Comment

A Critical Review of Anaesthetised Animal Models and Alternatives for Military Research, Testing and Training, with a Focus on Blast Damage, Haemorrhage and Resuscitation

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Summary — Military research, testing, and surgical and resuscitation training, are aimed at mitigating the consequences of warfare and terrorism to armed forces and civilians. Traumatisation and tissue damage due to explosions, and acute loss of blood due to haemorrhage, remain crucial, potentially preventable, causes of battlefield casualties and mortalities. There is also the additional threat from inhalation of chemical and aerosolised biological weapons. The use of anaesthetised animal models, and their respective *replacement* alternatives, for military purposes — particularly for blast injury, haemorrhaging and resuscitation training — is critically reviewed. Scientific problems with the animal models include the use of crude, uncontrolled and non-standardised methods for traumatisation, an inability to model all key trauma mechanisms, and complex modulating effects of general anaesthesia on target organ physiology. Such effects depend on the anaesthetic and influence the cardiovascular system, respiration, breathing, cerebral haemodynamics, neuroprotection, and the integrity of the blood–brain barrier. Some anaesthetics also bind to the NMDA brain receptor with possible differential consequences in control and anaesthetised animals. There is also some evidence for gender-specific effects. Despite the fact that these issues are widely known, there is little published information on their potential, at best, to complicate data interpretation and, at worst, to invalidate animal models. There is also a paucity of detail on the anaesthesiology used in studies, and this can hinder correct data evaluation. Welfare issues relate mainly to the possibility of acute pain as a side-effect of traumatisation in recovered animals. Moreover, there is the increased potential for animals to suffer when anaesthesia is temporary, and the procedures invasive. These dilemmas can be addressed, however, as a diverse range of *replacement* approaches exist, including computer and mathematical dynamic modelling of the human body, cadavers, interactive human patient simulators for training, *in vitro* techniques involving organotypic cultures of target organs, and epidemiological and clinical studies. While the first four of these have long proven useful for developing protective measures and predicting the consequences of trauma, and although many phenomena and their sequelae arising from different forms of trauma *in vivo* can be induced and reproduced *in vitro*, non-animal approaches require further development, and their validation and use need to be coordinated and harmonised. Recommendations to these ends are proposed, and the scientific and welfare problems associated with animal models are addressed, with the future focus being on the use of batteries of complementary *replacement* methods deployed in integrated strategies, and on greater transparency and scientific cooperation.

Key words: *aerosolised biological weapons, anaesthetised animal models, animal welfare, chemical weapons, computer modelling, coordination, haemorrhage, harmonisation, increased transparency, mathematical modelling, military research, organotypic cultures, resuscitation training, surgical training, traumatic brain injury, validation*.

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Introduction

Overview of military animal use

The principal categories of animal use in military research and testing are: a) weapons testing for efficacy and developing safety measures (1); and b) the study of battlefield injuries (from both ballistic and blast incidents) to make more-effective weapons, and to improve the treatment of traumatised and injured victims via the provision of life support measures. Animals are also used for surgical and resuscitation training (Table 1; 2). This

Table 1: The use of animals in military research, testing and training

Category of use	Endpoint/mechanism	Notes
Weapons testing for efficacy and development of safety measures	Whole body destruction; organ damage; diverse histopathological, physiological and biochemical changes, some linked to biomarkers of exposure and effect (usually with terminally-anaesthetised animals)	Conventional weapons, especially explosive and penetrating devices; ultrasound; tasers; WMD (biological, nuclear and chemical); data also used for developing improved protection (e.g. Advanced Combat Helmet)
Study of battlefield injuries (from both ballistic and blast incidents)	Traumatic organ injury (often anaesthetised animals allowed to recover)	Especially to the brain (TBI; see Table 2), but also to other target organs, including the lungs, eyes, auditory system and GI tract; data also used to assess strategies for improving survival and recovery; most work undertaken with rodents, but some with NHPs
Surgical and resuscitation training and assessment of injury treatment	Surgically-induced haemorrhage models involving anaesthetised animals (some of which are allowed to recover)	Usually 'large' animals (Table 2) are used

review covers issues raised by the use of anaesthesia with animal models of blast-induced trauma, germ warfare (3, 4), haemorrhage, and wound healing, as well as resuscitation and surgical training (5, 6). It also discusses other scientific and animal welfare limitations of the animal models, as well as the many available alternatives. In addition, some recommendations are made concerning the use of anaesthetics and for optimising the future development, validation and greater deployment of non-animal approaches.

Undeniably, this area of laboratory animal experimentation is highly controversial, both for its covert nature and in view of the high potential severity of many of the procedures often involved. The use of animals for weapons testing, in particular, has prompted a series of emotive reports and commentaries (see, for example, 7, 8). On the one hand, it is claimed that the use of animals is essential for weapons development and testing, as well as in trauma and wound treatment and training, while, on the other, it is argued that experimenting on, and sacrificing animals, for the improvement of methods to kill other humans is inherently unethical, and should be banned. Indeed, animal rights pressure has had some success in stopping certain types of military experiments (9). The present review aims to provide a reasoned and objective assessment of the limitations of anaesthetised animal models for military use, and the extent to which non-animal methods can be used instead.

That the use of animals in military research remains controversial is illustrated by a Sunday Times article, which appeared in November 2012, entitled *MOD Blows Up Live Pigs*. The article stated: "*Live pigs have been blown up and monkeys exposed to anthrax in a series of live animal tests at Porton Down... In one [experiment], funded by the US Department of Defence, live pigs were exposed to lethal chemical warfare agents. In another, they were blown up by high explosives. In a third, marmoset monkeys were made to inhale anthrax. Those animals were anaesthetised, but conscious guinea-pigs were poisoned with VX, a toxic nerve agent*".

The extent of animal use

According to Animal Aid (http://www.animalaid.org.uk/h/n/campaigns/experiments/all/763/), the number of animal experiments undertaken for weapons research in British laboratories quadrupled between 1997 and 2007, from 4,500 to more than 18,000. In 2006, it was reported that the number of animals used in British military experiments had doubled in the five previous years (10). The official Home Office statistics for procedures conducted on laboratory animals in Great Britain do not show separate figures for experiments undertaken for military purposes (11). In the UK, most of this work is conducted at the Government's establishment at Porton Down, Wiltshire (now known as DSTL — Defence Science and Technology Laboratory; 12), on behalf of the Ministry of Defence (MoD). It is alleged that, in 2006, over 21,000 animals, including monkeys, ferrets and pigs, were used, representing a 76% increase in numbers since 2000 (www.animalliberationfront.com). Figures for 2011 reveal that almost 10,000 experiments were conducted on animals at DSTL, a number that showed an increase of 300 in one year, and of more than 1,000 in the two years since 2009 (see 13–16).

During verbal answers on the use of animals in defence research procedures, given by the

Parliamentary Under Secretary of State for Defence in April 2002 to the House of Lords Select Committee on Animals in Scientific Procedures (17), it was noted that, while training exercises on resuscitation and surgical techniques for treatment under battlefield conditions of wounds inflicted by high and low velocity weapons are not offered in the UK, they are conducted bi-annually by the Danish Armed Forces' Medical Services for NATO and Partnership for Peace countries (18). It was stated that: "*They [the animals] are deeply anaesthetised throughout the exercises, and are attended at all times by veterinary staff, who may withdraw them at any time for any reason. The pigs are put down at the end of the day without recovering consciousness.*" The MoD also uses animals to undertake research on wound healing. These animals are also rarely allowed to recover from anaesthesia.

The latest available statistics on animal usage by DSTL are available for 2011 (19) as part of a response to a Freedom of Information (FOI) request. The response stated: "*During the calendar year 2011, Dstl returned the following numbers of species to the Home Office under the Animals (Scientific Procedures) Act: 652 guinea-pigs; 8,801 mice; 37 rats; 76 rabbits; 88 pigs; and 68 NHPs (including marmosets)*". It is totally unsatisfactory that one cannot tell whether or not these figures provided to the Home Office have been incorporated into the overall statistics. If they have, then it must be assumed that some of them account for those animals used for protection of man, animals and the environment, a heading used in the supplementary tables (www.gov.uk/government/uploads/system/uploads/attachment_data/file/224128/spanimals12-supptabs.ods). There is also a table headed *Techniques of particular interest*, which includes two categories relevant to military research — *Interference with brain* (the numbers of animals for 2012 being 10,739 for the mouse, and 10,455 for the rat respectively) and *Aversive training* (the respective animal numbers being 3,001 for the mouse, and 3,238 for the rat). The latter category might well relate to military training as it also included 130 in the entry for pigs, sheep and all other ungulates.

The US Congress in 1983 restricted the use of dogs and cats in training exercises, but the use of other animals, such as goats, monkeys and pigs, is still permitted. In 2007, the US Department of Defense (DoD) used over 3,500 pigs and 5,000 goats in combat trauma courses. People for the Ethical Treatment of Animals (PETA) surveyed military officials of 28 NATO countries about their use of animals in training programmes (18). Twenty-two of the countries stated that they do not use animals for training purposes, while the remaining six (one of these being the USA) indicated that they do use them.

The need for anaesthesia

The vast majority of animals used for military purposes are anaesthetised before experimentation, due to the often highly invasive nature of the procedures they are subjected to (Table 2). In some experiments, anaesthesia is terminal, while, in many others, the animals are allowed to recover to enable further investigations to be performed; for example, to assess the effects of protective measures and therapeutic interventions. The application of anaesthetics to laboratory animals is an important method for alleviating the pain and distress that they have to endure during and following experimentation. As such, anaesthesiology is part of the toolbox that researchers have available to refine animal experiments (20). *Refinement* is an integral part of the Three Rs first proposed by Russell and Burch in 1959 (21); the other two Rs being *Reduction* and *Replacement* (22–25).

The Dilemma of Using Anaesthetics

The application of anaesthetics to laboratory animals has to be carefully controlled, so that they have the desired effects, while ensuring that they interfere with the experimentation and compromise data interpretation as little as possible. However, it is well-known, if not nearly as widely acknowledged, that anaesthesia can substantially alter the responses of the body and its organs to externally-induced damage. This is particularly so with regard to the cardiovascular and central nervous systems, which are major targets during military action, causing, for example, haemorrhage and traumatic brain injury (TBI). Research on these two areas has involved the extensive use of animals. It is also relevant for exposure via inhalation to chemical and aerosolised biological weapons. In addition, the use of temporary anaesthesia is a further concern with regard to animal welfare, since it increases the risk that the animals are only partially unconscious, and can therefore feel some pain, especially when they are being subjected to highly invasive procedures.

Animal Models of Traumatic Brain Injury (TBI)

Background

Nowhere is the dilemma of using anaesthesia better illustrated than in the use of animal models of traumatic brain injury (TBI). TBI still represents the leading cause of morbidity and mortality in individuals under the age of 45 in the world (26–29). In the USA, TBI and spinal cord injury (SCI) together are responsible for an estimated 90,000 disabled persons

Table 2: Anaesthetised animal models used for military purposes discussed in the text

Endpoint involved	Model characteristics	Species mainly involved	Example refs.
TBI	Exposure with/without protective devices to overpressure from explosive blasts via shock tubes or under 'open-field' conditions	Rodents, sheep	27, 10, 11
	Trauma also induced with a howitzer, a bazooka, an automatic rifle, and underwater explosives	Pigs	46
Mainly unconstrained impact models of head trauma	Impact acceleration via a 1kg mass impact piston striking the head, with partial head restraint	NHPs	36
	Marmarou's weight drop model (impact produced by brass weights falling from a designated height through a Plexiglas tube)	Rodents	36
	Ovine head impact model (impact via captive bolt)	Sheep	48
Constrained head models	Movement of head confined to one plane	Rodents	34
Non-impact head acceleration	Rotational/angular acceleration induced by pneumatic shock	Rats, rabbits, pigs and NHPs	49
Controlled cortical impact (CCI) model	A transient pressure wave generating a controlled impact is delivered to the intact dura after opening the skull, by a compressed pneumatic piston causing deformation of the underlying cortex	Rodents	55
Gas-filled organs (lungs, ears, and gastrointestinal tract as target organs for blast injury)	Whole body exposures followed by organ function and histopathology studies	Rodents, but larger animals (e.g. pigs) favoured, especially for lung studies	58
Inhalation models of biological warfare research	Aerosol of the infectious agent to be delivered directly to the lungs via the mouth by using an endotracheal tube	Mice	61
	Aspiration (a known amount and concentration of solution/suspension pipetted into one of the nares or the distal oropharynx)	NHPs	62
Haemorrhage models (testing of potential new treatments)	Complex groin injury involving surgical intervention	Pigs	64
	Splenectomised animals (controlled haemorrhage by blood removal)	Pigs (immature females)	31
		NHPs	71
	Liver injury model	Pigs	61, 104
	Splenic injury model	Rats	69
Haemorrhage models (for resuscitation and live-tissue trauma training; LTTT)	Large, sudden blood loss by one of above methods, or infliction of penetrating/blast damage and trauma; training conducted in Advanced Trauma Life Support (ATLS) animal laboratories	Dogs, pigs, goats	18

annually. Approximately 4,000 individuals per day, in the USA alone, experience a TBI (30). Blast-induced traumatic brain injury (bTBI) is a major medical concern, both in civilians (where it is caused either accidentally, or deliberately by criminal attack) and in the armed forces as a result of combat (recently in Iraq and Afghanistan, in particular). The use of improvised explosive devices (IEDs) has resulted in greatly increased numbers of such casualties.

Traumatic head injuries produce two types of brain damage (31). Primary injury occurs in the first few milliseconds of the trauma, and consists of the biomechanical effects of forces applied to the brain. Primary effects include: skull fracture, parenchymal damage, contusions, and lacerations of the brain, as well as diffuse vascular damage with petechial (capillary) haemorrhages. Potential also exists for direct damage from intra-cerebral haemorrhage. There are no effective treatments for these injuries. The secondary injury stage of TBI occurs minutes to hours after the original trauma, and arises from complications of the processes initiated by the primary injury, causing delayed, non-mechanical damage. Common features of secondary damage, depending on the nature and intensity of the original trauma, are: cerebral ischaemia (both focal and general), hypoxia, hypotension, arterial/venous hypertension, hyperglycaemia, and raised intra-cranial pressure (ICP; 32). Accompanying events may include: haemorrhage, brain swelling, inflammatory excitotoxic brain oedema, hypercapnia, seizures, and vasospasms.

The extent of the damage from any given external trauma is influenced by changes in cerebral blood flow (CBF; hypo- and hyper-perfusion), impairment of cerebrovascular autoregulation, cerebral metabolic dysfunction and inadequate cerebral oxygenation. Furthermore, excitotoxic cell damage and inflammation can lead to apoptotic and necrotic neuronal cell death. The changes occurring during the secondary injury stage of TBI are amenable to treatment (33).

The use of animal experiments in blast trauma research

The use of shock tubes

Information on the stages of TBI has been obtained from human and animal studies, and the latter, which involve the application of experimentally-induced blast damage, have been used to identify potentially promising therapies (34–39). In some experiments, the pressure due to an explosion is directed toward the heads of the animals via shock tubes (27, 40–42). These devices are cylindrical metal tubes, closed at one end. For obvious reasons, anaesthesia is necessary for the humane care of animals subjected to experimental TBI (26). This is clearly different from the situation at the time that brain trauma normally occurs in humans exposed to blast injury. In TBI experiments, it is customary for anaesthetised animals, usually rats, to be immobilised individually in special holders designed to prevent any movement of their bodies in response to the blast. The blast overpressure and underpressure waves are then generated, either by detonation of plastic explosive or compressed air, in the closed end of the shock tube.

Examples of such experimental animal work include early studies conducted by Clemedson and Criborn (43), in which a charge of plastic explosive (penta-erythritol tetranitrate; PETN) was used for the exposure. The system was composed of a cylindrical 400mm-wide cast iron tube, with a cone shaped tip where the charge was placed. The effects of the blast on the nervous system and cerebral vasculature of rabbits were studied. The blast tube was modified for work with rodents in the 1990s. In one such application (44), anaesthetised rats were mounted in a blast tube at a distance of 1m from the charge. Five grammes of PETN resulted in a peak pressure exceeding 10 bar during detonation. The animals were mounted in metallic nets or fixed to a body-protection device, in order to limit acceleration movements.

In shock tube experiments, the position of the animal in the blast tube can greatly affect the results. Sundaramurthy *et al.* (45) used a blast tube with rats to examine the influence of animal placement location on the biomechanical load and subsequent damage to the brain and organs in the thoracic cavity, including the lungs. Anaesthetised animals were placed inside, outside, or near the exit of the shock tube. The biomechanical load on the brain and internal organs in the thoracic cavity (lungs and heart) varied significantly, depending on the location of the animals. Surprisingly, the greatest effects on organs in the thoracic cavity were experienced when the animals were placed outside the tube.

Open-field experiments

Shock tubes and small charges are not always used. For example, Säljö *et al.* (46) used a variety of other methods, including a howitzer, a bazooka, an automatic rifle, and underwater explosives, in so-called 'open-field' experiments. The induced pressure changes were recorded with a hydrophone sensor in the brains of pigs, and with an optical fibre sensor in rats. These systems were used to investigate the neuropathological effects of overpressure.

Impact acceleration models

TBI has also been induced by exposing the heads of animals to impact acceleration. In one study (see 36), anaesthetised primates were injured by a 1kg mass impact piston, which struck the head at a designated location on the skull. Head motion in these models was constrained only by the neck, which allegedly allowed close reproduction of the

acceleration–deceleration forces experienced in human head injury. Apparently, this model simulates some of the major characteristics of human TBI, such as short-term loss of consciousness, as well as histopathological, systemic and cerebral metabolic responses.

Marmarou's weight-drop model is an example of a constrained rodent model of impact acceleration head injury (36). The impact is produced by a column of brass weights falling freely by gravity from a designated height through a Plexiglas tube. After exposing the animal's skull with a mid-line incision, a stainless steel disc (10mm in diameter and 3mm in depth) is rigidly fixed with dental cement to the animal's skull, centrally, between the lambda and bregma fissures. The magnitude of the impact on the disc can be altered by using heavier or lighter weights, and by varying the height from which they are dropped, resulting in graded brain injury. This TBI model, with the improved method to control impact (36), activates pro-inflammatory mediators/modulators, and induces both apoptotic and necrotic neuronal cell death, as well as motor and cognitive deficits, in animals allowed to recover from anaesthesia.

Cats and sheep have also been used in TBI research; for example, there is a report on the induction of axonal damage in cats exposed to brain trauma (47). An example of the use of sheep concerns the ovine head impact model (48), in which anaesthetised sheep are placed in the sphinx position, with their heads positioned on a support to allow free rotational and lateral movement after impact (fully unconstrained model). The impact was generated by using a 'humane stunner' (captive bolt) that was aimed at the left temporal region of the unrestrained skull. Unsurprisingly, widespread axonal injury was induced in the brain, the degree of which was closely correlated with systemic and cerebrovascular responses.

To overcome the problem of lack of injury reproducibility, particularly in terms of outcome, of fully unconstrained injury models due to a lack of control of the response of the head to impact, constrained models have been developed. In these models, head motion is confined to a single plane, to retain some leeway for head movement.

Non-impact head acceleration models

Non-impact head acceleration models have also been developed, to simulate movement, such as rapid rotation, of the brain within the skull, which is a key cause of head injury (36). Rotational acceleration induces shear stresses that cause diffuse axonal injury, accompanied by various pathophysiological and behavioural effects. Apart from traumatic axonal injury, diffuse brain oedema and diffuse white-matter haemorrhage can be induced by weight-drop or rapid-acceleration models.

Gennarelli *et al.* (49) studied the effects of pure angular acceleration on brain injuries (see also 50). Their exhaustive research with non-human primates (NHPs), by using pneumatic shock testers and physical models, led to the identification of rotational acceleration thresholds for varying levels of brain injury. Angular acceleration thresholds for diffuse brain injuries were defined in a mathematical equation, in which injury severity values were expressed in terms of the length of unconsciousness according to the Abbreviated Injury Score (AIS) 1998 version (51).

Other acceleration models

Numerous other head acceleration models have been developed with different species apart from NHPs, including pigs, rabbits, and rats. Several of the models involve some level of head movement constraint, while others permit free head motion (36) — the former reduce variability, whereas the latter simulate more-closely the effects of this type of injury seen in humans. Inter-animal variability is also increased, because there is little control over the extent of the damage induced (52). This, in turn, increases the need for larger group sizes. Achieving *reduction* by decreasing animal numbers, is also compromised, as trauma outcome determinations at tissue and cell levels require sacrifice and tissue extraction from donor animals. In addition, as the amount of inertia to rotation determines the level of brain tissue damage, and since inertia depends on brain mass, it is necessary to use scaling, based on the relationship between brain mass and acceleration, when extrapolating from results with animal models to the human situation.

In yet another rotational head model, diffuse brain trauma was induced in miniature swine by securing the head to a pneumatic actuator through a snout clamp (53). Activation of the device rapidly rotates the animal's head over the designated angular excursion of 110 degrees in 20 milliseconds. Diffuse axonal injury, mainly in the hemispheric white-matter and brain-stem, was observed.

CCI (controlled cortical impact) model of TBI

Pneumatically-driven equipment is also used in the controlled cortical impact (CCI) model of TBI, which involves opening the skull and exposing the dura of the brain to a transient pressure wave. Controlled impact is delivered to the intact dura by a compressed air-driven metallic piston, causing deformation of the underlying cortex. In rodent

CCI models (54), a pneumatic cylinder, usually with a 4–5cm stroke, is mounted on a cross bar to adjust the position of the impactor. Impact velocity and duration of impact (dwell time) can be varied to give differing depths of cortical deformation.

In a CCI study (55) involving a mouse model of TBI, animals received 5% isoflurane in air and were placed in a stereo-tactic frame. Anaesthesia was maintained with 2.5% isoflurane via a nose cone. A CCI injury to an exposed area of the brain was produced with an integrated electromagnetic impact accessory. This produces a 'moderately severe' injury with pronounced behavioural deficits, but virtually no mortality. After injury, a plastic skull cap was secured over the impact site, and the skin incision was sutured. The animals were allowed to fully recover consciousness, but the effects of the surgery and traumatisation could have had lasting adverse welfare consequences after recovery.

Effects on gas-filled organs

Exposure to blast overpressure also affects organs containing air or structures with different densities (such as the ear, eye, lungs and the intestine; 56, 57). Animal models of lung damage due to blast-induced trauma are particularly susceptible to the effects of anaesthetics on breathing and respiration (see later). The effects of injury to gas-filled organs have been studied extensively, especially in rodents (58). However, due to their diminutive body size and variations in physiology, small animals are not always the best models to extrapolate to man in the evaluation of lung injury. Large animal models, such as the pig, are considered to offer significant scientific advantages over smaller animals. The lung physiology of the pig is more-comparable to that of man than is that of rodents, and the relatively large size of pigs and similarities in the tracheobronchial tree and vascular architectures mean that human intensive care unit (ICU) equipment can be used. However, the choice of the pig over a rodent species raises the disadvantage, from an animal welfare viewpoint, that a species with increased capacity to suffer will be involved.

In addition to chemical weapons testing (59), the lung is also a target in biological warfare research, and the use of models for this purpose, to develop and evaluate protective measures and potential antidotes, involves the exposure of anaesthetised animals (60). In such studies, it is customary for an aerosol of the infectious agent to be delivered directly to the lungs via the mouth, by using an endotracheal tube (61). In addition, anaesthesia is used with aspiration, as another alternative to inhalation delivery. With this approach, a known amount and concentration of a solution, or suspension, can be pipetted either into one of the nares, or into the distal part of the oropharynx (62).

Animal Models of Haemorrhage

Significance of the endpoint

Haemorrhaging is one of the major causes of death in war, and the improvement of surgical techniques and other methods to stop bleeding is crucial to preserving the lives of combatants (62). Uncontrolled haemorrhage is the most common preventable cause of death for soldiers wounded in combat (2). The vast majority of these deaths occur in the field, before the injured can be transported to a treatment facility. Therefore, early control of haemorrhage remains the most effective strategy for treating combat casualties.

Animal models

Traditionally, animals have been used extensively as models of haemorrhaging, and in battlefield trauma and resuscitation training. For example, the former type of model has been used by Alam *et al.* (64, 65), to develop better methods for controlling bleeding — such as the development of haemostatic agents, several of which have been deployed to the war-front for use in arresting bleeding before surgical, or other appropriate intervention, is feasible. A model of battlefield wounding was used by these authors, in which a complex groin injury was created in 72 swine, a procedure that included semi-transection of the proximal thigh and complete division of the femoral artery and vein. Resuscitation (500ml of Hespan® over 30 minutes) was started 15 minutes after injury and haemodynamic monitoring was performed for 180 minutes. The primary endpoints were survival and blood loss. In addition, maximum wound temperatures were recorded, and histological damage to artery, vein, nerve and muscle was documented.

Hess *et al.* (66) developed a porcine model of the anticipated military use of oxygen-carrying resuscitation solutions. The objective was to determine whether toxicity under adverse conditions could limit further development of haemoglobin-based products. Splenectomised immature female pigs were used. Five days prior to each experiment, central vascular catheters and a renal arterial flow probe were surgically placed in the animals. After recovery, and when weight gain had resumed, the animals were placed in metabolism cages and deprived of water for 48 hours, to produce hyperosmolar dehydration resulting in the loss of approximately 7% of body weight. In addition, the animals lost some 38% of estimated blood volume

over one hour by a controlled logarithmic haemorrhage. Resuscitation was by administration of a fixed volume of test solution. Haemodynamic function was observed, but no further therapy was given for three hours (a period corresponding to the average evacuation time in the field to a hospital). After this period, the animals' blood volume was restored. All the animals survived, despite the high probability that they suffered, following recovery, as a result of the procedures performed on them.

In a later study, Pusateri *et al.* (67) assessed the effects of nine haemostatic dressings on blood loss, by using a model of severe venous haemorrhage and hepatic injury in swine. Pigs were treated with one of nine different haemostatic dressings. Standardised liver injuries were induced, dressings were applied, and resuscitation was initiated. Blood loss, haemostasis, and 60-minute survival were measured. It was found that the haemorrhage model allowed differentiation among topical haemostatic agents for their suitability for treating severe haemorrhage. Kheirabadi *et al.* (68) also used a porcine model, with anaesthetised animals, to assess the efficacy of resuscitation preparations. Haemorrhage was achieved by isolating the right femoral artery after splenectomy, and performing a 6mm arteriotomy to cause unrestricted bleeding for 45 seconds.

Other species have also been used to assess resuscitation after haemorrhage. For example, Abu-Hatum *et al.* (69) used rats in which they induced extensive splenic injury to simulate haemorrhage. In addition, a model of lethal extremity haemorrhage in the goat (*Capra hircus*) has been developed, and a polymeric dressing agent (BioFoam) was tested (70). After tourniquet application to the thigh, a soft tissue and vascular injury was created by transecting muscles and the femoral artery. In another study (71), 17 cynomolgus monkeys, under N_2O analgesia and sedation, were subjected to severe volume-controlled haemorrhagic shock (their blood volume lost was up to 27ml/kg).

Despite their extensive use, Madje (72) observed that current animal models of haemorrhage and resuscitation vary substantially from one laboratory to another, and are not based on clinical experience. He noted that protocols are arbitrarily determined, and there is no consensus on predictive endpoints.

Animal Use in Surgical, Trauma and Resuscitation Training

Live-tissue trauma training (LTTT)

Trauma and resuscitation-training laboratories still use large animals (mainly dogs, pigs and goats) to more-closely approximate the size of humans, and to allow the use of human medical instruments. In LTTT, animals are used in the training of physicians and paramedics in the treatment of severe traumatic injuries, including substantial blood loss. Typically, such training takes place in the so-called Advanced Trauma Life Support (ATLS) animal laboratory (73), a facility that is widely used for training by the armed forces of NATO countries (18). The use of the ATLS animal laboratory follows strict protocols, and is closely monitored by certified veterinary and other appropriately-trained personnel. On completion of the procedures, or if they exhibit signs of distress, whichever is the sooner, the anaesthetised animals are killed.

Public concern

Concern in the USA about the way in which the DoD uses animals for trauma training has been raised, particularly because, during training simulations with live animals, there is the possibility that the animals are not fully anaesthetised, and could therefore feel pain, which might not be obvious to veterinary staff supervising the simulations. This concern prompted calls for a change in federal legislation in the form of the *Battlefield Excellence Superior Training Practices Act* or BEST Practices Act, *H.R. 1417*, (formerly *H.R. 403*) (74). This legislation outlaws the use of live animals to train military personnel to respond to chemical or biological attacks, or to treat battlefield injuries. However, according to the US 'govtrack' website (75), the current status of the BEST Practices Act is that it has been referred back to committee. Nevertheless, while the bill did not pass into legislation, Congress passed the *National Defense Authorization Act* in December 2012, which included language requiring the Secretary of Defense to report to Congress by 1 March 2013 on a strategy, including a detailed timeline, for replacing the use of animals with human-based methods. No information was found with regard to the current situation.

Issues Caused by the Use of Anaesthesia

Background

The above discussion demonstrates that anaesthesia is used routinely for several key animal models used in military research, testing and training, not to mention many other biomedical science applications. On the face of it, this would seem to be a necessary application of *refinement*, and should therefore be welcomed. However, there are both

scientific and welfare concerns relating to the use of animals in the types of experiments involved, particularly when animals are allowed to recover. These issues include the choice of type of anaesthetic and the dose, the possibility that anaesthetic directly interacts with a test agent, and the potential modification of the physiology and/or biochemistry of the animals, such that the data obtained might be misleading. A detailed discussion is beyond the scope of this review, but some examples are provided below.

A good illustration of how some of these scientific problems arise concerns the work on chemical warfare agents published by Jugg *et al.* (76). In these studies, apart from the possibility of species differences in responses to phosgene and furosemide — the test agents under investigation — and the possibility that these agents might interact with the anaesthetic, an anaesthetised animal has a fundamentally different pattern of breathing from a fully-conscious one (77). Anaesthetics affect the chemical control of breathing and behavioural control, or, most often, both. Centrally-mediated respiratory depression is common to both inhalational and intravenous agents, and both have a direct effect on lung physiology (78).

Effects on respiration and breathing

General anaesthesia, with or without the use of neuromuscular blocking drugs, results in the loss of airway patency due to the relaxation of the pharyngeal muscles and posterior displacement of the tongue (79). The ability to manage secretions is lost, and saliva and mucus can obstruct the oropharynx. In addition, loss of the cough reflex allows secretions (or refluxed gastric contents) onto the vocal cords, causing laryngospasm, or it allows them to enter the trachea and lungs, causing bronchospasm. These effects combine to obstruct the airways and prevent the passage of gases into and out of the lungs, resulting in hypoxia and hypercapnia (79). While many of these effects have been observed in patients, there is no reason why they should not occur also in experimental animals.

Therefore, anaesthesia reduces oxygenation, and hypoxaemia is a common occurrence (80). General anaesthesia also induces atelectasis formation (i.e. partial or full lung collapse), a reduction in lung volume, and respiratory mechanical impairment that may be combined with gas exchange abnormalities (81). Furthermore, reductions in functional residual capacity (FRC) can occur in recumbent subjects after the induction of anaesthesia. In addition, some anaesthetics reduce respiratory tract ciliary activity, while dry gases result in mucus plugging; several of them are directly irritant to the airways (79). Certain anaesthetics increase saliva and mucus production. In addition, most anaesthetics cause direct depression of the respiratory centre in the brain, reducing ventilation.

The above effects are complicated by other factors that may interfere with respiration. When an animal is in lateral recumbency, the lung that is at the bottom is being compressed by the rest of the body. Likewise, animals in dorsal recumbency may experience compression of the diaphragm by abdominal viscera (82).

Strategies for the use of anaesthetised animals in inhalation models of biological warfare, and for other purposes, need to take into account the above effects of anaesthetics on respiration, even though they are not innately harmful to the anaesthetised subject.

Effects on neural function

With respect to the use of animal models of TBI and haemorrhage, it should be remembered that, while the precise details of the modes of action of anaesthetics are obscure, it is clear that their action is due to their ability to modulate the physiology of the nervous system, particularly that of the brain (83). Since sensory and neural circuits are involved in all types of animal responses to insult, toxic damage and normal functioning, the use of anaesthetised animals can substantially complicate data interpretation. Therefore, the relevance of results obtained from anaesthetised animals to real-life situations, such as battlefield injury, can be compromised.

Some anaesthetics block the NMDA (*N*-methyl-D-aspartate) receptor (NMDAR), which is especially located in the post-synaptic region of brain cortical neurons. The NMDAR is a subclass of ionotropic glutamate receptor that mediates excitatory transmission throughout the central nervous system (CNS). The receptor is normally activated on release of the excitatory neurotransmitter, glutamate, by glial and neuronal cells from their pre-synaptic terminals. NMDA, a modified amino acid, mimics this effect of glutamate. Low level glutamate activation of NMDAR is a major mechanism for maintaining synaptic plasticity and long-term potentiation (LTP) — two key mechanisms in learning and memory (84, 85) — via a process of controlled calcium influx into the post-synaptic area of neurons, due to specific ion channel opening (86). Normal operation of the NMDAR allows individuals to respond to excitatory stimuli through the inter-related functioning of NMDA receptors, glutamate and dopamine. However, over-activation of the receptor, which occurs with increased release of glutamate following brain tissue trauma, promotes excessive neu-

ronal calcium influx, and, concomitantly, a cascade of abnormal neuronal processes, including neuronal apoptosis. This results in a phenomenon termed excitotoxicity or NMDA over-activation (87, 88). Although excitotoxicity can occur following brain trauma, this is not the case if the receptor is blocked, as would be the situation in an experimental model of TBI in which animals are administered an anaesthetic antagonistic to the NMDAR. In these instances, the anaesthetic would be neuroprotective, thereby reducing the fidelity of the model for human TBI.

On the other hand, in the absence of anaesthetic and trauma, the levels of glutamate, and therefore NMDAR activity, would both be 'normal', as would the cognitive and learning potential of the animals following their recovery. However, administering the same NMDAR antagonistic anaesthetic to negative control, untraumatised animals as that given to the respective traumatised animals, would prohibit the beneficial activity of the NMDAR in LTP and synaptic plasticity. The result would be an apparent potentiation in the difference in cognitive deficits between treatment animals and their respective controls. In other words, it might be impossible for an anaesthetic that acts as an NMDAR antagonist to be used as a true negative control with animal models of TBI.

That blocking NMDAR can affect neural function was shown by treating neonatal rats with MK-801, a non-competitive NMDAR antagonist. Hippocampal slice cultures from pre-treated animals were significantly more responsive to the addition of NMDA to activate the receptor, as measured by evoked electrical activity, than were cultures from animals that had not been treated with MK-801 (89). Of note is the fact that ketamine (90) is the only anaesthetic with this mode of action that is listed, together with 11 others, by Flecknall (20) for use with animals. Also, the injection of NMDAR inhibitors into the brains of animals reduces learning ability (84).

The fact that the use of anaesthesia in animal models of TBI can alter the results obtained was recognised by Ren *et al.* (91) who noted: "*The methods involved in most rodent models of TBI, including head fixation, opening of the skull, and prolonged anaesthesia, likely alter TBI development and reduce secondary injury*".

Neuroprotection and other effects on brain activity

Most anaesthetic agents are neuroprotective, when administered before an injury, which evidently is not the case in real-life situations (92, 93). Isoflurane is the most commonly used anaesthetic in experimental TBI, due mainly to its relative ease of administration and its ability to facilitate rapid recovery. Its impact on neuropathology and outcome has been studied extensively in the rat CCI model, where it was neuroprotective, in a comparison with fentanyl anaesthesia, against both neuropathology and adverse functional outcomes (94). Isoflurane is commonly used in experimental TBI, both before and early after injury, yet it is rarely used clinically. Some other anaesthetics, such as pentobarbital, have also been shown to be neuroprotective.

Apart from their intended effects, anaesthetics exert several other direct changes on the brain, some of which might interfere with the use of anaesthetised animal models of TBI. For example, there is evidence that general anaesthesia can decrease cognitive performance in humans (95). This might be related to observations that alternate treatments for five days, of mice with two volatile anaesthetics (halothane and isoflurane), up-regulated the synthesis of amyloid beta protein. This protein accumulates in neurons of the brains of Alzheimer's disease patients and of genetically-altered mice acting as models of that condition (96). Interestingly, the same effect was seen in cultured human neuronal cell cultures treated with isoflurane (95).

Indirect effects on the brain

Anaesthetics also exert widely variable effects on the blood supply to the brain, thereby influencing neurological outcome following trauma (97). It is increasingly being recognised that the support of cerebral perfusion during anaesthesia contributes significantly to a positive outcome for trauma patients. Such support is crucial, in order to adequately meet the stringent metabolic demands of the brain. Perturbations in systemic blood flow are usually prevented from causing significant alterations in cerebral blood flow (CBF), by a feedback mechanism called cerebral pressure autoregulation (CPAR), a process that can be disrupted by brain trauma. Moreover, under normal circumstances, the vasomotor tone of the cerebral blood vessels is linked to the oxygen requirement of the brain by another mechanism, called flow metabolism coupling (FMC). Provided FMC remains intact, a reduction of brain metabolic activity would mean lower oxygen demand, followed by vasoconstriction and a concomitant decrease in ICP. However, FMC can also be disrupted by brain trauma, leading to a loss of control of ICP.

The effects of anaesthetic agents on intra-cranial haemodynamics and neuronal injury are clearly complex. Observations on human TBI patients suggest that the overall outcome is dependent on several specific effects that include: ICP elevation, vasomotor changes, disruption of autoregulation, and secondary effects via alterations of cardiovascular and respiratory function (98).

A further complication arising from the use of anaesthetics in experimental models of TBI concerns changes to the blood–brain barrier (BBB). An acute increase in BBB permeability, as observed by IgG immunoreactivity, has been detected in rat brain following exposure of the head to a shock wave (41, 88). This acute disruption of the BBB is consistent with findings showing that the transepithelial electrical resistance (TEER) of endothelial monolayers — an indicator of BBB integrity — decreased immediately after exposure to overpressure. Disruption of the BBB results in cerebral oedema formation (COF), a major cause of high mortality in TBI patients. Thal *et al.* (99) studied the effects of different anaesthetics on COF, both *in vivo* (healthy mice and animals subjected to CCI) and *in vitro* (murine brain endothelial monolayers and neurovascular co-cultures of the BBB), by using three markers of BBB integrity — tight junction proteins (TJ), zonula occludens-1 (ZO-1), and claudin-5 (cl5) — as well as TEER and brain oedema *in vivo*. The volatile anaesthetics, sevoflurane and isoflurane, both significantly reduced the TEER of *in vitro* endothelial monocultures within 24 hours after exposure, although they did not alter TEER in BBB co-cultures mimicking the neurovascular unit (NVU). In healthy mice, anaesthesia did not influence brain water content and TJ expression, while brain water content increased significantly 24 hours after CCI, particularly when isoflurane was used. Corresponding changes in ZO-1 expression were also detected, and immunohistochemical analysis indicated disruption of ZO-1 at the cerebrovascular level. These data indicate that several anaesthetics (one of which is used in animal models) can influence the formation of brain oedema after experimental TBI. Therefore, the use of an anaesthetic, such as isoflurane, could render an animal model of TBI over-sensitive to trauma. It is noteworthy that the use of different anaesthetics in animal models of TBI can significantly affect neurological outcome (100).

Gender-specific effects

Anaesthetics can also influence gender-specific outcomes for TBI, a phenomenon which has been noticed in animals and humans (see O'Connor *et al.* [38]). These authors used three different anaesthetic protocols, and four different outcome measures, in a TBI model with male and female rats. Diffuse TBI was induced in adult animals by using an impact-acceleration brain injury model. Mortality in female animals was no different from that in males when halothane was used, and it was slightly better than males with isoflurane. However, when pentobarbital was used, female mortality was significantly greater than that of males. In cognitive tests, conducted by using the Barnes Maze, only isoflurane-anaesthetised females performed better than their male counterparts. Similarly, in an open-field activity task, females always performed better than males after trauma, with isoflurane-anaesthetised females also performing significantly better than the halothane-anaesthetised female group after injury. Therefore, gender specificity is another factor that needs to be considered, together with effects on cardiovascular and ventilatory function, intra-cranial haemodynamics, and potential neuroprotective properties, when selecting an anaesthetic to use with animal models of TBI.

Cardiovascular effects

With respect to haemorrhage models, both inhalational and intravenous anaesthetic agents affect the cardio-respiratory system, as well as the CNS, in a dose-related manner (78). A further factor that needs to be taken into account when using anaesthetics in both humans and animals, is that the cardiovascular effects of the anaesthetic differ substantially according to the type used (101). The actual effect exerted by a specific anaesthetic could depend on its ability to prolong cardiac re-polarisation by blocking ion currents (102).

Anaesthetics also have depressant effects on the myocardium and vascular smooth muscle, leading to a fall in cardiac output and hypotension, as well as direct effects on the heart or vasculature, decreasing cardiac output and blood pressure (103). The specific effects of certain anaesthetics on the cardiovascular system and blood pressure would be expected to modulate the resuscitation and wound healing of animals in experimental models, thereby complicating the interpretation of the resulting data. Therefore, as with other trauma targets, the nature of the anaesthetic is important with regard to the animal's response to, and recovery from, haemorrhage and resuscitation.

Potential Consequences of the Effects of Anaesthetics

The need for careful selection of anaesthetic

Clearly, there are several reasons why the selection of an anaesthetic for use with an animal model should be undertaken with care. The criteria used for selecting anaesthetics for TBI models should take account of the need to support cardio-respiratory function, in addition to neuroprotectivity and potential effects on BBB function.

Anaesthetics have complex and multiple effects on several key bodily functions — respiratory, cardiovascular, haemodynamic and neural systems being

of particular relevance to the present discussion — as well as on the general physiology of an animal, depending on the type of agent used, the dose, the duration of treatment, and many other parameters, such as the recumbency of the body during anaesthesia. Variations in the basic parameters of anaesthesiology are likely to substantially affect the characteristics and thus the data provided by many of the key animal models used in military research, testing and training.

Consequences for model predictivity

It is difficult to calculate the consequences for model predictivity from the available information, especially in view of the complex interplay between the different effects of anaesthetics on the ability of animals to withstand the detrimental effects of the test procedure. With regard to respiratory effects, it would be expected that a depression of breathing and gas exchange, induced by a state of anaesthesia, would endow an animal with more resistance to biological agents or chemicals entering the body via inhalation than a non-anaesthetised one, since a lower concentration of the agent would be inhaled under the same conditions of dosing. Moreover, an animal with depressed blood pressure from anaesthetisation would be expected to be more resistant to haemorrhaging and more responsive to recovery measures, than an animal that has not been anaesthetised, all other conditions being the same. If these hypotheses were true, it would be expected that anaesthetised animal models of respiratory infection and haemorrhaging could generate false-negative data, compared with the results that would have been obtained had non-anaesthetised animals been used, all other conditions being equal.

Importantly, Madje (72) noted that the use of anaesthetics in most animal models obscures crucial haemodynamic responses and environmental variables, and he also stressed that influences on outcomes, caused by modulating the stress state of the animal, are not controlled. He suggested that conscious animal models that can minimise anaesthesia artefacts, and prognostic endpoints that have been defined in the clinic, should form the basis of a standardised predictive preclinical animal model. In fact, the use of conscious pigs in resuscitation research is not a new practice (see 104, for example). However, the use of such models would raise a host of new issues, and is certainly not being advocated by the author of this review.

Brain trauma in humans involves an organ that is not usually under the influence of anaesthesia, at least initially. So, anaesthetised animal models of TBI more-closely simulate the situation when individuals with severe TBI are rapidly subjected to anaesthesia, as part of normal clinical practice. As explained earlier, the effects on CBF of administering anaesthetics to animals and then exposing them to brain damage, depend on whether or not the auto-compensatory mechanisms controlling cerebral blood flow are disrupted by the trauma sustained. If they remain intact, a reduction in oxygen demand as a result of anaesthesia will not increase ICP, due to the phenomenon of autoregulation. However, if the regulatory system(s) are disrupted, ICP would be expected to be lower in an anaesthetised brain than in a non-anaesthetised one, as the effect of the anaesthetic to lower oxygen demand of the brain would not trigger a compensatory drop in CBP. While this is better for the clinical outcome, it suggests that animal models of TBI could generate false-negative results, depending on the effects of the experimentally-induced trauma.

Assessing the relevance of animal models of TBI

Hicks *et al.* (31) noted the difficulties in validating the above models of TBI in the absence of more-precise human data. It is claimed that the models appear, either together or individually, to reproduce many of the overt neuropathological and behavioural deficits that have been described following human exposure. These claims would seem unlikely, given the wide diversity of TBI symptoms (including vasospasm, oedema, contusion, axonal injury, haemorrhage, transient alterations in electro-encephalograms, tympanic membrane perforations, and cognitive deficits; 105).

One area in which suitable data for assessing the fidelity of animal models of TBI are available, is the analysis of their success in identifying potential therapeutic interventions that work in humans. Such an analysis was undertaken by Guha (106), who concluded that, while animal models of head injury have contributed significantly to an understanding of TBI, the excellent therapeutic benefits demonstrated in some of these models, often have not been realised in the clinic. The same conclusion was reached by Morales *et al.* (30). Guha (106) attributed the lack of correlation between laboratory and clinical success to the fact that animal models rarely reflect the extent of heterogeneity of damage induced by trauma. Indeed, it has become clear, after decades of research, that some evidence must first be gathered to support the fact that the mechanisms governing animal models of injury apply to humans as well — a maxim well-known and followed by those who develop alternatives to animal experiments. In addition, Risling and Davidsson (44) noted that improved methods of translation between animal experiments and the clinic are needed; for

example, by employing the same methodology for analysis in both situations. They suggested that this could take the form of MRI imaging, or the systematic use of biomarkers. The latter possibility is being investigated by using microarray and proteomics technologies to link the development of blast damage, in the form of axonal changes, to modulations in specific gene expression (107, 108).

Risling and Davidsson (44) also highlighted a need for comparison between exposure data from actual clinical situations and the test conditions employed in animal experiments. In an effort to make animal models of TBI more relevant, Pick *et al.* (109) developed a method that results in graded levels of injury, as measured by a variety of endpoints, including behavioural and cognitive tests. This model was used to identify the ability of a long-acting glucagon-like peptide-1 receptor (GLP-1R) agonist, which can enter the brain and activate anti-apoptotic pathways to reverse the cognitive effects of mild TBI, as much as 30 days post-trauma, by using the novel object recognition test.

Animal Welfare Issues

Invasive procedures

As for the welfare of animals used in military research, training and testing, there are conflicting opinions concerning the capacity of, and the degree to which, an anaesthetised animal can experience pain. The extent to which this is an issue, under different conditions of anaesthesiology, remains poorly understood. Given the largely crude and excessive procedures involved in many military animal experiments, particularly for simulating trauma induction in TBI, one must sincerely hope that animals are always properly anaesthetised — a procedure that, in itself, is highly specialised and has several potential pitfalls. Moreover, as with all procedures conducted on animals under temporary anaesthesia (i.e. from which it is intended that animals should recover), there are possibilities for animal suffering both during and after anaesthesia — the former occurs, if the anaesthesia is not fully effective.

The need to use recovered animals

It is noteworthy that, while brain injury is inflicted when the animals are anaesthetised, it is necessary to allow them to recover in order to investigate fully the effects of trauma. This is particularly the case, when behaviour is being investigated and when potential new therapeutic treatments are being assessed.

The importance of recovery is illustrated by the work of Jugg *et al.* (76). These authors argued for experiments that involve recovery from acute respiratory distress, in order to provide a long-term recovery pig model. They asserted that the available terminally-anaesthetised model is limited in its application, since it cannot easily be taken beyond 24 hours post-exposure. Thus, any long-term beneficial effects in terms of survival, as well as physiological and pathological parameters, cannot be determined, and no assessment can be made as to whether the clinical benefits are real or simply delaying the outcome. However, the use of such a recovery model would be likely to have significant adverse welfare consequences for the animals concerned, the severity of which would depend on the extent and nature of the injury. This is because, although laboratory animals might experience little or no pain directly from the surgery and the induced trauma event itself, due to anaesthesia, the consequences on pain perception elsewhere in the body, and of behavioural deficits due to brain damage following recovery, could result in the imposition of numerous severe welfare costs. Such costs are likely to be complex, and resemble some, at least, of those experienced by human brain trauma patients during recovery (see 110). This is especially the case, if the claims made are correct, that animal models of TBI reproduce several of the overt neuropathological and behavioural deficits, that have been described following human brain trauma (29, 108, 111, 112), which are considered to increase the chance of mortality among post-deployment service personnel (113).

Significant acute and chronic pain problems in TBI patients are quite common (114), as are changes in brain functioning that affect sensory and motor function, and also, possibly, the perception of pain stimuli. Several pain conditions are common among patients with TBI, including headache and neuropathic pain, as well as pain arising from such conditions as psychiatric disorders, spasticity, heterotopic ossification, deep vein thrombosis, genito-urinary and gastrointestinal disorders, and orthopaedic trauma resulting from fractures and other musculo-skeletal injuries (115). The latter would assume greater importance, if any skull reconstruction required is delayed following TBI (116).

Chronic *versus* acute pain

Admittedly, much of this post-trauma pain is chronic in nature, developing some time after the injury, and, as such, would not manifest itself before animals used in experimental TBI studies would normally be sacrificed. However, some pain, such as headaches, can be acute. If acute neurodegeneration occurs as a result of TBI, it could result in acute conditions that mimic the

effects of chronic disorders, such as Parkinson's disease (attributed to loss of dopaminergic neurons in the substantia nigra), giving rise to the possibility of considerable pain, particularly in limbs and joints. Furthermore, it is sometimes necessary to allow animals to survive for extended periods following recovery from TBI — studies in the rat have shown the dose-dependent induction of axonal damage, activation of apoptotic transcription factors, and cell death in the hippocampus, 21 days after blast exposure-generated overpressure (117, 118).

Alternatives to Animal Models

Overview

A wide range of alternatives has been used in military studies, including the use of passive dummies and cadavers for crash and impact studies, complex interactive mannequins for trauma training, complex biomechanical, computer-controlled multi-elemental models, and *in vitro* organotypic cell cultures to investigate the effects of mechanical trauma on the body (Table 3; 119). Many of these methods have been available for several years, but there is an urgent need for their further development and validation, as well as for the generation and assessment of new systems to be formalised and coordinated, so that appropriate alternatives can be deployed more widely and more effectively (120).

Models for impact trauma research were categorised by Pince (121), as long ago as 1970, into four types: a) the human body (e.g. a trauma victim); b) anthropanalogous models (AAMs); c) live animals; and d) non-physical models. AAMs are anatomically-realistic mannequins or dummies that are considered by Galloway (73) to be passive or complex and interactive. This author also included cadavers in the categorisation. King *et al.* (122) assessed the contribution of the use of human cadaveric subjects in injury biomechanics research as being strongly positive.

Cadavers

Experimental cadaver data, in particular, have been widely used to validate computational impact-related TBI models. The first data set was derived from frontal head impact experiments with a series of cadavers (123). Further data were obtained in 1992, in the form of ICP measurements, from head impact experiments in which repressurised cadavers were hung in a sitting position with a harness, and impacted under varying conditions (124).

Human patient simulators

Human patient (procedure-based) simulators have an important role in trauma and resuscitation training, including in haemorrhage control (125, 126). Two examples of these are Cut Suit and TraumaMan®. The former was developed by Strategic Operations Inc. (San Diego, CA, USA), and simulates human organs, arteries and blood vessels, in an effort to provide a realistic experience for trainees, that includes haemorrhage control. Cut Suit is a human-worn partial task surgical simulator in the form of a synthetic prosthetic torso, on which surgical interventions can be performed, involving interaction with a real live subject (www.strategic-operations.com; 127). TraumaMan is another patient simulator (128), approved by the American College of Surgeons (ACS) on the premise that it could replace the use of animals in medical training programmes. Two further simulators are SimMan® and SimMan 3G® (Laerdal Medical Ltd, Orpington, UK; www.ahec.hawaii.edu/misc/Patient_Simulators.pdf; 129), which are used at the Army Medical Services Training Centre (AMSTC) in Strensall, York, UK, where the so-called Hospital Exercise (HOSPEX) takes place. ComputerMan (130) and METI Human Patient Simulator® are yet further simulators. The latter is manufactured by Medical Education Technologies, Inc., (Sarasota, FL, USA; www.ahcsimcenter.umn.edu/prod/groups/ahc/@pub/@ahc/@simcenter/documents/asset/ahc_asset_030078.pdf; 131), and is used in the Triservice Anaesthetic Apparatus Simulation Course (TAASC), at the Cheshire and Merseyside simulation centre in the UK — this model can actually be given an anaesthetic, under near-clinical conditions. Wireless versions of these systems represent the current pinnacle of simulation in healthcare and the culmination of over 50 years of research (132).

Computer models

Computer models for trauma research were first used to study human head–neck system kinematics in the 1970s (133). The three main types of computational simulations applied to dynamic modelling of the human body in flexible motion are: a) multibody (MB) models; b) finite element (FE) models; and c) combination models, comprising both MB and FE approaches. Combination models involve the use of the best properties of both of the two models, without requiring too much computing power. In both MB and FE models, the human body is broken down into a number of elements or segments, that can move relative to one another. An MB system is used to model the dynamic behaviour of interconnected rigid or flexible bodies, which can undergo substantial transla-

tional and rotational displacement relative to one another. In FE models, the system is divided into a number of finite volumes, surfaces or lines, to provide more complexity, for example, accounting for stress points and constituent material properties (134).

Several FE head models have been developed for use in impact-related TBI simulations, many of which have been refined by benchmarking against the cadaver data published by the Nahum and Troisseau research groups (see 135–137). These computational models have been used to study injury mechanisms, indicators of effect, and threshold doses in impact-related TBI studies, some of which closely simulate real-world accidents. However, their development for studying blast-related TBI is less extensive, and more-biofidelic models are urgently needed. One successful application of a bTBI computer model was undertaken by Nyein (58), who investigated the mechanical response of the human brain to blasts and the protective effects of the Advanced Combat Helmet (ACH). An 11-element human head model was developed from medical imaging, and evidence was obtained for direct brain injury, as revealed by ICP readings. The data were then used to improve the ACH.

Roberts and co-workers have undertaken extensive studies on the development of human surrogate FE models of blast and ballistic impact (see, for example, 138, 139). Their surrogate models are biofidelic, in that they simulate the biomechanical properties of human soft (organ) and hard (bone) tissue. Such models for the human torso are listed in Roberts and Merkle (140), while a review of other human torso and head–neck systems can be found in Roberts *et al.* (141), Merkle *et al.* (142) and Roberts *et al.* (143). The need to use validated systems cannot be emphasised strongly enough, since many publications describe models that have not been properly validated against either human surrogate models or *post-mortem* human subjects (PMHS), and have been used to make predictions of injury that could be incorrect. In most cases, such models have been validated with a loading situation (low blunt impact) that differs from that arising due to ballistic impact or a blast wave. The latter two loading situations are very high rate, and the blast produces pressure waves with many different frequency components. The studies of Roberts *et al.* (143) and Roberts *et al.* (141) involved validation of FE human surrogate torso and human head–neck models against human data for the same body regions that had been exposed to ballistic impact and blast damage.

Most computational models of blast trauma target the head and brain. However, models of other organs, such as the lung, have been developed (144).

Mathematical modelling

Mathematical modelling has also been applied in military research. For example, Stuhmiller *et al.* (145) generated a mathematical model of chest wall dynamics to predict the effects of blast-induced damage to the lungs of a range of animals (see 146 for a review). Also, Wright *et al.* (147) produced a series of algorithms for estimating the location and degree of diffuse axonal injury (DAI) under inertial loading of the head. The model takes account of white-matter microstructure (where DAI mostly occurs). A novel injury analysis method was developed to quantify the degree of axonal damage, and to highlight brain areas at greatest risk of DAI occurrence.

The use of lower organisms

The potential of lower organisms to act as models of blast injury has been investigated. However, due to large differences in anatomy and morphology between such species and humans, lower organisms have usually been utilised to investigate some specific aspect of the process. For example, a bone crush injury model in zebrafish adult caudal fin, which consists of the precise crush of bony rays with no tissue amputation, has been developed (148). This model has been used to study the serial expression of several key wound-healing markers. It was found that the brain of adult zebrafish has extensive capacity to regenerate neurons following the direct insertion of a needle into the telencephalon region to simulate brain damage, by stimulating production of neuronal precursor cells (NPCs). It has been observed in animal models that endogenous neural stem cells persisting in the subventricular zone (SVZ) are stimulated by TBI in adults, suggesting that these adult stem cells might have therapeutic potential. Therefore, neurogenesis in the ventricular zone (VZ) of the adult zebrafish telencephalon mirrors that which occurs in neurogenesis in the adult mammalian SVZ following injury (149).

In addition, a blast injury model has been developed in *Drosophila* (150). Flies, housed in a rectangular mesh enclosure and placed in a larger enclosed test area, were exposed to overpressure waves generated by a custom-built blast wave simulator (ORA Inc., Andover, MA, USA). Lifespan and negative geotaxis, indicative of motor function, were measured in flies after the blast injury. Mild blast resulted in death of 28% of the flies, while, in survivors, motor function was initially reduced and then fully regained by eight days post-injury, which is consistent with extensive neuronal regeneration occurring following blast injury in this species.

Table 3: Overview of *replacement* alternatives for military research, testing and training

Objective	Description	Notes	Reference(s)
Resuscitation and surgical trauma training (including haemorrhage control)	Cadavers	Can be difficult to obtain; ethical/religious issues; obvious differences in cardiovascular systems *versus* living body	73, 122, 125–127, 130
	Interactive mannequins	Computer-operated, interactive, responsive to intervention, realistic life signs and programmable scenarios; human patient simulators, e.g. Cut Suit, TraumaMan, SimMan and SimMan 3G, METI (Human Patient Simulator)[a]	73, 122, 125–127, 130
	Anthropanalogous models (AAM)	e.g. intubation dummies, catheterisation arms, bones (for fracture stabilisation training)	73, 122, 125–127, 130
	Computer modelling	Computer simulation/virtual reality systems	73, 122, 125–127, 130
TBI; crash and impact damage; assessment of protective clothing	Passive dummies; surrogate models of human torso and limbs	e.g. GelMan (torso); QuadGard (arm and leg protection simulation); ComputerMan (simulating wounding from ballistics — database/expert prediction systems developed — TraumAid and TraumaScan)	129, 177–179
	3-D biomechanical models[b]	Multibody, finite element (FE) and combination models (e.g. multi-body computational model of head and neck for whiplash injury uses series of rigid bodies to represent vertebral bone, and springs and dampers to represent passive muscles, ligaments and cervical discs); in FE models the geometry of the body is defined as a discrete grid of elements with stresses and strains calculated within elements (e.g. 50,000 element 'Brain Trauma Model' with elements for scalp, three layer skull, dura, CSF, pia, falx, tentorium, cerebral hemispheres, cerebellum, and brainstem)	58, 133
,	Mathematical modelling, including fluid dynamics[c]	Of blast pressure waves and their consequences to different body parts, including chest wall, limbs and head, brain and vascular supply; modelling of diffuse axonal injury under inertial loading of head	31, 44, 145–147, 180, 181
	Biomarker detection	Biomarkers of brain injury-glial fibrillary acid protein (GFAP), neuron-specific enolase, and ubiquitin C-terminal hydrolase (UCH)-L1 in brain, cerebrospinal fluid (CSF), and blood	182
	In vitro systems	Involving physical damage to cells in culture	Table 5

[a]The NORINA database lists 13 simulators (http://oslovet.norecopa.no/fag.aspx?fag=57&mnu=databases_1); [b]see text for further details; [c]many of these models were assessed by using data obtained with cadavers (123, 124).

Table 3: continued

Objective	Description	Notes	Reference(s)
Biological warfare	*In vitro* systems; PBPK modelling	Wide range of tissue culture models for inhalation toxicity	183–185
	Human studies	Epidemiology — computer modelling of exposure and response; use of Knowledge Acquisition Matrix Instrument (KAMI), an expert system that analyses bioagent-induced diseases that are wartime or terrorist threats, but for which only limited human response data are available (e.g. from accidents or modelling of influenza epidemics)	169, 170, 186
	Rapid detection of biohazardous agents by using biosensors	Optical wave-guide array biosensor	187
Multi-endpoints (especially blast-related injury and protection)	Computer/mathematical modelling/*in vitro*/human studies	Imperial Blast initiative	172, 173

[a]*The NORINA database lists 13 simulators (http://oslovet.norecopa.no/fag.aspx?fag=57&mnu=databases_1);* [b]*see text for further details;* [c]*many of these models were assessed by using data obtained with cadavers (123, 124).*

In vitro approaches

Range of models

A wide diversity of *in vitro* models are available for use in military research (Table 4). Some of these systems, such as those used for specific target organs for biological and chemical warfare, are similar to the ones used in toxicity testing. Others use similar tissue culture systems, but are subjected to specific external procedures in an effort to simulate the effects of trauma to *in situ* tissue, as a result of penetrating and blast injuries. Experimental injuries have been performed on fresh preparations of brain from the rat frontal cortex, guinea-pig cerebellum and rat hippocampus (see 52, 151).

In the case of blast injury, Morrison *et al.* (152) noted that it is possible to reproduce *in vitro* many of the resulting neuropathological changes that arise *in vivo* following TBI in patients. Moreover, the mechanical stress and strain experienced by tissues in the body, can also be simulated. These changes at the cellular level include ultrastructural alterations, ionic derangements, alterations in electrophysiology, and free-radical generation. Not only have *in vitro* models of the brain proven to be highly predictive of the brain's response to injury *in vivo*, they also allow for the precise control and characterisation of injury biomechanics (52, 153).

Organotypic cultures of both spinal cord and brain have been injured with a variety of devices (81, 154, 155; Table 4). Balentine *et al.* (154) used small pieces of mouse spinal cord cultures containing astrocytes and mature neurons with myelinated axons. Also, in electrophysiology studies, organotypic CNS slice preparations have been maintained for a maximum of approximately eight hours *in vitro* after injury in a barotrauma device (156). Primary axotomy (axonal breakage) has been studied *in vitro*, by using a plastic stylet to scrape adherent cells from a culture dish, to induce a tearing force on the cell layer. This method has been modified by using a rotating scribe, controlling the severity of the injury by varying the number of scribes used simultaneously (52).

Simulating compression and inertial loading

To simulate injury to the CNS by compression, an *in vitro* model has been developed, which involves dropping different weights from varying heights onto an organotypic culture of spinal tissue. Two

Table 4: Some *in vitro* models for TBI

System	Trauma mechanism	Notes
Fresh slice explants of CNS tissue (e.g. rat frontal cortex, guinea-pig cerebellum and rat hippocampus)	Variety of experimental injuries, including barotrauma	—
Organotypic cultures of brain and spinal cord; small chunks of cerebral cortex; coronal slices of rat brain	Barotrauma	—
Neuronal cell cultures	Transection with plastic stylet or rotating scribe, causing scraping/tearing of cells	To study axotomy
Organotypic spinal cell culture	Compression model	Dropping weights of varying mass from different heights
Flask of cultured cells	Trauma via impacting pendulum	Designed to simulate head acceleration
Cells growing on one plate of a parallel viscometer	Rotation of plate induces hydrodynamic forces to shear and stretch cell layer	Simulates inertial loading of the head; force applied to the cells controlled by rotation speed and distance between the plates
Cultured cells (e.g. hippocampal slices) adhered to surface of a thin silicone membrane	Vacuum pressure pulse deforms plate, stretching the cells; compressed gas can also be used (Flex Plate® apparatus)	—
Liquid cell culture in flask; organotypic hippocampal slice culture (OHSC) and the blood–brain barrier (BBB)	Compressed gas	Simulation of fluid-filled reservoir surrounding brain parenchyma

models of inertial loading (acceleration/deceleration) of the head have also been developed. Lucas and Wolf (157) designed an *in vitro* system that applies an acceleration as high as 220*g* to a flask of cultured cells, via an impacting pendulum. The system produced cellular damage after a minimum of three successive accelerations, provided that the acceleration was tangential, rather than perpendicular, to the layer of cells. This system has the advantages of increased speed and ease of use, although it models injuries from multiple impacts, while cellular deformation in response to acceleration cannot be measured.

Another *in vitro* model of inertial loading of the head utilises hydrodynamic forces to shear or stretch cultured cells (158), via the use of rotating plates in a parallel plate viscometer with cells grown on one of the plates. The hydrodynamic force applied to the cells is controlled by the speed of rotation and the distance of the gap between the plates. In yet another *in vitro* system, brain deformation is modelled by stretching a substrate onto which cells (organotypic rat hippocampal cells, for example) are strongly adhered. Several injury models have been developed by exploiting this principle (see 152), and a commercially available piece of apparatus, called Flex Plate®, can be used (159). This consists of a six-well cell culture plate, with the bottom of each of the plastic wells replaced by a 2mm-thick silicone substrate which can be deformed with a pressure pulse; see also an analogous system used by Cargill and Thibault (160). This *in vitro* model has been shown to reproduce many of the biochemical deficits associated with human head injury (see 161 for a summary).

Simulating blast pressure effects

Examples of other *in vitro* trauma models include one developed by Panzer *et al*. (162), to simulate the blast-induced effects of intra-cranial shock waves, by using tissue cultures submerged in a fluid-filled reservoir to simulate the surrounding brain. This model gave rise to pressure effects that closely resembled those detected within the brain in experimental studies. In addition, Effgen *et al*. (153) used an organotypic hippocampal slice culture (OHSC) to represent the brain parenchyma in an *in vitro* model of bTBI. This model is comprised of a compressed gas shock tube, used in conjunction with a fluid-filled receiver that contains the OHSC. The air shock wave, generated by compressed gas and delivered via a shock tube, hits the receiver and is transformed into fast-rising pressure transients, similar to intra-cranial pressures experienced from bTBI. The effects of the trauma are quantified in terms of cytotoxicity, and other damage, that was observed in both pyramidal and granule cells, similar to cell death patterns detected in *in vitro* models of inertial injuries (i.e. stretch or shear). The authors have also used the same system to investigate disruption and loss of function in a model of the BBB. Shock tubes have also been used with cell culture systems comprising mouse neuroblastoma/rat glioblastoma hybrid cells (NG108-15), or SH-SY5Y human neuroblastoma cells in tissue culture plates (163, 164).

The reader is referred to several comprehensive reviews (52, 151, 165, 166) for further information on *in vitro* and related methodologies for modelling TBI. Morrison *et al*. (52), assessing the use of the above types of *in vitro* models of TBI to discover new neuroprotective agents, noted that they have the potential for predicting which compounds may warrant further study *in vivo*. These authors discussed some examples of *in vitro* identification of neuroprotective agents, including: metalloporphyrins acting as inhibitors of haeme-oxygenase, to ultimately reduce free-radical generation; various antioxidants; iron chelators; and methyl-L-arginine, an inhibitor of nitric oxide synthetase. In addition, Ring *et al*. (167) were able to use an *in vitro* system, comprised of OHSCs from the rat, to detect potential neuroprotectants against excitotoxicity that act by inhibiting the NMDAR. In addition, following a detailed analysis of the data for targets available in *in vitro* models and the response of corresponding ones affected during TBI *in vivo*, Morrison *et al*. (52) concluded that 23 out of 28 targets gave results that correlated.

The use of human data

Obviously, the acquisition of prospective human data for military research is constrained by the small number of effects and processes that can be studied, due to ethical concerns about the often extremely invasive nature of the experiments. However, it is possible to use a wide range of non-invasive technologies for studying the progression of, and recovery from, brain trauma (168). Also, epidemiological data can be used to inform future research and development. Such data have been used to model the effects of weapons of mass destruction (WMD), due to biological and chemical warfare, and the effectiveness of countermeasures against them.

One such application is an expert system called Knowledge Acquisition Matrix Instrument (KAMI). This predicts threats from wartime and terrorist usage of biological warfare (169). KAMI takes into account information on infectivity, lethality, onset, illness severity profiles, and time to death or recovery, obtained as a result of accidental laboratory exposures and naturally occurring disease. KAMI also makes use of animal data on the effects of exposure to agents such as anthrax, plague, botulism, and Venezuelan Equine Encephalitis (VEE).

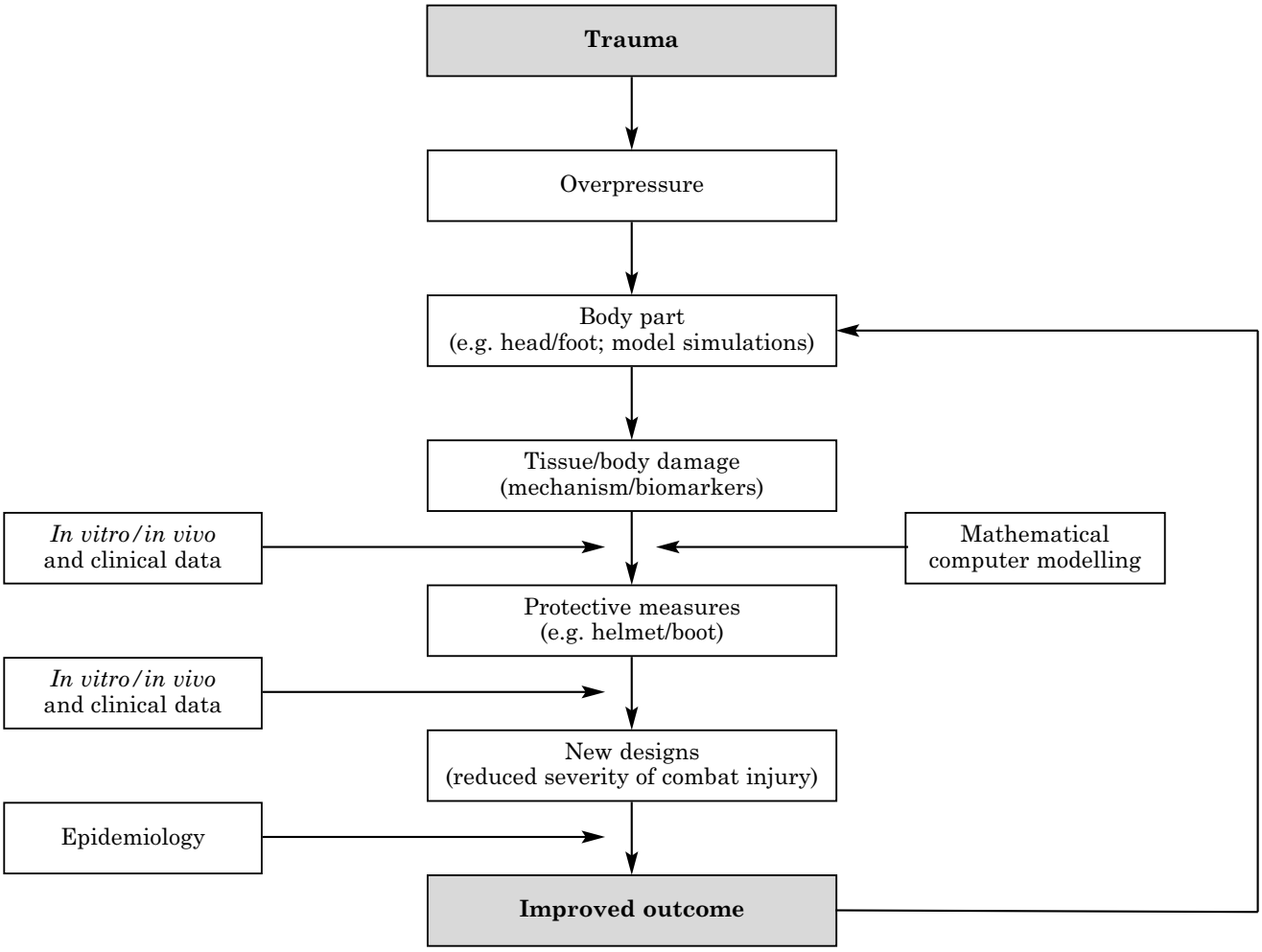
The system has been used for studying and combating the potential effects of chemical and nuclear weapons (170).

A holistic approach

Imperial Blast is a project that combines computational, *in vivo*, *in vitro* and human clinical and epidemiological approaches to analyse the effects of blast waves on the human body, from cellular to skeletal functions, with the aim of decreasing the severity of battlefield injuries (Figure 1; 171, 172). This collaborative, multidisciplinary project was initiated by a UK consortium, which includes Imperial College London, the Royal Centre of Defence Medicine (Birmingham) and DSTL.

One of the studies being undertaken by Imperial Blast is the assessment of the cellular and molecular basis of overpressure damage, by using live tissue under extreme mechanical impacts caused by compression waves. For this purpose, a biocompatible chamber that can be used in a split-Hopkinson pressure bar device (for testing the dynamic stress-strain response of materials) has been designed to house a tissue culture system of mouse mesenchymal stem cells (MSCs). These cells were chosen because they are precursors for many tissues commonly injured by explosions.

Figure 1: An overview of the Imperial Blast Initiative



The initiative involves an integrated approach to investigating the molecular changes and biomechanical aspects of brain injury, utilising a combination of in silico, in vitro, in vivo *and human data, with the aim of reducing the severity and effects of trauma through the development of better treatment modalities and improved protective measures (see main text for further details).*

Discussion and Conclusions

The dilemma of using anaesthetised animal models

The ongoing need to protect civilians and servicemen from the effects of warfare, and the scientific and welfare issues raised by the use of animal models for this purpose, create a significant dilemma. There is a perceived need for continuing military research, testing and training, in order to adequately and effectively protect armed forces and civilian populations from the consequences of conventional and unconventional warfare. Currently, a wide variety of animal models are used for military purposes, ranging from studies on TBI and haemorrhage damage, to resuscitation/surgical training and biological warfare. In almost all cases, the animals involved are anaesthetised before experimentation, and are often allowed to recover, in order to permit investigations on the effectiveness of various therapeutic, preventive and protective modalities.

However, both scientific and animal welfare problems arise from the use of anaesthesia in this way, particularly when it is not terminal. The first scientific problem relates to the numerous and variable effects of anaesthetics on basic physiology and on the functioning of key target organs and systems, such as the brain and the lungs, the cardiovascular and respiratory systems, and the CNS. At worst, the effects of anaesthesia could alter the responses of these systems and organs substantially, thereby compromising the fidelity of the animal models, while at best, these effects need to be investigated and controlled, so as to minimise variability, and to facilitate repeatability and data interpretation.

The second main scientific problem relates to the varied methods for applying trauma to experimental animals in TBI investigations. Ritzel *et al.* (173) noted: "*...blast injury research has seen a range of irregular and inconsistent experimental methods for simulating blast insult generating results which may be misleading, cannot be cross-correlated between laboratories, or referenced to any standard for exposure. Although conventional laboratory shock tubes offer many advantages over field trials using actual explosive charges, these can only partially replicate blast conditions from explosive events even when carefully configured*". These authors describe an advanced blast simulation apparatus with a modified shock tube for addressing these issues, but it has not been widely used due to its complexity.

Welfare problems arise by allowing the recovery of treated animals, since it is likely that they endure pain and discomfort from the injuries and trauma experienced as part of the experimentation, even though they might be completely insentient during the conduct of the procedures. In addition to the above possibilities for adversely affecting animal welfare directly, is the potential for levels of humane concern for the animals to deteriorate as a consequence of the psychological effect on laboratory staff of the routine use of severe procedures, such as subjecting restrained animals to explosive blasts, or life-threatening surgery to simulate haemorrhaging, albeit all under anaesthesia. Such conditions and practices, conducted regularly by the same individuals, might well hinder the development of a culture of care in an establishment involved in this kind of work, more so than the utilisation of less-invasive procedures.

Potential solutions

There are three potential solutions to the above dilemma, namely: a) continue to use anaesthetised animal models, but assess the ways in which the chosen methods of anaesthesiology affect the performance of each model, and its outcomes, to facilitate data interpretation; b) minimise the complicating effects of anaesthesiology by careful selection of the type of anaesthetic, as well as its dose and the duration of treatment; and c) avoid the use of sentient animals (*replacement*). In practice, a sensible approach would be to adopt all three options, with a view to achieving *replacement* as soon as is feasible. While the first option appears to be the most immediate and practical way forward, despite all of the issues concerning anaesthesia that have been raised in this paper (none of which should be novel to any veterinarian), there is little or no discussion of these problems in the published military studies analysed for this review. Moreover, individual papers invariably lack sufficient details concerning the methods used for anaesthesia for the reader to judge the validity of the model involved in the context of the many potential effects of anaesthetics. At the very least, published guidelines on animal models for military purposes should recommend that, if an animal is anaesthetised, the anaesthetic agent(s), dosage and route of administration should all be documented. Clearly, an investigation of the detailed effects of anaesthesia on each animal model should be an integral part of its development and validation, before it is used routinely.

The possibility of minimising the effects of anaesthesiology (option b above) without compromising animal welfare, was investigated by Ren *et al.* (91). These authors developed a closed-skull mouse model of TBI, in which the effects of cerebral oedema were assessed. The new method minimises the time of anaesthesia, allows monitoring of ICP, and can be modulated to produce mild and

moderate grade TBI. After the induction of mild trauma in the model, BBB permeability, cerebral oedema and ICP had largely normalised within seven days.

Adopting either option a or b would not avoid any adverse welfare consequences that might be experienced by the animals after recovery. Only the avoidance of sentient animals altogether (option c) would achieve that end. Fortunately, a diverse range of *replacement* methods for military purposes exists. However, it is clear that their further development, validation and use need to be coordinated, harmonised and improved, much along the lines that have been adopted for new alternatives to toxicity testing. This analogy becomes closer when it is noted that *in vivo* models for TBI have not had much success in identifying therapies that have proven to be equally effective in the clinic. In addition, the extensive progress that has been made in computer and mathematical modelling of blast-induced damage, and the development of ever more-sophisticated human patient simulators for training purposes, should mean that the requirement to use animals, in what are often imprecise and unrealistic experiments, will be substantially reduced.

The existence of a combat trauma database in the USA, dubbed the Joint Theater Trauma Registry (JTTR; 174), should provide a source of useful human data against which the relevance of existing and new alternative methods can be assessed, instead of employing animal data. The JTTR is based on information obtained from deployed medical and surgical units, which has been pooled in a central databank at the Army Institute of Surgical Research at Brooke Army Medical Center in San Antonio.

In addition, there are many phenomena that occur as a result of trauma, which can be reproduced at the cellular level, while new biomarkers of trauma are being discovered by using *in vitro* systems. For example, the production of a group of specific injury proteins has been demonstrated in an *in vitro* cell culture model of TBI, after a standardised injury was induced by scalpel cuts through a mixed cell culture of astrocytes, oligodendrocytes and neurons (175). Protein identification was by mass spectrometry, and time-lapse microscopy and immunostaining showed that most of the *de novo* proteins were linked to specific cellular processes that occur in response to trauma — including cell death, proliferation, lamellipodia formation, axonal regeneration, actin remodelling, cell migration and inflammation (176).

It should be noted, however, that adequate biomarkers for TBI are sorely lacking and a search for better ones is the subject of a new project, which was launched by the National Institutes (NIH) at the end of 2012 (International Traumatic Brain Injury Research Initiative: NIH Cooperative Program for Comparative Effectiveness of Clinical Tools and Therapies; see http://grants.nih.gov/grants/guide/rfa-files/RFA-NS-13-008.html).

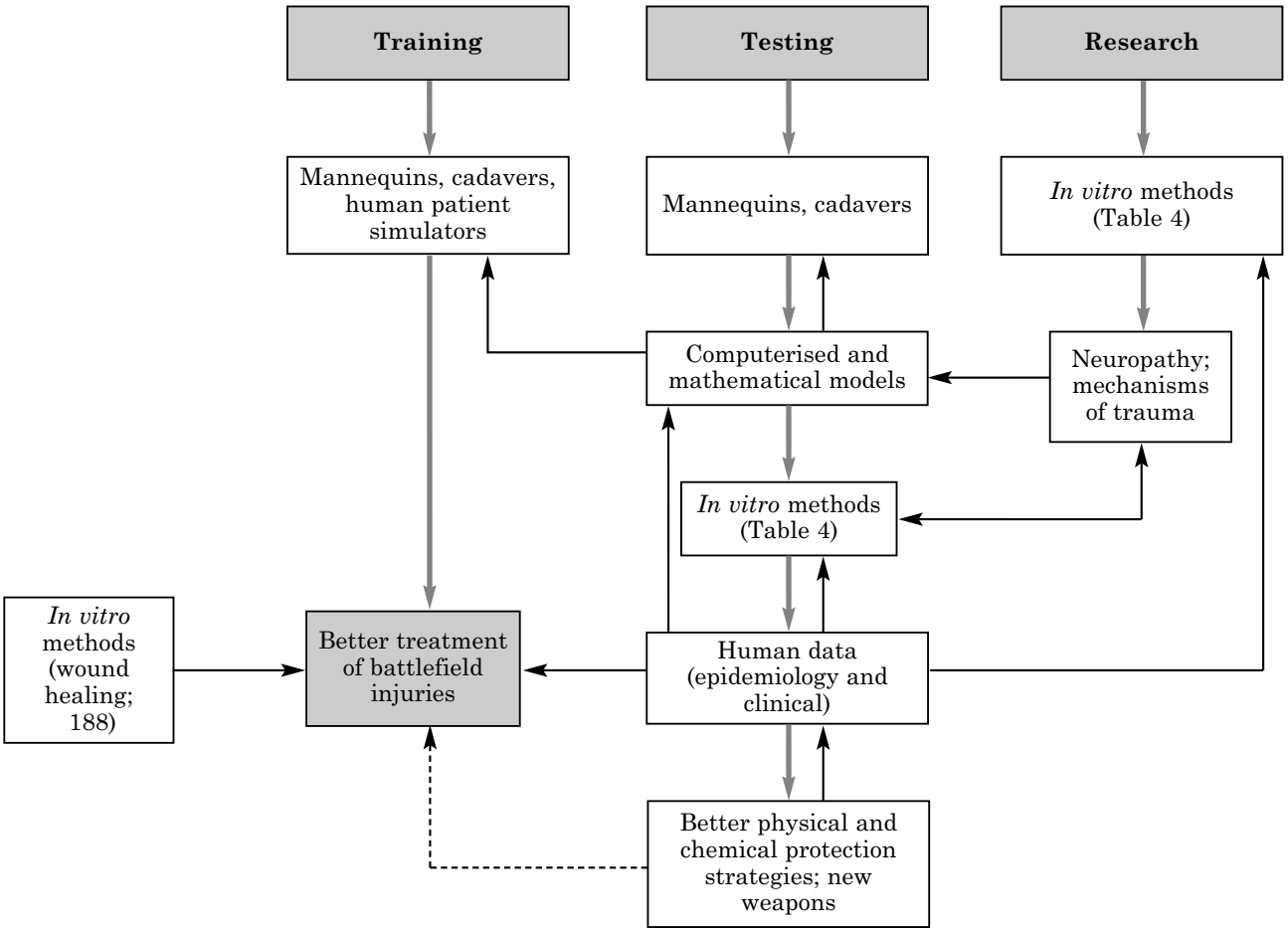
The potential of non-animal alternatives

It has been necessary to describe *in vivo* models used for military purposes in some detail, to indicate the wide range of procedures to which experimental animals are exposed, the need for anaesthesiology due to the severity of these procedures, and the purposes for which the animal models are used, in order to emphasise the dilemmas posed by this area of animal experimentation (see Table 2). From this, it can be appreciated that the major problems faced when attempting to implement *replacement* in the form of *in vitro* methods are: a) simulation of the infliction of penetrating and blast trauma on tissues and organs; and b) the assessment of the efficacy of physical protective methods, such as clothing (e.g. helmets and boots) and other measures (e.g. armoured vehicles). With respect to the former, it has been observed that the available techniques used to stress tissue cultures, the use of shock tubes, scraping the surface of tissue cultures, or the imposition of physical forces on organotypic culture systems, result in many effects that resemble those detected at the cellular level arising in animal models of TBI.

Tissue cultures are highly useful for assessing the effects of chemical and biological weapons and of the various antidotes and therapeutic interventions, as well as for studying wound healing. In the case of the evaluation of physical protective methods, tissue cultures have obvious limitations, but several well-developed mathematical and computerised models are available for this purpose instead — these have provided definitive data for many years (Figure 2).

With regard to the requirement for more *in vivo* models of TBI, it is noteworthy that several physicians that treat trauma patients on a regular basis do not believe that there is any need for further animal models (30). They also noted: "…*since the neuropathology of most head-injured patients is complex and comprises a mixture of diffuse and focal damage with a variable degree of secondary insults, no single experimental model can be expected to reproduce all aspects of clinical TBI*". This might be overcome by using batteries of animal models, but it would be unnecessarily complex, costly and lengthy, as well as increasing the total numbers of animals required, while the use of *in vitro* tests with overlapping complementary endpoints in batteries, is a very well-established concept in toxicity testing.

Figure 2: Potential and actual roles for *replacement* alternatives in military research, testing and training



Grey arrows (⟶) indicate the sequence of use of methods and data; horizontal and up arrows (⟶) represent opportunities for data input and feedback; dashed arrows (⇢) indicate possibilities for data transfer. Replacement techniques, particularly the use of in vitro *methods, exist in all three areas of military research. Information from the identification of mechanisms of toxicity and from human data is useful for improving the use of alternatives for testing new approaches to protection and treatment, as well as for developing better artificial models of the human body used for training (see main text for more details).*

That attitudes to the use of animals in military research are firmly entrenched in the traditional paradigm, with its focus on animal experimentation, is illustrated by the following statement: "*Some aspects of BINT [blast-induced trauma] can conceivably be studied* in vitro*. However, factors such as systemic response, brain oedema, inflammation, vasospasm, or changes in synaptic transmission and behaviour must be evaluated in experimental animals.*" This was written by Risling and Davidsson in 2012 (44), at a time when such dogma should have had no place in modern and flexible approaches to biomedical research and testing. The issues they raise should now be used as criteria for developing new improved *in vitro* methods. In the meantime, the use of integrated experimental strategies, involving *in silico*, *in vitro*, *in vivo* and human studies, as is being used in the Imperial Blast initiative, should be encouraged.

Recommendations

1. Papers reporting on results obtained by using anaesthetised animal models utilised for military purposes should always include full details of all aspects of anaesthesiology, as well as information regarding any relevant effects of the anaesthetic(s) used that might conceivably affect data interpretation. Such a strategy would be greatly facilitated by involving a vet-

erinarian at all stages of project planning, and by the rejection by journals of manuscripts that lack sufficient information.

2. Information on the potential modulating effects of anaesthetics on animal models for military research, testing and training should be sought and documented as an integral part of new model development, characterisation and validation.

3. The criteria for selecting an anaesthetic for use with animal models should take into account potential effects on the CNS, including the BBB, as well as on the cardiovascular and respiratory systems, including cerebral haemodynamics. The possibility for gender-specific effects should also be considered.

4. Anaesthetics acting as antagonists of the NMDAR should be avoided.

5. At the expense of developing yet more animal models of TBI and haemorrhage, the emphasis should be on developing more complementary *in vitro* models, each of which covers a specific mechanism responsible for conditions in patients, such that these can be used together in experimental strategies that cover the breadth and diversity of clinically relevant endpoints.

6. Despite issues of secrecy, ways should be found to harmonise and coordinate the development, validation, acceptance and deployment of new and existing alternative methods for military purposes, according to principles and criteria analogous to those agreed internationally for new toxicity testing methods.

7. There needs to be more transparency about the use of animals for military purposes. To this end: a) the numbers of animals and procedures used for military purposes should be itemised separately in published statistics, such as those produced by the Home Office in the UK and the EU (this would also facilitate monitoring of progress in implementing alternatives); and b) the DSTL in the UK should become a member of the Concordat on Openness on Animal Research (www.understandinganimalresearch.org.uk/policy/concordat-on-openness-on-animal-research).

8. The advantages and limitations of existing alternatives to animal models for military use should be discussed at an international workshop to: a) assess their current utility as part of integrated (i.e. *in silico*, *in vitro*, and *in vivo* animal and human studies) schemes; and b) provide information for guiding test improvement and new test development to fill gaps in the arsenal of non-animal methodologies.

9. While all staff using and handling animals should be monitored to ensure that their standards of, and attitudes toward, animal care are maintained at a satisfactory and high level, those employing highly invasive animal procedures, which are an integral part of many uses of animal models for military research, should receive especially close monitoring in this respect.

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