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Evaluation of a novel rodenticide: welfare assessment of fatal methaemoglobinaemia in adult rats (Rattus norvegicus)

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Abstract

*This study assessed the welfare of rats (*Rattus norvegicus*) poisoned with a lethal dose of the methaemoglobin (MetHb) inducing compound* para*-aminovalerophenone (PAVP). Twenty rats were orally gavaged with either PAVP (treated) or the vehicle only (control). Spontaneous and evoked behaviours were recorded and blood samples collected post mortem for analysis of MetHb%. Female and male rats received a mean (± SEM) dose of 263 (± 3) and 199 (± 6) mg PAVP kg^{-1}, respectively. Mean (± SEM) time to death was 67 (± 16) and 354 (± 71) min for female and male rats, respectively. Control animals did not show any signs of intoxication. The time to death from methaemoglobinaemia in rats was significantly shorter than that reported for anticoagulants and there were no obvious signs of distress or pain.*

Keywords: *animal welfare, death, hypoxaemia, methaemoglobin (MetHb), methaemoglobinaemia, rat*

Introduction

Rodenticides are the predominant method of controlling rodent pests, such as rats and mice. Following the withdrawal of several rodenticide products from the UK market, there is now more reliance on anticoagulants. While often very effective (PSD 1997; Mason & Littin 2003), there is widespread concern about the humaneness of anticoagulant poisons (PSD 1997; Littin *et al* 2000; Mason & Littin 2003; Gregory 2004a; Fisher *et al* 2010) and the risk that second-generation anticoagulants, in particular, pose to non-target species through greater persistence and through primary and secondary poisoning routes (Eason *et al* 2002; Sanchez-Barbudo *et al* 2012; Langford *et al* 2013). Specific welfare issues associated with anticoagulant intoxication are: the prolonged time to death, dehydration, pain associated with bleeding in joints and other enclosed spaces within the body, haemorrhage in the respiratory tract and associated laboured breathing, episodic struggling, reduced activity, effects on general condition and paralysis (PSD 1997; Littin *et al* 2000). The mean time from ingestion of a second-generation anticoagulant (brodifacoum) to the beginning of clinical signs of toxicosis (reduced feed intake) has been reported as four days in rats (Littin *et al* 2000), and the period from the onset of clinical signs to death is between three to four days (PSD 1997; Littin *et al* 2000). It has also been reported in electroencephalographic (Rowsell *et al* 1979) and behavioural (PSD 1997; Littin *et al* 2000) studies that generally the animals remain conscious and responsive during the sickness period until immediately before death.

Novel vertebrate pesticides are now being developed, with the aim of improving humaneness and minimising the risk to non-target species without compromising efficacy. Methaemoglobin-inducing compounds have been evaluated as vertebrate pesticides for the control of feral pigs (*Sus scrofa*) (Anon 2010), stoats (*Mustela erminea*) (Fisher *et al* 2005; Eason *et al* 2010; Dilks *et al* 2011), ferrets (*M. furo*) (Fisher & O'Connor 2007), brushtail possums (*Trichosurus vulpecula*) (Fisher *et al* 2008) and feral cats (*Felis catus*) (Murphy *et al* 2007). In mammalian species, these compounds target red blood cells and induce the formation of methaemoglobin (MetHb), which reduces the capacity of blood to carry oxygen to tissues causing hypoxia and respiratory depression leading to death over a shorter time-period than anticoagulant agents (Eason *et al* 2014). The mode of action of MetHb inducers is the oxidation of the haem iron in red blood cells from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}) to form MetHb (Rennison *et al* 2013). However, rodents appear to have a high MetHb reductase activity after treatment with sodium nitrite (Stolk & Smith 1966) or *para*-aminopropiophenone (PAPP) (Scawin *et al* 1984), and this could reduce the effectiveness of those pesticides in this group of animals. More recently, analogues of PAPP have shown promise during *in vitro* and *in vivo* testing as being more toxic than PAPP in rodents. Of these analogues, *para*-aminovalerophenone (PAVP) has so far been found to have the highest toxicity *in vivo* with reported oral LD50 values of 84 (CI 56–126) mg kg^{-1} (Pan *et al* 1983) and 85 (± 31) mg kg^{-1} (Rennison *et al* 2013) in

rats. To date, there has been no comprehensive assessment of the welfare of rodents poisoned with MetHb-forming compounds, such as PAPP or PAVP.

The aim of this study was to examine the behaviours and welfare of adult laboratory rats (*Rattus norvegicus*) orally gavaged with a lethal dose of the MetHb-inducing compound, PAVP. The laboratory rat was used as a model for the wild rat.

Materials and methods

All procedures were carried out in the same laboratory in the United Kingdom (UK) under the provisions of the Animals (Scientific Procedures) Act 1986 and with the approval of the institute's Animal Welfare and Ethical Review Board. Wistar (*R. norvegicus*) rats were obtained from a commercial supplier. Prior to experimentation, rats were acclimatised to the laboratory conditions for three weeks. They were housed in groups of three in colony cages (45 × 28 × 13 cm; length × width × height) in an automatically temperature-controlled room, and kept under a 12:12 h, light:dark cycle with white lighting. Water and feed were available *ad libitum* during the acclimatisation period. All rats were fasted overnight prior to dosing. The active compound PAVP used in the study was synthesised at the University of Auckland, New Zealand, and purity was confirmed by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR).

Twenty rats (ten female, ten male) were randomly selected and dosed with either PAVP (treated) or the vehicle only (control). Animals were matched for dosing time and sex. The numbers were based on the minimum required to get a representative data set for male and female rats, while allowing for individual animal variation. There were no existing data on the welfare of methaemoglobinaemia in rats to allow the performance of a power analysis for sample size. The mean (± SEM) bodyweight of control and treated female rats was 194 (± 5) and 190 (± 2) g, respectively, while the mean (± SEM) weight of the male rats was 248 (± 7) and 253 (± 8) g for controls and treated animals, respectively. All rats were gavaged unanaesthetised and without sedation. Treated rats were given a set dose 1 ml of 50 mg ml^{-1} PAVP via oral gavage, dissolved in a 9:1 mixture of polyethylene glycol 200 (PEG) and triethanolamine (TEA) (PEG:TEA). Treated female (n = 5) and male (n = 5) rats received a mean dose of 263 (± 3) and 199 (± 6) mg kg^{-1} PAVP, respectively. Matched controls (female five; male five) received the vehicle only.

Post-dosing, rats were immediately placed in individual wire cages (35 × 28 × 25 cm) and observed under white lighting during the normal 12-h light period. After dosing, the time to onset of behavioural toxicosis, the duration and frequency of abnormal behaviours (spontaneous and evoked), changes in posture, and the time to unconsciousness and death were sequentially recorded every 10 min for the first 2 h and then every 30 min thereafter. Table 1 describes the behaviours (spontaneous and evoked) and signs of methaemoglobinaemia evaluated in PAVP-treated and control rats. Spontaneous behaviours and external signs of methaemoglobinaemia were recorded for a 1-min period for each animal using continuous focal sampling. The observed signs and behaviours included: repositioning; drinking; exploratory behaviour; grooming; cyanosis of the feet and nose; pale ears; dark eyes; whisker-twitching; recumbency; resting of the head against structures; lethargy; ataxia and respiration rate. Evoked behaviours were tested after the recording of the spontaneous behaviour of each animal. When an animal gave an overt physical response to a stimulus it was recorded as a positive reaction. The following stimuli or responses were assessed separately: ear, foot, and tail pinches applied using finger and thumb nails; righting reflex and response to handling, tested only when the animal was prostrate in sternal or lateral recumbency; corneal reflex; air being blown down a metal tube onto the face or body; cleat response, stimulation with a fingernail between two digits on a hind limb. Death was determined by the loss of the corneal reflex, plus absence of rhythmic breathing and heart beat as assessed by palpation of the chest. Immediately post mortem, blood was collected via cardiac puncture in pre-heparinised (Multiparin, Heparin Sodium, CP Pharmaceuticals Ltd, Wrexham, UK) syringes (0.1 ml, 500 IU ml^{-1}). As treated animals died, a corresponding matched control was dispatched by cervical neck dislocation. Death as an endpoint is avoided where possible under UK regulations on animal experimentation. However, in this study it was necessary because: (i) the welfare of fatal methaemoglobinaemia had yet to be assessed in rodents; (ii) it was unknown which signs would be reliable indicators of pending mortality; and (iii) it was important to document any signs of severe suffering and to determine if these occurred in fatally or sub-lethally dosed animals.

Immediately post mortem, blood samples were collected to assess circulating total haemoglobin (tHb) g dL^{-1}, oxyhaemoglobin (O_2Hb)%, carboxyhaemoglobin (COHb)%, and methaemoglobin (MetHb)% (GEM OPL CO-oximeter, Instrumentation Laboratories Ltd, Warrington, UK). Three replicate readings were taken, with the mean taken as the value. Post mortem examinations were conducted on each animal to assess gross pathological signs of methaemoglobinaemia. Specific regions and tissues examined included: nose, lips, ears, mucous mucosa, eyes, tail/feet, diaphragm, trachea, lungs, oesophagus, stomach, pericardium/myocardium, blood, spleen, liver, bladder, flank and skeletal muscle. The colour of tissues post mortem was assessed using the *Methuen Handbook of Colour* (Kornerup & Wanscher 1978). Blood collected from the abdominal cavity (1 ml) was placed on white filter paper and assessed for colour. All colour assessments were conducted under yellow fluorescent lighting.

As there were differences in spontaneous and evoked behaviours between the sexes, each sex was analysed separately. However, the frequency of behavioural episodes was pooled for the two sexes due to the low number of observations. Where appropriate, data were analysed using 6.0 Prism (GraphPad Software

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Table 1 The behaviours (spontaneous and evoked) and external signs of methaemoglobinaemia evaluated in PAVP-treated and control rats.

Behaviours/signs	Description
Spontaneous behaviours and external signs of methaemoglobinaemia	
Drinking	Animal raises head to lick water from water bottle
Whisker-twitching	Spontaneous whisker movements
Grooming	Scratching, wiping or licking the fur, face, ears, limbs, genitals or tail
Repositioning	Adjustment or shifting of bodyweight, limbs or head position
Exploratory/active behaviour	Inquisitive movements of the head and/or body including normal locomotion (non-escape), investigation of the environment, standing on hind legs, climbing and sniffing of the surroundings
Sleep-like posture	Curled posture with head and limbs tucked into the abdomen, eyes closed
Cyanosis of the feet and nose	Bluish colouration of the skin of the feet and mucous membranes of the nose
Pale ears	Pale colouration of the ears
Dark eyes	Darkening of the eyes and surrounding mucosa
Recumbency	The animal ceases to support weight on all four feet and exhibits either lateral, dorsal or sternal recumbency
Lethargy	Sluggish activity or unresponsiveness
Ataxia	Gait instability and inco-ordination of muscle movements
Head resting against cage	Loss of neck muscle tone with resting of the head against structures (often associated with recumbency)
Respiration rate	The number of breaths taken per minute
Evoked behaviours	
Loss of reaction to blowing on body/face	Air blown down a metal tube onto the face or body to provoke a movement response
Ear, foot and tail pinch	Movement or vocalisation in response to a noxious pinch of the ear, tail or foot. Applied using finger and thumbnail, without causing tissue damage
Righting reflex*	Animal manually placed in dorsal recumbency, with the effort to regain sternal recumbency assessed. Effort includes movement of the legs, head and torso
Response to handling*	Physical movement or repositioning in response to handling
Corneal reflex	Involuntary blinking of the eyelids in response to stimulation of the cornea
Cleat response	Stimulation with a fingernail of the skin between two digits on a hind limb, evokes a kick response
Heart beat	Assessed by palpation of the chest
Death	Determined by the loss of the corneal reflex, plus absence of rhythmic breathing and heart beat as assessed by palpation of the chest

* Tested only when the animals was prostrate in sternal or lateral recumbency.

Incorporated, San Diego, CA, USA) and 20.0 SPSS (IBM Corporation, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to determine the distribution of the data. The number of behavioural episodes recorded in the control and treated groups were compared with an unpaired *t*-test with Welch's correction. Blood-based measures were normally distributed and were analysed with a two-way ANOVA. Comparisons between control and treated values were made using the bonferroni *post hoc* test. Associations between treatment and colour of tissues and organs post mortem were made with Fisher's exact test. The level of significance for all tests was $P < 0.05$.

Results

All ten rats treated with PAVP died and the mean (± SEM) time to death was 67 (± 16) and 354 (± 71) min for female and male rats, respectively. Control animals did not show any signs of intoxication and were all dispatched by cervical neck dislocation.

The signs of methaemoglobinaemia followed a general cascade (Tables 2 and 3). In the first phase after dosing, rats treated with PAVP showed signs of cyanosis of the hind feet and nose, the ears became pale, and whisker-twitching stopped or was reduced. This phase lasted, on average,

Table 2 General sequence of behaviours and loss of physical responses in female and male rats gavaged with PAVP.

Time to:	Female (min)			Males (min)		
	Mean (± SEM)	Range	n	Mean (± SEM)	Range	n
Onset of eyes becoming dark	11 (± 3)	4–20	5	11 (± 2)	7–15	5
Onset of recumbency	13 (± 3)	4–20	5	12 (± 2)	10–20	5
Hind feet becoming blue	23 (± 4)	10–35	5	22 (± 11)	7–65	5
Onsert of ataxia	12 (± 8)	4–20	2	33 (± 9)	20–60	4
Loss of reaction to blowing on body/face	27 (± 9)	10–40	3	245 (± 71)	45–395	5
Loss of response to tail pinch	62 (± 18)	20–120	5	231 (± 41)	180–395	5
Loss of response to ear pinch	59 (± 16)	20–105	5	267 (± 48)	180–395	5
Marked fall in respiration rate	67 (± 20)	35–105	3	286 (± 66)	165–420	4
Loss of response to handling	72 (± 19)	38–120	4	292 (± 66)	180–428	4
Loss of righting reflex	59 (± 16)	20–105	5	345 (± 74)	180–566	5
Loss of response to cleat pinch	65 (± 20)	44–105	3	333 (± 70)	180–566	5
Loss of corneal reflex	52 (± 18)	20–105	4	347 (± 73)	180–566	5
Death	67 (± 16)	38–120	5	354 (± 71)	192–566	5

Table 3 Mean (± SEM) number of behaviour episodes during the recording periods. Data for female and male rats were pooled due to the low number of observations.

Factor	Treated	Control	*P*-value
Mean (± SEM) number of repositioning episodes	2.4 (± 0.6)	0.1 (± 0.1)	0.001
Mean (± SEM) number of unco-ordinated movements (ataxia)	1.9 (± 0.7)	0.0 (± 0.0)	0.05
Mean (± SEM) number of drinking episodes	0.0 (± 0.0)	0.7 (± 0.3)	0.01
Mean (± SEM) number of active behaviour episodes*	0.2 (± 0.2)	3.1 (± 0.7)	0.001
Mean (± SEM) number of grooming episodes	0.0 (± 0.0)	1.2 (± 0.3)	0.01
Mean (± SEM) number of times observed with head resting against the cage#	6.6 (± 2.1)	0.0 (± 0.0)	0.01
Time spent in sleep-like posture (min)	0 (± 0)	97 (± 28)	0.01

* Including climbing and general exploratory behaviour;
# Including the resting of the head against the vertical bars of the feeder.

Table 4 Mean (± SEM) percentage of circulating of total haemoglobin (tHb) g dL^{-1}, oxyhaemoglobin (O_2Hb)%, carboxyhaemoglobin (COHb)%, and methaemoglobin (MetHb)%, at death in treated and control female and male rats.

Factor	Female			Males		
	Control mean (± SEM)	Treated mean (± SEM)	*P*-value	Control mean (± SEM)	Treated mean (± SEM)	*P*-value
MetHB (%)	0.0 (± 0.0)	76.0 (± 1.9)	< 0.001	0.0 (± 0.0)	74.0 (± 3.1)	< 0.001
tHb (g dL^{-1})	13.5 (± 1.1)	11.8 (± 1.4)	ns	13.0 (± 1.3)	16.1 (± 0.8)	ns
O_2Hb (%)	25.9 (± 5.0)	13.9 (± 3.5)	ns	34.4 (± 10.8)	6.7 (± 2.1)	< 0.05
COHb (%)	0.4 (± 0.2)	5.2 (± 1.2)	< 0.001	1.2 (± 0.5)	5.4 (± 0.3)	< 0.01

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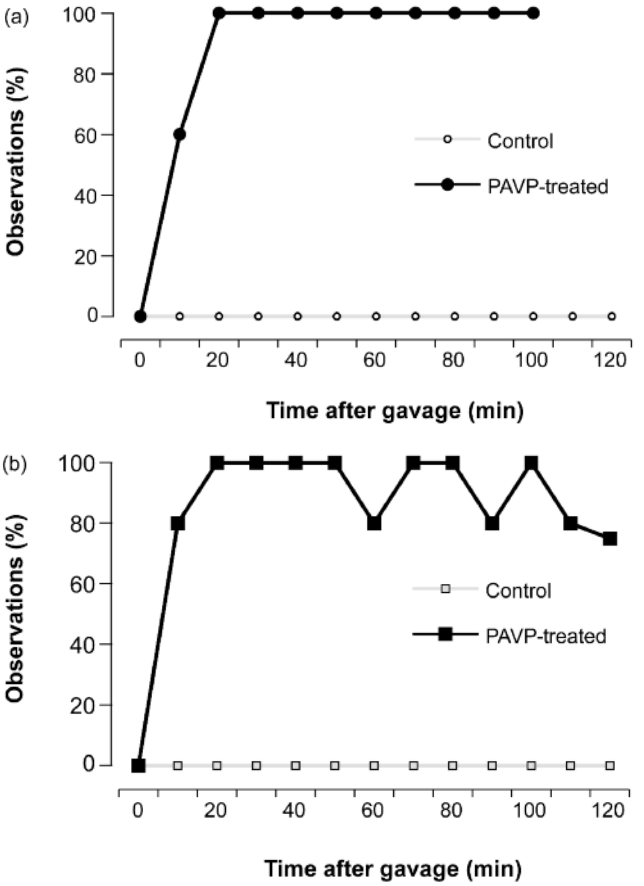
23 (± 4) and 22 (± 11) min for female and male rats, respectively (Table 2). During the second phase the animals became lethargic (often in a prostrate posture) with slowed body movements. This was followed by 60% of treated rats (female n = 2; male n = 4) displaying signs of ataxia after 12 (± 8) and 33 (± 9) min post-dosing for female and male rats, respectively. The animals then became unresponsive to air being blown on the back or face (female 27 [± 9]; male 245 [± 71] min), followed by no response to a manual tail (female 62 [± 18]; male 231 [± 41] min) or ear pinch (female 59 [± 16]; male 267 [± 48] min). When animals were close to death they became unresponsive to handling (female 72 [± 19]; male 292 [± 66] min) and lost their righting reflex (female 59 [± 16]; male 345 [± 74] min). At this point many of the rats went into lateral recumbency. Immediately prior to death the corneal reflex was absent (female 52 [± 18]; male 347 [± 73] min). Seven rats (70%) showed marked hypopnoea with the respiration rate dropping below 48 breaths per min (normal respiration rate was 85–110 breaths per min). Prior to this, there were no obvious signs of dyspnoea or tachypnoea. Control animals did not show any signs of intoxication. In both the control and treated animals there were no signs of excessive salivation or vocalisation.

Between dosing and death, PAVP-treated rats had a significantly higher frequency of recorded repositioning episodes (treated 2.4 [± 0.6]; control 0.1 [± 0.1]; $P < 0.001$), uncoordinated movements (ataxic episodes) (treated 1.9 [± 0.7]; control 0.0 [± 0.0], $P < 0.05$) and significantly ($P < 0.01$) more episodes of supporting the head against the cage wall (6.6 [± 2.1]) compared with the control group (0.0 [± 0.0]) (Table 3). No rats treated with PAVP were observed either drinking or grooming during the recording periods, unlike the control animals. Furthermore, no rats in the treated group assumed a sleep-like posture, while the control animals spent, on average, 97 [± 28] min in this position ($P < 0.01$).

Female and male rats treated with PAVP became inactive and spent increasingly more time in sternal or lateral recumbency compared with matched controls (Figure 1). After 10 min, 60 and 80% of female and male rats, respectively, were observed as recumbent (Figure 1[a] and 1[b]). Twenty minutes after dosing, all females were observed as recumbent and they remained in this position until death, while some male rats had brief periods of activity involving repositioning (Figure 1[b]).

Post mortem, the blood of all treated rats was dark brown in colour with MetHb levels of 76 (± 1.9) and 74 (±3.1)% in female and male rats, respectively, control animals had no detectable levels of MetHb 0% ($P < 0.001$) (Table 4). There was no significant difference in tHb between the female or male rats and their controls. The % of O_2Hb was significantly different in treated male rats (6.7 [± 2.1]%) compared with their matched controls (34.4 [± 10.8]%) ($P < 0.05$), whereas the difference between treated and control female rats was not significant. The percentage of COHb was significantly higher in treated female (treated 5.2 [± 1.2]; control 0.4 [± 0.2]; $P < 0.001$) and male rats (treated 1.2 [± 0.5]; control 5.4 [± 0.3]; $P < 0.01$) compared with their matched controls.

Figure 1



Percentage of observations where (a) female or (b) male rats were in sternal or lateral recumbency after gavage with 200 mg kg^{-1} PAVP in PEG:TEA (PAVP-treated) or PEG:TEA alone (untreated). Treated female ● and control ○ rats; treated male ■ and control □ rats.

All animals underwent pathological examination immediately following death (treated) or cervical dislocation (control). In both groups, there were no signs that the dosing material was aspirated into the trachea or lungs. Specific tissues were examined for changes in gross pathology and colour. The tongue, oesophagus, trachea, liver, spleen, diaphragm, and bladder showed no differences in pathology or colour between the treatment groups. Also, there were no obvious signs of infectious disease in any of the bodies (treated or control). There was a significant association between the colour of the hind feet ($P < 0.001$), blood ($P < 0.001$), myocardium ($P < 0.001$), flank ($P < 0.01$) and skeletal muscle ($P < 0.01$) and treatment group. Table 5 displays the recorded tissue colours, post mortem, for the PAVP-treated and control groups (data from females and males pooled). The skin of the feet of the rats that received PAVP (n = 10) was bluish grey (Methuen range 20B2–21C2) in colour compared with reddish grey (Methuen range 8C2–10B3) in the control group (n = 10). All rats in the treated group had blood that was reddish brown (Methuen range 7E5–9F6), while the control group

Table 5 Colour of tissues post mortem using the Methuen colour-coding scheme. Only data from tissues that showed a significant association between treatment and colour are displayed.

	Treated	Control	*P*-value
Hind feet colour	Bluish grey (10)	Reddish grey (10)	< 0.001
Blood colour	Reddish brown (10)	Dark red (9) Red (1)*	< 0.001
Myocardium	Reddish brown (8) Violet brown (2)	Violet brown (10)	< 0.001
Flank muscle	Light brown (6) Reddish brown (2) Reddish grey (2)	Dark red (1) Reddish grey (9)	< 0.01
Skeletal muscle	Light brown (3) Reddish brown (6) Reddish grey (1)	Reddish grey (8) Reddish brown (2)	< 0.01

* Note this sample had to be reanalysed 24 h post mortem.

was either classified as dark red (n = 9) (Methuen range 10C8–10D8) or red (n = 1) (Methuen colour 9B7) in colour. The myocardium of the treated or control rats was reddish brown (Methuen range 8F5–9F6) or violet brown (Methuen range 10E6–10F6) in colour, respectively. The flank muscles of treated rats were light brown (n = 6) (Methuen range 7C5–7D5), reddish brown (n = 2) (Methuen range 8D4–8D5) or reddish grey (n = 2) (Methuen colour 8C4), compared to reddish grey (n = 9) (Methuen range 8C4–9C5) and dark red (n = 1) (Methuen colour 9C6) in the control group. Skeletal muscle in treated animals was reddish brown (n = 6) (Methuen range 8D4–8E5), light brown (n = 3) (Methuen range 7C3–7D4), or reddish grey (n = 1) (Methuen colour 8C3) in colour compared with reddish grey (n = 8) (Methuen range 9B4–9C5) and reddish brown (n = 2) (Methuen range 9D5–9D6) in the control group.

Discussion

The toxic accumulation of MetHb in the bloodstream reduces the capacity of blood to carry oxygen to tissues and the central nervous system (CNS), resulting in hypoxia (generalised and cerebral), CNS and respiratory depression, leading to death at toxic concentrations. From an animal welfare perspective, the rapid induction of cerebral hypoxia and depression of CNS function leading to unconsciousness and death should minimise the potential for distress and suffering compared with anticoagulant agents. The MetHb-inducing agent PAPP has already been registered in New Zealand (NZ) for stoat and feral cat control (Eason *et al* 2011; Blackie *et al* 2014).

The time to death from methaemoglobinaemia in rats was significantly shorter (38–556 min) to that previously reported for anticoagulants (5.6–8.5 days) (PSD 1997; Littin *et al* 2000), but not as rapid as for potassium cyanide (2–45 min) (Chadha *et al* 1991). Signs of methaemoglobinaemia depend on the concentration of MetHb in the circulation. Immediately after dosing, the rats appeared asymptomatic, but from 4 min post-dosing cyanosis was observed followed by lethargy and ataxia (mean onset for both sexes 26 min). In humans, it has been reported that levels below 30% produce minimal or no symptoms, then consciousness becomes increasingly depressed with MetHb concentrations of between 45 and 55%, and at levels of 55–70% there is circulatory failure, respiratory depression, cardiac arrhythmias and coma, levels above 60% can be lethal, while above 70% in humans usually results in death (Hall *et al* 1986; Gowans 1990; Sachdeva *et al* 2003; Matteucci *et al* 2008; Harvey *et al* 2010). In dogs dosed with PAPP, Vandenbelt *et al* (1944) reported ataxia occurring with MetHb concentrations of 60%, salivation and prostration at 75%, loss of consciousness at 85 and death at 95%.

From a welfare perspective, hypoxaemia is now considered one of the more benign ways in which an animal can die, and it forms the basis for some new humane killing methods (Raj & Gregory 1995; Gregory 2004b). However, Niel and Weary (2007) reported aversion in rats to argon-induced hypoxia at static concentrations of 90%. Suggesting that acute hypoxia causes distress from a sensation of dyspnoea in rats. Furthermore, rats treated with PAVP had a significantly higher number of repositioning episodes compared to the matched controls. Repositioning or postural adjustments have been used previously as indicators of pain in laboratory animals (Mayer 2007). Potentially, in the early stages of methaemoglobinaemia, these repositioning episodes could have been associated with pain and/or distress prior to the onset of insensibility. In humans, cephalalgia (headache) has been reported at MetHb concentrations above 20% (Hall *et al* 1986; Matteucci *et al* 2008). It is possible that similar concentrations in rats could have resulted in cephalalgia, which may be associated with the increased repositioning episodes.

Based on the results of the current study, it was concluded that the rats started to experience CNS depression from PAVP-induced hypoxaemia from 26 min onwards (mean time to onset of lethargy and ataxia for both sexes). The mean time to unresponsiveness to a noxious stimulus (tail or ear pinch) was 147 (males: 62; females: 231) min and total unconsciousness was achieved by 163 (females: 59; males: 267) min. It appears from these results that the events leading to death from methaemoglobinaemia are relatively more humane than those from anticoagulant intoxication, based on the reduced time to death, the relatively benign signs of hypoxia-induced cerebral depression and absence of obvious signs of distress or pain. This is further supported by the lack of pathological signs or conditions that could have been associated with potential suffering. Generally, tissues at post mortem showed signs of methaemoglobinaemia in the treated animals, in contrast to the controls. The blood was chocolate brown in colour, which in humans occurs when MetHb concentrations exceed 15–20% (Gowans 1990).

However, unlike the current results, Scawin *et al* (1984) reported convulsions and salivation almost immediately after intravenous injection of PAPP (dose range 76–423 mg kg^{-1}) in mice, with most deaths occurring within 1 min. Fisher *et al* (2008) reported that possums orally dosed with PAPP had rapid respiration, with blood from the nose, anus and in the urine. In addition, PAPP-dosed

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tammar wallabies (*Macropus eugenii*) often had excessive salivation (Fisher *et al* 2008). Convulsions, respiratory distress and salivation can be signs of potential suffering if occurring prior to the onset of insensibility. Similar signs and behaviours were not observed during the recording periods in the current study and presumably the convulsions reported after intravenous injection of PAPP were anoxic in nature and occurred after the onset of hypoxia-mediated insensibility. It is also relevant to note that a MetHb concentration of 94% has been reported in a human following an overdose of amyl nitrite (Edwards & Ujma 1995). Throughout the period of elevated MetHb, the patient was unconscious and unresponsive to pain, and following recovery the patient had no memory of the overdose or treatment (Edwards & Ujma 1995). This suggests that severe acute methaemoglobinaemia induces insensibility that is incompatible with pain and distress and can produce a period of retrograde amnesia.

Para-aminovalerophenone like PAPP is an indirect MetHb inducer, requiring hepatic modification before it becomes biologically active. The metabolised active toxin *para*-hydroxylaminovalerophenone is taken up by erythrocytes, and in the presence of oxygen the haem iron undergoes a two-stage reaction, where $HbFe^{2+}$ is oxidised to MetHb ($HbFe^{3+}$) (Rennison *et al* 2013). Methaemoglobin is unable to bind oxygen and under normal physiological conditions is formed continuously within the bloodstream, with normal circulating levels in humans of 0.5 to 2% (Hagler *et al* 1979; Sachdeva *et al* 2003). Within the bloodstream MetHb is reduced to deoxyhaemoglobin (deoxyHb) by the nicotinamide adenine dinucleotide phosphate dependent (NADPH) MetHb reductase (Sachdeva *et al* 2003). The reductase normally prevents excessive accumulation of MetHb (Kohn *et al* 2002). The goal with using MetHb-inducing toxicants as rodenticides is to overwhelm the MetHb reductase activity, resulting in acute accumulation of MetHb in the circulation beyond the rate that can be reduced by the reductase.

In the current study, there appeared to be a sex difference with female rats being more susceptible to PAVP-induced methaemoglobinaemia compared with males. However, due to the method of formulation of the doses (mg ml^{-1}), the heavier males received a lower dose (mg kg^{-1}) of PAVP compared to the lighter female rats. Suggesting that dose as opposed to sex was responsible for the reported difference. However, unpublished findings by the authors have found the LD50 for PAVP to be 43.3 and 60.0 mg kg^{-1} for female and male rats, respectively, confirming that there is a sex difference in susceptibility. Similar findings have been previously reported for PAPP-induced methaemoglobinaemia in Beagle dogs (Bright *et al* 1987) and rats (Scawin *et al* 1984). Bright *et al* (1987) suggested that the probable reason for the sex difference was a lower rate of N-hydroxylation in males compared with females of PAPP to the final methaemoglobinaemia-inducing active metabolite N-hydroxylaminopropiophenone (HAPP). By contrast, Coleman *et al* (1990) reported that male rats were more susceptible compared with female rats to dapsone-induced methaemoglobinaemia. Their results suggest that N-hydroxylation of dapsone is a prerequisite for toxicity and that the lack of metabolism of dapsone to its N-hydroxy derivatives in female rats protects them from dapsone-induced methaemoglobinaemia (Coleman *et al* 1990).

Although MetHb inducers appear to have advantages in terms of welfare, there are a number of potential issues relating to their effectiveness as rodenticides. Firstly, rats often make short visits to feeding stations during which only small quantities of food are consumed. This, combined with the rapid onset of action of MetHb inducers and the apparent ability of rats to sense the early effects of toxicosis (Greaves *et al* 1974), can result in insufficient ingestion of the toxin to be lethal. This could result in sub-lethal poisoning and learned aversion towards the poison in the bait (bait shyness). Bait shyness has been previously reported for other fast-acting toxicants, for example, cyanide (O'Connor & Matthews 1997), zinc phosphide (Sridhara & Srihari 1980), and cholecalciferol (Gould & Holmes 2008).

Secondly, although the MetHb inducers PAPP and PAVP appear highly toxic in other mammalian species (LD50 PAPP, ferrets:15.52 and stoats: 9.3 mg kg^{-1}) (Fisher *et al* 2005; Fisher & O'Connor 2007), they seem to be less toxic in rats (LD50 PAPP, females: 233; males: 474; PAVP 84–85 mg kg^{-1}) (Pan *et al* 1983; Scawin *et al* 1984; Rennison *et al* 2013) and mice (LD50 PAPP > 5,000 mg kg^{-1}) (Scawin *et al* 1984). This, combined with their rapid onset of action, makes it essential that a lethal dose is consumed in a single meal, prior to the onset of toxicosis. A potential approach would be micro-encapsulation of a lethal dose to improve toxicity. Thirdly, the reported difference in toxicity for female and males rats, makes PAVP less ideal as a rodenticide. The LD50 of 60 mg kg^{-1} in male rats is considered insufficiently toxic for PAVP to be practical as a rodenticide.

Finally, with any toxicant that causes hypoxia in the CNS and other tissues, there is the potential for sub-lethal poisoning and temporary or permanent neurological impairment or damage that could compromise welfare or impair the animal's ability to survive in the wild. In the medical literature, an amyl nitrite poisoned man was reported as making a complete recovery following medical intervention with no residual neurological deficits after developing MetHb levels of 94% (Edwards & Ujma 1995). It has also been reported that full recoveries have occurred within 1 to 2 days post-dosing of PAPP in stoats, ferrets, brushtail possums, tammar wallabies and mallards (*Anas platyrhynchos*) (Fisher *et al* 2005, 2008; Fisher & O'Connor 2007). This work was anecdotal without precise assessment of potential physical or neurological deficit. The accompanying paper by Quy *et al* (2015; this issue) objectively examines the potential for neurological impairment following sub-lethal poisoning with 40 mg kg^{-1} PAVP (based on estimates of LD50). Sub-lethally poisoned rats were behaviourally impaired within 12 min of consuming PAVP (based on acute toxicity data), but all treated rats were normal in appearance and mobility within 48 h. Mild, non-significant neurological deficit lasted up to 11 days, but it was not considered to warrant welfare concern.

Animal welfare implications and conclusion

The major finding of the study is that death from methaemoglobinaemia is more humane than that with anticoagulant poisoning. This is based on the reduced time to death, hypoxia-induced cerebral depression and absence of obvious signs of distress or pain. These results could be regarded as proof of concept for this class of compounds and serves as a model for more humane rodenticides in the future.

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