was judged minimal and limited to the moving of the animals from the holding cage to the perspex box. The stress response during induction was assessed as mild in guinea pigs and mice and as mild-moderate in rats. The level of stress response was estimated from the animals' reactions of uneasiness, laboured breathing, urination and defecation. These responses were limited to 10–20 seconds in mice and rats and up to 40 seconds in guinea pigs.

Carbon dioxide plus oxygen

Guinea pigs lost their righting reflex after an average of 29 seconds, rats and mice after 27 and 13 seconds, respectively.

Compared to pure CO_2, the adding of 20 % oxygen during the first minute of exposure did not change the time required for induction of unconsciousness in guinea pigs. This was in contrast to rats and mice, where the induction time approximately doubled. As assessed from the animals' responses to CO_2 exposure (Table 1–3), the animals appeared less distressed when oxygen was added.

Postmortem findings

The results of the necropsies are shown in Tables 4–6. It is obvious that the majority of the postmortem findings concern the circulatory system as shown by the distribution of blood and fluids, i.e. when the latter is not directly connected with the use of a physical method such as stunning or cervical dislocation.

Guinea pig (Table 4). The lung changes varied considerably with the killing method used. Blood and fodder aspiration was a common finding when the animals were stunned. The use of pentobarbital and CO_2 resulted in a lung emphysema, whereas CO_2 + O_2 produced a severe lung oedema and haemorrhages (Figure 1). A marked dilation of lymph vessels surrounding the hepatic arteries was found when pentobarbital and CO_2 were used but was absent when CO_2 + O_2 was used.

Rat (Table 5). Similarly in the rat the lung response to the different methods used varied considerably. Decapitation and pentobarbital resulted in emphysema while CO_2

Table 4. Post mortem findings in guinea pigs in connection with different killing methods.

		KILLING METHOD			
		Stunning	Pentobarbital i/p	CO_2	CO_2 + Initial O_2
MACROSCOPICAL FINDINGS		Epistaxis 3/6	Pale carcass 3/6	Pale carcass 6/6	Cyanotic carcass 6/6
		Traumatic lesions to head and brain 6/6	Fluid in abdominal cavity 6/6	Lung emphysema 2/6	Sanguineous fluid from nostrils 3/6
		Blood aspiration 5/6	Hyperemia of peritoneum 4/6	Lung emphysema and petechial haemorrhages and small hyperemic areas 4/6	Severe lung oedema and haemorrhages 6/6
		Lung emphysema 3/6	Lung oedema, emphysema and petechial haemorrhages 4/6	Lung oedema 3/6	Bilateral dilation of the heart 3/6
		Lung emphysema and petechial haemorrhages in lungs 3/6	Lung oedema 1/6	Bilateral dilation of the heart 6/6	Hyperaemic liver 5/6
			Hyperaemic kidney 3/6	Hyperaemic liver 4/6	Hyperaemic kidney 3/6
MICROSCOPICAL FINDINGS	Lung	Moderate to severe emphysema 5/6	Moderate to severe emphysema 2/6	Moderate emphysema with areas of collapsed alveoli 6/6	Very severe oedema and haemorrhages 6/6
		Moderate emphysema and moderate haemorrhages 1/6	Moderate emphysema in superficial parts of the lung 3/6	Contracted vessels and bronchi 6/6	Alveoli and bronchi filled with blood and oedema fluid 6/6
		Blood in alveoli and bronchi, blood aspiration 6/6	Focal areas of emphysema 1/6	Mild oedema, small amounts of blood in bronchi and alveoli 6/6	
		Contracted vessels and bronchi 2/6	Central parts mildly collapsed with mild to moderate hyperemia, oedema and haemorrhages 4/6	Mild to moderate perivascular oedema 4/6	
		Dilated vessels and bronchi 2/6	Small amounts of blood in occasional alveoli 1/6	Mild to moderate, focal and diffuse haemorrhages 6/6	
		Fodder aspiration 2/6	Contracted arteries and bronchi 6/6		
			Dilated veins 4/6		
	Brain	Randomly distributed bleedings of different size and extent in brain-tissue and meninges 6/6	No changes 6/6	No changes 6/6	Moderate hyperemia and occasional haemorrhages in brain and meninges
		Disrupted brain-tissue 6/6			
		Moderate perivascular oedema 6/6			
	Myocard	Uneven blood distribution 4/6	Uneven blood distribution 6/6	Dilated subepicardial vessels 6/6	Occasional degenerated muscle cells 5/6
		Small diffuse intramural bleedings 2/6	Several small intramural haemorrhages 5/6	Occasional degenerated muscle cells 3/6	Numerous degenerated muscle cells 1/6
		Dilated vessels 2/6	Small focal areas with muscle degeneration 5/6	General mild hyperaemia 1/6	Dilated subepicardial vessels 3/6
				Uneven blood distribution 1/6	

caused emphysema, oedema, and occasionally extravasation of blood. $CO_2 + O_2$, on the other hand, caused a marked hyperemia, oedema, and haemorrhages. In addition, free blood was present in alveoli and bronchi.

Mouse (Table 6). Cervical dislocation and pentobarbital produced only minor changes, while CO_2 produced a mild to moderate lung emphysema. $CO_2 + O_2$, on the other hand, caused oedema and haemorrhages.

Table 4 (cont.). Postmortem findings in guinea pigs.

		KILLING METHOD			
		Stunning	Pentobarbital i/p	CO_2	Initial $CO_2 + O_2$
MICROSCOPICAL FINDINGS	Kidney	Moderate hyperaemia in the cortex 1/6 Mild hyperaemia in the cortex 3/6 Uneven blood distribution 1/6	Moderate to severe hyperaemia in the cortex 6/6	Mild hyperaemia in the cortex 5/6	Moderate hyperaemia in the cortex 3/6 Mild hyperaemia in the cortex 2/6
	Liver	Mild contraction of arteries with mild dilatation of surrounding lymph vessels 1/6 Depleted of blood 4/6	Moderate to severe dilation of lymph vessels surrounding arteria hepatis 6/6 Mild to moderate acute stasis 4/6	Moderate to severe dilation of lymph vessels around arteria hepatis 5/6 Moderate contraction of arteries 5/6 Depleted of blood 2/6	Mild to moderate acute stasis 4/6 Focal haemorrhages and oedema 1/6
	Muscles	No changes 6/6	No changes 6/6	No changes 6/6	Occasional degenerated muscle cells 2/6
	Spleen	Mild hyperaemia 4/6	No changes 6/6	No changes 6/6	No changes 6/6
	Intestine	No changes 6/6	Mild-moderate dilation of vessels 5/6	No changes 6/6	No changes 6/6

All methods produced different degrees of hyperemia and haemorrhages in the renal cortex.

A mild – moderate hyperemia of the spleen, but no splenomegaly, was recorded in all animals killed by means of decapitation, pentobarbital, and CO_2, but was not present in animals killed with $CO_2 + O_2$.

Discussion

The method of killing to be chosen is dependent on whether or not the animal or parts of it are required for investigation after death and also on the kind of investigation in mind (*Iwarsson et al.* 1985b). An illustrative example of how common euthanasia methods including cervical dislocation, pentobarbital and CO_2 can influence certain immunological parameters, in mice, like mitogen induced lymphocyte proliferation, was reported by *Howard et al.* (1990). It is obvious that if animals are killed just to terminate their lives, any method could be used as long as it fullfils the definition of euthanasia given by *Green* (1979); "killing of an animal with a minimum of physical and mental suffering".

Physical methods

In the present investigation we studied the response and the postmortem changes in guinea pigs, rats and mice subjected to some routinely used methods of killing.

The three physical methods used in the present study were judged to be acceptable and are widely recommended. In guinea pigs, stunning has often been used prior to exsanguination. Stunning in guinea pig produced morphological changes mainly in brain tissue and respiratory organs. Blood and fodder aspiration is presumably connected with uncontrolled spinal cord mechanisms resulting in fits. It is obvious that stunning should not be used in guinea pigs utilized for investigations concerning respiratory organs and brain. In addition, stunning should not be used in connection with health monitoring, due to the obvious risk of microbiological contamination by fodder aspiration (Table 4).

In the rat, killing by means of stunning is reported to produce much lower catecholamine values than sacrifice by decapitation (*Sadjak et al.* 1983).

It is reported that decapitation releases a massive symphathetic neuronal discharge (spinal mechanisms activated by decapitation) and adrenal medullary secretion of catecholamines, including a hypovolaemic shock (*Depocas & Behreus* 1977, *Roizen et*

Table 5. Postmortem findings in rats in connection with different killing methods.

		KILLING METHOD			
		Decapitation	Pentobarbital i/p	CO_2	Initial CO_2 + O_2
MACROSCOPICAL FINDINGS		Mild lung haemorrhages 4/6 Oedema fluid and blood in bronchi 2/6 Haemorrhages in the tissues of the neck around cut surface 6/6	Mild-moderate hyperaemia of the peritoneum 4/6 Fluid mixed with blood in the abdominal cavity 4/6 Moderately hyperemic carcass 1/6 Moderate lung emphysema 6/6 Moderate-severe lung oedema 1/6	Moderately hyperemic carcass 2/6 Foci of cyanosis in the lung 4/6 Foci of lung emphysema 2/6	Moderately hyperemic carcass 1/6 Cyanotic lungs 3/6 Petechial lung haemorrhages 6/6 Hyperaemia 6/6
MICROSCOPICAL FINDINGS	Lung	Mild emphysema 1/6 Moderate-severe emphysema 5/6 Focal haemorrhages 4/6 Blood aspiration 5/6	Moderate emphysema 6/6 Moderately dilated vessels 4/6 Small amounts of blood in alveoli 3/6	Moderate emphysema and oedema 5/6 Foci of moderately collapsed alveoli 4/6 Moderate general emphysema 1/6 Blood in alveoli and bronchi 3/6	Moderate-severe hyperaemia, oedema and haemorrhages 6/6 Blood in alveoli and bronchi 6/6 Foci of emphysema 2/6
	Brain	Mild-severe hyperaemia and haemorrhages in the meninges 5/6 Bleedings in the brain 4/6 Dilated congested vasa vasorum of larger arteries 1/6	Moderate hyperaemia in the meninges 1/6	Mild-moderate hyperaemia in meninges 4/6 Occasional perivascular haemorrhages in brain 2/6	Moderate general hyperaemia in the meninges 1/6
	Myocard	Dilated subepicardial vessels 6/6 Occasional intramural bleedings 1/6 Occasional degenerated muscle cells 1/6	Moderate hyperaemia and dilated subepicardial vessels 5/6 Occasional intramural haemorrhages 3/6 Occasional degenerated muscle cells 4/6	General moderate-severe hyperaemia and dilated vessels 6/6 Mild-severe intramural haemorrhages 6/6 Foci of mild-moderate muscle degeneration 4/6	Occasional degenerated muscle cells 5/6 Moderate muscle cell-degeneration 1/6 Dilated subepicardial vessels 4/6
	Kidney	No change 6/6	Moderate hyperaemia in the cortex 6/6 Occasional small haemorrhages in the cortex 2/6	Mild-moderate hyperaemia in the cortex 6/6 Occasional small haemorrhages in the cortex 4/6	Moderate-severe hyperaemia in the cortex 6/6 A moderate number of small haemorrhages in the cortex 5/6
	Liver	No change 6/6	Mild acute stasis 1/6	Mild acute stasis in some lobuli 2/6	Mild acute stasis in some lobuli 4/6
	Muscles	No change 6/6	No change 6/6	No change 6/6	No change 6/6
	Spleen	Mild-moderate hyperaemia 4/6	Mild-moderate hyperaemia 6/6	Mild-moderate hyperaemia 6/6	No change 6/6
	Intestine	No change 6/6	No change 6/6	No change 6/6	No change 6/6

al. 1978). This effect is markedly potentiated if the animals have been subjected to stress before the decapitation, presumably as a result of a change in spinal cord mechanisms controlling the sympathetico-adrenal medullary activation (*Kvetnansky et al.* 1978).

The morphological changes in rats resulting from decapitation were, in spite of presumably increased catecholamine levels, rather restricted, most probably due to the rapid exsanguination. Alterations were restricted to the cut surfaces of the neck and brain and to the lungs. The brain changes were limited and of a circulatory nature and would in most instances not affect brain examination, while the lung changes (emphysema, hae-

Table 6. Postmortem findings in mice in connection with different killing methods.

		KILLING METHOD			
		Cervical dislocation	Pentobarbital i/p	CO_2	Initial CO_2 + O_2
MACROSCOPICAL FINDINGS		Bleedings around the cranium 6/6 Occasional petechial haemorrhages in the lung 1/6	Blood mixed fluid in the abdominal cavity 6/6 Mild-moderate hyperaemia in the peritoneum 6/6 Dilated peripheral vessels of carcass 6/6 Mild lung emphysema 6/6	Moderately hyperemic carcass 6/6 Mild-moderate lung emphysema 6/6	Markedly dilated peripheral vessels 6/6 Severe lung oedema and haemorrhages 6/6 Severe focal areas of lung emphysema 2/6
MICROSCOPICAL FINDINGS	Lung	Mildly dilated vessels 6/6 Mild haemorrhages 2/6	Mild dilatation of peripheral vessels 5/6 Mild, partly focal areas of emphysema 6/6	Mild-moderate lung emphysema 6/6 Dilated peripheral vessels 2/6	Severe bleedings. Rich amounts of blood in alveoli and bronchi 6/6 Severe oedema 6/6 Focal moderate emphysema 3/6
	Brain	Occasional small-large haemorrhages in the meninges 4/6 Occasional perivascular, mostly mild haemorrhages 2/6 Mild hyperaemia 2/6	Mild hyperaemia and small haemorrhages in the meninges 4/6 Occasional mild perivascular haemorrhages in brain tissue 2/6	Occasional minor haemorrhages in the meninges 1/6	Minor haemorrhages and hyperemia in the meninges and brain 2/6
	Myocard	Occasional mild haemorrhages 1/6 Occasional degenerated muscle cells 4/6	Occasional degenerated muscle cells 5/6	Moderately dilated vessels and occasional degenerated muscle cells 1/6	Moderately dilated vessels 4/6 A moderate amount of small intramural haemorrhages 1/6 Occasional degenerated muscle cells 5/6
	Kidney	Mild hyperaemia and occasional mild interstitial haemorrhages in the cortex 6/6	Mild-severe hyperaemia and interstitial haemorrhages in the cortex 6/6	Mild-severe hyperaemia and interstitial haemorrhages in the cortex 6/6	Moderate-severe hyperaemia and interstitial haemorrhages in the cortex 6/6
	Liver	No change 6/6	No change 6/6	No change 6/6	No change 6/6
	Spleen	No change 6/6	Moderate hyperaemia 1/6	Mild-moderate hyperaemia 2/6	Moderate hyperaemia 2/6
	Intestine	No change 6/6	Moderately dilated subserous vessels 2/6	No change 6/6	No change 6/6

morrhages and blood aspiration) may disguise other morphological changes and could affect microbiological investigations in connection with health monitoring (Table 5).

It has been recommended that, until additional information is available as to whether guillotined animals perceive pain and whether cervical dislocation is followed by immediate unconsciousness, animals to be killed by these techniques should be sedated or lightly anaesthetized prior to killing (*AVMA* 1986). It seems likely that the update by the AVMA Panel on Euthanasia (5th ed.) will classify stunning not followed by an additional method as an "unacceptable condition" (*Krulisch* 1992).

Cervical dislocation in mice produced mostly minor changes except for traumatic haemorrhages of the cervical region and in meninges. Notable findings were the generally slightly dilated lung vessels and the degenerative changes in heart muscle cells in 4 out of 6 animals (Table 6).

Intraperitoneal injection of pentobarbital sodium

Pentobarbital depresses catecholamine levels below normal, including the sympathetic neuronal and adrenal medullary discharge

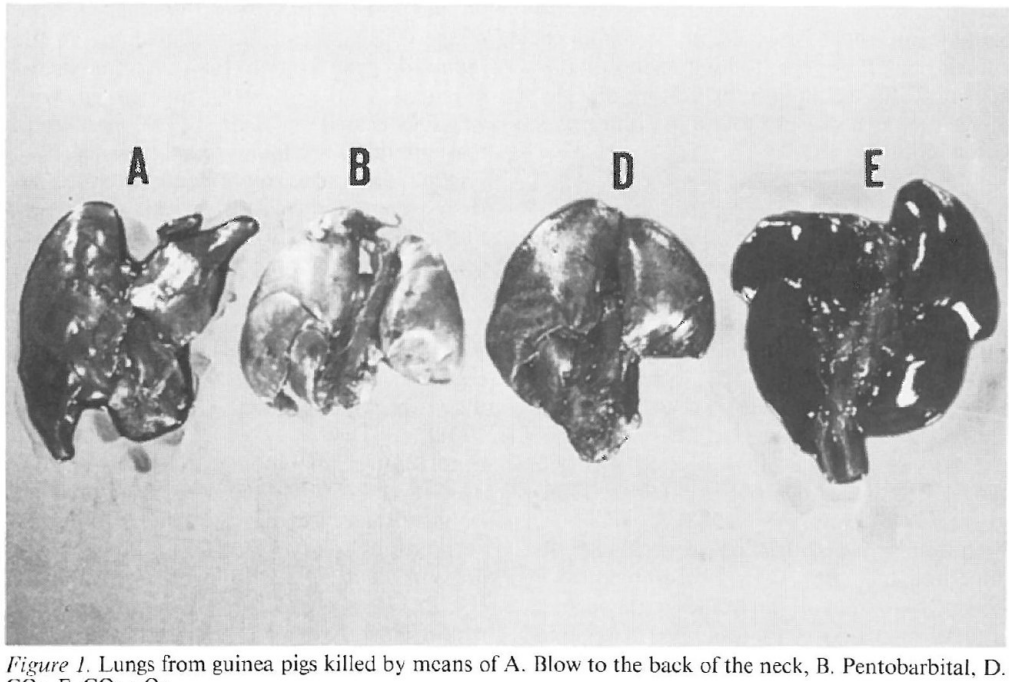

Figure 1. Lungs from guinea pigs killed by means of A. Blow to the back of the neck, B. Pentobarbital, D. CO_2, E. $CO_2 + O_2$.

(*Roizen et al.* 1978, *Sadjak et al.* 1983). In addition, pentobarbital depresses the CNS, respiration, O_2 uptake, and the mean systemic arterial systolic and central venous pressure, but elevates the partial pressure of CO_2 in arterial blood (*Green* 1979).

In studies on post-mortem changes in the rat lung, *von Messow et al.* (1987), compared 6 different killing methods and found the longest time proceeding to death to be 372 ± 95 sec. for i p pentobarbital (Nembutal) and the next longest, 144 ± 27 sec. for killing by overdosing with ether.

In the present study, intraperitoneal injection of an overdose of pentobarbital in all the three species resulted in the longest mean time for induction of unconsciousness and death: 80 sec. in mice, 152 sec. in rats, and 210 sec. in guinea pigs (Tables 1–3). However, soon after the injection, excitement and motor activity were suppressed and within minutes the course of narcosis deepened to profound unconsciousness and death.

In almost all animals there was sanguineous fluid in the abdominal cavity and congestion of the peritoneum. The lung changes found were limited in all 3 species and would probably not interfere with histological evaluation. Myocardial lesions, characterized by acute degenerative changes, probably due to circulatory failure and ischaemia, were a constant feature without species differences.

Notable also were the circulatory changes in the renal cortex of all species and the lack of splenomegaly in all animals. Marked splenomegaly is a common feature in many species, as a result of disturbances of the systemic and portal circulation (*Jubb & Kennedy* 1970).

A finding confined to guinea pigs (Table 4) was marked dilation of the lymph vessels surrounding the hepatic arteries. This was also found in the guinea pigs killed with CO_2

but to a much lesser extent, related to the contraction of the vessels of the stunned guinea pigs. It appears to be a lesion of guinea pigs affected mainly by respiratory hypoxaemia, as it was not found in guinea pigs killed with $CO_2 + O_2$.

CO_2

The rapid depressant and anaesthetic effects of CO_2 in concentrations of 40 % or higher are well established (*Green* 1979, *Woodbury et al.* 1958) and CO_2 is recommended for the euthanasia of adult, small laboratory animals (*Green* 1979, *CCAC* 1984, *AVMA* 1986).

Among inhalational agents, CO_2 is considered a more humane alternative to nitrogen gas (*Hornett & Haynes* 1984, *AVMA 1986) or ether (Blackshaw et al.* 1988).

It should be noted that, based on studies in mice and rats, *Britt* (1987) recommended a slower induction of anaesthesia through a gradual increase in CO_2 (in air) as compared to high concentrations.

The CO_2-chamber design used in the present investigation allowed precharging with CO_2, as recommended by *AVMA* (1986), to make the induction of unconsciousness as fast as possible. The time for loss of consciousness (judged as loss of the righting reflex) was on an average 13 seconds in rats and 10 seconds in mice and corresponds well with results reported by *Britt* (1987) in studies on CO_2 euthanasia of rats and mice and by *Fenwick & Blackshaw* (1989) using CO_2 as a short-term anaesthetic for rats.

The results obtained in this study (cf Tables 1–3), confirm reported signs of distress during the brief time of induction with high CO_2 concentrations (*Britt* 1987). Based on these results he recommended to use a slower induction of anaesthesia through a gradual increase of CO_2 (in air). A relatively slow introduction of CO_2 was also recommended by *Jaax* (1988), using a mobile carbon dioxide inhalation chamber, with resulting times for induction on unconsciousness of 40 seconds in rats and mice and one minute for guinea pigs.

As shown by *Britt* (1987) a gradual increase of the CO_2 concentration also increased the induction time to more than the double that obtained with a system precharged with CO_2. As stated by *Britt* (1987) neither of these methods are found to be stress-free.

Carbon dioxide is reported to produce effects on haemodynamics e.g. initial contraction and later on dilation of capillaries and veins, except for lung vessels, and also acidosis.

The gas also depresses the cerebral cortex but stimulates chemoreceptors, the mesencephalic reticular formation, and initially the respiratory center (*Woodbury et al.* 1958).

Extravasation of blood in the lung in connection with CO_2 killing is reported to occur in rats, mice, guinea pigs, and rabbits by *Feldman & Gupta* (1976), in rat by *von Messow et al.* (1987) and by *Fawell et al.* (1972). *Fawell et al.* (1972) also reported perivascular oedema as a constant finding in rat. In the present study we thought a mild (mice and guinea pigs) or a mild-moderate (rats) level of stress response ocurred before the quick onset of collapse. The signs of stress response recorded (cf. Tables 1–3) are probably to be attributed to a local irritation of the mucous membranes combined with the unnaturally deep respiration provoked by the CO_2. Thus, when using pure CO_2 for euthanasia, it is essential to use systems which allow precharging with the gas before and between animals in order to reduce the time of onset of effect.

In this investigation all three species developed lung emphysema.

Extravasation of blood was noted in the lungs of 4 out of 6 guinea pigs, while in 5 out of 6 rats a general lung oedema was present.

The lung changes, however, would not significantly influence histological investigation. The differences observed could be due to the mode and time of exposure to the gas. The above cited authors did not describe their technical devices, nor whether they were

precharged with CO_2 or whether the animals were initially exposed to a mixture of CO_2 and air.

Other notable findings were the marked dilation of myocardial vessels and the rather common degenerative changes of myocardial muscle cells seen in guinea pig and rat but rarely in mouse. The difference between species presumably depends on the time of exposure to the gas, and thus to the extent of acidosis and hypoxia.

Carbon dioxid + oxygen (CO_2 + O_2; 80/20)

Signs of longer and more obvious distress (restlessness, deep respiration, salivation, pawing at noses, tear flow) in rodents during CO_2 exposure in old, primitive systems, incompletely filled, or not precharged with CO_2, were our stimulus for adding oxygen, thereby relieving the discomfort of hypoxia during the induction of unconsciousness (*Iwarsson et al.* 1985b).

A CO_2 + O_2 gas mixture has been recommended as a nonexcitatory replacement for pure CO_2 in euthanasia systems for cats and other small animals (*Mac Arthur* 1978) as well as ether for anaesthesia/euthanasia in terminal toxicological studies (*Thuring et al.* 1983).

Mixtures of carbon dioxide and oxygen have been used by several scientists for induction of anaesthesia in mice, rats and guinea pigs. It was found, however, that hypertension, with increased venous return and an initial increase in respiratory rate and amplitude commonly occurred, leading to capillary bleeding (*Green* 1979).

Fenwick & Blackshaw (1989) concluded that CO_2 without O_2 was not a suitable short-anaesthetic for rats. Comparing CO_2-mixtures of 50:50 and 80:20 respectively, as a short-term anaesthetic in rats with subclinical respiratory disease, they recommended the 50:50 mixture.

In studies on rats, using a gas mixture equivalent to the one of the present study (CO_2/O_2 80:20), *Forslid et al.* (1986) demonstrated rapid transient effects upon the CNS, depressing afferent signal transmission in the nervous system, with the EEG changing into a slow wave pattern within 30 sec. and the sensory evoked potentials (SEP) amplitude decreased to almost zero within 45 sec. From these studies in rats *Forslid* (1987) concluded that the rapid loss of sensory neocortical response, together with the fact that EEG changes occurred typical for anaesthesia in man, meant that exposure to 80 % CO_2 + 20 % oxygen cause an anaesthetic state comparable to unconsciousness after about half a minute. The times for induction of unconsciousness in rats, reported in the present paper are in good agreement with the results reported by *Fenwick & Blackshaw* (1989) using a CO_2:O_2 mixture of identical proportions for induction of anaesthesia. The mean time period for the pedal reflex to disappear was on an average 30 seconds compared to a mean of 27 seconds reported for controlled loss of the righting reflex in the present study.

Pigs exposed to 68 % CO_2 + 32 % air showed an initial increase in arterial and venous blood pressure lasting around 2 minutes and then giving way to a marked decrease in heart rate, blood pressure and respiratory rate (*Mullenax & Dougherty* 1963).

It has also been shown that the blood O_2 content changes little in pigs given CO_2 + O_2 (68 resp. 32 %) but decreased markedly in those given CO_2 + air (68 resp. 32 %). Blood CO_2 content increased and pH decreased in both treatment groups (*Mullenax & Dougherty* 1963). It may be assumed that when the mixture of CO_2 and O_2 is used, hypoxia is minimized and that the direct and continuous depression of cerebral cortex activity, by CO_2, is the major factor leading to the death of the animal, while, when using pure CO_2, hypoxia has to be considered a very important factor. Hence the use of a mixture of CO_2 and O_2 will produce death principally due to the effect of CO_2.

These findings in combination with the subjectively noted less distressing animal situa-

tion recorded in the present study suggest initial CO_2 plus O_2 induction to be a recommendable method for routine euthanasia in rodents (Tables 1–3). In this conclusion, however, compatibility with the basic requirements for an adequate method of euthanasia listed in the introduction has been disregarded.

The adding of oxygen during the first minute did not increase the time for loss of the rightening reflex in guinea pigs, which is in contrast to the case of mice and rats. This may be due to species differences in sensitivity to CO_2 per se or to the induced hypoxia, acidosis, and hypercapnia.

A common feature in all three species investigated was the lung changes characterized by lung oedema and marked extravasation of blood. This was prominent in guinea pigs and to such an extent that in this species the lesions produced may easily conceal other lesions. These findings support the statement of *Green* (1979) that the use of a mixture of carbon dioxide and oxygen for anaesthesia should be limited to short periods, preferably less that 2 minutes. Possibly due to the prolonged exposure to CO_2 and the extent of acidosis. Myocardial muscle cell degeneration was a general feature in all three species. In two guinea pigs, skeletal muscle cell degeneration was also found. In general, guinea pigs appeared to be more sensitive to the direct effect of CO_2 than mice and rats.

Several factors causing stress, excluding the mode of euthanasia, may influence the physiological status before and during the killing procedure (*Gärtner et al.* 1980, *Sadjak et al.* 1983). To, as far as possible, avoid misinterpretations caused by the handling of the animals, all of them were exposed to the same careful treatment by the same person before the killing procedure. For the same reason the animals were individually killed in a separate room. The variations obtained within and between species are thus to be considered individual or species dependent differences in the response to the various killing methods used. It is important for scientific accuracy that researchers using laboratory animals recognize the lesions produced by different euthanasia methods.

It is apparent from the results obtained that the postmortem findings, in general and with few exceptions, are connected with effects on the circulatory system. Similar observations were reported by *Fawell et al.* (1972) and *Feldman & Gupta* (1976). It is also evident that different species of animals may respond differently when exposed to the same or a similar method. In addition it is clear that different killing methods produce different gross- and microscopic alterations.

Summary

Methods for euthanasia in laboratory animals should primarily be chosen with regard to animal welfare, safety of personnel and the purpose of the experiment. In the present study different killing techniques for guinea pigs, rats and mice were compared with regard to the animal response as well as post-mortem changes.

Stunning by a blow to the back of the neck (guinea pigs), decapitation with guillotine (rats) and cervical dislocation (mice) were judged to be followed by immediate unconsciousness rapidly followed by a cessation of breathing. If possible, animals should be sedated or lightly anaesthetized before euthanasia using a physical method. Physical methods induced local traumatic damage (neck, brain, meninges) as well as changes in the respiratory organs, especially the lungs (emphysema, bleeding, blood and fodder aspiration).

Intraperitoneal overdose of pentobarbital (150 mg/kg bw) was followed by a calm induction within 2–3 minutes and a cessation of breathing within 8–11 minutes, with considerable individual variation. Morphologically, acute degenerative lesions in myocardial muscle cells and circulatory changes in the kidney cortex as well as limited lung changes were demonstrated in all species.

Pure carbon dioxide in an equilibrated system induced unconsciousness within 10–20 sec. in rats and mice and within 40 sec. in guinea pigs, followed by rapid death. Rats especially showed a moderate uneasiness during the induction. All species developed lung emphysema while myocardial cell changes and extravasation to alveoli were found in guinea pigs and rats.

Induction with CO_2/O_2 (80:20) for 1 minute followed by pure CO_2 was judged to be the most humane method in all species from the animal welfare point of view. By adding oxygen the time for induction of unconsciousness was doubled in

rats and mice but not much changed in guinea pigs. Breathing ceased within 4 min in rats and mice and within 7 min in guinea pigs. In all species this method induced lung oedema and considerable extravasation to alveoli. This method cannot be recommended for studies including morphological investigations of lungs.
From a strict animal welfare point of view the CO_2/O_2-method is the most recommendable of the methods studied, followed by the pure CO_2-method and next pentobarbital i/p. The equipment for inhalation euthanasia should be equilibrated with the actual gas or gas mixture before introduction of the animals. From the animal welfare point of view it is clear that the handling of the animals and technical efficiency of the person in charge are crucial factors for a good result. Ethically, all euthanasia techniques call for properly trained personnel and knowledge about post-mortem changes for an optimal scientific outcome.

Sammanfattning
Avlivningsmetoder för försöksdjur skall primärt anpassas till djurskydd, personalsäkerhet och försöksmotiv.
I föreliggande studie jämfördes olika avlivningsmetoder för marsvin, råtta och mus med avseende på djurens reaktion och postmortala förändringar. Nackslag (marsvin), dekapitering med giljotin (råtta) och cervikal dislocering (mus) bedömdes leda till momentan medvetslöshet och snabbt åtföljande andningsstillestånd. De fysiska metoderna medförde lokala traumatiska skador (hals, hjärna, hjärnhinnor) jämte förändringar i respirationsorganen, speciellt i lungorna (emfysem, blödningar, blod- och foderaspiration).
Intraperitoneal överdos av pentobarbital (150 mg/kg) medförde hos alla djurslag lugnt insomnande inom i medeltal 2–3 min och upphörd andning inom 8–11 min, med avsevärd individuell variation. Morfologiskt påvisades akuta, degenerativa myokardskador och cirkulationsrubbningar i njurbark jämte begränsade lungförändringar hos samtliga species.
Ren koldioxid i ekvilibreret system medförde medvetslöshet inom 10–20 sek (råtta och mus) respektive 40 sek (marsvin) och snabbt åtföljande död. Speciellt hos råtta noterades viss oro under induktionstiden. Samtliga djurslag utvecklade lungemfysem medan alveolärt vätskeutträde och myokardförändringar enbart påvisades hos marsvin och råtta.
Induktion med CO_2/O_2 (80:20) under 1 minut följt av ren CO_2, bedömdes ur djurskyddssynpunkt som den skonsammaste metoden för samtliga djurslag. Inblandningen av oxygen förlängde anslagstiden för medvetlöshet hos råtta och mus men ej hos marsvin. Andningen upphörde inom 4 min hos råtta och mus och inom 7 min hos marsvin. Metoden medförde hos samtliga species lungödem och höggradigt vätskeutträde i alveoli. Metoden kan f.f.a. hos marsvin ej rekommenderas i studier inkluderande morfologisk undersökning av lunga.
Av de undersökta avlivningsmetoderna rekommenderas ur strikt djurskyddssynpunkt CO_2/O_2-metoden, därnäst ren CO_2 samt pentobarbital i/p. Utrustning för avlivning genom inhalation bör vara ekvilibrerad med den aktuella gasblandningen innan djuren exponeras.
Djurskyddsmässigt är det uppenbart att djurens behandling och den tekniska skickligheten hos eksekutorn spelar en avgörande roll för ett gott resultat. Ur etisk synvinkel kräver alla avlivningsmetoder utbildad personal och kännedom om postmortala förändringars betydelse för forskningsuppgiften i fråga.

Yhteenveto / K. Pelkonen
Koe-eläinten eutanasiamenetelmät pitää valita erityisesti huomioiden eläinsuojelunäkökohta, henkilökunnan turvallisuus ja kokeen tarkoitus. Tässä tutkimuksessa vertailtiin marsun, rotan ja hiiren eutanasiamenetelmiä ottaen huomioon eläimen reaktiot ja kuolemanjälkeiset muutokset.
Voimakas isku marsun takaraivoon, rotan pään katkaisu giljotiinilla ja hiiren niskavenytys; näiden arvioitiin aiheuttavan välittömän tajunnanmenetyksen ja nopean hengityksen loppumisen. Mikäli mahdollista., eläimet tulee rauhoittaa tai kevyesti nukuttaa ennen mekaanista eutanasiaa. Fysikaaliset menetelmät aiheuttivat paikallisia kudosvaurioita (niska, aivot, aivokalvot) ja muutoksia hengityselimissä, erityisesti keuhkoissa (ilmapöhö, verenvuodot, verta ja rehujäänteitä keuhkoissa).
Vatsaontelonsisäinen pentobarbitaalin yliannostus (150 mg/kg) aiheutti rauhoittumisen 2–3 minuutissa ja hengityspysähdyksen 8–11 minuutissa. Yksilöiden välillä oli huomattavia eroja. Kaikilla kolmella lajilla oli havaittsvissa akuutteja degeneratiivisia sydänlihasvaurioita ja verenkiertomuutoksia lisämunuaiskuoressa samoin kuin muutoksia keuhkoissa.
Puhdas hiilidioksidi aiheutti rotilla ja hiirillä tajunnanmenetyksen 10–20 sekunnissa ja marsilla n. 40 sekunnissa, jota seurasi nopea kuolema. Erityisesti rotat olivat levottomia. Kaikilla lajeilla havaittiin keuhkopöhöä ja rotissa ja marsuissa muutoksia sydämen lihassoluissa ja verenpurkaumia keuhkorakkuloihin.
Kaikissa lajeissa vaikutti eläinsuojelullisesti humaanimmalta, kun annettiin minuutin ajan ensin hiilidioksidi.happiseosta (80:20) ja tämän jälkeen puhdasta hiilidioksidia. Happilisä kaksinkertaisti nukahtamisajan rotissa ja hiirissä, mutta ei paljon vaikuttanut marsuissa. Rotilla ja hiirillä hengitys pysähtyi 4 minuutissa ja marsuilla 7 minuutissa. Kaikilla lajeilla menetelmä aiheutti keuhkoturvotusta ja merkittävästi verenpurkautumia keuhko-

rakkuloihin. Tätä menetelmää ei voi suositella tutkimuksiin, jossa on osana keuhkojen morfologian tutkiminen.

Puhtaasti eläinsuojelulliselta kannalta hiilidioksidi-happi -menetelmä on suositeltavin tässä tutkituista, seuraavaksi puhdas hiilidioksidi ja seuraavaksi pentobarbitaali i.p. Inhalaatioeutanasialaitteisto pitää ensin täyttää kaasulla ennen eläinten altistusta. Eläinten käsittelijöiden taidolla on keskeinen merkitys hyvän tuloksen kannalta. Ectitisesti olennaista on, että eutanasian suorittaa teknisesti osaava henkilökunta ja menetelmän aiheuttamat kuolemanjälkeiset muutokset ovat tiedossa tieteellisesti oikeiden päätelmien tekemiseksi.

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Added in proof

After this paper was accepted for publication two important documents on laboratory animal euthanasia have been published:

AVMA, American Veterinary Medical Association Panel of Euthanasia. 1993 Report of the AVMA Panel on Euthanasia. J. A. V. M. A. 1993, *20,* 229–249.

Commission of the European Communities. Recommendations for Euthanasia of Experimental Animals. EEC DG XI/A/2. Final Report, May 1993, 84 pp.