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Refinement of the use of non-human primates in scientific research. Part III: refinement of procedures

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Abstract

There is an ethical and scientific need to minimise the harm experienced by animals used in scientific procedures and to maximise their well-being. Welfare can be improved by the refinement of practice, particularly if these refinements are applied to every aspect of the life of an animal used in the laboratory, from birth to death. Primates are considered likely to have a greater capacity for suffering than other sentient species and therefore refinement of their use is particularly important. The refinement of the human impact on laboratory-housed primates and of housing and husbandry practices are dealt with in parts I and II of this three-part review. In part III, methods of refinement that can be applied specifically to the use of primates in procedures, are summarised and discussed, together with a description of some current practices, and the scientific evidence that suggests that they should no longer be used. Methods of refinement of identification, capture and restraint, sampling, administration of substances, humane endpoints, and euthanasia are included. If these methods are used, taking into account species-specific differences and needs, it is concluded that harm can be minimised and primate welfare improved.

Keywords: *animal welfare, humane end-points, identification, non-human primates, refinement, scientific procedures*

Introduction

In accordance with the harmonised definition of refinement proposed by Buchanan-Smith *et al* 2005 the concept should be considered to encompass not only the need to minimise the degree of inhumanity, where inhumanity is defined as the infliction of distress, after Russell and Burch (1992), experienced by animals used in laboratories, but also the need to promote well-being. This principle should be applied to every aspect of the life of the animal in the laboratory from birth to death. In this way, ethical concerns about the use of animals in science can be reduced and the validity of the animals as scientific models increased. The application of refinement techniques to minimise harm and maximise well-being of animals during use in procedures is therefore critically important. Information regarding the application of refinement techniques to primate use is published in a diverse selection of journals, and therefore a comprehensive summary is important. In order to widen awareness, refinement techniques that can be applied to all aspects of laboratory use of primates and those that apply specifically to the housing and husbandry of these animals have been discussed in parts I and II of this three-part review (Rennie & Buchanan-Smith 2006 a, b). In this final part of the review we concentrate on methods of refinement that can be applied specifically to the use of primates in scientific procedures in order to both increase the ability of primates to cope with such procedures and to reduce their need to cope.

Refinement of procedures

In the European Directive an experiment is defined as: "any use of an animal for experimental or other purposes that may cause pain, suffering, distress or lasting harm…an experiment starts when the animal is first prepared for use and ends when no further observations are to be made for that experiment…" (European Union [EU] 1986, Article 2[d]).

It is also a requirement that any experiment carried out: "…shall be designed to avoid distress and unnecessary pain and suffering to the experimental animals…"(EU 1986, Article 7 Paragraph 4).

There are many methods by which the execution of experimental procedures can be refined. Many of these can be used to refine routine procedures carried out on the majority of experimental animals. However, the potential benefits of these refinements will only be fully realised if they are executed in the context of each specific experimental manipulation and, most importantly, adapted to suit the species and the individuals concerned. Several important refinements are outlined in this report, in relation to the progression of an experimental procedure from preparation through to termination of the experiment and re-use.

Identification

In Europe, the identification of every individual primate held in laboratories, by the least painful available method, is

a requirement of the European Directive (EU 1986). Many researchers and animal care workers are able to identify individuals by facial features and behavioural characteristics (Poole *et al* 1999). Identification of individuals by this method can be carried out with a minimum of interference. However, the International Primatological Society (IPS) guidelines (1993) recommend that a permanent identification mark should be used, so that every individual can be identified and matched to his or her medical and research records. This is necessary because members of staff at research premises may change, the care of individuals may be transferred between staff, either on the same site or on different sites, and because welfare inspectors must be able to accurately match individuals with records. Marking of animals is not considered to be an experimental procedure, provided that it is carried out by the most humane available method, and therefore does not have to be regulated by designated authorities (EU 1986). Three permanent methods of marking have been used in laboratory primates, with varying success.

Freeze branding

Freeze branding produces scar tissue, and therefore white hair growth, by the application of a small branding iron, cooled to around -60ºC using dry ice or a dry ice ethanol mixture to the skin (Sherwin *et al* 2002). The method has been used in vervet monkeys (*Cercopithecus aethiops*) (Griffin 1988). In cattle, the method results in lower cortisol and fewer behavioural responses than hot branding, although the response is still greater than that in control animals (Schwartzkopf-Genswein *et al* 1997a, b; 1998). However, hyperalgesia was produced at the site of branding for 7 days after treatment. In order to reduce pain and tissue sensitivity, sedation and local anaesthetic should be used during the procedure and a programme of analgesia to control pain following the procedures should be considered. In other species, freeze branding produces clearly recognisable marking, however, in primates, difficulty in seeing the white brand hair in long-haired species has meant that the method is not commonly used (Fortman *et al* 2002) and is not recommended.

Tattooing

Tattooing is widely recommended in laboratory husbandry guidelines (The Canadian Council on Animal Care [CCAC] 1993; IPS 1993; Baskerville 1999; Bearder & Pitts 1999; Erkert 1999; Fritz *et al* 1999; Visalberghi & Anderson 1999; Mendoza 1999). It is generally recommended that the tattoo should be placed in a largely hairless area of skin, but different areas have been recommended for different species. For example, the inside of the ear or lips is recommended for prosimian species (Bearder & Pitts 1999), whilst the inner thigh is recommended for chimpanzees (*Pan troglodytes*) (Fritz *et al* 1999) and squirrel monkeys (*Saimiri spp*.) (Mendoza 1999), and the inner thigh or the chest is recommended for Old World monkeys (Baskerville 1999). One of the main disadvantages of the technique is that the tattoo can be difficult to read at a distance, although this depends on its positioning and skin pigmentation. Tattoos can even be difficult to read when the animal has been captured, particularly as the animal gets older, as the ink can fade as the animal grows (Fortman *et al* 2002). The tattoo may therefore have to be renewed periodically to ensure that identities are not confused (Fritz *et al* 1999). However, it is noted that in many toxicological studies the primates are used and killed before the fading of the tattoo becomes as issue. In order to ensure the accuracy of the tattoo mark and to minimise pain the pain and distress of the procedure, all primates should be anaesthetised for tattooing. Subsequently, the animal must be monitored to ensure that any adverse effects of the identification procedure are detected early on. However, microchips are a refinement of this identification technique.

Microchip identification

Since the early 1990s, subcutaneously implanted microchips holding unique codes for each individual, have been used to permanently identify primates. These microchips are widely used in pet animals. The unique individual code on the microchip must be read using a scanner. Following sedation, the microchip is inserted under the skin using a specially designed hypodermic needle, usually supplied ready loaded with a microchip. Insertion can therefore be achieved very quickly. The microchip may be placed under the interscapular skin or behind the ear (Taylor *et al* 1993), but placement under the skin at the elbow or wrist has proven to be the most accessible for scanner reading in both long-tailed (*Macaca fascicularis*) and rhesus (*Macaca mulatta*) macaques (Wolfensohn 1993). Microchip identification has been recommended for all species of primates. There are disadvantages to the use of microchips for identification. Firstly the microchips and scanning equipment are expensive and thus may significantly add to the cost of an experiment. Further as microchips are different in Europe and the USA and Canada, different readers are required. Secondly, the microchips can only be picked up by the scanner when the scanner wand is around 5-10 cm away from the chip (Poole *et al* 1999). Although primates can be trained to stay still whilst the microchip is scanned (Savastano *et al* 2003), untrained animals may need to be restrained in order to obtain a successful reading. Finally migration of the microchip under the skin is possible and may reduce the efficiency of identification, although chips usually move only a short distance (approx 5 cm) (Wolfensohn 1993).

Temporary identification

Because of difficulties with reading tattoos and microchips, the combination of a permanent method of identification with a more easily distinguishable, temporary method of identification is often used (Fortman *et al* 2002). The provision of a means of identifying the animal easily and immediately enables the caregiver to quickly determine which animals they are observing and therefore to determine whether the behaviour of that animal is normal for that individual, thus facilitating welfare assessment. Easy identification also facilitates the development of relationships between the caregiver and the animal which is

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thought to increase the overall standard of care (Scott 1990). Temporary methods of identification include hair dyes, fur clipping and collars with identity tags. Care must be taken with the choice of dye, particularly in young infants, to ensure that its application does not cause irritation (Anon 2001) and measures should be taken to minimise potential injury from collars and clipping equipment. Collars and tags are only appropriate for use with animals that have stopped growing. They are commonly used in squirrel monkeys (Mendoza 1999), callitrichids (Poole *et al* 1999) and owl monkeys (*Aotus spp*.) (Erkert 1999) and specially adapted collars used to help manipulate and restrain Old World monkeys can be colour coded to facilitate identification of group housed animals (Baskerville 1999). They must be kept clean, and primates may be trained to accept collar cleaning without restraint. Fur dying is commonly used to identify dependent infants. Alternatively, the hair on the tail may be clipped to distinguish between dependent infants. Dye and hair clipping generally may last less than a month.

The combined use of highly visible temporary identification methods, with permanent, but less visible methods, will help to minimise the frequency of intrusive handling and facilitate group housing of primates of all species. However, where handling is necessary for identification, for example, in order to read microchips or to renew temporary marking, the effects of handling can be minimised if the animals are trained to co-operate with the process. For example macaques can be trained to extend a wrist to allow scanning of microchips (Wolfensohn 1993), whilst with regular interaction with humans, group housed cotton-top tamarins (*Saguinus oedipus*) accepted dye marking of their infants without capture, using long cotton-tipped swabs (Halloren *et al* 1989).

Restraint

In order to successfully achieve experimental objectives (eg draw blood, administer test-substance) and in many cases to minimise the risk of injury to the subject or handler, restraint is generally considered to be a prerequisite for the use and maintenance of primates in the laboratory (Klein & Murray 1995; Reinhardt *et al* 1995). Many means of restraining primates have been developed, some of which are specifically adapted to suit the species concerned. However, there is evidence that these methods result in changes in physiology and behaviour (Reinhardt *et al* 1995). The extent of these effects varies with the duration and degree of restraint (Pare & Glavin 1986). Often, restraint and procedures are carried out in a room separate from the colony room (Reinhardt *et al* 1990), in order to minimise disturbance to other animals in the colony (Grant & Doudet 2003). It is now well recognised that removal from the colony room and restraint by any method can constitute an uncontrolled experimental variable (Reinhardt *et al* 1995), especially as it is common practice in toxicology research to use each animal as its own control (Landi *et al* 1990).

No direct reference to the use of restraint is made in the European Directive (86/609/EEC) (EU 1986) or in any of the European national legislation. However, restraint is mentioned in the IPS International Guidelines for the Acquisition, Care and Breeding of Non-human Primates (IPS 1993) and in the Canadian Council on Animal Care (CCAC) Guidelines on the Care and Use of Laboratory Animals (CCAC 1993). In these guidelines it is recommended that restraint of any sort should only be used when no less stressful alternative is available. Many of the most commonly used restraint techniques, their relative merits and pathological, physiological and behavioural effects have been described in recent reviews (Klein & Murray 1995; Reinhardt *et al* 1995; Sauceda & Schmidt 2000). No attempt will therefore be made to repeat these reviews here, but an overview of the most important issues will be given, together with more recent literature.

Catching techniques

Both the impact on the primates, and the safety of the personnel must be considered in relation to capture and handling. Capture and handling of laboratory-housed primates requires experience and skill. Several methods are regularly used within the research community across Europe including hand and net catching, capture of animals by containing them in their nest box or moving them into transport cages and by attaching a pole to a collar fitted to the animal under anaesthesia. When catching by hand the use of protective gloves has been highly recommended (Klein & Murray 1995; Fortman *et al* 2002), but the weight of glove used must be carefully gauged to ensure that excessive pressure is not applied to the animal and that the handler is sufficiently well protected (Sainsbury *et al* 1989). Species differences should also be considered. Fortman *et al* (2002) recommend double gloving when handling macaque species, but the use of heavy gloves to handle marmosets may result in damage to their delicate teeth if they attempt to bite. In contrast tamarins (similar in size to marmosets) have long upper canine teeth and should not be handled without protective gloves (Poole *et al* 1999). The handler must be aware that injury to the primate can arise as a result of forceful removal from the cage, especially when individuals cling to the mesh sides of the cage.

Sainsbury *et al* (1989) recommended that nets may be used in conjunction with hand capture, particularly with groups of animals housed in large cages. However, this method of capture can result in entanglement and injury during removal from the net. Stress resulting from net capture can cause acute diarrhoea, rectal prolapse and lacerations (Luttrell *et al* 1994). Furthermore, the method potentially places the handler in danger from both the subject and other animals in the cage if they must enter the home cage and chase the target animal, causing fear and arousal in the whole group (Luttrell *et al* 1994). Feasible, less stressful alternatives to capture are available and therefore net capture is not recommended here.

Pole and collar capture and restraint is widely used in larger primates including macaques and baboons, with little risk to

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the handler (Klein & Murray 1995). A plastic or metal collar is fitted to the animal either under anaesthesia or sedation. A spring loaded pole is attached to the collar through the door or bars of the cage at the time of capture and the animal is moved from the pen and walked to a grooved restraining chair into which the collar can be slotted (Klein & Murray 1995). For larger species two handlers can attach poles to the collar increasing the level of control. Whilst in this chair, the animal's limbs can be restrained just as in a normal restraint chair (see below) and the whole body is accessible for physical examination, blood sampling and administration procedures. Positive reinforcement training (PRT) and desensitisation can be used in order to get the animal to accept capture by this method and to walk on the end of the pole using treats to reward co-operative behaviour (Marks *et al* 2000; Sauceda & Schmidt 2000; McGuffey *et al* 2002). Acceptance of the full capture and restraint procedure can be achieved in 4 or 5 ten minute training sessions (Klein & Murray 1995) and thus, the use of pole and collar restraint without training must be considered unacceptable.

In some research establishments, marmosets are captured by trapping them in their nest box. The animals move into the box in response to the threat of capture (eg showing a gloved hand or even just opening the cage). This method of capture reduces the risk of injury during removal from the cage and may reduce the stress of capture overall. However, this method of capture is considered less than ideal as it is inappropriate to use the nest box for capture. The nest box should provide the animal with a safe retreat in which it can rest securely without the threat of capture. Also, the marmosets move into the nest box because of the negatively reinforcing threat of capture, thus introducing an unnecessary source of stress.

In other research establishments transport cages are used to capture individual primates for routine examination and procedures. Different methods have been used to induce the animals to enter the transport cage, for example Luttrell *et al* (1994) used negative reinforcement training, by shouting and waving the arms at rhesus macaques to induce them to move into a holding pen before releasing them through a chute from which they could be filtered off into a transport cage. After five weeks of training the animals could reliably be caught one at a time in the transport cage. PRT is preferable and has also been used to induce entry to transport cages for capture (Reinhardt 1992a, b; Klein & Murray 1995; Scott *et al* 2003) either attached to the home cage or placed in a chute configuration. Reinhardt (1992b) found that rhesus macaques trained by positive reinforcement would enter the transport crates in a reliable order each time capture was initiated. This order appeared to be influenced more by age than dominance hierarchy, but ensured that specific animals could reliably be caught (Reinhardt 1992b). Positive reinforcement has also been used to train marmosets to enter transport cages. Despite the relative ease with which animals can be caught by this method, Line *et al* (1987) found that singly-housed, female, rhesus macaques confined in a transport cages for 5 minutes showed elevated plasma cortisol 15 minutes after confinement had taken place. Also Clarke *et al* (1988) demonstrated that there are species-specific differences between rhesus, long-tailed and bonnet (*Macaca radiata*) macaques in their physiological and behavioural responses to transport cage training. Furthermore, care must be taken that the design of the transport cage enables the handler to catch the animal safely, without risk of injury. For example, for marmosets the transport box should be easily attached to the front of the cage, allowing caregiver's hands to be free to deliver the reward through a mesh front, and simultaneously close a sideways sliding door. Given their weight, a macaque transport box may be most suitable on a trolley. From these studies, it cannot be concluded that training to enter a transport cage is a means of stress-free capture of laboratory-housed primates, but if carried out correctly using habituation and positive reinforcement (Rennie & Buchanan-Smith 2006a) the method can significantly reduce capture stress and must be considered the most humane method of capture.

Manual restraint

Hand-restraint of primates also has risks and a high proportion of accidents in the laboratory occur when primates are restrained in this way (Klein & Murray 1995). For example when restraining an adult macaque it is recommended that at least two handlers should be involved (Reinhardt *et al* 1995) and that the arms should be held behind the back (Sauceda & Schmidt 2000). However, shoulder joints and muscles can be overstrained (Sainsbury *et al* 1989) and fracture of the humerus can occur as a result of excessive force used to hold the arms of the monkey (Klein & Murray 1995). Macaques may also be restrained in a cradle, using only one arm to immobilise the animal. Marmosets and tamarins should be held around the chest between thumb and forefinger. The thumb can be placed under the chin to prevent biting (Sainsbury *et al* 1989). Landi *et al* (1990) found that, in long-tailed macaques, manual restraint induced changes in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) depending on the method of restraint. ALT and AST are used as measures of liver pathology, but the effects of restraint alone on these parameters illustrates the scientific need to find alternative methods for obtaining samples from these animals. Many other changes in physiological parameters caused by manual restraint have been reported (see Reinhardt *et al* 1995 for a review).

Squeeze-back cages

Another commonly used restraint device for all larger species of primates used in laboratories is the squeeze back cage (Fortman *et al* 2002). As the name would suggest, this caging system incorporates a manual or automatic mechanism which moves the back panel of the cage towards the front. Thus, the animal in the cage is slowly moved to the front of the cage and can be partially or totally immobilised, allowing access for experimental procedures.

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Because the method is easy to use, staff can be easily trained to use it correctly (Sauceda & Schmidt 2000). Animals can be restrained quickly and safely for routine procedures like injections and the need for physical handling is reduced (Sauceda & Schmidt 2000). The method is not recommended for more complicated techniques however, when accuracy is essential (eg intubation) (Sauceda & Schmidt 2000). Squeeze-back cages are most commonly used as a home cage when animals are housed singly. Whilst this reduces the need for handling, squeeze cages are often designed to meet cage size requirements and because of their size and the squeeze mechanism of the cage, structural and inanimate enrichments must be kept to a minimum (Dexter & Bayne 1994), although primates can be housed in this system in pairs. Although the method is generally considered to be safe, fast and relatively stress free for most individuals, in comparison with other methods of restraint, significant increases in cortisol and persistent decreases in testosterone have been reported following squeeze cage capture (Reinhardt *et al* 1995). Blank *et al* (1983) found a decrease in serum prolactin accompanied by a decrease in growth hormone and an increase in cortisol. Grant and Doudet (2003) reported behavioural signs of fear and distress in some of their rhesus macaque subjects, including circular pacing, bouncing, baring teeth and grimacing, just prior to squeeze-back restraint even though their subjects had been trained to accept the procedure. However, the macaques in this study were brought to a procedure room before restraint took place and previous studies have shown that removal from the home cage and colony room itself may induce more distress than restraint in the home cage (Reinhardt *et al* 1990). Together this evidence suggests that squeeze-back cages may induce less stress than some other methods of restraint, if the cage is used as a home cage. However, in these circumstances the animal is continually exposed to the threat of capture within its home environment. Thus the use of a squeeze cage alone as the animals' home cage is not recommended. As a compromise, banks of squeeze cages can be incorporated into group housing systems for macaques and the animals can be trained, preferably using PRT, to move into them when required. Non-human primates can be trained to present a specific side to the front of the cage when the squeeze mechanism is started, thus reducing the need for repositioning and reducing potential stress (Luttrell *et al* 1994; Reinhardt 1997).

Restraint chairs

Restraint chairs maintain the animal in a sitting position during restraint. Perspex or plastic circular restraints surround the neck and waist to hold the animal in place but allow full access to the body for experimental manipulations (Reinhardt *et al* 1995). Modern chairs can be adjusted to accommodate anatomical differences in different species, taking account of position and length of tail, position of ischial callosities, crown to rump length, limb length and variations in posture (Klein & Murray 1995). Restraint chairs have been described as 'comfortable' (Klein & Murray 1995), and some evidence indicates that in comparison with other methods of restraint, including manual restraint, restraint on a board and restraint in a box or stocks, the chair is less stressful (Landi *et al* 1990). However, chairs have also been associated with many physical problems including skin abrasions, necrosis of the ischial callosities, position-dependent oedema, inguinal hernias, laryngeal air sacculitis and rectal prolapse (Morton *et al* 1987). Fractures and decubital ulcers have also been reported (Klein & Murray 1995). Chair restraint also has effects on physiology, inducing persistent elevations in adrenocorticotrophic hormone (ACTH) and cortisol within 15 mins of restraint and after rhesus macaques were returned to their home cages (Norman & Smith 1992; Norman *et al* 1994). Restraint was also shown to inhibit pituitary luteinising hormone (Norman *et al* 1994) and testosterone release (Norman & Smith 1992) in female and male rhesus macaques respectively, thus having the potential to inhibit reproduction. Morrow-Tesch *et al* (1993) found changes in white blood cell counts and the percentage of neutrophils increased during the restraint period in rhesus macaques, whilst the number of lymphocytes and monocytes declined. Habituation to chair restraint, giving rewards for calm behaviour, can reduce the response of animals to chair restraint (Castro *et al* 1981) and the inclusion of some social contact may reduce the stressful nature of the procedure even more (Hennessy 1984). Smith *et al* (1998) showed that in black tufted-ear marmosets (*Callithrix kuhli*), relocation to a novel cage produced no measurable change in urinary cortisol over a four day period provided that the move was made in pairs. Isolated individuals showed elevated urinary cortisol in response to separation and relocation, in comparison with baseline. However, empirical data on the effects of procedures on the untested 'buddy' animal has yet to be provided and the use of partners to reduce stress in study animals should only be carried out with caution until this information becomes available. Where chair restraint for extended periods is unavoidable, manipulation of the individual's legs can help to maintain circulation, reducing the effects of the abnormal position on the animal.

Tethering

The chair method of restraint allows the subject little or no freedom of movement. An alternative to chair restraint is the use of the vest and tether system developed by Chatham (1985). This system allows continual administration and sampling via surgically implanted cannulae, which are enclosed in a tether protected by a flexible stainless steel casing (Klein & Murray 1995). Subjects are able to lie down and turn around, thus their movement within a small cage is not impeded (Reinhardt *et al* 1995). Tethers can also contain lead wires attached to recording devices on the exterior of the cage. The tether is held in place in a nylon mesh or leather vest.

Using tethering, samples are obtained remotely so that the animal does not need to be physically restrained and the method may therefore be safer and less stressful for the primate and the handler. However surgery is required prior

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to initiation of the study and additional time must be allowed to acclimatise the animal and ensure that the cannulation procedure has been successful. Morton *et al* (1987) found that after pre-experiment conditioning without the cannula in place, subjects may take as little as a week to accept the vest and tether and stated that 95% of subjects can be successfully adapted to the system. Wheeler *et al* (1990) found that the cortisol response to tethering was lower than that recorded in response to chair restraint in rhesus macaques. However, it is likely that this difference is associated with the presence of handlers to obtain samples from chaired individuals, whilst tethered animals can be sampled remotely. Also cortisol was measured in animals tethered for 4 weeks, whilst cortisol from chair restrained animals was measured after 8 hours, thus lower cortisol (chronically elevated) responses would be expected in tethered animals.

One of the main problems with tethering is the use of a transcutaneous cannula which can become infected, potentially resulting in septicaemia. The best means of infection prevention is by use of sterile procedures and the best treatment is by complete removal of the cannula. Antibiotics should be used after cannulation and to aid recovery if infection occurs. However, septicaemia can still arise despite best efforts to prevent its occurrence (Morton *et al* 1987). The risk of complications with tethering are greatly increased by housing animals in pairs because of the potential for interference with the vest and tether (Kinter & Johnson 1999). For this reason animals are usually singly housed during the instrumentation period which, in chronic studies, can last weeks or months (Crockett *et al* 1993) and single housing is viewed as unacceptable when avoidable. Studies have shown that tethering is associated with chronic activation of the adrenal cortex (Crockett *et al* 1993), and sympathetic nervous system (Adams *et al* 1988; Crockett *et al* 1993) despite acclimatisation procedures (Crockett *et al* 1993). Adaptations to the system, including improving the fit of vests (Morton *et al* 1987), use of sterile procedures (Morton *et al* 1987), improvement of the acclimatisation procedure (Crockett *et al* 1993) and the presence of, and access to, companions in an adjacent cage (Coelho *et al* 1991) have been shown to reduce the stress induced by tethering. Furthermore, Reinhardt (1997) reported that juvenile rhesus macaques were successfully housed in compatible pairs whilst tethered. If tethering is required, such practices should be used where possible to refine its use.

Other methods of restraint

Tubes, chutes, boards and boxes are further means of restraint that are used in laboratories to restrain non-human primates (eg Greig *et al* 2006 for marmosets). All these methods have been reported to induce physiological and behavioural changes associated with stress (Reinhardt *et al* 1995). It is clear from the literature that, although all methods of restraint can be highly stressful, much of this stress can be eliminated if the method is used sympathetically with the needs of subject in mind (Fortman *et al* 2002). For example, Moseley and Davis (1989) adapted two tube restraint devices for use in marmosets and owl monkeys. By using PRT and acclimatisation procedures the subjects apparently adapted well to the procedures without any obvious behavioural signs of distress. Animals which were considered unable to adapt were not used in the study. Similarly Greig *et al* (2006) consider a marmoset restraint device to be a refinement over manual restraint as only one handler is required (as opposed to two to three) to carry out the procedure. However, no objective measurements of behaviour or physiological signs of stress were made in these studies and it should be noted that assumptions about the stress experienced by individuals is no substitute for empirical measurement. In a study by Yasuda *et al* (1988), crested black macaques (*Macaca nigra*) were restrained in plexiglas cylindrical restraint tubes and appeared behaviourally relaxed. However, glucose clearance and insulin secretion were found to be impaired and further restraint resulted in elevations of glucose and cortisol suggesting that animals may in fact have experienced stress as a result of the restraint procedure.

Section summary

• Microchipping is the best available method of permanent identification of laboratory-housed primates. PRT can be used to facilitate chip reading.

• The use of a second more obvious temporary method of marking (eg a collar) is beneficial for rapid identification.

• Dye marking or tail-clipping is more appropriate for infants until weaning when permanent identification is required.

• Restraint is often considered a prerequisite for the use of primates in laboratories.

• It is also widely recognised that capture and removal from the colony to the procedures room and restraint can represent uncontrolled experimental variables.

• The use of protective gloves is recommended for personnel safety, but care must be taken not to injure the animals through lack of sensation.

• Net capture is highly stressful, resulting in marked stress-related physiological responses and often in injury. This method should be avoided where possible.

• The pole and collar technique can be used to capture and move larger primates, but should only be used with PRT.

• Nest-box trapping is not recommended for any species as the box is used as a secure retreat by the animals during resting hours and should not be associated with the threat of capture.

• Training to enter a transport cage (using PRT) is considered to be the least stressful means of capture for all species and in New World species should be used instead of nest box capture. The design of the transport cage must be such that removal of the animal can be achieved without risk of injury.

• The full time use of squeeze-back cages to facilitate restraint and capture is considered unacceptable as these

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cages are typically small, limit the scope for enrichment and are associated with the constant threat of capture. The use of squeeze back cages as part of a group housing system and the use of positive reinforcement to train primates to enter the system is considered a more acceptable use of the squeeze-back system.

• Chair restraint has been associated with injury and stress-related changes in physiology. The use of PRT and habituation as well as adaptations of design to suit the species, and minimisation of the duration of restraint, are essential to minimise stress associated with this restraint method.

• Tethering has been associated with a lesser degree of stress than chair restraint, but unacceptable single housing is commonly a consequence of its use.

• Most other methods of restraint are also associated with stress-related changes in physiology and behaviour. These effects can be minimised by sympathetic use and handling and PRT.

Sedation and anaesthesia

Sedation and anaesthesia may be used to minimise the effects of capture and restraint and are often called 'chemical restraint' methods. The agent can be delivered using a syringe for restrained animals or those trained to present a limb for procedures, a pole and syringe for animals in transport crates and small cages, and capture pistols, rifles and blow darts for animals in large group rooms or outside (Fortman *et al* 2002). Pistols and rifles expel the anaesthetic dart very fast in order to ensure penetration of the skin of animals that are far away, however in most laboratory situations non-human primates are close enough to dart using a blow pipe and the pistol and rifle darts may cause excessive bruising without increasing the accuracy of darting (Fortman *et al* 2002). An overview of the most commonly used anaesthetics for laboratory primates is provided by Rensing (1999) and only a summary of key points will be reported here, together with more recent literature.

Ketamine is the most commonly used anaesthetic agent in primates administered at a dose of between 5 and 40mg kg^{-1} (Castro *et al* 1981; Erkert 1999; Poole *et al* 1999). Accurate dosing should result in dissociative anaesthesia, analgesia and superficial sleep (Castro *et al* 1981). However, if too weak a dose is administered, increased tonic activity of muscles may occur, making the subject difficult to control. Overdosing can result in excitation of the central nervous system and cramping (Ghaly *et al* 2001). The advantage of ketamine anaesthesia is that it does not depress swallow, breathing and eyelid reflexes ensuring that the state of the subject can be more easily monitored and animals will not stop breathing unexpectedly (Fortman *et al* 2002). A distinct disadvantage to its use is that tolerance can develop over a period of time. Ketamine is often used in combination with xylazine to counteract any central excitation (Erkert 1999; Poole *et al* 1999).

The use of anaesthetic agents themselves may have effects on physiological parameters. The potential for effects varies with the species and drug applied and care must be taken when selecting both the species and drug for each specific study and during interpretation of results (Reinhardt *et al* 1995). Particular caution must also be exercised when anaesthetics are administered to pregnant or lactating females as some agents may cross through the placenta to the foetus or be released in milk (Klein & Murray 1995). Yasuda *et al* (1988) described unwanted effects of ketamine in black-crested macaques, including excessive salivation, slow hand and limb movements and uncoordinated eye movements. Wall *et al* (1985) found that ketamine injection had significant effects on blood cell parameters and cardiovascular function in vervet monkeys. The changes in physiological parameters and the behavioural effects of anaesthetics may well be indicative of stress in non-human primates and thus, the use of anaesthesia to minimise stress may be counterproductive. Because of the risks involved, the use of anaesthesia, either to reduce stress or to facilitate procedures, should only be considered in cases where it is not possible to train the primate to co-operate with the procedure voluntarily. Stress associated with restraint for administration of ketamine can be greatly reduced or eliminated by using PRT to encourage the individual to co-operate (Philipp 1996).

Section summary

• Ketamine is most commonly used for anaesthesia in primates and is used both to immobilise the animal during invasive procedures and to reduce stress associated with restraint.

• Anaesthesia of primates is always associated with risks and it may affect physiological parameters which must be taken into account in experimental procedures.

• Where possible PRT should be used as an alternative to anaesthesia to reduce stress associated with procedures.

• Where anaesthesia is necessary to achieve the procedure, PRT should be used to minimise stress associated with its administration.

Sampling

Blood sampling

Blood sampling is carried out routinely on experimental animals. The measurement of the blood biochemistry, hormones and haematological parameters are well established protocols, providing reliable measures of physiological status. Venous blood samples are the most commonly used sample, but arterial and cardiac samples and the use of cannulae may also be used, although some consider that sampling by these methods requires special justification (Morton *et al* 1993). With poor management, inexperience, unsympathetic handling and the use of the wrong or poor technique, blood sampling may become unnecessarily stressful for the animal (Morton *et al* 1993) and hypovolaemia, bruising, haemorrhage and thrombosis may be the result. Arterial blood sampling is most commonly used to collect large samples of blood quickly, because arterial blood is under more pressure than blood in veins. However

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adverse effects of blood sampling are more common when blood is collected in this way. Care must be taken to ensure that pressure is applied to puncture sites until bleeding has definitely stopped. If thrombi (fibrinous blood clots) form at the site of puncture of an artery they may dislodge and can cause severe pain if they become lodged in a major vessel (eg the femoral artery). Care must be taken, especially with smaller animals that the volume of blood taken is not excessive and that time is given for blood volumes to recover in prolonged trials. It has been recommended that, for one-off sampling 10% of the animal's circulating blood may be taken, but for repeated sampling only 1% may be taken every 24 hours (Morton *et al* 1993). Phlebitis (inflammation of the vessel) may also occur if cleanliness is not ensured. However, excessive cleaning of the skin's surface before sampling may be counterproductive as clipping and shaving have the potential to increase the stressfulness of the procedure and repeated sterilisation of the skin can result in inflammation, dryness and discomfort (Morton *et al* 1993).

Under good conditions involving PRT, the actual act of venipuncture has been achieved in Old World species with no adverse response (Priest 1991; Reinhardt *et al* 1991). Rhesus macaques, stump-tailed macaques (*Macaca arctoides*), drills (*Mandrillus leucophaeus*) and chimpanzees (*Pan troglodytes*) have all been trained to accept blood sampling in their home cages (Reinhardt 1989; Reinhardt *et al* 1990; Priest 1991; Fritz *et al* 1999). Reinhardt *et al* (1991) compared cortisol in trained, male, rhesus macaques, sampled in their home cage and a second group of venipuncture-trained animals sampled, whilst unrestrained, in a restraint apparatus. Animals sampled in their home cages showed less change in cortisol than those sampled in a restraint chair. It was concluded that venipuncture is not stressful *per se* but that stress can be induced when it is carried out in association with removal from the home cage (Reinhardt *et al* 1991). Capitanio *et al* (1996) found that cortisol in rhesus macaques was unaffected either by the time taken to draw blood or the duration of disturbance prior to sampling. This suggests that the presence of experimenters in the colony room is not necessarily stressful, adding weight to the argument that non-human primates should be trained and sampled in their home cage wherever possible. However, Capitanio *et al* (1996) did find changes in haematological parameters independently of a hypothalamo-pituitary adrenal (HPA) axis stress response. The authors concluded that disturbance in the colony room may cause experimental variation in haematological parameters, but that stress is not necessarily induced.

Priest (1991) describes a procedure by which a diabetic adult male drill was trained to accept regular blood samples. The drill was trained to hold a handle until the signal was given to let go and a reward was given. Venipuncture was accepted by the drill whilst holding the handle. Training to accept venipuncture is becoming more widely used in Old World primates and is recommended as a less stressful option to restraint for all Old World species (Baskerville 1999). Although training to accept laboratory procedures is possible in New World primates (Poole *et al* 1999; Visalberghi & Anderson 1999; McKinley *et al* 2003), acceptance of venipuncture has not been achieved, partially because their small size makes it difficult to access blood vessels. However, with training and desensitisation the stress of capture (Reinhardt 1992a, b; Klein & Murray 1995; Scott *et al* 2003) and restraint can be minimised and thus the overall stress of routine procedures can also be reduced in New World species (Moseley & Davis 1989).

Venipuncture itself may be refined by carefully selecting the gauge of hypodermic needle. The gauge should be such that the minimum size of puncture wound is applied but that blood will flow through the needle reasonably swiftly to avoid extending the duration of the procedure and to reduce the possibility of haemolysis of the sample (Morton *et al* 1993). The details of suitability of different lengths and gauges of hypodermic needle for different species of laboratory animals are described in the BVA (AWG)/ FRAME/ UFAW/ RSPCA report on the removal of blood from laboratory mammals and birds (Morton *et al* 1993).

The femoral vein is considered to be the most appropriate vessel for blood sampling marmosets (Morton *et al* 1993; Poole *et al* 1999) but the coccygeal (tail) vein has also been recommended (Morton *et al* 1993). The saphenous and brachial veins are recommended for drawing blood from Old World monkeys (Morton *et al* 1993; Baskerville 1999). These vessels are large enough to allow easy venous access and to ensure that blood may be taken relatively quickly and are superficial enough to be easily accessed in conscious or lightly sedated animals (Morton *et al* 1993). The femoral vein has also been recommended for venipuncture in anaesthetised, heavily sedated animals or in those that are well trained for sampling, as it is located deeper below the skin (Baskerville 1999). Larger volume samples should be taken from the femoral vein (Baskerville 1999).

Damage to blood vessels as a result of regular blood sampling may be minimised by alternating between blood vessels on either side. However, if frequent sampling is required, some authors recommend that cannulation be used to reduce the stress of sampling, primarily by eliminating the need for frequent skin puncture but also to minimise the duration of handling (Wheeler *et al* 1990; Morton *et al* 1993). Early cannulation techniques required that anticoagulant be placed in the 'dead space' of the cannula to maintain patency. This had to be removed at the start of sampling and replaced afterwards, thus increasing the duration of restraint. More modern cannulae incorporate multiple entry ports such that anticoagulant can remain in the tube continually so that the draw-time is reduced and cannula patency is better maintained (Morton *et al* 1993). The use of cannulation must be carefully considered before undertaking the procedure as the adverse effects of the technique are potentially numerous. In non-human primates only the jugular and femoral veins and arteries are recommended for cannulation (Morton *et al* 1993). Placement of the cannula requires anaesthesia, which in itself induces

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stress (Wall *et al* 1985). Subsequently the cannula will require protection from manipulation by the animal, to help prevent accidental or deliberate removal of the cannula (Morton *et al* 1993). Jackets with a swivel and tether have been used to protect cannulae long-term in primates, but this method of restraint has been associated with long term arousal of the sympathetic nervous system (Adams *et al* 1988) and may result in gastric ulcers (Brodie & Hanson 1966) as a result of stress. Restraint of this sort usually requires that animals are kept in single cages because of the assumption that cagemates will interfere with the jackets (Kinter & Johnson 1999), although pair housing of tethered juvenile rhesus macaques has been achieved (Reinhardt 1997). Single housing should be avoided whenever possible as most laboratory-housed primates are highly social species. However cannulae have been successfully maintained in group-housed marmosets (Morton *et al* 1993). Cannulae can become blocked and efforts must be made to unblock it if patency is to be restored. However, unblocking the cannula may require prolonged handling, thus minimising one of the potential benefits of its use. Cannulae can be a focus of bacterial infection and although the use of aseptic conditions can minimise the risks, infection can occur resulting in thrombus formation, increased body temperature and loss of appetite. Thus, the cannula may have to be removed and the animal's involvement in the experiment terminated.

Non-invasive sampling

Methods by which physiological parameters can be measured without venipuncture and therefore in a less invasive manner are gaining in popularity, as validation of their use for a wider range of parameters progresses. Hormones including gonadal steroids, adrenal steroids, ACTH and many others can be measured in urine and faeces of non-human primates. The suitability of these sampling techniques must be considered with respect to the species studied and the requirements of the experiment as the ability to reliably detect parameters in urine or faeces is variable in different species and there is a time lag, of variable duration, between the appearance of parameters in the blood and their detection in the excreta. For example, in squirrel monkeys hormones are excreted predominantly in the faeces and not in urine (Visalberghi & Anderson 1999). Although invasive methods of collection of urine exist (including catheterisation and cytocentesis), samples can be collected by positioning a cup under a urinating animal or by training the animals to urinate when and where required. Training for urine collection has been used with success in marmosets (Anzenberger & Gossweiler 1993; McKinley *et al* 2003), chimpanzees (Stone *et al* 1994; Fritz *et al* 1999), capuchins (Visalberghi & Anderson 1999), and vervet monkeys (Kelley & Bramblett 1981). Analyses of hormones in urine and faeces provide a measure of the hormone over a period of time. Thus, it is particularly useful for determination of reproductive status and for assessment of chronic arousal or stress. However, physiological responses to instantaneous stressors cannot be measured in this way (Lutz *et al* 2000). The measurement of salivary hormones can provide a less invasive (although potentially intrusive) means for measurement of short-term responses than blood sampling. Again the potentially intrusive nature of sampling saliva can be minimised by training non-human primates to co-operate with the sampling procedure by positive reinforcement with reasonable reliability. For example (Lutz *et al* 2000) collected saliva successfully from 21 out of 23 rhesus macaques, and found two potentially useful means of obtaining the sample. They found that licking a small piece of absorbent mesh fabric and chewing a piece of cotton rope both produced sufficient samples although disadvantages were identified with both methods. A method for non-invasive sampling of saliva was developed and reported by Cross *et al* (2004). In these studies, marmosets were tempted to chew on a cotton wool swab by dipping the tip in banana and allowing the animal to chew on the swab for 5 minutes. In field conditions, genetic information about subject animals is commonly obtained through collection of faeces and hair samples (eg Bradley *et al* 2002). Such work can also be carried out on laboratory non-human primates.

Telemetry

The intrusive nature of many physiological data collection methods has been reduced by the use of radio- or bio-telemetry. Bio-telemetry is the term used to describe the collection of physiological data using sensors in close contact with the animal. Data picked up by the sensors is relayed through a transmitter to a receiver. The data can thus be collected remotely without disturbing the animal. The basic concept of this technology has been developed widely and many methods currently exist by which this technology can be applied in animal studies in the laboratory.

Non-invasive telemetry devices

Externally applied telemetric devices have been used for many years for the study of the behavioural ecology of wild animals. Transmitters are attached to the fur or skin of the trapped animal or are designed to attach to a collar. The system can remain viable for periods of up to several months. The transmitters must either be removed by recapturing the animal or will become detached when the animal moults or as old skin cells slough off. Externally applied telemetry devices may also be used in laboratory animals. In this case the logger/transmitter is usually enclosed in a jacket worn by the animal (Kramer *et al* 2001). Electrodes or sensors are attached to the animal and linked up to the logger and the data are transmitted to receivers held nearby. This method has the advantage that the animal can move around freely and that data may be collected 24 hours a day. Also the equipment may be transferred between animals and batteries can easily be changed. This equipment can only be used in larger non-human primates as well fitted vests and jackets are not available for smaller species (Visalberghi & Anderson 1999). Also the risk of damage to the equipment and removal of sensors is high because of interference with the equipment by the wearer (Kramer *et al* 2001). Despite these problems externally applied radio-telemetry devices have been used successfully. For example

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Novak (2003) recently described a study in which a remote telemetry system was used to determine heart rates in singly-housed rhesus macaques that were engaging in self-injurious behaviour. It was found that monkeys engaging in self-biting behaviour had higher than normal heart rates prior to biting, and that their heart rate became even more elevated during biting. However, when these animals finished a bout of biting, their heart rates declined to baseline levels. It was argued that the act of self-biting has a calming influence that is self-reinforcing and will therefore persist despite changes in the environment (Novak 2003). The measurement of heart rate using this method can be carried out without difficulty despite interference with the exact positioning of the sensor, movement and the resistance of skin and underlying tissues. However, the measurement of some physiological parameters may require more accurate placement of sensors and the minimisation of resistance in order to achieve usable results. Thus, the number of measures that may be made using externally applied telemetry devices is comparatively limited, and single housing is unacceptable. Therefore alternative techniques should be used.

Capsule telemetry devices

The data-logger/transmitter component of the telemetry system can be made small enough to fit into a capsule, although the data recorded by these devices tends to be simpler. Such devices have been used to measure gastric pH and transit time (Barrie 1992; Mojaverian 1996), deep body temperature (Hoffman *et al* 1969), subcutaneous body temperature (Cilia *et al* 1998). The most commonly used capsule telemetry device is the identity microchip used widely in pets and in laboratory-housed animals. Implantation of these capsules may be achieved quickly by injection through a specially designed hypodermic needle, without sedation or surgery. Restraint of the animal is however required. Also, the needle required to implant the capsule is of a much larger gauge than that used for venipuncture or drug administration. The use of local anaesthetic would reduce the aversive nature of the procedure itself, but would increase the need and duration of handling (particularly in untrained animals). Also capsule telemetry devices of any kind must be close to the receiver in order to be read and thus in untrained animals handling may be required (Kinter & Johnson 1999).

Invasive telemetry systems

More complex transmitters and sensors have been developed that can be implanted (partially or fully) surgically within the animal. These systems have the advantage that minor movements of the body do not result in movement of the sensors. The sensor is applied directly to the target area so that there is less resistance between the sensor and the tissue. Such devices have been termed 'pre-invasive' because they require invasive surgery in order to provide telemetered subjects, but these animals are given time to recover before initiation of experimental protocols that may be both non-invasive and non-intrusive (Schnell & Gerber 1997). Partially implanted systems require initial surgery in which the sensors, electrodes or cannulae are implanted at the required site, sutured in place and then tracked back subcutaneously to the transmitter. At a suitable position (eg through interscapular skin) the wires are led through the skin to be connected to the transmitter. The transmitter can be worn by the animal in a jacket allowing unrestricted movement or the animal is restrained using a tether and swivel system through which the wires run to reach the transmitter outwith the cage. As with externally applied telemetry devices, partially implanted telemetry systems can only be used in larger primates because of difficulties fitting vests and jackets in smaller species (Visalberghi & Anderson 1999) and the resulting increase in the risk of interference with the equipment. However partially implanted radio-telemetry systems have been used successfully to collect data 24 hours a day from common marmosets (Crofts *et al* 2001). Because of the transcutaneous components of partially implanted telemetry systems, infection and injury from interference with equipment are serious risks. Because of these risks, primates carrying partially implanted telemetry systems are often housed singly, which is also likely to cause stress (Kinter & Johnson 1999; Visalberghi & Anderson 1999).

Fully implantable devices also require surgery, but here both the transmitter and the wires are implanted within the animal. Equipment is available for use in both large and small primates. In marmosets (and other small primates) the transmitter (often adapted from transmitters designed for the similarly sized rat) is placed in the peritoneal cavity and sutured to the mucosa (Einstein *et al* 2000; Crofts *et al* 2001). In larger species including rhesus, Japanese and long-tailed macaques and baboons the transmitter may either be placed under the interscapular skin or inserted into the peritoneal cavity (Kaufman & Detweiler 1999). The electrode wires or cannulae can then be tunnelled subcutaneously to the appropriate position and sutured into place (Kaufman & Detweiler 1999). Fully implantable telemetry devices require no restraint during the test protocol and have been successfully used to record parameters 24 hours a day. Alternation between subjects over a 24 hour period allows multiple subjects to be housed in pairs or groups (Crofts *et al* 2001). These devices leave animals less open to infection as, after the implantation incisions heal, there is no route by which infection can cross from the external environment. Battery power can be saved by turning the device off remotely when not in use. Thus, working instrumentation has been successfully maintained in common marmosets for up to 2 years (Crofts *et al* 2001). Such devices have been used successfully in non-human primates to collect cardiovascular, blood pressure, temperature, motor, vocalisation, locomotion and pH data, and to record electrocardiograms (ECG), electromyograms (EMG), electroencephalograms (EEG) and electrocorticograms (ECoG) (Kinter & Johnson 1999). The data obtained by this method can be used in a diversity of experiments in which behaviour and physiology may be monitored with or without manipulations. For example, telemetry has been used to detect diurnal rhythms in baboons (Stark *et al* 1999)

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and squirrel monkeys (Robinson & Fuller 1999a, b). It has also been used to determine the aetiology of cardiovascular changes occurring in anticipation of locomotion in baboons (Smith *et al* 2000) and in response to social interaction in rhesus macaques (Aureli *et al* 1999), and social challenge in common marmosets (Gerber *et al* 2002a, b). After implantation in both mother and foetus, radio telemetry has been used to assess the development of neurobehavioural, growth and circadian rhythms in neonatal baboons (Mirmiran *et al* 2001). Studies have also been carried out to determine normative values for heart rate (Schnell & Wood 1993; Kaufman & Detweiler 1999) and ECG (Schnell & Wood 1993; Kaufman & Detweiler 1999), blood pressure (Schnell & Wood 1993), sleep patterns (Crofts *et al* 2001) and body temperature (Cilia *et al* 1998). The information obtained using this technique can also be used for example to accurately determine the effects of new procedures or drugs (Horii *et al* 2002), to examine psychological (Novak 2003) and physiological conditions (Kerl 1997) or to determine the relative stressfulness of everyday husbandry procedures (Kerl & Rothe 1996; Schnell & Gerber 1997; Bowers *et al* 1998; Gerber *et al* 2002a, b). The critical advantage of the technique is that all measurements are taken from animals that can be group housed and maintained in their home environment without the need for handling or restraint. For example a fully implanted system described by Schnell and Wood (1993) was used to measure blood pressure, heart rate and motor activity in conscious, unrestrained group-housed marmosets over a period of 11 months.

Summary of the costs and benefits of the use of telemetry

The costs and benefits of the use of telemetry in animal studies are discussed in detail in reports on the use of telemetry by the BVAAF/FRAME/RSPCA/UFAW Joint Working Group on Refinement (Morton *et al* 2003; Hawkins *et al* 2004). In these reports it is stressed that the use of telemetry in laboratory-housed animals will always have some negative impact and the costs and benefits of its use must be continually reviewed throughout studies. There is no doubt that, in comparison with early efforts, modern fully implanted telemetry devices are considerably improved. Also, after the implantation procedure, no handling is required and data can be obtained from 'resting' animals rendering the system less intrusive than traditional methods of data collection (Einstein *et al* 2000). This in turn increases the accuracy of data collection, as physiological parameters are unaffected by changes associated with restraint to obtain the sample. For example Schnell and Gerber (1997) found that the heart rates of macaques and marmosets trained to accept restraint for sampling were at least 100 beats per minute faster than those measured in unrestrained telemetered animals in their home cage. Blood pressure was also lower in telemetered animals. The use of telemetry can significantly reduce the numbers of animals required to obtain reliable results, because of such reductions in stress-related variation (Brockway *et al* 1993). It is also possible to use animals for more than one study without the need for further intervention.

Despite these benefits, the need for and extent of surgery required to implant the devices in the first place cannot be ignored and, in primates, re-use of instrumented animals is common because of their relative value, particularly if they are trained for operant tests. These animals may be re-operated in order to replace batteries or other components during the course of an experiment or series of experiments (eg Gerber *et al* 2002a). Schnell and Gerber (1997) found that marmosets involved in mild drug studies showed elevated HPA activity in comparison to baseline measurements despite the use of telemetry to minimise the intrusiveness of these studies. Thus it was demonstrated that the repeated re-use of animals in even very non-intrusive experimental protocols may introduce a certain degree of stress and therefore unwanted variation into studies. Fully implanted telemetry devices have been found to significantly increase the reactivity of the sympathetic nervous system in mice (Einstein *et al* 2000). It is likely that such effects of carrying telemetry devices are less in primates because the devices used are comparatively smaller in relation to the size of the animal, however such chronic effects cannot be discounted. Although the development of fully implantable devices has facilitated group housing of telemetered animals, many users may continue to favour single housing because the majority of telemetry devices transmit on the same frequency (Hawkins *et al* 2004). Hawkins *et al* (2004) recommend that rather than single housing telemetered primates, they be housed in compatible pairs with only one pair-mate being telemetered or that the animals should be group housed and that data should be collected in turn from each device whilst the other devices are turned off. Significant benefits of using radio-telemetry and the results that can be gained from these studies must be seriously considered with respect to the considerable costs to the animal.

Sonophoresis

Cook *et al* (2000) reported the development of a system of sonophoresis, an innovation with the potential to considerably reduce the need for minor sampling procedures on laboratory animals. This sampling method works on the basis that low-frequency ultrasound can increase the permeability of the skin to drugs and high molecular weight proteins. Thus, movement of blood constituents could be induced in the opposite direction from blood to skin surface, by applying an osmotic gradient to the skin and increasing skin permeability using sonophoresis. The method has been used to successfully measure glucose in humans (Tamada *et al* 1995) and cortisol, testosterone, insulin, 17β estradiol and glucose in humans and sheep (Cook 2002). In order to collect a sample, a small quantity of ultrasound gel is applied to the skin and the transdermal collection device is held against it for a total of two minutes (Cook 2002). Results indicate that the concentrations of parameters measured by transdermal collection were comparable with those measured in blood, were higher than those in saliva, and did not encompass the time lag (10-20 min) associated with salivary hormone responses (Cook 2002). Human

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volunteers reported that the methods did not cause any pain and thus, frequent sampling is facilitated (Cook *et al* 2000). Although the time taken to obtain samples using this method is comparatively long, primates could be trained to accept this sampling technique, especially as it does not cause pain.

Section Summary

• Venous blood samples are the most commonly taken sample from primates used in research.

• Adverse effects of blood sampling are likely to be more severe if taken arterially. Cleanliness is essential during both venous and arterial sampling.

• Evidence indicates that stress associated with blood sampling can be greatly reduced or eliminated by the use of PRT.

• The most appropriate sample site varies with species.

• Some parameters can be measured in urine, faeces and saliva, as well as in blood,

• The invasiveness and intrusiveness of sampling by these methods can be reduced by PRT.

• Biotelemetry can be used to collect physiological data remotely without disturbing the animal, which reduces stress associated with the experiment and, because accuracy is increased, reliable results can be obtained using fewer animals.

• Implanted telemetry devices allow group housing and are therefore a refinement over those requiring single housing.

• The most extensive results have been obtained using telemetry devices that are implanted surgically. Thus, there is a conflict between the reduction of animal numbers and the severity of the measurement technique.

• Sonophoresis is a non-invasive technique by which an extensive range of blood parameters can be measured. It has potential for use in primates and could be further refined using PRT.

Administration

The selection of the species, substance to be administered and the route of its administration is determined to a great extent by the objectives of the experimental procedure concerned. However, certain basic practices must also be considered in order to ensure that the principle of minimum harm is upheld. A complete review of refinement techniques for administration of substances has been provided by the BVA (AWG)/FRAME/RSPCA/UFAW working party (Morton *et al* 2001). Only an overview of the main points will therefore be presented here.

The nature of the selected species must be considered if, for example, multiple administration sessions are to be required. Certain species (for example marmosets (Poole *et al* 1999) are known to be particularly stressed by restraint and, due to their small size, may be more difficult to train for intravenous (iv) administration and other administration procedures than other primates. The expertise of staff must also be considered and training and advice sought in order to ensure that staff are capable of handling the animal, administering the substance, recognising adverse effects and initiating emergency procedures should unexpected effects or severity occur (Hau 1999). If the effects of any programme of administration are unknown, the use of pilot studies to inform on potential consequences must be considered (CCAC 1998; Wong 1998; Morton 1999; Wallace 1999). Commercially available drugs (or medicines) are supplied in a form considered suitable for the route of administration such that both active substance and its vehicle are appropriate. However, if a substance is experimental or a commercially available substance is to be administered by an unconventional route, the suitability of both the substance, the vehicle and their interaction must be considered with respect to the species concerned (Morton *et al* 2001). Non-compatibility has the potential both to increase the severity of the procedure and to adversely affect results. The solubility, viscosity, pH, irritancy, stability and purity of the substance must be considered and may be affected by the frequency of administration, dose and concentration and therefore the volume to be administered. Some routes of administration are more stressful than others (Morton *et al* 2001). Generally the administration of substances by oral or iv routes are likely to cause less irritation than administration into tissues, including intraperitoneal (ip), subcutaneous and intramuscular injections (Morton *et al* 2001). A summary of the severity of different routes of administration is provided by Morton *et al* (2001). Most routes of administration have the potential to be refined or can be refined by using the least severe method of administering by that route. For example, intra-articular, intra-cerebral and intra-tracheal routes of administration should only be used under anaesthetic. If the primates are not trained to accept the dose, the requirement for restraint during administration by, for example, iv, subcutaneous, intramuscular and oral routes of administration, increase the overall severity of the procedure. Thus training to voluntarily present for injection (eg Wolfensohn & Honess 2005) or even habituation to the restraint device (Reinhardt *et al* 1991) would greatly reduce the severity of the procedure. Oral administration by gavage is considered to be of medium severity (Morton *et al* 2001), partially because of the need for restraint in order to achieve administration and also because poor placement of the tube has the potential to harm or kill the subject animal. However, in rats, Wheatley (2002) found that the technique could be refined using flexible catheters and a fixed multidirectional dosing apparatus which prevented movement of the tube once placed and allowed accurate dosing of substances. This refinement is likely to apply to primates also. Extra care must be taken when using oral gavage in marmosets as their teeth are delicate and are easily damaged (Poole *et al* 1999). Alternatively substances could be incorporated into feed or fluids and offered to the animal, a method considered to have minimal impact on the animal (Morton *et al* 2001). However, care should be taken with the use of this method of administration as aversion to common laboratory feeds could result if an association between the consumption of

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the feed with subsequent adverse effects was learned (Matsuzawa & Hasegawa 1983). This response could subsequently be passed on socially to other group members (Hikami *et al* 1990).

Section summary

• Species-specific considerations and staff expertise must also be considered in the design of the experiment.

• Pilot studies should be carried out to evaluate methods if novel substances are to be administered.

• The vehicle, viscosity, solubility, pH, irritancy, stability, purity, frequency of administration, dose, concentration and volume of the substance must be considered, particularly when novel substances are administered.

• Intra-articular, intra-cerebral and intra-tracheal administrations are considered most stressful and require anaesthesia.

• Restraint is likely to result in stress and PRT should be used to reduce the stress of administration and the associated restraint.

Surgery

Surgical procedures should be carried out under anaesthesia, using the best available animal care techniques. During the design phase of each experiment, the timing, surgical technique and the use of analgesia (pre- and post-operatively) and anaesthesia should be carefully considered with reference to the best available advice in order to prevent or alleviate adverse effects. The availability of technical staff to oversee recovery must always be ascertained before surgery begins and training in post-surgical treatment must be given. The use of anaesthetics and analgesics is beyond the scope of this review but information on anaesthesia and analgesia is available elsewhere (eg see Flecknell 1996, 2000; Flecknell & Waterman-Pearson 2001) for overviews for all laboratory animals.

Refinement of experimental endpoints

In studies of toxicity, vaccines and disease, the induction of potentially severe pain and/or distress is often prerequisite (Goldberg 2000). Lethality or moribundity are accepted or required endpoints by law in procedures used to test and licence new chemicals and vaccines that are carried out on primates (Council of Europe 2001). Pain and distress are rarely treated in these studies because of the potential for interactions with the test compound (Stokes 2000a). Alternative procedures with less severe endpoints are being developed (Lipnick *et al* 1995) and it is widely recognised, in the laboratory animal welfare research community, that the incorporation of earlier endpoints into the experimental design of toxicological experiments would constitute significant refinement and would greatly improve welfare.

Non-rodent mammals used in testing is expected to be a more accurate model of the effects of a chemical in humans (Sass 2000). Primates are considered to be particularly useful models for human responses because their anatomy and physiology are very similar to that of humans and they share 85-92% of human DNA (Garg 2000). Primates are mainly used in studies of reproductive toxicology, immunity (vaccines) and disease (primarily parasitic). The UK, France and Germany are the three principle users of non-human primates in research (Council of Europe 2003; Rennie & Buchanan-Smith 2005). Forty percent of non-human primates used in science in Europe in 2001 were used in toxicology and safety evaluations (Council of Europe 2003; Rennie & Buchanan-Smith 2005). Thus, the identification, validation and use of earlier endpoints which apply specifically to primates are of the utmost importance and should be considered a priority in these species.

The European Directive (86/609/EEC) dictates that when it is deemed necessary to use animals in scientific procedures arrangements should be made to prevent the occurrence of pain or avoidable suffering, distress or lasting harm (EU 1986). Thus, where more humane endpoints are fully validated and accepted, their use is a legal obligation. This is not reflected in some monographs of the Council of Europe (2001) in which both death and alternative more humane endpoints are acceptable.

Definition of humane endpoints and the principles of their use

In November 2000 the Organisation for Economic Co-operation and Development (OECD) published guidelines on the use of humane endpoints (OECD 2001). In this document the term 'humane endpoint' was defined as "the earliest indicator in an animal experiment of severe pain, severe distress, suffering, or impending death" (OECD 2000 p 10).

Ultimately the aim of endpoint studies is to identify endpoints that accurately predict death, pain or distress as an outcome (Wallace 1999; OECD 2001), so that results may be obtained and the experiment terminated **before** the animal experiences these conditions. It must be recognised that, at present, we have limited ability to predict the outcome of toxicological tests before the onset of pain or disease. It is therefore recommended that, until criteria are validated that can be used to predict prognoses, every effort must be made to use currently available tools to identify pain, distress or suffering at, or as soon as possible after, onset (OECD 2001) and steps must be taken to alleviate pain, distress or suffering at this stage. The early identification of these conditions will allow measures to be taken to minimise their effects.

Determination of more humane endpoints

Important criteria for the determination of humane endpoints are discussed in great detail in the literature and are recommended in the OECD guidelines on the use of humane endpoints (OECD 2001). Only a summary of the main points will be described here.

Humane endpoints must be considered in the context of the experiment. The potential benefits of the experimental outcome must be considered sufficiently important to warrant the induction of an expected degree of pain and distress in the animal subject and for the proposed experiment to go ahead. In order to effectively weigh up both scientific and welfare consequences of a study, the specific

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experimental objective must be carefully considered and clearly defined (Wallace 1999). The criteria that will be used to indicate that the experimental objective has been reached (scientific endpoints) must also be defined (Wallace 2000).

The experiment should be terminated as soon as the scientific objective has been attained (OECD 2000). Thus the earliest possible endpoint is used without jeopardising the objective of the study, and therefore reducing its benefits. It is also necessary to consider how the endpoints themselves will be detected and whether the assessment of endpoints alone will add to the severity of the experiment (Wallace 1999) (eg if more blood samples are required or the means of measurement requires an operation (telemetry)). The least invasive means of detecting an endpoint must always be chosen (Olfert & Godson 2000).

Humane and scientific endpoints must also be specific to the study species. Differences in anatomy and physiology are likely to result in variations in response to experimental challenges (Richmond 1999). Also species-specific diurnal and seasonal rhythms will influence biochemical and behavioural patterns (Scharmann 1999). It must also be remembered that the time course of the response will vary between individuals of the same species (Richmond 1999).

In order to select species-specific, scientific and humane endpoints, the results of previous studies, using the test parameter or related parameters, must be examined where that information is available. Consultation with staff and outside experts may also be an essential source of information (Richmond 1999). However, in toxicological research, the outcome of a particular testing procedure may be unknown and in many cases the determination of effects is the objective. In these cases, the use of pilot studies with a small number of animals, examined intensively throughout the trial period has been strongly recommended (CCAC 1998; Wong 1998; Morton 1999; Wallace 1999). In pilot trials endpoints can be determined and experience of their use gained; morbidity, time course of effects, inter-sample intervals and monitoring intervals can all be assessed. In this way the earliest indicators of toxicological effects, dose rates, time course and unexpected outcomes of testing can be determined.

Detection of endpoints

In the majority of scientific manipulations a 'normal' healthy animal with potentially good welfare is compromised such that its state deviates from normal (Morton 2000). In order to use both scientific and humane endpoints effectively, we must in some way be able to recognise when an animal's condition has deviated from normal (Morton 1999). Further, the ability to assess the extent of this deviation provides us with a measure of the severity of the manipulation (Morton 1999). The assessment of the effects of experimental procedures on laboratory animals and thus recognition that endpoints have been reached must be carried out without the benefits of verbal self-report of pain and distress. The use of unprovoked and provoked behaviour, clinical signs (observable physical indicators) and biochemical markers may be objectively recorded and used to assess deviations from normal. The measurement of all of these parameters must be based on sound background knowledge of what is normal for that species and individual.

The occurrence of certain behaviours, clinical signs and biochemical markers are non-specific in that they occur in response to many experimental manipulations. These include general sickness behaviours like anorexia, reduced movement and increased sleeping, weight loss (as a result of anorexia), hyper- and hypothermia and variations in systemic cortisol (general measure of the activation of the endocrine stress response). Changes of a certain magnitude in these parameters, for example persistent anorexia causing 10% weight loss or a reduction of body temperature by 4-6ºC, can be used as reliable endpoints (Wallace 1999, 2000; Farah *et al* 2001) indicating presence of general illness. However, signs of this sort cannot be used to detect effects on specific organ systems, nor can they be used to discriminate between pain, distress or illness. More specific signs of the onset of effects may include guarding behaviour in response to manipulation of a limb, changes in quality of faeces (eg diarrhoea or presence of blood), cramping of limb or stomach, and biochemical markers of organ damage or disease (Poon & Chu 1999).

Validation of endpoints

In order to be scientifically and legally accepted, novel endpoints must be validated against established endpoints to ensure that the same degree of scientific relevance and to ensure inter- and intra-laboratory reproducibility (Morton 2000; Stokes 2000b). Schlede *et al* (2000) reviewed the clinical signs leading up to death in acute toxicity studies incorporating 4000 rats. Analysis indicated that animals showing convulsions, lateral position, tremor, gasping and vocalisation died in over 90% of cases. Thus these signs could be used as reliable alternative endpoints. Olfert *et al* (1998) questioned the use of death as an endpoint in the study of disease. They argued that by using death as an endpoint and not monitoring clinical signs as the disease progresses, significant differences between treatment groups may be missed. This view is in accord with the views of Mench (1999) who argues that, particularly in chronic studies, death may not in fact be caused by the experimental variable, but is simply a secondary result caused by dehydration or starvation. If the objective of the study is not to assess the effects of a variable but to determine whether or not it is toxic or disease is induced, much earlier endpoints may be used. In disease research, the rate of release of acute phase proteins has proven to be prognostic of the severity of infection and can therefore be used as a reliable alternative endpoint very early after infection (Olfert *et al* 1998). Once established, it is critical that the use of endpoints is retrospectively assessed to ensure that they are relevant to the study in hand and that the results of such analysis are reported (Fentener van Vlissingen *et al* 1999).

Monitoring for endpoints

Many protocols are regularly used by personnel in laboratories to structure the monitoring procedure. One of the most

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frequently used in the UK is the clinical observation sheet in which possible signs of abnormality prompt the observer (Hawkins 2002). Score sheets listing clinical signs specific to the experiment are also commonly used (Hawkins 2002). The presence or absence of a sign or a score of the severity of the sign is recorded during every scheduled check providing an accurate record of changes in the condition of subjects. A method of structured assessment of pain and distress was described by Morton and Griffiths (1985) in which the animal is initially observed from a distance before being approached and finally examined clinically. Morton later describes how these principles of assessment can be incorporated into the design of score sheets including both general and specific clinical signs (Morton 1999, 2000). This method provides a valuable and practical tool to ensure that the assessment of laboratory animals is objectively and clearly recorded.

The assessment of pain and distress in animals and how to record it requires training. Observers must have extensive knowledge of the boundaries of normal behaviour of animals, species and individuals on the study. They must also be trained to recognise the critical signs indicating pain and distress (Hau 1999). All members of the animal care team (veterinarian, scientist, animal care worker) must be aware of their roles and responsibilities. Further, they must be aware of both the scientific and humane endpoints of the study and have the power and skill to execute the designated treatment should the endpoints be reached. All personnel must be aware of contingency plans in the event of unexpected pain and distress (Hau 1999).

Variations in the amount of abnormal behaviour recorded between establishments reflect differences in training and therefore differences in the ability to detect endpoints in the experimental situation (Schlede *et al* 2000). This has implications for the welfare of the animals concerned.

In order to ensure that humane endpoints are not overstepped, observations may need to be carried out more frequently than when death or moribundity is the endpoint. During critical periods of time (eg the first 24 hours after a procedure has begun) the frequency of observations must be further stepped up (Schlede *et al* 2000). The frequency of observation required may be determined during pilot trials.

Use of earlier endpoints in practice

In practice the use of humane endpoints is limited, particularly in the testing of chemicals for use in humans. Hendriksen conducted an informal and unpublished survey of research institutions that tested vaccines (Cussler *et al* 1999) and found that many establishments were found to use death as the preferred endpoint when a less inhumane alternative was legally acceptable (Council of Europe 2001). Specific descriptions of the endpoints used in research on primates are unusual in the literature. The reasons for this are unclear although it is likely that the need for endpoints that are specific to the experiment in hand and the sensitivity of the use of primates in research are contributing factors.

Endpoints can be used to significantly minimise the effects of experimental manipulation and the welfare of laboratory primates may be compromised when the use of endpoints is disregarded. The following two papers illustrate these points. Farah *et al* (2001) describes the use of rodent and primate models of schistosomiasis and describe methods by which the procedures can be refined and alternatives incorporated. Farah *et al* (2001) recommended the use of the baboon as the main model of schistosomiasis infection in humans, because of ethical restrictions on the use of chimpanzees. Numerous limits were recommended for use in experimental manipulations. Farah *et al* (2001) maintained that, although few animals display clinical signs of disease, certain limits should be applied to studies. These included restrictions on the level of experimental infection (1000 cercariae per 5 kg baboon), dosing (several small doses of cercariae reduced morbidity and was a better model of natural infection than one large dose), and restrictions on the frequency of blood samples and liver biopsies. Treatment with antibiotics was provided when clinical signs of disease occurred. The recommendation that animals be humanely killed using an overdose of barbiturate if the occurrence of severe dysentery (blood in faeces) and abdominal cramping was not alleviated by 5 days of treatment. The same was recommended if weight loss reached 10% of the starting weight.

A second paper by Atzpodien *et al* (1997) described oral safety toxicity studies of a novel non-steroidal anti-inflammatory drug (NSAID) 'Lornoxicam', using wild-caught, long-tailed macaques, in both a 6-week dose finding study and a 52-week study with a 4-week recovery period. In the introduction to this study it was explained that Lornoxicam had potentially greater anti-inflammatory, anti-pyretic and analgesic activity than other NSAIDs. The well-known potential complications of the use of NSAIDs were also described, indicating that gastro-intestinal (GI) mucosa erosions and ulcerations and kidney complications were all possible and that in serious cases, in predisposed patients, these effects could cause death. In the methodology, a description of the clinical signs of NSAID treatment was provided, including blood in faeces and weight loss associated with anorexia. The objective of the experiments was simply to evaluate the oral toxicity of the drug and no humane endpoints were defined. The animals were to be humanely killed at the end of the trials and autopsies performed. The animals were observed twice daily for novel clinical signs and for mortality. Clinical signs of NSAID toxicity were present in the monkeys in the dose-finding study, including faecal blood, diarrhoea, hypoactivity, anorexia, and weight loss, indicative of GI lesioning. Four animals died as a result of toxicity, two of them from gut perforation, and all these animals showed blood in the faeces before they died. Despite this the authors claimed that no clinical signs of toxicity had been present. In the 52-week study no clinical signs of disease were observed despite GI lesioning found during autopsy. These lesions healed with NSAID withdrawal in a small group of animals during 4 weeks of recovery. The results of the 52 week trial

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were compromised because the wild-caught subjects were infected with GI parasites, despite anthelmintic treatment at the start of the study. A number of GI lesions with associated parasites were identified at autopsy. However, autopsies on the recovered animals, allowed discrimination of the extent to which lesions were caused by the NSAID and not the parasites. The contrasting use of endpoints in these two European studies is dramatic and is illustrative of the variation in the use of more humane techniques in science across Europe.

Barriers preventing use earlier endpoints

The principal barrier preventing the more widespread uptake of earlier endpoints in research is the potential cost. The standard of training required to recognise the critical signs that indicate that endpoints have been reached is much higher than that required to recognise death or moribundity. Also the detection of earlier endpoints requires more frequent checks and there is an associated increase in the workload required to accomplish these checks (Cussler *et al* 1999).

Novel endpoints must be approved for use by the OECD (Schlede *et al* 1999). Validation and licensing of alternative, more humane endpoints is expensive and time-consuming. The financial and time costs of this procedure are sufficient to deter scientists from undertaking this essential work (Hendriksen & Steen 2000). To make matters worse, there is considerable prejudice against endpoints validated in other laboratories or countries. The result is that the same clinical signs are interpreted and described in different ways by different authors and in different countries and validation is repeated unnecessarily (Hendriksen 1999; Morton 1999; Schlede *et al* 1999). In order to reduce barriers preventing the use of humane endpoints, international harmonisation of training (Hau 1999) and the validation and use of clinical signs is needed (Fentener van Vlissingen *et al* 1999). Published work on the use of animals in science should describe the endpoints used and how they were validated. Welfare problems encountered should also be described (Richmond 1999).

Section summary

• Currently death and moribundity are legally acceptable endpoints in obligatory toxicity and safety tests, and pain and distress are not routinely treated in these studies.

• Toxicology and safety evaluations are one of the main uses of primates in research.

• Humane endpoints are defined as the earliest indicator of pain, distress or ensuing death.

• Use of more humane endpoints allows animals to be treated and reduces the need for euthanasia.

• Severity of endpoints should be taken into account in cost/benefit analysis and the most humane methods of detection should be used.

• Endpoints must be species- and experiment-specific.

• In novel studies advice on endpoints should be sought and, if no information is available, pilot studies should be used to identify suitable early endpoints.

• Endpoints may be detected using general clinical signs or indicators specific to the study.

• The scientific validity of novel endpoints must be determined.

• Detection of more humane endpoints requires more detailed and more frequent monitoring and more extensive staff training.

• In practice the use of more humane endpoints varies considerably between studies.

• Barriers preventing wider uptake of more humane endpoints include perceived financial and time investment, the need for validation, unwillingness to accept novel endpoints and poor dissemination of information.

Recommendations

• Reduced tolerance of death and moribundity as endpoints where alternatives exist.

• Validation guidelines or better dissemination of methods to widen acceptance of externally validated endpoints.

• Guidelines for ethical review committees to ensure Europe-wide consistency in the use of more humane endpoints.

• A legal requirement for research to identify, validate and then use more humane endpoints.

Refinement of euthanasia

Euthanasia of laboratory animals may be required if animals are found to experience an unexpected level of pain or suffering during procedures or at the end of an experiment (EU 1986). The European Directive does not specify methods of euthanasia, but states that an animal should be 'killed by a humane method' (EU 1986). The definition of a 'humane method of killing' is 'the killing of an animal with a minimum of physical and mental suffering, depending on species' (EU 1986). In the European Convention (European Commission [EC] 1986), training of staff in methods of euthanasia is recommended. In guidelines recognised across Europe, overdose of anaesthesia is the only method that is considered acceptable for non-human primates (IPS 1993; Close *et al* 1997; Baskerville 1999; Bearder & Pitts 1999; Erkert 1999; Fritz *et al* 1999; Mendoza 1999; Poole *et al* 1999; Visalberghi & Anderson 1999; Beaver *et al* 2001).

In order to ensure that euthanasia is carried out humanely it is generally recommended that unconsciousness should be induced as quickly as possible, should occur prior to the loss of motor function and that no signs of pain, distress or panic should be observed (CCAC 1993; IPS 1993; Close *et al* 1997; Home Office 1997; Beaver *et al* 2001). For these reasons the most commonly recommended method of euthanasia for laboratory primates is iv injection of barbituric acid derivatives (mainly sodium pentobarbitone but also thiopentane and secobarbital) (Baskerville 1999; Bearder & Pitts 1999; Erkert 1999; Fritz *et al* 1999; Mendoza 1999; Poole *et al* 1999; Visalberghi & Anderson 1999). The use of ketamine anaesthesia prior to euthanasia is also recommended to minimise necessary restraint, whilst ensuring that the injection is successfully administered (IPS

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1993; Erkert 1999; Poole *et al* 1999; Beaver *et al* 2001; Fortman *et al* 2002). Euthanasia of Old World monkeys by ip injection of sodium pentobarbitone was recommended as an alternative to iv administration by Baskerville (1999). Close *et al* (1996) recommended that ip injection may be simpler to perform and the use of this route may reduce stress caused by extended handling. However, elsewhere the ip route of administration is only recommended for the administration of relatively large volumes of non-irritant anaesthetics to laboratory rodents, because other routes are potentially less hazardous and are more easily accessible, especially in larger animals (Morton *et al* 2001). Ip administration is also likely to cause more pain than iv administration as it is necessary to puncture the peritoneum.

Overdose of barbiturates causes death by inducing descending depression of the central nervous system (CNS), starting from the cerebral cortex, resulting in loss of consciousness and anaesthesia (Beaver *et al* 2001). Death in the unconscious animal occurs as a result of cardiac arrest when respiratory and cardiovascular centres are depressed (Beaver *et al* 2001). The onset of effects occurs very rapidly after iv administration (Close *et al* 1996) and death is induced smoothly (Beaver *et al* 2001). Sodium pentobarbitone is also cheap and is therefore very widely used (Beaver *et al* 2001) although in some countries it can only be obtained under licence (Close *et al* 1996). However, concern has been expressed about the indiscriminate use of pentobarbitone for euthanasia, particularly if administered extravascularly (Ambrose *et al* 2000).

In pharmacological literature, evidence of hyperalgesic effects of sub-anaesthetic doses of barbituric acid and its derivatives were first reported in humans over 40 years ago (Dundee 1960; Neal 1965). The conclusions of these studies have been confirmed by more recent work in mice (Carmody *et al* 1986; Carmody *et al* 1991a, b), rats (Ossipov & Gebhart 1984) and in rhesus macaques (Hori *et al* 1984). It has been concluded that the occurrence of hyperalgesia following administration of low doses of barbiturates is the result of a mechanism involving GABA receptors in nerve cells in the spinothalamic tract (Carmody *et al* 1986). It has further been concluded that at sub-anaesthetic doses hyperalgesia is likely to occur after administration of any drug which facilitates the movement of chloride at the $GABA_A$ receptor/chloride channel complex (Ewen *et al* 1995). Following pentobarbitone administration at doses as low as 20% of the anaesthetic dose, nociceptive sensitivity has been reported to double (Carmody *et al* 1986). In rats, ip administration of sodium pentobarbitone for euthanasia caused writhing and induced inflammation (Wadham cited in Ambrose *et al* 2000), two classically recognised responses to known irritants. Indeed, the ip administration of any irritant chemical can cause pain, swelling and adhesions (Morton *et al* 2001). Close *et al* (1996) also recognise that sodium pentobarbitone can cause irritation of the peritoneum, but note that irritation can be reduced by diluting the drug. Following ip administration of any drug, there is some temporal delay before an efficacious concentration of the drug reaches the blood (although after ip administration absorption is relatively rapid). Thus, for a limited period of time (dependent on dosage and size of animal) after ip administration of sodium pentobarbitone, the concentration of the drug in the blood will be below that required to induce anaesthesia. This will be exacerbated by dilution of the drug. Thus ip administration of sodium pentobarbitone has the potential to cause pain or irritation as a direct result of the injection and also to cause hyperalgesia as a result of sub-anaesthetic doses of the drug reaching the blood. The same argument is true if iv administration of the drug is attempted but some, or all, of the dose is administered outwith the blood vessel as a result of poor restraint, handling or technique. This evidence supports the assertion that sodium pentobarbitone should be only be administered intravenously and that ketamine anaesthesia should be used to facilitate its administration (IPS 1993; Erkert 1999; Poole *et al* 1999; Beaver *et al* 2001; Fortman *et al* 2002).

Chloral hydrate and T-61 are euthanatising agents which could be used to induce death in laboratory primates. However chloral hydrate induces unconsciousness more slowly than barbiturates, increasing the potential requirement for restraint and resulting in more muscular spasms (Beaver *et al* 2001). T-61 is a combination of an anaesthetic, a hypnotic and a curariform drug. There is some debate concerning the possibility that this agent may induce respiratory arrest before unconsciousness and for this reason the drug is banned in the USA (Beaver *et al* 2001). However, there is also evidence that loss of consciousness and muscle activity occur simultaneously (Hellebrekers *et al* 1990), making the use of the drug more acceptable. Thus T-61 is still available in Canada and Europe (except Sweden) and is not restricted by the same controls as barbiturates, making it more easily available (Close *et al* 1996). T-61 must be administered intravenously and care must be taken not to inject the agent too quickly as this can cause pain (Beaver *et al* 2001). The disadvantages of the use of chloral hydrate and T-61 are generally considered to exceed those of barbiturates (Beaver *et al* 2001) and sodium pentobarbitone remains the most acceptable method of euthanasia (Close *et al* 1996).

When specific tissue samples are required for the experimental technique, inhalation anaesthesia followed by exsanguination and perfusion has been reported to be an acceptable method of euthanasia of primates (Close *et al* 1997). Refinement of inhalation anaesthesia for primates is discussed in detail by Morton *et al* (2001) and is summarised in the 'administration' and 'restraint' sections above. The procedure may require short-term restraint, either manually or in a restraint chair and the use of specialised masks and anaesthesia equipment is necessary. Anaesthetic induction following inhalation of an anaesthetic agent is slower than that induced by direct administration into the blood (Beaver *et al* 2001). Volatile inhalant anaesthetics, including halothane, isoflurane, desflurane and enflurane may be used for euthanasia and induce unconsciousness reliably and rapidly (Close *et al* 1996). Death is

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caused by cardiovascular and respiratory centre depression and liver metabolism of isoflurane and enflurane is only slight making them particularly useful in toxicology studies (Close *et al* 1996). However, some of these agents smell strongly and may cause breath holding, thus delaying onset of anaesthesia (Beaver *et al* 2001). Further, evidence suggests that both halothane and isoflurane can cause hepatotoxicity, especially if used repeatedly in close succession (De Groot & Noll 1983; Brunt *et al* 1991), but this should not affect their use as euthanatising agents. The stress of this method of euthanasia may be minimised if the animals are competently handled and carefully trained to accept the required restraint, mask and the sound of rapidly flowing gas (Morton *et al* 2001). Adequate training may take up to three weeks and unless the animals are trained to accept the procedure for other experimental purposes, the financial and time investment in training for the one-off procedure of euthanasia is likely to be considered too high to be feasible. After anaesthesia has been induced, careful monitoring is essential to ensure that an adequate level of anaesthesia is maintained until death from exsanguination is confirmed. The use of this procedure should be limited to circumstances where the collection of perfused tissue is absolutely necessary for the attainment of the experimental objective. The training of staff is essential to achieve euthanasia by this method.

Section summary

• Euthanasia of laboratory-housed primates is required if the animals experience pain or suffering beyond that necessary to reach the agreed objective of the study and in some cases at the end of experiments.

• European Directive (86/609/EEC) (EU 1986), requires that the most humane methods of euthanasia are used for all animals used in science, but does not specify acceptable methods.

• The administration of the euthanatising agent should result in rapid loss of consciousness, before death ensues. There should be no evidence of pain or distress.

• Overdose of anaesthetic is the only method considered acceptable for primates. Ketamine can be used to minimise the need for restraint during administration.

• Iv, but not ip administration of barbiturates is recommended.

• Chloral hydrate and T-61 are not recommended.

• Inhalation of anaesthetics is used in studies where perfused tissue must be collected but is not recommended for routine use.

• Stress prior to euthanasia may be minimised by training primates to co-operate with relocation to the procedures room and with the process of restraint.

• Staff administering euthanatising agents must be extremely competent, for their own and the animals' safety.

Re-homing – an alternative to euthanasia

Re-homing or retirement is an alternative to indiscriminate euthanasia of laboratory-housed primates on completion of non-lethal studies or when their existence in the laboratory is no longer required. As the use of great apes in research has become unacceptable in Europe, retirement homes have been found for all the laboratory-housed chimpanzees that were used in Europe. Indeed most of the emphasis on re-homing primates has centred upon this species (Brent *et al* 1997; Brent 2004). Re-homing of other species of laboratory-housed primate is also becoming an option (Seelig & Truitt 1999). Once the decision to re-home the animals has been made, it is imperative that the suitability of potential homes is rigorously assessed to ensure that standards of housing, husbandry, staff training and veterinary care are good and that the financial stability of the establishment is secure. There is no European legislation to protect re-homed primates, although each country may protect the animals in national legislation. For example in the UK, any person or persons keeping animals listed under the Dangerous Wild Animals Act 1976 must have a licence. The licence is only granted if the licensing authority is satisfied that the premises and potential licensee are suitable. The animals are also protected under the Protection of Animals Act 1911 and the Protection of Animals (Scotland) Act 1913 which makes it illegal to carry out acts of cruelty towards animals. If a sanctuary intends to allow members of the public to visit they must also be licensed under the Zoo Licensing Act 1981 and are subject to assessment by zoo inspectors and the animals should be kept in accordance with the Standards of Modern Zoo Practice. European Directive 1999/22/EC similarly requires that if animals kept in captivity are to be viewed by the public they must be licensed and require that the animals are kept in accordance with conservation strategies and housing conditions. As the number of primates retired to sanctuaries from research establishments grows, the need to regulate sanctuaries more carefully is also increasing (Seelig & Truitt 1999)

Conclusions and animal welfare implications

Refinement of the use of primates in scientific procedures can be achieved by avoiding, alleviating or minimising the adverse effects of experimental procedures and by maximising well-being. This document provides a sample of the types of refinements that could be incorporated into routine and experimental protocols. With careful use, based upon knowledge of species-specific needs, these refinements have the potential to greatly improve the welfare of laboratory-housed primates, whilst greatly reducing inhumanity in accordance with Russell and Burch's 3R's model (Russell & Burch 1992).

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