THROMBOLYTICS TO AID CLOT LYSIS IN INTRACEREBRAL HAEMORRHAGE: IDENTIFYING STRATEGIES TO MAXIMISE BENEFIT

A thesis submitted to the University of Manchester for the degree of Master of Philosophy (MPhil) in the Faculty of Biology, Medicine and Health.

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Contents

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Abbreviations

Blood Brain Barrier (BBB)
Central Nervous System (CNS)
Clot Lysis:
Evaluating Accelerated Resolution of IVH (CLEAR)
Damage-associated molecular pattern (DAMP)
Foetal Bovine Serum (FBS)
Haem Oxygenase (HO)
Haemoglobin (Hb)
High mobility group protein B1 (HMGB1)
Interleukin-1 (IL-1)
Interleukin-1 receptor antagonist (IL-1Ra)
Intracerebral Haemorhage (ICH)
Intraventricular Haemorrhage (IVH)
Matrix metalloproteinases (MMP)
Minimally Invasive Surgergy (MIS)
Minimally Invasive Surgery Plus rt-PA for ICH Evacuation (MISTIE)
N-methyl-D-aspartate (NMDA)
Nitric Oxide (NO)
NMDA Receptor (NMDAR)
Nuclear Factor Kappa-B (NF-κB)
Nuclear factor-erythroid 2-related factor-2 (Nrf2)
Peroxisome proliferator activated receptor gamma (PPARy)
Phosphate Buffered Saline (PBS)
Plasminogen Activator Inhibitor (PAI1)
Reactive Oxygen Species (ROS)
Standard Deviation (SD)
Subarachnoid Haemorrhage (SAH)
Surgical Trial in Lobar Intracerebral Hemorrhage (STICH)
Tissue-type plasminogen activator (tPA)
Toll-like Receptor (TLR)
Tumour necrosis factor (TNF)
Urokinase-type plasminogen activator (uPA)

Abstract

Thrombolytics to Aid Clot Lysis in Intracerebral Haemorrhage: Identifying Strategies to Maximise Benefit

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The University of Manchester

Master of Philosophy

Intracerebral haemorrhage (ICH) is a subtype of stroke caused by bleeding into the brain. ICH has a high case fatality rate of 42% at 1 month and only 20% of survivors regain independence. Large clinical trials are currently underway to assess the potential benefit of minimally invasive surgery (MIS) in combination with the thrombolytic alteplase in ICH. Although preliminary results are promising alteplase is known to be neurotoxic and may therefore exacerbate damage when administered in ICH, reducing its overall effectiveness. Alternative thrombolytics to alteplase do exist and the initial aim of this project is to establish the toxicity of these compounds in comparison with alteplase in cell culture. Here the optimisation of a cell culture model of neuronal injury is described. This utilizes a glial-neuronal rat cortical co-culture with 5μM of FUDR added during seeding. After 12 days of culture cells are treated with the pro-inflammatory cytokine interleukin-1β as a full media change with serum free medium. LDH release is used to measure viability after 72h and this resulted in a low basal cell death which is increased moderately with 0.1ng/mL of IL-1β. The activity of each thrombolytic has also been determined using a clotting assay. These can now be combined to compare toxicity in culture. Optimising the original MIS approach represents a 'back-translation' of what has been shown to be effective in the clinic and how this can be improved further using basic research. All drugs used are already licenced for use in patients and as such, the use of an alternative thrombolytic could be implemented relatively easily to improve mortality and functional outcomes after ICH.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Introduction

Stroke is defined by the WHO as 'an interruption of the blood supply to the brain...This cuts off the supply of oxygen and nutrients, causing damage to the brain tissue'[1]. There are two subtypes of stroke, ischaemic and haemorrhagic. Ischaemic stroke is the result of a blocked artery, preventing blood flow, and causing downstream oxygen deprivation and ultimately tissue death, or infarction. Haemorrhagic stroke refers to bleeding into the brain parenchyma caused by vessel rupture and accounts for 15% of all cases of stroke[2] (Fig. 1b). There are 2 main subtypes of haemorrhagic stroke based on the location of the bleed; subarachnoid haemorrhage (SAH) is bleeding on the surface of the brain and intracerebral haemorrhage (ICH) occurs within the brain parenchyma. Intraventricular haemorrhage (IVH) refers to bleeding within the ventricles which rarely occurs in isolation and is most commonly an extension of ICH. ICH is the most common form of haemorrhagic stroke, accounting for approximately 10% of all stroke cases[3] (Fig. 1b).

Incidence and Outcome of Stroke

Stroke is the second most common cause of death worldwide, after heart disease[4]. ICH has a high mortality rate of 42% at 1 month compared to 16% for ischaemic stroke[5]. Although ischaemic stroke is responsible for more death globally (5.97%) than ICH (5.79%)[6]. However mortality rates underestimate the burden of stroke, both financially and socially, with over half of stroke survivors becoming dependent on others upon discharge[7]. ICH in particular is associated with serious neurological deficits with just 20% of survivors regaining independence[8]. The overall stroke incidence is decreasing and this can be attributed to improved preventative measures, for example, diagnosis and treatment of hypertension[9]. However the incidence of ICH and case fatality at 30 days post stroke, remained unchanged between 1983 and 2003 (Fig. 1a and c)[10]. This may be attributed to the lack of specific treatment for ICH.

Location of Haemorrhage

Deep ICH refers to haemorrhage located in the ganglionic regions, cerebellum and brain stem[11], with the former being the most common. Haemorrhage occurring in the cortical-subcortical areas is referred to as lobar[11]. Location of the initial bleed has implications for treatment and prognosis. Larger

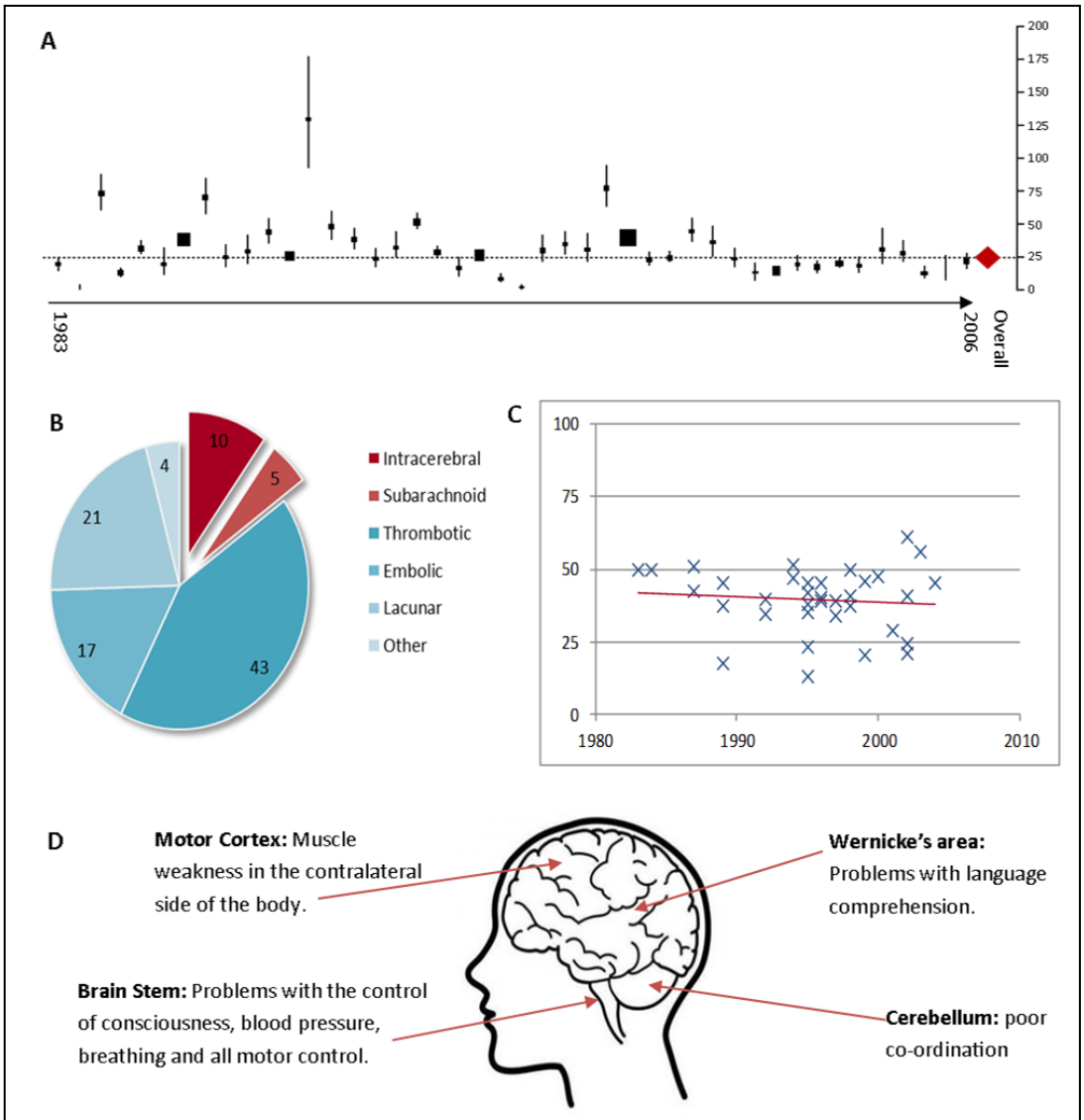


Figure 1: Stroke Statistics

A) Incidence of ICH per 100,000 person-years from 44 study periods. Size of the point estimates is proportional to the weight of the studies. Overall incidence was 24.6 per 100,000 person years (95% CI 19·7–30·7)[10]

B) Breakdown of stroke incidence by subtype. Haemorrhagic stroke in red and ischaemic stroke in blue.

C) Case fatality at 1 month post-ICH. Median (indicated by red line)was 40.5% (range 13·1–61·0)[10]

D) Typical symptoms of ICH by location.

haematomas are associated with poor outcomes and high mortality rates partially because they often spread into the ventricles causing secondary IVH; a predictor of early mortality[12]. Surface haematomas are more easily operable and so may be associated with positive outcomes. A subgroup analysis of the STICH (International Surgical Trial in Intracerebral Haeamorrhage) trial suggested that early surgical evacuation, within 24 hours, may be beneficial for patients with superficial bleeds but without extension into the ventricles. However a further trial, STICHII, to test the hypothesis was undertaken and the overall effect was neutral[13].

Location of the bleed also has implications for the symptoms at onset and the phenotype of longer-term disabilities (Fig. 1d). For example, bleeding within the motor cortex is likely to cause muscle weakness, bleeding within Wernicke's area will produce aphasia and haematoma within the cerebellum is associated with a loss of motor co-ordination. The brainstem is essential for communication between the cerebral cortex and the body via the spinal cord, and is therefore necessary for many vital functions, including consciousness, breathing, cardiac rhythms and blood pressure regulation. As such, haemorrhage in the brainstem can be devastating.

Causes and Risk Factors for ICH

ICH can be divided into spontaneous, traumatic, and non-traumatic ICH dependent on the aetiology. Non-traumatic ICH is most common in older patients as a result of hypertension, cerebral amyloid angiopathy (CAA) or the use of anticoagulants[11]. The major cause of spontaneous ICH is hypertension, accounting for two thirds of cases of haemorrhagic stroke[2]. Chronic hypertension causes the formation of microaneurysms, although these are not the bleeding point in the majority of cases, and degeneration of the vessel walls. This results in weakening and eventually vessel rupture. It has been estimated that between 17 and 28% of hypertension related ICH cases could be prevented by treatment of hypertension[14]. CAA is the deposition of the protein β-amyloid in the walls of the arteries within the brain where it causes degeneration, fibrinoid necrosis, and microaneurysm formation[15]. Carriers of the ε2 or ε4 alleles of the apolipoprotein E gene are at a higher risk for CAA-associated haemorrhage (CAAH) and risk of recurrence

compared to the more common ε3. The incidence of amyloid angiopathy increases with age and is strongly associated with Alzheimer's disease; the causes however are currently not completely understood and no treatment is available. Spontaneous ICH risk factors also include a reduced clotting ability, which can be caused by, for example, the use of anticoagulants and coagulopathies such as disseminated intravascular coagulation[11,16,17]. Alcohol and drug abuse are also associated with an increased risk of ICH, particularly cocaine and amphetamine which cause intense spikes in blood pressure. The risk of stroke is 700% greater in the 24 hours after the use of cocaine[9].

Traumatic and spontaneous ICH predominates in younger sufferers where it is caused by traumatic brain injury or brain malformation, respectively. Malformations which can result in spontaneous ICH include, brain tumours, aneurysms, arteriovenous fistula (AVF) and arteriovenous malformations (AVM). AVMs and AVFs are congenital vascular lesions causing blood to bypass capillaries and move directly from arteries to veins. This causes an abnormally high blood flow and weakened vessel walls which can result in rupture and haemorrhage[18].

Diagnosis and Treatment of ICH

There is a strong correlation between the time of stroke diagnosis and intervention and the chance of mortality or morbidity. The recognition of this, and the launch of the National Stroke Strategy in 2007, has led to better hospital procedures to fast track patients for diagnosis and treatment by specialised stroke response teams. In addition the public health campaign, act F.A.S.T, has facilitated the early recognition of stroke, and reduced time taken to seek medical attention. Current guidelines for the management of ICH state that in order to determine the best course of treatment patients presenting with stroke-like symptoms require neurological imaging, magnetic resonance imaging (MRI) or computerized tomography (CT) scans, which confirm the presence and type of stroke i.e. ischaemic or haemorrhagic (Fig.2). Anticoagulant therapies will be discontinued and blood pressure medications administered in cases of acute hypertension[19]. Immediate and intensive blood pressure lowering (below 140mm/Hg within 1 hour) is safe and effective in improving patient outcomes after ICH. Tests can also be undertaken to determine the likely cause of the bleed and action taken to reverse this and avoid a repeat

haemorrhage, for example, identification and treatment of vascular abnormalities[19]. Depending on the cause of ICH surgery may be required; rebleeding of aneurysms can be prevented by clipping or coil embolisation. However, acute surgery is dependent on the clinical condition of the patient, premorbid health and, as discussed, the location of the bleed. Haematoma evacuation is commonly used to reduce intracranial pressure (ICP). Hemicraniotomy may also be used where clot removal is difficult or incomplete[20]. Surgical removal of the haematoma is usually only considered in patients with significant deterioration or in the case of large (>30ml), superficial bleeds[19].

Pathophysiology of ICH

ICH causes brain injury in 3 major ways (Fig. 3). Firstly there is the mass effect of the initial bleed, haematoma expansion and oedema formation[21]. This increases intracranial pressure causing distortion of brain structures, and mechanical damage to cells. The distortion and stretch of brain cells results in glutamate release, this in turn leads to excitotoxicity and ultimately necrosis. It has been recognised

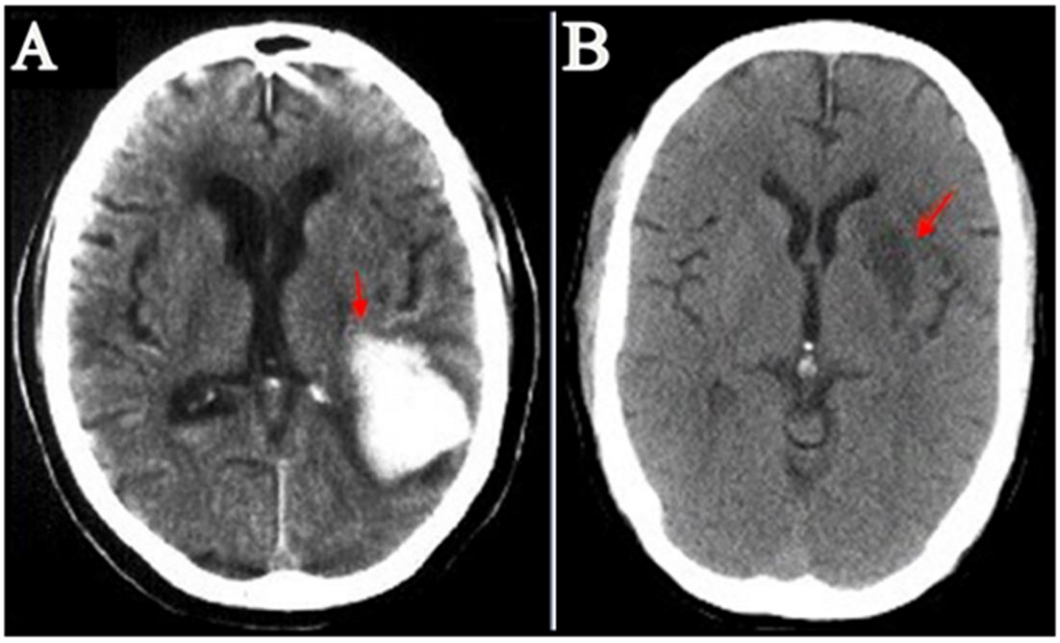


Figure 2: Detecting Ischaemic and Haemorrhagic Stroke by MRI

A) Ischaemic stroke apparent as an area of hypodensity.
Arrow indicates ischaemic infarct.
B) Haemorrhagic stroke apparent as an area of hyperdensity. Arrow indicates haematoma.

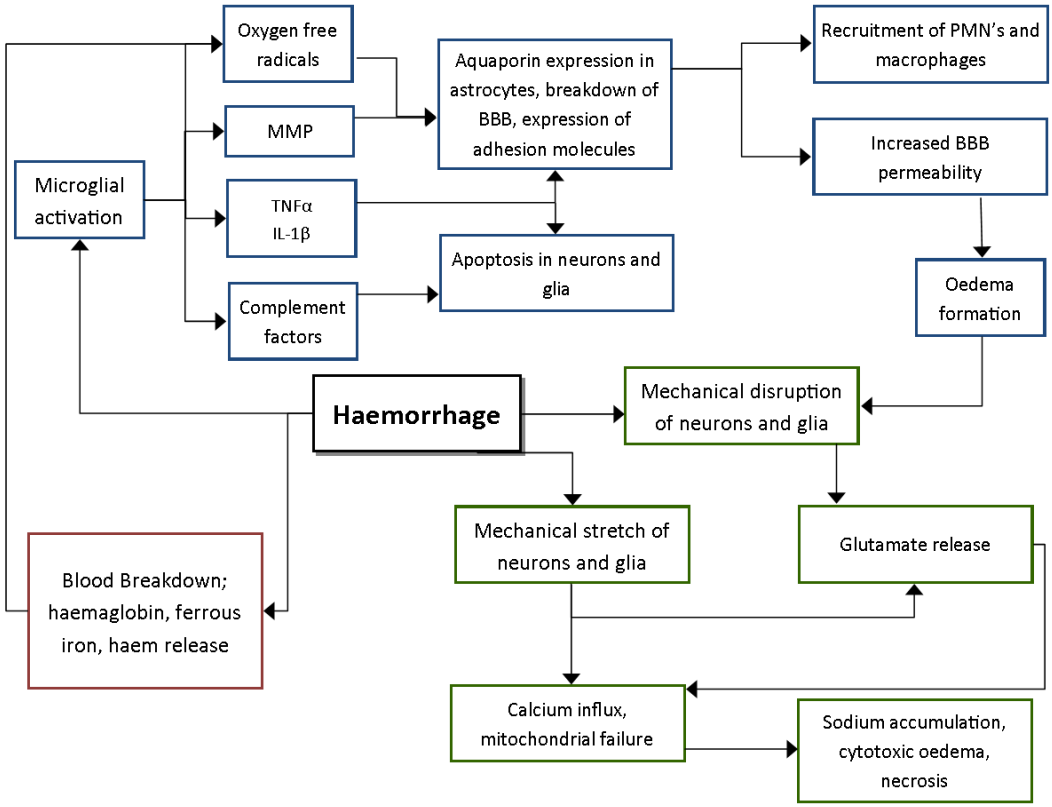


Figure 3: Pathophysiology of Intracerebral Haemorrhage

Boxes outlined in green relate to the mass effect of the haematoma. Boxes outlines in red relate to the products of haemolysis and boxes outlined in blue relate to neuroinflammation following ICH. Adapted from Qureshi, 2009.

since 1916 that relieving pressure and evacuating the haematoma are crucial to prevent this mass effect[22]. However, it is now known that there is also a large role for neuroinflammation causing secondary brain damage following ICH. This is mediated by DAMPs and the downstream mediators such as MMP's (matrix metalloproteinases), TNFα (tumour necrosis factor-α), and IL-1β (interleukin-1β) which are released upon microglial activation. DAMP's induce apoptosis and the breakdown of the blood brain barrier (BBB)[21]. Finally, the rapid breakdown of clotted blood causes the release of toxic breakdown products, such as haemoglobin (Hb), haem, and iron. These cause oxidative stress through the production of free radicals and promote brain inflammation[21]. Each of these facets of ICH pathophysiology feeds into one another, for example, neuroinflammation causes oedema which contributes to the mass effect. Therefore a treatment plan that addresses both the neuroinflammation and toxicity of blood products in addition to

the mass effect caused by ICH is likely to offer the greatest preservation of brain function and reduction in mortality.

Minimally Invasive Surgery in the Treatment of ICH

A History

Conventional surgical evacuation of ICH involves a craniotomy whereby a large area of skull is removed and the brain is cut into to reach the haematoma. This causes a significant amount of damage to the brain. A randomised trial comparing the outcome of ICH patients who underwent craniotomy with medically managed patients found no difference in mortality rates or functional outcome[23]. More recently the STICH and STICHII trials found no overall benefit with early haematoma evacuation[13]. The advent of imaging techniques, such as CT, allowed for a new approach to ICH treatment utilising CT-directed minimally invasive surgery (MIS) to evacuate the haematoma. The first attempt involved a helical screw, which was rotated within a cannula to first disrupt the blood and then draw it up through the cannula[24]. This provided a good model to which other groups made several modifications. For example, a stereotactic frame was designed to allow intraoperative guidance[25]. Later the screw itself was motorized to improve clot disruption and an aspirator was attached to facilitate liquid removal[26]. However despite the improvements these techniques still resulted in incomplete clot removal and significant mechanical trauma to the brain.

Thrombolytics and MIS

Haemostasis is the process by which bleeding is controlled in the event of vessel damage; this involves a 3 step process of vasoconstriction, platelet aggregation and clot formation[27]. The coagulation cascade results in the thrombin catalysed conversion of soluble fibrinogen into insoluble fibrin which forms a stable fibrin plug[27]. Fibrinolysis is the process by which the fibrin clot is degraded once damage has been repaired[27]. This process utilizes endogenous plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which cleave the proenzyme, plasminogen, to form active plasmin which degrades fibrin[27]. Alteplase (recombinant tPA) and urokinase (recombinant

uPA) belong to a class of drugs known as thrombolytics, which are administered to facilitate clot lysis, for example, in ischaemic stroke[28].

It was Doi and colleagues (1982) who first used the thrombolytic, urokinase, in combination with MIS for the treatment of ICH (Cited by Samadani, 2009)[29,30]. The use of a thrombolytic facilitates catheter patency and aids in clot resolution to allow maximal removal with minimal mechanical trauma. Preclinical studies in a rabbit model of ICH compared urokinase treatment in combination with MIS alone[31]. MIS in combination with urokinase gave a reduction in BBB permeability, reduced water content and lower brain concentration of excitatory amino acids all of which are associated with negative outcomes after ICH[31]. A similar study using a canine model of ICH found the combination of urokinase and MIS led to improved functional outcomes[32].

Following this a protocol was described for the CT-directed stereotaxic haematoma evacuation aided by urokinase in ICH patients[33]. First a small hole is drilled in the skull, significantly smaller than is necessary during craniotomy. Then a soft, narrow diameter, silicone tube is fed into the centre of the haemorrhage using co-ordinates obtained from 3D CT scans. A syringe is attached to the tube and used to remove the liquid blood before the administration of 5ml of urokinase in saline via the tube. Following this the system is closed to allow the drug to act. This is repeated 6-12 hourly until haematoma evacuation is complete as determined by repeat CT scans. No significant adverse effects were observed using this method. However the sample size was small and unmatched with regards clinical grade which prevented statistical comparison of CT-directed evacuation and the more invasive craniotomy method[33]. A preliminary trial showed that administration of urokinase via an intraventricular catheter significantly improved 30-day mortality in ICH patients[34].

The positive results in this trial secured funding for a phase II trial. The Clot Lysis: Evaluating Accelerated Resolution of Intraventricular Hemorrhage (CLEAR) trial began in 2005. However, withdrawal of urokinase from the market due to production issues necessitated a change of thrombolytic and alteplase was chosen. The primary aim of CLEAR was to determine the safety of low-dose alteplase administered via a ventricular catheter in a small cohort of patients[35]. The cohort consisted of patients who had a small ICH with significant extension into the

ventricles. Critically patients were required to have an external ventricular device (EVD) in place for the treatment of hydrocephalus as per normal clinical practice. Patients were given either 3ml saline or 3mg/ml alteplase via the EVD every 12 hours until either clot resolution was sufficient for EVD removal, or a safety endpoint was reached. Daily CT scans were performed to monitor clot resolution until EVD removal and a final scan at day 28-32 post-ICH.

The pre-specified safety limits of death at 30 days, ventriculitis and symptomatic events were not met and were not significantly different between placebo and alteplase treated patients (Fig. 4a). Although a nonsignificant trend towards bleeding in the alteplase treated patients was observed (p=0.106). The secondary aim was to assess the rate of clot lysis with alteplase compared to placebo. Clot resolution was significantly faster with alteplase (18% per day) than placebo (8%), although it was not as fast as predicted by preclinical work. This translated to a shorter treatment time with an average dosing of alteplase of 7 days compared to 12 days with placebo. However, although long-term functional outcomes were improved with alteplase, statistical significance was not reached.

A phase III trial has been completed with a primary outcome measure of modified Rankin scores between 0 and 3 at 180 days (NCT00784134). Here a lower dose of 1mg was administered to reduce the risk of bleeding events. Results have not yet been published but have been reported by Medscape[36]. The article reports that just the insertion of an IVD appears to have been beneficial with 50% of patients in this study regaining independence compared to the 20% in other studies. When compared with saline, flushing the clot with alteplase conferred an additional 10% decrease in mortality with no change in the number of patients requiring full-time care. However there was no significant improvement in the primary outcome between saline and alteplase. Notably, positioning of the IVD determined the success of clot removal, which in turn correlated with good outcome and rate of mortality.

A similar approach was used in the MISTIE (Minimally invasive surgery and rt-PA in ICH evacuation) trial[37]. Patients were randomized into 3 groups: medical management, MIS alone and MIS in combination with alteplase. Alteplase was administered via the catheter following image-guided, catheter insertion. The system was closed for 1 hour to allow drug interactions and then opened again for gravitational drainage. This process was repeated every 8 hours for a maximum of 9

doses or until a prespecificed clot resolution endpoint was reached. Once daily CT scans were used to establish a pre-intervention baseline, monitor clot lysis throughout treatment and at the end of treatment. Semi-automated volumetric analysis of the oedema and ICH volume was employed.

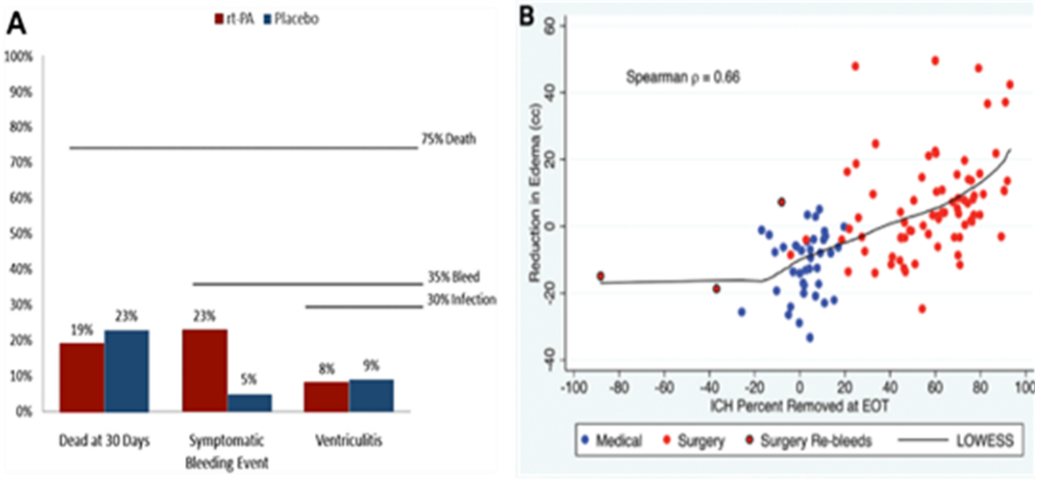


Figure 4: Primary Outcomes of CLEAR and MISTIE Trial.

A) Pre-specified safety limits in CLEAR trial. No limit was reached in either alteplase (red) or placebo (blue) treated patients. No statistical significance between groups. Death at 30 days (p=1.00), symptomatic bleeding event (p=0.106), ventricilitis (p=1.00). Taken from [35] B) Percentage of ICH removed vs. reduction in oedema for patients receiving medical management (blue) and MIS (red). Taken from [37]

The haematoma volume after treatment was significantly reduced in the surgical group (19.6±14.5 compared to the medical group (40.7±13.9cm, p=<0.001) (Fig 1b). The haematoma volume was also decreased with MIS and alteplase compared to MIS alone though this did not reach statistical significance (p=0.46). Similarly at the end of treatment oedema volume in the surgical group was significantly reduced compared to the medically managed group (41.7±14.6cm³, p=<0.001). Oedema volume was slightly increased in alteplase treated patients compared with MIS alone however; again, this did not reach significance (p=0.46). Following these promising results a 500-patient phase III trial has received funding and enrolment began in 2013 (NCT01827046).

Thrombolytics

Alteplase is known to be neurotoxic and so may not represent the best thrombolytic for use in combination with MIS for the treatment of ICH[38–41]. In addition to the endogenous thrombolytics, alteplase and urokinase, there are a

number of exogenous thrombolytics such as streptokinase and the genetically engineered tenecteplase (also known as TNKase). Their benefit in disease involving thrombosis has been extensively studied and numerous clinical trials have been completed. Thrombolytics are currently licensed for the treatment of myocardial infarction, deep vein thrombosis, pulmonary embolism, acute ischaemic stroke and for maintenance of catheter patency. Here the history, properties, and applications of each will be discussed. These properties are summarized in table 1.

Tissue-type Plasminogen Activator: Alteplase

tPA was first discovered in 1979 and alteplase was the first recombinant tPA to be cloned into an expression vector in 1983, allowing it to be mass-produced initially for the treatment of myocardial infarction and later for ischaemic stroke[42,43]. After successful trials tPA was approved for use clinically in North America in 1996 and 2002 in Europe for the treatment of ischaemic stroke[44]. However, there are significant issues with alteplase. It is only possible to administer it up to 4.5h after stroke onset because of the increased risk of haemorrhage and the earlier the treatment the greater the benefit, particularly if administered within 90 min[45]. Although this is a concern with thrombolytics as a whole not specifically tPA. There is rapid clearance of alteplase from the blood so infusion is necessary to maintain

Table 1. Key Characteristics of Thrombolytics.
Summary of Alteplase, Urokinase, Tenecteplase and Streptokinase properties in terms of half-life, fibrin specificity, PAI1 inhibition, neurotoxicity and allergenic properties. Adapted from Ross, 1999.[47]

Thrombolytic	Half-Life	Fibrin Specificity	PAI1 Inhibition	Neurotoxicity	Antigenic
Alteplase	4-6 min	++	No	Yes	No
Urokinase	15 min	++	Yes	Yes/No	No
Tenecteplase	20 min	+++	Yes	No	No
Streptokinase	Biphasic: 16 min, 90 min	+	Yes	Unknown	Yes

activity; this results in a complicated administration protocol. However bolus injection is also problematic as it causes a sudden and dramatic increase in alteplase concentration in the blood so the risk of bleeding is high. In theory alteplase has a high specificity and affinity for fibrin meaning it should only convert plasminogen to plasmin in the presence of a fibrin clot which reduces the risk of systemic haemorrhage[46]. However in practice alteplase treatment does cause systemic lysis[47]. Endogenous plasminogen activator inhibitors (PAI1) exist to maintain haemostasis and prevent excessive clot lysis[27]. Alteplase is susceptible to inhibition by PAI1 and so it's efficacy *in vivo* is limited[48]. Importantly, alteplase is known to be neurotoxic through its interaction with the NMDA receptor (N-methyl-D-aspartate; NMDAR). In a rat model of ICH an antibody was administered which specifically inhibits the interaction between endogenous tPA and the NMDAR, without altering NMDAR neurotransmission[38,39]. This caused a reduction in oedema volume, cell death, microglial activation and functional deficits[38]. In addition endogenous tPA acts as a pro-inflammatory cytokine to activate microglia[40]. Alteplase does not appear to be neurotoxic in isolation but does exacerbate damage. For example, alteplase significantly increases cell death in neuronal cultures treated with low doses of Hb compared with Hb alone[41] .

Urokinase-type Plasminogen Activator

Urokinase is a recombinant form of the endogenous, plasminogen activator, uPA which is produced in human kidneys and found in blood, urine and the extracellular matrix. The fibrinolytic potential of urine was first reported in 1947 and a protocol for the isolation of a pure, stable enzyme was published in 1956 [49,50].

Urokinase has a lower rate of clearance than alteplase with a half-life of 15 minutes[51]. As urokinase is cloned from an endogenous plasminogen activator it is susceptible to inhibition by PAI1[48]. Additionally, as it is an endogenous, human protein it is not antigenic. Unlike alteplase, urokinase is known not to be neurotoxic through any interaction with the NMDAR[52]. However both alteplase and urokinase are known to induce the expression of MMP-9 which causes the degradation of laminin and exacerbates neuroinflammation[53,54]. Recent research has also suggested that urokinase may be neuroprotective[55]. In *in vitro* models of venous thrombolysis results suggested a slower rate of lysis but higher fibrin specificity with urokinase

compared to alteplase[56]. It has therefore been suggested that urokinase may represent a compromise between the poor safety and high efficacy of alteplase.

Urokinase was initially tested in various animal models of intracranial haematoma; in a rabbit model of ICH and IVH. Urokinase injected directly into the clot was shown to be safe and effective in clot removal compared to a control saline injection[57]. Urokinase was used in many of the early studies using thrombolysis in combination with MIS and was originally intended for use in the CLEAR trial however problems with supply meant it was withdrawn from the market.

Tenecteplase

Tenecteplase is the product of genetic point mutations of alteplase to achieve higher fibrin specificity, a longer half-life and greater resistance to PAI1[58]. All of which suggest tenecteplase may be a safer and more effective alternative to alteplase. A longer half- life allows for administration via bolus injection and the high initial concentration may decrease the time to clot lysis. This increased half-life is conferred by the rearrangement of glycosylation sites in kringle 1[58]. At position 117 asparagine is substituted with glutamine to remove a glycosylation site and at position 103 threonine is replaced by asparagine to reintroduce a glycosylation site[58]. This reduces clearance of the drug and thus increases half-life to 20 minutes. 4 alanine residues replace the lysine 296 –histidine297 –arginine298 –arginine299 sequence to confer resistance to PAI1, which should improve efficacy, and the higher fibrin specificity[58]. Higher fibrin specificity should improve safety profiles by decreasing the risk of bleeding because of administration whilst also improving efficacy. In addition, injection of tenecteplase into the outer retina of pig eyes suggest no toxic effect[59]. Furthermore there is less than one third of the L arginine content of alteplase in the tenecteplase vehicle. L-arginine is involved in the production of free radicals so there may be a reduced oxidative stress associated with tenecteplase administration.

The in vivo properties of tenecteplase were first investigated in a rabbit model of carotid artery occlusion. When compared with alteplase, tenecteplase administered as a bolus caused a significant reduction in time to reperfusion and clot lysis was much more complete despite no increase in incidence of haemorrhage[60]. Despite these encouraging results tenecteplase was not trialled in patients for another

decade. A small pilot study in 2005 found doses of 0.1mg/kg, 0.2mg/kg, and 0.4mg/kg to be safe when administered as an IV bolus to ischaemic stroke patients[61]. Subsequent to this a phase IIB/III trial was funded to directly compare the outcomes following treatment with either tenecteplase or alteplase. Unfortunately, slow enrolment terminated the trial early and no definitive conclusion was provided[62].

An alternative pilot trial used CT scans to select patients for tenecteplase treatment and assess reperfusion and infarct growth[63]. Outcomes were then compared to treatment with alteplase and shown to be improved in the former with regards reperfusion, functional outcome, and incidence of haemorrhage. However alteplase patients were not selected for using CT so results were not directly comparable. A phase II trial was initiated to confirm and expand upon these results. Using CT perfusion and angiographic imaging, patients were selected for because of mismatch between small infarct volume and large perfusion lesion and the presence of a large vessel occlusion. Patients were then randomized to 0.9 mg/kg alteplase, 0.1mg/kg tenecteplase, or 0.25mg/kg tenecteplase with a mean time of treatment of 2.9 hours. Significant improvements in reperfusion and NIHSS scores at 24 hours were reported in the tenecteplase treated patients compared to alteplase. Long-term improvement was also seen with significantly higher proportion of excellent or good recovery at 90 days with tenecteplase (72%) compared to alteplase (44%). The highest dose of tenecteplase appeared to be the best in terms of primary outcomes but was not associated with an increase in spontaneous ICH incidence suggesting this as the optimal dose. In this trial the relatively small cohort necessitated strict inclusion criteria in order to target individuals for whom tenecteplase would be most beneficial. Future trials will need to broaden the criteria for inclusion to accommodate all those who would usually be eligible for alteplase treatment.

As yet no studies have been conducted, either in animal models or patients, to assess the safety and efficacy of tenecteplase in ICH. However enhanced activity, potential reduced toxicity and apparent low incidence of spontaneous ICH suggests this may be a worthwhile avenue to pursue.

Streptokinase

Streptokinase was the first thrombolytic to be discovered and considered for clinical use. It was also integral to elucidating the mechanism and key players of

fibrinolysis[64]. It was observed in 1933 that haemolytic streptococci cultures secrete a fibrinolytic substance capable of dissolving blood clots in human plasma[65]. The substance was eventually named as streptokinase and, once purified, was administered to patients[46]. Streptokinase is licensed for the treatment of acute myocardial infarction (AMI). However, alteplase has since replaced streptokinase in AMI treatment following the publication of trial data showing reduced mortality over 6-12 months when compared with streptokinase[66,67]

Streptokinase has a biphasic half-life with an initial rapid phase half-life of 16 minutes and then a slow phase half-life of 90 minutes[46]. It does not activate plasminogen directly but instead forms a complex, which facilitates the activation of further plasminogen[68]. Plasminogen activation by streptokinase is not fibrin specific, which increases the risk of bleeding. Furthermore, as streptokinase is a bacterial enzyme there can be issues with allergies with prolonged or repeated use[67]. The GUSTO (the Global Utilisation of Streptokinase and alteplase in Occluded Coronary Arteries) trial compared alteplase with streptokinase and found a rate of allergic reaction to streptokinase of 5.7%[67]. However, analysis of the GUSTO trial data found that efficacy of streptokinase was not compromised by allergic reaction[69]. Comparison of alteplase, urokinase and streptokinase in a venous thrombosis model found streptokinase to have the slowest rate of clot lysis and be the least fibrin-specific but was the most cost effective[56]. Streptokinase is not resistant to inhibition by PAI1 and a negative correlation between pretreatment PAI1 levels and successful reperfusion in patients with AMI has been reported[70].

Streptokinase has been extensively trialled in ischaemic stroke but has been associated with significant bleeding events and uncertain efficacy, with three major trials investigating streptokinase in ischaemic stroke being suspended due to high rates of haemorrhage[71–73].However it is still being pursued as an alternative thrombolytic due to its low cost. This is of particular importance in developing countries where incidence of stroke is high, not improving, and correlated with patient wealth[74]. The Asia Africa Ischaemic Stroke Trial (ASSIST) is planned to assess safety of streptokinase in carefully selected patients in developing countries. If the safety profile is acceptable the trial will continue with a larger cohort to determine efficacy[74].

Summary and Aims

MIS in combination with a thrombolytic drug appears to be a promising novel approach to the treatment of ICH. However the use of alteplase, which is known to be neurotoxic, may exacerbate damage after ICH. Alternative thrombolytics exist and could improve the potential benefit of this approach. First, a direct comparison of the neurotoxicity of each thrombolytic is necessary. As alteplase does not appear to be toxic to healthy cells an *in vitro* model of neuronal injury is required. IL-1β is a pro-inflammatory mediator of neuronal injury which has been shown to cause a 2-fold increase in cell death over basal at 0.1ng/mL and a 3-fold increase with ≥1ng/mL in neuronal-glial co-cultures[75]. It is necessary for the level of cell death induced by IL-1β to be significantly higher than basal but not approaching 100% to allow a significant difference to be seen in the presence of the thrombolytic. Therefore, a 2-3 fold increase would be appropriate to allow the synergistic effect of IL-1β and thrombolytics to be assessed. The use of IL-1β is relevant as its concentration is elevated in the brain after ICH due to its release by apoptotic cells and activated glia[76,77]. Furthermore, after ICH there is the rapid accumulation of blood breakdown products, such as Hb, haem and iron, as the clot is lysed[78]. In addition Hb (E. Ismail, personal communications) and haem also cause the release of IL-1[79].

Therefore the aims of this project are as follows:

1. To develop a model of neuronal injury using neuronal-glial co-cultures treated with IL-1β based on a previously published method[75].
2. To compare the neurotoxicity of each thrombolytic in the presence and absence of IL-1β.

Methods

Murine Cortical Co-Cultures

Cortices were isolated from embryonic day 18 Wistar Han rat embryos (Harlan Laboratories, UK). Cells were dissociated in seeding media (Minimum essential medium (MEM), 2mM Glutamine, 100U/mL penicillin, 100µg/mL streptomycin (all Sigma-Aldrich, UK), 10% foetal bovine serum (FBS) (PAA Laboratories, UK) and B27 supplement with antioxidants (Invitrogen, UK)). Meninges were removed by pipetting cell suspension onto 80µm sterile nylon mesh (John Stanier and Co, UK) and allowing the cells to filter through. Cells were then collected by centrifugation at 250xg for 10min and suspended in seeding media. At this point if necessary 5-Fluoro-2′-deoxyuridine (FUDR) (Sigma-Aldrich, UK) was added at a concentration of either 5 or 20µM. Co-cultures were seeded at a density of $2.5x10^4$ cells/cm^2 in 48-well plates (Corning, UK). These plates had been pre-coated with poly-D-lysine (Sigma-Aldrich, UK) for 24 h at 4°C. A full media change was done 24 and 72 h after explantation with change media (MEM, 2mM glutamine, 100U/mL penicillin, 100µg/mL streptomycin and 10% FBS). A half media change with starve media (MEM, 2mM glutamine and 100U/mL penicillin, 100µg/mL streptomycin) was done on days 7 and 10 after seeding. Co-cultures were treated on day 13. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

Interleukin-1β Treatment

Previous work has demonstrated that treating rat cortical neuronal-glial co-cultures with IL-1β causes a concentration-dependent increase in cell death, as measured by LDH release[75]. To replicate this result the same protocol was followed. Co-cultures were treated with 0.01-10ng/mL mouse recombinant IL-1β or vehicle (0.4% sterile-filtered BSA (Sigma-Aldrich, UK) w/v in Phosphate buffered saline (PBS; Sigma-Aldrich, UK)) for 72h in triplicate. 500µM NMDA (Sigma-Aldrich, UK) served as a positive control. Unless otherwise stated treatments were prepared in starve media and given as a full media change.

LDH Assay

Lactate dehydrogenase (LDH) is released into the media by damaged cells. This was used to assess cytotoxicity in response to treatments using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, UK). 72h after treatment 50µl of supernatant was removed from each well in triplicate and transferred to a 96-well plate (Corning, UK). 50µl of cell lysate was used as a positive control. To each well 50µl of substrate mix was added and the plate incubated for 30 min at room temperature on a shaker in the dark. 50µl of stop solution was added to each well to stop the reaction and the plate read at 490nm using the plate reader (Biotech NorthStar, UK). Wells containing media only served as background absorbance and this was subtracted from the cell-containing wells. LDH release was expressed as a percentage of total lysis. Triplicate values from the same culture were averaged and each data point is representative of the average LDH release from separate cultures.

Thrombolytic Functional Assay

Rats (Sprague-Dawley or Wistar Han, male and female) were anesthetised under 3% isoflurane in oxygen and placed in a supine position. Blood was obtained using a 1.5" 21 gauge syringe inserted into the heart. Cardiac blood (100µl) was quickly aliquoted into pre-weighed sterile 0.5mL microcentrifuge tubes (Starlab, UK) and allowed to clot for 90min at 37°C. Serum was removed from each tube and then each was weighed to determine the weight of the blood clot. Clots were then treated with 20µl of thrombolytic (100-100,000IU) or PBS and incubated for 1h at 37°C. Again the serum was removed from each tube and then each weighed to determine the weight of the blood clot. The amount of clot resolution was then expressed as a percentage for each treatment. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

Effect of Interleukin-1 on Coagulation

Recombinant rat IL-1α (20pg; R&D Systems Europe Ltd, UK), recombinant rat IL-1β (20pg; National Institute for Biological Standards and Controls, UK), IL-1 receptor antagonist (IL-1Ra) (20ng; Amgen, USA) or vehicle (0.1% sterile filtered BSA w/v in PBS) was added to 8-12x sterile, pre-weighted 0.5mL microcentrifuge tubes (Starlab, UK). All tubes were kept on ice. Male Sprague-Dawley rats (Harlan

Laboratories, UK) were anesthetised under 3% isoflurane in oxygen and placed in a supine position. Blood was obtained using a 1.5" 21 gauge syringe inserted into the heart. 100µl of cardiac blood was quickly aliquoted into the prepared tubes and allowed to clot for 90min at 37°C. Serum was removed from each tube and then each was weighed to determine the weight of the blood clot. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

Effect of Interleukin-1 on Clot Lysis

Male Sprague-Dawley rats (Harlan Laboratories, UK) were anesthetised under 3% isoflurane in oxygen and placed in a supine position. Blood was obtained using a 1.5" 21 gauge syringe inserted into the heart. Cardiac blood (100µl) was quickly aliquoted into the prepared tubes and allowed to clot for 90min at 37°C. Serum was removed from each tube and then each was weighed to determine the weight of the blood clot. Recombinant rat IL-1α (20pg; R&D Systems Europe Ltd, UK), recombinant rat IL-1β (20pg; National Institute for Biological Standards and Controls, UK) or vehicle (0.1% sterile filtered BSA w/v in PBS) was added to 8x sterile, pre-weighted 0.5mL microcentrifuge tubes (Starlab, UK). Alteplase (20 IU) was then added to all tubes. All microcentrifuge tubes were incubated at 37°C for 1h to allow clot lysis to occur. Serum was again removed from each tube and then each was weighed to determine the post-treatment weight of the clot. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

Preparation of Thrombolytics

All thrombolytics (Alteplase (Actilyse; Boehringer Ingelheim, Germany), Urokinase (Syner-KINASE; Syner-Med (Pharmaceutical Products) Ltd, UK), Streptokinase (Biofactor Streptokinase; Biofactor GmbH, Germany), Tenecteplase (Metalyse; Boehringer Ingelheim, Germany)) were reconstituted and diluted in sterile PBS (Sigma-Aldrich, UK) and stored at -80°C until use.

Statistical Analysis

Data were analysed using GraphPad Prism Software (San Diego, CA) and are expressed as the mean ± standard deviation (SD). Data were analysed using one-way

ANOVA or two-way ANOVA with a Tukey or Dunnet's *post-hoc* test as appropriate. Statistical significance was considered as $p = <0.05$.

Results

In Vitro Model of Neuronal Injury

Previous work has demonstrated that treating rat cortical neuronal-glial co-cultures with IL-1β causes a concentration-dependent increase in cell death, as measured by LDH release[75]. However, when this protocol was followed the basal level of cell death was high (70-85%) and there was no change in LDH release with IL-1β treatment compared with basal levels therefore no effect of IL-1β could be seen (Fig 5a).

The original protocol administered IL-1β with a half-media change in serum free media. To reduce the high levels of LDH IL-1β was instead administered as a full media change in either serum free or serum containing medium (Fig 5b). A full media change reduced the basal cell death to 40% with serum starvation or 47% with serum containing medium (Fig 5b). Moreover a dose-dependent increase in cell death was observed with IL-1β treatment (Fig 5b). However this was only evident with serum starvation. When IL-1β treatment was given as a full media change with serum containing medium there was no difference in cell death with any concentration of IL-1β when compared to basal levels (~50%, Fig 5b). Therefore a full media change with serum free medium provides the optimum conditions for measuring LDH release, and thus cell death, in response to IL-1β treatment in neuronal-glial co-cultures. NMDA was included as a positive control.

FUDR is an antineoplastic drug which inhibits mitosis and thus at appropriate concentrations will abolish glial cells whilst preserving neurones in culture[80]. FUDR at a concentration of 20μM is sufficient to almost completely abolish glia in cortical cultures (Fig 6biii). The magnitude of this reduction was not quantified here but previous work suggests glia are reduced to less than <5%[80]. With this proportion of glia IL-1β treatment only has a very moderate effect on cell death (Fig 6a). With a lower concentration of FUDR (5μM) the ratio of glia to neurones appears to be qualitatively reduced compared to having no FUDR (Fig 6bi,ii), however, again this was not quantified here. With 5μM of FUDR the response to IL-1β was dose-dependent and ranged from 50% cell death at a low concentration of 0.1ng/mL IL-1β to 68% cell death at a high concentration of 10ng/mL IL-1β (Fig 6a). The reduced proportion of glia also reduced the basal cell death to 30%, compared with 38% in

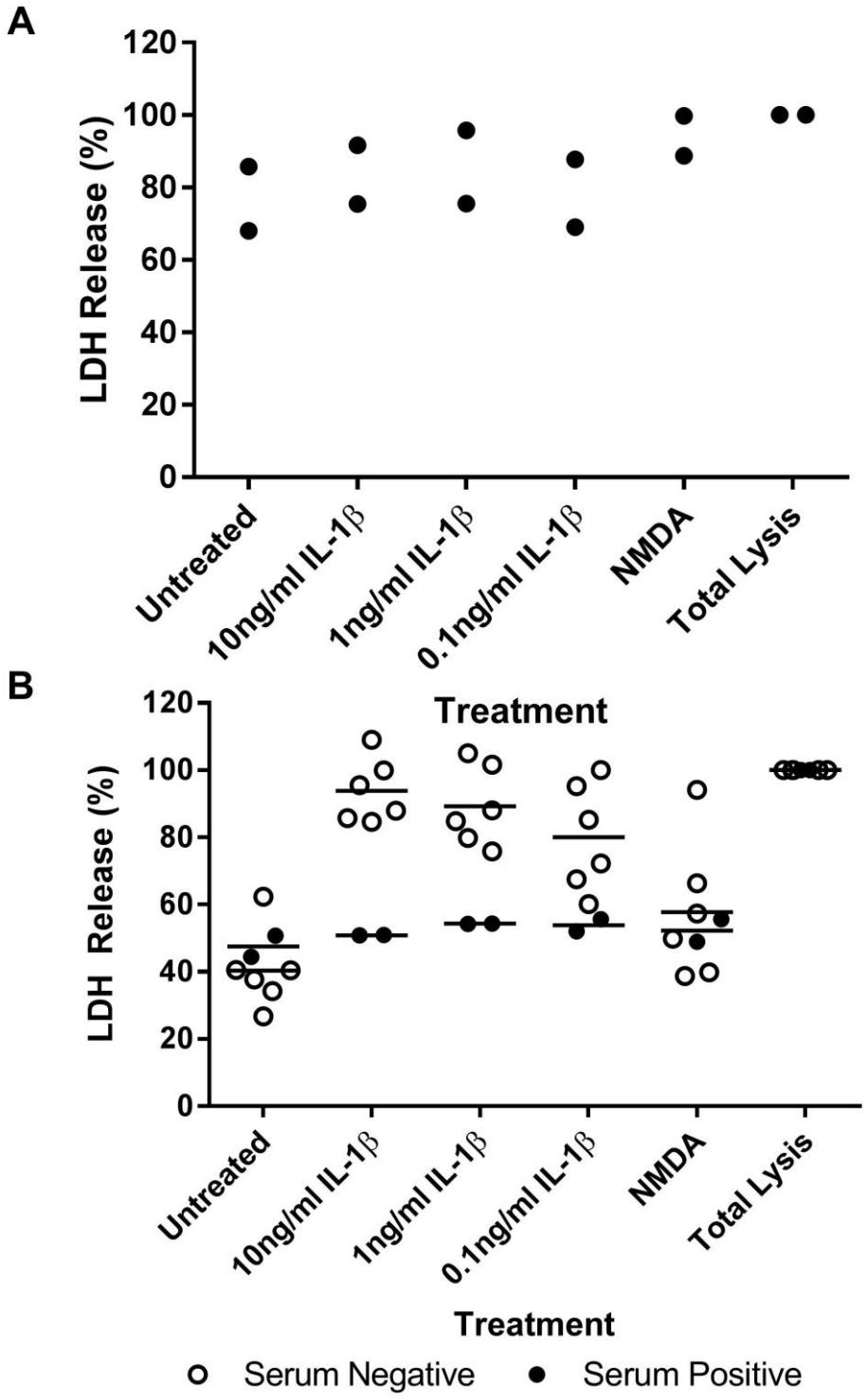
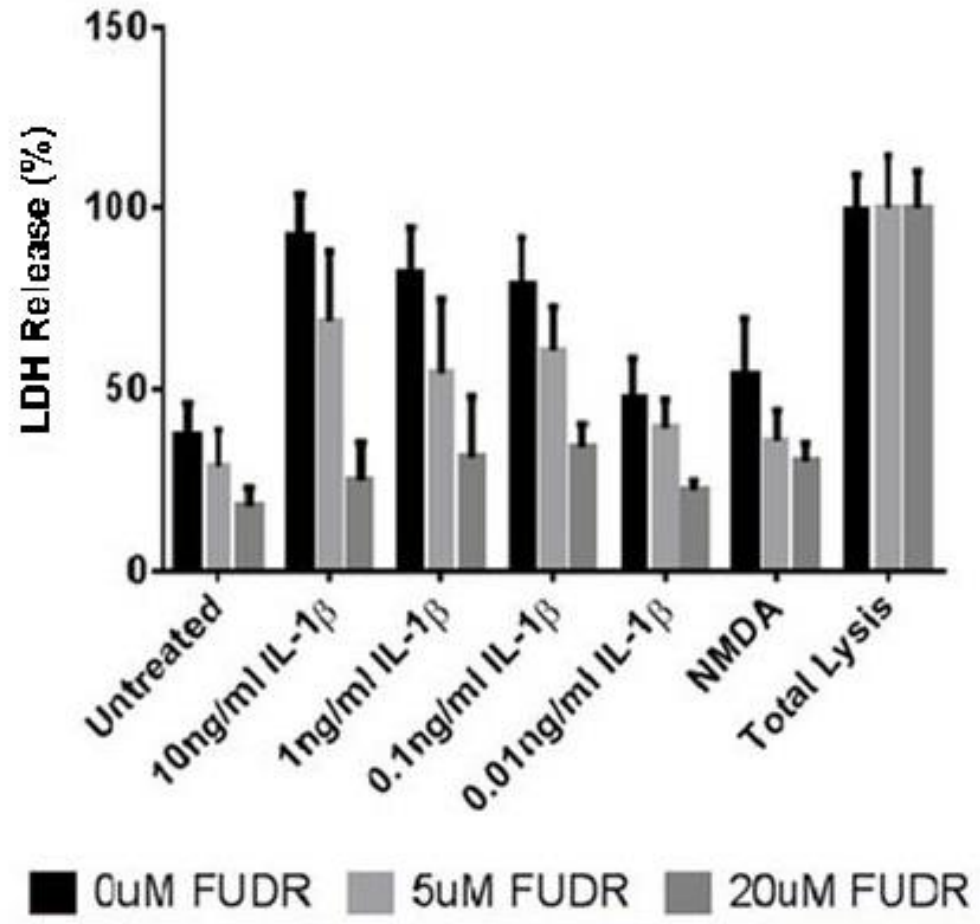


Figure 5: IL-1β Treatment of Co-cultures.

Cell death in rat cortical neuron-glia co-cultures in response to IL-1β treatment for 72h as measured by LDH release. LDH release expressed as percentage of total cell lysis ±SD.

A) IL-1β treatment was given with a half media change in serum free media. n = 2

B) IL-1β treatment given with a full media change with serum positive or serum negative media.



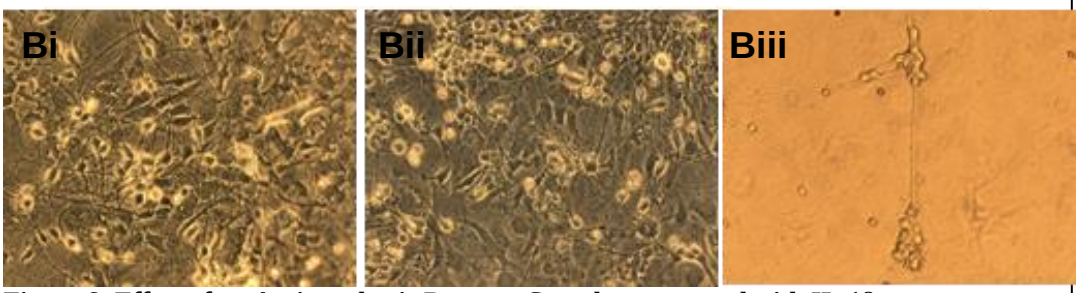


Figure 6: Effect of an Antineoplastic Drug on Co-cultures treated with IL-1β.

Rat cortical neurone-glia co-cultures seeded with varying concentrations of 2′-Deoxy-5-fluorouridine (FUDR); Control, 5µM or 20µM.

A) Cell death in response to IL-1β treatment for 72h as measured by LDH release. IL-1β treatment given with full change in serum free media. LDH release is reported as a percentage of total cell lysis. Mean percentage plotted ±SD n = 3-6.

B) Representative images of co-cultures 12 days after seeding. Bi) Control, Bii) 5µM and Biii) 20µM.

the absence of FUDR (Fig 6a). This is a more suitable level for assessing the effect of thrombolytics on neurones. Therefore seeding cortical co-cultures with 5µM of FUDR achieves an optimum ratio of neurones to glia.

Comparing Thrombolytic Neurotoxicity

As expected, alteplase alone does not cause any cell death above basal levels (Fig 7a). With co-treatment of 0.1ng/mL IL-1β and alteplase of varying concentrations there may be a trend towards increased cell death compared to 0.1ng/mL IL-1β alone (Fig 7b). However the basal cell death here was higher than expected at 58%.

Thrombolytic Functional Assay

To test the activity of each thrombolytic after preparation and storage at -80°C an *in vitro* assay of blood clotting and clot lysis was used. This was based on a previously published method but adapted to utilise rat blood as opposed to human blood[81]. In the *in vivo* rat model of ICH the volume of autologous blood utilised is 100ul. Therefore 100ul of blood was used in this assay. The concentrations (100 IU, 1000 IU, 10,000 IU and 100,000 IU where possible) were chosen to represent the widest range possible, with the highest concentration being dependent on the concentration of the stock solution. The assay may not be sensitive enough that any difference would be seen between concentrations less than one order of magnitude apart. Each thrombolytic was reconstituted and diluted in PBS so PBS was included as a negative control.

PBS alone caused 21% clot lysis (Fig 8a-d). Alteplase gave a dose-dependent increase in clot lysis with no significant increase above basal levels at 100 IU (27%), a significant ($p<0.05$) increase to 46% at 1000 IU and reaching a maximum of 58% with greater than or equal to 10,000 IU (Fig 8a). Tenecteplase also showed a dose dependent increase in clot lysis. There was no statistically significant increase in clot lysis at 100 IU or 1000 IU, but there was significance ($p<0.05$, 43%) at 2500 IU (Fig 8c). A higher concentration was not achievable due to the low stock concentration. Urokinase also showed a dose-dependent increase in clot resolution following a similar trend to Tenecteplase. At 100 or 1000 IU there was no significant increase above basal clot lysis (27%). However a significant increase to 40% with a concentration of 10,000 IU was observed ($p<0.05$, Fig 8b). Streptokinase did not show a significant increase above basal clot lysis with any concentration including the maximum concentration of 10,000 IU (Fig 8d).

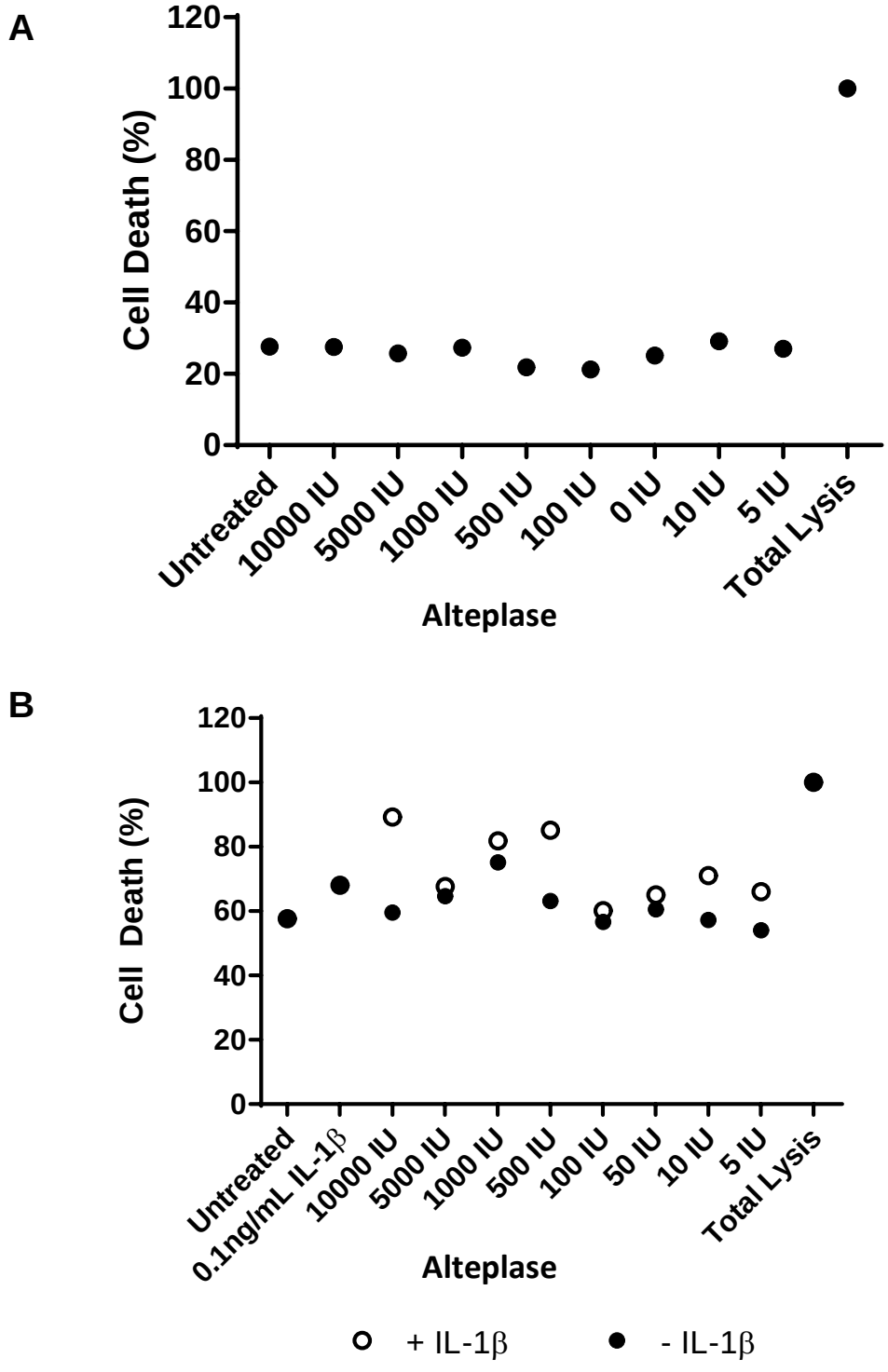


Figure 7: Neurotoxic Effect of Alteplase.

A) Rat cortical neurone-glia co-cultures were treated with varying concentrations of alteplase for 72h. LDH release is reported as a percentage of total lysis. N = 1.

B) Rat cortical neurone-glia co-cultures were treated with varying concentrations of alteplase for 72h in the presence or absence of 0.1ng/mL IL-1β. N = 1

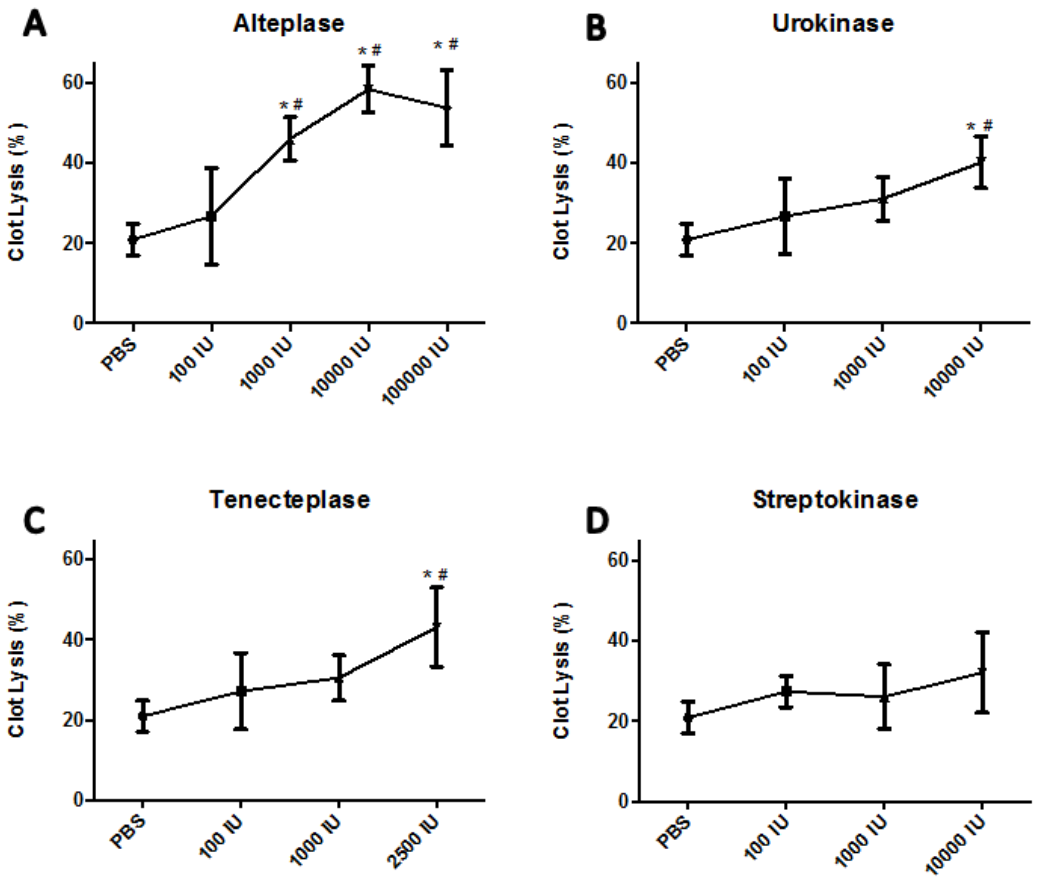


Figure 8: Effect of Different Thrombolytics on Clot Resolution.

Clot lysis of clots prepared from 100uL of whole rat blood by different concentrations of A) alteplase (100 IU, 1000 IU, 10000 IU and 100000 IU), B) Tenecteplase (100 IU, 1000 IU, 2500 IU), c) Urokinase (100IU, 1000IU, 10000IU) and D) streptokinase (100IU, 1000IU, 10000IU). An equal volume of PBS served as a negative control. Average percentage plotted ±SEM. One-way ANOVA and Tukey's *post hoc* test *p = <0.05 vs. PBS, #p = <0.05 vs. 100 IU. N = 3.

Interleukin-1, Blood Coagulation and Clot Lysis

IL-1 has been implicated in thrombosis through the increased aggregation of platelets induced by thrombin or collagen[82]. There is also evidence that IL-1 induces the synthesis of fibrinogen leading to coagulation[83]. Here a simple blood coagulation and clot lysis assay has been utilised to determine how IL-1α and IL-1β alter both clot formation and clot lysis. First the effect of IL-1 on clotting was established by incubating fresh whole blood with IL-1α, IL-1β, IL-1Ra or vehicle and assessing the effect on clot size (Fig 9a). Interestingly there is little variation in clot size between samples taken from the same animal but there is great variation in the average clot

size between animals. With animal one clot size is significantly smaller with both IL-1α and IL-1β pre-treatment compared with vehicle (Fig 9a). Whilst with animal two there is no difference (Fig 9a). However when data are combined there is no significant difference between control clot size and clot size when pre-treated with IL-1α, IL-1β or IL-1Ra. As there are only two repeats it is impossible to say from this data which is the true effect.

Next the effect of IL-1 on clot lysis was studied by incubating clotted blood with 1000 IU alteplase, to cause clot lysis, in combination with IL-1α, IL-1β, IL-1Ra or vehicle. 1000 IU of alteplase was chosen as previous experiments have shown this to cause a 46% decrease in clot size that is significant compared to the negative control but not large enough that any increase in clot lysis caused by co-treatment would not be observable (Fig 9a). Again there was no difference in percentage clot lysis between any treatment group and the negative control (Fig 9b).

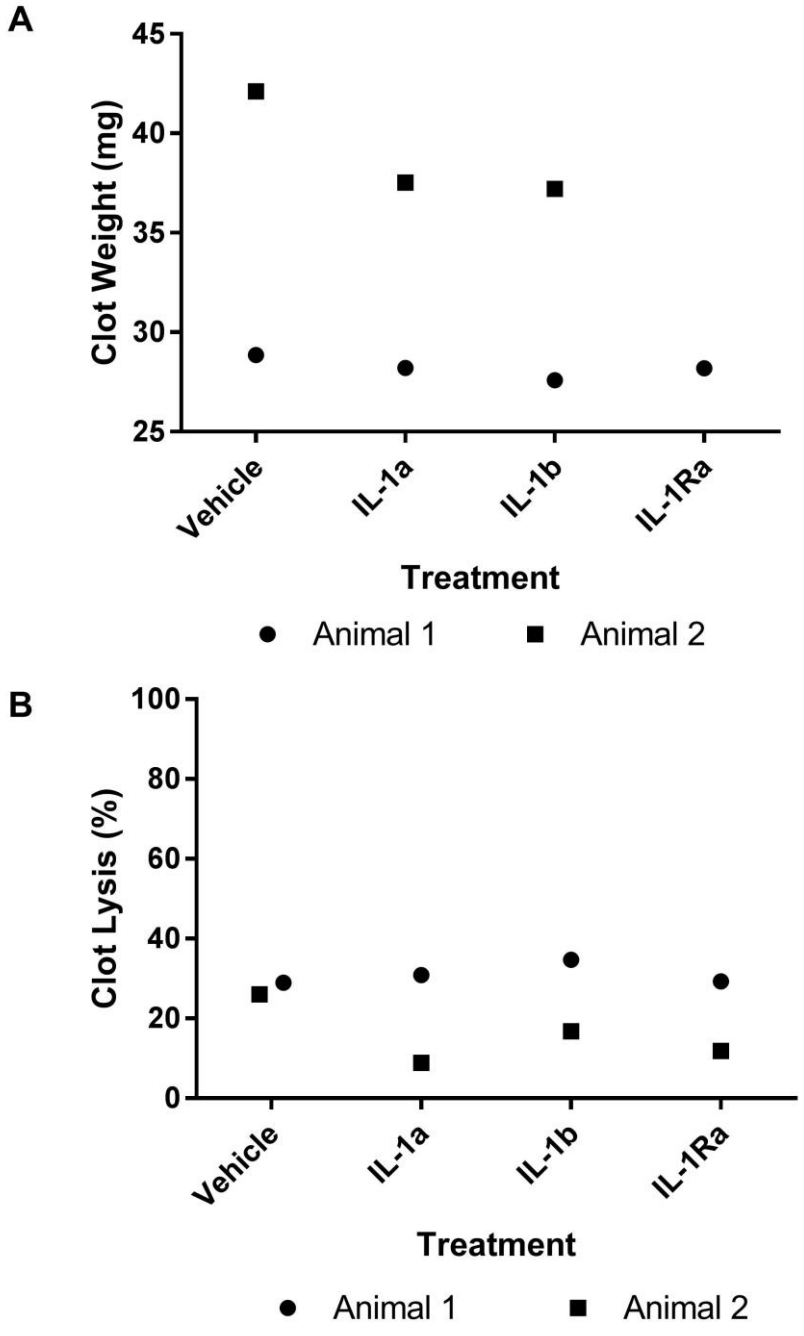


Figure 9 Effect of Interleukin-1 on Clot Formation and Lysis.

A) The weight of the clot (mg) formed in 1h 30min by 100uL of rat blood in the presence of 1ng/mL IL-1α, 1ng/mL IL-1β, 1µg/mL IL-1Ra or an equal volume of vehicle. Mean for each animal used presented. N = 2 (12 repeats for each).

B) Clot lysis of clots prepared from 100uL of whole rat blood by 20µL 1000 IU alteplase co-administered with 20µL 1ng/mL IL-1α, 1ng/mL IL-1β, 1µg/mL IL-1Ra or an equal volume of vehicle. Mean presented. N = 2 (8 repeats for each).

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Discussion

In Vitro Model of Neuronal Injury

Here the development of an *in vitro* model of neuronal injury is described. This model was based on previous work, which has shown that treatment of rat cortical co-cultures with IL-1β induces moderate, dose-dependent cell death[75]. IL-1β is a relevant stimulus in this context due to its role in the pathogenesis of ICH. IL-1β is released by cells in the brain which are mechanically disrupted by the bleed, this released IL-1 causes the neuroinflammation which occurs rapidly after haemorrhage[76]. In addition the breakdown of extravascular blood causes the release of products including haem, Hb, and bilirubin. All three of which have been demonstrated to induce IL-1β release in cardiac macrophages[84] and organotypic slice culture[79], glia (E.Ismail, personal communications) and neonatal neutrophils[85] respectively. Therefore neurones will be exposed to and affected by IL-1β after ICH.

The original protocol had to be modified to reduce basal levels of cell death. This was achieved by providing IL-1β treatment as a full media change as opposed to a half media change. Half media changes are used during culturing to prevent neuronal stress, as primary neurones in culture do not tolerate media changes well. This includes administration of treatments as a half media change. LDH will accumulate in the media during the period between seeding and treatment of the cells. This LDH will then contribute towards the results after IL-1β treatment and so increase the apparent cell death. A full media change before IL-1β treatment removes all LDH from the media meaning only LDH released during treatment contributes towards the observed cell death. In addition evaporation occurs within the incubator and this can be uneven between wells. Therefore with only half media changes the volume in each well will differ and this alters the concentration of the IL-1β treatment.

The original protocol was altered further, by the use of serum free medium, in order to see a dose-dependent effect with IL-1β treatment. Serum contains an ill-defined mixture of proteins that vary from batch to batch, it can also contain undesirable factors which may interfere with the IL-1β treatment[86]. Removing the serum therefore removes this variation making results more reproducible. Serum starvation also synchronises the cell cycle and prevents or reduces proliferation[87].

This may help to reduce glial proliferation and prevent them from growing beyond confluence during the 72h treatment. An excess of glia in the co-cultures may alter how the cultures respond to IL-1β exposure. However the high metabolic demand of neurons renders them intolerant of nutrient deprivation. Consequently serum starvation causes rapid cell death in cultured neurons[88].

Despite these improvements the level of death induced by IL-1β treatment was still high and any exacerbating effect of the thrombolytics with co-administration may have been difficult to interpret. It was therefore necessary to reduce the basal cell death and the level of cell death induced by IL-1β treatment. Glial cells are proliferating cells and are more tolerant of culture conditions than post-mitotic neurones. This increased proliferation may cause an excessive ratio of glia to neurones in co-cultures that masks the effect of IL-1β on neurones. To better control the glial cell density the use of a transwell insert to allow separate culturing of neurones and glia was considered. However this was discounted, as it would require the use of twice as many animals as glia are isolated from 1-2 day old pups whilst neurones are isolated from E18 foetuses. An alternative approach was to limit the number of glia in the co-culture with the addition of an antineoplastic drug, FUDR, during cell seeding. FUDR arrests mitosis in proliferating cells, glia, but has no effect on post-mitotic cells, neurones. In order to obtain a pure neuronal culture a concentration of 20μM FUDR was added to the cells during seeding and removed 24 hours later. The literature suggests that this results in a culture containing less than 5% glia[80]. However in the absence of glia there is no neuronal response to IL-1β, this was shown previously and supported here[75]. Moreover it has previously been demonstrated that astrocytes are the key mediators of this response as toxicity was restored in astrocyte-neurone cultures[75]. However, in this assay a low concentration of FUDR caused a qualitative reduction in the ratio of glia to neurones in culture and consequently a reduction in both basal and IL-1β induced cell death. This gives a greater range within which any synergistic effect of IL-1β and a thrombolytic can be detected.

The LDH assay simply measures the release of LDH into culture media during treatment; this occurs when a cell membrane is ruptured or permeabilised and is not cell specific as all cells contain LDH. A neurone specific measure of cell death would be useful to isolate the neuronal response from that of the glia. One such

method has been described which utilizes a neurone-specific marker, microtubule associated protein 2, and immunofluorescence[89]. This assay was considered, however previous work has shown that the LDH released in response to IL-1β treatment is likely neuronal due to the co-localisation of caspase activation with neurones as determined by immunohistochemistry[75].

NMDA acts on the NMDA receptor and causes cell death through excitotoxicity; it was included as a positive control. 500μM NMDA was expected to cause LDH release similar to that of total lysis, however it did not. Neurons from juvenile rats are less susceptible to NMDA neurotoxicity due to differential subunit composition of the NMDA receptor[90]. However 500uM should still be enough of an excess to overcome this[91]. Therefore there may have been an issue with the preparation from lyophilised stock and/or freeze thawing and its effect on NMDA structure and thus ability to interact with the NMDA receptor.

In summary an *in vitro* model of neuronal injury was developed. This utilizes a glial-neuronal rat cortical co-culture with 5μM of FUDR added during seeding. After 12 days of culture cells are treated with IL-1β as a full media change with serum free medium. LDH release is used to measure viability after 72h and this resulted in a low basal cell death which is increased moderately with 0.1ng/mL of IL-1β.

Comparing Neurotoxicity of Thrombolytics

Unfortunately due to supply issues and repeated contamination of cell cultures it was not possible to compare the neurotoxicity of alteplase, urokinase, tenecteplase, and streptokinase using the above-described model. However in a single experiment alteplase did not appear to increase IL-1 induced cell death.

A recent study directly compared alteplase and urokinase in an *in vitro* model of excitotoxicity and an *in vivo* model of IVH. Here cortical neurones were co-treated with NMDA and either alteplase or urokinase. Alteplase potentiated neuronal death however urokinase did not. Furthermore, urokinase treatment resulted in a reduction in inflammation and neurological deficits in mice after the induction of IVH when compared with those treated with alteplase. This was despite no difference in ventricular enlargement, haematoma volume or oedema between alteplase and urokinase treated mice[92]. The interaction between alteplase and the

NMDAR is mediated through its kringle-2 domain; this domain is absent in urokinase which would explain its lack of effect in potentiating excitotoxic damage[93,94]. Although not compared in this study structurally the kringle-2 domain of tenecteplase is identical to that of alteplase, however point mutations at different sites within the protein may have altered the 3D structure sufficiently to prevent interaction with the NMDAR[58]. Furthermore, streptokinase is structurally very distinct from alteplase, urokinase and tenecteplase and has no kringle-2 domain[95]. NMDA was used to simulate neuronal injury in this study however IL-1β may be more relevant in the context of ICH. Additionally it is not clear exactly how IL-1β mediates neurotoxicity but interaction with the NMDAR is unlikely to be the only mechanism. For example, release of MMPs and nitric oxide (NO) from astrocytes have also been implicated[96,97]. In fact, activation of MMP-9 by IL-1 is mediated by endogenous urokinase bound to neuronal membranes[96]. Therefore thrombolytics that do not interact with the NMDAR, such as urokinase, may potentiate cell death with IL-1β despite no effect being seen with NMDA. The interaction between endogenous urokinase and IL-1β should not interfere with this models as both the membrane bound urokinase and endogenous IL-1β would be present in the brain after ICH.

Thrombolytic Activity Assay

The thrombolytics used were clinical drugs that are packaged as lyophilised single doses; these are usually reconstituted and used immediately without the need for long-term storage. It was unclear therefore how these compounds would respond to dilution, storage at -80°c and subsequent thawing. It was also necessary to compare fibrinolytic activity between the thrombolytics. A simple assay to assess the clot lysing ability of the 4 thrombolytics was used. A similar percentage of clot lysis was seen here with urokinase when compared to the original study from which this protocol was taken and modified[81]. The original study used freshly prepared urokinase suggesting that freeze/thawing does not significantly affect activity. In support of this previous experiments have demonstrated that urokinase activity is unaffected by frozen storage[98]. It also suggests that urokinase has similar activity in rat blood compared with human blood. Data is unavailable for any of the other

thrombolytics using this particular assay to assess thrombolytic activity and the effect of freeze/thawing.

The fibrinolytic activity of each thrombolytic was compared at varying concentrations. Comparable clot lysis was achievable for Tenecteplase, Alteplase and Urokinase with different doses and activity is retained after freeze/thawing. However the fibrinolytic activity of streptokinase was much lower and did not increase in a dose-dependent manner. It was unclear if this was a result of frozen storage. However previous work has shown that streptokinase does have a lower rate of clot lysis in an *in vitro* venous thrombosis model when compared with alteplase and urokinase[56]. In summary it appears as though, with the exception of streptokinase, it is possible to achieve equivalent clot lysis with each thrombolytic by adjusting the dose. This will allow for the standardisation of doses for the use in *in vitro* model of neuronal injury when comparing thrombolytics.

Interleukin-1, Blood Coagulation and Clot Lysis

IL-1 has been implicated in thrombosis through the increased aggregation of platelets induced by thrombin or collagen[82]. There is also evidence that IL-1 plays a role in coagulation through the induction of fibrinogen synthesis[83,99]. A phase IIb trial was conducted to assess the effect of canakinumab, an antibody that neutralises IL-1β, on inflammation with atherosclerosis (Canakinumab Anti-inflammatory Thrombosis Outcomes Study, NCT01327846). The anti-IL-1β treatment reduced serum fibrinogen levels compared with placebo treated patients[99]. In support of this, fibrinogen synthesis in the lung has been demonstrated to be IL-1 dependent using IL-1 receptor knockout mice[83].

Here the effect of IL-1α and IL-1β on coagulation and lysis of rat blood was investigated. Although no effect was seen the large variation in clot size between animals and clot lysis within animals makes this data unreliable. It may not be possible to reduce the variation between animals, however here, although the same species and sex of rat was used (Male, Sprague-Dawley), the animals varied in age. Thus age would need to be better controlled for if this assay was repeated. To reduce within animal variability blood could be collected form the rat in syringes containing citrate. Citrate chelates Ca^{2+}, Ca^{2+} is necessary for the clotting cascade, and thus

citrate prevents coagulation. This can then be reversed with the addition of excessive Ca^{2+}. This would prevent coagulation whilst blood is collected and aliquoted and thus reduce the variability in the clot size obtained which in turn improves reliability of the results. The coagulation cascade is usually activated by contact with vessel walls or the presence of tissue factor[100]. Adding tissue factor to each blood sample may also improve results by promoting initial clot formation. If the clot sizes were more comparable then the percentage clot lysis may not be as variable. Alternatively the volume of treatment (IL-1α, IL-1β and IL-1Ra) could be standardised to the actual clot size instead of the initial blood volume.

However it may be that this assay is not sensitive enough for investigation of the effect of IL-1 on thrombosis and clot lysis. One alternative method utilises the fibrin degradation product, D-dimers. D-dimers are only present in the blood when the coagulation system has been activated and can therefore be used as a measure of fibrinolytic activity. D-dimers can be quantitatively detected in blood by ELISA.

Clinical Relevance

The combination of MIS and a thrombolytic to treat ICH has great potential to quickly, safely and almost completely resolve and remove the hematoma. Thus reducing the mass effect induced by ICH. However, the choice of thrombolytic is not the only factor which may limit its potential. Although the mass effect has a large role in the damage caused by ICH there are alternative mechanism by which cellular death occurs. Secondary injury occurs due to both neuroinflammation and the toxicity of the blood breakdown products.

Summary

A lack of specific treatment for ICH results in a high rate of mortality and morbidity. Clinical trials are underway to assess the effectiveness of a new ICH treatment that utilises MIS in combination with thrombolytics. Although a promising idea the benefit may be limited firstly by the choice of thrombolytic and secondly by the failure to address the secondary damage caused by neuroinflammation and blood toxicity. Here a protocol that will allow the toxicity of multiple thrombolytics to be compared has been developed and described. This takes the form of a cortical neuronal-glial co-cultures treated with the pro-inflammatory cytokine IL-1β. This

can now be used to determine which thrombolytic is least neurotoxic compared with the currently used alteplase. However in order to further optimise the use of MIS and thrombolytics to treat ICH it will be necessary to also address both neuroinflammation and blood breakdown product toxicity. With MIS the placement of a catheter directly into the haematoma offers a unique method of drug delivery into the site of action allowing lower doses to be administered than is possible *via* alternative routes. This can be taken advantage of in the treatment of all facets of ICH pathology by co-administering the thrombolytic with drugs to prevent neuroinflammation, such as the anti-inflammatory IL-1Ra, or drugs to combat the toxicity of blood breakdown products such as iron chelators.

Future Work

In Vitro

Toxicity of thrombolytics needs to be compared in the described model of neuronal injury. A range of concentrations of each thrombolytic, Alteplase, Urokinase, Tenecteplase and Streptokinase, from 10 IU up to the maximum for each thrombolytic should be tested in the presence or absence of 0.1ng/mL IL-1β. Both alteplase and tenecteplase contain L-arginine as a carrier. L-arginine is a substrate for NO synthase in the production of NO and NO at high concentrations can induce apoptosis and necrosis[101] . Therefore L-arginine alone will also be tested for neurotoxicity in this model.

In Vivo

The thrombolytic which is least neurotoxic in the described in vitro model should next be tested in vivo. The autologous blood injection model is an appropriate model for this as it faithfully represents the sudden accumulation of extravascular blood as it happens in patients with ICH and results in oedema and inflammation. However it does not cause a spontaneous bleed, small vessel rupture, or rebleeding and so fails to recapitulate the complete clinical presentation of ICH. A protocol for mimicking the MIS used in the clear trial has been described in mice. A similar protocol could be used here but the catheter placed within the brain parenchyma, at the site of autologous blood injection, rather than the ventricles.

Typically young, male rats are used as the use of female animals is complicated by the oestrous cycle, as oestrogen is known to be neuroprotective, and aged animals increase the cost of research[102]. However, gender is independently predictive of outcome after ICH with females being nearly twice as likely to have a bad outcome, as measured by modified Rankin score, compared to males[103]. Additionally the incidence of ICH is much greater in the aging population and the incidence of ICH risk factors, such as hypertension and CAA, also increase with age. Therefore both age and gender are an important consideration in preclinical studies.

As discussed although rapid removal of the haematoma is crucial in limiting neuronal death after ICH even a brief duration of extravascular blood presence is

sufficient for activation of neuroinflammatory pathways. The thrombolytic will also cause indirect toxicity through the release of blood breakdown products, which are damaging to the CNS. Therefore a combinatorial approach to ICH treatment may be necessary for the best outcomes to be achieved. The use of MIS provides a unique system of drug delivery into the centre of the haematoma; therefore lower doses can be administered directly to the site of action. Using this autologous blood injection model of ICH with a two-way catheter into the haematoma a combination of drugs may be administered and the outcomes assessed. These should include a thrombolytic, an anti-neuroinflammatory and a drug to protect against the blood breakdown products. Examples of drugs to be considered are discussed below.

Therapeutic Targets: Neuroinflammation

There is upregulation of IL-1 mRNA and protein in patients with ischaemic stroke and traumatic brain injury[104]. The IL-1 receptor has an endogenous antagonist; IL-1Ra. IL-1Ra has been shown to be neuroprotective in a variety of species and disease models such as ischaemic stroke in rodents[104] . As similar inflammatory reactions are observed following ICH it seems likely IL-1 will play a part in the pathogenesis of ICH and IL-1Ra may have therapeutic benefit. In support of this adenovirus-mediated overexpression of IL-1Ra reduced oedema in rats after autologous blood injection and reduced neutrophil infiltration in thrombin-treated rats[105] .

Peroxisome proliferator activated receptor gamma (PPARγ) is a transcription factor that regulates phagocytosis. Phagocytosis is important in repair after ICH by removing the extravascular red blood cells. Treatment with a PPARγ agonist, rosiglitazone, after ICH induction in a mouse resulted in enhanced phagocytosis, faster haematoma resolution and improved neurological outcomes[106]. PPARγ also has anti-oxidative and anti-inflammatory properties through regulation of catalase and NF-κB expression, respectively[106,107]. A pilot study assessing the safety of a different PPARγ agonist, pioglitazone, in ICH is currently on going (Safety of Pioglitazone for Hematoma Resolution In Intracerebral Haemorrhage, NCT00827892).

A further promising target is the chromatin binding factor high mobility group protein B1 (HMGB1). HMGB1 is released by immune cells in response to various

stimuli and during necrosis and has been implicated in inflammation[108]. HMGB1 inhibition by glycyhrrhizin reduces oedema, neuronal death and improved functional outcome in a rat collagenase model of ICH[109]. Glycyhrrhizin is the major component of liquorice. It is currently only clinically available in Japan for the treatment of Hepatitis C.

Additional potential therapeutics includes necrostatin and anti-TNFa antibodies. Both of which have had positive outcomes pre-clinically in ICH models but are not yet clinically available[110,111].

Therapeutic Targets: Blood Breakdown Product Toxicity

As the time course of haemolysis and thus breakdown product toxicity is delayed it offers a great opportunity for therapeutic intervention. The iron chelator, deferoxamine, is neuroprotective and reduced ROS in neuronal cultures treated with Hb[112] . In vivo deferoxamine reduced oxidative DNA damage, oedema and neurological deficits in rats following induction of ICH[113]. In light of these findings deferoxamine was trialled in ICH patients (High dose deferoxamine in ICH, NCT01662895) but recruitment was suspended due to a high incidence of acute respiratory distress. A second trial with a modified dosing regime is currently underway (iDEF; Intracerebral Hemorrhage Deferoxamine Trial, NCT02175225).

Alternatively edaravone, a free-radical scavenger, has been shown to be neuroprotective in multiple in vivo models of ICH. Intravenous edaravone administration reduced oedema formation, oxidative stress and cognitive deficits following induction of ICH when compared to placebo treated animals[114,115] . Furthermore, patients treated with MIS and urokinase in combination with edaravone had improved neurological scores when compared with MIS alone, this correlated with reduced serum MMP-9[116] .

Finally, through a combination of anti-oxidative and pro-Hb scavenging sulforaphane appears to have potential in ICH treatment. Sulforaphane is a nuclear factor-erythroid 2-related factor-2 (Nrf2) activator which induces the expression of haptoglobin in addition to catalase, superoxide dismutase and glutathione-S-transferase[117]. Treatment with sulforaphane after autologous blood injection in a mouse model of ICH resulted in reduced neutrophil infiltration, oxidative stress and

functional deficits compared to control animals[117]. Although not clinically available sulforaphane is a compound found in cruciferous vegetables and is available as a supplement.

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