In Vitro Models as a Platform to Investigate Traumatic Brain Injury

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Summary — Traumatic Brain Injury (TBI) remains a significant cause of mortality and morbidity, affecting individuals of all age groups. Much remains to be learned about its complex pathophysiology, with a view to designing effective neuroprotective strategies to protect sublethally injured brain tissue that would otherwise die in secondary injury processes. Experimental *in vivo* models offer the potential to study TBI in the laboratory, however, treatments that were neuroprotective in animals have, thus far, largely failed to translate in human clinical studies. *In vitro* models of neurotrauma can be used to study specific pathophysiological cascades — individually and without confounding factors — and to test potential neuroprotective strategies. These *in vitro* models include transection, compression, barotrauma, acceleration, hydrodynamic, chemical injury and cell-stretch methodologies. Various cell culture systems can also be utilised, including brain-on-a-chip, immortalised cell lines, primary cultures, acute preparations and organotypic cultures. Potential positive outcomes of the increased use of *in vitro* platforms to study TBI would be the refinement of *in vivo* experiments, as well as enhanced translation of the results into clinically meaningful neuroprotective strategies for the future. In addition, the replacement of *in vivo* experiments by suitable *in vitro* studies would lead to a welcome reduction in the numbers of animal procedures in this ethically-challenging field.

Key words: *head injury, in vitro models, neuroprotection, pathophysiology, Three Rs, traumatic brain injury.*

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Introduction

Traumatic Brain Injury (TBI) is defined as acquired, non-degenerative, acute brain trauma caused by an external mechanical force resulting in clinical or radiological evidence of injury. The incidence of TBI in Europe is estimated as 262 per 100,000 population, with most causes attributable to falls and road traffic accidents (1). TBI carries significant mortality, affects patients of all age groups, and results in severe disabilities in a substantial proportion of patients. Therefore, it is a significant socioeconomic burden to society, in addition to costs in human suffering (2).

The pathophysiological cascades that ensue TBI are not yet fully understood. Nonetheless, injury processes can be divided into primary injury, which occurs at the time of the mechanical insult, and secondary injury, which is the tissue damage that occurs in the minutes, hours or even days after the initial insult (3). The secondary injury involves several processes, such as inflammation, hypoxia, glutamate-mediated excitotoxicity, free-radical production, ion fluxes and the induction of enzymes, for example, caspases that lead to apoptotic cell death pathway initiation (4). Attenuation of the cellular and subcellular pathways that perpetuate the secondary injury, collectively known as 'neuroprotection', is being actively researched (5). Unfortunately, to date, no randomised controlled trials of neuroprotection in TBI have been successful (5). Of note, a large number of trials investigating the NMDA receptor antagonist class of drugs, carried out in the 1980s and 1990s, failed to translate to human clinical studies, despite promising data from animal studies (6). The reasons for this are multifactorial and have been reviewed elsewhere (7), but include:

— an incomplete understanding of the pathobiology of TBI in humans and TBI in animal models;
— the diverse aetiologies and clinical presentation of TBI;
— suboptimal translation of potentially neuroprotective agents because of issues related to drug pharmacokinetics, pharmacodynamics, safety, and brain-tissue penetration ability;
— the lack of due consideration of an appropriate time-window for treatment (e.g. should treatment be initiated at the accident scene?);
— unsuitable patient selection;
— inadequate patient consent;
— incomplete identification/definition of appropriate endpoints or therapeutic outcomes;
— statistical problems, including the misrepresentation of data; and

— publication and presentation bias, including missed opportunities to understand why other research might not have translated successfully.

However, a further important contributor to the failure in the development of neuroprotective agents could be the over-reliance on *in vivo* models of TBI, which do not accurately depict the complexity of TBI in humans (8). There are concerns surrounding not only the technical aspects of *in vivo* TBI models, in terms of their reliability and predictive outcomes, but also the ethics of the animal studies (9, 10). Although all animal research in the UK is controlled by Home Office Licence regulations, significant numbers of animal studies are performed. A number of missed opportunities for the *reduction*, *refinement* and *replacement* of experimental animal use have been identified (11, 12), which would, if addressed, fulfil the key principles of the Three Rs (13). Accordingly, there is an urgent need for the further development of new non-animal alternative methods to supplement animal studies in the exploration of the mechanisms underlying neurotrauma, with a view to subsequently developing hypothesis-based strategies that can be tested with more confidence (14).

Here, I suggest that *in vitro* models offer the possibility of dissecting out specific pathophysiological mechanisms, and only once specific mechanisms have been identified should clearer hypotheses be tested. This approach is more likely to subsequently translate into the neuroprotective therapies of the future. It is also important to note that, although *in vitro* models are not exempt from ethical issues, they certainly present significantly fewer such issues than *in vivo* models. In addition, *in vitro* models allow more data to be obtained in a shorter time-frame, especially if a high-throughput format is used and if systems, such as brain-on-a-chip systems, are further developed in the future (14).

Animal Models of TBI Are Not Accurate Representations of the Human Situation

Several animal models of TBI have been used extensively, and have been reviewed in detail elsewhere (15, 16). However, the consensus is that no optimal model exists (8). The commonest *in vivo* models include fluid percussion injury, cortical impact injury, weight-drop impact acceleration injury, ballistic/penetrating injury and blast injury methodologies (15). A variety of animal species, including mice, rats, rabbits, ferrets, cats, dogs, sheep, pigs and primates, have been subjected to these injuries. However, most animal studies are carried out on rodent models due to lower costs, simpler surgical procedures and more straightforward animal care. Rodent models are also more amenable to analysis through the use of transgenic or knockout animals (17). Conversely, studies on larger animals are expensive and necessitate more extensive veterinarian involvement for complex care after surgery. In addition, the resulting data are more difficult to analyse from a neurobehavioural point of view (8).

Animal models of TBI incompletely represent the human situation in a number of ways, particularly with regard to size considerations and extensive anatomical and histological differences. Firstly, several anaesthetic drugs that would need to be given around the time of surgery exert effects on cerebral blood flow, metabolism and intracranial pressure, which could confound experimental findings. Secondly, animal models are not able to recapitulate distinct focal or generalised TBI. As such, a number of clinical phenomena, such as seizures, reduced consciousness or coma, following diffuse TBI, are not accurately modelled in animal studies (8). Similarly, peri-lesional hypometabolic or ischaemic tissue is considerably underestimated, depending on the model. Finally, although the severity of the external injury (e.g. weight-drop or acceleration delivered) can be controlled in *in vivo* studies, the extent of internal injury (e.g. pressure to tissue or deformation) cannot be adequately controlled or verified. This lack of control accentuates animal-to-animal variability (18). Nonetheless, certain situations can only be studied in an *in vivo* context, such as neurobehavioural studies, advanced electrophysiological or vascular studies, and studies where systemic influences are present (e.g. cardiorespiratory or some immunological studies).

In Vitro Systems

As stated above, *in vitro* models of TBI can play a primary role in establishing and testing individual hypotheses in isolation, so that neuroprotective strategies can be investigated with more confidence (14). Such model systems permit tighter control of experimental variables, so that confounding factors can be eliminated more easily, leading to higher experimental fidelity (14). In addition, *in vitro* studies of TBI pose fewer ethical problems and are much more cost-effective, thus offering a potential high-throughput format for the screening of neuroprotective agents (19).

At the same time, one needs to be aware that brain injury and recovery in its entirety cannot currently be modelled through the use of *in vitro* models alone. *In vitro* studies offer snapshots of the situation under controlled conditions, and these must be studied in the real-life context. A putative therapeutic strategy is unlikely to translate in human studies without *in vivo* demonstra-

Table 1: *In vitro* models of TBI

TBI mechanism	Methodology	Comments
Static mechanical injury	Transection	Relatively straightforward technique, not technically demanding and inexpensive to carry out. Severity of injury can be adjusted. The extent of the injury is then quantified by assessing the proportion of damaged cells. Can be used to study secondary injury pathways. Models do not permit the estimation of certain mechanical variables, including force, strain and strain rate.
	Compression	Analogous to animal models of weight-drop or dorsal cord injury. The severity of the injury can be controlled by adjusting the force, depth, impactor shape and duration. Particularly useful in the study of secondary injury pathways. However, the strain field at the site of impact and the rate of application of the strain cannot be measured.
	Barotrauma (i.e. hydrostatic pressure)	Permits good control over injury parameters. Injury can be created by subjecting tissue to: a quasi-static barotrauma chamber (constant pressure can be applied for a given period); a modified fluid percussion device; or a weight dropped on a piston fluid-sealed chamber. Barotrauma is distinct from compression in that a constant pressure is exerted. Injury parameters are controllable, but tissue injury is difficult to verify. Neural tissue is relatively incompressible and it might not actually deform under hydrostatic pressure in most types of clinical TBI. Might be useful to model blast injury.
Dynamic mechanical injury	Acceleration/ Deceleration	The most important mechanisms of neuronal death in human TBI (manifest in shear forces resulting in contusions or diffuse axonal injury). Strain guidelines applied *in vitro* are adjusted accordingly, and can be derived from physical models of clinically relevant post-injury outcomes. Tissue deformation cannot be measured.
	Hydrodynamic	Permits direct visualisation of morphological changes and calculation of tissue shear strain; 3-D shear strain models that allow confocal microscopic visualisation have been developed. A technical shortcoming is that the temporal resolution of the strain measurements was limited to a strain rate of 30/second, which might lead to inconsistent injury modelling.
	Cell-stretch	Can replicate secondary (i.e. sublethal) brain injury. Include standardisable and reproducible injury, with direct, and real-time measurements of all biomechanical aspects. Physiological levels of tissue deformation are achievable and injury tolerance can be studied as well, if a standardised, minor injury is delivered. However, these systems are relatively expensive and require experienced users.
Chemical injury	Glutamate treatment	Administered to neural tissue *in vitro* has been used to study cell death in acute neuronal injuries, including stroke and TBI. Chemical injury can either be studied in isolation, or as an adjunct to mechanical injuries, for example, glutamate injury plus cell-stretch modelling.
	Peroxide treatment	Free-radical injury is a key feature in the propagation of secondary injury in TBI. Tissue exposed to hydrogen peroxide simulates oxidative stress and has been used to model several neurological diseases, including TBI, *in vitro*. As above, can be studied in isolation, or as an adjunct to mechanical injuries.
Various	Brain-on-a-chip	Offers an opportunity to model the disease process accurately, for example, by permitting the inclusion of free-radicals or through the use of genetically modified cells. Has been used to model a subtype of TBI (diffuse axonal injury) that occurs when the brain is exposed to rotational mechanical shear and strain. Currently, the ability to accurately model TBI on a chip is limited because of the complex and incompletely understood pathophysiology of the condition. The delivery of a mechanical injury at the microscopic level of axons and neurons could be challenging (e.g. acceleration/deceleration of microscopic amounts of tissue would be impracticable) and the fidelity with which it models human TBI could be questioned.

tion of efficacy. It is unlikely that a drug or therapeutic strategy that has not been first tested on animals will be tested on a trauma patient due to legal, ethical and consent issues. To emphasise, the current *in vitro* models are not scientifically superior to *in vivo* models, and must be used in conjunction to further understand TBI pathophysiology (14). However, on a positive note, one study reported that 88% of agents demonstrating neuroprotective efficacy with *in vitro* models of neurotrauma appeared to have similar effects *in vivo* (18). Despite these findings, demonstration of efficacy *in vitro* is not a prerequisite prior to obtaining ethical approval for *in vivo* studies.

The design of *in vitro* injury models

Accurately translatable injury models are key to understanding the pathophysiology of TBI. Newer *in vitro* models of neurotrauma have largely superseded older techniques. One of the older models is the rotating stir-bar model, in which 1mm^3 sections of rat cortex are immersed in nutrient medium and subjected to a mechanical injury by a rotating stir-bar for time periods between 3 minutes and 48 hours (20).

Although this was one of the pioneering *in vitro* models of TBI, which provided important information regarding the potential differential survivability of neurons and glia from anatomically different areas, contemporary TBI models attempt to accurately reproduce each distinct mechanical component of TBI in isolation. Mechanical stimuli can include a combination of transection (e.g. bone fragments/depressed skull fracture or shrapnel/bullet), compression (e.g. haematoma or oedema), barotrauma (e.g. blast, pressure waves due to bullet tracks), acceleration/deceleration (e.g. coup or contre-coup injuries), and hydrodynamic shear and strain (e.g. tangential/linear/rotational as brain shears and strains in a closed skull container). These models must provide repeatable, well-controlled, environmentally isolated experimentation, without systemic confounding factors such as inflammation, hypoxia and ischaemia (18).

The following sections include overviews of: a) static mechanical TBI (transection, compression and barotrauma); b) dynamic mechanical TBI (acceleration/deceleration models and hydrodynamic models and cell-stretch models); c) chemical TBI; d) the use of brain-on-a-chip systems.

Static mechanical systems

Transection

Transection models, first described by Ramón y Cajal (21), permit the study of axotomy and penetrating white matter injury. Transection at tissue level can be modelled by using a plastic stylet (22), a rotating scribe (23) or blades (24) to scrape adherent neurons (with or without glial cells) in culture plates. The severity of the injury can be increased as necessary by increasing the number of lesions or the spatiotemporal duration of the injury. The extent of the injury is then quantified by assessing the proportion of damaged cells.

These injury models, collectively termed macrotransection models, involve a relatively straightforward technique, are not technically demanding, and are inexpensive to carry out. These characteristics make them ideal platforms for high-throughput drug discovery testing (25). They have been used to study secondary injury pathways, including glutamate-mediated excitotoxicity and glial activation, and are representative models of missile and penetrating brain injuries (18).

A more precise micro-transection model, which delivers microlesions on single cells, has also been developed (26, 27). This system can be so precise that specific subcellular particles or organelles can be targeted, for example, neurites or nuclear structures (28). Injury parameters can be accurately controlled, although increasing sophistication can result in increasing costs and a higher level of operator skill being required due to the use of single-cell molecular biology techniques. This can result in an inevitable decrease in experimental throughput.

Transection models have some disadvantages: they do not permit the estimation of certain mechanical variables, including force, strain and strain rate (14). Furthermore, the relative significance of primary axotomy as compared to secondary axotomy, in the clinicopathology of non-penetrating TBI, is still uncertain (29).

Compression

Compression phenomena are a feature of focal brain contusions, traumatic intracranial haemorrhage and spinal cord injury. *In vitro* compression models are analogous to animal models of weight-drop or dorsal cord injury (14). Essentially, they involve a standardised insult delivered by using weight-drop or impactor methods (30, 31). The severity of the injury can be controlled by adjusting the force, depth, impactor shape and duration (18). Compression models are particularly useful in the study of secondary injury pathways (32). However, a downside to this type of model is that the strain field at the site of impact and the rate of application of the strain cannot be measured (14).

Barotrauma

Barotrauma, or hydrostatic pressure, can be modelled *in vitro*, and it permits good control over

injury parameters. This type of injury system can be created by subjecting the tissue to: a) a quasi-static barotrauma chamber, where a constant pressure can be applied for a specified amount of time (33); b) a modified fluid percussion device (34); or c) a weight dropped from a given height on a piston fluid-sealed chamber (35). Barotrauma is distinct from compression, in that a constant pressure is exerted.

Although injury parameters are controllable, tissue injury is difficult to verify (18). To induce cellular injury in the quasi-static barotrauma model, prolonged exposure to very high pressures (e.g. 15 atmospheres) over a prolonged time period (e.g. 10 minutes) is needed, which raises questions about the translatability of this model. Furthermore, whereas the fluid percussion and weight-drop models depict injury duration accurately, they overestimate the pressure changes, as estimated by computational analysis such as finite element modelling (36). Additionally, neural tissue is relatively incompressible (compared to, for example, cardiac or skeletal muscle) and it is uncertain whether it is actually subject to deformation under hydrostatic pressure in most types of clinical TBI (37). However, as there is increasing interest in the study of blast injury, barotrauma models might be useful to model this specific subtype of TBI (38).

Dynamic mechanical systems

Acceleration/deceleration

Acceleration/deceleration forces are arguably the most important mechanisms of neuronal death in human TBI, as they manifest in shear forces resulting in contusions or diffuse axonal injury (14). A fairly user-friendly method that has been developed can subject brain tissue to tangential accelerations at high G-forces, which closely mimics human TBI (39). However, as with the models mentioned above, tissue deformation cannot be measured.

The manner in which *in vitro* tissue deformation is produced does not necessarily need to mimic the *in vivo* scenario, as long as similar magnitudes and rates are achieved (14, 19). Strain guidelines applied *in vitro* are adjusted accordingly, and can be derived from physical models of clinically relevant post-injury outcomes. For example, Margulies *et al.* (40) filled skulls of non-human primates with material mimicking the properties of the brain and exposed them to inertial loading forces seen in diffuse axonal injury. The data obtained then became the basis of further *in vitro* experiments.

Hydrodynamic models

Inertial loading of the brain, causing shear or stretch forces, can be studied through hydrodynamic models of TBI. The *in vitro* cell shearing injury device consists of a parallel plate viscometer that has cells growing on one plate and a variable speed rotating plate that permits the application of a range of hydrodynamic forces on the cells by adjusting the speed of the rotating plate and the gap distance between the plates (41). Individual cell strain can be accurately calculated, as the cells can be observed directly by light microscopy, and indirectly by measurements on microbeads attached to the plates. However, a technical shortcoming is that the temporal resolution of the strain measurements was limited to a strain rate of 30/second, which limits the flexibility of injury modelling (14, 19). A similar model of milder head injury has been achieved through the use of air or fluid micropipetted onto the cell layer to generate mild fluid shear stress. This approach has allowed improved visualisation and determination of cellular hydrodynamic pressures (42).

Despite these technical shortcomings, hydrodynamic shear strain-based TBI modelling might be more relevant to the pathological processes in human TBI than models based on acceleration/deceleration shear stress — for example, when cultured tissue is placed in an inertial loading module and then subjected to linear impact-induced inertial loading in the form of an acceleration over a 9cm distance, followed by deceleration, to create a predefined shear strain (43). However, the advantages of this latter model include the direct visualisation of morphological changes and calculation of tissue shear strain. Three-dimensional shear strain models that allow confocal microscopic visualisation have also been developed (44). A relative disadvantage of hydrodynamic shear-stress based models is that, although hydrodynamic forces are thought to be more relevant to the pathological processes in human TBI than shear strain-based, they are likely to be less clinically relevant in most types of human TBI than other acceleration/deceleration injuries.

Cell-stretch models

A common disadvantage of all of the above mentioned models is that they do not completely depict the current understanding of clinical TBI. To investigate secondary injury processes, a model that significantly injures the cells, but does not kill them (i.e. sublethal), is more appropriate. Sublethal traumatic neuronal injury *in vitro* triggers a range of distinct signalling pathways leading to specific secondary injury, rather than to a generalised increase in vulnerability to delayed injury (45). As such, models that use cell-

stretch strategies are at the present time considered to be the gold standard for mimicking sublethal traumatic neuronal injury. The advantages of this type of model include standardisable and reproducible injury, with direct, and real-time measurements of all biomechanical aspects. However, these systems are relatively expensive and require experienced users to carry out the experiments.

Early modelling was performed on cultured rat astrocytes in six-well culture plates with a flexible silastic bottom that deformed when a graded pressure pulse of predetermined amplitude and duration was applied (46). Cell, or tissue, strain could be indirectly measured as a proportion of substrate strain (14).

This was followed by a model of NG108-15 'neuroblastoma cross glioma' cells grown in custom-built wells, which were deformed with a vacuum pulse (47). This model offered an important advancement: it allowed strain rates to be a modifiable parameter, with rates between < 1/second and 10/second achievable. This approach has progressed further, to achieve higher strain ratios and a wider range of strain rates, and more accurate measurement and delivery of injury via a computer-controlled solenoid valve (48). Tissue injury is measured through a laser displacement transducer, which allows dynamic membrane deflection to be calculated in a non-contact manner. Physiological levels of tissue deformation are achievable and injury tolerance can be studied as well, if a standardised, minor injury is delivered (14).

Uniaxial, biaxial and 3-D injury

In vivo brain tissue injury occurs in 3-D, so 3-D modelling ostensibly offers greater simulation potential and translatability (44). One of the pitfalls, however, is the sheer complexity of the data obtained, which cannot be completely deciphered by automated or programmable methods and thus necessitates the use of tensor field mathematics — a postgraduate subspecialty in its own right. On the other hand, uniaxial injury could better simulate axonal injury, because it can cause injury to cell processes, such as axons and neurites, without the confounding effects of cell body injury (18, 49). A collaborative effort with mathematicians, or those experienced in tensor field mathematics, would be of considerable interest.

Biaxial injury is more damaging than uniaxial injury by about one order of magnitude, as determined through the measurement of intracellular free-calcium changes, which are a hallmark of neuronal injury (50). By extrapolation, 3-D injury would be expected to further increase the extent of the trauma to *in vitro* tissue. A computer-generated strain pattern has been developed for this purpose, and the 'heterogeneity' of the substrate strain field (or the change of rate of strain) has been found to be the most injurious agent out of the other variables (51). Once again, physiological considerations of injury must be borne in mind, in order to accurately model TBI *in vitro* (14). Studying the injury under conditions as close as possible to reality is always a preferred option.

Milder injury

As above, the addition of further variables to *in vitro* models seeks to increase the magnitude of tissue injury. However, there is much interest in modelling milder head injuries, as they offer a more realistic target for intervention (14). Sublethally injured neurons are more susceptible to further injury, and their subsequent death constitutes secondary injury. They appear to exhibit DNA fragmentation, and mitochondrial and nuclear abnormalities, suggesting the role of apoptotic rather than necrotic processes, and thus offering an opportunity for neuroprotection (45).

To model mild injuries, representative neural tissue was placed on coated silastic membranes on a six-well plate, which was subjected to automated injury with the Flexcell® FX-3000™ Strain Unit (Flexcell International, Burlington, NC, USA), which is a computer-driven instrument that delivers a vacuum pulse to deform the silastic membranes for a duration of 1 second to a stretch of 130%. Although the actual strain rate or tissue strain could not be measured definitively, it was presumably much less than that delivered by previous models (14, 45). Crucially, this experiment successfully demonstrated neuroprotection — it convincingly showed decreased apoptosis through the use of a free-radical scavenger.

Chemical injury

In addition to the models above, which confer a mechanical injury to tissue, generic neuronal injury can be simulated through the application of neurotoxins. A detailed discussion is beyond the scope of this paper; here we refer to two examples — glutamate injury and peroxide injury.

Glutamate-mediated excitotoxicity is one of the key mediators of neuronal cell death in several neurological diseases, including neurotrauma (4). Extracellular glutamate administered to neural tissue *in vitro* has been used to study cell death in acute neuronal injuries, including stroke and TBI (52). Similarly, free-radical injury is a key feature in the propagation of secondary injury in TBI (4). Tissue exposed to hydrogen peroxide simulates

oxidative stress and has been used to model several neurological diseases, including ischaemia and TBI, *in vitro* (53).

Chemical injury can either be studied in isolation, or as an adjunct to mechanical injury, for example, glutamate injury plus cell-stretch modelling.

Brain-on-a-chip systems

There is considerable interest in the development of 'brain-on-a-chip' systems to investigate neurotrauma. The brain-on-a-chip system, a subtype of body-on-a-chip systems, consists of 3-D cell cultures that attempt to model the physiological responses of brain tissue in a microfluidic environment, i.e. at a scale and volume very much smaller than other models (54). Currently, the main use of brain-on-a-chip systems is in the high-throughput screening of chemicals in pharmacological and toxicology studies (54). In other words, a micro-model of a neurological disease can be created on a chip and a large number of chemicals screened to evaluate positive physiological effectiveness. Brain-on-a-chip models also offer an excellent opportunity for the detailed study of disease processes — for example, by adding free-radicals, causing inflammation, or using modified cell lines to simulate the disease process. Currently, the ability to accurately model TBI on a chip is limited because of its complexity and incompletely understood pathophysiology. In addition, the delivery of a mechanical injury at the microscopic level of axons and neurons could be challenging (e.g. acceleration/deceleration of microscopic amounts of tissue would be impracticable), and the fidelity with which it models human TBI could be questioned. Therefore, further development of this approach is warranted.

Nevertheless, a subtype of TBI — i.e. diffuse axonal injury — that occurs when the brain is exposed to rotational mechanical shear and strain has been successfully modelled. To achieve this, Dollé *et al.* (55) developed a brain-on-a-chip system where pneumatic pressure was applied through a deformable plate upon which the cultures had been placed, and which delivered a uniaxial strain injury. Axonal injury and biochemical changes were accurately replicated and mitochondrial membrane potentials were studied in this context (56). This model can be thought of as a variation of a cell-stretch system — albeit on a smaller scale. Further work on these models is eagerly anticipated.

Other cell culture systems

A range of cell types and tissue preparations can be used to model TBI and serve as alternatives to *in vivo* experimentation. However, it is crucial that the most appropriate sources of cells and tissues are selected. Some examples — including immortalised cell lines, primary cultures, acute preparations (i.e. *ex vivo* tissue), and organotypic cultures — are outlined below.

Models of extraparenchymal tissue, which could be used in neurotrauma studies, have also been developed. These include cerebral vascular explants (57), blood–brain barrier (58), neural stem cells (59), meninges and bone fragments (60). Obvious limitations are the lack of a circulatory system and the lack of influence of infiltrating inflammatory cells. On the other hand, these could be seen as strengths, in that the influence of inflammatory pathways and circulatory factors can be studied separately and under controlled conditions, by their precise addition following injury (18). It must be emphasised that the cell culture systems described are not immune from ethical problems — the Three Rs principles must still apply, as discussed above.

Immortalised cell lines

A number of immortalised cell lines are available commercially, and can provide a suitable system in which to study neurotrauma *in vitro*. Their main advantages are that they tend to be inexpensive, robust and low-maintenance, affording the ability to be freeze-thawed and passaged several times. Certain cell lines can model neuronal or glial characteristics fairly accurately. For example, the NT2 human teratocarcinoma cell line treated with retinoic acid yields a high proportion of post-mitotic neuron-like cells (41). Similarly, NG108-15 'neuroblastoma cross glioma' cells can be terminally differentiated into neuron-like cells (47), and PC2/PC12 cells and SH-SY5Y neuroblastoma cells can be differentiated into neuron-like cells with processes that often express ion channels (61, 62). In addition, microglial cells can be modelled by using the BV-2 cell line (63) and endothelial cells modelled with ECV304 (64). Certain cell lines, such as the C6 glioma line, can be effectively transfected to express particular genes of interest (14).

However, among the disadvantages of immortalised cell lines is that their mitotic and immunological behaviour can differ considerably from that of neural tissue and, as such, their response to trauma, including paracrine signalling, might also be distinct (14). During the process of immortalisation, the inherent cell death cascades, including apoptotic mechanisms, are altered — for example, glutamate-mediated excitotoxicity is not adequately modelled in immortalised cell lines. Additionally, patterns of gene and protein expression may differ considerably from actual brain tissue, and there can be disruption of contact

dependency, as well as poor adhesion to plates, which can make some injury paradigms difficult to represent (18).

Primary cultures

Primary neuronal or glial cells are cultures that have been dissociated from brain tissue. They can be used to provide insight into the responses of a particular cell type or culture mix to mechanical injury. Astrocytes and microglia are the most agreeable cell types for culture, owing to relatively lesser apoptosis in culture, with neurons and oligodendrocytes somewhat less robust, although protocols are in place to increase the *in vitro* viability of the latter (65). Primary cultures can either be homogenous cell types (34, 47), or a mixture of neurons and glia (22, 23). A disadvantage of a mixed culture of neurons and glia is that the presence of glia has been shown to be a hindrance in laser microtransection studies — laser microtransection preferentially injures neuronal processes (39). Nonetheless, tissue engineering methods can optimise injury models by culturing lower densities of mixed populations of cells within hydrogels (66).

The development of primary cultures is somewhat technically challenging, particularly their harvesting, which is often from embryonic tissue for best results. Indeed, it could be argued that experiments on such tissues might better reflect the developing or immature CNS, and therefore do not model adult neurotrauma accurately (14). For example, neurons derived from embryonic tissue do not express glutamatergic receptors, and, by themselves (i.e. unless they are maintained in long-term culture), do not exhibit glutamate-mediated excitotoxicity, which is the key mediator of cell death in adult neural tissue (67). As immature tissue is more plastic and adaptive relative to adult tissue, it might be that this methodology underestimates the response to injury. A possible way to mitigate this shortcoming is to add mature astrocytes to primary cultures derived from embryonic tissues (14).

Another consideration is that, in establishing primary cultures, the necessary mechanical dissociation and enzymatic digestion processes can disrupt intracellular connections and possibly lead to direct cell injury. This concern is relevant, as the role of neural networks and neuron–glia interactions in neurotrauma are currently a subject of keen investigation.

Acute (ex vivo) preparations

Freshly explanted animal brain tissue can be prepared in thin slices. Most experimentation needs to be completed rapidly (within eight hours or so), before the tissue begins to deteriorate and lose viability (68). The types of animal tissue commonly used include the frontal cortex (69), cerebellum (70) and hippocampus (68), mostly derived from rodents.

There are a number of advantages in studying acute preparations. Firstly, acute preparations derived from adult animals give a more accurate depiction of neurotrauma in adults (compared to tissue derived from embryonic sources). Secondly, the absence of tissue dissociation requirements preserves the original anatomical structure (including basement membrane and extracellular matrix), neuronal circuitry and heterogenous cell populations (18).

The major shortcoming of acute preparations is that it is unclear whether the tissue isolation process modifies the subsequent injury responses. Furthermore, the culture medium could dilute injury mediators and thus alter the injury response and the data obtained (18).

Organotypic cultures

Organotypic cultures are thin explants of tissue which are cultured *in vitro* as a whole, i.e. without cell dissociation, and are then maintained in culture for extended periods of time (typically in excess of seven days), where they can undergo maturation. The anatomical structures and neuronal circuitry are well preserved (as in acute preparations), and this permits the analysis of specific brain regions, typically the hippocampus (14), although coronal brain sections (71), thalamocortical mixed cultures (31), spinal cord (33) and slices of cortex (72) have also been employed.

Histological, cytological and proteomic characteristics are accurately represented *in vitro*, as tissue architecture and biochemical properties are maintained (73–77). In addition, synapses mature appropriately *in vitro* (78), and this permits electrophysiological studies, including those on long-term potentiation (14). However, the biggest advantage of organotypic cultures is that the tissue has had the opportunity to recover from the trauma of harvesting (as compared to acute preparations). As tissues are maintained in culture over extended periods of time, damaged cells and processes are cleared, denuded dendritic fields are repopulated, and a quiescent state is established, upon which trauma effects can then be studied (14, 18). In other words, the trauma of the explant process does not confound the injury administered.

The disadvantages of organotypic cultures are that the source-animal tends to be younger than postnatal day 11, and that maturation *in vitro* is slower than *in vivo*, which might skew the results obtained (18). As mentioned earlier, there is the concern that tissue from younger animals may

have more plasticity and relative resilience to injury (14). At present, the long-term culture of adult brain is not possible (79).

Other disadvantages are that organotypic culture tends to be expensive and technically demanding. Furthermore, the problems associated with modelling injury in 2-D compared to a more realistic 3-D system remain, because organotypic cultures must be restricted in thickness.

Conclusions and Perspectives

TBI still remains a significant cause of disease and disability. While strategies to prevent TBI have proven successful (for example, protective equipment and road safety initiatives), there are no neuroprotective medications or strategies that might help to reduce the secondary injury that occurs after the initial brain injury (80, 81). Incomplete or inaccurate experimental models of human TBI have contributed to some of the problems associated with the successful development of neuroprotective agents.

As described above, *in vitro* models can accurately depict certain aspects of the complex pathophysiological cascades involved in neurotrauma. Therefore, it is to be expected that *in vitro* studies are currently used to complement *in vivo* studies. Close approximation of clinical scenarios through *in vitro* studies allows the design of more-appropriate *in vivo* experiments, and thus decrease some of the inherent ethical problems associated with laboratory animal use. The search for potential neuroprotective agents in TBI continues, as more is learned about the complex pathophysiology of neurotrauma. This approach could lead to the development of drugs and strategies that might translate better in human studies.

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