Mobilisation of endogenous haematopoietic stem cells and their use as treatment for subacute stroke

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GLOSSARY

AE	Adverse event
AF	Atrial fibrillation
ASC	Adipose stem cell
AXIS	AX200 for the treatment of ischaemic stroke
BI	Barthel index
BNP	β-natriuretic peptide
CBF	Cerebral blood flow
CI	Confidence interval
CSF	Colony stimulating factor
CT	Computerised tomography
DWI	Diffusion weighted imaging
EM	Electron microscopy
EPO	Erythropoietin
ESC	Embryonic stem cell
G-CSF	Granulocyte colony stimulating factor
G-CSFR	Granulocyte colony stimulating factor receptor
GFAP	Glial fibrillary acid protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRID	Gadolinium-rhodamine dextran
HSC	Haematopoietic stem cell
HTI	Haemorrhagic transformation of infarction
ICAM	Intercellular adhesion molecule-1
ICH	Intracerebral haemorrhage
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPS	Induced pluripotent stem cell
IQR	Interquartile range
LACS	Lacunar syndrome
MCAo	Middle cerebral artery occlusion
M-CSF	Macrophage-colony stimulating factor
MMP-9	Matrix metalloproteinase-9
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
mRS	Modified Rankin scale

MSC	Mesenchymal stem cell
NEADL	Nottingham extended activities of daily living
NICE	National Institute of Clinical Excellence
NIHSS	National Institutes of Health stroke scale
NMDA	N-methyl D-aspartate
NPC	Neural progenitor cell
OR	Odds ratio
PACS	Partial anterior circulation syndrome
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PET	Positron emission tomography
PI	Propidium iodide
PICH	Primary intracerebral haemorrhage
POCS	Posterior circulation syndrome
RCT	Randomised controlled trial
rt-PA	Recombinant tissue-plasminogen activator
SAE	Serious adverse event
SCF	Stem cell factor
SD	Standard deviation
SDF-1	Stromal cell derived factor
SNSS	Scandinavian neurological stroke scale
SPIO	Super paramagnetic iron oxide
STAIR	Stroke Therapy Academic Industry Roundtable
STEPS	Stem cell Therapy as an Emerging Paradigm for Stroke
STEMS-2	Stem cell Trial of recovery EnhanceMent after Stroke-2
SMD	Standardised mean difference
TACS	Total anterior circulation syndrome
TIA	Transient ischaemic attack
TOAST	Trial of Org 10172 in acute stroke treatment
TPO	Thrombopoietin
VEGF	Vascular endothelial growth factor
WMD	Weighted mean difference

ABSTRACT

The potential application for stem cell therapy is vast. Despite a limited understanding of their mechanisms of action, clinical trials assessing stem cells in human stroke are underway. Colony stimulating factors (CSF) such as granulocyte colony stimulating factor (G-CSF), which have been used to mobilise haematopoietic stem cells (HSC), also show promise in treating stroke.

Preclinical experiments evaluating the effect of G-CSF in stroke were meta-analysed; G-CSF significantly reduced lesion size in transient but not permanent models of ischaemic stroke. Further studies assessing dose-response, administration time, length of ischaemia and long-term functional recovery are needed.

Tracking iron-labelled cells with MRI may help to establish migratory patterns following transplantation. Our systematic review of iron-labelled stem cells in experimental stroke suggests that compounds already licensed for humans (ferumoxide and protamine) may potentially be used in clinical trials.

In a phase IIb single-centre randomised controlled trial (n=60), the safety of G-CSF in recent stroke was assessed (STEMS-2). G-CSF appears safe when administered subacutely and may reduce stroke lesion volume. Phase III trials are required to test efficacy.

An updated Cochrane review on CSFs in stroke shows that G-CSF was associated with a non-significant reduction in early impairment but had no effect on functional outcome in 6 small studies. In two trials, erythropoietin therapy

was significantly associated with death by the end of the trial. It is too early to know whether G-CSF could improve functional outcome in stroke.

In 8 recruits randomised into STEMS-2, mobilised CD34+ HSCs were paramagnetically labelled, re-infused and tracked with serial T2* MRI. Post-stroke HSC labelling appears safe and feasible. There is suggestive evidence in one patient that labelled HSCs migrate to the ischaemic lesion.

Our in vitro evaluation of CD34+ HSCs has revealed that uptake of superparamagnetic iron oxide (SPIO) is enhanced but not dependent upon a transfection agent. Iron labelling of CD34+ cells in this manner did not affect cell viability or inhibit growth. This methodology could be applied to clinical trials.

We have established the expression of G-CSF protein, its receptor (G-CSFR) and CD34 antigen in post-mortem brain tissue from participants recruited to STEMS-2. Areas of angiogenesis and expression of G-CSFR in acute and chronic infarction suggest potential targets for therapy.

There are many preclinical studies reporting the effects of stem cells in treating stroke (with a noticeable lack of neutral or negative articles). Despite the wealth of literature there remain many unanswered questions and patients should not undergo stem cell therapy unless it is as part of a well designed clinical trial.

ACKNOWLEDGEMENTS

The Medical Research Council funded the Stem cell Trial of recovery EnhanceMent after Stroke 2 (STEMS-2) (grant G0501997). Professor Philip Bath conceived the trial and provided me with guidance and support over the years.

Research Nurses from the Stroke Research Network (SRN) helped identify recruits and both Fiona Hammonds (Research Nurse) and Dr Gillian Sare (funded by the British Heart Foundation (grant PG/05/137)) performed blinded follow up assessments. We thank Sally Anderson and the Stem Cell Team for advice and performing CD34+ counts; Lucy Kerr for optimising and performing immunohistochemical specimens and Jim Lowe for supervision in histopathology; Dorothee Auer and Maryam Abaie, for vital support in neuroradiology including lesion volume analysis and scan interpretation; Dr Naranjargal Dashdori and the Radiographers for performing magnetic resonance imaging; and Dr Rhodri Jones for his help with CD34+ cell labelling in the Clinical Tissues Laboratory (Queen's Medical Centre, Nottingham) and designing the Endorem study (chapter 7). The Data Monitoring Committee for STEMS-2 comprised Professor Martin Dennis (Edinburgh) and Associate Professor Nikola Sprigg. Medical Statistician Cheryl Hogg gave me statistical advice and the Nottingham City Hospital Stroke Unit nurses and Pharmacist Emma Grace were invaluable in helping to administer the trial drug.

Undoubtedly I would not have managed to complete this thesis without the patience and support of my wife and family.

CHAPTER 1

INTRODUCTION

Publications contributing to this chapter:

England T, Martin P, Bath PM. Stem cells for enhancing recovery after stroke: a review. *International Journal of Stroke*. 2009;4:101-110

Background

Definition

The most widely accepted definition of a stroke is 'a clinical syndrome characterised by rapidly developing clinical symptoms and/or signs of focal (and at times global) loss of cerebral function with symptoms lasting for more than 24 hours or leading to death, with no apparent cause other than that of vascular origin'.[1] This encompasses stroke secondary to its three pathological subtypes: cerebral infarction, primary intracerebral haemorrhage and subarachnoid haemorrhage.

Symptom duration of greater than 24 hours is an arbitrary cut-off point primarily developed for epidemiological purposes to distinguish a stroke from a transient ischaemic attack (TIA). The definition of a TIA is therefore a sudden focal neurological deficit of the brain or eye, presumed to be of vascular origin and lasts less than 24 hours. Whilst this definition is valid and useful, it is based on the assumption that TIAs are associated with complete resolution of cerebral ischaemia with no permanent brain injury. Advances in acute stroke imaging have shown this to be false and a new 'tissue-based' definition has been proposed: 'a TIA is a brief episode of neurological dysfunction caused by focal brain or retinal ischaemia, with clinical symptoms typically lasting less than one hour, and without evidence of acute infarction'.[2]

Epidemiology

Stroke is the third leading cause of death worldwide [3] and is devastating to both patients and carers. Its incidence (the number of new cases per unit of person-time) and prevalence (the total number of cases at a given time) vary according

to the population studied; in the United Kingdom there are 110,000 first strokes and 30,000 recurrent strokes each year.[4] Incidence rises with age with less than one quarter of cases aged less than 65.[5, 6] Apart from the very elderly, males have a higher prevalence than females.[6] Incidence of stroke also varies according to ethnic background; for example, there is a higher incidence of stroke in black compared to white populations,[7] blacks are more susceptible to stroke caused by small vessel disease,[8] and far eastern countries have higher rates of primary intracerebral haemorrhage.[9] Overall stroke affects approximately 1% of the population; this is going to increase as the population ages but whether incidence will change remains unclear.

Mortality

Stroke mortality is dependent upon aetiological subtype (table 1).[10] In the Oxford Community Stroke Project (OCSP), 675 patients were followed up for 6.5 years after their first ever stroke;[11] 19% were dead within 30 days and those who survived 30 days had an annual risk of death 2.3 times higher than the general population. After 30 days, non-stroke cardiovascular disease becomes an increasingly important cause of death. Very broadly, 1 year after a first ever stroke, one third of patients have died, one third are independent and one third are dependent on others for everyday activities.[10] The risk of recurrent stroke among survivors is about 2-4% in the first month, and 10-16% within the first year, thereafter this falls to 4-5% per year.[12]

Table 1. Case fatality, recurrence and functional status from the Oxford Clinical Stroke Project.

	Subtype				
	TACI (n=92)	PACI (n=185)	POCI (n=129)	LACI (n=137)	All Patients (n=543)
30 Days					
Dead	39	4	7	2	10
Dependent	56	39	31	36	39
Independent	4	56	62	62	50
6 months					
Dead	56	10	14	7	18
Dependent	39	34	18	26	29
Independent	4	55	68	66	52
1 year					
Dead	60	16	19	11	23
Dependent	36	29	19	28	28
Independent	4	55	62	60	49
Recurrence	6	17	20	9	14

Independent = modified Rankin scale (mRS) 0-2; Dependent = mRS 3-5; TACI, total anterior circulation infarct; PACI, partial anterior circulation infarct; LACI, lacunar infarct; POCI; posterior circulation infarct. Data given as %.

Aetiological classification

Stroke and TIA are merely symptoms and signs of a number of possible underlying disease processes. In Caucasians, 80-85% of strokes are due to cerebral infarction, 10-15% to primary intracerebral haemorrhage (PICH) and 5% to subarachnoid haemorrhage (SAH).[13] Black and South East Asians have a higher incidence of PICH.[14] The division in classification is important it has implications on treatment, management and prognosis. Ischaemic stroke and PICH are considered in turn but SAH is clinically distinct from these and will not be considered further in this thesis.

Ischaemic stroke

Though there are clinical indicators that might differentiate between ischaemic stroke and PICH (e.g. presence of headache and decreased conscious level in PICH), the only reliable method is with brain imaging such as a computerised tomography (CT) scan. The aetiology of ischaemic stroke is best considered using the following subtypes, the TOAST (Trial of org 10172 in acute stroke treatment) classification,[15] which identifies the most probable pathophysiological mechanism on the basis of clinical findings and the results of investigations.

Large artery atherosclerosis

Atherosclerosis is a chronic inflammatory disorder that results in hardening and thickening of arterial walls. Though it inevitably accompanies aging, it is not a degenerative process. The initial insult, called a 'fatty streak', is a purely inflammatory lesion and has been observed in infants.[16] Over many years circulating monocyte-derived macrophages adhere to and invade the arterial wall.[17] An inflammatory response and deposition of cholesterol and other lipids create arterial plaques. Over time, these plaques narrow the arterial lumen (and at times dilate it) and subsequently rupture, causing platelet activation, aggregation and resultant thrombus (and embolus) formation.[18] It remains unclear as to what causes a stable plaque to rupture but it may be due to mechanical stress (e.g. hypertension) and the large lipid core redistributing shear stress over weakened areas in a thin fibrous cap.[19]

Atherosclerosis mainly affects large and medium sized arteries at places of arterial branching (e.g. carotid bifurcation) and it accounts for approximately 50% of ischaemic strokes in white people.[20] Atherosclerotic TIA or ischaemic

stroke occurs when there is insufficient blood flow to part of the brain as a result of (i) in-situ thrombotic arterial occlusion, (ii) low flow distal to an occluded or severely narrowed artery or (iii) embolism from an atherosclerotic plaque or thrombus. Many factors (such as the nature of the stroke, the presence of bruits on examination) can implicate large artery atheroma as a cause, but in reality it is often difficult to prove since there are multiple potential sites where atheroma can develop and imaging is often performed after the vessel has been given the chance to spontaneously re-canalise. Nonetheless, it is known that increasing age, male sex, hypertension, lipid abnormalities, diabetes and cigarette smoking increase the risk of atheroma formation, making it reasonable to assume atherothromboembolism as a potential cause in many cases.

Small vessel disease

Strokes caused by occlusion of the smaller arteries in the brain (less than approximately 500μm in diameter) account for between 11% and 20% of ischaemic strokes.[20, 21] These infarcts are usually less than 15mm in size (a lacunar infarct) and it is felt that microatheroma is the commonest underlying mechanism. Lipohyalinosis accounts for many of the smaller lacunes, especially those that are asymptomatic.[22] Patients with a lacunar stroke have a similar risk factor profile to those with large vessel disease with diabetes, hypertension, hyperlipidaemia and current smoking most prominent.[23, 24] The high risk associated with hypertension but absent risk with heart disease supports the hypothesis of a unique pathophysiological mechanism for lacunar stroke.[24]

Cardio-embolism

Embolism of cardiac origin accounts for about 20% of ischaemic strokes.[25] These are generally severe and prone to early recurrence. The most common source of embolism from the heart is non-rheumatic atrial fibrillation (AF) with a prevalence of 12%.[26] Other major sources include mitral incompetence (6%), myocardial infarction within 6 weeks (5%), mitral stenosis (1%) and paradoxical embolism (1%).[26] Rarer causes include endocarditis and intracardiac tumours. The average absolute risk of ischaemic stroke in unanticoagulated non-valvular AF is 5% per annum (6 times greater than those in sinus rhythm) and 12% per annum in unanticoagulated AF in patients with previous stroke or TIA.[27] Cardio-embolic strokes are more prone to haemorrhagic transformation, reported in up to 71% of cases.[28]

Primary intracerebral haemorrhage

Primary intracerebral haemorrhage (PICH) causes 10-15% of strokes in white people [29, 30] and the risk of PICH appears higher in black Caribbean and black African groups compared with whites.[31] Patients with PICH have a high early case fatality but survivors have a similar outcome to patients with cerebral infarction.[29] Most cases of PICH are related to hypertension and intracranial small vessel disease.[32] These haemorrhages are usually deep in the brain in the areas supplied by small penetrating arteries. Pathophysiologically, hypertension causes early proliferation of arteriolar smooth muscle followed by smooth muscle death and collagen deposition; collagen is non-elastic and is liable to break under pressure and hence lead to haemorhage.[33] In older individuals, amyloid angiopathy is more common and is typically associated with multiple (typically lobar) haemorrhages and cognitive decline. Underlying is

a progressive fibrillosis of arterioles and deposition of beta-amyloid, increasing the fragility of these vessels.[33] If the patient is younger, underlying vascular abnormalities such as an arteriovenous malformation should be suspected and investigated appropriately. Intracerebral haemorrhage may also be secondary to conditions such as intracranial tumour (primary or secondary), intracranial venous thrombosis, inherited bleeding diatheses, iatrogenic causes (e.g. thrombolysis, anticoagulation) and drug use (e.g. cocaine [34, 35] or amphetamines [35]).

Other causes

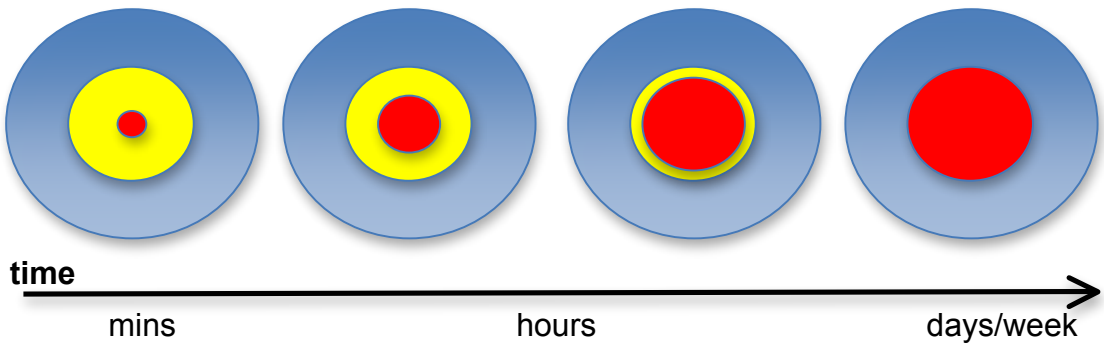
Conditions such arterial dissection, vasculitis and thrombophilia causing stroke are rare but should be suspected in the young and those presenting with unusual features.

The ischaemic penumbra

Occlusion of a major cerebral artery leads to a severe reduction in cerebral blood flow (CBF). Cell death then occurs in a spatial and temporal continuum; CBF reduction is greatest at the core of the ischaemic area where there is metabolic failure and cell death within minutes. Surrounding this area is tissue at risk of death but the reduction in CBF is less severe due to collateral blood supply. The area of salvageable tissue is known as the penumbra (figure 1).[36] Brain tissue within the penumbra is dysfunctional (occurs when CBF falls below 0.16-0.17mls/g/min) but it has the potential to recover hence many therapeutic and experimental interventions attempt to re-establish CBF sufficiently to prevent cell death in the penumbra.

Figure 1. Schematic of the ischaemic penumbra

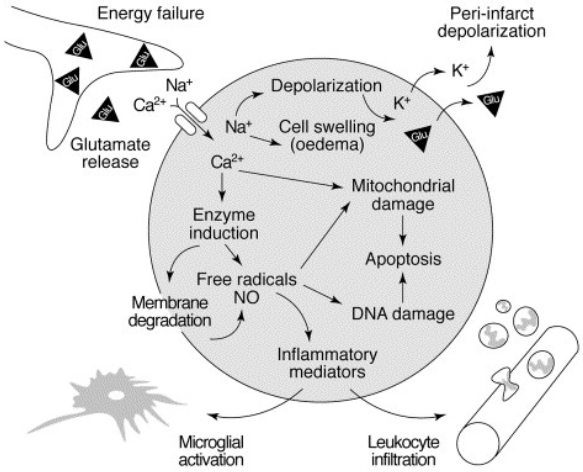
An area of ischaemic but potentially viable tissue surrounds an infarct core. Over time, the core grows at the cost of the penumbra. The blue area represents the brain; yellow, the punumbra; red, the infarct core.



Mechanisms of cell death

The figure below summarises the pathophysiological mechanisms in focal ischaemic brain. The mechanisms are then discussed further.

Figure 2. Overview of the mechanisms of cell damage and cell death after ischaemic injury [37]



Excitotoxicity

After focal ischaemia, the loss of oxygen and glucose leads to depolarisation in neurons and glia, activation of voltage gated calcium channels and subsequent release of toxic amino acids, including glutamate, into the extracellular matrix. Reuptake of glutamate via the N-methyl-D-aspartate (NMDA) receptor leads to cellular calcium influx, triggering mitochondrial depolarisation and eventual cell death.[38] In addition, the influx of sodium (followed by water) regulated largely by the α-amino-3hydroxyl-5-methyl-4-isoazole propionic acid (AMPA) receptor, causes cytotoxic intracellular oedema. Antagonism of both of these receptors leads to neuroprotective effects [39, 40] and are therefore possible targets for therapeutic intervention.

Oxidative stress

Further direct tissue damage following reperfusion is caused by the generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and nitric oxide, via mitochondrial apoptotic mechanisms.[41] Endogenous ROS scavengers (e.g. superoxide dismutase, glutathione), which mitigate the effects of ROS,[42] are insufficient in reducing the damage caused by significant focal ischaemia.

Peri-infarct depolarisation

Cells within the ischaemic penumbra experience an exchange of potassium and neurotransmitters across the cell membrane leading to repolarisation after the initial depolarisation. This can occur repetitively, at the expense of further energy consumption, originating at the ischaemic core and passing to the periphery in a wave-like fashion. The infarct grows larger as the number of depolarisations increase.[43] These events may propagate ischaemic injury and it is suggested that some neuroprotective molecules (e.g. NMDA-antagonists) reduce infarct volume by inhibiting this process.

Inflammation

An inflammatory reaction can last for days to weeks after an ischaemic insult and can contribute to exacerbation of focal neurological deficits. Several cell populations, including endothelial cells, neurons, astrocytes and microglia are able to secrete proinflammatory cytokines including tumour necrosis factor-α (TNF-α), interleukins (ILs) IL-1ß and IL-6 and interferon.[44] Research strategies aim at reducing the potential detrimental effects of inflammation within a potentially wide therapeutic window. It, however, needs to be balanced with

potential protective effects of inflammation, for example, neuroprotection mediated through ischaemic tolerance (brain cells preconditioned to induce a state of resistance to a subsequent 'challenge' that would otherwise be lethal).[45]

Proinflammatory cytokines induce adhesion molecule expression, such as vascular adhesion molecule (VCAM), intercellular adhesion molecule-1 (ICAM-1), intergrins and selectins on endothelial cells, leucocytes and platelets.[44] Signalling through adhesion molecules leads to leucocyte infiltration into the brain parenchyma (via 'leucocyte-rolling') after an ischaemic insult and usually occurs within 4-6 hours, starting with an infiltration of neutrophils. This process may exacerbate stroke injury since depleting stroke rats of circulating neutrophils will lead to a reduction in infarct volume and improve functional outcome.[46] The significance of lymphocyte recruitment into the brain remains unclear.

Multiple other molecules are implicated in regulating inflammation post-stroke, including inducible nitric oxide synthase (iNOS), which catalyses the production of nitric oxide (a free radical);[47] iNOS and its neuronal form (nNOS) are damaging under ischaemic conditions, whereas a third isoform, endothelial nitric oxide synthase (eNOS) may play a protective role in improving cerebral blood flow. Other important molecules implicated in post-ischaemic inflammation include cyclooxygenase-2 (a rate limiting enzyme in the production of prostaglandins) and toll-like receptors (TLRs).[48, 49]

Clinical Diagnosis

The diagnosis of a stroke is initially based on a detailed history (taken from the patient or witness) and medical examination, and confirmed with diagnostic radiological imaging. A typical history is one of a sudden loss of neurological function determined by the site of the brain that has been damaged by ischaemia or haemorrhage. Symptoms are usually maximal at onset but occasionally they worsen gradually or in a stepwise fashion. Alternative diagnoses (such as intracerebral malignancy) should be considered if ictus is not sudden. Table 2 summarises typical clinical features following a stroke or TIA.

Table 2. Typical clinical features of stroke and TIA

Symptom	Descriptive term
Motor Symptoms	
Weakness of one side of the body	Hemiparesis
Difficulty swallowing	Dysphagia
Imbalance	Ataxia
Inability to perform certain actions not due to weakness	Dyspraxia
Sensory Symptoms	
Altered feeling on one side of the body	Hemisensory disturbance
Neglect of one side	Tactile or visual inattention
Loss of vision in one eye	Monocular blindness or amaurosis fugax
Loss of vision in a visual field	Hemianopia or quadrantanopia
Double vision	Diplopia
A spinning sensation	Vertigo
Speech or language disturbance	
Difficulty understanding or expressing spoken language	Receptive or expressive dysphasia
Difficulty writing	Dysgraphia
Difficulty calculating	Dyscalculia
Slurred speech	Dysarthria

Symptoms in isolation (dysphagia, ataxia, vertigo, dysarthria) or non-focal neurological symptoms (generalised weakness, dizziness, loss of consciousness, confusion, incontinence) suggest an alternative cause other than stroke. Moreover, if there are unusual clinical features such as a young age or unexplained fever then underlying diagnoses must be sought.

Clinical Classification

Stroke syndromes based on clinical features, established by the Oxfordshire Community Stroke Project (OCSP),[50] allow the clinician to estimate information on the anatomical and vascular location of the stroke, its aetiology and prognosis. All strokes do not fit perfectly into these subtypes but they are categorised as follows:

1) Total Anterior Circulation Syndrome (TACS)

A TACS is a combination of all three of:

i. Hemiparesis involving at least two of face, arm and leg, with or without hemisensory loss.
ii. Homonymous hemianopia
iii. Cortical (or 'higher cerebral') dysfunction, usually a dysphasia if involving the dominant hemisphere or inattention / neglect if involving the non-dominant hemisphere.

These are usually large infarcts involving the middle cerebral or anterior cerebral artery or due to large lobar haematoma. The patient is often drowsy and confused as a consequence. Prognosis is poor with 60% dead and 35% dependent at 1 year post stroke.[50]

2) Partial Anterior Circulation Syndrome (PACS)

A PACS consists of

a. A combination of two of the three features of a TACS or
b. Isolated high cerebral dysfunction or
c. Sensory motor deficit isolated to one of the face, arm or leg.

PACS are usually secondary to occlusion of one of the branches of the middle cerebral or anterior cerebral arteries or due to lobar haemorrhage. Risk of recurrence is high but prognosis significantly better than TACS;[50] 15% dead and 30% dependent at 1 year.

3) Lacunar Syndrome (LACS)

There are 4 main lacunar syndromes:

a. Pure motor hemiparesis (up to 50% of lacunar cases) causes unilateral weakness of two of three of face, arm or leg. The lesion occurs where the motor pathways are tightly packed such as in the internal capsule or pons.
b. Pure hemisensory loss is less common and consists of unilateral sensory symptoms with or without signs. The lesion is usually in the thalamus.
c. Hemisensorimotor loss (35% of lacunar cases) is a combination of unilateral motor and sensory loss with no other features. The lesion may be in the thalamus, internal capsule, corona radiata or pons.
d. Ataxic hemiparesis (10% of lacuna cases) is a combination weakness and ataxia in the arm and/or leg. The responsible lesion is usually in the pons, internal capsule or cerebral peduncle. This category also includes clumsy hand dysarthria syndrome. Prognosis is fair with 10% dead and

30% dependent at 1 year.[50] A LACS syndrome is usually due to a lacunar infarct in 80-90% of cases.[51]

4) Posterior Circulation Syndrome (POCS)

Strokes involving the posterior circulation include those affecting the brainstem, cerebellum, thalamus or occipital lobe and can cause multiple signs and syndromes (e.g. isolated hemianopia and cranial nerve palsies, lateral medullary syndrome, 'locked-in' syndrome). 60% are independent at 1 year.[50]

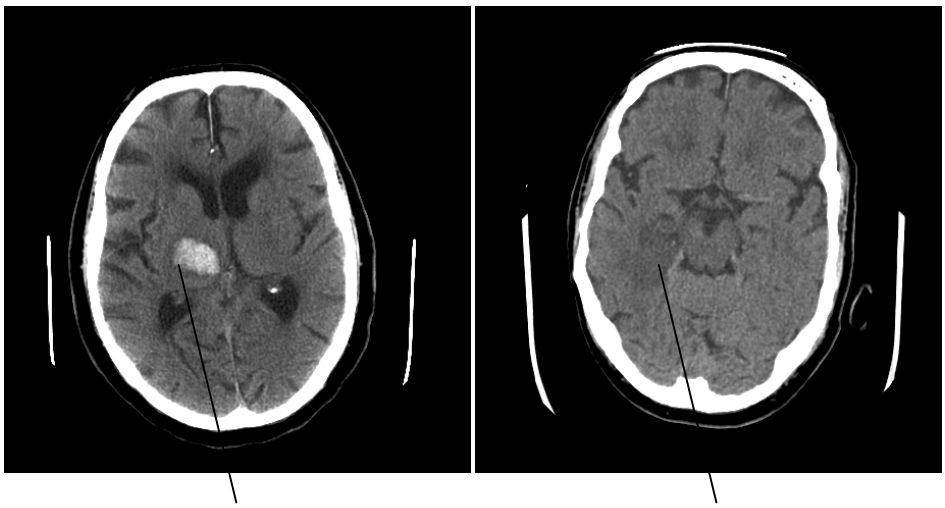
Diagnostic Imaging

A CT or MRI (magnetic resonance imaging) brain scan should be performed as soon as possible and immediately (within 1 hour) in suspected stroke if any of the following apply:[52]

- indications for thrombolysis or early anticoagulation
- a depressed level of consciousness (Glasgow coma score <13)
- on anticoagulation treatment or a known bleeding tendency
- unexplained progressive or fluctuating symptoms
- severe headache at onset
- papilloedema, fever or neck stiffness.

Both CT and MRI are equally effective at detecting acute PICH [53] but a CT scan is usually the initial investigation of choice because it is more widely available, less expensive and safer to use in acutely ill stroke patients. Subtle signs of early ischaemia on CT include loss of grey-white differentiation (especially of the insular ribbon or lentiform nucleus), succal effacement and the hyperdense artery sign, which are all associated with a poor outcome.[54, 55]

Figure 3. The appearance of acute haemorrhage and ischaemic stroke on CT imaging.



Nonetheless, particular MRI sequences have specific uses, for example, in determining whether a stroke is acute or chronic (diffusion weighted MRI) or by adding information to help establish the underlying mechanism (MR angiography). If imaging is delayed for more than 1-2 weeks after stroke onset it becomes increasingly difficult to distinguish infarct from haemorrhage. As the blood is gradually reabsorbed, CT appearances change from a white hyperdense area to a black hypodense area. To make the distinction, an MRI (gradient echo / T2*) can detect haemosiderin deposition secondary to haemorrhage months or even years after the event. Overall, relative to the final clinical diagnosis of acute stroke, MRI has a sensitivity of 83% and CT 26%.[53]

The role of brain imaging in patients who have suffered a TIA is less clear. A wide variety of brain pathologies can present with transient focal neurological deficits and brain imaging is important to rule these out. Guidelines from the National Institute of Health and Clinical Excellence (NICE) state that people who

have had a suspected TIA in whom the vascular territory or pathology is uncertain should undergo urgent brain imaging.[52] This should be done more urgently if they are at high risk, for example, those with an ABCD2 score (based on age, blood pressure, clinical features, duration of symptoms and presence of diabetes) of 4 or above,[56] or with crescendo TIA, and preferably with diffusion-weighted MRI. Up to 10% of people with TIA will have a recurrent stroke within 48 hours.[57]

Other investigations

All stroke and TIA patients should have 'baseline' tests performed including full blood count, erythrocyte sedimentation rate, biochemistry, glucose, lipid profile, and electrocardiogram. Further specific tests are aimed at establishing an underlying cause and future treatment options. These depend upon the patient's symptoms, age and pre- and post-stroke condition and may include an echocardiogram, duplex carotid ultrasound, immunological assays or thrombophilia testing.

Acute Management

Pre-hospital management

Stroke is a medical emergency and should be treated with the same urgency as acute myocardial infarction. Acute stroke care begins in the community and ambulance system with recognition of a 'brain attack'; educating paramedics can increase their diagnostic accuracy from 60 to 80% and lead to an increase in the rates of thrombolysis.[58] A useful tool to enhance recognition within the community and for paramedics is the FAST test (Face Arm Speech Test) which shows good agreement with physician assessment.[59, 60]

General Measures

Once the diagnosis of stroke is suspected or established there are a number of general measures that should be addressed for both ischaemic and haemorrhagic subtypes.

Maintenance of the airway to help avoid aspiration and adequate oxygenation are fundamental measures in initial management. Supplementary oxygen does not appear to be beneficial in mild and moderate strokes [61] but further trial evidence is awaited to determine whether there is benefit in severe strokes.[62] The presence of a fever is likely to be due to infection but can be secondary to the stroke itself. Nevertheless, a raised temperature is associated with increased morbidity and mortality [63] and should be treated with anti-pyretics for the patient's comfort and perhaps to improve outcome.[64] Other general measures aim to prevent complications of stroke such as avoiding pressure sores with consideration for positioning and continence, and early intervention with hydration and nutrition in those with an unsafe swallow.[65] The routine use of compression stockings to prevent deep vein thrombosis (DVT) has been abandoned since the recent completion of the CLOTS (Clots in Legs Or sTockings after Stroke) trial, revealing no significant reduction in DVT and an associated increase in skin tears and necrosis.[66] Assessment of serum glucose is also important as hypoglycaemia can mimic stroke but whether routine treatment of hyperglycaemia is beneficial remains unclear. NICE guidelines suggest maintaining blood glucose between 4 and 11 mmol/L.[52]

Hypertension is present in approximately 80% of acute stroke patients and is independently associated with a poor outcome.[67] Its management is

controversial with recommendations based on opinion rather than evidence. Lowering blood pressure may be of benefit by reducing brain oedema and haemorrhagic transformation but this needs to be balanced against the potential reduction of perfusion to the ischaemic penumbra (an area of salvageable tissue surrounding an infarcted core). More recent guidelines recognise the lack of evidence and suggest treating blood pressure when post-stroke conditions are stable.[68, 69] Others recommend blood pressure control only if there is a hypertensive emergency (for example, aortic dissection) [52] or if the systolic blood pressure exceeds 200mmHg in haemorrhagic stroke [52]. There appears to be no benefit in lowering BP gradually with candesartan within 30 hours of ictus,[70] and a lower blood pressure produced by continuing pre-stroke anti-hypertensive drugs does not appear to be harmful.[71] Hypotension, on the other hand, is less common after acute stroke and other potential factors such as hypovolaemia, co-existent heart failure and underlying sepsis must be recognised and treated.

Acute ischaemic stroke

Despite numerous clinical trials, there are only two effective drug interventions for acute ischaemic stroke: aspirin and thrombolysis.

Antiplatelet therapy

Aspirin has been assessed in two mega-trials. The Chinese Acute Stroke Trial (CAST) used 160mg of aspirin versus placebo given within 48 hours of stroke onset in 21,106 patients,[72] and the International Stroke Trial (IST) examined aspirin (300mg), heparin, both or neither within 48 hours in 19,435 patients.[73] Treatment increased the odds of making a complete recovery (odds ratio, OR

1.06; 95% confidence interval, CI 1.01-1.11) and at the end of follow-up 13 more patients were alive and independent for every 1000 patients treated. This was offset by a small increase in symptomatic intracranial haemorrhage (SICH) occurring in 2 for every 1000 patients treated.

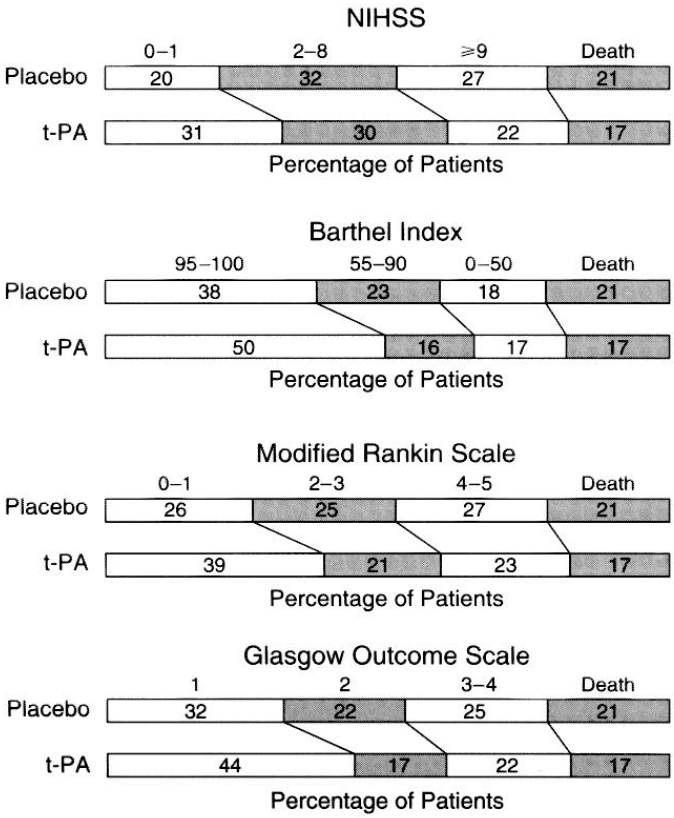
Two other commonly used antiplatelet agents, dipyridamole (a phosphodiesterase inhibitor) and clopidogrel (an ADP antagonist), have not been assessed for use in acute ischaemic stroke but trials are underway (e.g. TARDIS). A phase III trial assessing abciximab, a glycoprotein IIb IIIa inhibitor, was stopped early because of increased rates of SICH.[74]

Thrombolysis

If a stroke is due to blockage of an artery by a blood clot then prompt treatment with thrombolysis can restore the blood flow before significant brain damage has occurred. In the first, pivotal, thrombolysis trial, treatment with intravenous rtPA within three hours of the onset of ischemic stroke improved global clinical outcome at 3 months (OR 1.7, 95% CI 1.2-2.6) despite an increased incidence of SICH (6.4% of patients given rtPA but only 0.6% of patients given placebo).[75] Figure 4 below illustrates the outcome at three months from this trial - scores of $\underline{<}$1 on the National Institutes of Health Stroke Scale (NIHSS), 95 or 100 on the Barthel index, $\underline{<}$1 on the modified Rankin scale, and 1 on the Glasgow outcome scale were considered to indicate a favorable outcome). The ECASS (European Cooperative Stroke Study) thrombolysis trials, however, did not show benefit if treatment was given within 6 hours.[76, 77] Pooled analysis of rtPA trials have suggested that treatment is effective up to 4.5 hours post ictus; this has been confirmed with the recent ECASS 3 trial.[78] Furthermore, the sooner that rtPA is

given to stroke patients, the greater the benefit, especially if started within 90 min.[79] Overall, in 18 thrombolysis trials (with significant heterogeneity and not including ECASS 3) including 5727 patients, there is a net reduction in the proportion of patients dead or dependent in activities of daily living at a cost of an increase in deaths within the first seven to ten days, SICH, and deaths at follow-up at three to six months.[80] Ongoing trials (IST-3) are assessing the effectiveness of rtPA beyond 4.5 hours. Other thrombolytic agents,[81, 82] intra-arterial thrombolysis [83] and mechanical clot retrieval devices under are evaluation.[84] Streptokinase is associated with an unacceptable risk of haemorrhage and death.[85]

Figure 4. Outcome at 3 months according to treatment from the National Institute of Neurological Disorders and Stroke (NINDS) rtPA trial.[75]



Anticoagulation

Currently, the evidence does not suggest any role for early anticoagulation in acute ischaemic stroke. In a meta-analysis of 22 trials, anticoagulant therapy was associated with about 9 fewer recurrent ischaemic strokes per 1000 patients treated but it was also associated with a similar sized 9 per 1000 increase in symptomatic intracranial haemorrhages.[86]

Neuroprotection

Neuroprotectants are drugs that protect the brain from injury. Early trials that have shown initial promise, for example with the free radical scavenger NXY-059,[87] have failed to demonstrate any benefit in follow up studies.[88] This is discussed in more detail later.

Decompressive Surgery

In a selected group of patients with malignant middle cerebral artery infarction, trials have shown that hemicraniectomy, when compared to control, can improve outcome and survival.[89] In those not suitable for surgery, medical therapy with mannitol [90] or glycerol [91] can be used to reduce intracranial pressures.

Rehabilitation and Regeneration strategies

Motor, sensory and cognitive dysfunctions are common consequences of stroke and cause considerable disability and social distress. Restoration of movement and function form the focus of rehabilitation based on physical therapy. Recovery of motor and cognitive function occurs to a variable degree through a

number of pathways:[92-96] unmasking – recruitment of existing but latent connections; sprouting – development of new neural connections (including synaptogenesis); long term potentiation – enhancement of memory and learning; resolution of diaschisis (remote functional depression); and neurogenesis – replacing lost neurones. The addition of drugs that might help improve this process of neural plasticity has provided some interesting results.

The use of oral amphetamines could potentially modulate synaptic function, and studies in rats with stroke have suggested possible benefit.[97] However, the results from a meta-analysis of 11 small clinical trials (n=329) of amphetamines and stroke showed no benefit on functional outcome (despite a trend toward improved motor scores), a non-significant trend toward death and an increase in adverse events in patients treated with amphetamines.[98] The dopaminergic system could also be stimulated with levodopa, a precursor of dopamine used routinely in the treatment of Parkinson's disease. The DARS trial (Dopamine Augmented Rehabilitation in Stroke) is ongoing and aiming to recruit 572 patients to receive co-careldopa or placebo in conjunction with routine rehabilitation.[99] Other drugs licensed for use in humans for other conditions also show promise. In the FLAME study (fluoxetine for motor recovery after acute ischaemic stroke), 118 patients were randomly assigned to 20mg of fluoxetine (an anti-depressant) or placebo for 3 months 5 to 10 days after stroke onset.[100] The primary outcome, improvement in the Fugl-Meyer motor scale, was significantly better in the treatment group. These findings now need to be confirmed in larger studies.

The brain can also be stimulated via physical means, and in its simplest form, comes as physiotherapy and repeated practice. Animal models of recovery of function after stroke reveal that repetitive practice or exercise can evoke endogenous neurogenesis and the expression of signalling molecules such as brain derived neurotrophic factor (BDNF), which then promotes neuronal repair, enhancing learning and memory.[101] Repetitive task training in stroke survivors can result in modest improvements in limb function, and task orientated exercise can help restore gait, balance and strength in a paretic limb.[102] Other approaches to physical manipulation include constraint induced movement therapy (forced use of the affected arm by restraining the unaffected arm), for which there may be value in the short term but further larger trials are needed to show evidence of persisting benefit.[103] The brain may also be manipulated by non-invasive brain stimulation such as transcranial magnetic stimulation (TMS); these techniques can act to either stimulate or inhibit somatosensory processing and could be used in adjunct with rehabilitation.[104] Interestingly, a recent meta-analysis of 18 randomised controlled trials (RCT) (n=392) assessing repetitive TMS in stroke found a significant effect on improving motor function,[105] calling for further trials in larger populations.

Ideally, all stroke patients should be cared for on a specialist stroke unit containing of both acute and rehabilitation wards; compared to general medical wards, care in a stroke unit decreases the likelihood of death, dependency and the need for institutionalised care.[106] It is also more cost-effective. Furthermore, there is increasing interest (often from a financial perspective) in 'early supported discharge' for patients with mild to moderate handicap,[107] which reduces hospital length of stay as well as reducing long-term dependency. The

stroke unit facilitates recovery by using organised multidisciplinary care, involving doctors, nurses, physiotherapists, occupational therapists, speech and language therapists, dieticians, social workers and pharmacists. The length of stay should be tailored to individual needs, using goal-based therapy, allowing progress to be measured. Complications such as deep vein thrombosis and pulmonary embolus, infections, recurrent stroke, seizures, pressure ulcers and post-stroke depression can often inhibit targeted goals. The overall aim is to minimise handicap and return the patient to an optimal functional state.

Haemorrhagic stroke

No specific medical treatment has been shown to improve outcome in haemorrhagic stroke and treatment is largely supportive care. The use of recombinant activated factor VIIa (a haemostatic agent) showed early promise in a phase II trial demonstrating reduced mortality and improvement in functional outcomes at 90 days.[108] These findings were not reproduced in the larger phase III trial.[109] Surgical evacuation is unproven but early surgery for large cerebellar or superficial bleeds should be considered.[110]

Animal models of stroke

A pre-clinical stroke model should ideally mimic the pathophysiological mechanisms that occur in human stroke, which is inherently difficult considering its heterogeneous presentation– the anatomical location, aetiology, severity and co-morbidities are all factors that will confound interpretation and produce variability in outcome. There is therefore no single stroke model that will fairly represent all forms of human stroke. Various models are considered.

Focal models of ischaemic

Focal models of ischaemia involve occluding a specific vessel, usually the middle cerebral artery, and can be either permanent or transient (reversible). Permanent models allow examination of ischaemic core and surrounding area of less damaged tissue (penumbra), whereas transient models build in the consequences of reperfusion on ischaemic brain (in the clinical setting, recanalization occurs to a variable degree hence both transient and permanent models hold value). Transient ischaemia will produce varying degrees of infarction, depending on the length of occlusion, and there are more likely to be higher survival rates than permanent models of ischaemia. Surgical techniques that induce vessel occlusion include cauterisation, clips, threads, use of an intraluminal filament [111] and endothelin-1 administration.[112] Surgery can be extensive and therefore reduce the ability to perform advanced behavioural testing. Some anaesthetic agents can also confound matters by producing their own neuroprotective effects (e.g. ketamine [113]).

Intraluminal middle cerebral artery occlusion involves inserting a monofilament suture into the internal carotid artery to block blood flow to the middle cerebral

artery inducing either permanent or transient occlusion. It produces a reproducible infarct size with a substantial penumbra, making it appropriate for studies of neuroprotection. Potential complications include subarachnoid haemorrhage secondary to suture induced arterial rupture and spontaneous hyperthermia when occlusion is longer than 2 hours.[114]

Thrombo-embolic models utilise homologous blood clot fragments injected directly into the carotid artery.[115] This produces infarcts predominantly in the area of the middle cerebral artery. The main disadvantage is that the location of the infarct can be inconsistent due to micro-embolisation and re-canalisation. Reproducibility of the model is influenced by the size and length of the clot and improved by using a fibrin-rich thrombus.[116]

A photochemical model of stroke is attractive since it is relatively non-invasive.[117] A photosensitive dye (rose bengal) is injected intravenously and the brain (no craniectomy required) is exposed to light of a specific wavelength. A photochemical reaction between the light and the dye generates thrombosis within the vessel. The size of the stroke is dependent on the beam diameter. A rapid ischaemic insult develops with significant vasogenic oedema and no penumbral region as seen in other models of ischaemia. This lack of representation to human stroke is probably why the model is less often used.

Global models of ischaemia

This involves occluding all major blood vessels to the brain producing a large area of infarction. It is generally used to analyse cerebral ischemia after cardiac arrest rather than stroke.

Haemorrhagic stroke model

Intracerebral haemorrhage can be induced via direct injection of a bacterial collagenase (a metalloproteinase that degrades interstitial and basement membrane collagen).[118] The size of the haematoma is dependent on the dose injected. A pronounced inflammatory response occurs with neutrophils evident at 12 hours and lipid-filled macrophages infiltrating after 7 days. Consolidation of the necrotic mass results in a cyst by three weeks.

Animal size

The use of small (rodents) over large animals (dogs, cats, monkeys) offers a number of advantages. Small animals (especially mice) are genetically homogenous and can be more easily manipulated to investigate mechanisms of disease, such as producing genetic knock-out models or over expression of certain proteins. The small brain size in mice and rats may be considered an advantage for quicker fixation and analytical procedures. However, anatomical and functional aspects may be better represented by larger gyrencephalic brains. Small animals are less costly and easier to maintain but sophisticated physiological monitoring is more challenging compared to larger animals.

Species

The majority of studies assessing ischaemic brain have used rat models, probably reflecting their cheaper cost but also due to their greater similarity to the human cerebral circulation compared to other species.[119] Several species should be studied if possible when addressing the effects of a drug due to potential differences. For example dizocilpine (MK-801, a glutamate antagonist) is neuroprotective in rabbits,[120] rats [40] and gerbils,[121] but not in primates.[122]

Behavioural testing in animal models of stroke

Assessment of neurological status in animals is difficult. Simple measures of motor function in rodents are available, including neurological stroke scales that correlate with the size of the infarcted area.[123] Generally, however, there is a poor correlation between behavioural testing and reductions in infarct size.[114] Tests assessing sensorimotor function include rotarod, grid walking, limb placement, beam walking and the adhesive label test.[124] Some of these behavioural tests appear more likely to yield a positive treatment effect than others (such as rotarod, adhesive tape removal and neurological scales [125]), but it must also be acknowledged that improvement during the tests may simply be due to compensatory learning (e.g. gradual improvement on a rotarod because the animal learns to rely on other limbs for balance).[126] Cognitive tests are also available, for example, the Morris water maze test assessing memory and learning. The table below provides examples of commonly used behavioural outcome tests.

Table 3. Behavioural tests used in experimental models of stroke

(adapted from [125])

Test	Assessment
Neurological stroke scale	Motor and sensory functions, balance, reflexes
Adhesive tape removal	Forelimb sensory asymmetry
Rotarod	Coordination, balance, motor function
Limb placement	Response to tactile and proprioceptive stimulation
Cylinder	Spontaneous use of forelimbs
Treadmill	Gait, motor function
Beam-walking	Hind-limb function
Morris water maze	Cognitive function

Combining the results of behavioural testing with histological assessment and infarct volume measurements are important in determining the effectiveness of a drug in the pre-clinical setting.

Therapeutic interventions and pre-clinical studies of neuroprotection

Sodium and potassium channel blockers

Sodium channel inhibition is neuroprotective in experimental stroke, but in a dose escalation phase II trial of sipatrigine (619C89) administered within 12 hours of stroke, an excess of adverse events was observed, including vomiting, neuropsychiatric effects and hyponatraemia.[127] BMS-204352, a potassium channel modulator that decreases neuronal excitability and neurotransmitter release, reached a phase III trial and failed to demonstrate efficacy in stroke patients.[128]

Calcium stabilising drugs

During ischaemia, influx of intracellular calcium via glutamate receptors and voltage-dependent calcium channels eventually leads to cell death and multiple clinical trials (mostly assessing nimodipine) have assessed the use of calcium channel blockers in treating stroke, both as a neuroprotectant and anti-hypertensive strategy. A meta-analysis of 29 randomised controlled trials investigating a calcium channel antagonist administered within 12 hours of ischaemic stroke found no overall effect on functional outcome.[129] A subsequent systematic review on nimodipine in animal model experiments of focal cerebral ischemia concluded that there was no convincing evidence to substantiate the decision to perform clinical trials of nimodipine in large numbers of patients.[130]

Anti-excitotoxic agents

Several agents that inhibit glutamate/NMDA receptors have been tested in experimental models and humans. Dizocilpine (MK-801), a non-competitive NMDA antagonist is neuroprotective in some animal models [131, 132] but investigations were halted due to safety concerns including finding evidence of neuronal vacuolation in rats [133] and psychotic side effects in human clinical trials. Other competitive and non-competitive NMDA antagonists have been found to be neuroprotective in animals but have failed to demonstrate efficacy in humans; selfotel administered within 6 hours of stroke onset was assessed in a phase III clinical trial and was stopped early (n=576) due to a trend in increased mortality.[134] Similarly, a trial using aptiganel (n=628), another NMDA antagonist, was terminated for the same reasons. AMPA antagonists, such as YM-872 have also been studied in humans following initial promise in preclinical studies without yielding any positive results.[135] Magnesium also inhibits the NMDA receptor and is neuroprotective through other mechanisms including inhibiting neurotransmitter release, blocking voltage-gated calcium channels and promoting vasodilation. Trials assessing its hyperacute administration are ongoing.[136]

Antioxidants

Free radical scavengers have also shown promise in experimental stroke and again have failed to demonstrate efficacy in human trials. The most recent example is that of NXY-059. In an RCT of 1722 patients, NXY-059 given within 6 hours of stroke onset was well tolerated and improved outcome as measured by the modified Rankin scale at 90 days.[87] Unfortunately, the follow up trial in

3195 patients demonstrated no evidence of efficacy in any of the primary or secondary end points.

Anti-apoptotic agents

Caspases are a family of proteins essential in the regulation of cell apoptosis. Inhibition of these enzymes in experimental stroke may be beneficial. Granulocyte-colony stimulating factor, a glycoprotein hormone involved in the regulation of granulopoeisis (and discussed in more detail later) is a candidate neuroprotective agent thought to reduce apoptosis and possess anti-inflammatory properties.[137, 138]

Anti-inflammatory drugs

Inflammatory cells can exacerbate stroke injury through production of cytotoxins and by forming aggregates that re-occlude vessels. Inhibiting interaction between inflammatory cells and adhesion molecules is another potential therapeutic approach. Anti-p-selectin antibodies can decrease infarct volume by up to 70% in mice (when administered before stroke induction) [139] and anti-ICAM-1 antibodies will reduce lesion volume in transient but not permanent rat models of ischaemia.[140] The clinical trial assessing enlimomab (a murine ICAM-1 monoclonocal antibody) in 625 patients was negative, causing an increase in mortality and a significantly poorer outcome in the treatment group.[141] A follow-up preclinical experiment investigating the cause of this negative trial suggested that enlimomab causes host antibodies to exacerbate the inflammatory reaction.[142] The ASTIN (Acute Stroke Therapy by Inhibition of Neutrophils) trial investigated UK-279,276 (a selective CD11b/CD18 antagonist, a neutrophil inhibitory factor) in 966 patients with ischaemic stroke and was also

administered within 6 hours on onset.[143] The trial was stopped early on grounds of futility, though the drug was well tolerated. Other anti-inflammatory approaches have shown success in animal models of stroke including mitogen activated protein kinase (MAPK) inhibitors, [144] which alter cytokine production, but to date there have been no successful trials of anti-inflammatory drugs in stroke.

Corticosteroids and immunotherapy

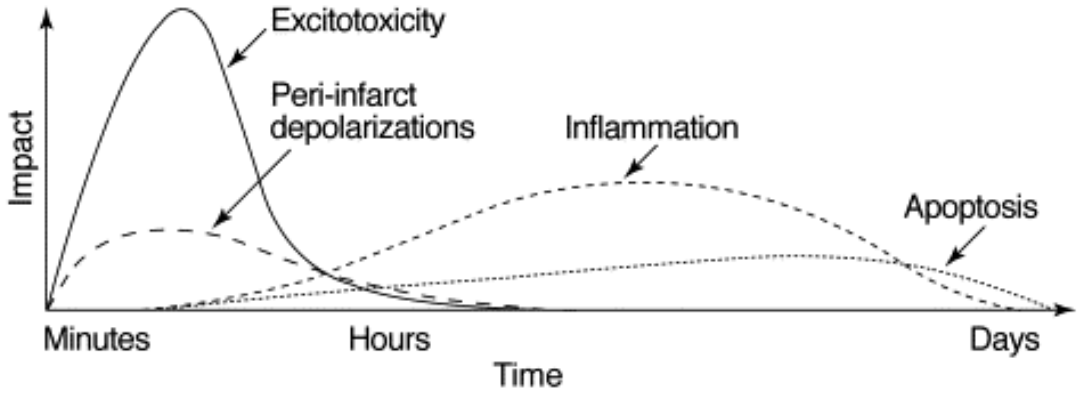
Vasogenic oedema associated with intra-cerebral tumours responds well to steroids.[145] There is no current evidence that corticosteroids are beneficial in reducing stroke associated cytotoxic oedema. A meta-analysis of eight trials and 466 people showed no difference in outcome but pooling the data was difficult due to large inconsistencies between trials.[146] Tacrolimus is a potent immunosuppressant used in transplant medicine for many years and has shown promise in experimental stroke; a systematic review of 1759 animals reported a 31% improvement in outcome compared to placebo but concerns were raised with regards to study quality and publication bias.[147] Another potential immunotherapy in stroke is the use of intravenous immunoglobulin (IVIG), which is used widely in clinical practice, particularly in neuro-autoimmune diseases. Administration of IVIG to experimental stroke mice reduces infarct volume, possibly by reducing production of cell adhesion molecules thereby decreasing the inflammatory infiltrate.[148]

Why have clinical trials failed despite favourable pre-clinical data?

Therapeutic window

It seems reasonable to assume that drugs designed to work on a specific mechanism should be administered when that mechanism is active. For example, glutamate release occurs seconds after the ischaemic event and NMDA-antagonists are effective in animal models up to 90 minutes after ischaemia.[149] Despite this, most of the trials evaluating NMDA-antagonists allowed inclusion of patients up to 6 hours post stroke. This could be considered a reasonable time window, however, since the cascade of events in the penumbra is often delayed compared to the infarct core due to collateral blood flow. The figure below represents the cascade of damaging events in focal ischaemia over time.[37] Nonetheless rt-PA (thrombolysis) is the only compound licenced for use in acute ischaemic stroke when given within 4.5 hours and it is effective in animal models over a similar time frame.[150] This suggests trials of novel compounds should mimic the time window used in the pre-clinical experiments. The duration of treatment should also reflect the mechanism to which the drug is targeting. Previous neuroprotectant trials have varied from using a single bolus to 12 weeks of oral therapy;[151] neuroprotective drugs should probably cover the first 72 hours of excitotoxicity. In addition to drug pharmacology, administration will also depend on tolerability, practical issues of administration and interaction with other therapies.

Figure 5. Cascade of events in focal ischaemia depicting the impact of each event over time [37]



Dose of drug

A drug dose that is effective in animal models may not be effective in humans, and it may not be sufficient to scale up a dose of a drug in mg/kg from rodent models to larger animals and humans. In addition, drug delivery to the brain at risk is affected by the blood brain barrier, cerebral blood flow and drug pharmacokinetics which may vary between species.

Monotherapy

Targeting just one component of the ischaemic cascade may not be enough. Using 2 or more compounds, however, is likely to increase the risk of adverse events. The ideal neuroprotective drug might be a single compound that targets greater than one component of the pathophysiological process.

Animal models

As discussed, there are multiple animal models of stroke, and there is no clear 'best' model to use. It is important to study the same strain of rodent throughout the experimental stage due to variations in infarct size and it is suggested that

determining efficacy in a permanent model of ischaemia will maximise the chances of clinical success.[152] Ideally, the models should incorporate other co-morbidities such as including older animals and animals with diabetes and hypertension, to more closely mimic the clinical setting. Furthermore, the most appropriate animal model should be selected; for example, using reperfusion models if a drug is to be used in patients with thrombolysis would strengthen the validity of the experiment.

Stroke Therapy Academic Industry Roundtable (STAIR)

Despite multiple drugs demonstrating efficacy in experimental stroke, none have been translated into clinical use. To help identify and address barriers to translation of animal studies to human clinical trials, the Stroke Therapy Academic Industry Roundtable (STAIR) produced a series of recommendations in 1999, outlined in the table below.[152]

Table 4. Initial STAIR pre-clinical recommendations

1. Establish an adequate dose response curve
2. Define the time-window in a well characterised model
3. Use blinded, physiologically controlled reproducible studies
4. Assess histological and functional outcomes acutely and long-term
5. Initially use rodent studies, then consider gyrencephalic species
6. Assess permanent occlusion models, then transient in most cases

Since these initial recommendations, data explored from 1026 neuroprotective strategies (and 8516 experiments) in a systematic review [153] were analysed using a simple checklist derived from STAIR I and provided an overview of the

quality of the data. 550 drugs were reported to be effective in pre-clinical animal models but only 5 fulfilled the criteria. There was a relationship between increasing study 'quality' score and declining efficacy (i.e. poor quality studies over-estimated efficacy). However, absence of the above recommendations in a pre-clinical experiment does not necessarily mean that it was carried out to a poor standard; these experiments have to explore mechanisms of action and it would be inappropriate to involve each of the components to in order for it to represent a good quality study. The impact of various 'quality' items on efficacy has further been assessed;[154] the presence or absence of randomisation to a treatment group, blinding of drug allocation and blinding of outcome assessments were the most powerful determinants of outcome. Additional recommendations from STAIR in 2009 are described in the table below.[155]

Table 5. Summary of the updated STAIR recommendations

1. Use sample size calculations
2. State inclusion and exclusion criteria
3. Randomisation – explain the methods of allocation into experimental groups
4. Allocation concealment – explain methods of investigator blinding
5. Report animals excluded from analysis
6. Blinding of outcome assessment
7. Report conflicts of interest and study funding

Stem cells for enhancing recovery after stroke

One potentially important area of development in the treatment of stroke is the use of stem cells to enhance recovery. The definition of a 'stem cell' continues to evolve. The current definition requires a cell to demonstrate two properties: the capacity for self-renewal and the ability to differentiate into multiple cell types (potency).[156] A 'progenitor cell' also has these characteristics but with a more restricted potential, i.e. it can only differentiate into a limited number of cell types and has a reduced capacity for self-renewal.[156] Stem cell transplant trials are already underway in conditions such as diabetes, cancer, neurodegenerative disease (e.g. Parkinson's disease) and heart disease.[157-160]

The mechanism by which stem cells may improve recovery is still poorly understood. There are two theories: neuroprotection - preventing damaged neurons undergoing cell death in the acute phase of cerebral ischaemia; and neurorepair - the repair of broken neuronal networks in the chronic phase of cerebral ischaemia. It seems probable that stem cells promote recovery with elements of both neuroprotection and neurorepair.

Reduced apoptosis and inflammation

An intervention that reduces stroke lesion size, usually by decreasing cell death within the ischaemic penumbra, indicates neuroprotective activity. This has been seen with intravenous administration of haematopoietic stem cells and via intracerebral grafting of human neural stem cells in ischaemic stroke models.[161, 162] However, a reduction in lesion volume is not always observed with transplantation of stem cells (from a variety of sources [163-166]), this perhaps relating to the method and time of administration post stroke.

Neuroprotection may also be achieved by reducing inflammatory responses. It is likely that stem cells play a pivotal role in the regulation of the inflammatory cascade through the production of cytokines and growth factors and it might be expected that a cell transplant would induce or exacerbate inflammation. An exaggerated inflammatory response in the peri-infarct area has been observed in a murine model of stroke treated with G-CSF.[167] Conversely, the use of human cord blood cells in a rat model of stroke had the opposite effect by decreasing the inflammatory infiltrate as indicated by a reduction in the expression of pro-inflammatory cytokines.[168]

Recruitment of endogenous neural progenitor cells (NPC)

Since progenitor cells exist within the brain, and neurones, glia and vascular cells can be renewed, it might be thought that significant neurogenesis would take place after stroke. Although there are a number of rodent models investigating endogenous neurogenesis in cerebrovascular disease,[169-171] few studies have been reported in humans. In one, histological examination was performed in an 84 year old patient who suffered a stroke 1 week prior to death.[172] Using a neural stem cell label, large numbers of neural stem cells, vascular endothelial growth factor-immunopositive cells, and new blood vessels around the region of infarction were identified suggesting the presence of ischaemia induced NPC recruitment and neovascularisation. Other studies have also shown the presence of ischaemia-induced endogenous neurogenesis.[173]

Growth factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are involved in the regulation of brain stem cell division and

differentiation. Augmenting these naturally occurring neurogenic factors through exogenous administration offers a potential treatment for stroke. A recent Japanese study investigated neurotrophic factors released in rat brain tissue following intravenous transplantation of human mesenchymal stem cells (MSCs) one day after middle cerebral artery occlusion (MCAo);[174] compared to controls (iv phosphate buffered saline), treated rats produced increased exogenous (human origin) insulin-like growth factor (IGF-1) and endogenous (rat origin) vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). However, only one molecule has been tested to date in humans; in a phase II/III trial (286 patients) of bFGF, treatment was ineffective, even showing a trend to hazard, and caused leucocytosis and hypotension.[175] The use of treatments targeting a single growth factor pathway in pathophysiological states may be limited, as has been seen with numerous neuroprotectants with unimodal mechanisms of action.[176] Stem cells offer a potential multimodal mechanism of action, including recruitment of endogenous progenitor cells via secretion of growth factors.

Neurogenesis

Whether stem cells can improve recovery by replication and differentiation ('direct neurogenesis'), and thereby replace damaged brain cells and reconstruct neural circuitry, remains unclear, with limited and conflicting evidence. In rats with induced MCAo, transplanted neurospheres survived 4 weeks post transplant with the majority of migrating cells expressing a neuronal phenotype (including doublecortin, β-tubulin and glial fibrillary acid protein [GFAP] markers).[163] Neurospheres (non-adherent spherical clusters of neural stem cells) derived from adult stroke rats can also differentiate into glia (as well as neurons), and can migrate towards the ischaemic lesion.[177] A human neural

stem cell line (CTX0E03, ReNeuron Group plc [178]), derived from human somatic stem cells following genetic modification with an immortalising gene, can differentiate into neurons and astrocytes, and induce significant improvements in both sensorimotor and gross motor function in the rat MCAo model of stroke 6 to 12 weeks post grafting;[179] this cell line has now reached safety trials in human stroke.[180] In mice incapable of developing cells of myeloid and lymphoid lineages, transplanted adult bone marrow cells (BMCs) migrated into the brain and differentiated into cells that expressed neuron specific antigens (NeuN, a nuclear protein that is found exclusively in neurons).[181] In contrast, no evidence of neural-like cells was found in another study of mice transplanted with BMCs.[182] Other groups have also failed to detect differentiation of haematopoietic stem cells into neural tissue.[183] In the positive studies showing cell differentiation into a neural phenotype, only small numbers of cells survived, suggesting that integration into host circuitry is not the only or main mechanism of action to enhance functional recovery. However, the finding that response may depend on the number of cells administered ('dose response') is compatible with beneficial effects being mediated by cell replacement.[184]

Angiogenesis

Neurogenesis alone will not lead to recovery since neurones need nutritional support and new blood vessels will need to develop. Hence, a rich vascular environment mediated by CD34+ cells might enhance subsequent neuronal regeneration. Angiogenesis has been found experimentally, as shown when bone marrow-derived CD34+ cells were administered intravenously to mice 48 hours after stroke induction;[161] accelerated neovascularisation occurred in comparison with mice injected with CD34- cells. Furthermore, administration of an antiangiogenic agent prevented the beneficial effect of CD34+ precursors.[161]

While angiogenesis could be the result of differentiation of haematopoietic stem cells into blood vessel wall cells, an indirect effect is also likely since human CD34+ cells have been shown to secrete numerous angiogenic factors, including vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1).[185]

Plasticity

Neuroplasticity refers to organisational changes in the brain so that individual neurones or networks adapt their function. This theory forms the basis for goal-directed, therapeutic rehabilitation. In stroke, stem cells may augment this process through enhancing various mechanisms of recovery, including sprouting and unmasking, but the evidence remains limited. Sprouting has been seen in experimental stroke following intravenous administration of human bone marrow stromal cells to rats; a 50% reduction in lesion volume was seen in comparison to saline-treated controls.[186] In contrast, treatment with human umbilical cord blood cells did not improve sensorimotor or cognitive outcome in stroke rats in one of the few reported negative studies.[166]

Sources of stem cells for stroke therapy

A number of cell sources for stem cell transplantation are available and these can be categorised in several dimensions: exogenous or endogenous; embryonic, foetal or adult derivation; neural or non-neural origin; and pluripotential (which can divide indefinitely) or multipotential (which usually regenerate their 'own tissue' but have the ability to transdifferentiate into other tissue cell types). However, to date, there is no set of markers that precisely identifies a neural stem cell and distinguishes it from other more limited

progenitors, precursors or differentiated cells. The isolation of true neural stem cells and the regions in which they exist remains under investigation. For example, cells cultured from areas outside the striatal subventricular zone in the post-natal brain demonstrate a more limited capacity for self renewal than well characterised ventricular subependymal neural stem cells.[187] The formal identification of cells as stem cells still requires functional demonstration of multipotency, self-renewal and longevity. Potential stem cell sources are considered below.

Neural progenitor cells (NPCs)

Although a commonly held belief that 'the brain does not regenerate', it is now accepted that spontaneous post-natal (adult) neurogenesis can occur.[188] In both human and animal adult brain there are multiple sites of ongoing neuronal and glial formation. During development human neural stem cells reside in the periventricular regions and cerebral cortex and have been shown to persist into adulthood in a number of sites including the dentate gyrus of the hippocampus, substantia nigra and olfactory bulb.[188-192] Animal models demonstrate that ischaemic stroke is associated with differentiation of cells into neurons phenotypically similar to those lost in the ischaemic lesion,[173] a finding suggesting that the adult brain has capacity for self repair. As observed in rodents, subventricular and hippocampal cells from adult human brain can be expanded *in vitro*, can differentiate into all three neural cell lineages (neuronal, astrocytic and oligodendroglial) [193] and were shown to improve functional recovery when administered intravenously into a rat model of stroke.[194] Conflicting results were been seen when human foetal derived neural stem cells were transplanted into stroke rats;[195] though the cells were shown to migrate

throughout the damaged striatum, cells within the transplant core were undifferentiated and of immature neural lineage. Virtually none of the grafted cells differentiated into astrocytes or oligodendrocytes.

Embryonic and foetal stem cells

Embryonic stem cells are capable of producing large quantities of neural progenitors and, in principle, are pluripotent with unlimited expandability; murine embryonic stem cells have been observed to differentiate into neurons and glial cells when grafted into stroke rats [196, 197] and survive for up to 12 weeks. One potential drawback is that they tend to develop a heterogeneous mix of neural precursors and differentiated neurons or glia as well as residual stem cells and a small percentage of non-neural cells;[198] the challenge here is to direct differentiation into producing a homogenous cell population. Another disadvantage is their potential for malignant transformation. For example, when undifferentiated murine embryonic stem cells were xenotransplanted into a rat model of stroke, neuronal differentiation was observed; when the same cells were transplanted into the homologous mouse brain, the cells did not migrate and they produced a highly malignant teratocarcinoma.[199]

Sufferers of Parkinson's disease appear to have benefited from human foetal stem cell transplantation,[200] but these cells are in limited supply and their use is fraught with ethical issues in obtaining human tissue for the purpose of obtaining stem cells. Patients with Parkinson's disease and Huntington's disease also have undergone stem cell transplantation with foetal porcine neurons [201, 202] and this donor source is considered as relatively safe. However, the insertion of foreign cells brings the risk of rejection and the need for chronic

immunosuppression. Furthermore, xenotransplantation may lead to transmission of porcine viruses, such as the porcine endogenous retrovirus, although one study did not find any evidence of this problem.[203]

Immortalised cell lines

Cells derived by either genetic transformation or cultured embryonic and adult tissue offer a ready and unlimited source of cells and thereby remove the ethical concerns of obtaining aborted foetal tissue. For example, LBS-Neurons (Layton Bioscience, Inc) were produced from a NT2/D1 human precursor cell line and were induced to differentiate into neurons by the addition of retinoic acid. This cell line was originally derived from a human testicular tumour more than 20 years ago.[204] The final product gives a neuronal cell population virtually indistinguishable from terminally differentiated post-mitotic neurons.[205] Malignant transformation following therapeutic transplantation of such cells is a key concern for this approach.

Induced pluripotent stem cells

Somatic cells (such as fibroblasts) can be programmed, with the addition of transcription factors, to pluripotent stem (iPS) cells.[206] These cells exhibit the morphology and growth properties of embryonic stem (ES) cells, and like ES cells, they have the potential to form tumours when transplanted into the ischaemic brain;[207] in this particular study, transplanted iPS cells formed much larger tumours in post-ischaemic brain than in sham operated animals. Nonetheless, transplanted iPS cells have been shown to induce functional recovery in the stroke-damaged mouse in a more recent study, without evidence of tumour formation.[208] Improvement was probably due to enhancing

endogenous plasticity (with evidence of increased vascular endothelial growth factor levels) rather than through direct neuronal replacement. Functional neurons can also be generated through reprogramming somatic cells without 'de-differentiating' to pluri-potent stem cells.[209] These induced neuronal (iN) cells may, therefore, eliminate the risk of tumour formation posed by iPS cells. Both cell types offer a potential patient-specific approach to autologous cell transplantation.

Stem cells derived from the blood and bone marrow

Bone marrow stromal cells, umbilical cord blood and peripheral blood stem cells (PBSCs) are alternative sources of stem cell and their use carries minimal ethical unease when transplanted in an autologous manner. Bone marrow and umbilical cord blood are composed of multiple cell types containing haematopoietic and endothelial precursors (CD34+ cells) and non-haematopoietic cells (mesenchymal stromal or CD34- cells). Approximately 10-20% of bone marrow derived stem cells (BMSCs) are multipotent, with the remainder representing more differentiated committed cells.[210]

The CD34 molecule is a cell surface glycoprotein expressed on haematopoietic stem cells (HSCs) and used to facilitate their identification, though CD34-ve HSCs may exist.[211] An ischaemic stroke leads to mobilisation of CD34+ cells (as also seen in myocardial infarction [212]), which occurs in bursts over the first 10 days post stroke;[213, 214] those with higher levels of CD34+ cell mobilisation have a better neurological outcome.[213] Furthermore, levels of stromal derived factor (SDF-1α) and stem cell factor (implicated in stem cell signalling) are correlated with increases in endothelial progenitor cells post ischaemic

stroke.[215] The origin and fate of these cells are not known. Interestingly, circulating CD34+ counts are inversely related to subsequent recurrent cerebral infarction [216] and cardiovascular events.[217] Considering these changes, it is reasonable to hypothesise that promoting the mobilisation of CD34+ cells may be of therapeutic benefit. In one recent study, mononuclear cells were isolated and cultured *ex vivo* from the peripheral blood of 30 acute stroke patients.[218] These 'outgrowth' cells were a heterogeneous population of cells with endothelial and neuronal morphologies; the neuronal outgrowth cells, transplanted 4 days post ischaemia into rat brains, survived (for over 6 months), differentiated into neuronal phenotypes and helped improve functional recovery.

It is contentious as to whether BMSCs can transdifferentiate into neural cells; they can adopt neural characteristics [181, 219, 220] but the cells are atypical (spherical in nature with few processes [221]) and it is argued that transplanted cells spontaneously fuse with recipient cells and subsequently adopt their phenotype.[222] Additionally, murine haematopoietic stem cells demonstrate an age-dependent diminution of self-renewal, increased apoptosis and functional exhaustion under conditions of stress.[223] Nevertheless, BMSCs improved outcome in experimental models of stroke,[164, 224] and cognitive function was seen to be preserved with intravenous transplantation of mesenchymal stem cells into rat models of MCAo.[225]

The stem cell marker CD133, a transmembrane cell surface antigen, is specifically expressed on 30-75% of CD34+ cells and has potential benefit for stem cell transplantation since they are less differentiated. Behavioural and neurological improvement has been demonstrated with intravenous infusion of

CD133+ cells in stroke rats;[226] however, in this particular study,[226] behavioural improvement was only apparent using intravenous transplantation within 1 hour of the stroke (compared to delivery at 3 days post stroke) and reduction of cerebral infarct size was only seen when the cells were transplanted intracerebrally.

Bone marrow derived stem cell trials have progressed further in cardiac patients. Preliminary safety studies have evaluated stem cell use in both acute and chronic ischaemic heart disease but data are limited.[227] In a recent Cochrane Review of 33 randomised controlled trials (1765 participants),[228] stem/progenitor cell treatment of acute myocardial infarction improved left ventricular function, which was maintained in the long-term, but there was no effect on morbidity and mortality. There was significant clinical heterogeneity and further work addressing optimum cell type, cell dose and administration time are still required before large scale clinical trials are commenced.

Mobilisation of stem cells

Production of stem cells derived from bone marrow is stimulated by hormones called colony stimulating factors (CSFs). Stem cell factor (SCF) regulates differentiation of CD34+ stem cells; granulocyte-colony stimulating factor (G-CSF) – neutrophils; erythropoietin (EPO) – red blood cells; granulocyte-macrophage-colony stimulating factor (GM-CSF) – macrophages and neutrophils; macrophage-colony stimulating factor (M-CSF or CSF-1) – monocytes; and thrombopoietin (TPO) – platelets. SCF, G-CSF, EPO, GM-CSF and M-CSF have all been evaluated in pre-clinical models of ischaemic stroke; [229-233] studies using G-CSF and EPO have advanced to human stroke trials.

Intentional recruitment of haematopoietic CD34+ stem cells from bone marrow to peripheral blood with G-CSF is a clinical process termed peripheral blood stem cell (PBSC) mobilisation. Although the mechanism is poorly understood, G-CSF alone or with chemotherapy is used routinely in clinical practise to reduce the duration of neutropenia in patients with haematological disease, or for mobilising and harvesting PBSCs for subsequent autologous or allogenic infusion. Its use in stroke is novel and under investigation in both animals and humans. For therapeutic purposes, autologous marrow stem cells could be obtained but this is unattractive in view of the need for multiple marrow punctures.

The mechanisms of action of G-CSF are probably multimodal. Work on the effects of G-CSF revealed that neurons and adult neural stem cells express a G-CSF receptor and its expression is induced by ischaemia.[137] It is thought that the neuroprotective effect of G-CSF is mediated by anti-apoptotic activity via up-regulation of Stat 3 (signal transducer and activator of transcription 3) and the JAK/STAT signaling pathway.[234] Pre-clinical studies also show that G-CSF may have an anti-inflammatory role as it causes suppression of inducible nitric oxide synthase (iNOS) [234] and other inflammatory mediators such as interleukin-1 beta.[138] If G-CSF is administered subacutely then mechanisms of recovery are more likely to relate to PBSC cell migration, enhancing neurorepair via angiogenesis and neurogenesis. Despite the paucity of preclinical studies addressing this time-window of drug administration, G-CSF has been shown to be effective in promoting long-term functional recovery at various time points up to one week post stroke.[235, 236] These mechanisms should be distinguished from stem cell trials assessing direct intracerebral implantation since it is not clear

that mobilised PBSCs target, migrate and remain in the stroke lesion, although it has been observed in animal models following intravenous administration of CD34 cells 48 hours post-stroke.[161]

In rodent models of ischaemic stroke, a number of groups have demonstrated G-CSF to be neuroprotective at various doses,[237] in the presence of thrombolysis, [238, 239] induce functional recovery [231, 240] and promote angiogenesis [235, 240, 241] and neurogenesis.[137, 240, 241] All have illustrated that G-CSF causes a reduction in stroke lesion volume. However, G-CSF can also lead to impaired behavioural function [167] and may have no benefit when given in the chronic phase of stroke [242] or in global ischaemic models.[243] Interestingly, G-CSF deficient mice have larger infarcts and these effects are reversed by G-CSF administration.[244] G-CSF induced improvements in functional outcome appeared to be preserved in aged stroke rats.[245] No deleterious effects were seen with co-administration of thrombolysis in one study.[246] Although G-CSF increased total leukocyte counts, there was no evidence of neutrophils infiltrating the stroke lesion, important since this might lead to microvessel occlusion and the release of free radicals and interleukins.[237] G-CSF improved sensorimotor recovery in a rat model of cerebral haemorrhage [247] and, in vitro can protect human cerebral neurons from ischaemia.[248] G-CSF is a candidate treatment for stroke [249] and is being assessed in phase III clinical trials.

SCF is a haemopoietic cytokine produced by bone marrow (BM) stromal cells. It has limited clonogenic activity when used alone but augments the other colony stimulating factors, including G-CSF.[250] When administered following middle cerebral artery occlusion in pre-clinical studies, SCF induced neuroproliferation

and neurogenesis,[229] the former by a combination of inducing migration of BM derived cells to the peri-infarct area and stimulating proliferation of intrinsic neural progenitor cells.[251] Alone and in combination with G-CSF, SCF can reduce infarct size and induce functional improvement in rat models of acute stroke.[252] Similarly, in experimental chronic stroke (3.5 months post ictus), SCF + G-CSF treated rats had an improved outcome and reduced infarct volume when compared to control.[242]

EPO controls red cell production and has been available in recombinant form since 1985; it has been utilised for the treatment of anaemia in patients with end-stage renal disease since 1988. EPO was neuroprotective in experimental stroke [230] and increased functional recovery,[253] effects possibly mediated by inhibiting apoptosis in the penumbra.[254] In a small clinical trial, EPO was safe and well tolerated [255] but in the following phase III trial safety concerns were raised.[256] Derivatives of EPO, which do not alter red cell kinetics but retain their neuroprotective activity, have been developed;[257, 258] clinical studies of these have yet to be reported.

GM-CSF can be used to mobilise stem cells (CD34+) and activate macrophages, and has been shown to induce vascular proliferation. GM-CSF improved collateral flow in patients with coronary artery disease.[259] In stroke patients, baseline GM-CSF levels have been seen to be higher when compared to control, but no correlation has been found with clinical or neurological outcome.[260] Pre-clinical studies have found that GM-CSF stimulates arteriogenesis, improves cerebral blood flow,[261] and protects against haemodynamic injury in a rat model of cerebral artery occlusion.[262] Intra-carotid

injection of GM-CSF increased numbers of activated microglial cells and protected against neuronal apoptosis after transient middle cerebral artery ischaemia in rats;[263] GM-CSF given intravenously crossed the blood-brain barrier and counteracts programmed cell death,[264] and intra-peritoneal administration decreased infarct volume and improved locomotor recovery in rats with ischaemic stroke.[265]

M-CSF (colony stimulating factor-1, CSF-1) selectively stimulates the proliferation and differentiation of monocytes, as well as granulocytes and platelets, indirectly through the generation of interleukin-6 and G-CSF. M-CSF has also been reported to prevent the progression of atherosclerosis. Serum levels of M-CSF were elevated in patients with previous cerebral infarction compared with healthy people.[266] In vitro, M-CSF is neuroprotective, inhibiting excitotoxic neuronal apoptosis.[233] In mice, recombinant M-CSF increased neuronal survival and reduced the size of the cerebral infarct.[267]

Development of stroke trials using stem cells

Although many studies assessing stem cells in pre-clinical stroke have been reported, and limited clinical trial data are now available, many questions remain unanswered. It is also important to recognise the lack of published studies reporting a negative or neutral outcome. This publication bias can perpetuate the development of inappropriate clinical trials that will not answer the uncertainties that remain in this field.[268] It is vital that future experimental studies are of high quality (for example, conforming to the STAIR criteria [269]) and have standardised protocols and outcome measures so that they can be fairly compared.

One possible advantage of treating stroke with stem cells is their potentially wide therapeutic window. The optimal time of administration post stroke may relate to the micro-environment of the damaged area, i.e. should stem cells be administered during the acute phase of stroke (exerting a neuroprotective effect) at a time when inflammatory responses are maximal or will delayed treatment be effective at a time when scar tissue has formed? The inflammatory response can last up to several weeks and it may be that stem cells could exacerbate or attenuate this process. It may be that stem cells will work by directly replacing damaged neurons and supporting cells, or, more likely, by stimulation of endogenous regeneration through secretion of growth factors. It may therefore be of benefit to transplant cells when the initial inflammatory response has settled giving the cells an opportunity to survive and integrate with the host tissue. A recent study, evaluating aged stroke rats (24 months old) with distal MCA occlusion,[270] transplanted human-embryonic stem cell derived neural precursor cells intracerebrally as late as 3 weeks post infarct and still induced a reduction in infarct volume and an improvement in functional outcome. Another study assessed human neural stem cells transplanted as neurospheres into a rat model of ischaemic stroke at various time points post-ictus;[271] cells transplanted 48 hours after stroke demonstrated better survival than those grafted at 6 weeks, although the delay did not appear to cause any difference in magnitude of cell proliferation, differentiation and migration (it is not clear, however, that increased cell survival translates into functional benefit). Further preclinical stroke studies designed specifically to address timing of administration of stem cells are therefore required.

The ideal route for stem cell delivery is also unclear. One pre-clinical study compared intra-striatal, intra-ventricular and intravenous administration of neural precursor cells to rats with cerebral ischaemia. All routes of delivery were associated with cells targeting the lesion.[272] Other studies have found benefit from intravenous delivery of stem cells;[273] in a rodent MCAo model, intravenous administration of human umbilical tissue-derived cells improved neurological severity scores when compared to control at doses $\geq$ 3 x10^6 cells/injection up to 30 days post stroke. In contrast, a study assessing intravenous administration of human umbilical cord cells in stroke rats did not detect any evidence of stem cells in the target lesion.[274] Furthermore, concern has been raised with intra-arterial administration of cells leading to an increased risk of vascular events, potentially caused by clumping of cells and micro-emboli formation. The STEPS (Stem Cell Therapy as an Emerging Paradigm for Stroke) reports have called for more research into this area.[275, 276] If intracerebral administration is the most efficient route [272] (though probably the most hazardous) then should cells be transplanted directly into the ischaemic lesion or distant to it (reducing the chance of damaging vital structures) and relying on spontaneous stem cell migration?[163, 277, 278] Existing human safety trials used intracerebral injections of stem cells directly into the peri-infarct area,[279, 280] a decision appearing not to be based on any substantial pre-clinical work. The role of immunosupression in such exogenous transplants also remains unclear.

Relatively few studies have assessed the effects of stem cells in intracerebral haemorrhage (ICH). A reduction in inflammation was observed in one study using intravenous human neural stem cells in experimental ICH;[281] the majority of the transplanted cells were detected in the marginal zones of the spleen with

only very few seen in the brain sections. Adipose derived stem cells (ASCs) have also been trialled in experimental ICH,[282] and again, a reduction in the inflammatory response was seen but ASCs did not reveal any evidence of neuronal transdifferentiation. In contrast, allogenic bone marrow stromal cells (BMSC) transplanted into female rats subjected to ICH expressed neuronal phenotypes;[283] the rats receiving BMSCs 2 months post-ICH improved neurologically compared to rats receiving saline only. Further work is needed to explore the role of stem cells in treating human ICH.

Clinical trials of stem cell transplantation after stroke

There have been no large-scale clinical trials of stem cell transplantation in stroke. However, a number of safety studies have been reported (table 6).

In one non-controlled study, cultured neuronal cells, derived from an immortalised cell-line (LBS-Neurons), were transplanted into 12 patients (age 44-75) with stroke (primarily involving basal ganglia).[280] Patients received one of 2 doses of cells via CT-guided, stereotactic-targeting of cell implants; immunosuppression was achieved with intravenous methylprednisolone (during surgery) and then cyclosporine for 8 weeks. Although functional outcome could not be assessed since the study was too small and had no control group, positron emission tomography (PET) in 6 patients showed increased metabolic activity, suggesting either cell viability or the presence of inflammatory cells.[280]

Table 6. Summary of observational and randomised controlled trials involving stem cells after stroke

Author, year	Cells	Patients (active / control)	Stroke type	Administration	Immunosuppression	Comments
Kondziolka 2000 [280]	Immortalised neuronal	12 / 0	Basal ganglia infarct	Stereotactic transplantation into region of the stroke	Methylprednisolone during surgery. Cyclosporine 1 week prior to surgery and continued for 8 weeks	No effect on functional outcome. PET showed increased metabolic activity.
Kondziolka 2005 [279]	Immortalised neuronal	14 / 4	Basal ganglia infarct	Stereotactic transplantation into region of the stroke	Methylprednisolone during surgery. Cyclosporine 1 week prior to surgery and continued for 6 months	No effect on functional outcome.
Savitz 2005 [284]	Foetal porcine	5 (of planned 12) / 0	MCA infarct	Stereotactic transplantation into region of the stroke	None (cells pre-treated with anti-MHC antibody)	Study stopped early after 2 SAEs
Bang 2005 [285]	Autologous, mesenchymal	5 / 25	MCA infarct	Intravenous	None	Questionable study quality, e.g. 10 patients lost to follow-up
Rabinovich 2005 [286]	Human foetal	10 / 11	Ischaemic and haemorrhagic stroke	Intra-thecal	None	Improvement in 'quality of life' in treated group. Poor quality report
Suarez-Monteagudo 2009 [287]	Autologous, mesenchymal	5 / 0	Ischaemic and haemorrhagic stroke	Stereotactic transplantation into region of the stroke	None	Open label. No adverse events. Timing of implant unclear
Lee 2010 [288]	Autologous, mesenchymal	16 / 36	MCA infarct	Intravenous	None	Open label, observer blinded.
Honmou 2011 [289]	Autologous, mesenchymal	12 / 0	MCA infarct	Intravenous	None	Open label. No adverse events

PET, positron emission tomograpgy; MCA Middle Cerebral Artery; SAE Serious Adverse Event; BI Barthel Index

Interestingly, detailed histopathological study of the brain from one of the study patients who died at 18 months after cell implantation identified survival of transplanted neuronal cells with no evidence of malignancy.[290] The potential for de-differentiation of transplanted cells back to a malignant state is a theoretical concern with the use of transformed stem cells derived from tumours, although pre-clinical studies in mice with this cell line (NT2) have demonstrated no toxicity or tumourgenicity.[291]

Subsequent to this study, the same researchers performed a phase two randomised trial, using the same cell line, in 18 patients.[279] Patients were between 18 and 75 years and had a fixed motor deficit that was stable for at least 2 months. The treated group (n=14) received one of two doses of implanted cell and a rehabilitation programme; the control group (n=4) had rehabilitation alone. The primary outcome measure, the change in the European Stroke Scale motor score at 6 months, did not differ between the two groups.[279]

A safety and feasibility study of the use of foetal porcine cells in patients 1.5-10 years after a middle cerebral artery infarct (affecting the striatum) has also been performed.[284] To prevent rejection, cells were pre-treated with anti-MHC1 antibody and no immunosuppressive drugs were given to the patients. Of the 12 planned patients, only 5 patients entered the study following which the US Federal Drug Administration halted the trial prematurely as 2 patients developed serious adverse events. One patient suffered a cortical vein occlusion secondary to the surgical procedure, and the other experienced generalised and partial seizures while hyperglycaemic. Such observations

highlight the potential risks with intracranial transplantation of stem cells and suggest that other modes of administration should be considered.

One such alternative is to use autologous stem cells intravenously. A recent randomised controlled trial tested mesenchymal stem cells (MSCs) in patients with middle cerebral artery territory infarction.[285] 5 patients with persistent neurological deficit 7 days after their ischaemic stroke were allocated to receive intravenous MSCs and 25 patients acted as controls (no additional intervention and no sham procedure). MSCs were acquired from bone marrow aspirates and then cultured *ex vivo* to obtain sufficient quantities before re-injection into each patient 4-6 and then 7-9 weeks after symptom onset. At 1 year, 10 patients in the control group were lost to follow-up but no serious adverse events such as infection or tumour formation were reported. The study has demonstrated feasibility in administering *ex-vivo* cultured MSCs but the authors were heavily criticised for both its methods and conclusions reached [292] and have since issued an apology for having plagiarised sections of their discussion.[293] More recently, the same authors have completed a long-term randomised controlled observer-blinded safety trial using the same cell isolation and administration methods in 16 patients with MCA infarct (day 7 NIHSS $\geq$ 7) compared to 36 controls.[288] Mean follow up was 118 weeks during which time there were no significant concerns regarding tumour formation or adverse events related to cell delivery, and improved survival and functional outcome in the MSC treated group. Autologous bone marrow stem cells have also been assessed in an open study of 5 patients where cells were stereotactically implanted intraparenchymally [287] and in a further trial of 12 patients receiving 1 $x10^8$ cells

intravenously 36-133 days post ischaemic stroke;[289] again, no significant adverse events related to cell delivery or tumour formation occurred in either.

Yet another potential route of cell administration was reported in a Russian study, in which 2 $x10^8$ human foetal cells obtained from spontaneous or prostaglandin-induced abortions were injected intra-thecally into 10 patients with ischaemic or haemorrhagic stroke.[286] A significant improvement in 'quality of life' (as measured by a Karnofsky score) in the treatment group was reported. No significant adverse events were described although "some patients developed meningism and fever" during the 48 hours post transplantation.[286] Unfortunately, the quality of the publication is questionable as important details are missing including the timing of administration of stem cells and a description of adverse events.

G-CSF in clinical stroke

Five small, randomised controlled trials have assessed the safety of recombinant G-CSF after ischaemic stroke (table 7). A recently completed phase IIa dose-escalation trial assessed G-CSF in 36 patients (G-CSF, n=24; placebo, n=12) with sub-acute ischaemic stroke.[294] Patients were treated between 7-30 days post ictus with increasing doses of G-CSF (1-10μg/kg given either once or daily for 5 days) given subcutaneously. G-CSF increased circulating CD34+ counts 10-fold (as measured by flow cytometry) in a dose-dependent manner and with the peak level occurring at day 5.

Table 7. Summary of human clinical trials in G-CSF and ischaemic stroke

Author / year	Trial Design	G-CSF Regimen	Time after stroke	Patients (active / control)	Comments
Shyu 2006 [295]	Single-blind controlled	15μg/kg/day s/c For 5 days	Within 7 days	7 / 3	No thrombotic complications. Improved outcome in G-CSF group but a majority of lacunar strokes
Sprigg 2006 [294]	Double-blind placebo-controlled	Dose escalation 1-10μg/kg s/c For 1 or 5 days	7-30 days post ictus	24 / 12	No difference in SAEs although non-significant increase in infection rates in active group
Zhang 2006 [296]	Double-blind placebo-controlled	2 μg/kg/day s/c For 5 days	Within 7 days	15 / 45	No difference in adverse events reported. Significant reduction in NIHSS
Shabitz 2010 [297]	Double-blind placebo-controlled	Dose escalation 30-180μg/kg iv Over 3 days	Within 12 hours	30 / 14	No difference in adverse events reported
Floel 2011 [298]	Double-blind placebo-controlled	10μg/kg s/c For 10 days	>4 months	21 / 20	Feasible and tolerated. No effect on efficacy

SAE serious adverse event; s/c subcutaneous; iv; intravenous; NIHSS National Institutes of Health Stroke Scale

A dose-dependent increase in white cell count at day 3 and decrease in platelet count also occurred (expected effects of G-CSF). Serious adverse event rates did not differ, although the frequency of infection was non-significantly higher in G-CSF treated patients (29% vs. 25%); stroke recurrence rates did not differ between the treatment groups.

In a second trial, 10 patients with a NIHSS score between 9 and 20 and within 7 days of stroke onset were randomised to 5 days of G-CSF (15 μg/kg per day, n=7) or control (routine care, n=3).[295] Over a 12-month follow-up, patients who received G-CSF had a significantly greater improvement in neurological function and less disability than those in the control group. There was no aggravation of stroke symptoms or thrombotic complications. Unfortunately, the results are confounded since many patients receiving G-CSF had a lacunar infarction, a type of stroke that often improves spontaneously. In a third trial, 15 patients were randomized within 1 week of stroke onset to receive G-CSF and 30 to placebo [296]. A non-significant reduction in NIHSS was noted at day 10, which became significant at day 20.

In a Cochrane systematic review (2006) of these trials of G-CSF (as well as other colony stimulating factors) for stroke, G-CSF did not significantly alter functional outcome although there was a trend to reduced impairment.[299] Furthermore, G-CSF was well tolerated and appeared to be safe, and significantly increased white cell count.[299] More recently, the AXIS (AX200 for the treatment of Ischemic Stroke) trial has assessed hyperacute intravenous administration of G-CSF in 44 patients, using doses between 30μg/kg and 180μg/kg over 3 days within 12 hours of ischaemic stroke in the middle cerebral artery.[297] G-CSF was well tolerated and there were no reported concerns

regarding safety even at the higher doses. Similarly, no safety issues were raised in a randomised placebo controlled trial of G-CSF (10 μg/kg subcutaneously od for 10 days) in 41 patients (21 G-CSF, 20 placebo) with chronic stroke (4 months post ictus).[298] The primary efficacy outcome, hand motor function, was not affected by treatment.

The long term safety of G-CSF has been monitored in 101 normal donors who received G-CSF for the purpose of mobilising PBSCs prior to allogenic transplantation; there were no obvious adverse effects over a 3-6 year follow-up period and, in particular, the rates of cancer and vascular disease were not increased.[300] Nevertheless, G-CSF may cause a hypercoagulable state,[301] a potential mechanism for increasing the risk of recurrent stroke. In healthy donors receiving G-CSF, *in vitro* bleeding time is decreased with increased levels of Factor VIII and fibrinogen, and reduced protein C and S activity.[301]

Further trials of G-CSF are underway, including a phase III efficacy trial (hyperacute),[302] a safety trial of acute administration (within 48 hours, n=20),[303] and STEMS-2, a phase IIb randomised placebo-controlled trial of subacute treatment with G-CSF (n=60),[304] described in full detail in subsequent chapters. Data from completed and ongoing trials will suggest whether larger phase III trials of G-CSF in subacute stroke are warranted. Although other colony stimulating factors such as stem cell factor (SCF), granulocyte-macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF or CSF-1) also mobilise bone marrow-derived stem cells (and recombinant forms are available), trials in patients with stroke have not been reported.[299]

Outcome Measures

There is no single accepted outcome measure after stroke that encompasses assessment of all its potential consequences. Several tools are therefore required to measure multiple outcomes including motor and sensory function, vision, language, cognition, affect and function. This lack of consensus regarding the best choice of primary outcome measure, which affects the design, interpretation and comparison of all stroke trials.[305] A number of measures used in subsequent chapters are discussed below.

An outcome measure needs to be reliable, reproducible, standardised, valid (measure what it intends to measure) and sensitive. Examples of such tools includes the National Institutes of Health Stroke Scale (NIHSS) [306] and Scandinavian Neurological Stroke Score (SNSS or SSS) [307] which measure specific neurological deficits. They are reproducible, easy and quick to perform and are useful in predicting outcome.[307, 308]

The NIHSS is a 15-item scale providing key components of the neurological examination (table 8). It offers quick and accurate assessments of patients with stroke and can be implemented by all types of health care provider.[306] Video and online training is available, important for standardisation for multi-centre clinical trials. Both intra- and inter-observer reliability are excellent [309] and it yields high correlation coefficients with infarct volume (one measure of validity).[310] However, for equivalent NIHSS scores, stroke volume in the right hemisphere may be larger when compared to the left hemisphere.[311] The scoring system also favours the left hemisphere. Of a total of 42 points, 7 are dedicated to language function, whilst there is only a maximum of 2 for neglect. Another pitfall is the lack of detailed assessment of the cranial nerves – patients

with brainstem or cerebellar infarcts can be quite disabled yet produce a relatively low NIHSS score. Nonetheless, baseline NIHSS scores strongly predict outcome at both 7 days and at 3 months.[312]

Table 8. The National Institutes of Health Stroke Scale

1a Level of consciousness	**1b Questions**	**1c Commands**
0=Alert	0=Answers both correctly	0=Performs both tasks correctly
1=Not alert, arousable	1=Answers one correctly	1=Performs one task correctly
2=Not alert, obtunded	2=Answers neither correctly	2=Performs neither task
3=Unresponsive		
2 Gaze	**3 Visual fields**	**4 Facial palsy**
0=Normal	0=No visual loss	0=Normal
1=Partial gaze palsy	1=Partial hemianopia	1=Minor paralysis
2=Total gaze palsy	2=Complete hemianopia	2=Partial paralysis
	3=Bilateral hemianopia	3=Complete paralysis
5a Left motor arm	**5b Right motor arm**	**6a Left motor leg**
0=No drift	0=No drift	0=No drift
1=Drift before 10 s	1=Drift before 10 s	1=Drift before 5 s
2=Falls before 10 s	2=Falls before 10 s	2=Falls before 5 s
3=No effort against gravity	3=No effort against gravity	3=No effort against gravity
4=No movement	4=No movement	4=No movement
6b Right motor leg	**7 Ataxia**	**8 Sensory**
0=No drift	0=Absent	0=Normal
1=Drift before 5 s	1=One limb	1=Mild loss
2=Falls before 5 s	2=Two limbs	2=Severe loss
3=No effort against gravity		
4=No movement		
9 Language	**10 Dysarthria**	**11 Extinction**
0=Normal	0=Normal	0=Normal
1=Mild aphasia	1=Mild	1=Mild
2=Severe aphasia	2=Severe	2=Severe
3=Mute or global aphasia		

The Scandinavian Stroke Scale was developed following a trial of haemodilution in ischaemic stroke.[313] The maximum score is 48 (table 9), and unlike the NIHSS, the higher the score, the better the outcome. Scores correlate well with 3-month outcome.[307] Inter-observer agreement is very good but disadvantages include the absence of assessing neglect and it was initially intended for use in conscious patients.[313] Motor power is assessed on the affected side only.

Table 9. The Scandinavian Stroke Scale

Consciousness	**Eye movement**:	**Speech**:
6=fully conscious	4=no gaze palsy	10=no aphasia
4=somnolent, can be awaked to full consciousness	2=gaze palsy present	6=limited vocabulary or incoherent speech
2=reacts to verbal command, but is not fully conscious	0=conjugate eye deviation	3=more than yes/no, but not longer sentences
0=unconscious		0=only yes/no or less
Arm, motor power	**Leg, motor power**	**Hand, motor power**
6=raises arm with normal strength	6=normal strength	6=normal strength
5=raises arm with reduced strength	5=raises straight leg with reduced strength	4=reduced strength
4=raises arm with flexion in elbow	4=raises leg with flexion of knee	2=some movement, fingertips do not reach palm
2=can move, but not against gravity	2=can move, but not against gravity	0=paralysis
0=paralysis	0=paralysis	
Gait	**Orientation**	**Facial palsy**:
12=walks 5 m without aids	6=correct for time, place and person	2=none/dubious
9=walks with aids	4=two of these	0=present
6=walks with help of another person	2=one of these	
3=sits without support	0= completely disorientated	
0=bedridden/wheelchair		

The Barthel Index (BI) assesses activities of daily living (ADLs) and is a commonly used, validated, primary outcome measure in stroke trials.[314] It is a 10-item scale assessing feeding, bathing, personal grooming, bowel and bladder control, toileting, transfers, ambulation and stair climbing.

Table 10. The Barthel Index

Bowels	**Bladder**	**Transfer**
0=Incontinent (or needs to be given enema)	0=Incontinent, or catheterised and unable to manage	0=Unable, no sitting balance
5=Occasional accident (once/week)	5=Occasional accident (max once per 24 h)	5=Major help (one or two people, physical), can sit
10=Continent	10=Continent	10=Minor help (verbal or physical)
		15=Independent
Toilet use	**Feeding**	**Stairs**
0=Dependent	0=Unable	0=Unable
5=Needs some help, but can do something alone	5=Needs help cutting, spreading butter, etc	5=Needs help (verbal, physical, carrying aid)
10=Independent (on and off, dressing, wiping)	10=Independent (food provided in reach)	10=Independent up and down
Mobility	**Dressing**	**Grooming**
0=Immobile	0=Dependent	0=Needs help with personal care
5=Wheelchair independent, including corners, etc.	5=Needs help, but can do about half unaided	5=Independent face/hair/teeth/shaving (implements provided)
10=Walks with help of one person (verbal or physical)	10=Independent (including buttons, zips, laces, etc)	**Bathing**
15=Independent (but may use any aid—eg, stick)		0=Dependent
		5=Independent (or in shower)

Again, it is simple to use but it does not help to discriminate between patients at the upper end of the scale; that is a maximum score can be reached before functional improvement has stopped (a 'ceiling' effect). This can be overcome with more detailed functional assessments such as the Nottingham Extended Activities of Daily Living (NEADL), which is validated and sensitive to an effective therapeutic intervention.[315, 316] The BI can also be susceptible to the

'floor effect' in the setting of acute stroke – patients with minor stroke are often bed-bound in the first few hours and will score very low on the BI. In addition, many aspects of functional living are not included in the BI, such as language, vision and emotional impairment. For example, a patient with severe aphasia may sore a maximum BI but is unable to function at home without assistance.

The modified Rankin Score (mRS) [317] is now the commonest assessment tool used in stroke trials and is a measure of disability and dependency on a 7 level ordinal scale ranging from no symptoms to death (shown below).

Table 11. The modified Rankin scale

0=no symptoms	
1=no significant disability, despite symptoms	Able to perform all usual duties and activities
2=slight disability	Unable to perform all previous activities but able to look after own affairs without assistance
3=moderate disability	Requires some help, but able to walk without assistance
4=moderately severe disability	Unable to walk without assistance and unable to attend to own bodily needs without assistance
5=severe disability	Bedridden, incontinent, and requires constant nursing care and attention
6=dead	

Its simplicity allows it to be applied to multi-centre trials with consistent results. Compared to the Barthel Index, the mRS is a better instrument for differentiating between changes in mild-moderate disability; it may also reflect disability from an emotional context.[318] However, it is subject to 'interpretation variability',[319] especially in the middle of the scale, which could potentially lead to reporting negative or neutral trials that are actually positive. Training

programmes (DVD or internet based) and centralised adjudication are strategies that may reduce interpretation variability. A structured interview can also improve reliability, with agreement in 81% of cases.[320] Attempts to measure the mRS by telephone will significantly reduce reliability and is not recommended.[321] Other disadvantages include the scale's lack of specificity, not including domains such as cognition, vision and language, but also the source of disability. If a patient is bed-bound due to a hip fracture, they will score highly on the mRS even if their stroke is minor.

The incidence of dementia is high after ischaemic stroke [322] and the Mini-mental State Examination (MMSE) is a 30 point questionnaire assessing orientation, memory, language, attention, and construction. It is a valid tool and correlated with educational level.[323, 324] Depression is also common after stroke [325] and is often difficult to distinguish from dementia. Various tools can be utilised to measure its severity including the Geriatric Depression Scale [326] and the Zung depression scale.[327] These scales are not stroke specific and suffer when patients with aphasia or visual disturbance are included. More specific depression assessment tools for patients with aphasia have also been developed; the SADQ-H10 (10-item Hospital version of the Stroke Aphasic Depression Questionnaire is valid and reliable in measuring depressive symptoms in stroke patients with aphasia.[328]

Systematic review and meta-analysis methodology

Three of the subsequent chapters are based on systematic review and meta-analysis and a review of its methodology is described.

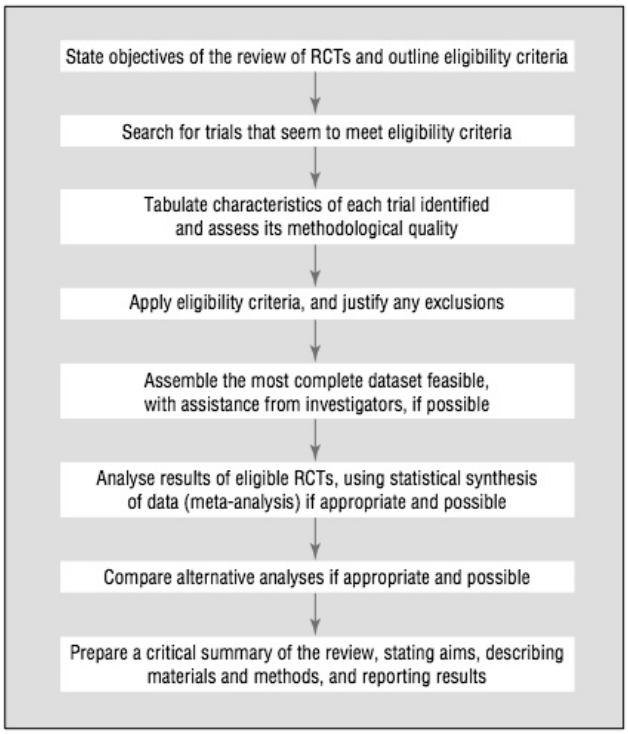
Traditional review articles summarise the medical literature in a particular field, forming a narrative of the existing evidence. This approach is subject to a biased interpretation since the overview of the primary studies is unlikely to have been approached in a thorough, objective and standardised way. A systematic review attempts to reduce bias and offers the following advantages:[329]

- Explicit methods reduce bias in study identification.
- More reliable and accurate conclusions.
- Accumulation and summary of large amounts of information.
- May reduce the delay between identification of an effective strategy and its implementation.
- In studies of generalisable results (lack of heterogeneity), findings can be formally compared.
- Identification of reasons for heterogeneity
- Subgroup hypothesis generation
- Quantitative assessment (meta-analysis) can increase the precision of the result

Systematic reviews can be performed on any important clinical question, which must be defined precisely, so papers identified following a literature search can be explicitly included or excluded. The figure below, derived from the Cochrane

Collaboration Handbook (http://www.cochrane-handbook.org), identifies the methods for a systematic review of randomised controlled trials.

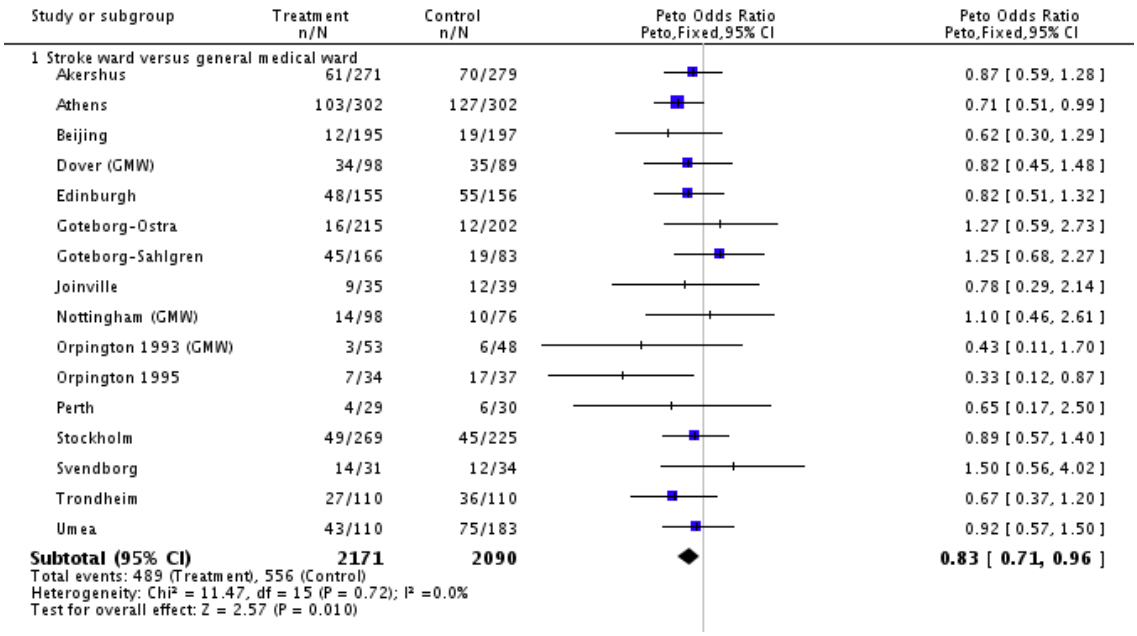
Figure 6. Methodology for systematic review of randomised controlled trials [329]



The search clearly needs to be comprehensive, and attempts made to identify studies that may be missed by standard database search engines, including reviewing references of published articles, searching conference proceedings and 'grey literature' (e.g. pharmaceutical industry files), and attaining unpublished sources of information via personal communication. Once selected, the quality of the studies should be assessed. Various systems can be used, for example the Jadad scoring system for RCTs, which provides an overall score for evidence of randomisation and blinding (and their methods) and how data is managed following participant withdrawals.[330] Cochrane review methods also take into account evidence of 'reporting bias' (evidence of selective outcome reporting).

Comparable data from selected studies can be extracted, tabulated, synthesised and meta-analysed. A pictorial representation of this data is a 'forest plot', an example of which is shown in figure 7. The data are comparing the effects of stroke ward care versus a general ward on stroke patient outcome (death by the end of scheduled follow up). The horizontal lines represent each trial, the square in the middle is the point estimate of the difference between the groups, and the width of the line is the 95% confidence interval of the estimate. If the confidence interval (CI) crosses the 'line of no effect' (the vertical line down the middle) then either there is no difference between groups or the sample size is too small to be confident of where the true result lies. The diamond at the bottom of the plot represents the pooled data of all of the trials and will have a narrower CI. In this particular example, data from the pooled trials shows stroke ward care reduces death, odds ratio 0.83 (95% CI 0.71-0.96); this translates to a number needed to treat (NNT) of 25.

Figure 7. Example of a forest plot. Effects of stroke ward care versus general ward care on death (by the end of scheduled follow up).[331]



One of the main problems with meta-analysis is comparing studies of different designs ('comparing apples with pears'), resulting in heterogeneity. Statistical software will measure statistical (not clinical) heterogeneity using the χ^2 and the I^2 tests. χ^2 tests whether there is greater spread of results between studies than is due to chance, and a value of <0.1 usually suggests this. The I^2 test tries to quantify any heterogeneity present, a value of >40% usually suggests this.[332] In the example forest plot above there is no statistical heterogeneity. If it is present, sources of heterogeneity should be sought. Furthermore, if clinical heterogeneity is thought to be present then a 'random-effects' model of analysis should be used; this model assumes that individual studies are estimating different treatment effects and that there is variability of distribution around a central value. In contrast, a fixed-effect model assumes a single common effect of every study with no statistical heterogeneity. Sensitivity analyses can also be performed on meta-analysis results, which tests whether the findings are robust. This can be done by removing certain studies from the analysis (e.g. those of poor quality) and reviewing the effect on the overall result.

An additional factor to take into account is the potential presence of publication bias, i.e. studies that are negative or neutral are less likely to be published. This can be visualised on a funnel plot (or Egger's plot) which plots the precision of the estimate of each study (such as the standard error) on the vertical axis against the treatment effect on the horizontal axis. If there is no publication bias, the studies will be distributed uniformly within a 'V' shape (in the absence of heterogeneity, 95% of the trials should lie within the funnel). If there is bias, funnel plots will appear asymmetrical.[333, 334] Funnel plots can be misleading, however, since the scale of the y-axis will change the appearance of the chart.

AIMS: THE CURRENT INVESTIGATION

The potential application for stem cell therapy is vast and its development for use in stroke is still in its infancy. Trials are also underway evaluating haematopoietic precursors mobilised with G-CSF, an approach offering an autologous means of administrating stem cells for therapeutic purposes. This thesis will concentrate on the use of G-CSF in stroke with the following aims:

1. To systematically analyse the use of granulocyte-colony stimulating factor in experimental stroke and its effects on infarct size and functional outcome**.**
2. To systematically review stem cell labelling with iron based techniques in the context of experimental ischaemic stroke.
3. To perform a pilot randomised placebo-controlled trial assessing the use of granulocyte-colony stimulating factor in mobilising bone marrow stem cells in sub-acute stroke.
4. To update a systematic review assessing the use of colony stimulating factors (including erythropoietin, granulocyte colony stimulating factor and analogues) for stroke.
5. To assess the potential for stem cell migration using paramagnetic labelling of CD34+ haematopoietic cells and their identification using MRI after stroke.
6. To assess CD34+ cell uptake of superparamagnetic iron oxide (SPIO) with and without a transfection agent.
7. To determine the histopathological characteristics of stroke brain post treatment with G-CSF.

CHAPTER 2

GRANULOCYTE-COLONY STIMULATING FACTOR IN EXPERIMENTAL STROKE AND ITS EFFECTS ON INFARCT SIZE AND FUNCTIONAL OUTCOME: A SYSTEMATIC REVIEW.

Publications contributing to this chapter:

England TJ, Gibson CL, Bath PM. Granulocyte-colony stimulating factor in experimental stroke and its effects on infarct size and functional outcome: A systematic review. *Brain Res Rev*. 2009;62(1):71-82

ABSTRACT

Background

Granulocyte-colony stimulating factor (G-CSF) shows promise as a treatment for stroke. This systematic review assesses G-CSF in experimental ischaemic stroke.

Methods

Relevant studies were identified with searches of Medline, Embase and PubMed. Data were extracted on stroke lesion size, neurological outcome and quality, and analysed using Cochrane Review Manager using random effects models; results are expressed as standardised mean difference (SMD) and odds ratio (OR).

Results

Data were included from 19 publications incorporating 666 animals. G-CSF reduced lesion size significantly in transient (SMD -1.63, $p<0.00001$) but not permanent (SMD -1.56, $p=0.11$) focal models of ischaemia. Lesion size was reduced at all doses and with treatment commenced within 4 hours of transient ischaemia. Neurological deficit (SMD -1.37, $p=0.0004$) and limb placement (SMD -1.88, $p=0.003$) improved with G-CSF; however, locomotor activity ($\geq$4 weeks post ischaemia) did not (SMD 0.76, $p=0.35$). Death (OR 0.27, $p<0.0001$) was reduced with G-CSF. Median study quality was 4 (range 0-7/8); Egger's test suggested significant publication bias ($p<0.001$).

Conclusions

G-CSF significantly reduced lesion size in transient but not permanent models of ischaemic stroke. Motor impairment and death were also reduced. Further studies assessing dose-response, administration time, length of ischaemia and long-term functional recovery are needed.

INTRODUCTION

Stroke has enormous consequences both for the individual and society. Finding an effective treatment for this burden is proving challenging and protection of the neurovascular unit [335] might be achieved through enhancing reperfusion, modifying neuronal activity and augmenting neurorepair. Of these, reperfusion is effective with alteplase [336] whilst neuroprotection has not been shown to be effective to date.[88, 176, 337] One neuroprotectant showing promise is recombinant granulocyte colony stimulating factor (G-CSF). Its pharmacological and side effect profile is well known since G-CSF is already licensed for use in other indications in humans.

G-CSF is a 207 amino acid glycoprotein cloned more than 20 years ago.[338] Its recombinant form is usually administered to patients with neutropenia to reduce the risk of sepsis, or to volunteers willing to donate haematopoietic stem cells (mobilised by G-CSF) for allogenic or autologous infusion. Endogenous G-CSF is produced by numerous cell types including monocytes [339] (the most abundant source), fibroblasts,[340] mesothelial and endothelial cells.[341] G-CSF and its receptor are expressed in the penumbral region of ischaemic stroke [137] and recent studies have highlighted its neuroprotectant properties as a possible therapy for cerebrovascular disease. G-CSF also stimulates the release of stem cells from the bone marrow and it could, therefore, also promote neurorepair.[342]

In light of ongoing human clinical trials assessing G-CSF in stroke, the purpose of this chapter is to review systematically the effects of G-CSF in experimental stroke and, in particular, its effect on infarct size, motor impairment and death.

MATERIALS AND METHODS

Experimental (non-human) studies assessing the effects of G-CSF in ischaemic models of stroke (any species, age, sex and model) were sought with searches of Medline, Pubmed and Embase; search keywords included: 'stroke', 'cerebrovascular', 'thrombosis', 'brain', 'cerebral', 'cerebellum', 'middle cerebral artery', 'ischaemia', 'embolism' and 'G-CSF'. Searches were limited to animal studies. The reference lists of included articles and review articles were searched, and abstracts used to select relevant articles. Pre-specified exclusion criteria were used to aid selection and prevent bias and studies were included if the following were met: (i) a focal ischaemic stroke model, not global; (ii) treatment was in the acute/subacute phase i.e. <7 days, not chronic; (iii) no other potential neuroprotectants were administered with G-CSF (i.e. confounded); (iv) measures on infarct size or functional outcome were performed; (v) there was a control group; and (vi) data were from an original article, not a letter or review article. Decisions on study inclusion and exclusion were made by TE and PB.

Data Extraction

Summary data on total infarct size, measured as volume or area (mm^3, percentage of normal brain, or mm^2) were extracted from all eligible papers up to June 2009. If given, infarct volume from total brain, cortex and subcortex was obtained separately. Volumes corrected for oedema were chosen in preference to uncorrected data. Similarly, information on vital status, weight (grams), Rotarod test (time spent on Rotarod expressed in seconds or percentage compared to baseline), motor impairment (low scores indicate a better outcome), foot fault (number of errors and percentage of total errors made),

limb placement test (neurological scores) and locomotion (vertical movement and rearing) were also collected. If published studies used multiple groups (e.g. to assess dose-response relationships) then the number of animals in the control group was divided by the number of comparison groups. When numerical values were not available and contacted authors were unable to provide necessary information, published graphs were enlarged from the article and the size of axes and position of data points estimated using *Grab* (version 1.3.1) on an Apple Mac. Methodological quality was assessed using methods previously described [343, 344] based on an eight-point STAIR criteria with one point given for evidence of each of the following:[152] presence of randomisation, monitoring of temperature (not just maintenance), assessment of effect by G-CSF dose, assessment of effect by time between stroke onset and treatment, masked outcome measurement, assessment of outcome at days 1–3, assessment of outcome at days 7–30, and assessment of outcome other than just lesion size. Two authors (TE and CG) independently assessed methodological quality and data extraction.

Analysis

Data were grouped before analysis by (i) model type (permanent or transient ischaemia); (ii) species; (iii) time to treatment with G-CSF; and (iv) total dose of G-CSF. Data from each of these groups were analysed as forest plots using the Cochrane Review Manager software (version 4.2.10), as used in previous animal meta-analyses.[343, 344] Since heterogeneity was expected between study protocols (different species, stroke models, dose, time), random effect models were used. The results of continuous/ordinal data are expressed as standardised mean difference (SMD), with 95% confidence intervals (CI), which

allows data measured on different scales and in different species to be merged. The results of binary data are expressed as odds ratios (OR) with 95% CI. Studies were weighted by sample size and statistical significance was set at $p<0.05$. Publication bias was assessed by visually examining Begg's funnel plot (standard error of SMD against the SMD); asymmetry in the plot was formally assessed using Egger's test.[333]

RESULTS

Design of Studies

The initial search for studies identified 220 relevant publications (figure 8). Once pre-specified exclusion criteria were applied, a total of 19 publications were chosen for analysis (table 12); these came from 12 laboratories in 8 countries (China, France, Germany, Japan, South Korea, Taiwan, UK and USA). Only one negative study was identified in which G-CSF had a detrimental effect on behavioural function, caused brain atrophy and exaggerated the inflammatory response in the infarcted area.[167] Studies excluded (table 13) were those administering G-CSF in combination with other agents such as stem cell factor (SCF) and those assessing chronic (i.e. >7 days post stroke) and global (transient bilateral common carotid artery occlusion) ischaemia. Of note, no functional improvement was seen with G-CSF in the studies of chronic [242] and global [243] ischaemia; studies co-administering SCF reduced infarct volume in female mice (permanent ischaemia) by 50% [345] and improved functional recovery in acute and subacute phases.[251]

Of the 19 included publications, 14 studied rats, and 5 studied mice; hypertensive (1 of 18) and diabetic (1 of 18) rats were investigated but no studies in primates were identified. Some publications included more than one experimental condition, giving 44 studies in total (table 12). Studies included transient models of ischaemia (n=29) with vessel occlusion time ranging between 45 and 180 minutes. Permanent models were used in 12 studies; and a photothrombotic model, which is less likely to negatively affect survival, was used in 3 studies, each of which only assessed functional outcome.

Figure 8. Summary of trial identification process

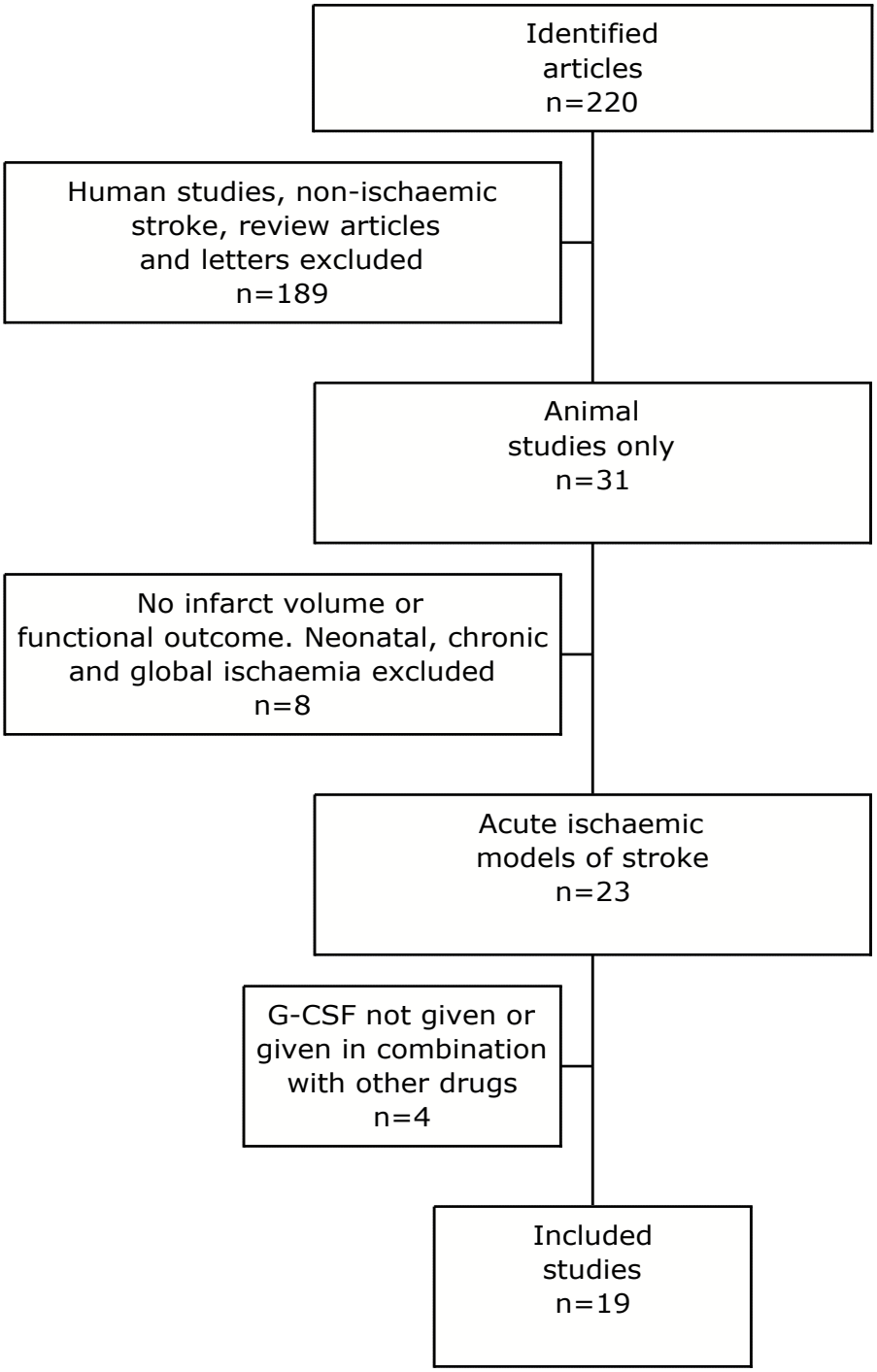


Table 12. Included Studies

Study	Parameters assessed [time of assessment]	G-CSF dose (µg/kg)	Time to Rx (hours)	Species	Model	Route	STAIR score
Gibson 2005a Experiment 1 Experiment 2	Infarct volume (mm^3) [48 hours] Rotarod, foot fault [daily for 7 days], Morris water maze [days 15 to 20]	50	1	Adult male CL57 BL/6 mice	T 60mins	s.c.	6
Gibson 2005b	Infarct volume (mm^3) [48 hours], survival	50	0	Adult male CL57 BL/6 mice	P	s.c.	4
Han 2008 Experiments 1-3	Infarct volume (mm^3) [4, 16 and 24 hours]	60	0.5	Male Wistar rats	T 60mins	i.v.	3
Kobayashi 2006 Experiments 1-2	Infarct volume (mm^3), neurological deficit (0-3 scale) [24 and 72 hours]	50	0.5	Adult male CL57 BL/6 mice	T 60mins	i.v.	4
Lan 2008 Experiment 1-3	Infarct volume (mm^3), NSS (0-18 scale) [days 7, 14 and 21]	50 for 7, 14 and 21 days	0	Male Sprague Dawley diabetic rats	P	s.c.	4
Lee 2005 Experiment 1 Experiments 2-4	Infarct volume (mm^3) [day 1] Rotarod (% of baseline), MLPT (0-7 scale) [1 week before and weekly for 5 weeks post ischaemia]	50 for 3 days	2 2, 24, 96 and 168	Male Sprague Dawley rats	T 90mins	i.p.	7
Schneider 2005 Experiment 1 Experiment 2 Experiment 3	Infarct volume (mm^3), survival Infarct volume (mm^3), Rotarod (seconds and AUC), NSS* (0-16 scale) [72 hours] Rotarod (seconds and AUC), NSS* (0-16 scale) [weekly, 2 to 6 weeks post-ischaemia]	60 50 15 for 5 days	2 1 1	Male Wistar rats	T 90mins T 180mins Photo-thrombotic	i.v.	5
Schneider 2006 Experiment 1 Experiments 2-3	Infarct volume (mm^3) [24 hours] Rotarod (seconds and AUC) [weekly, 1 to 6 weeks post ischaemia]	60 10 for 10 days	4 24 and 72	Male Wistar rats	T 90mins Photo-thrombotic	i.v.	6
Schabitz 2003	Infarct volume (mm^3) [24 hours], survival	60	0.5	Male Wistar rats	T 90mins	i.v.	4

Sehara 2007a Experiment 1 Experiment 2	Infarct volume (mm^3) [72 hours] Neurological deficit (0-3 scale) [24 and 72 hours]	50	1.5	Adult male Wistar rats	T 90mins	s.c.	4
Sehara 2007b	Infarct area (mm^2) [day 7]	50	1.5	Adult male Wistar rats	T 90mins	s.c.	2
Sevimli 2009 Experiment 1 Experiment 2 Experiment 3	Infarct volume (mm^3) [48 hours] Neurological deficit (0-5 scale) [24 and 48 hours] Rotarod (seconds), survival	250 bd for 6 days	-96	G-CSF deficient female C57BL/6 mice	T 45mins	s.c.	5
Shyu 2004 Experiment 1 Experiment 2	Infarct volume (mm^3) [day 7], Body swing test (% recovery) and locomotor activity (vertical movement) [days 1 to 28]	50 for 5 days	24	Adult Sprague Dawley male rats	T 90mins	s.c.	4
Six 2002	Infarct volume (mm^3) [4 days], survival	50	24	Male CL57 BL/6 Mice	T 60mins	s.c.	0
Solaroglu 2006 Experiment 1 Experiment 2 Experiment 3	Infarct volume (mm^3) [24 hours] Infarct volume (mm^3) [72 hours] Neurological deficit [24, 48 and 72 hours]	50 50 for 2 days 50 for 2 days	1.5	Adult male Sprague Dawley rats	T 90mins	s.c.	5
Solaroglu 2009 Experiment 1	Infarct volume (mm^3) [24 hours]	50	1.5	Adult male Sprague Dawley rats	T 90 mins	s.c.	5
Experiment 2	Neurological score (scale 3-18) [24 hours]						
Taguchi 2007 Experiment 1	Infarct area (mm^2) [72 hours]	50 for 3 days	24	CB-17 mice	P	s.c.	6
Experiments 2-3	Locomotor activity (rearing) and neurological deficit [35 days]	0.5, 5, 50 or 250 for 3 days					
Yanqing 2006 Experiments 1-3	Infarct volume (mm^3), survival, NSS (0-18 scale) [7, 14 and 21 days]	10 for 5 days	5	Male Sprague Dawley rats	T 60mins	s.c.	4
Zhao 2007b Experiment 1	Infarct volume (%) [12 weeks]	50 for 7 days	3	Male hypertensive rats	P	s.c.	4
Experiments 2-3	Foot fault, limb placement test [1, 4, 7 and 10 weeks]						

T, transient ischaemia; P, permanent ischaemia; s.c., subcutaneous; i.v., intravenous; i.p., intraperitoneal; NSS, neurological severity score; AUC, area under the curve; MLPT, modified limb placement test

Table 13. Excluded studies

Study	Reason for exclusion and comments.
Toth 2008 [345]	G-CSF given in combination with SCF in a permanent MCAo mouse model. Treatment induced infarct volume reduction and enhanced angiogenesis.
Morita 2007 [346]	Assessed inflammatory marker expression in mice with permanent ischaemia treated with a combination of G-CSF and SCF in acute (1 to 10 days) and subacute (11 to 20 days) phases. No assessment on functional recovery or infarct volume
Zhao 2007a [242]	Treatment given to rats with chronic ischaemia (3.5 months post stroke). No benefit seen in functional outcome in rats given G-CSF alone. SCF alone and in combination with G-CSF improved functional outcome.
Yata 2007 [347] Kim 2008 [348]	Rat models of neonatal hypoxia. No assessments of infarct volume or functional recovery. Treatment with G-CSF improved quantitative brain weight [347] and inhibited apoptosis [348].
Kawada 2006 [251]	G-CSF given in combination with SCF acutely and subacutely. Permanent MCAo model used. Reduction in infarct volume and enhanced functional recovery.
Matchett 2007 [243]	Effect of G-CSF on global cerebral ischaemia. No long-term (2 weeks) protection seen in neurobehavioural studies.
Zhoa 2007b [349]	Observed that G-CSF and SCF pass through an intact blood-brain barrier in intact rats. No stroke induced.
Chen 2005 [350]	Article in Chinese and unable to acquire to translate.
Willing 2003[351]	Transplanted peripheral blood progenitor cells (mobilised by G-CSF) and human umbilical cord-blood derived stem cells in rats 24 hours after permanent MCAo. Compared to control, a reduced stroke-induced hyperactivity was observed in the transplanted animals.
Hokari 2009 [352]	Bone marrow stromal cells (BMSC), pre-treated with G-CSF, transplanted into mouse stroke brain, enhanced motor function earlier than mice treated with 'non-treated' BMSC.
Muller 2009 [353]	Compared neurotransmitter profile in rats subjected to photothrombotic ischaemia treated with either G-CSF or brain derived neurotrophic factor (BDNF). No infarct volumes or functional outcome measures.
Sehara 2007b [354]	In a rat model of transient MCAo, G-CSF enhanced cell proliferation in the dentate gyrus. No measures of infarct volume or functional outcome.

SCF Stem Cell Factor; MCAo middle cerebral artery occlusion

All photothrombotic models used direct illumination of the cortex after sensitisation with the dye rose bengal to produce a focal infarct. G-CSF was given via various routes (subcutaneously n=30, intravenously n=12, and intra-peritoneum n=4) and at various dose regimens (total dose range 50 to 3000 mcg/kg) and time from onset of ischaemia (range from -96 hours to 1 week with 68% of animals having ischaemia for $\leq$2 hours). Infarct size was measured either histologically (staining with triphenyltetrazolium chloride [TTC], toluidine blue, haematoxylin and eosin or cresyl violet) or with MRI evaluation (T2 weighted images).

Infarct Size

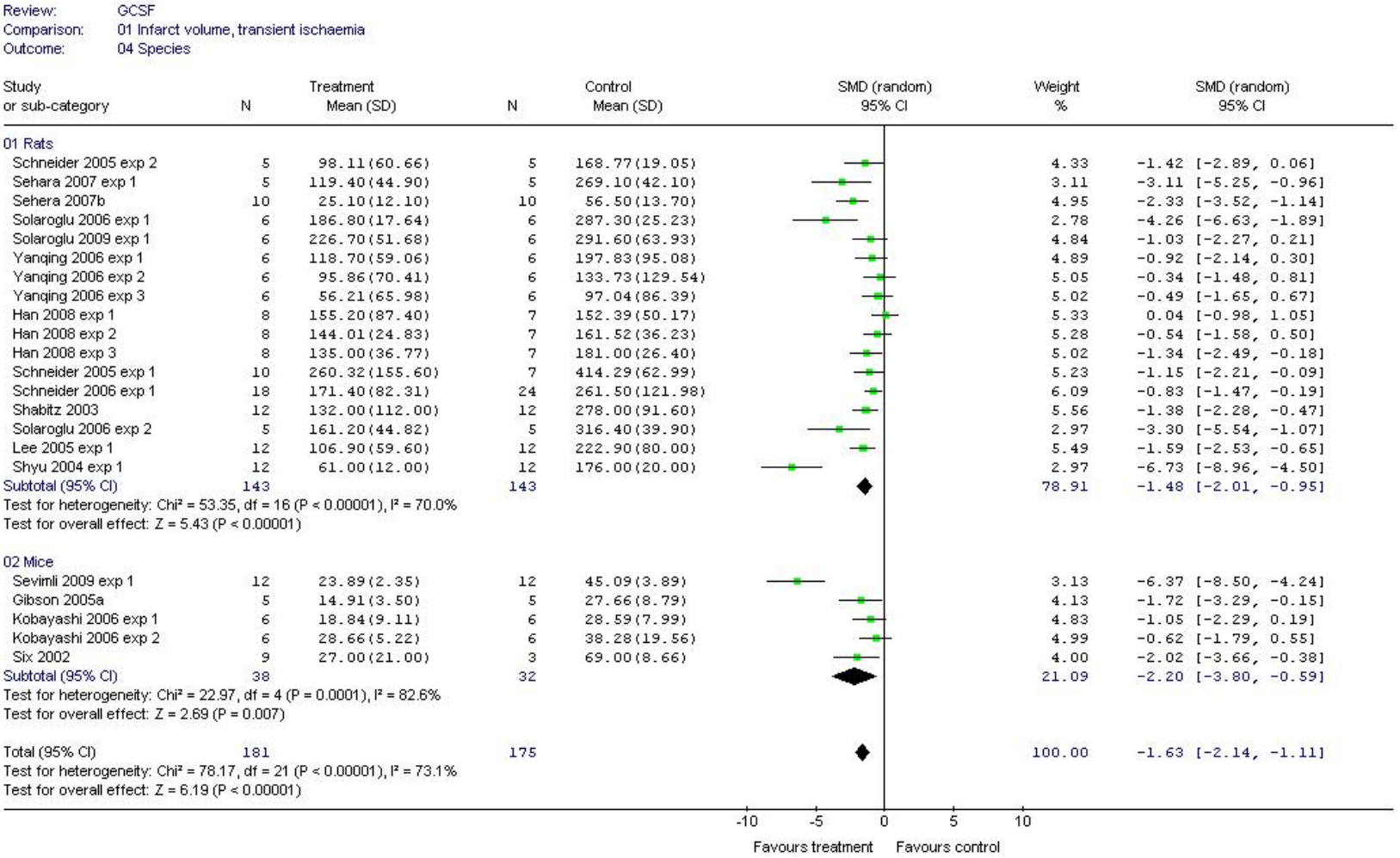
26 studies from 19 publications (table 14) assessed the effects of G-CSF on lesion size in a total of 412 animals (318 rats, 94 mice). All studies measuring infarct volume had protocols which required that G-CSF be administered within 24 hours. 22 of 26 studies measured these effects following transient ischaemia (table 14, figure 9). The presence of publication bias is suggested by a positive Egger's test of asymmetry ($p<0.001$, figure 10). Overall, G-CSF significantly reduced lesion size in transient ischaemia (SMD -1.63, 95% CI -2.14 to -1.11, $p<0.00001$). In the 4 studies using a permanent model (56 animals), G-CSF did not significantly reduce lesion size (SMD -1.53, 95% CI -3.42 to 0.36, $p=0.11$). Significant reductions in lesion size were seen in both rats and mice in transient (figure 9) but not permanent ischaemia. When only including the 21 studies that reported lesion volume (in mm^3) in transient ischaemia, the weighted mean difference was -62.32 (95% CI -79.6 to -45.1); equivalent to a SMD of -1.59 (95% CI -2.12 to -1.06).

Table 14. Effect of G-CSF on lesion volume, functional outcome and survival

(SMD, standardised mean difference; OR, odds ratio; 95% CI, 95% confidence interval; IS, ischaemic stroke)

	N° of Studies	N° of Animals	SMD 95% CI	p-value
Lesion Volume				
Transient IS	22	356	-1.63 (-2.14, -1.11)	< 0.00001
Permanent IS	4	56	-1.53 (-3.42, 0.36)	0.11
Mice	7	94	-1.61 (-2.81, -0.40)	0.009
Rats	19	318	-1.58 (-2.12, -1.04)	< 0.00001
Motor Impairment				
Neurological deficit	11	108	-1.37 (-2.13, -0.61)	0.0004
Rotarod (≤1 week post IS)	5	87	1.11 (0.16, 2.06)	0.02
Rotarod (5 weeks post IS)	6	115	3.24 (1.63, 4.85)	< 0.0001
Foot fault (1 week post IS)	2	32	-0.45 (-2.10, 1.19)	0.59
Limb placement (1 week post IS)	5	95	-0.97 (-1.91, -0.04)	0.04
Limb placement (4 weeks post IS)	5	95	-2.17 (-3.61, -0.72)	0.003
Locomotor activity (≥4 weeks post IS)	5	54	1.75 (-1.89, 5.38)	0.35
	N° of Studies	**N° of Animals**	**OR 95% CI**	**p-value**
Survival				
Transient ischaemia	6	230	0.27 (0.14, 0.451)	< 0.0001

Figure 9. Effect of G-CSF on lesion volume in transient middle cerebral artery occlusion.



(N, number of animals; SMD, standardised mean difference; 95% CI, 95% confidence interval; SD, standard deviation; MCAo, middle cerebral artery occlusion)

Figure 10. Begg's funnel plot.
The relationship between lesion volume (standardised mean difference, SMD) and standard error of SMD. Smaller neutral or negative studies (i.e. where lesion volume was not altered by G-CSF or increased with it) are missing suggesting the presence of publication bias (Egger's test $p<0.001$).

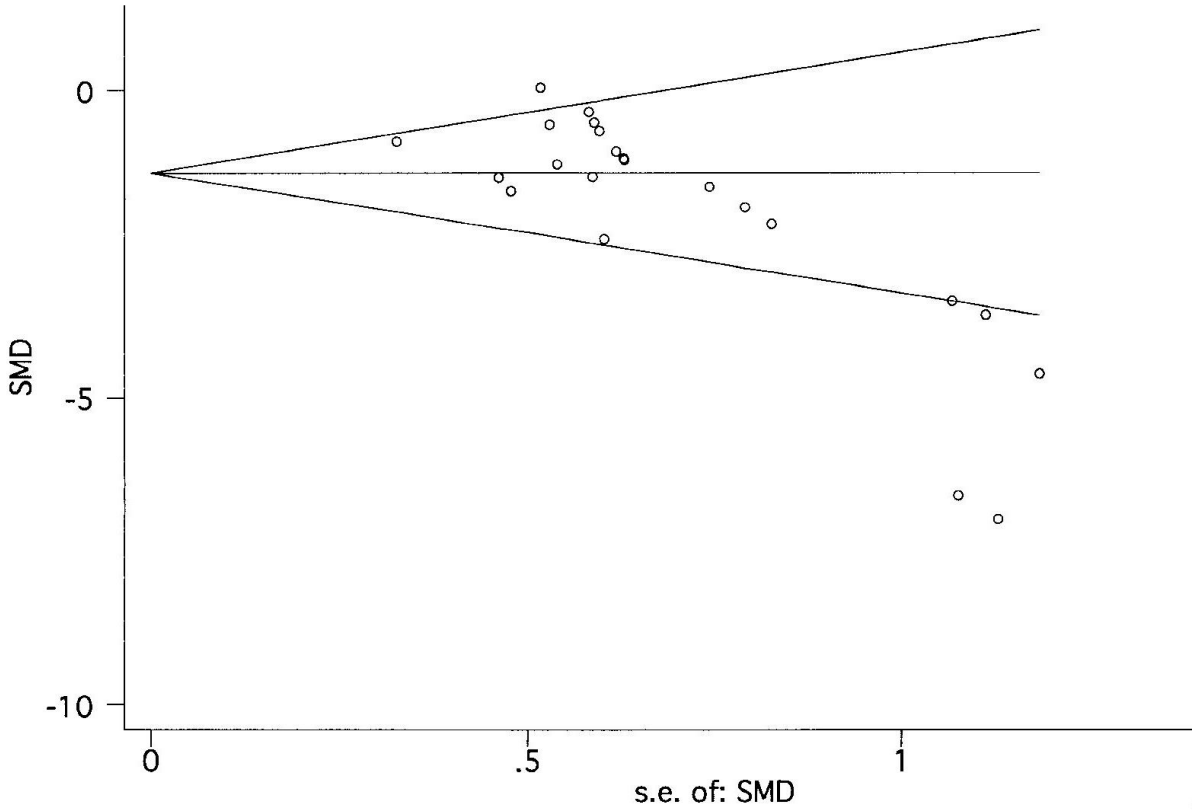
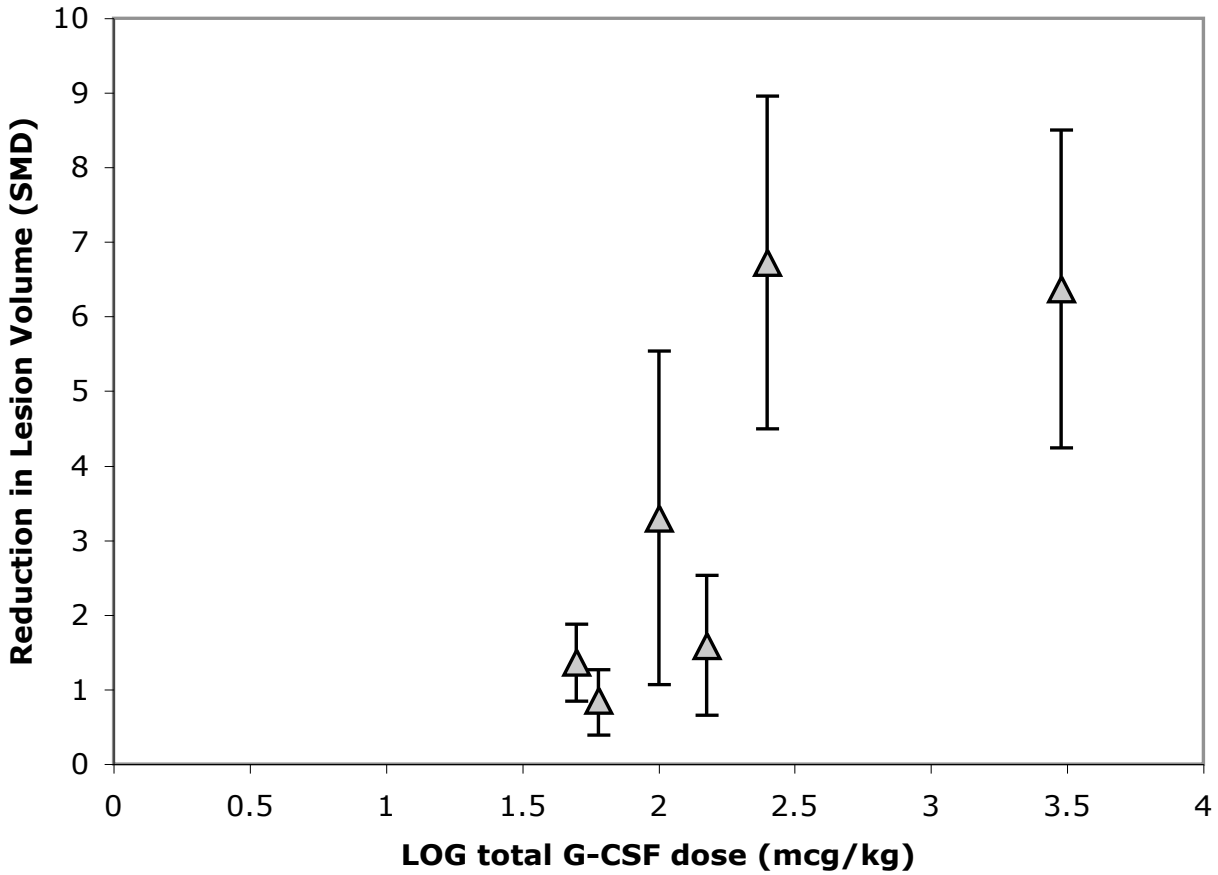


Figure 11. Effect of total G-CSF dose (logged) on lesion volume in models of transient ischaemia.

Data expressed as standardised mean difference (SMD) and 95% confidence intervals. Significant reductions in lesion volume: * p<0.05; ** p<0.001, # p<0.0001, ## p<0.00001



Motor Impairment

G-CSF significantly reduced neurological deficit (SMD -1.37, 95% CI -2.13 to -0.61, p=0.0004) in 11 studies.[167, 234, 244, 355-357] Impairment was measured at various time points post stroke (1, 2, 3, 7, 14, 21 and 35 days); beneficial effects of G-CSF were seen at all times but there was no correlation between reduction in impairment and time to measurement (r_s= 0.37, p=0.47). G-CSF increased time that animals stayed on a Rotarod [231, 235, 236, 244] (table 14) at 1 and 5 weeks post ischaemia. Similarly, improvements were seen in limb function as assessed by limb placement tests at 1 and 4 weeks post stroke. This was not reflected, however, in locomotor activity (at 4 and 5 weeks), as assessed by vertical movements / rearing, where there was no difference between G-CSF and control (SMD 0.76, 95% CI -0.98 to 2.51, p=0.35).[167, 240]

Survival

Data on vital status were available in 6 studies;[137, 231, 237, 244, 355, 358] G-CSF reduced the odds of dying almost 4-fold (OR 0.27, 95% CI 0.14 to 0.51, p<0.0001).

Total G-CSF dose

G-CSF administration varied considerably between studies: in studies using higher doses, regimens divided the G-CSF dose over a number of days. In an indirect comparison assessing total administered dose in transient ischaemia, a significant reduction in lesion size occurred at all doses (dose range 50 to 3000 mcg/kg) (figure 11). None of the doses used in permanent ischaemia (50 to 350 mcg/kg) had a significant effect on infarct size. No studies compared lesion size

with 2 or more doses of G-CSF but one study displayed impaired behavioural function with increasing doses of G-CSF (0.5 to 250 mcg/kg).[167]

Timing of treatment

G-CSF was administered pre-ischaemia in one study [244] and only one study compared time to treatment.[235] Significant reductions in lesion size in transient models of ischaemia were seen with treatment started within 4 hours post-ischaemia; trends to efficacy were also seen with commencement at 5 and 24 hours post-onset of ischaemia (data not shown).

Study Quality

The median study quality score was 4 (out of 8) with range 0 to 7. The majority of included studies were randomised [137, 138, 231, 234-237, 241, 244, 252, 355-357, 359-361] and used blinded outcome assessments [137, 138, 167, 231, 234-237, 240, 244, 252, 355-357, 359, 360] and although each study varied in G-CSF dose regimen, only one study specifically addressed the optimal time window of drug administration.[235] One study assessed dose response.[167] After adjusting for the number of animals in each study, the effect of G-CSF on lesion size was not related with study quality (Spearman's rank correlation, r_s=-0.16, p=0.7).

DISCUSSION

This systematic review finds that G-CSF reduces lesion size in transient ischaemia within 4 hours of administration, motor impairment (neurological severity and limb function) and death in experimental stroke. No significant effects were seen in permanent ischaemic models or in long term locomotor activity. Of note, the identified studies are biased towards those which might be expected to be positive, that is most studies assessed transient (32 experiments) rather than permanent (12 experiments) ischaemia, and short rather than long ischaemia.

The mechanisms of action of these potential effects are under investigation and are probably multi-factorial. First, neuroprotective activity may be secondary to suppression of oedema formation,[138] reduction of inflammation [138, 235, 356] (although G-CSF has also been reported to exacerbate the inflammatory response within the peri-infarct area [167]), and anti-apoptotic effects (with reduced cell death in the ischaemic penumbra).[137, 234, 360] Second, CD34+ haematopoietic stem cells (which are mobilised by G-CSF) are able to migrate to the site of injury when administered either intracerebrally or intravenously.[362, 363] Transplanted human bone marrow cells have been shown to generate neurons and astrocytes [364] but the ability of migratory stem cells to help restore functional and structural recovery post stroke has been questioned; in one study, bone marrow derived cells spontaneously fused, in vivo, with recipient Purkinje neurons in the cerebellum with no evidence of transdifferentiation.[365] Neurogenesis [137, 240, 241] needs to be sufficient and angiogenesis [235, 240, 241] is also necessary. In rats, G-CSF is able to cross the blood brain barrier when administered exogenously [137] and both G-CSF and its receptor are widely

expressed from neurons in the CNS.[137] In addition, a recent post mortem series highlighted G-CSF receptor upregulation in human ischaemic stroke.[366] Though these findings are encouraging, species differences in the expression profile of G-CSF should be considered which could alter the effect it has on infarct volume reduction and functional recovery.

The optimal time of administration of G-CSF relative to stroke onset (0-4 hours) supports a role of neuroprotective mechanisms. Whether neuroreparative actions occur is far less clear, since only trends to efficacy were seen between 5 and 24 hours. However, these data were limited and neurorepair may need stimulation after 24 hours when inflammatory responses have declined.[367] With the exception of 2 studies, [235, 236] pre-clinical experiments of G-CSF did not assess the effects of the drug given beyond 24 hours, which has implications on human trials; the AXIS study group are assessing hyperacute administration,[368] whereas all other human safety trials administer the drug during the sub-acute phase.[294, 295, 303, 304] Further studies are thus required to determine the extent of the therapeutic window. Moreover, the most appropriate G-CSF dose and administration regimen still needs to be established. A positive correlation was seen between total G-CSF dose and lesion size reduction but the true dose-response relationship requires confirmation in a study designed to answer this.

Furthermore, the infarct type (transient or permanent ischaemia) requires consideration. Our analysis confirms efficacy of G-CSF in rodents with transient ischaemia within 4 hours of administration but a better model for treatment in the majority of human stroke would be a permanent ischaemic model treated beyond 4 hours – a combination without evidence in this review. However, the

data are limited and the smaller number of animals tested in the permanent ischaemic model may account for the lack of significance.

Human stroke is also increasingly treated with thrombolytic agents and no articles were identified (except for one abstract [239]) assessing G-CSF in combination with tissue plasminogen activator (t-PA). This is especially important for potential neuroprotective agents which have to be administered early. The paucity of the number of publications assessing a combination of t-PA and G-CSF is likely to be related to the difficulty of reproducing embolic pre-clinical models. The procedure is technically very difficult to perform (in only a few laboratories worldwide) and the resulting infarct from embolism can be very inconsistent. Other stroke models could also influence lesion volume and recovery, for example, the presence of co-morbidities such as diabetes, hypertension, hypercholesterolaemia, female gender and increasing age. Ideally, all should be tested for their confounding effects.[155]

It is important to note that the potential beneficial effects observed here could be artefactual in view of the presence of potential publication bias, detected for the effect of G-CSF on lesion size caused by transient ischaemic models. This suggests that neutral or negative studies were not published, or at least not identified despite using a comprehensive search strategy. It is possible that these studies have been published in a journal not included in Medline or Embase, or that investigators deliberately did not publish neutral/negative data, or that such studies are not attractive to journal reviewers and editors.

We assessed study quality on the basis of methodological recommendations derived from the STAIR consortium [152] and found no relationship between study quality and effect on lesion size. STAIR standards have been developed by an expert panel to address why so many clinical trials of neuroprotectants have failed, and we have previously used this scale in other meta-analyses of experimental regimes for stroke.[343, 344] Although the majority of studies in this review had written evidence of randomisation (16/19 publications) and blinded outcome assessment (16/19), important sources of bias if unused,[369, 370] other key methodological criteria were missing, including assessment of dose-response (1/19) and time-response (2/19). It is, of course, possible that these criteria were not reported rather than not being performed. Furthermore, the majority of studies reporting randomisation and blinded outcome assessment was significantly higher than in other stroke reviews [371] and may explain why no study quality effect was observed here. It is also important to highlight, however, that absence some of the 'quality' items do not necessarily mean that the study was carried out to a poor standard; criteria such as randomisation, blinding and monitoring temperature are likely to reflect the standard to a greater degree than, for example, assessment of lesion volume and outcome at two different time-points.

Research involving animals is subject to strict regulation and adhering to the high standards is increasingly difficult in the context of the '3 Rs'. 'Reduction' of the number of animals in each experiment is encouraged by the use of sample size calculations and avoiding inefficient use of animals. 'Refinement' methods are alternatives that minimise distress and pain as to improve animal well-being, for example, ensuring use of adequate analgesia, proper handling and

appropriately sized caging. ‘Replacement’ involves utilising other methods such as computer simulation and *in-vitro* studies; human volunteer studies are also encouraged. The NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) have recently published the ARRIVE guidelines (Animial Research: Reporting *In Vivo* Experiments) to help improve the quality of reporting pre-clinical experiments, similar to the CONSORT (Consolidated Standards of Reporting Trials) statement for reporting randomised controlled trials.[372] ARRIVE guidelines contain a 20-item checklist describing the minimum information required in a publication to allow accurate critical review of methods, results and conclusions.

Meta-analysis methodology has a number of limitations and there are several caveats for this review. First, its findings depend on the success of identifying all relevant studies; the non-inclusion of some studies, perhaps due to non-publication (‘publication bias’) means that the estimated treatment effects may be an over- or an under-estimate. Second, the results depend on study quality (so assignment and observer bias are minimised). Third, study design determines what data are available for each included study; e.g. Rotarod and limb placement assessments largely came from one study [235] thereby restricting interpretation. Fourth, differences in methodology and study quality limit interpretation and introduce heterogeneity in findings, a problem that is addressed, in part, by using random effects models; e.g. analysing using standardised mean differences allows for comparison of infarct size whether measured by volume, area or percentage. Last, the selection process of suitable publications has the potential to introduce its own bias.

Of note, a similar systematic review was published whilst this one was being written. The former observed a 0.8% reduction in infarct volume per 1 μg/kg increase in G-CSF dose.[373] However, it included fewer articles (13 publications), excluded studies appropriate for analysis [167, 241, 374] and found no evidence of publication bias.

In summary, G-CSF appears to have neuroprotective qualities, although the evidence is limited in nature and potentially biased. Nonetheless, G-CSF offers a potential multimodal therapy for ischaemic stroke and it is possible that meaningful reductions in infarct volume and improvements in functional recovery may be translated into human trials. However, further experimental studies are required; in particular, to assess permanent models of ischaemia, length of ischaemia, other species (such as primates), both sexes, and animals with other co-morbidities and of increasing age (mimicking patients with stroke).[155] Studies also need to address optimal dose and route regimens, and elucidation of time responses. Acquiring this information is key since clinical trials assessing the safety of G-CSF in human stroke are already underway.[294, 295, 299]

CHAPTER 3

TRACKING IRON LABELLED STEM CELLS IN STROKE: A SYSTEMATIC REVIEW

Publications contributing to this chapter:

England TJ, Sprigg N, Bath PMW. Tracking iron labelled stem cells in stroke: a systematic review. International Journal of Stroke 2010;5(s3):43.

(Abstract poster presentation at UK Stroke Forum)

ABSTRACT

Background

Labelling stem cells with iron and tracking their movement with non-invasive magnetic resonance imaging (MRI) may help improve our understanding of the mechanism of action of stem cells. This review identifies iron-labelling techniques used in experimental stroke models and their potential application to human clinical trials.

Methods

Two authors independently searched Medline and Embase (to June 2010) for studies examining experimental stroke with iron-labelled stem cells. Data were collected on label technique, administration regimen (cell type, time post ictus, dose, route), stroke type (transient, permanent, photothrombotic) and outcome

(evidence of migration, transdifferentiation, function, lesion volume). Study quality was assessed using an 8-point scale.

Results

17 publications containing 25 experimental paradigms were identified. Mesenchymal stem cells (MSC) (n=11) and neural progenitor cells (n=8) were used most commonly and the majority were labelled with ferumoxide (n=16); the use of a transfection agent (e.g. protamine sulphate) was inconsistent. Iron labelled MSCs did not appear to transdifferentiate into neurons or glia. Study quality was low so potentially introduced significant bias; no studies reported blinded outcome assessments and only one utilised randomisation for animal selection.

Conclusion

Ferumoxide is useful for tracking stem cells, is already licensed for humans, and could be used in clinical studies. Protamine sulphate may be used as a transfection agent. Further research is needed using higher quality experimental models that mimic human stroke. The incorporation of iron into the cell to provide sufficient MRI contrast needs to be balanced with its potential negative effects on cell viability and function.

INTRODUCTION

Stem cells offer therapeutic potential for ischaemic stroke, a condition with few effective interventions. Numerous animal experiments have suggested functional benefit following treatment with a variety of stem cell types, but the mechanisms of action underlying improvements are poorly understood.[367] It is likely that the role of a stem cell is multifactorial orchestrating neurogenesis, angiogenesis and supporting the neurovascular unit via secretion of chemokines and growth factors; whether stems cells contribute directly to neuronal replacement remains unclear. One growing area of interest in helping to understand stem cell function during cerebral ischaemia is establishing stem cell migration patterns and tracking their movement with non-invasive MR imaging.

The majority of animal stroke models assessing stem cells determine the fate of a particular cell by using histological 'snap-shot' assessments. In contrast, the potential advantage of MRI in cell tracking is to provide near real-time cell movements. This can be achieved by labelling the cells of interest with an iron-containing contrast agent which will 'negatively enhance' T2* weighted images.[375] Numerous techniques are used, each with varying degrees of success; some of the agents applied in these experiments are already licensed for use in humans and therefore could be applicable to clinical studies.

The aim of this systematic review is to assess stem cell labelling with iron based techniques in the context of experimental ischaemic stroke.

METHODS

Search strategy and selection

Experimental models of stem cell labelling and ischaemic stroke were sought by systematically searching Medline and Embase (June 2010) using keywords 'stem cell', 'progenitor cell', 'tracking', 'migration', 'MRI', 'stroke', 'cerebrovascular', 'thrombosis', 'brain', 'cerebral', 'cerebellum', 'middle cerebral artery', 'ischaemia' and 'embolism'. Two independent authors (TE, NS) reviewed each article and their reference list, and articles were included in the analysis providing they met the following criteria: (i) stroke model involving transient or permanent ischaemia; and (ii) analysis of stem/progenitor cells labelled with iron. There were no limitations on species, timing of administration relative to stroke ictus, or whether there was a control group.

Data Collection

Information was gathered on cell labelling technique, cell type and number, route of administration and differentiation capacity. Where available, data on functional outcome was collected, specifically the adhesive tape removal test (time in seconds, less time indicating better function) and foot fault test (number of faults expressed as a percentage of the total number of steps). When numerical values were not available, published graphs were enlarged and the size of axes and position of data points estimated using *Grab* (version 1.3.1) on an Apple Mac.[376] Meta-analysis was performed with *Review Manager* (version 5.0.21); continuous data is expressed as a weighted mean difference (WMD) with 95% confidence intervals (95% CI) and random effects models were used due to expected heterogeneity between studies. Median and interquartile range are given for non parametric data. Statistical significance was set at $p<0.05$.

Quality

The quality of the included studies was assessed using an 8-point scoring system based on the STAIR report,[152] as previously used in systematic reviews.[343, 376] Each publication scored one point for evidence of (i) randomisation, (ii) monitoring (not measuring) physiological parameters (i.e. temperature), (iii) assessment of dose-response, (iv) assessment of optimal time of administration, (v) blinded outcome assessments, (vi) assessment of outcome at days 1 to 3, (vii) assessment of outcome at days 7 to 30, and (viii) combined measurement of lesion volume and functional outcome. A higher score indicates a higher quality experiment.

RESULTS

Study Characteristics

The initial search up to June 2010 identified 216 articles. Once pre-specified exclusion criteria were applied, 17 articles (containing 25 experimental paradigms) were selected for analysis (figure 12). These publications were presented from 10 laboratories across 7 countries (USA, Korea, Singapore, China, Czech Republic, Belgium and Germany). A total of 291 animals were studied. The majority of animals used were male rats; male mice (n=10) and female rats (n=47) were also studied.[377-379] Studies not selected for analysis included those assessing haemorrhagic stroke [380] and those using alternative labelling methods (for example, Gadolinium,[240, 278] and bioluminescence [381]). One study provided insufficient detail in two of its three *in vivo* experimental paradigms to be fully included.[382]

The primary aim of most studies identified was qualitative, that is to determine migrational cell dynamics and the use of iron as a marker for non-invasive MR tracking.

Infarct type

Eleven of 25 experiments used a photothrombotic stroke model (table 15), a method that is less likely to affect the survival of the rodents concerned. Two studies used an embolic method whilst the remainder utilised transient models of ischaemia with occlusion time ranging between 1 and 3 hours. None used a permanent model of ischaemia.

Figure 12. Summary of database search

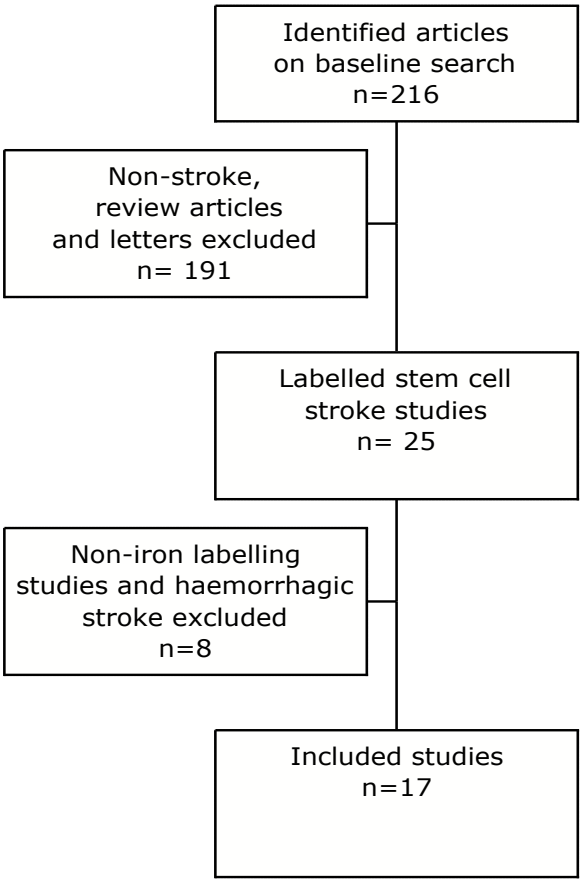


Table 15. Characteristics of included studies

Article	Species	Stroke type	Transplant time	Cell type	Cell N°	MRI field	Active, Control	Label	Transf'n agent	Route	Outcomes
Hoehn 2002	Male Wistar rats	T - 1 hr	2 weeks	ESC (m)	$3x10^4$	7T	11,3	Sinerem	FuGENE	ic	Cell culture, IHC (GFP), cell viability
Jendelova 2003 A	Male Wistar rats	Photo-chemical	24 hours	MSC (r)	$3x10^5$	4.7T	15,?10	Ferumoxide - BrdU	-	ic	EM, IHC (GFAP, Neu N), iron content
Jendelova 2003 B	Male Wistar rats	Photo-chemical	24 hours	MSC (r)	$2x10^6$	4T	12, 6	Ferumoxide - BrdU	-	iv	
Zhang 2003	Male Wistar rats	Embolic MCA	48 hours	NPC (r)	$1x10^5$	7T	8,?2	Ferromagnetic gun	-	ic	Function: foot fault test, adhesive removal test, body weight at 7 to 35 days
Jendelova 2004 A	Male Wistar rats	Photo-chemical	1 week	ESC (eGFP)	$2x10^5$	4.7T	$12,3^{ß}$	Ferumoxide	-	ic	
Jendelova 2004 B	Male Wistar rats	Photo-chemical	1 week	ESC (eGFP)	$2x10^6$	4.7T	7,0	Ferumoxide	-	iv	EM, IHC (GFAP, Neu N), iron content
Jendelova 2004 C	Male Wistar rats	Photo-chemical	1 week	MSC (r)	$2x10^5$	4.7T	$15,3^{ß}$	Ferumoxide - BrdU	-	ic	
Jendelova 2004 D	Male Wistar rats	Photo-chemical	1 week	MSC (r)	$2x10^6$	4.7T	12,0	Ferumoxide - BrdU	-	iv	
Jiang 2005	Rats	Embolic MCA	48 hours	NPC (r)	$1x10^5$	7T	8,0	Ferromagnetic gun	-	ic	Cerebral blood flow, IHC (vWF) assessing angiogenesis,
Jendelova 2005	Male Wistar rats	Photo-chemical	1 week	CD34+ (h)	$7.5x10^6$	4.7T	8,4	Extracellular micro beads	-	ic	EM, IHC (GFAP, Neu N), iron content
Jiang 2006	Male Wistar rats	T - 3 hrs	48 hours	NPC (h,f)	$1x10^6$	7T	17,9	Ferumoxide	Protamine	ia	Foot fault test, adhesive removal test at 1, 7 to 35 days
Li 2006	Male Wistar rats	Embolic MCA	48 hours	NPC (r)	$1x10^5$	7T	8,3	Ferromagnetic gun		ic	CBF, IHC (B-tubulin, GFAP, nestin, vWF), lesion volume
Rice 2007	Male balb C mice	T - 1 hr	2 weeks	ASC (m)	500 to 5000	7T	8,2	Ferumoxide	PLL	ic	IHC (GFP), cell viability
Guzman 2007	Male Sprague Dawley rats	T - 1 hr	7 days	CNS-SCns	$1x10^5$ (x 3)	4.7T	10,0	Ferumoxide	Protamine	ic	MRI characterisation IHC (GFAP, B-tubulin)

Guzman 2008	Male Sprague Dawley rats	T - 1 hr	minus 7 days	NPC (m)	$2x10^5$	4.7T	12,3 (6,6)*	Ferumoxide - BrdU	Protamine	ic	Cell viability, labelling efficiency, DCX, B-tubulin, GFAP, iba-1
Kim 2008	Male Sprague Dawley rats	T - unclear	7 days	MSC (h)	$1x10^5$	4.7T	$11^{\dagger}$,2	Ferumoxide	Protamine	ic	MRI characterisation IHC (human specific nuclei, nestin, tubulin, MAP2, GFAP)
Walczak 2008 A	Adult female Wistar rats	T - 2 hrs	0.5 hrs	MSC (r)	$1x10^6$	4.7T	$17,4^{\$}$	Ferumoxide - BrdU	PLL	ia	IHC (BrdU), survival
Walczak 2008 B	Adult female Wistar rats	T - 2 hrs	0.5 hrs	MSC (r)	$1x10^6$	4.7T	$4,4^{\$}$	Ferumoxide - BrdU	PLL	iv	
Zhu 2008	Rats	T- 1 hr	2 weeks	NPC (r)	$2x10^6$	4.7T	unknown	Ferumoxide - BrdU	PLL	ic	IHC (GFAP, Map2, BrdU)
Song 2009 A	Male Sprague Dawley rats	T - 2 hrs	24 hours	NPC (h)	$4x10^5$	1.5T	3,3	Fe-BrdU	-	ic	Viability, proliferation, iron content, IHC (Neu N, GFAP, MHC class II)
Song 2009 B	Male Sprague Dawley rats	T - 2 hrs	24 hours	NPC (h)	$4x10^6$	1.5T	3,3	Fe-BrdU	-	iv	
Lee 2009 A	Female Wistar rats	Photo-chemical	48 hours	MSC (h,f)	$2x10^4$	1.5T	$9,7^{\mu}$	MGIO	-	ic	IHC (Vimentin, ED1), cell characterisation, iron content, viability
Lee 2009 B	Female Wistar rats	Photo-chemical	48 hours	MSC (h,f)	$2x10^4$	1.5T	$4,7^{\mu}$	Ferucarbotran	-	ic	
Lee 2009 C	Female Wistar rats	Photo-chemical	48 hours	MSC (h,f)	$2x10^6$	1.5T	$2,7^{\mu}$	MGIO	-	iv	
Crabbe 2010	Male Fisher rats	Photo-chemical	24 hours	MAPC (r)	$1x10^5$	9.4T	8,?0	Ferucarbotran	PLL	ic	MRI characterisation, IHC

T, transient; MCA, middle cerebral artery; MSC, mesenchymal stem cell; ESC, embryonic stem cell; NPC, neural progenitor cell; ASC, adipose stem cell; CNS-SCns, central nervous system stem cell neurospheres; MAPC, multipotent adult progenitor cells; (m) murine; (r), rat; (h) human; f (foetal); PLL, poly-L-lysine; MGIO, microgel iron oxide; BrdU, Bromodeoxyuridine; ic, intracerebral; ia, intra-arterial; iv, intravenous; EM, electron microscopy; IHC, immunohistochemistry.

*12,3 - cells v placebo (6,6 - stroke v non-stroke)

†6 ipsilateral, 5 contralateral to the stroke; $^{ß,\$,\mu}$ same control

Stem cell type

Real-time tracking using iron labelled cells and MRI was assessed in experimental stroke using CD34+ haematopoietic stem cells (HSC) (n=1),[362] rodent and human mesenchymal stem cells (MSC) (n=10),[378, 379, 383-385] rodent and human neural stem/progenitor cells (NSC) (n=8),[386-391] embryonic stem cells (ESC) (n=3),[277, 384] adult progenitor cells (n=1),[382] adipose derived stem cells (n=1) [377] and human CNS neurospheres (n=1).[392]

Dose, route and time of administration

The total number of cells injected ranged between 500 and 7.5 x 10^6. Assuming an average rat weight of 300g, this is an equivalent dose of between 1.7 x 10^3 and 2.5 x 10^7 cells per kilogram. Most studies injected the cells via a direct intracerebral route (n=17), whilst the remainder used intravascular routes (intravenous, n=6; intra-arterial, n=2). There was marked variation in the time of administration with cells injected between minus 7 days and 2 weeks post stroke ictus, though this did not appear to affect cell migration as all studies analysed detected positive evidence of this. Median time of cell administration was 48 hours.

Label type

Cells were labelled with ferumoxide (superparamagnetic iron oxide nanoparticles, SPIO, particle size 120-150nm containing a crystal approximately 5nm in size) in 16 experiments; ferumoxtran (ultrasmall superparamagnetic iron oxide, USPIO, particle diameter 20-40nm) in one experiment;[277] ferucarbotran (particle diameter 60nm) in two; or a ferromagnetic gun (which ‘shoots’ dye-coated iron into clusters of cells using compressed gas

and a nylon filter) in three studies.[387, 388, 393] Only one publication directly compared two different labels, a larger 600nm particle (microgel iron oxide, MGIO) and the smaller ferucarbotran.[379] Extracellular microbeads attached to human CD34+ cells were also evaluated as an effective iron label but this required 7.5 x 10^6 cells, the highest number, injected intracerebrally to produce a weak hypointense signal on 4.7 Tesla (T) MRI.[362] MRI strengths ranged from 1.5T to 9.4T. In 8 experiments, a co-label (bromodeoxyuridine, BrdU, a thymidine analogue that labels cells in the S-phase of the cell cycle) was used to aid identification of migrating cells.

Eight studies used a transfection agent such as protamine sulphate (n=4) or poly-l-lysine (PLL) (n=5) to enhance cellular uptake during incubation. Transfection agents were not consistently required. For example, both MSCs and NPCs were assessed with and without a transfection agent (table 15).

Outcomes

All experiments confirmed that cell migration was detectable with MRI using iron labelled stem cells – there were no negative studies. Quantification of differentiated iron labelled stem cells (into neurons or glia) was measured in 8 of 25 experiments, and assessed but not quantified in 4 more. Interestingly, iron labelled MSCs did not appear to produce any response in developing new neurons or glia,[383, 385] whereas 70% of iron labelled ESCs differentiated into astrocytes, 5% into neurons and, ominously, 10% produced tumour-like cells.[384] 13% of labelled NPCs produced detectable new neuron formation and human CNS neurospheres produced neurons and glia *in vitro* (56% and 19%

respectively).[392] Labelled CD34+ cell differentiation was not observed in one study.[362]

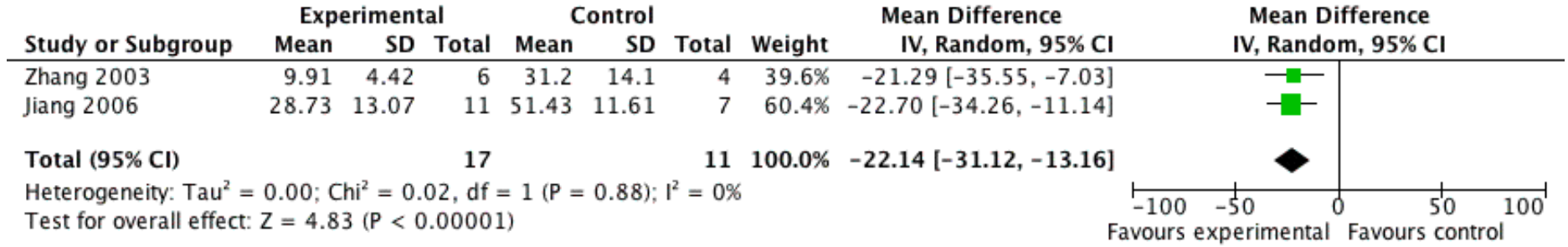
Lesion volume was evaluated in one study only.[393] Functional outcome measures were evaluated in 2 studies;[387, 389] labelled SVCs [387] and NPCs [389] improved both the tape adhesion test (WMD -22.14, 95% CI -31.12 to -13.16, p<0.00001) and foot fault test (WMD -4.67, 95% CI -8.15 to -1.2, p=0.008) (figure 13). Only one study reported survival in which a higher mortality was observed in the group treated with intra-arterial MSCs when compared to control (67% vs 7%).[378] Conversely, the other study assessing intra-arterial delivery (of NPCs) showed an improvement in functional outcome.[389]

Quality

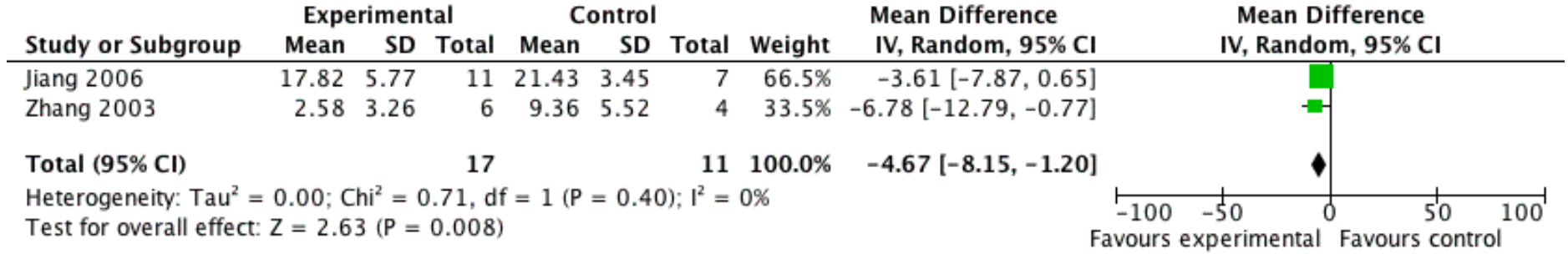
Control groups were not provided in 5 of 25 experiments (and may not have been present in another [382]). Only one study reported that the animals were randomised into treatment or placebo groups.[389] No studies specified that outcome assessments were blinded to treatment allocation. Assessment of dose-response and optimal time of therapeutic administration was not assessed in any of the identified studies, although this may not be surprising considering that the main outcome assessments were not focused on function or infarct volume analysis (these are key parameters in experimental stroke studies [152]). Overall the median quality score was 2 (interquartile range 2-3). The low quality scores suggest that significant bias may be present and findings should be treated with caution.

Figure 13. The effects of iron labelled stem cells on functional outcome: (a) the adhesive tape removal test and (b) the foot fault test.

(a)



(b)



DISCUSSION

Stem cell labelling with iron based compounds allows real-time tracking of migration patterns of implanted cells. The primary intention of this review was to describe labelling techniques used in experimental stroke models with a view to considering whether such labelling methods could be translated into clinical practice. However, none of the articles analysed reported a failure to display migration capacity and hence it would be challenging to select a superior technique based on this outcome alone. The absence of negative studies suggests the presence of publication bias.

The most commonly used compound was ferumoxide (n=16), a dextran-coated superparamagnetic iron oxide nanoparticle (SPIO), which is commercially available and licensed for human use. Its particle size is in the region of 120-150nm, containing a crystal approximately 5nm in size. Incorporation into stem cells can be enhanced using a transfection agent such as poly-L-lysine (PLL) or protamine sulphate, which works by altering electrostatic interactions across the cell membrane and transferrin receptor. Protamine sulphate is also licensed for human use (for reversal of the effects of heparin) and it is apparent that its combination with ferumoxide is increasingly used in labelling experiments. However, it is evident from this review that there has been inconsistency in the type of transfection agent used across cell types or even whether a transfection agent has been used at all.[378, 384, 385]

Loading a cell with internal iron can potentially attenuate its function by interfering with cell signalling pathways; an adequate cell iron concentration for maximum tissue contrast on MR imaging is desirable but it must be balanced with its effects on

cell viability and differentiation capacity.[394] MSCs labelled with ferumoxide [383, 385] and implanted intravenously or intracerebrally in ischaemic stroke models failed to demonstrate significant cell differentiation into neurons or glia suggesting either the cells cannot differentiate into neural-type cells or they have impaired function. Nevertheless, other non-stroke studies have shown that labelling MSCs with SPIO may not inhibit cell differentiation but can cause subtle yet significant cell phenotypical alterations.[395] Bone marrow stem cells are capable of adopting neural characteristics [396] but it has been suggested that they simply fuse with recipient cells and adopt their phenotype.[222]

With the exception of one, all studies in this review utilised intracellular iron labelling techniques. The remaining study used an extracellular CD34+ antibody containing iron oxide nanobeads.[362] *In vitro*, it was observed that 6050 cells/μl were required to produce a visible contrast difference on 4T MR scanning, each cell containing, on average, 0.275 pg of iron. Higher cell numbers were transplanted intracerebrally *in vivo* in this particular study ($7.5x10^6$ cells).[362] In contrast, much smaller doses of cells were used in studies using intracellular labelling; ESCs containing 13.6 pg of iron per cell required only 315 cells/μl (17 cells per voxel) to be visualised with 7T MRI (in vitro). The higher the cellular iron concentration, the more likely it is to effect cell viability, function and differentiation capacity. If a cell's functional capacity were limited to a significant degree, cell labelling experiments in human clinical trials would have to be designed with 'proof of concept' in mind rather than as an assessment of the cells for therapeutic purposes, since it would be unethical to transplant dysfunctional cells. However, the two studies in this review that observed

improved functional outcome when using labelled cells suggest that the potential therapeutic benefit is unaffected.

One major concern is the high mortality rate in one study transplanting cells via the intra-arterial route,[378] an issue also highlighted in the 'Stem Cell Therapies as an Emerging Paradigm in Stroke' (STEPS) report.[276] A suggested mechanism for excess mortality could be intercellular adhesion, forming microemboli to cause recurrent stroke, a factor also influenced by total cell dose. The least invasive, and therefore preferable, route of administration is intravenous, but more cells must be injected for adequate numbers to reach the cerebral infarct.[272] Further concern has also been raised in a study that assessed transplanted MHP36 labelled neural stem cells in a rat MCAo model.[397] Cells labelled with the contrast agent GRID (Gadolinium-Rhodamine Dextran conjugate) did not significantly improve functional behavior compared to animals transplanted with the same cells labelled with a red florescent dye. In addition, the GRID-labelled group demonstrated an increase in lesion volume growth over 1 year; it is possible that GRID mediated inflammation leads to exacerbation of the stroke lesion, effects that are not seen in the short term or *in vitro*.[397] The same authors have also demonstrated that transplantation of dead stem cells can lead to an increase in lesion volume.[398] These results highlight the need more for studies assessing labelled stem cell grafts in the chronic phases of stroke and the significant implications for clinical studies. Labelling cells may affect their function and potential therapeutic benefit but they may also cause harm. Ethical and regulatory authorities are likely to view a labelled cell as a different therapy compared to the same unlabelled cell and therefore expect equivalent demonstrations of preclinical safety and efficacy before human clinical trials.

This systematic review has limitations. First, it is important to recognise that studies were only included if they described ischaemic stroke models and iron based stem cell labelling; no conclusions can therefore be drawn on the treatment effects of stem cells in stroke models. Moreover, non-iron labelling studies such as those assessing gadolinium cell labelling (providing MRI contrast on T1-weighted images),[278, 399] were not included but these methods still offer the potential to label cells in a human clinical trial setting. Second, heterogeneity between the methodologies of studies such as timing of administration and dose of cells transplanted makes comparison challenging. In particular, only transient or photochemical models of stroke were included. A alternative paradigm for human stroke would be to use a permanent model of ischaemia in addition to hypertensive, diabetic and aged rodent models. Third, the 'quality' of the studies was low and important methodological quality markers (randomisation and masked outcome assessment) were notably absent, or at least not reported. As a result, significant selection, measurement, and publication bias may be present.[371] Finally, it may be that iron based cell tracking techniques will be inappropriate in human stroke. Up to 85% of ischaemic strokes can undergo haemorrhagic transformation.[400] Iron contained in blood will show as hypodense regions on MR gradient echo imaging and will significantly confound scan interpretation.

In summary, it is currently unclear which labelling technique is best to translate into human trials, and further research is required using consistent stroke models in higher quality studies. It would be sensible to use agents with established pharmacological profiles in humans, such as ferumoxide or ferucarbotran (SPIOs),

and protamine sulphate as a transfection agent if required. The incorporation of iron into the cell to provide sufficient MRI contrast needs to be balanced with cell viability and function.

CHAPTER 4

STEM CELL TRIAL OF RECOVERY ENHANCEMENT AFTER STROKE 2 (STEMS-2): PILOT RANDOMISED PLACEBO-CONTROLLED TRIAL OF GRANULOCYTE-COLONY STIMULATING FACTOR IN MOBILISING BONE MARROW STEM CELLS IN SUB-ACUTE STROKE.

Publications contributing towards this chapter

England TJ, Abaei M, Auer DP, Lowe J, Russell N, Walker M, Bath PMW. Stem-cell trial of recovery enhancement after stroke 2 (STEMS-2). Randomised placebo-controlled trial of granulocyte-colony stimulating factor in mobilising bone marrow stem cells in sub-acute stroke. *Stroke*. In Press

Winner of the Warlow Prize for best oral abstract from a trainee or consultant of less than 3 years standing. Presented by the British Association of Stroke Physicians (BASP). Presented to the Stroke Society of Australasia Annual Scientific Meeting, 14 – 16 September 2011, Adelaide Convention Centre.

Winner of the McCallum Prize for the best written research paper by medical staff of non-consultant level in the Nottingham area. Presented by Nottingham Medico-Chirurgical Society 2011.

ABSTRACT

Background

Granulocyte-colony stimulating factor (G-CSF) is neuroprotective in experimental stroke, and mobilises CD34+ peripheral blood stem cells into the circulation. We assessed the safety of G-CSF in recent stroke in a phase IIb single-centre randomised controlled trial.

Methods

G-CSF (Filgrastim 10 μg/kg [=1x10^6 U/kg]) or placebo (ratio 2:1) was given subcutaneously for 5 days to 60 patients 3-30 days after ischaemic or haemorrhagic stroke. The primary outcome was the frequency of serious adverse events (SAE). Peripheral blood counts, CD34+ count and functional outcome were measured. MRI assessed stroke lesion volume and atrophy.

Results

60 patients were recruited at mean 8 days (SD 5) post ictus, with mean age 71 (12) years, and 53% male. In 'on-treatment' analysis, the groups were well matched for baseline minimisation/prognostic factors. There were no significant differences between groups in the number of participants with a SAE: G-CSF 15/38 (40%) versus placebo 7/22 (32%), death or dependency (modified Rankin Scale: G-CSF 3 (IQR 2-4), placebo 3 (IQR 2-4)) at 90 days, or the number of injections received. G-CSF increased CD34+ and total white cell counts 9.5 and 4.2-fold respectively. There was a trend towards reduction in MRI ischaemic lesion volume with respect to change from baseline in G-CSF treated patients (p=0.06).

Conclusions

This randomised double-blind placebo-controlled trial suggests that G-CSF is safe when administered sub-acutely.

INTRODUCTION

Few interventions aid recovery for patients with acute stroke and although earlier trials of putative neuroprotectants failed (e.g. NXY-059 [88]), new studies are ongoing, including focussing on colony stimulating factors such as granulocyte-colony stimulating factor (G-CSF) and erythropoietin where promising data exist in pre-clinical experimental models of stroke.[376]

G-CSF is a glycoprotein hormone encoded by a single gene located on chromosome 17 q11-22;[401] it plays a key role in the regulation of granulopoiesis and is responsible for the terminal maturation of neutrophils. Recombinant G-CSF is used for treatment of neutropenia and for production of CD34+ haematopoietic stem cells (HSC) for bone marrow transplantation. When considering G-CSF as a potential treatment for stroke, the mechanisms of action appear to be multimodal. Experimentally, neuroprotection occurs by reducing apoptosis in the ischaemic penumbra and attenuating the inflammatory cascade.[137] It also appears to be neuroreparative through potentiating angiogenesis [235] and neurogenesis,[137] in part by activating brain endothelial cells [402] and mobilising HSCs to migrate to the ischaemic lesion.[362] More contentiously, bone marrow derived stem cells have been shown to differentiate into neurons and glia [222] and experimental transplants of haematopoietic stem cells have been observed to improve outcome post-stroke.[403]

The purpose of the present trial, STEMS-2, was to further evaluate the safety of G-CSF in stroke with emphasis on its effects on bone marrow derived HSCs and their fate in the brain.

METHODS

Design

We performed a prospective, single-centre, double blind, randomised, placebo-controlled, phase IIb trial of G-CSF in patients with subacute stroke. The trial was conducted in accordance with the Declaration of Helsinki and the International Conference of Harmonisation of Good Clinical Practice (ICH-GCP), received authorisation from the Medicines and Healthcare products Regulatory Agency (EudraCT 2006-005345-11), the Local Research Ethics Committee and was a registered clinical trial (International Standard Randomised Controlled Trial Number 63336619).

Patients

Patients 3-30 days post ischaemic and haemorrhagic stroke were recruited from the stroke service at Nottingham Universities Hospital NHS Trust from July 2007 to January 2010 (see table 16 for inclusion and exclusion criteria). Written informed consent was obtained from each patient. If the patient was unable to consent (e.g. due to confusion or dysphasia), proxy consent was obtained from a relative or carer.

Intervention

Participants were randomised 2:1 to receive subcutaneous human recombinant G-CSF ($1x10^6$ units/kg, equivalent to 10μg/kg, Neupogen, Amgen, the maximum dose in STEMS-1 [294]) or matching subcutaneous saline once per day for 5 days. Treatment was prepared centrally and both the participants and those administering the injections were blinded to treatment and its assignment.

Randomisation involved computerised minimisation (table 16) and randomised treatment was administered in addition to best medical care.

Table 16. Inclusion and exclusion criteria for STEMS-2 and computerised minimisation factors used for randomisation.

Inclusion criteria
Age > 18
Clinical diagnosis of stroke
Limb weakness present at randomisation (SNSS score in arm and/or leg <4)
Brain scan compatible with ischaemic or haemorrhagic stroke
Time from ictus 3-30 days
Exclusion criteria
Pre-morbid dependency (modified Rankin scale, mRS >3),
Coma (SNSS consciousness <4)
Dementia
Malignancy
Sickle cell disease
Pregnancy
Neutropenia
Computerised minimisation factors
Age (<70, ≥70 years)
Sex
Time since stroke (<8, ≥8 days)
Stroke type (ischaemic, haemorrhage)
Stroke severity (SNSS <30, ≥30),
Cortical signs (cortical, sub-cortical)
Presence of infection
Intention to perform MRI

SNSS, Scandinavian Neurological Stroke Score

Clinical outcomes

The primary outcome measure was safety, assessed as the number of participants having a serious adverse event (SAE) by day 90. Clinical secondary outcomes included tolerability, feasibility, impairment (NIHSS), grip strength, dependency (mRS), disability (Nottingham Extended Activities of Daily Living, NEADL; Barthel Index, BI), cognition (Mini Mental State Examination, MMSE) and mood (Zung depression score); measurements were made at days 10 and 90. All assessments were blinded to treatment assignment. The Data Monitoring Committee assessed unblinded safety data after recruiting 20 and 40 patients into the trial.

Laboratory Measures

Peripheral blood CD34+ cell count was measured at day 5 using flow cytometry (FACScalibur, Becton Dickinson, Oxford, UK) and complied with ISHAGE guidelines.[404] Full blood counts and putative markers of neuroprotection efficacy (D-dimer, B-natriuretic peptide (BNP), matrix metalloproteinase-9 (MMP-9) and protein S-100β, (Triage Stroke Panel, Biosite Inc, San Diego, US)) were assessed on days 0, 5 and 10.

Volumetric analysis

Magnetic resonance imaging (MRI) of the brain was performed at baseline and on day 90 (±7) in patients with ischaemic stroke, except in those who were intolerant, had contraindications to MRI, or refused imaging. The volume of acutely cerebral infarcted tissue was calculated at baseline by measuring DWI hyperintense lesion outlines at day 0 and at day 90 at which time point intermediately T2 weighted scans were used (table 17). Contralateral ventricular

volume on DWI images (including the lateral ventricles to the level of the third ventricle) was also assessed as an indirect measure of cerebral atrophy. Two assessors blinded to treatment allocation (TJE, MA) were trained by an experienced neuroradiologist (DPA), and measured volumes independently. Scans were performed on a 3T Achieva (Philips, Netherlands) using an 8-channel phased array coil or on a 1.5T Signa Excite (GE Medical Systems, US) for baseline scans when participant could not be transferred for 3T imaging. Lesion volume was measured using semi-automated software developed locally (NeuRoi, Dr C Tench, Department of Clinical Neurology, Nottingham, UK [405]).

Table 17. MRI sequence parameters

MRI Scan Sequence	Parameter	3T Achieva	1.5T Signa Excite
Diffusion	Echo Time (ms)	53	92
weighted	Repetition time (ms)	6605	8000
(baseline, day 0)	Slice thickness (mm)	3	3
	Field of View (mm)	240×240	256x256
	Scan Matrix	96×96	128x128
	Voxel Size (mm)	2.5x2.5x2.5	2x2x3
	Diffusion weighting (s/mm^2)	1000	1000
T2 weighted	Echo Time (ms)	32	74
	Repetition time (ms)	2000	4120
	Slice thickness (mm)	3	3
	Pixel size (mm)	0.38x0.38	0.47x0.47

Statistical analysis

The primary outcome was the number of participants with one or more SAEs; this was chosen since G-CSF was associated with a non-significant increase in SAEs in our earlier phase IIa trial.[294] The sample size was calculated as 60 assuming alpha 5%, power 80%, SAE rate G-CSF 50% vs. placebo 20%, ratio

G-CSF:placebo 2:1, losses 5%. Data were analysed using Fisher's Exact test, Mann-Whitney U test, repeated measures ANOVA, or ANCOVA as appropriate. Data are presented as number (percentage), mean (± standard deviation, SD), or median (interquartile range, IQR) and are presented as 'on-treatment' analysis. Stroke lesion volume data was positively skewed; it was therefore transformed using log(10) to normalise the distribution and anti-logged to present the geometric mean. All analyses were performed using PASW statistics data editor (version 18.0) and MediStat; analysis was by intention-to-treat (according to randomisation) and statistical significance was taken at $p<0.05$.

RESULTS

Subjects

Of 205 screened participants who were eligible, 60 were included and the groups (40 G-CSF, 20 placebo) were well matched at baseline (figure 14, table 18). The first dose of trial drug was received, on average, 10 days after stroke onset. The mean number of treatment doses received was 4.5 (90.5% in total) in participants randomised to G-CSF, and 5 (100%) for placebo; 2 participants randomised to G-CSF refused any treatment (but agreed to continued follow-up) before they received their first dose. The data for these 2 recruits were analysed in the placebo group. Other participants stopped treatment after receiving G-CSF due to lower back pain (n=1, 2 of 5 doses received), acute illness not related to the trial drug (n=1) and administration error (n=2, missed doses). One patient withdrew from the trial; whilst they refused follow-up, they allowed information on vital status and mRS to be obtained from the General Practitioner.

Clinical outcomes

No significant difference in the number of patients with SAE (the primary outcome) or in the mean number of SAEs and adverse events (AEs) per patient was observed between treatment groups (tables 19 and 20). There were 3 deaths in the G-CSF group (pulmonary embolus, ischaemic bowel, community acquired pneumonia) but all occurred after the end of the treatment phase; no deaths occurred in the placebo group. There were no differences in vascular events (non-fatal stroke, non-fatal MI, pulmonary embolus, deep vein thrombosis) or infection rates by day 90 (table 19). 15% (6 G-CSF, 3 placebo)

of the participants were included with a primary intracerebral haemorrhage and no safety concerns were present in this subgroup (table 20).

Figure 14. Flow of participants into the trial

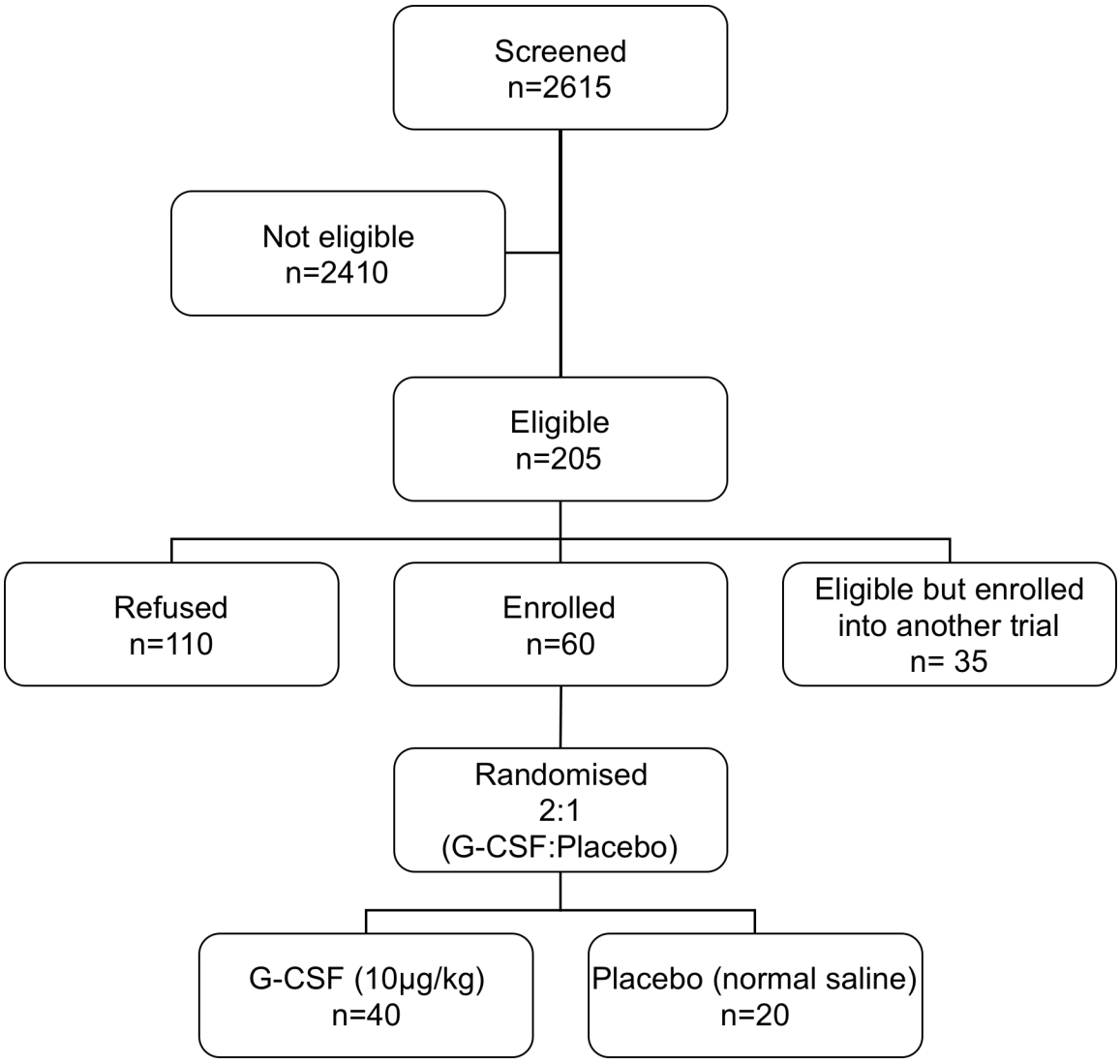


Table 18. Baseline patient characteristics by treatment group

Characteristic	All	G-CSF	Placebo
Number	60	38	22
Age (years) †	71.5 (11.8)	72.0 (12.3)	70.6 (11.1)
Male (%) †	32 (53)	20 (53)	12 (55)
NIHSS	10 [5-15]	10 [4-15.3]	8.5 [6-15.3]
Time to randomisation (days) †	8.1 (4.8)	8.0 (4.8)	8.3 (4.8)
Stroke type (%) †			
Ischaemic	51 (85)	34 (90)	17 (77)
Haemorrhagic	9 (15)	4 (11)	5 (23)
Clinical syndrome (%) †			
Lacunar	13 (22)	8 (21)	5 (23)
Partial anterior circulation	19 (32)	10 (26)	9 (41)
Total anterior circulation	27 (45)	19 (50)	8 (36)
Posterior circulation	1 (2)	1 (3)	0 (0)
TOAST subgroup (%) ‡			
Small Vessel	8 (16)	7 (18)	1 (5)
Large Vessel	15 (29)	8 (21)	7 (32)
Cardio-embolic	19 (37)	14 (37)	5 (23)
Unknown	8 (16)	5 (13)	3 (14)
Past Medical History (%)			
Hypertension	31 (52)	17 (45)	14 (64)
Hyperlipidaemia	27 (45)	16 (42)	11 (50)
Diabetes mellitus	6 (10)	2 (5)	4 (18)
Atrial Fibrillation	9 (15)	5 (13)	4 (18)
Stroke	9 (15)	5 (13)	4 (18)
TIA	6 (10)	4 (11)	2 (9)
Ischaemic Heart Disease	17 (28)	10 (26)	7 (31)
Peripheral Vascular Disease	0	0	0
Thrombolysis at admission (%)	4 (7)	3 (8)	1 (4)
Antiplatelet treatment (%)			
Aspirin	49 (82)	34 (90)	15 (68)
Dipyridamole	40 (67)	28 (74)	12 (55)
Clopidogrel	1 (2)	0 (0)	1 (4)
Statin treatment (%)	48 (80)	32 (84)	16 (73)
Infection present (%) †	14 (23)	10 (26)	4 (18)
Time stroke to first dose (days)	9.7 (4.9)	9.4 (4.9)	10.3 (4.9)

Data: Number (%), mean (standard deviation), median [interquartile range]; NIHSS: National Institutes of Health Stroke Scale; TOAST (Trial of org 10172 in acute stroke treatment) classification; † minimisation variables; ‡ haemorrhagic stroke excluded

Table 19. Clinical outcome encompassing serious adverse events (primary outcome), functional measures and lesion volume by treatment group ('on-treatment' analysis)

Event	G-CSF	Placebo	p
	N=38	N=22	
Mean N° serious adverse events	0.6 (0.9)	0.6 (1.1)	0.91
Mean N° adverse events	1.2 (1.2)	1.0 (1.1)	0.42
N° of patients with SAE	15 (40)	7 (32)	0.59
Non-fatal	12 (30)	7 (35)	1.0
Death	3 (8)	0 (0)	0.29
Vascular event	6 (16)	3 (14)	1.0
Fatal	2 (5)	0 (0)	0.53
Non-fatal stroke	1 (3)	0 (0)	1.0
Non-fatal MI	1 (3)	1 (5)	1.0
Venous thromboembolism †	3 (8)	2 (9)	1.0
All Infections	7 (18) ‡	5 (23)	0.74
Lower respiratory tract	6 (16)	2 (9)	0.70
Urinary tract	2 (5)	2 (9)	0.62
Other	0 (0)	1 (5) ¶	0.37
Timing			
During treatment/wash-out	5 (13)	5 (23)	0.47
During follow-up	10 (26)	2 (9)	0.18
Functional measures	N=38	N=22	
Modified Rankin Scale (/6)	3 [2-4]	3 [2-4]	0.68
Barthel Index	75 [33-98]	75 [40-93]	0.65
NIHSS	4.0 [2-9]	5.0 [2.5-11]	0.38
Δ NIHSS (Day 0 – Day 90)	4.0 [2-6]	4.0 [2.5-5]	0.72
Motoricity Index (/100)	77 [13-100]	67 [18-81]	0.46
Grip Strength (kg)	5.5 (7.5)	4.1 (4.3)	0.45
NEADL (/66)	25 [5-40]	16 [4-32]	0.60
Zung Depression score ∞	50 [40-64]	50 [38-63]	0.95
MMSE ∞	27 [20-29]	24.5 [21-29]	0.87
Stroke Lesion volume (cm^3) ††	N=14	N=6	
Day 0 (DWI)	9.8 (9.0)	26.9 (4.2)	0.32
Day 90 (T2-weighted)	5.2 (10.2)	27.6 (4.0)	0.51
Difference (day 90 - day 0) ∂	-1.9 (2.0)	1.03 (1.2)	0.06
Contralateral ventricular vol (cm^3)			
Day 0 (DWI)	23.1 (12.6)	22.7 (8.2)	0.96
Day 90 (DWI)	24.3 (13.5)	23.2 (8.9)	0.87
Difference (day 90 – day 0) ∂	1.2 (1.8)	0.5 (2.0)	0.47

Number (%), mean (standard deviation); comparison by Fisher's Exact Test, Mann-Whitney U test, t-test, or Analysis of Covariance. ∂ adjusted for baseline lesion volume (ANCOVA). NEADL, Nottingham Extended Activities of Daily Living; MMSE, Mini-Mental State Examination; SNSS, Scandinavian Neurological Stroke Scale; DWI: Diffusion weighted imaging; † Includes deep vein thrombosis and pulmonary embolism; ‡ One participant had both urinary tract and lower respiratory tract infections; ¶ Clostridium difficile diarrhoea; ∞ Dysphasic participants who were unable to answer questions were excluded; †† geometric means and SD.

Table 20. (i) Serious adverse events (SAE) and (ii) adverse events (AE) by treatment group and their relationship to study drug (blinded adjudication).

(i)

Group	Subject N°	Serious Adverse Event	Days post Rx	Relationship to drug
Placebo	9	UTI	15	Unlikely
	10	Aspiration pneumonia	2	Possible
	17	Functional weakness	8	Possible
		Clostridium difficile diarrhoea	16	Not related
	30	UTI	0	Not related
		Urinary retention	4	Unlikely
		Lower GI bleed	17	Unlikely
		DVT	70	Unlikely
	31	Aspiration pneumonia	8	Possible
	36	Neurological deterioration	6	Possible
		PE	9	Possible
		DVT	20	Possible
	37 †	STEMI	15	Unrelated
		Seizure	66	Possible
G-CSF	5	Aspiration pneumonia	26	Not related
	6	ARF 2^{o} to outflow obstruction	15	Possible
		UTI	16	Possible
		Post stroke psychosis	7	Possible
		NSTEMI	11	Possible
	7	Muscular chest pain	58	Not related
		Ischaemic stroke	62	Not related
	8	Aspiration pneumonia	0	Not related
		DVT	17	Unlikely
	13	Cardiac arrest 2^{0} to massive PE	15	Unlikely
	14	Admission for inguinal hernia	11	Not related
	15 †	Aspiration pneumonia	2	Unlikely
	20	Aspiration pneumonia	1	Possible
		UTI	44	Not related
	23	Anaemia under investigation	13	Possible
	25 †	DVT	22	Not related
		PE	26	Not related
	26	Community pneumonia causing death	86	Not related
	35	Ischaemic bowel causing death	24	Unlikely
	41	UTI	31	Unlikely
		Aspiration pneumonia	49	Unlikely
	45	Admitted for investigation of PE	26	Not related
	46	Hyperaesthesia	6	Unlikely

† Haemorrhagic stroke; UTI, urinary tract infection; DVT, deep vein thrombosis; PE pulmonary embolus; STEMI, ST elevation myocardial infarction; ARF, acute renal failure; NSTEMI, non-ST elevation myocardial infarction.

(ii)

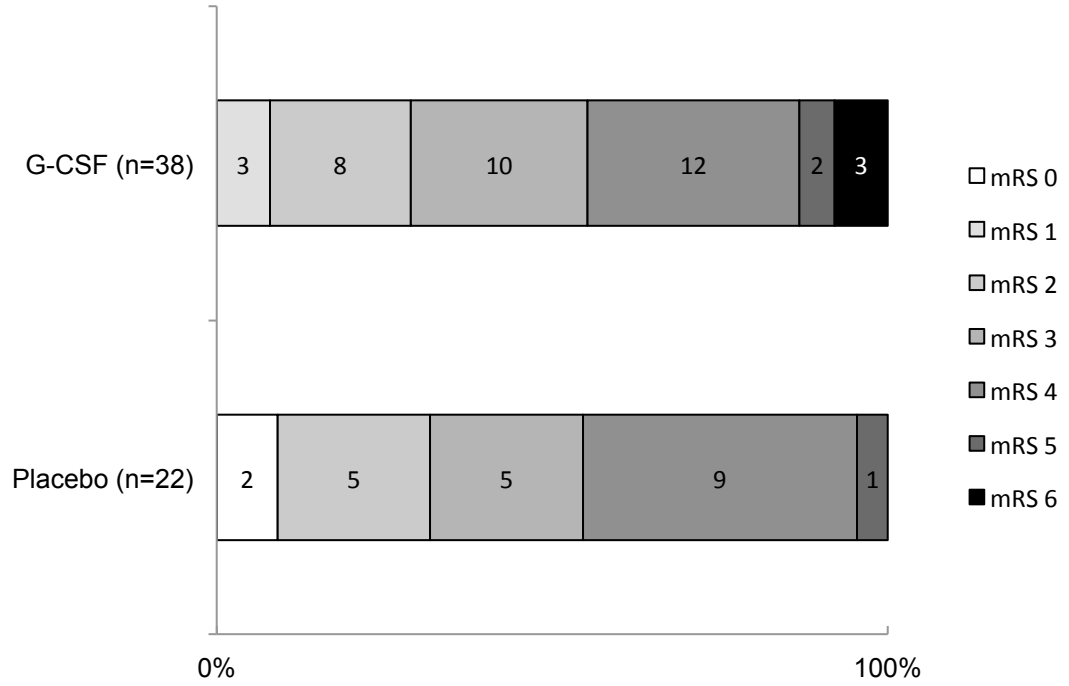
Group	Subject N^{o}	Adverse Event (not serious)	Days post Rx	Relationship to drug
Placebo	4	Depression	18	Unlikely
	10	Neuropathic pain	7	Unlikely
		Depression	20	Unlikely
	11	Exacerbation COPD	34	Not related
		Oral candidiasis	45	Not related
	17	Angina	4	Possible
		Functional weakness	75	Not related
		Functional weakness	80	Not related
	19	UTI	17	Unlikely
	22	Abdominal bruising	0	Definite
	24	Hypokalaemia 2^{0} to thiazide	5	Unrelated
		UTI	7	Possible
		Neuropathic pain	22	Not related
		Spasticity	56	Not related
	30	LRTI	10	Possible
		LRTI	67	Not related
	31	Hypothyroidism	5	Not related
	40 †	Intertrigo	5	Possible
		Back pain	67	Not related
	52	Bad dreams	4	Unlikely
G-CSF	2	Minor epistaxis 2^{0} anticoagulation	8	Unlikely
		UTI	18	Unlikely
		Hypokalaemia 2^{0} to thiazide	24	Not related
		Statin induced rise in liver enzymes	25	Not related
	3	Back pain post trial drug injections	0	Probably
	8	Dizziness post injection	0	Probable
		Oral candidiasis	5	Possible
		Abdo pain 2^{0} constipation	16	Unlikely
	12	UTI	53	Not related
		Vasovagal syncope	41	Not related
	13	Abdominal bruising	0	Definite
	16	Headache after 3rd dose only	2	Possible
	18 †	Viral diarrhoea	21	Not related
	20	UTI	52	Not related
	21 †	UTI	7	Possible
		UTI	46	Not related
		UTI and haematuria	67	Not related
	21	Deranged LFTs	67	Not related
	25 †	Deranged LFTs	9	Possible
		Oral Candidiasis	15	Possible
	29 †	Muscular shoulder pain	55	Not related

32	UTI	3	Possible
38	UTI	6	Possible
	Abdominal bruising	1	Definite
39	Transient hypotension	5	Possible
41	UTI	89	Not related
43	Back pain	0	Probable
	Frozen shoulder	66	Not related
	Swollen hand 2^0 to osteoarthritis	40	Not related
45	Thrombocytopenia	7	Probable
	Leucopoenia	18	Possible
46	UTI	6	Possible
	UTI	20	Unlikely
49	UTI	10	Possible
	Transient left arm pain	4	Possible
50	Epistaxis	8	Possible
	UTI with renal impairment	12	Possible
	UTI	33	Not related
51	Simple fall	9	Unlikely
54	Photopsia	4	Possible
	Muscular chest pain	6	Unlikely
	Allergic rash to morphine	7	Unrelated
55	Dizziness	3	Possible
57	Neuropathic pain	19	Unlikely
	UTI	26	Unlikely
	Deranged LFTs	33	Unlikely
58	Gout	90	Not related

† Haemorrhagic stroke; UTI, urinary tract infection; COPD, chronic obstructive pulmonary disease; LFT, liver function tests.

No significant difference emerged between treatment and placebo groups in respect of measurements of dependency (mRS) (figure 15), disability (BI, NEADL), impairment (NIHSS, Motoricity index, grip strength), cognition (MMSE) or mood (Zung depression score) (table 19). When mRS is dichotomised (poor outcome >2), no difference is observed between groups (G-CSF 27/38 vs placebo 15/22, p=0.82).

Figure 15. Distribution of the modified Rankin scale (mRS) by treatment group ('on-treatment' analysis)



Volumetric analysis

Stroke lesion volume was analysed at baseline and day 90 in 20 participants (14 G-CSF, 6 placebo) (table 19). Scans were not indicated in 9 recruits (primary haemorrhage) and not performed due to patient refusal (n=17), contraindication (n=4), scan intolerance (n=1) and severe illness or death (n=5). It was not possible to analyse 4 scans due to poor quality and the acute lesion becoming embedded in chronic ischaemic change. Stroke lesion territory and size varied considerably but there was no significant difference in baseline DWI lesion volume (table 19) between placebo and treatment groups. When adjusted for baseline DWI lesion volume, a trend towards reduced lesion volume at day 90 was present in G-CSF treated patients (ANCOVA, p=0.06). With respect to change in ventricular volume over 90 days, no significant difference was seen between groups (table 19).

Laboratory measures

In comparison with placebo, G-CSF significantly elevated CD34+ cell count (peak 31.4 versus 3.3 cells/μL), white cell count (peak $40.2x10^9$ vs $9.5x10^9$ cells/L), and white cell components including neutrophils (peak $34.1x10^9$ vs $6.9x10^9$ cells/L), at day 5 (table 21, figure 16). By day 10, counts were returning to normal. There were apparent absolute differences between groups in baseline values in BNP and D-dimer but they did not reach statistical significance (p=0.08 and 0.07 respectively). Change in haemoglobin, platelet count, red cell count, haematocrit, D-dimer, BNP, MMP-9 and protein S100-β did not differ significantly between the treatment groups.

Table 21. Laboratory measures by treatment group: G-CSF versus placebo.

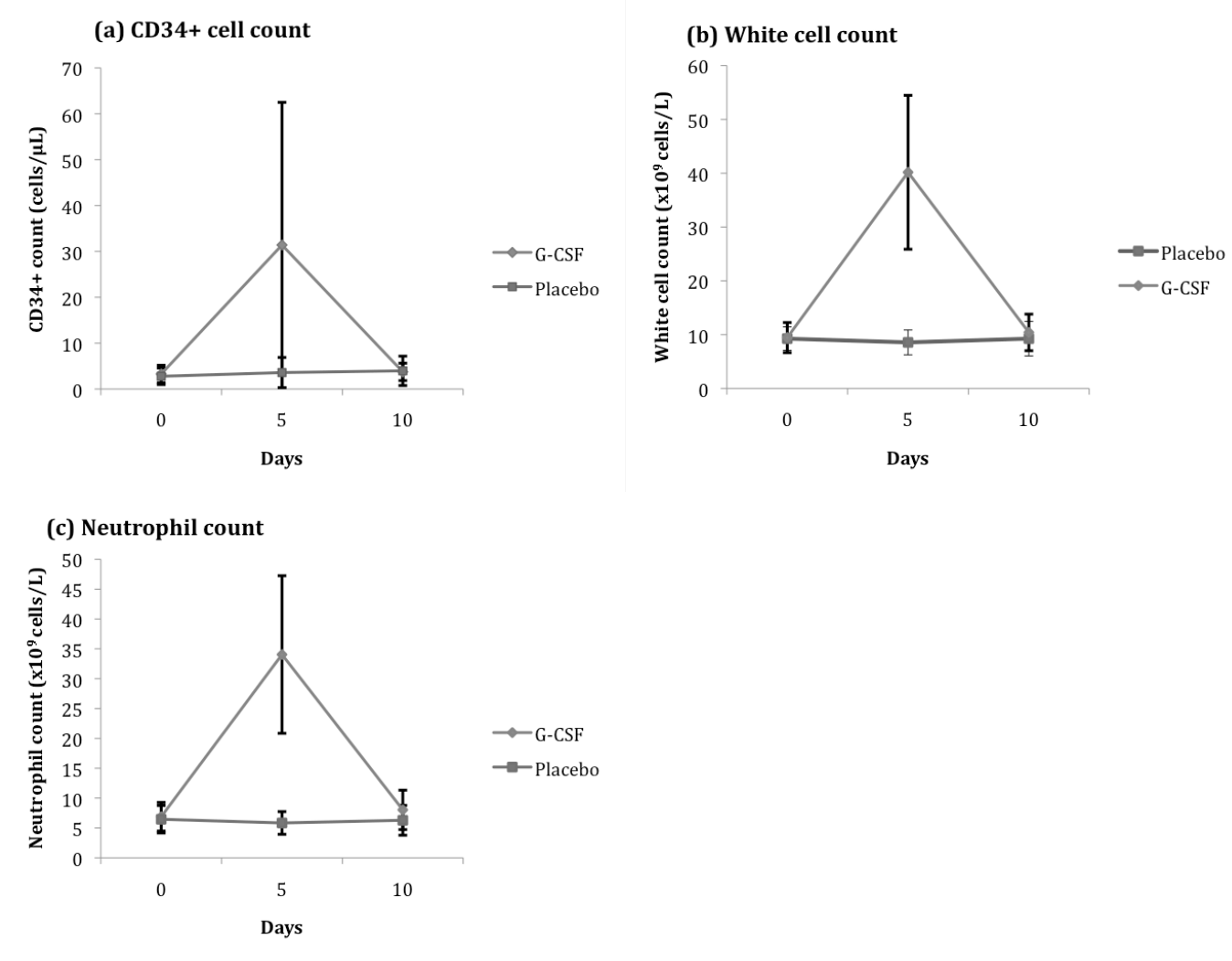
Mean (standard deviation); comparison by repeated measures analysis of variance

	G-CSF			Placebo			ANOVA
Day of treatment	**0**	**5**	**10**	**0**	**5**	**10**	**p**
Haemoglobin (g/dL)	13.6 (1.9)	13.4 (1.8)	13.4 (1.9)	14.1 (1.2)	13.8 (1.4)	13.6 (1.3)	0.49
White cells ($x10^9$/L)	9.45 (2.8)	40.2 (14.3)	10.41 (3.4)	9.26 (2.2)	8.56 (2.3)	9.26 (3.2)	<0.001
Neutrophils ($x10^9$/L)	6.88 (2.4)	34.1 (13.2)	8.03 (3.3)	6.46 (2.3)	5.84 (1.9)	6.29 (2.5)	<0.001
Lymphocytes ($x10^9$/L)	1.56 (0.5)	2.74 (0.9)	1.58 (0.6)	1.81 (0.5)	1.80 (0.4)	1.85 (0.5)	0.22
Monocytes ($x10^9$/L)	0.79 (0.3)	2.39 (1.1)	0.78 (0.9)	0.77 (0.2)	0.71 (0.3)	0.76 (0.3)	<0.001
Eosinophils ($x10^9$/L)	0.15 (0.2)	0.36 (0.4)	0.15 (0.1)	0.23 (0.2)	0.22 (0.2)	0.20 (0.1)	0.99
Basophils ($x10^9$/L)	0.02 (0.04)	0.07 (0.16)	0.01 (0.03)	0.02 (0.04)	0.03 (0.05)	0.03 (0.05)	0.61
CD34+ (/µL)	3.27 (1.9)	31.40 (31.1)	3.74 (1.9)	2.78 (1.8)	3.59 (3.3)	3.97 (3.2)	<0.001
Platelets ($x10^9$/L)	240 (93)	279 (89)	238 (93)	260 (63)	272 (42)	302 (61)	0.17
Red cell count ($x10^{12}$/L)	4.53 (0.6)	4.43 (0.6)	4.51 (0.6)	4.55 (0.5)	4.46 (0.5)	4.22 (1.0)	0.63
Haematocrit (L/L)	0.41 (0.05)	0.41 (0.05)	0.40 (0.05)	0.42 (0.04)	0.41 (0.04)	0.41 (0.04)	0.51
D-dimer (ng/ml) †	2952 (2009)	2560 (1819)	2440 (1811)	1844 (1682)	1635 (1768)	1586 (1789)	0.10
BNP (pg/ml) †	281.9 (501.6)	219.9 (315.9)	206.8 (303.4)	89.9 (101.7)	97.1 (102.5)	118.1 (134.6)	0.15
MMP-9 (ng/ml) †	284.2 (325.3)	259.1 (265.3)	231.6 (174.4)	374.8 (407.4)	202.5 (204.1)	163.9 (102.8)	0.87
S100-β (pg/ml) †	107.4 (30.7)	105.5 (17.0)	113.0 (34.0)	103.1 (11.1)	109.1 (31.0)	104.9 (18.8)	0.60

BNP: ß-natriuretic peptide; MMP-9: matrix metalloproteinase-9. †, n=41 (G-CSF 26, placebo 15)

Figure 16. CD34+ cell, white cell and neutrophil count at days 0, 5 and 10 by treatment group.

Mean (standard deviation). Analysis by repeated measures ANOVA, p<0.001 for a, b and c.



DISCUSSION

The STEMS-2 trial was designed to test the safety of G-CSF and explore mechanisms by which it might work in patients with subacute stroke. There were no significant differences in serious adverse events (primary outcome) between two well-matched groups, supporting the view that treatment with G-CSF is safe. Of note, there were no differences in either the number of patients with a SAE or the average number of SAEs per patient. The inclusion of 9 haemorrhagic strokes also provides initial safety data, though further phase II trials designed specifically for this subgroup are warranted. The majority of subcutaneous injections were received suggesting that the treatment is also feasible to administer, at least when given subacutely. Adverse events experienced that were probably or definitely related to the study drug were expected side effects of G-CSF (musculoskeletal pain, dizziness). Despite significant increases in white cell count, no difference in the frequency of vascular events (arterial and venous) or infection, were seen. Two of three deaths in the G-CSF group were vascular in nature and did not appear to be secondary to active treatment; the overall vascular event rate was identical in each group (15%).

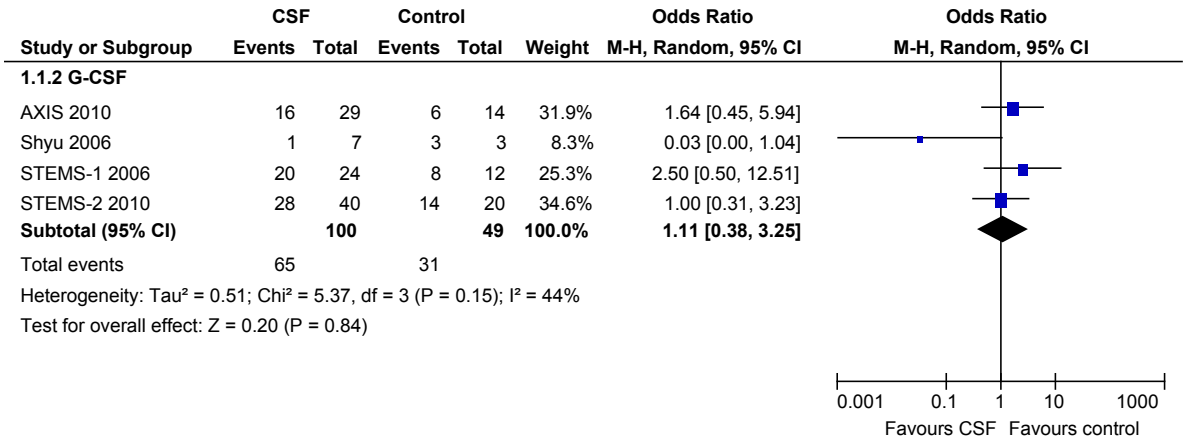
Although no significant differences between groups were seen with respect to functional outcome (and the trial was not powered to detect this), a trend towards improvement was observed in NIHSS at 90 days in G-CSF treated subjects. This is consistent with other measured clinical parameters (motoricity index, grip strength, NEADL) whereby the median value in the G-CSF group was non-significantly better when compared to placebo. Further phase III trials are therefore required to test efficacy of G-CSF. Similarly, a trend to reduced

stroke lesion volume and growth in the G-CSF group was observed. This is unexpected considering G-CSF administration in the subacute phase is thought to improve outcome via promotion of neurogenesis [137] and angiogenesis [235] rather than through neuroprotection. Such volume differences should be interpreted with caution due to the small sample size and heterogeneity of case mix, though these were accounted for, in part, by adjusting for baseline stroke volume. In addition, preclinical concern about the effects of G-CSF on cerebral atrophy [167] was not borne out here; subgroup analysis revealed no significant difference between groups in change in contralateral ventricular volume, though the numbers are small and 90 days may be an insufficient follow-up period.

Altogether, four trials have been published evaluating G-CSF in ischaemic stroke – this and the first STEMS study, and two others.[295, 368] Of these, the first assessed 10 patients (7 in the G-CSF arm dosed at 15μg/kg od for 5 days, 3 in the control arm) randomised within 7 days of ictus and no safety concerns were raised.[295] In the AXIS trial (n=44), where treatment was instituted with 12 hours of stroke onset at a cumulative dose range of 30 to 180 μg/kg,[368] there was no significant difference between treatment groups in respect of serious adverse events and thromboembolic complications. Although G-CSF transiently increases total white cell count and therefore has potential to lead to vascular complications, there is currently no evidence to suggest that G-CSF causes thomboembolic events or aggravates stroke symptoms.[299] Each of the four trials was too small individually to assess the effect of G-CSF on functional outcome; when data from the four trials are aggregated, no safety concern is obvious (odds ratio 1.11, 95% confidence intervals 0.38 to 3.25, figure 17). In light of the above evidence and a recent negative trial assessing administration of EPO

(another colony stimulating factor [249]) within 6 hours of ischaemic stroke [256] further phase II/III trials are required to evaluate the safety and efficacy of G-CSF.

Figure 17. Meta-analysis of the effect of G-CSF on functional outcome events assessed as dependency (modified Rankin score>2 or Barthel index<60)



Whilst the trial provides robust clinical data on the safety of G-CSF in stroke, a number of limitations need to be taken into account. First, the small sample size means that any findings may be due to chance; although there are no significant safety concerns in the current investigation, trends that are present here (e.g. higher number of deaths in the G-CSF group, though not thought to be related to treatment) may become significant in a larger phase II/III trial. Another caveat is the wide time-window of inclusion post stroke onset, which has implications on interpreting stroke lesion volume and serum biomarker results. The variable time of inclusion (between 3 and 30 days) leads to heterogeneity within the trial and it becomes difficult, therefore, to make fair comparisons between groups. Since patients were enrolled into the trial during the subacute phase of stroke (mean time to first dose of 10 days), any potential

effects of treatment on biomarker expression may have been missed; the peak serum levels of S100β occur within 2-3 days, and MMP-9 within early hours after a stroke, and are both related to infarct volume.[406, 407] The trial was designed, however, to evaluate the potential 'neuroreparative' qualities of G-CSF (via CD34 cell migration) and not early neuroprotective mechanisms.

In summary, this randomised double-blind placebo-controlled trial suggests that G-CSF is safe when administered in sub-acute stroke.

CHAPTER 5

COLONY STIMULATING FACTORS (INCLUDING ERYTHROPOIETIN, GRANULOCYTE COLONY STIMULATING FACTOR AND ANALOGUES) FOR STROKE

Publications contributing to this chapter:

Bath PM, Sprigg N. Colony stimulating factors (including erythropoietin, granulocyte colony stimulating factor and analogues) for stroke. *Cochrane Database of Systematic Reviews*. 2006;3:CD005207

This chapter is an update of the above review and has been submitted for publication. The chapter is laid out in the CDSR format.

ABSTRACT

Background

Colony stimulating factors (CSFs), also called haematopoietic growth factors, regulate bone marrow production of circulating red and white cells, and platelets. They have been shown to be neuroprotective in experimental stroke. Some CSFs also mobilise the release of bone marrow stem cells into the circulation.

Objectives

To assess the effects of CSFs on functional outcome and haematology measures in patients with acute or subacute stroke.

Search methods

We searched the Cochrane Stroke Group Trials Register (last searched December 2010), the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library Issue 2, 2006), MEDLINE (1985 to December 2010), EMBASE (1985 to December 2010), and Science Citation Index (1985 to December 2010). In an attempt to identify further published, unpublished and ongoing trials we contacted manufacturers and principal investigators of trials (last contacted January 2011). We also searched reference lists of relevant articles and reviews.

Selection criteria

Unconfounded randomised controlled trials recruiting patients with acute or subacute ischaemic or haemorrhagic stroke were included. CSFs included stem cell factor (SCF), erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF),

macrophage-colony stimulating factor (M-CSF, CSF-1), and thrombopoietin (TPO), or analogues of these. The primary outcome was functional outcome (assessed as combined death or disability and dependency using scales such as the modified Rankin Scale or Barthel Index) at the end of the trial. Secondary outcomes included safety at the end of treatment (death, impairment, deterioration, extension or recurrence, serious adverse events, infection), death at the end of follow up, infarct volume and haematology measures (blood counts after treatment had been completed). Two review authors (TE and NS) independently extracted data and assessed trial quality. Study authors were contacted for additional information.

Results

In two trials (n=562), EPO therapy was significantly associated with death by the end of the trial (odds ratio 1.98, 95% confidence interval 1.17 to 3.33, p=0.01) and a non-significant increase in serious adverse events. EPO significantly increased red cell count with no effect on platelet or white cell count, or infarct volume. Two small trials of carbamylated EPO have been completed but have yet to be reported. In six small trials (n=215), G-CSF was associated with a non-significant reduction in early impairment and early death but had no effect on functional outcome at end of trial. G-CSF significantly elevated white cell count and CD34+ cell count, but had no effect on infarct volume. Further trials of G-CSF are ongoing.

Authors' conclusions

No large trials of EPO, G-CSF or other colony stimulating factors have been performed and it is too early to know whether CSFs improve functional outcome.

BACKGROUND

Colony stimulating factors (CSFs), also called haemopoietic growth factors, regulate the bone marrow production of circulating red cells, white cells and platelets. CSFs act on stem cells leading to lineage specific differentiation. Stem cell factor (SCF) regulates differentiation of CD34+ stem cells, whilst other factors modulate the synthesis of more specific cell types: erythropoietin (EPO) for red cells; granulocyte colony stimulating factor (G-CSF) for neutrophils; granulocyte-macrophage colony stimulating factor (GM-CSF) for macrophages and neutrophils; macrophage colony stimulating factor (M-CSF, also called colony-stimulating factor-1 (CSF-1)) for monocytes; and thrombopoietin (TPO) for megakaryocyte maturation and platelet synthesis. Evidence now suggests that these factors may be candidate treatments for stroke, either as potential neuroprotectants or as neuroreparative agents.[408]

The background to CSFs for use in stroke has been discussed in the introduction. Their physiological actions and positive data relating to their use in experimental stroke justify the assessment of recombinant haemopoietic growth factors in clinical stroke. This report systematically reviews the effects of CSFs in patients with acute or subacute stroke.

OBJECTIVES

Hypothesis

Colony stimulating factors (haemopoietic growth factors) may improve functional outcome after ischaemic or haemorrhagic stroke.

Aims

To assess:

(1) the safety and efficacy of CSFs in patients with acute or subacute ischaemic or haemorrhagic stroke;

(2) the effect of CSFs on circulating stem and blood cell counts.

METHODS

Types of studies: unconfounded randomised controlled trials.

Types of participants: People with recent (within 30 days of onset) ischaemic or haemorrhagic stroke. This time period allows studies investigating either acute neuroprotection (for example, through possible anti-inflammatory or anti-apoptotic effects) or recovery enhancement (for example, through putative stem cell mediated events) to be included.

Types of interventions: we considered unconfounded trials of:

- stem cell factor (SCF) or analogue versus control;
- erythropoietin (EPO) or analogue versus control;
- granulocyte colony stimulating factor (G-CSF) or analogue versus control;

- granulocyte-macrophage colony stimulating factor (GM-CSF) or analogue versus control;
- macrophage-colony stimulating factor (M-CSF, CSF-1) or analogue versus control; and
- thrombopoietin (TPO) or analogue versus control.

Types of outcome measures:

Primary outcome

- Functional outcome at end of follow up, assessed as combined death or disability and dependency (using scales including the modified Rankin Scale and Barthel Index).

Secondary outcomes

- At the end of treatment: death; impairment (using a stroke severity scale, for example, the Scandinavian Stroke Scale or the National Institute of Health Stroke Scale); deterioration (death or worsening in stroke severity scale); extension or recurrence; number of patients with a serious adverse event; number of patients with an infection; stroke lesion volume.
- At the end of follow up: death.
- Haematology measures (during or soon after treatment): CD34+ count; red cell count (RCC); white cell count (WCC); platelet count.

Search methods for identification of studies

We searched the Cochrane Stroke Group Trials Register, which was last searched by the Review Group Co-ordinator in December 2010. In addition, we performed electronic searches of the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library Issue 12, 2010), MEDLINE (1985 to

December 2010), EMBASE (1985 to December 2010), and Science Citation Index (1985 to December 2010) (Appendix 1). These searches commenced from 1985, the date when EPO (the first recombinant CSF) became available. Additionally, we contacted the manufacturers of SCF, EPO, G-CSF, GM-CSF, M-CSF and CSF-1, and TPO and the principal investigators of any identified trials (last contacted January 2011). Finally, we searched the reference lists of relevant articles and clinical reviews of these colony stimulating factors.

We used the following search strategy for MEDLINE and adapted it for the other databases.

1. Hematopoietic Cell Growth Factors/
2. Colony-stimulating factors/ or exp colony stimulating factors, recombinant/ or exp erythropoietin/ or exp granulocyte colony-stimulating factor/ or exp granulocyte-macrophage colony-stimulating factor/ or interleukin-3/ or macrophage colony-stimulating factor/
3. Stem Cell Factor/
4. Thrombopoietin/
5. (Haematopoieti$ or hematopoieti$ or erythropoietin or epoetin or darbepoetin or colony stimulating factor or colony-stimulating factor or interleukin-3 or Filgrastim or thrombopoietin or stem cell factor).tw.
6. (G-CSF or M-CSF or GM-CSF or CSF-1 or HGFs or IL-3 or MGI-1 or TPO or SCF or E21R).tw.
7. or/1-6
8. Cerebrovascular disorders/ or exp basal ganglia cerebrovascular disease/ or exp brain ischemia/ or exp carotid artery diseases/ or exp cerebrovascular accident/ or exp hypoxia-ischemia, brain/ or exp intracranial arterial diseases/ or

intracranial arteriovenous malformations/ or exp "intracranial embolism and thrombosis"/ or exp intracranial hemorrhages/ or vasospasm, intracranial/ or vertebral artery dissection/

9. ((Brain or cerebr$ or cerebell$ or vertebrobasil$ or hemispher$ or intracran$ or intracerebral or infratentorial or supratentorial or middle cerebr$ or mca$ or anterior circulation) adj5 (isch?emi$ or infarct$ or thrombo$ or emboli$ or occlus$ or hypoxi$)).tw.

10. ((Brain$ or cerebral or cerebell$ or intracranial or subarachnoid) adj5 (haemorrhage or hemorrhage or hematoma or haematoma or bleed$ or aneurysm)).tw.

11. or/8-10

12. 7 and 11

13. Limit 12 to human

Data collection and analysis

Selection of studies

We independently selected trials for inclusion. Disagreements between authors were resolved by discussion.

Quality

We assessed the methodological quality of trials using standard Cochrane criteria including determining methods of randomisation, concealment of allocation, blinding of outcome assessment, analysis by intention to treat, and participants lost to follow up. An intended sensitivity analysis assessing the primary outcome in high quality trials alone was not performed since only two EPO trials and six G-CSF trials had been completed.

Data extraction

We independently extracted data from publications on quality parameters (as above), drug, dose, route and timing, the numbers of events or status at end of treatment and end of follow up (including death, dependency), and haematological measures. If necessary, we sought additional information from the chief investigators of identified trials and pharmaceutical companies. We defined the primary outcome to be poor if there was a modified Rankin score of >2 (7 of 8 trials) or Barthel Index <60 (1 trial [295]). To enable comparison of neurological impairment, data from one trial [294] was converted from the Scandinavian Neurological Stroke Score to the National Institutes of Health Stroke Score using a recognised formula.[409] Early neurological impairment included data collected between 3 and 10 days post randomisation and data extracted on mortality was defined as early (within 14 days of randomisation) or 'end of trial'.

Data synthesis

We analysed data by intention to treat where available, tested for heterogeneity using I-squared (I^2), and planned to calculate a weighted estimate of the typical treatment effect across trials. Random-effects models were used since between-trial sources of heterogeneity were expected, for example, due to biological differences in CSFs, and clinical and statistical differences in the trials. Treatment effects were determined using the odds ratio for dichotomous data and weighted or standardised mean difference for continuous data. The Cochrane Collaboration's Review Manager software, Review Manager 5 (Apple Mac), was used for data entry and analysis.

RESULTS

Description of studies

Erythropoietin

Two completed trials of EPO were identified (table 24). The first involved 40 participants with hyperacute (less than seven hours of onset) ischaemic stroke; data from 13 participants treated in an uncontrolled phase of this study were excluded.[255] A further phase III trial of EPO included 522 patients with middle cerebral artery ischaemic stroke.[256] Two small safety trials of carbamylated erythropoietin, a derivative of EPO without haematopoietic activity, have been completed.[410, 411]

Granulocyte-colony stimulating factor

Six completed trials of G-CSF were found (table 22). The first involved 10 participants within seven days of ischaemic stroke;[295] STEMS-1 involved 36 participants seven to 30 days post ischaemic stroke;[294] STEMS-2 included 60 patients three to 30 days post ischaemic or haemorrhagic stroke in a phase IIb trial.[304] Another included 45 participants within seven days of ischaemic stroke but has been published in abstract form only.[296] AXIS was a hyperacute trial (randomisation within 12 hours of ictus) involving 44 patients with ischaemic stroke,[297] whilst STEMTHER was an unblinded safety trial with 20 participants.[303] Another more recent randomised controlled trial performed in Thailand was identified in abstract form but without enough information to include in the analysis.[412] Results from an uncontrolled study of G-CSF in 20 participants are apparently still awaited (Bogdahn 2005). 2 other trials of G-CSF are ongoing: a safety trial assessing 20 patients with non-dominant hemisphere

stroke,[413] and a larger hyperacute trial with a recruitment target of 328 patients.[302]

Other CSFs

No completed or ongoing studies of SCF, GM-CSF, M-CSF, or TPO in patients with stroke were identified.

Risk of bias in included studies

The two published trials of EPO and four of the G-CSF trials were triple blind with treatment compared with placebo, with adequate allocation concealment, and all measures were made blinded to treatment assignment (Ehrenreich 2002; STEMS-1 2006; Zhang 2006; Ehrenreich 2009; AXIS 2010; STEMS-2 2010).[255, 256, 294, 296, 297, 304] Shyu 2006 was single blind, non-placebo controlled, with blinded outcome assessments.[295] STEMTHER was an open label randomised trial with no placebo;[303] the method of randomisation is unclear. In two trials investigators held a patent on the effects of the respective colony stimulating factor in the treatment of stroke (table 23).[256, 297] Sample size justification was given in two trials.[256, 304]

Effects of interventions

EPO non-significantly reduced combined death or dependency, as measured by the modified Rankin Scale, in two trials including 562 patients,[255, 256] but with 92% of the weight resulting from the later phase III trial. Conversely, death by the end of the trial was significantly increased almost two-fold (odds ratio 1.98, 95% confidence interval 1.17 to 3.33, p=0.01, table 24, figure 19, analysis 1.2).

There was also a non-significant trend towards increased number of serious adverse events in the EPO group. EPO significantly increased red cell count (table 24, figure 25, analysis 1.8) with no effect on platelet or white cell count. EPO had no discernible effect on stroke infarct volume.

G-CSF did not alter combined death or dependency, early death or end of trial death; however, a trend to reduction in early impairment as measured by NIHSS was seen in G-CSF treated patients. G-CSF was associated with a non-significant increase in serious adverse events but no effect on the rate of infection. G-CSF significantly increased white cell (table 24, figure 24, analysis 1.7) and CD34+ count (table 24, figure 27, analysis 1.10) but had no effect on other blood count variables (table 24, figures 25 and 26, analyses 1.8 and 1.9). G-CSF had no discernible effect on infarct volume (table 24, figure 29, analysis 1.12).

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Table 22. Characteristics of included and ongoing studies

Included studies

Study	Methods	Participants	Interventions	Outcomes
AXIS 2010	National, multicenter, randomised, placebo-controlled dose escalation study	44 patients (6 German centres) with acute (< 12 hours of onset) ischaemic MCA-territory stroke and diffusion/perfusion 'mismatch' on MRI	G-CSF, dose-escalation (4 levels, cumulative dose range between 30µg/kg and 180µg/kg over 3 days) versus control	(1) Thromboembolic complications by day 4 (2) Severe infection; SAEs; function (NIHSS, mRS, BI) at 4 weeks and day 90; lesion growth MRI day 90
Ehrenreich 2002	Double-blind placebo-controlled trial	40 patients (1 German centre) with acute (< 8 hours of onset) ischaemic stroke	rhEPO 3.3E4 IU iv x 3, or saline placebo	(1) NIHSS, SSS, BI, mRS (day 30) (2) serum (EPO); impairment (NIHSS, SSS); function (BI, mRS); imaging (MRI: DWI, FLAIR); biomarkers
Ehrenreich 2009	Randomised, double-blind, placebo-controlled	522 patients (German multicenter trial) with acute ischaemic stroke within 6 hours of onset.	Epoietin-alpha 40 000 IU iv within 6 hours of symptom onset, at 24 and 48 hours	(1) NIHSS, BI, mRS (day 30 and day 90) (2) Lesion volume (MRI), adverse events.
Shyu 2006	Single-blind controlled trial	10 patients (1 Taiwanese centre) with acute ischaemic stroke within 7 days of onset	G-CSF (Filgrastim) 15µg/kg sc for 5 days	(1) NIHSS, ESS, EMS, BI (12months) (2) FDG-PET
STEMS-1 2006	Double-blind placebo-controlled dose escalation trial	36 patients (2 UK centres) with acute ischaemic stroke within 7 days of onset	G-CSF (Filgrastim) 1-10µg/kg sc for 1 or 5 doses	(1) CD34+ (0 - 10 days) (2) Safety, clinical, efficacy; SSS (day 10), BI, mRS (day 90)
STEMS-2 2010	Single-centre, double-blind, randomised, placebo-controlled, phase IIb trial	60 patients (1 UK centre), 3 - 30 days post ischaemic or haemorrhagic stroke	G-CSF (Filgrastim) 10µg/kg versus control sc for 5 days	(1) Serious adverse events (2) Efficacy; SNSS, mRS, BI, EADL at days 10 and 90 (3) Lesion volume (MRI) at baseline and day 90
STEMTHER 2010	Prospective, single-centre, unmasked, randomised controlled trial	20 patients (1 Russian centre), <48hours post ischaemic stroke	G-CSF (Leukostim) 10µg/kg for 5 days versus control (no placebo)	(1) Safety (2) Efficacy; NIHSS, mRS, BI (day 90) (3) Lesion volume (MRI) and laboratory measures
Zhang 2006	Double-blind placebo-controlled trial	45 patients (1 Chinese centre) with acute ischaemic stroke within 7 days	G-CSF (Filgrastim) 2µg/kg sc for 5 days	(1) NIHSS, (2) WCC, (3) CD34+

Ongoing studies				
Study	**Methods**	**Participants**	**Interventions**	**Outcomes**
AXIS-2	A multinational, multicenter, randomised, double-blind, placebo-controlled phase II trial	328 patients aged 18-85 with acute middle cerebral artery ischaemic stroke with lesion size on MRI DWI >15cm3	G-CSF 135 mcg/kg or placebo (sodium chloride solution), short term infusion (20 to 30 minutes) and continuous infusion over 3 days	Primary: mRS at day 90; secondary: NIHSS by day 90
CEPO 2008	Randomised, double-blind, placebo-controlled, single-dose, dose-escalation study	16 patients aged 50-90 within 12-48 hours of ischaemic stroke	Dose escalation (0.005 mcg/kg to 50.0 mcg/kg) trial of Lu AA24493 or placebo	Safety, NIHSS and the mRS
CEPO 2009	Randomised, double-blind, placebo-controlled, dose-escalation study	Patients aged 50-90 within 0-48 hours of ischaemic stroke	Dose escalation (0.005 mcg/kg to 50.0 mcg/kg) trial of Lu AA24493 or placebo	Safety, NIHSS and the mRS, pharmacokinetics, immunogenicity and biomarkers
GIST 2008	Randomised, double-blind, placebo-controlled trial	20 patients with non-dominant ischaemic stroke (>3cm3) with hemiparesis	G-CSF 10 ug/kg sc once daily for 4 days, repeated once 6 weeks later, or placebo (normal saline)	Safety, feasibility, efficacy
Shabitz 2006	Randomised, double-blind, placebo-controlled trial	180 patients with a diagnosis of chronic stroke or amyotrophic lateral sclerosis	G-CSF for chronic stroke and EPO for amyotrophic lateral sclerosis (ALS)	Learning success in a word learning model; response time in motor function tests
Nilanont 2010	Block randomised according to stroke severity	36 participants with acute ischaemic stroke patients within 7 days of onset	G-CSF 15 ug/kg sc once daily for 5 days	Safety and efficacy
Studies awaiting classification				
Bogdahn 2005	Unknown	20 patients (1 German centre) with acute ischaemic stroke	G-CSF given for 5 consecutive days. Dose escalation up to 10ug/kg	Unknown

BI: Barthel index; CD34+: CD34+ cell count; DWI: diffusion weighted imaging; EADL: extended activities of daily living; EMS; European Motor Scale; ESS: European Stroke Scale; EPO: erythropoietin; FDG-PET: fluorodeoxyglucose positron emission tomography; FLAIR: fluid-attenuated inversion recovery; G-CSF: granulocyte-colony stimulating factor; iv: intravenous; MCA: middle cerebral artery; MRI: magnetic resonance imaging; mRS: modified Rankin Scale; NIHSS: National Institutes of Health Stroke Scale; rhEPO: recombinant human erythropoietin; SAE: serious adverse event; sc: subcutaneous; SSS: Scandinavian Stroke Scale; WCC: white cell count

Table 23. Risk of bias, included studies

Study	Adequate sequence generation?	Adequate allocation of concealment?	Blinding?	Incomplete outcome data addressed?	Free of selective reporting?	Free of other bias?
AXIS 2010	Yes	Yes	Adequate	Yes	Yes	Investigator holds a patent on the use of EPO for treatment of cerebral ischaemia
Ehrenreich 2002	Unclear	Yes	Adequate	Yes	Yes	None reported
Ehrenreich 2009	Yes	Yes	Adequate	Yes	No	Investigator holds a patent on the use of EPO for treatment of cerebral ischaemia
Shyu 2006	No	No	Adequate	Yes	Yes	None reported
STEMS-1 2006	Adequate	Adequate	Adequate	Yes	Yes	None reported
STEMS-2 2010	Adequate	Adequate	Adequate	Yes	Yes	None reported
STEMTHER 2010	Inadequate	Inadequate	Inadequate	No	Yes	Yes
Zhang 2006	Adequate	Adequate	Adequate	Unclear	Unclear	Unclear

Table 24. Data and analyses

Outcome or Subgroup	Studies	Participants	Effect Estimate
1.1 Functional outcome (death or dependency), end of trial			**OR [95% CI]**
1.1.1 Erythropoietin	2	562	1.01 [0.72, 1.42]
1.1.2 G-CSF	5	167	1.16 [0.50, 2.69]
1.2 Death, end of trial			**OR [95% CI]**
1.2.1 Erythropoietin	2	562	1.98 [1.17, 3.33]
1.2.2 G-CSF	6	215	1.20 [0.36, 4.07]
1.3 Early death			**OR [95% CI]**
1.3.1 Erythropoietin	2	562	1.50 [0.37, 6.05]
1.3.2 G-CSF	6	215	0.70 [0.07, 7.22]
1.4 Early Impairment, NIHSS			**WMD [95% CI]**
1.4.1 Erythropoietin	1	40	-2.20 [-10.01, 5.61]
1.4.2 G-CSF	5	203	-0.40 [-1.82, 1.01]
1.5 SAE, number of patients			**OR [95% CI]**
1.5.1 Erythropoietin	1	522	1.30 [0.89, 1.90]
1.5.2 G-CSF	4	160	1.31 [0.64, 2.68]
1.6 Infection			**OR [95% CI]**
1.6.1 Erythropoietin	0	0	-
1.6.2 G-CSF	4	160	0.91 [0.36, 2.28]

1.7 White cell count, 3 to 10 days			**WMD [95% CI]**
1.7.1 Erythropoietin	1	522	0.52 [-0.08, 1.12]
1.7.2 G-CSF	6	191	28.04 [23.73, 32.34]
1.8 Red cell count, 3 to 10 days			**WMD [95% CI]**
1.8.1 Erythropoietin	2	562	0.17 [0.06, 0.27]
1.8.2 G-CSF	3	94	0.04 [-0.19, 0.28]
1.9 Platelet count, 3 to 10 days			**WMD [95% CI]**
1.9.1 Erythropoietin	2	562	7.61 [-5.32, 20.55]
1.9.2 G-CSF	4	136	-4.21 [-35.75, 27.33]
1.10 CD34+ count, 5 to 10 days			**SMD [95% CI]**
1.10.1 Erythropoietin	0	0	-
1.10.2 G-CSF	4	140	1.77 [0.91, 2.63]
1.11 Serum S100-ß, 7 to 10 days			**WMD [95% CI]**
1.11.1 Erythropoietin	1	40	0.32[-0.82, 0.18]
1.11.2 G-CSF	1	40	0.01 [-0.01, 0.02]
1.12 Infarct volume, post treatment			**SMD [95% CI]**
1.12.1 Erythropoietin	2	485	-0.05 [-0.23, 0.13]
1.12.2 G-CSF	3	68	-0.02 [-0.52, 0.48]

G-CSF, granulocyte colony stimulating factor; OR, odds ratio; WMD, weighted mean difference; SMD, standardised mean difference

Forest plots

Figures 18 to 29 correspond to the data in table 24 (CSF, colony stimulating factor; 'random' , random effects model of analysis; M-H, Mantel–Haenszel analysis for repeated tests of independence, CI confidence interval)

Figure 18 (Analysis 1.1). Functional outcome (death or dependency), end of trial.

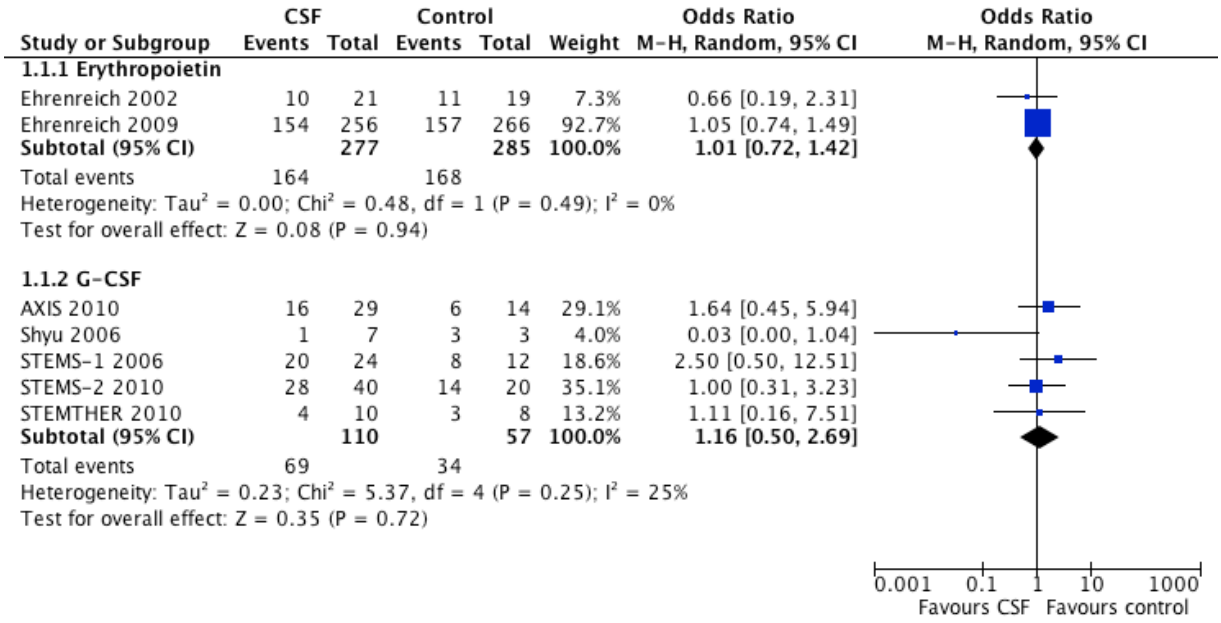


Figure 19 (Analysis 1.2). Death, end of trial.

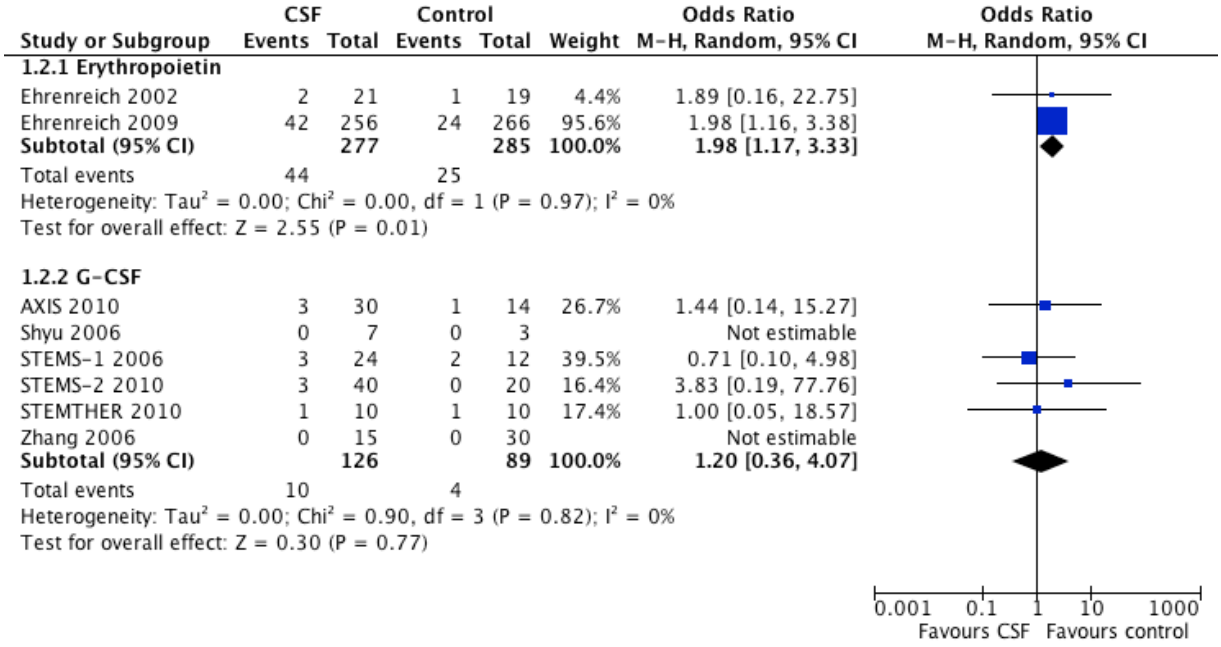


Figure 20 (Analysis 1.3). Early death.

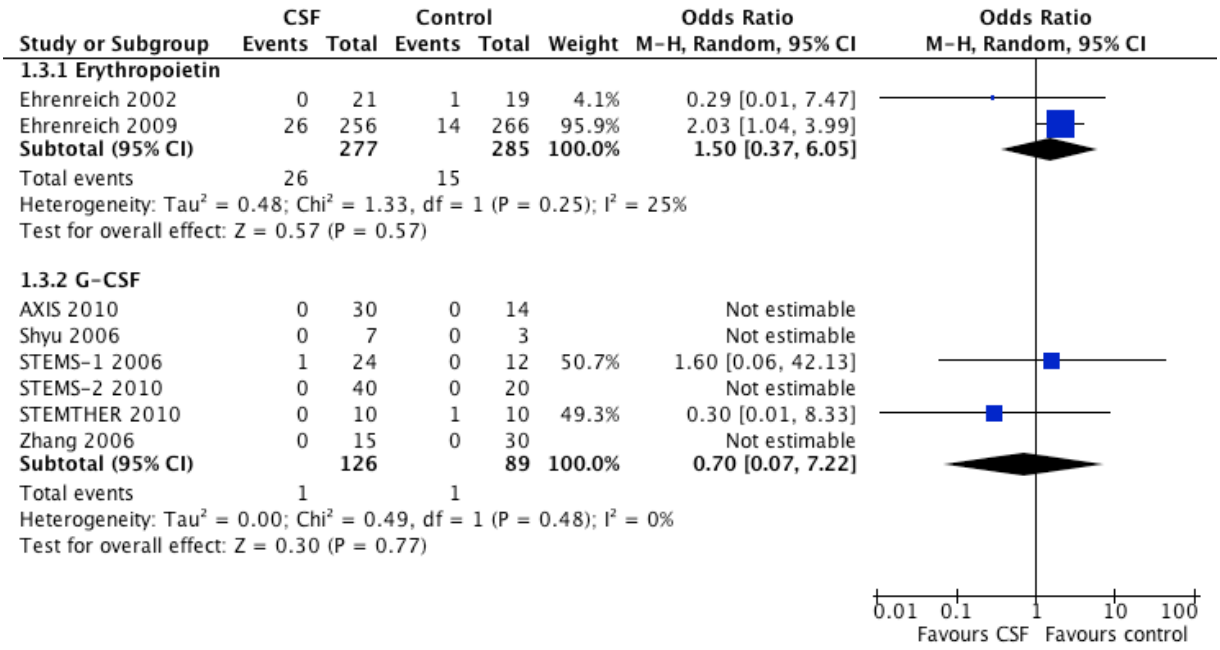


Figure 21 (Analysis 1.4). Early Impairment, NIHSS.

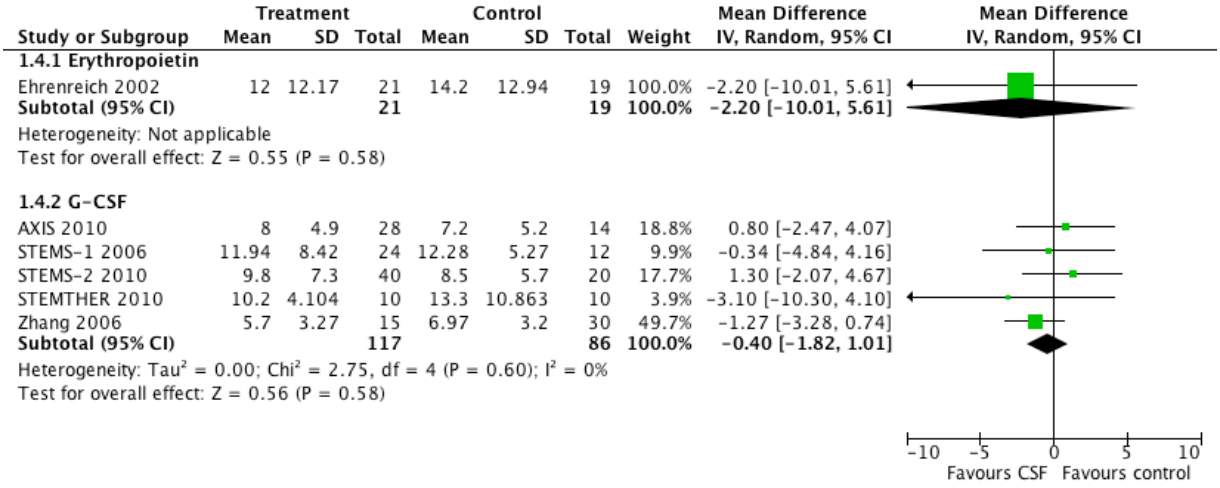


Figure 22 (Analysis 1.5). Serious Adverse Events, number of patients.

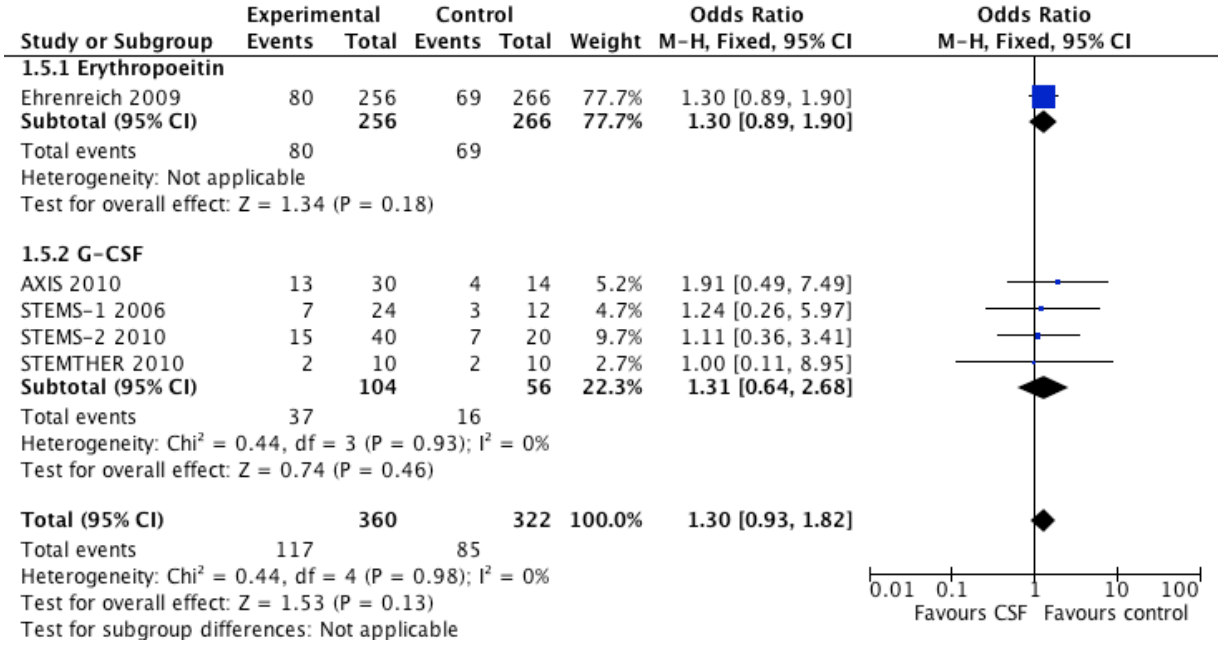


Figure 23 (Analysis 1.6). Infection.

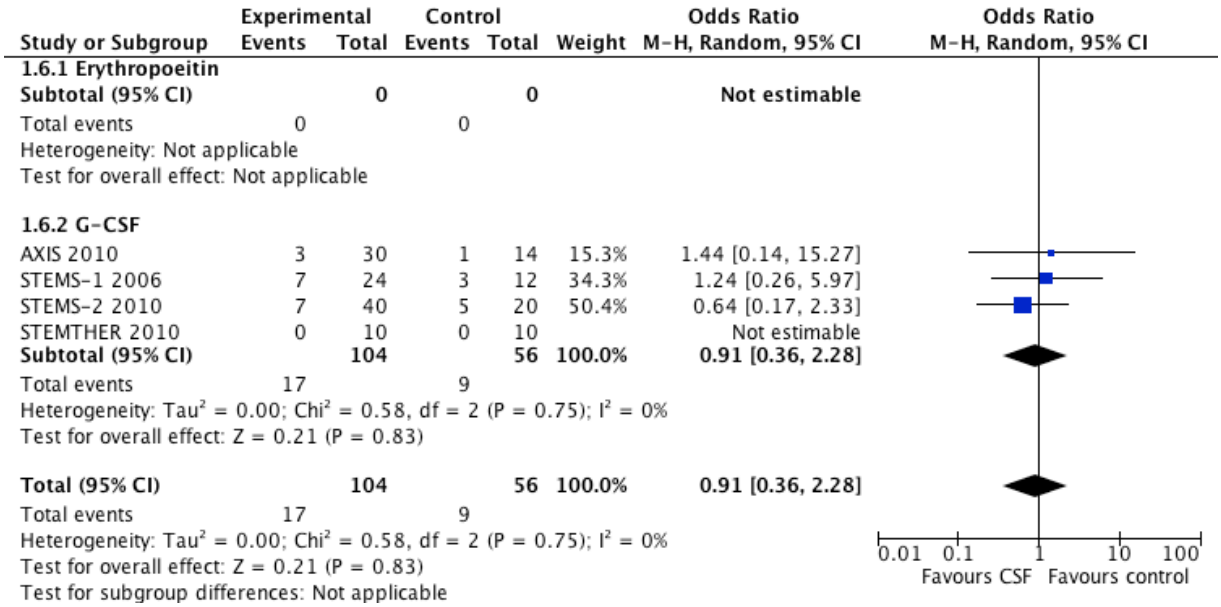


Figure 24 (Analysis 1.7). White cell count, 3 to 10 days.

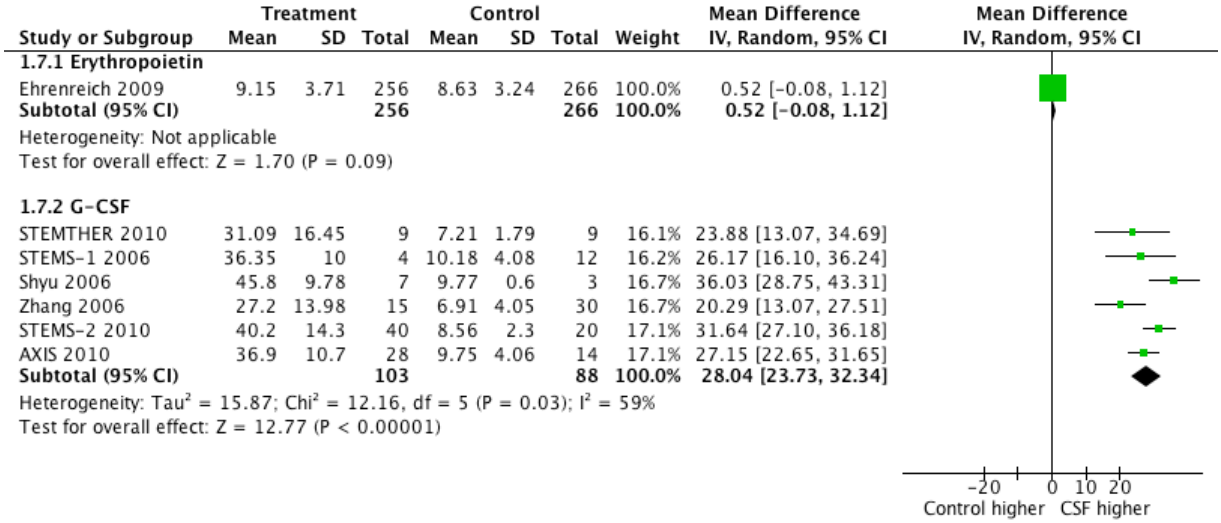


Figure 25 (Analysis 1.8). Red cell count, 3 to 10 days.

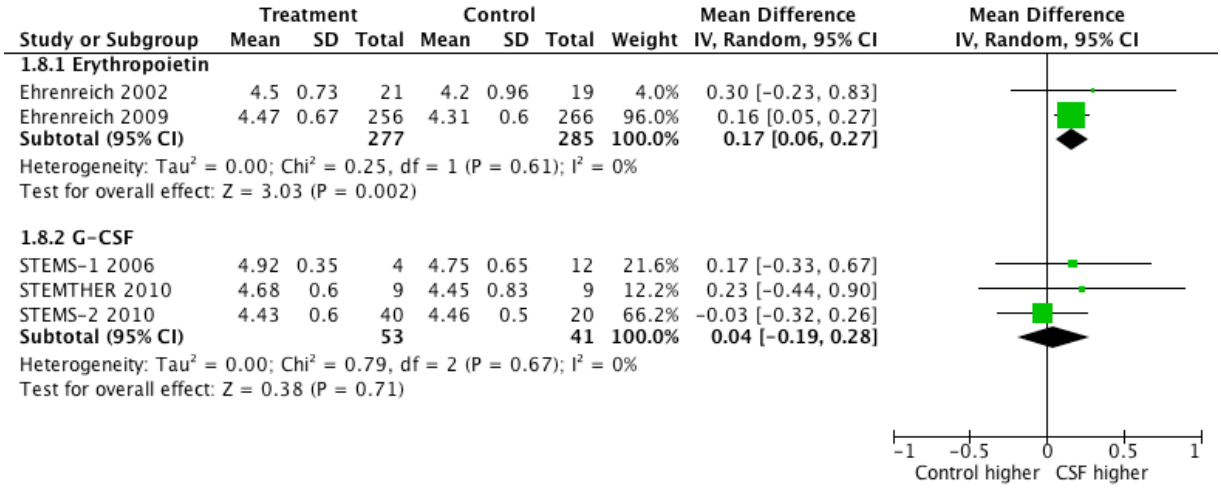


Figure 26 (Analysis 1.9). Platelet count, 3 to 10 days.

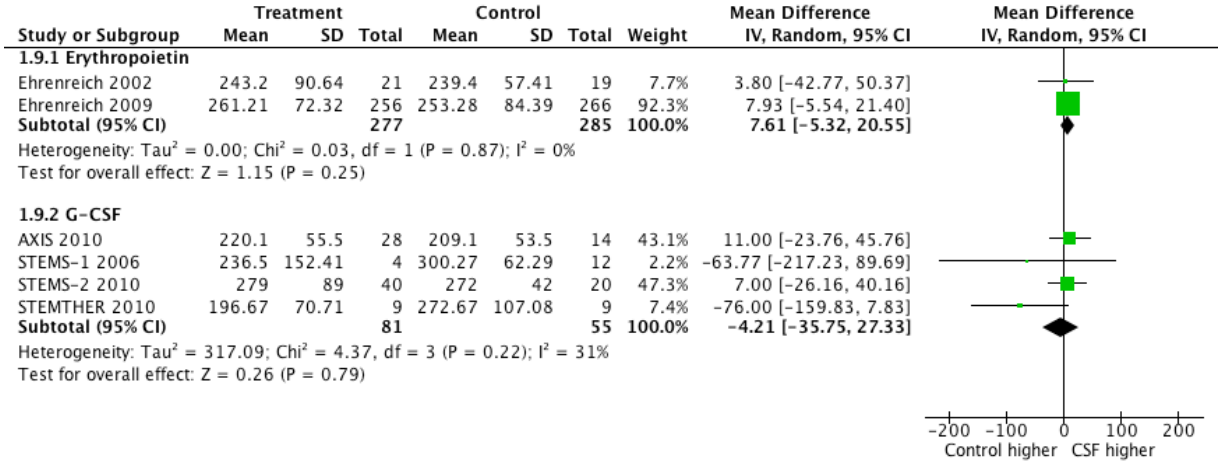


Figure 27 (Analysis 1.10). CD34+ count, 5 to 10 days.

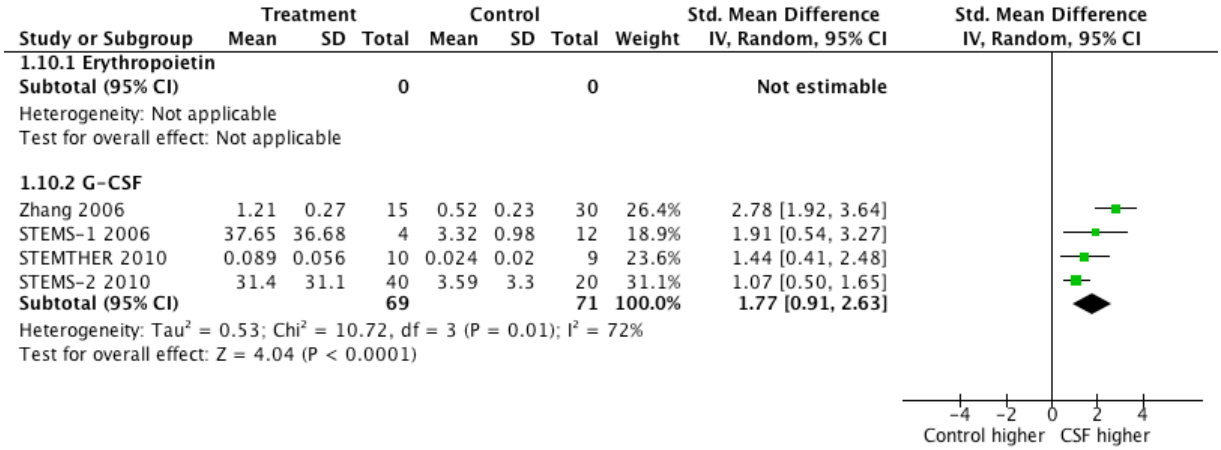


Figure 28 (Analysis 1.11). Serum S100-ß, 7 to 10 days.

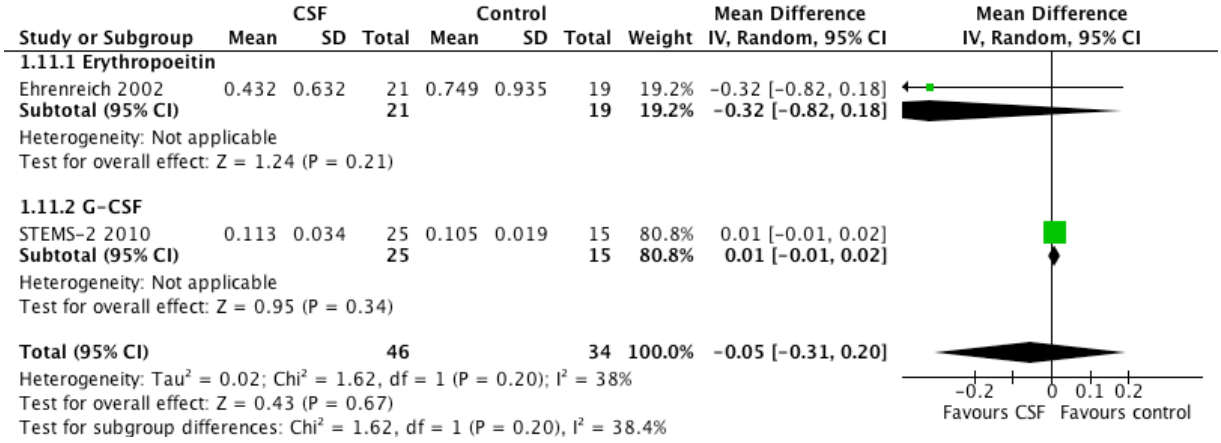
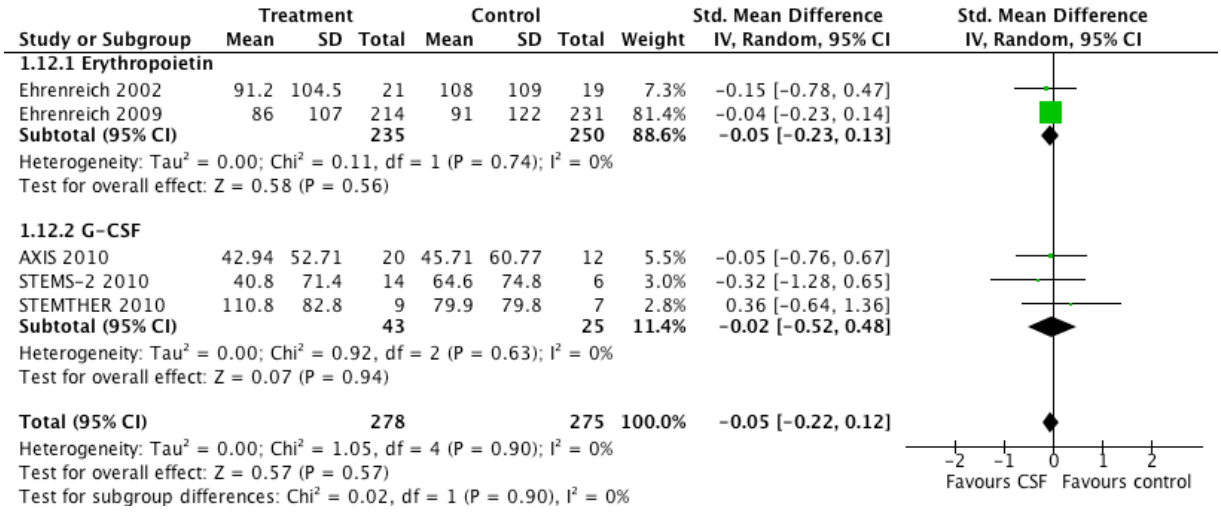


Figure 29 (Analysis 1.12). Infarct volume, post treatment**.**



DISCUSSION

Although there are a number of types of colony stimulating factors, only EPO and G-CSF have been studied in completed trials in patients with acute or subacute stroke. All were small safety studies except for one phase III trial of EPO.[256] EPO did not show a significant effect on combined death and dependency but there was an increase in death rate. The authors suggest that mortality was related to the abnormally high number of recruits receiving thrombolysis (63%), of which half demonstrated contraindications to rtPA administration. No particular mechanism of death was apparent, however, but an EPO-rtPA interaction could not be ruled out; additionally, the significant rise in red cell count caused by EPO might be a contributing factor.

Non-haematopoietic derivatives of EPO, which do not increase red cell count and haematocrit and a subsequent procoagulant state, are in clinical development; two trials of carbamylated EPO have been completed and their results are awaited.[410, 411]

G-CSF did not significantly alter functional outcome but its administration was associated with a non-significant reduction in early impairment and early death. G-CSF was well tolerated in all six trials and appeared to be safe, although it significantly increased white cell count.

Further randomised controlled trials of CSFs are underway, including two with G-CSF, a small safety study and a larger hyperacute safety and efficacy trial.[302, 413] No trials of SCF, GM-CSF, M-CSF, TPO were identified.

It is apparent that at least two paradigms are being studied with CSFs in the treatment of stroke. First, CSFs such as EPO and G-CSF are neuroprotective in animal models of acute stroke and this potential mechanism is under investigation in patients with acute stroke.[255, 256, 297, 302] Second, stem cell mobilising CSFs (as with SCF, G-CSF, and GM-CSF) could contribute to brain repair through neurogenic-related mechanisms, again as has been seen in experimental models of stroke; five trials have investigated this approach (Shyu 2006; STEMS-1 2006; Zhang 2006; STEMS-2 2010; STEMTHER 2010),[294-296, 303, 304] one of which attempts to track iron-labelled CD34+ cells with MRI.[304] All of these studies are focusing on recent stroke. No trials of CSFs in chronic stroke have been reported, though one is ongoing.[414]

There are a few limitations to consider. First, despite aggregating all available data, the total number of included patients is small, which restricts interpretation of the outcomes. When further data are available and the above mechanisms of action are considered, analysis may need to be reviewed in subgroups of time-window of administration, such as hyperacute, subacute and chronic. Second, common to most meta-analyses, the data are subject to clinical heterogeneity, though statistical heterogeneity was not apparent. This restriction is accounted for, in part, by using a random effects model of analysis. Third, there is potential bias in other forms, such as publication bias where all relevant studies completed may not be included in the analysis (e.g. due to unpublished neutral or negative studies). We also introduce an investigator bias since the questions we ask of the data are put forward after all of the trials are complete. The bias represented here is reduced (but not abolished) by ensuring the analysis is performed systematically and transparently.

CONCLUSIONS

Implications for practice

No conclusive evidence suggests that any type of CSF should be used in the routine management of stroke.

Implications for research

The completed trials provide evidence of the practicality and feasibility of administering CSFs. However, questions have arisen regarding the safety of EPO in the context of acute stroke, especially in patients who have received thrombolysis.[256] The mechanism explaining an EPO-rtPA interaction is unclear; an increase in blood brain barrier permeability may contribute [415] but further experimental work on stroke models is required.

Non-haematopoietic derivatives of EPO are in clinical development, including two ongoing trials of carbamylated EPO.[410, 411]

The mechanisms by which CSFs might work in acute or subacute stroke are unclear. Although CSFs could be neuroprotective, reductions in stroke lesion size were not seen in trials of EPO of G-CSF. This may reflect the difficulties in translating experimental stroke models into clinical medicine and animal models not sufficiently representing human stroke.[152] G-CSF may also be neuroreparative since its administration in the sub-acute period was associated with a non-significant reduction in early impairment and early death. Similar results have been seen in preclinical studies and mobilised stem cells could

enhance neurogenesis and angiogenesis. One clinical trial of G-CSF attempted to track the migration of CD34+ cells into the brain.[304]

Further studies need to address mechanisms by which CSFs might work, for example, using alternative cell labelling techniques or cerebral perfusion imaging. Understanding potential mechanisms of action will help investigators decide when to administer treatment relative to stroke onset. There is a need for adequately powered phase III trials of appropriately researched CSFs. Whether CSFs aid recovery in chronic stroke also needs to be addressed.

Contributions of authors

PB conceived and wrote the review, performed electronic searches, and is the guarantor. TE and NS wrote the review, and performed searches.

Declarations of interest

The authors (PMWB, NS, TE) performed two independent phase II trials of granulocyte colony stimulating factor (G-CSF) funded by The Stroke Association and UK The Medical Research Council.[294, 304] PB acted as a consultant to Axaron (now called Sygnis, who are developing G-CSF) and is a member of the Steering Committee for Lundbeck's trials of carbamylated EPO; no consultancy fees from Axaron or Lundbeck were used in any way for the development of the review, and neither company had any influence over the initiation, planning or production of the review.

CHAPTER 6

PARAMAGNETIC LABELLING OF HAEMATOPOIETIC CD34+ CELLS AND THEIR IDENTIFICATION WITH MAGNETIC RESONANCE IMAGING AFTER STROKE: DATA FROM THE STEMS-2 TRIAL

Publications contributing towards this chapter

England TJ, Auer DP, Abaei M, Lowe J, Russell N, Walker M, Bath PMW. Stem-cell trial of recovery enhancement after stroke 2 (STEMS-2). Randomised placebo-controlled trial of granulocyte-colony stimulating factor in mobilising bone marrow stem cells in sub-acute stroke (5th UK Stroke Forum). *International Journal of Stroke*. 2010;5 (Suppl 3):3. Oral presentation.

ABSTRACT

Background

In-vivo tracking of iron labelled stem cells with serial MRI is feasible in experimental stroke but has never been attempted in the human stroke brain.

Methods

Patients enrolled into the Stem cell Trial of recovery EnhanceMent after Stroke 2 (STEMS-2), a randomised double blind placebo-controlled trial assessing the safety of granulocyte-colony stimulating factor (G-CSF) in subacute stroke,

were invited to partake in a CD34+ haematopoietic stem cell (HSC) labelling substudy. Recruits received G-CSF (10μg/kg, subcutaneously) or placebo for 5 days 3-30 days post ictus. On day 6, mobilised HSCs were extracted from the patient, paramagnetically labelled and re-infused intravenously. Serial MRI T2* imaging (days 0, 10-12, 90) was used to track iron-labelled cells. HSC counts were measured using flow cytometry.

Results

8 participants (6 G-CSF, 2 placebo) with a mean age 75 (SD 7), of whom 50% were male, undertook cell labelling 14 days (SD 3.8) post ischaemic stroke. In G-CSF treated participants, between 50–430 $x10^4$ HSCs were harvested for re-infusion from 100-200 ml of whole blood. HSC harvest correlated significantly with day 5 peripheral blood HSC count (Spearman's rho 0.83, p=0.01). One recruit developed a hypodensity compatible with iron-deposition within the infarct evident on day 10 and 90 T2* scans. Three subjects had hypodensities in their infarct zones compatible with haemorrhagic transformation at days 0, 10 and 90. The remaining four participants had no hypodensities in their infarct zones at any time point.

Conclusions

Post-stroke paramagnetic CD34+ cell labelling appears safe and feasible. There is suggestive evidence from one patient that labelled HSCs migrate to the ischaemic lesion.

INTRODUCTION

Experimental stroke recovery is enhanced by cell therapy following implantation with a number of stem cell types.[162, 221, 416, 417] It is not yet known if this therapeutic promise can be translated into clinical practice. Clinical trials assessing stem cell transplantation in human stroke are already underway [418, 419] but there is still much to learn regarding how best to apply any potential treatment. Is it safe or feasible? Which stem cell and at what dose should be used? How, when and where should they be implanted?

Insights into stem cell dynamics and migration will contribute towards our understanding of mechanisms underlying the recovery process. Methods often applied in pre-clinical stroke to track migrating cells can be toxic and are not suitable for human use. However, labelling with intracellular [277] and extracellular [362] iron labels and subsequent monitoring with serial MRI has been successful in tracking cells in rodents for several weeks post transplantation.[384] To our knowledge, tracking stem cells using these methods has never previously been attempted in the human stroke brain.

Following stroke, mobilisation of CD34+ haematopoietic stem cells (HSC) into the peripheral circulation can be achieved with administration of granulocyte colony stimulating factor (G-CSF),[294] a glycoprotein hormone responsible for the production of granulocytic neutrophils. It is used routinely for acquiring HSCs for bone marrow transplantation and shows promise as a treatment for stroke. HSCs could migrate to the stroke through injury induced chemotaxis and contribute towards a recovery process, enhancing neurogenesis and

angiogenesis.[161] The Stem cell Trial of recovery EnhanceMent after Stroke 2 was a pilot, phase IIb safety trial evaluating G-CSF for the treatment of stroke.[304] Here we describe a substudy of STEMS-2 which aimed to determine the fate of mobilised HSCs using a CD34+ extracellular iron-oxide label and subsequent imaging with MRI.

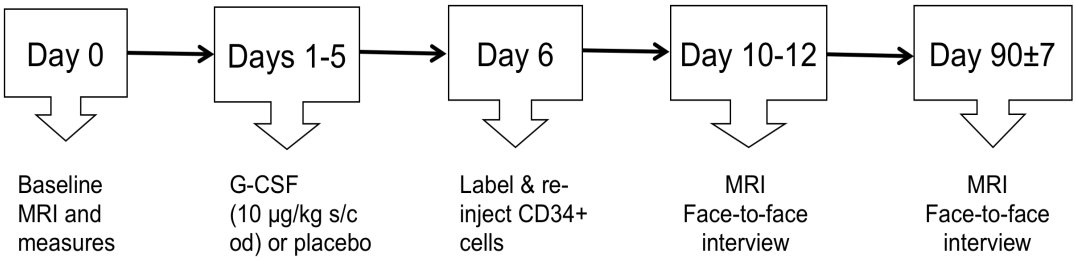
METHODS

Subjects

STEMS-2 was a prospective, single-centre, double blind, randomised, placebo-controlled, phase IIb trial of G-CSF in patients with subacute stroke (see chapter 4). Patients included were 3 to 30 days post ischaemic or haemorrhagic stroke and were randomised 2:1 to receive subcutaneous human recombinant G-CSF ($1x10^6$ units/kg, equivalent to 10μg/kg, Neupogen, Amgen) or matching subcutaneous saline once per day for 5 days. If there was no evidence of haemorrhage on baseline CT brain imaging then recruits were offered the opportunity to take part in the CD34+ cell labelling substudy. Recruitment for the main trial commenced in July 2007 and ended in January 2010. Due to procedural and logistical complexities, patients were recruited into the substudy from November 2008.

Once consent or assent from an appropriate legal representative was gained, baseline (Day 0) observations were obtained including age, sex, stroke severity (Scandinavian Neurological Stroke Score, SNSS) and stroke subtype based on the Oxford Community Stroke Project (OCSP) [50] (total anterior circulation infarct [TACS], partial anterior circulation stroke [PACS], lacunar infarct [LACS] and posterior circulation infarct [POCS]). Follow-up by face-to-face interview was performed on days 10-12 and 90 (±7) (figure 30). All assessments were made blinded to treatment allocation.

Figure 30. Time scheme for the trial and CD34+ cell labelling substudy



Stroke volume

Two independent investigators measured baseline stroke lesion volume (MR diffusion weighted images, echo time (TE) 53 ms, repetition time (TR) 6605 ms, slice thickness 2.5 mm, diffusion weighting 1000 s/mm^2, field of view (FOV) 240×240 mm^2) using semi-automated software developed locally (NeuRoi, Dr C Tench, Department of Clinical Neurology, Nottingham, UK [405]).

CD34+ cell labelling

Between 100 and 200 ml of blood was collected on day 6 and processed in a HTA licensed clean room facility. CD34+ cells were immunomagnetically separated from the blood using a CliniMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) (Appendix A). The CD34+ antibody used for immunoseparation contained a dextran-coated iron-oxide nanobead, effectively labelling the cells and allowing visualisation with gradient echo (T2* weighted) imaging.[362] Post labelling, the CD34+ cells were re-injected intravenously back into the donor patient on the same day and tracked with MR T2* imaging on day 10 and day 90 (3T: TE 16 ms, TR 1070 ms, slice thickness 3 mm, FOV

230×143 mm^2; 7T: TE 254 ms, TR 2500 ms, slice thickness 1.6 mm, FOV 220×220 mm^2). These images are highly sensitive to iron, and therefore to iron-labelled cells.

CD34+ cell counts were measured using flow cytometry (FACScalibur, Becton Dickinson, Oxford, UK) and complied with ISHAGE guidelines.[404]

A neuroradiologist blinded to treatment allocation and cell counts interpreted pre and post labelling scans. Scans were performed on a 3T Achieva (Philips, Netherlands) and in some instances, where clinically possible and in patients who tolerated MRI scans well, we scanned patients using Nottingham University's 7T Achieva (Philips, Netherlands).

RESULTS

Eight participants (six in the G-CSF group and two placebo) with a mean age of 74.6 (standard deviation, SD 6.9), of whom 50% were male and had moderate stroke severity (mean SNSS score 33.4, SD 13.2, a lower score representing a greater severity) underwent CD34+ cell labelling (figure 31, table 25). All stroke subtypes except POCS were included: 3 TACS, 2 PACS and 3 LACS with substantial differences in stroke lesion volume ranging between 1.2 cm^3 and 184.5 cm^3. There was marked variation in each participant's response to G-CSF; following 5 days of treatment, between 50–430 $x10^4$ cells were harvested from 100-200 ml of whole blood. Participants receiving placebo yielded between 2–7 $x10^4$ CD34+ cells. The process of white blood cell separation and then immunomagnetic CD34+ cell selection yielded a low proportion of CD34+ cells from the original sample of donated blood (mean 24%, table 26). The total quantity of CD34+ cells acquired for re-injection was positively correlated with patient peripheral blood CD34+ cell count on day 5, Spearman's rho coefficient, $r_s = 0.83$, p=0.01 (figure 32). Labelling occurred, on average, 14 (SD 3.8) days from stroke ictus.

Phantom model

A phantom model was used to demonstrate that iron-oxide microbead-labelled CD34+ cells (harvested from a volunteer) dispersed in 0.5% agar ($4x10^3$ cells/ml) were visible on 3T T2* MR scanning; agar alone, and unlabelled CD34- cells were not detectable (figure 33).

CD34+ cell labelling

Following review by an experienced neuroradiologist (DA), who was blinded to treatment assignment, the scans of one G-CSF treated participant (recruit number 50) developed a hypodensity compatible with iron-deposition within their left gangliocapsular infarct, evident on both day 10 and 90 T2* scans (figure 33). The hypodensity was not present at baseline and this participant had received the highest number (430×10^4) of labelled CD34+ cells with administration 14 days after stroke onset. Three (G-CSF 2, placebo 1) participants (recruits 30,46 and 51) had evidence of hypodensities compatible with haemorrhagic transformation at days 0, 10 and 90. The remaining 4 participants receiving labelled cells had no hypodensities in their infarct zones at any time point (figure 34).

Table 25. Baseline characteristics of patients undertaking CD34+ cell labelling

Patient trial N^{o}	Treatment assignment	Age	Sex	Stroke Region (ischaemic)	Clinical subtype	Stroke lesion volume (cm^3)	SNSS (_/58)	Day Labelled (days post ictus)	Presence of HTI
30	Placebo	84	F	Right MCA	TACS	165.1	19	13	+
52	Placebo	83	M	Multiple in left hemisphere	PACS	1.7	42	11	-
27	G-CSF	66	M	Left pontine	LACS	1.2	40	13	-
39	G-CSF	81	M	Right thalamic	LACS	Poor quality	44	20	-
43	G-CSF	71	M	Right thalamo-capsular	LACS	1.4	43	12	-
46	G-CSF	72	F	Left MCA	TACS	184.5	10	19	+
50	G-CSF	69	F	Left ganglio-capsular	PACS	56.0	26	14	-
51	G-CSF	71	F	Right striatal	TACS	16.1	43	9	+

SNSS: Scandinavian Neurological Stroke Scale; G-CSF: granulocyte-colony stimulating factor; HTI: haemorrhagic transformation of infarction; MCA, middle cerebral artery; TACS: total anterior circulation infarct; PACS: partial anterior circulation stroke; LACS: lacunar infarct;

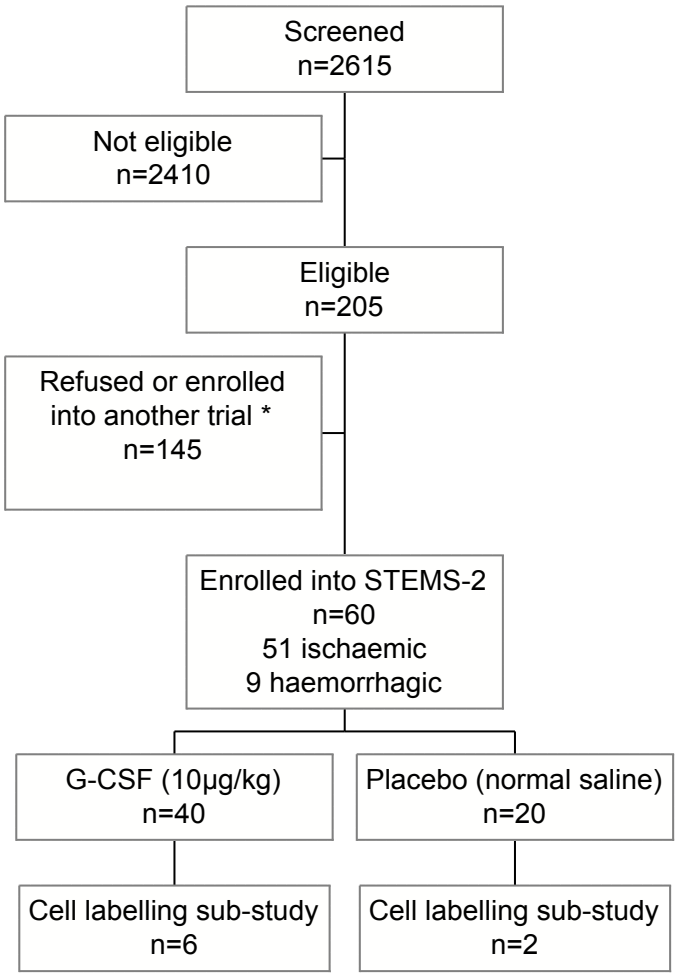
Table 26. CD34+ cell counts pre and post immunomagnetic labelling

Patient trial number	Treatment assignment	Day 5 peripheral blood [$CD34^+$] (cells/µl)	Total $CD34^+$ count * (blood collection bag)	Total $CD34^+$ count † (pre-immunomagnetic selection)	Total $CD34^+$ cell count (post-immunomagnetic selection)	Yield % (from original blood collection bag)
30	Placebo	2.8	Not measured	678×10^4	2.1×10^4	-
52	Placebo	2.82	18×10^4	15.3×10^4	6.9×10^4	38.3
27	G-CSF	177.8	Not measured	Not measured	246×10^4	-
39	G-CSF	46.7	503×10^4	369×10^4	125×10^4	23.9
43	G-CSF	51.6	737×10^4	610×10^4	145×10^4	20.4
46	G-CSF	22.7	555×10^4	224×10^4	45×10^4	9
50	G-CSF	47.8	1370×10^4	853×10^4	430×10^4	31.4
51	G-CSF	37.6	743×10^4	512×10^4	161×10^4	21.5

[ ], denotes concentration; * derived from 100 – 200 mls of whole blood taken from the patient on Day 6;

† post-labelling but pre-immunomagnetic selection

Figure 31. STEMS-2 trial flow and recruitment into CD34+ cell labelling sub study



G-CSF, granulocyte colony stimulating factor
* 110 refused, 35 enrolled into another trial

Figure 32. Correlation of Day 5 peripheral blood CD34+ cell count and total number of CD34+ cells harvested for labelling on day 6

Correlation is statistically significant, Spearman's rho coefficient, r_s = 0.83, p=0.01)

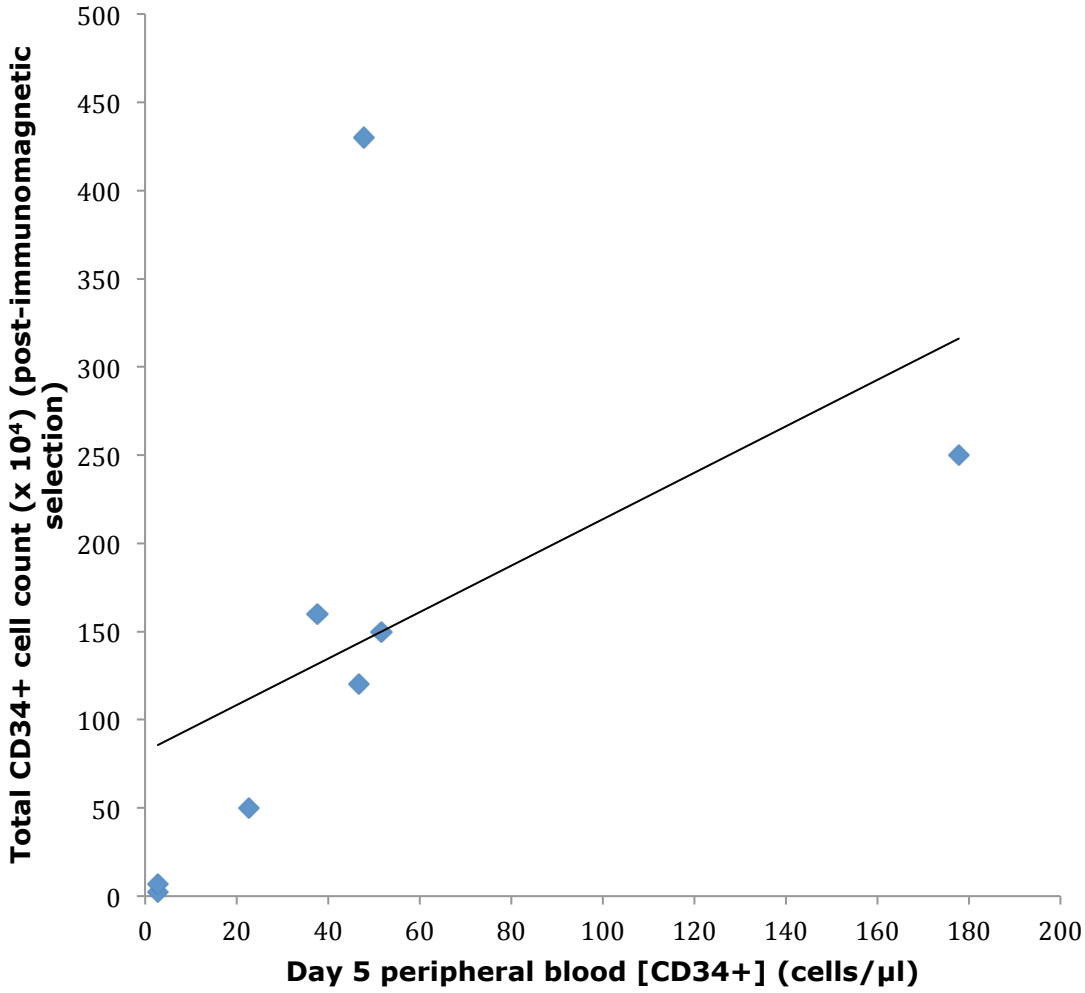


Figure 33. Gradient echo imaging at days (i) 0, (ii) 10, and (iii) 90 in patient 50 with left gangliocapsular infarction. (iv) Corresponding diffusion weighted image at day 0.

The participant received $4.3x10^6$ intravenous immunomagnetically-labelled CD34+ cells on day 6. Arrow indicates new area of negative enhancement. (v) Phantom model comparing (a) labelled CD34+ cells dispersed in 0.5% agar ($4x10^3$ cells/ml), (b) agar alone, (c) iron-oxide microbeads alone and (d) unlabelled CD34- cells.

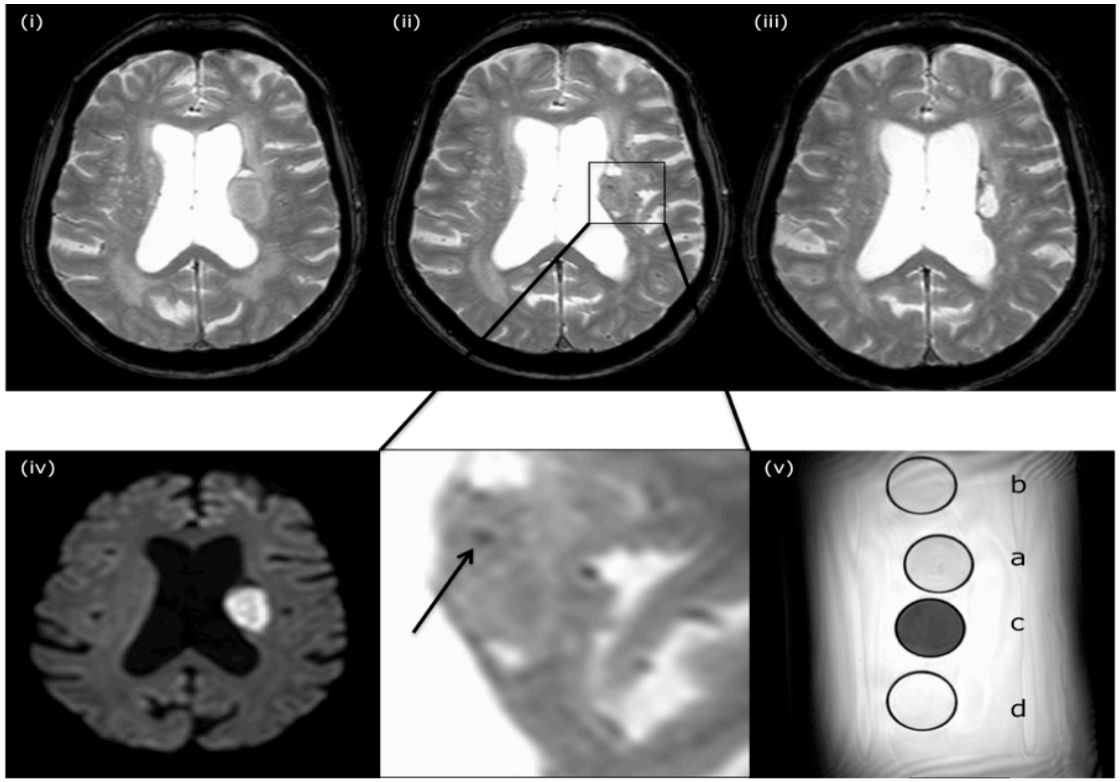
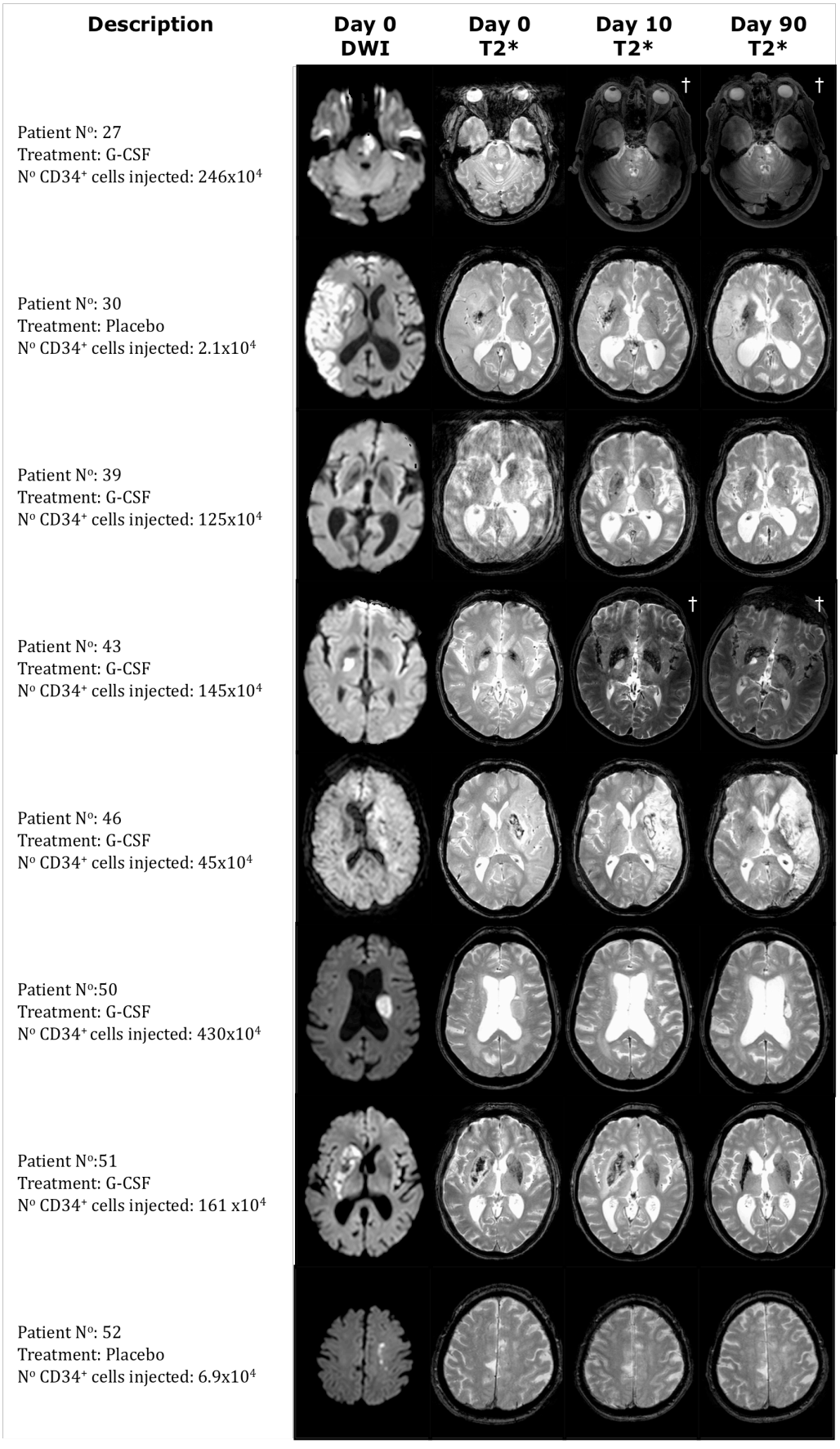


Figure 34. Gradient echo imaging at days 0, 10, and 90 and corresponding diffusion weighted image at day 0 in all recruits receiving iron labelled CD34+ cells (†=7T MRI, all other scans performed at 3T)



DISCUSSION

STEMS-2 was a high quality double blind placebo-controlled randomised trial. The CD34+ cell labelling substudy was designed to establish whether G-CSF mobilised iron-labelled CD34+ cells migrate to the site of the stroke lesion, thus potentially contributing to neurorepair, as seen experimentally.[161, 351] The complexity of recovering, labelling and re-administering CD34+ cells meant that we studied fewer participants than desired. Nonetheless, T2* MR imaging suggested the presence of migrated labelled cells in one participant as demonstrated by new signal loss within the infarct on days 10 (4 days after reinjection) and 90, and without evidence of baseline haemorrhagic transformation (at day 14 post stroke). This followed intravenous injection of 430 $x10^4$ labelled CD34+ cells (the highest number of cells recovered from any participant). There was no evidence of T2* signal loss in four participants, and haemorrhagic transformation occurred in three. Bone marrow derived stem cells have been tracked successfully in animal models of stroke by using extracellular and intracellular iron labels,[362, 385] a method that confers visibility on gradient echo MR imaging. Qualitative interpretation of T2* images was limited by scan quality and pre-existing signal loss (e.g. due to haemosiderin deposition from previous bleeds). New petechial or parenchymal bleeding from haemorrhagic transformation further limited specificity of interpretation of new signal loss; haemorrhagic transformation is present in up to 85% of ischaemic stroke in serial MR imaging studies.[400]

The failure to visualise MRI hypodensities compatible with CD34+ cell migration in more than one patient may reflect several issues. First, the total number of cells injected may have been too low; experimental rodent models used 3–750

$x10^4$ labelled stem cells,[277, 362] a significantly higher weight-adjusted dose than available to us. Second, the route of administration may be suboptimal; both intracerebral and intra-arterial injection will deliver a greater number of cells to the brain.[272] Nonetheless, intravenous administration has been shown to be capable of delivering migrating cells to the lesion of interest [384] although this finding is not consistent.[378] Third, it is possible that the cells migrate to a site other than the brain, such as an area of concomitant infection, or to other organs including liver, spleen and bone marrow.[420] Fourth, extracellular iron-oxide microbeads may not provide adequate contrast for visualisation using MRI *in vivo*, though the *in vitro* phantom data argues against this since as few as 4 $x10^3$ cells/ml were identifiable. Fifth, the microbeads may not remain attached to the CD34+ cell membrane after they have been re-infused into the patient; they could be internalised or detach and transfer to other cells (e.g. macrophages). Sixth, the microbeads may interfere with CD34+ activity,[362] e.g. attenuating cell migration. Further studies could consider the use of intracellular iron labels.[392] Seventh, the presence of haemorrhagic transformation will have prevented seeing any migrated cells in 3 of our G-CSF treated participants. Furthermore, even if the presence of negative enhancement on MR images were to represent iron within a cell, then this would not provide evidence on cell viability, which could be achieved, for example, with positron emission scanning (discussed below). Eighth, the timing of cell administration could be important and we may have treated either too early or too late. Last, it is conceivable that CD34+ cells do not naturally migrate to the ischaemic lesion, in which case they are unlikely to influence neurorepair.

There are no previous clinical data published involving iron-labelled stem cells and stroke; reports are described in melanoma (dendritic cells),[421] traumatic brain injury (neural stem cells),[422] islet cell transplantation [423] and spinal cord injury (CD34+ cells).[424] Other reports in stroke have attempted to address stem cell migration using alternative methods. In one non-controlled study, 12 patients with chronic stroke received CT-guided implantation of stem cells derived from an immortalised cell line;[280] follow-up FDG-PET (Fluorodeoxyglucose Positron Emission Tomography) scanning suggested either the presence of inflammation or the viability of implanted cells (FDG is an analogue of glucose labelled with fluorine-18 and its use as a marker for cell tracking is limited by its short half-life and increase of radiation exposure to the patient. Its uptake is also non-specific and the PET scan will be positive in cells with a high metabolic rate, for example, in an area of inflammation or tumour formation). Post mortem examination of one patient 27 months after cell implantation identified the survival of transplanted neuronal cells.[290]

Another trial assessed migration of intrathecal immunomagnetically-labelled CD34+ cells in 10 patients with chronic spinal cord injury in comparison with 6 controls who received intrathecal immunomagnetic beads only.[424] This study was non-randomised, patients received between 50-120 $x10^4$ labelled cells, and hypointense signals were observed in 50% of lesions in the treatment group 20 and 35 days after transplantation. An alternative labelling technique was employed in another non-randomised, phase I, open-label clinical trial:[425] 6 men received radio-labelled bone marrow derived mononuclear cells that had been cultured *ex-vivo*, which were intra-arterially injected into the middle cerebral artery between 59 and 82 days post ischaemic stroke. Whole body scintigraphy

and Single-Photon Emission Computed Tomography (SPECT) indicated cell homing to the brain at 2 hours but due to the short half-life of Technetium-99m (6 hours), scan interpretation was limited at 24 hours. Uptake was also seen in the liver, lungs, spleen and kidneys.

This is the first clinical trial in stroke that has identified possible CD34+ cell migration post intravenous implantation, though there are a number of limitations as described. Whilst iron-labelling techniques offer the potential to track cells over a long period of time (compared to the short half-life of radiolabelling techniques), it still may not be an optimal technique to use in human stroke. The common presence of haemorrhagic transformation of infarction (HTI), which can occur at variable time points post-stroke, will always confound interpretation of cell migration. If iron labels are used then careful patient selection is required, that is, excluding HTI before enrolment with MRI. Pre-clinical models of stroke will continue to provide insights into migratory patterns of stem cells but cell dynamics will inevitably vary in the human clinical trial setting. This underlies the importance of determining their behaviour in humans. Further research is therefore required to investigate the feasibility and safety of other potential stem cell labels.

CHAPTER 7

HAEMATOPOEITIC STEM CELL (CD34+) UPTAKE OF SUPERPARAMAGNETIC IRON OXIDE (SPIO) IS ENHANCED BUT NOT DEPENDENT UPON A TRANSFECTION AGENT

Publications contributing to this chapter:

TJ England, PM Bath, M Abaie, D Auer, R Jones. Haematopoeitic stem cell (CD34+) uptake of superparamagnetic iron oxide (SPIO) is enhanced but not dependent upon a transfection agent. Cerebrovascular Diseases 2012, Vol. 33, Suppl. 2. Poster presentation and European Stroke Conference, Lisbon 2012.

Submitted to Cytotherapy (under review)

ABSTRACT

Background

Superparamagnetic iron oxide particles (SPIO) can be internalised into stem cells to allow real-time tracking with gradient echo magnetic resonance (MR) imaging. Although there are many reports of haematopoietic stem cells being labelled using SPIO, there is some controversy regarding the outcomes and it is not clear whether undifferentiated CD34+ progenitor (stem) cells are able to take up iron in the absence of a transfection agent to enhance the process.

Methods

CD34+ cells were treated in vitro as follows: incubation with (i) medium only (control), (ii) ferumoxide (Endorem) and (iii) ferumoxide (Endorem) plus

exposure to a transfection agent (protamine sulphate). Cells were incubated for 2, 4 and 24 hours & assessed for viability, differentiation capacity and visualised *in vitro* with 3T magnetic resonance imaging. The cells were also analysed using flow cytometry and the morphology examined by electron microscopy.

Results

CD34+ haematopoietic progenitor cells can internalise ferumoxide (Endorem) independently of a transfection agent. However, uptake of ferumoxide is enhanced following exposure to protamine sulphate. Iron labelling of CD34+ cells in this manner does not affect cell viability and does not appear to affect the potential of the cells to grow in culture. Iron labelled CD34+ cells can be visualised *in vitro* on 3T MRI scanning.

Conclusions

Endorem and protamine sulphate can be combined to promote iron-oxide nanoparticle uptake by CD34+ cells and this methodology can potentially be used to track the fate of cells in a human clinical trial setting.

INTRODUCTION

Stem and progenitor cells provide increasing potential for treatment of a variety of conditions including Parkinson's disease,[200] multiple sclerosis,[426] spinal cord injury,[427] and vascular diseases such as myocardial infarction and stroke.[279, 428] Despite experiences gained from (human) clinical trials, stem cell fate in the context of tissue repair or regeneration in vivo is relatively poorly understood. Particular interest has been generated in the field of autologous haematopoietic stem cells since these can be obtained via bone marrow aspiration (CD34+ cells) or via mobilisation into the circulation using granulocyte-colony stimulating factor (G-CSF).

Labelling cells with iron and tracking them in vivo with magnetic resonance (MR) imaging creates the potential for 'real-time' migration patterns to be established. Superparamagnetic iron oxide particles (SPIO) can be taken up into cells to allow tracking with gradient echo MR imaging and although it has been reported that 'haematopoietic stem cells' have been labelled using SPIO,[420, 429-431] it is not clear whether undifferentiated CD34+ cells are able to take up iron particles without a transfection agent (substances that efficiently shuttle particles into the cell). SPIOs are negatively charged and do not adhere to cell surface membranes, so a transfection agent could be used to electrostatically combine with the dextran coating of the SPIO, modifying the surface charge and allowing adherence to the cell membrane, with subsequent internalisation.

However, most labelling techniques used in animal models are not applicable for, or are not licensed for, use in human patients. Both the SPIO, ferumoxide,

and protamine sulphate (which can be used as a transfection agent) are approved (separately) for use in humans. Endorem is an effective labelling agent in other cell types,[389, 391, 392] typically used as a MRI contrast reagent administered for detecting liver lesions.[432] Protamine sulphate is a low molecular weight polycationic peptide used for reversing the effects of heparin in vivo.[433]

The aim of this study was to determine whether human CD34+ cells are capable of intracellular uptake of SPIO in vitro and whether the process requires a transfection agent (e.g. protamine sulphate). The use of established compounds such as Endorem as an intracellular label and (if required) protamine sulphate might facilitate the transition to human clinical trials where data on cell fate are required.

MATERIALS AND METHODS

Adult CD34+ cells

CD34+ cells were purchased from a commercial supplier (Stem Cell Technologies, Vancouver, Canada) being originally pooled from different donors. The cells, prepared using immunomagnetic cell separation procedures, were transported from the supplier on dry ice and stored in liquid nitrogen (vapour phase) on receipt. When required, a frozen cell suspension was thawed by gentle agitation in a 37 ^{o}C water bath and the cells transferred to a sterile universal container. A viable cell count was performed using trypan blue exclusion. The cell suspension volume was made up to 20mls in a sterile universal container by dropwise addition of RPMI 1640 containing 10% heat inactivated foetal bovine serum (FBS); the whole washed twice by centrifugation (200g for 15 minutes) and then resuspended in a final volume of 1 ml (RPMI/10%FBS).

Preparation of ferumoxide solutions for cell labelling

Incubation medium contained RPMI 20% FBS and 10µl/ml glutamate (all Sigma Aldrich, Dorset, UK). For the SPIO-labelled cells, ferumoxide ('Endorem', Guerbet, France) supplied at 11.2mg iron per ml was added to incubation medium at a final concentration of 100µg/ml. When required, protamine sulphate was also added (to the incubation medium) at a final concentration of 5µg/ml (Fe-Pro solution).

Equal volumes of the cell suspension (containing 10^6 CD34+ cells) and the prepared medium were combined to a final concentration of 50µg ferumoxide/ml (total vol. 2ml) and incubated for 2, 4 and 24 hours (37 ^{o}C, 5% CO_2). Post incubation, the cells were washed twice in phosphate buffered saline

(PBS), the second wash containing heparin (10 U/ml) to inactivate any protamine sulphate in extracellular Fe-Pro complexes. Cells were incubated with either ferumoxide alone or with ferumoxide plus protamine sulphate. As controls, cells were incubated with medium alone or with medium containing only protamine sulphate (and heparin added post incubation). After incubation, the cells were fixed using 0.5% formaldehyde solution for analysis using flow cytometry.

Cell culture and viability

After incubation with ferumoxide and/or protamine sulphate, cells were analysed for any impact on differentiation ability and on viability. For growth and differentiation studies, 3 x 10^5 CD34+ cells were added to 3mls of haematopoietic culture medium (Methocult H4434: Stem Cell Technologies, Vancouver, Canada), as per the manufacturer's instructions and the whole incubated, in a humid atmosphere, at 37^0C, 5% CO_2 for 21 days. In addition to haematopoietic culture, cell viability was determined using propidium iodide (PI) uptake, analysed via flow cytometry. PI was added to 1ml of each cell suspension to a final concentration of 0.5 µg/ml. PI binds to DNA in cells with a damaged or disrupted cell membrane, thus conveying a relatively accurate measure of cell viability when this is analysed using flow cytometry.

MRI 'Phantom' Model

The cells derived from each experimental protocol were assessed using MRI (Philips Achieva at 3T, Philips, Eindhoven, NL) after dispersing in 4ml of an agar solution (0.5%) and allowing the whole to set. Gradient echo sequences were used (TE: echo time 16.1ms; TR: repetition time 643.9ms; slice thickness:

1mm; Field of View: 40mm x 230mm x 183.3mm, pixel size 0.5mm x 0.5mm, flip angle 18) since these are sensitive to iron, and are demonstrated as hypointense areas relative to the surrounding agar if iron is present.

Electron Microscopy (EM)

Aliquots of 3 x 10^6 CD34+ cells were divided equally and incubated in medium alone, ferumoxide or Fe-Pro for 24 hours following the methods described above. The resulting cells were prepared for EM by adding them to an active fixative solution containing 500µl 25% EM grade gluteraldehyde solution and 5mls of 0.1M sodium cacodylate buffer, pH7.4. The cell preparations were evaluated using a JEOL 1010 transmission electron microscope (JEOL UK Ltd.) performed at 100kV. Two investigators (TE and RJ) assessed the cells for any structural changes and for the presence of intracellular ferumoxide. As a reference material, a suspension of ferumoxide alone was also evaluated using EM. Images were recorded using iTEM, a digital camera system (Olympus Soft Imaging Solutions, Olympus SIS GMBH, Germany).

RESULTS

Viability of adult CD34+ cells after ferumoxide uptake

In order to determine whether ferumoxide has a measurable effect on cell viability, the CD34+ cells were analysed by flow cytometry after incubation with ferumoxide, FePro or with medium alone for 24h. Using propidium iodide (PI) as a marker of cell viability, a region was demarcated around the cell population showing a 'shift' in shape and size and the proportion of these non-viable cells (i.e. PI positive) was calculated. Overall cell viability was unaffected by incubation with ferumoxide (88.9%) and Fe-Pro (90.9%) when compared with medium alone (91.1%). In addition, incubation with protamine sulphate alone did not affect cell viability (89%) compared with medium alone (91.1%).

Internalisation of ferumoxide by adult CD34+ cells

Flow cytometric analysis of the cells following incubation with medium alone (control), ferumoxide or Fe-Pro is shown in figure 35. The size and granularity of the cell population in the control group generally remained unchanged over the 24 hour period of the study. However, as cells die within a medium they initially increase in size & granularity, which was evident in the small population with increased side scatter in the control group at 4h and at 24h (figure 35A). In the ferumoxide-only group, a gradual increase in side scatter (granularity) was apparent over the timescale of observations (2h – 24h: figure 35B) compared to the control; whereas in cells incubated with FePro granularity was pronounced at both 4h and 24 h (figure 35C). It is not clear whether this pattern of granularity was due to a subpopulation of cells undergoing apoptosis (for example) or whether it was due to cells taking up the iron particles. In total, 45.3% of the CD34+ cells in the 24 hour Fe-Pro group were contained within

the subpopulation showing increased granularity (figure 35C), compared to 17.3% in the control group (figure 35A) & 24.3% in the ferumoxide group (figure 35B). Cells incubated with protamine sulphate alone showed a similar pattern to those from the control (medium only) – data not shown. Overall, these data suggest that although exposure to ferumoxide alone has some effect on cell integrity in this culture system, this effect is more marked when cells are incubated with Fe-Pro.

CD34+ cell culture

The cells from the experimental groups (control, ferumoxide, FePro and protamine sulphate) were seeded in Methocult medium (to assess haematopoietic potential) and incubated for 21 days. Haematopoietic colonies were produced from CD34+ cells in all 4 experimental groups. Compared to the control culture (medium alone), there was no difference in the distribution or quantity of colonies derived from cells in all experimental groups (data not shown), indicating that there was no detriment to haematopoietic lineage commitment after incubation of the CD34+ cells with ferumoxide, FePro or with protamine sulphate alone.

Electron Microscopy (EM)

EM images of the ferumoxide preparation alone showed clusters of iron particles ranging between 216nm and 310nm in diameter with the dextran polymer coating surrounding the ferumoxide particles also visualised (figure 36a). The CD34+ cell preparation was evidently undergoing apparent ‘apoptotic’ transformation (figure 36b-d), with filaments protruding from the membrane and with some cytoplasmic vacuolation. When incubated with ferumoxide alone

(figure 36c) it was noted that particles were internalized to some extent (by the cells) - but that there was variation between cells. Cells incubated with FePro (ferumoxide plus protamine sulphate) showed a greater uptake of particles but there was also an increase in the number of vacuoles within the cells (figure 36d). These vacuoles were mostly empty but there were also some liposomes containing coalescent material suggestive of degraded ferumoxide particles.

MRI Phantom Model

The cells from each 24h experiment (medium alone, ferumoxide, Fe-Pro and protamine sulphate alone) were assessed with MRI at 3T (Philips Achieva, Philips, Eindhoven, NL) after dispersal in 4ml of 0.5% agar solution. An area of negative enhancement was observed in the cells incubated with ferumoxide and protamine sulphate, indicating the presence of iron (figure 37). Cells incubated in ferumoxide alone showed no such contrast difference.

Figure 35. Flow cytometric analysis of bone marrow-derived CD34+ve cells.

Incubated with (A) medium alone (control), (B) ferumoxide, and (C) ferumoxide-protamine sulphate (Fe-Pro) complexes for 2h, 4h and 24h. The data show forward vs side scatter and indicate relative granularity of the cell populations. At 24h, there is a change in granularity in the control cells (A: 17.3% in region R1) which is more pronounced in cells incubated with ferumoxide (B: 24.3% in region R1) and is marked in cells incubated with ferumoxide-protamine sulphate complexes (C: 45.3% in region R1).

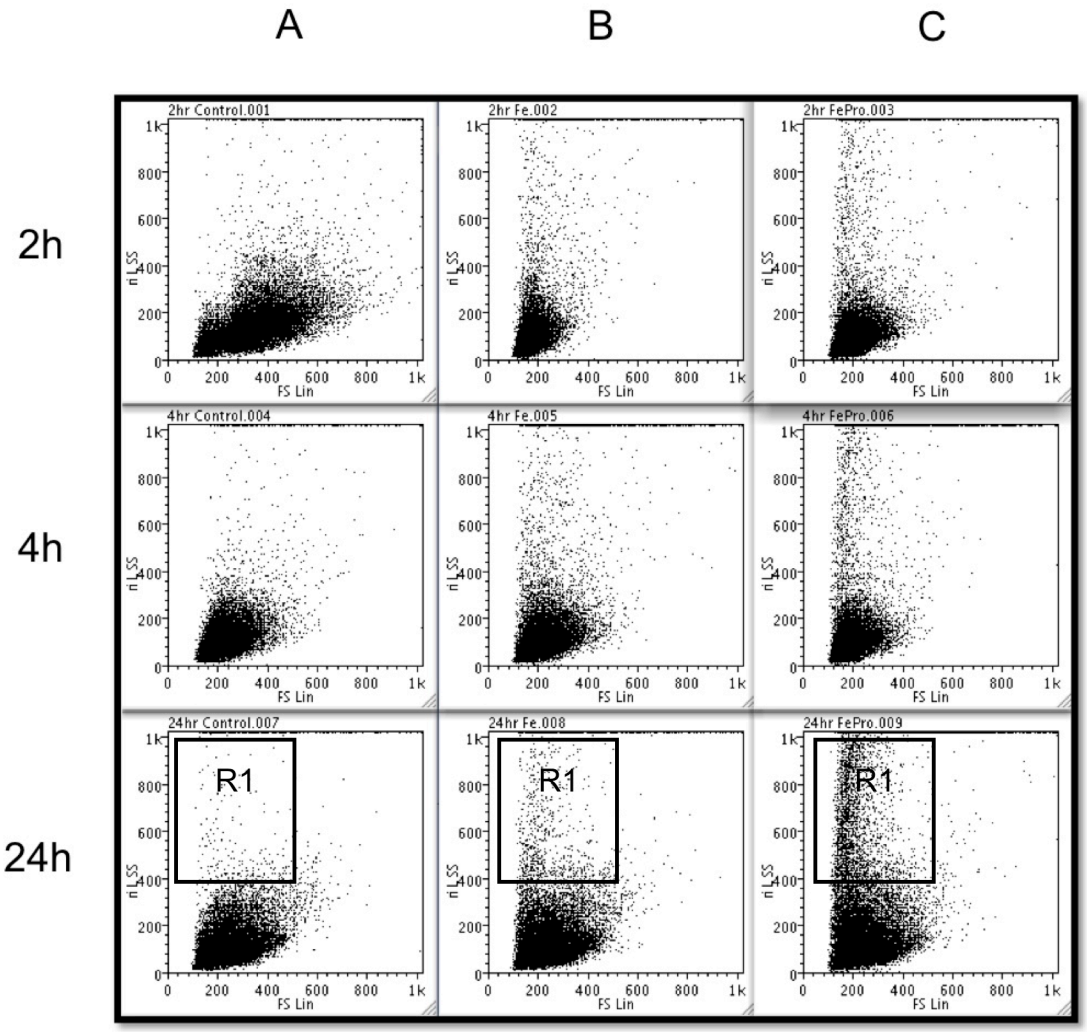


Figure 36. Electron micrographs of labelled CD34+ cells.

(a) Ferumoxide (Endorem) particle, (b) CD34+ cells incubated with medium alone, (c) CD34+ cells incubated with ferumoxide alone and (d) CD34+ve cells incubated with ferumoxide + protamine sulphate (Fe-Pro). Arrows indicate areas where ferumoxide has been taken up into the cell. Cells exposed to Fe-Pro (d) show coalescent material within liposomes, suggesting possible compromise of cell metabolism.

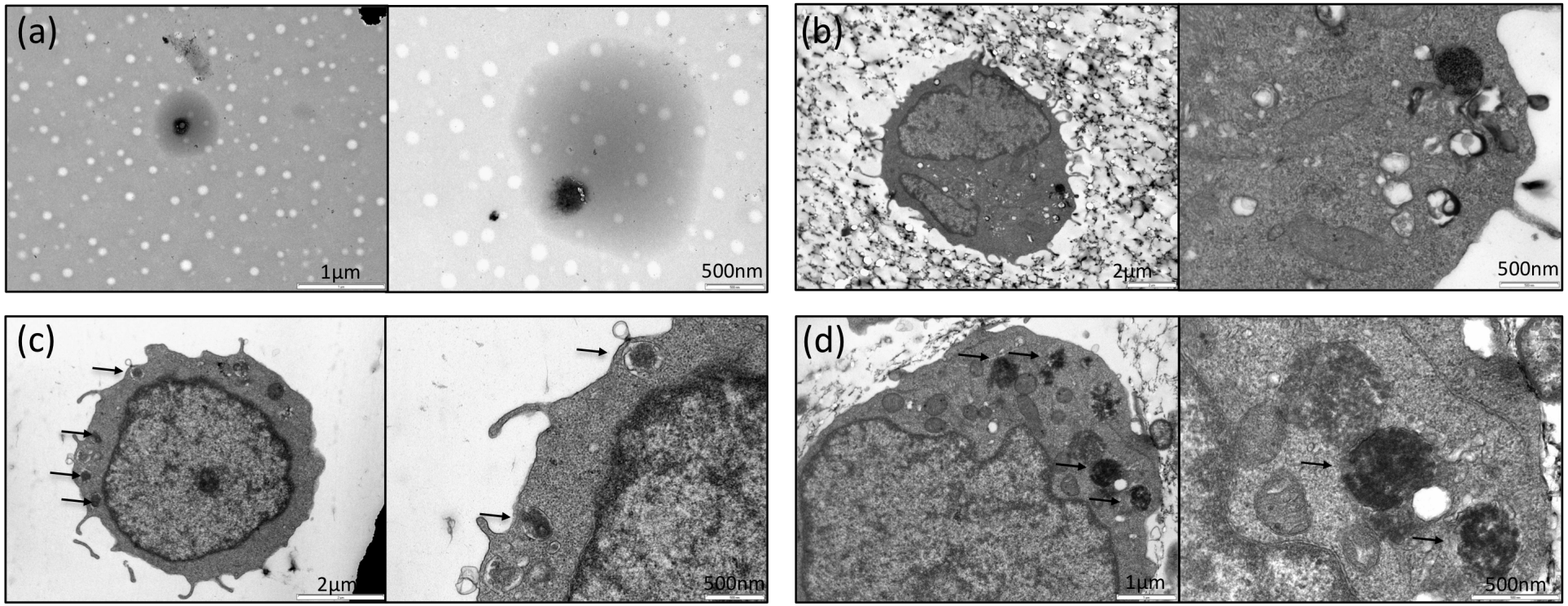
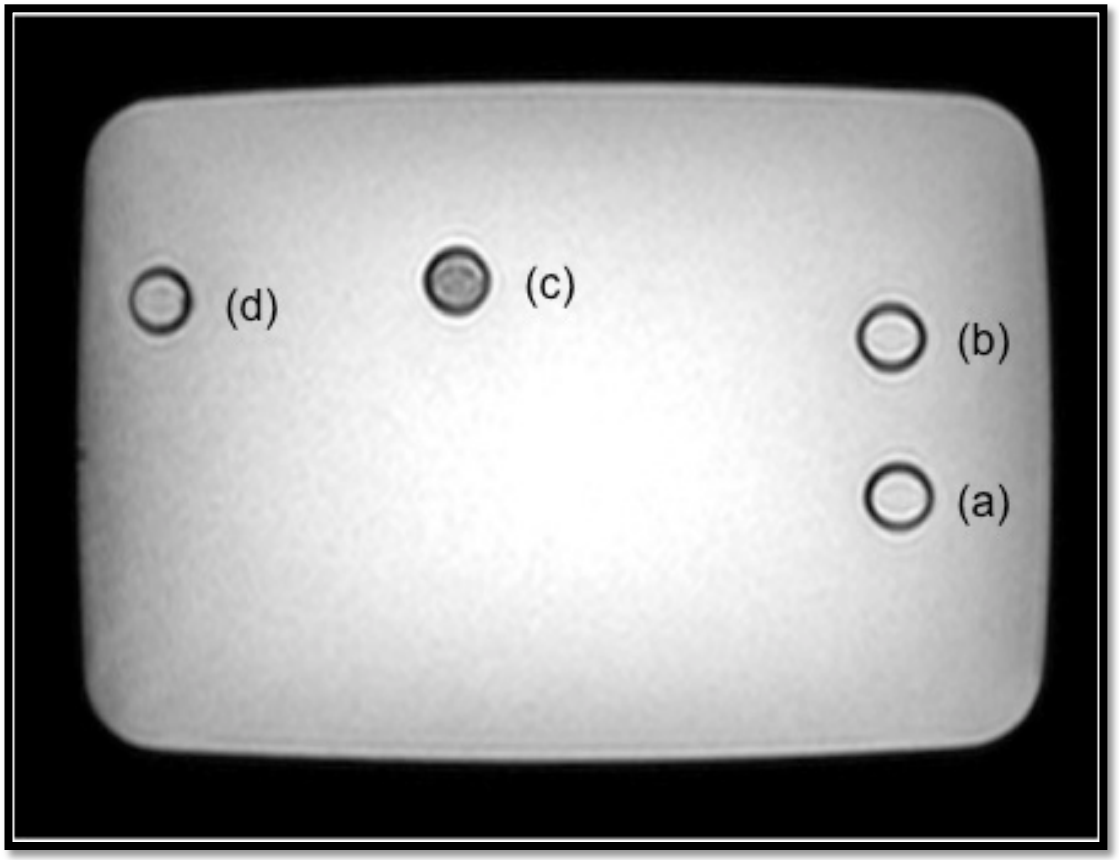


Figure 37. Gradient echo MRI of labelled CD34+ cells.

CD34+ve cells incubated for 24 hours with (a) medium alone, (b) protamine sulphate, (c) ferumoxide + protamine sulphate (Fe-Pro) and (d) ferumoxide alone. Each circle represents a vial containing $2x10^5$ CD34+ve cells dispersed in 4mls of agar.



DISCUSSION

We have demonstrated that CD34+ve haematopoietic progenitor cells can internalise superparamagnetic iron oxide particles (SPIO) in culture medium over a 24 hour period, and that the process of internalisation is enhanced when protamine sulphate is added to the medium. SPIO labelling, with or without protamine sulphate, does not affect cell viability and does not appear to affect the potential of the cells to differentiate in haematopoietic culture medium *in vitro*. Further, we have confirmed that CD34+ve cells labelled *in vitro* in this way can be visualised on 3T MRI scanning.

Other groups have previously addressed haematopoietic stem cell (HSC) labelling with iron-containing compounds (SPIO) of varying composition (such as ferumoxide or ferumoxtran) but the majority of studies have not labelled CD34+ve cells specifically; in those studies cells were incubated for a number of days [431] or weeks, before labelling [434] or were grown in culture until sufficient numbers were obtained.[430] Haematopoietic progenitor (CD34+ve) cells are likely to have differentiated (some to end-stage cells) during time spent in culture and the ability to take up SPIOs could, therefore, be expected to have altered. One study used mixed cell populations (CD34+ve with CD34- cells) obtained from cord blood,[429] making it unclear which cells were actually internalising the iron particles. Another study, using human cord blood as a source of HSCs, demonstrated that CD34+ve cells cannot take up iron without a transfection agent (P7228 with liposome).[435] However, since CD34+ve cells were separated from CD34- cells after the labelling process, it is possible that iron was internalised preferentially into one cell type. In the current study, a single defined cell population was selected and labelled, and this process

revealed that CD34+ve cells can internalise iron in the form of a SPIO (ferumoxide; Endorem), both with and without the addition of protamine sulphate.

Flow cytometric analysis of the CD34+ve cells at 24 hours incubation in the ferumoxide group identified a distinct cell subpopulation, presumably cells containing the iron particles. The uptake of SPIO was confirmed using electron microscopy (preferred to Prussian blue staining since the CD34+ve cells had been harvested by immunomagnetic selection and therefore had iron-containing particles on the cell membrane). It was noted that that cellular uptake of SPIO is far from uniform – ranging from 17.3% to 45.3% - indicating that not all of the CD34+ve cells internalise the ferumoxide. This observation is consistent with a previous report that used iron particle uptake by CD34+ve cells to assess cell migration, post infusion, in immunodeficient mice;[436] cells labelled with Endorem and Resovist (a carbodextran-coated SPIO with a diameter of 60nm) accumulated iron in 61% and 49% of cells respectively. Of interest, freshly isolated cells were compared with frozen/thawed cells and demonstrated up to 85% labelling efficiency if fresh cells were used.[436] Using quantification with spectrometry, other groups have indicated that the use of protamine sulphate and other transfection agents will load a CD34+ve cell with between 1.1pg/cell and 3pg/cell but using high concentrations of ferumoxide during incubation in culture medium.[420, 435] In contrast, lower levels of iron uptake have been observed in (unspecified) peripheral blood progenitor cells (0.8pg/cell) and CD34+ cells (0.01pg/cell) without the use of a transfection agent.[420, 435] In our study, we were unable to quantify the SPIO uptake and therefore cannot directly compare these data with these other reports.

Uptake of iron particles by cells *in vitro* is dependent upon incubation time (and iron concentration) with longer incubation times having a detrimental effect on cell viability.[429, 430] We did not observe this problem: after 24 hours in culture medium, the CD34+ve cells were ~90% viable (using propidium iodide). Despite this high level of viability, electron microscopy revealed the cells to be under stress, raising concerns about the labelling (or thawing) process on cell function. Nonetheless, when compared to unlabelled cells, it was clear that the SPIO-containing cells were able to form similar numbers of colony forming units in culture. Phenotype and migration capacity of HSCs also appears to be unaffected by ferumoxide- or FePro- labelling.[430, 434] However, the effect of SPIO uptake on function remains a controversial point as it has been reported to impede CD34+ve cell differentiation [436] or alter cell surface marker expression in, for example, mesenchymal stem cells.[395, 437] Electron micrograph (EM) data from our study indicate that although protamine sulphate enhanced SPIO uptake, this group of CD34+ve cells had intracellular changes not evident in the ferumoxide group. Since such changes only occurred in the Fe-Pro group (exposure to protamine sulphate without ferumoxide is comparable to medium alone), we suggest that protamine sulphate may enhance SPIO uptake but that the increased iron loading of the cells may ultimately compromise function.

The current study has a number of limitations. First, the commercially sourced CD34+ve cells were immunomagnetically selected, potentially influencing analysis of the iron content of the cells. It was not clear whether the iron containing microbeads on the cell membrane would either degrade or be internalised by the cell. The control groups did not reveal any indication of

internalised iron on EM images, though only a limited number of the cells were assessed. Second, the quantity of internalised iron oxide was not calculated, although analysis by flow cytometry indicated that there is considerable variability in cellular uptake of SPIO. Third, despite confirmation with EM that iron particles were internalised in the ferumoxide group, the magnetic resonance images revealed that only the cells incubated with ferumoxide plus protamine sulphate together were detectable on gradient echo imaging. The detection limit will clearly depend upon magnet field strength, cell number, iron label (size and quantity), site (in vitro or in vivo) and the level of dispersion of cells (concentration). The detection limit and iron load must be balanced with cell viability and functional capability: it is a process still undergoing optimisation.[394] Previously reported studies have shown that as few as 2.5 x 10^5 Fe-Pro labelled cells are needed to be visualised on 1.5T MRI [429] compared to 2 x 10^5 with 3T MRI in the current study.

In summary, the two products Endorem (ferumoxide) and protamine sulphate, individually licensed for human use (for other indications), can be combined to promote iron-oxide nanoparticle uptake by CD34+ve cells uptake without significantly affecting cell viability and proliferative capacity. Further, although the process is enhanced but is not dependent upon the use of protamine sulphate, it would be necessary to carry out further investigations *in vivo* to determine the fate of cells labelled in this way. However, these results suggest that, with adequate cellular concentrations, gradient echo MRI could visualise and therefore potentially track iron (SPIO)-containing cells in a human clinical trial setting.

CHAPTER 8

HISTOPATHOLOGICAL CHARACTERISTICS OF STROKE BRAIN POST TREATMENT WITH GRANULOCYTE COLONY STIMULATING FACTOR: DATA FROM THE STEMS-2 TRIAL

Publications contributing towards this chapter

England TJ, Auer DP, Abaei M, Lowe J, Russell N, Walker M, Bath PMW. Stem-cell trial of recovery enhancement after stroke 2 (STEMS-2). Randomised placebo-controlled trial of granulocyte-colony stimulating factor in mobilising bone marrow stem cells in sub-acute stroke (5th UK Stroke Forum). *International Journal of Stroke*. 2010;5 (Suppl 3):3. Oral presentation.

ABSTRACT

Background

Granulocyte colony stimulating factor (G-CSF) shows promise as a treatment for stroke through neuroprotection or neurorepair (via mobilisation of haematopoietic CD34+ stem cells). The expression of G-CSF protein, its receptor (G-CSFR) and CD34 has never been evaluated in the human brain following treatment with G-CSF.

Methods

In the event of death, patients from the Stem cell Trial of recovery EnhanceMent after Stroke 2 (STEMS-2), a randomised controlled trial

assessing the safety of G-CSF administration 3-30 day post stroke, were eligible to take part in a post mortem substudy. Lesion morphology from histological samples was described qualitatively by a consultant neuropathologist blinded to treatment. Immunohistochemical characterisation for astrocytes (GFAP), microglia (CD68) and cell proliferation (Mib-1) was performed in addition to CD34, G-CSF protein and G-CSFR. 'Non-trial' stroke brains were used as control cases.

Results

The brains of 3 patients treated with G-CSF were compared to 3 control brains (mean age 76 and 64 respectively) analysing areas of acute, subacute and chronic infarction. There was no discernable difference in immunoreactivity between groups for CD34 (weakly staining endothelium), G-CSF (also positive in endothelium) and G-CSFR (evident in neurons in acute stroke and microglia in chronic stroke). Mib-1 was positive in endothelium in acute infarction indicating ongoing angiogenesis.

Conclusions

G-CSF treatment did not clearly alter the cellular profile of ischaemic brain tissue though the sample size was small. Areas of angiogenesis and expression of G-CSFR in acute and chronic infarction suggest potential targets for therapy.

INTRODUCTION

Experimental stroke studies assessing the effects of granulocyte colony stimulating factor (G-CSF), a polypeptide glycoprotein hormone that regulates the production of neutrophilic granulocytes,[438] have demonstrated promise in developing the drug as a potential treatment for ischaemic stroke.[137] If administered hyperacutely, reductions in infarct volume suggest that improvements in recovery could be due to neuroprotection.[376] Furthermore, G-CSF induced mobilisation of haematopoietic (CD34+) stem cells migrating to the infarcted brain could explain why enhanced functional recovery is also observed when G-CSF is given beyond 24 hours post ictus [161, 235, 236, 439] (though fewer published studies have addressed this). G-CSF can cross the blood-brain barrier, reduces apoptosis in the ischaemic penumbra via upregulation of a neuronal G-CSF receptor and augments the neurovascular microenvironment via new vessel and neuron formation.[137, 235] G-CSF receptor is expressed on a number of haematopoietic (platelets, myeloid and lymphoid cell lines [440]) and non-haematopoietic cell types including neurons in the central nervous system [137] and endothelial cells.[402]

Despite the accumulating wealth of evidence, very little has been established about the expression of G-CSF and its receptor in human ischaemic brain tissue. One autopsy series of 21 stroke brains revealed evidence of G-CSF receptor expression on neurons in acute stroke, microglia and macrophages in subacute stroke and astrocytic expression in chronic infarction.[366] No studies have shown how the cellular profile alters following treatment with G-CSF. As part of the Stem cell Trial of recovery EnhanceMent after Stroke 2 (STEMS-2), which assessed the safety and feasibility of G-CSF treatment for subacute

stroke, [304] we aimed to establish the cellular profile in cerebral infarction in patients treated recently with recombinant G-CSF (Filgrastim), with reference to CD34, G-CSF and G-CSF receptor expression in the brain.

METHODS

Subjects

Patients were recruited as part of a substudy of the STEMS-2 trial. These methods are covered in earlier chapters. Briefly, STEMS-2 was a double blind, randomised placebo-controlled trial assessing the safety and feasibility of G-CSF in subacute stroke.[304] Patients received either 10μg/kg G-CSF or placebo (normal saline) subcutaneously once per day for 5 days and were followed up for 90 (±7) days. Participants were given the option to donate their brain to the trial in the event of their death for post mortem examination.

Ethics

Full ethical approval was obtained during the original application for the main trial (Local Research Ethics Committee, Nottingham LREC 22nd May 2007). Patients were approached once they had enrolled into the main part of the study and information on brain donation was left with them to consider involvement. Fully informed consent or assent from a legal representative was gained in cooperation with the Pathology Department, Queen's Medical Centre, Nottingham. In order to develop immunohistochemical techniques, we used brain retained from post mortem examinations performed under normal ongoing Trust diagnostic activities; the cases came with consent for use of retained organs and tissues for research and teaching in addition to the diagnostic component of the post mortem. For control cases, the standard post mortem consent form was used, which is aligned to the nationally advised form and complies with requirements under the Human Tissues Act.

Immunohistochemistry (IHC)

Brain slices were examined following formalin fixation. Histological samples were taken from representative brain areas to include both macroscopically pathological and normal tissue. Pathology was characterised using conventional histological techniques using formalin-fixed, wax-embedded tissue; the tissue was cut on a microtome set at 3μm and floated on to a water bath (45 ^{o}C) to iron out any creases. Sections were picked up on charged slides and drained for 20 minutes before baking at 60 ^{o}C for 2 hours prior to staining (this minimises the risk of sections lifting off the slide). IHC staining was performed using a panel of antibodies applied in accordance with the manufacturers recommendations using BondMax staining machines (Leica Microsystems). IHC characterisation was carried out using standard markers for astroglial (GFAP, 1/4000) and microglial (CD68, 1/2000) phenotypes.[290] Immunostaining for CD34 was performed as well as Mib-1 labelling to look for cell proliferation in neuronal and vascular cells.[290] In addition, staining and characterisation for G-CSF protein (1/50, Santa Cruz Biotechnology, Inc) and its receptor (G-CSFR, 1/50, Santa Cruz Biotechnology, Inc) were carried out.[366] Lesion morphology was described qualitatively and age of the infarct categorised into acute, subacute or chronic. Slides were interpreted by a consultant neuropathologist (JL) blinded to treatment allocation.

RESULTS

Subjects

6 patients donated their brain for analysis (figure 38), 3 in the G-CSF group (1 male and 2 female) and 3 non-trial controls (1 male and 2 female); mean age was 76 and 64 years respectively. The time of death from onset of neurological symptoms ranged between 5 and 92 days (table 27). Infarcts within the hippocampus (acute), cortex (subacute and chronic), cerebellum (chronic) and basal ganglia (chronic) were studied. In 3 patients, the cause of death was secondary to stroke or complications from it (aspiration pneumonia); the other 3 cases died from community acquired pneumonia, ischaemic bowel and hydrocephalus.

Immunohistochemistry

Regions of infarct were initially studied using H&E sections, markers for astrocytes (GFAP) and microglia (CD68) were used to further determine these areas. It became evident that 'normal' H&E sections demonstrated an abnormal GFAP pattern, indicating GFAP a more sensitive marker for ischaemia (figure 39).

Across all cases (table 28, figures 40-46), CD34 immunoreactivity was weak, often inconsistent, and stained endothelium in small to medium sized vessels within regions of infarction. No other cells were CD34 positive.

In control and G-CSF treated subjects, G-CSF receptor antibodies stained positively in microglia (and was weakly positive in astrocytes) in areas of

chronic stroke with no discernable difference between treatment groups. In one patient in the control group with an acute area of infarction in the hippocampus there was strong staining for G-CSF receptor, in neurons and astrocytes (figure 40).

The G-CSF protein antibody weakly stained endothelium in small vessels in all except one patient in the G-CSF group. This exceptional case demonstrated, interestingly, strong immunoreactivity in viable vessels within the area of infarction (figure 45).

Mib-1, which identifies proliferating cells, was consistently immunopositive in endothelium in one control case, indicating ongoing angiogenesis in regions of subacute and acute infarction (figure 46A). A proportion of cells of unclear type were Mib-1 immunopositive in the ischaemic border zone of the acute infarct (figure 46B). Cells with normal cerebral architecture in the peri-ischaemic tissue were Mib-1 negative. Unfortunately, the Mib-1 stain across the majority of remaining cases failed to highlight any proliferating cells; this may be secondary to modes of death (very large infarcts) and delay to post-mortem influencing tissue viability.

Table 27. Baseline characteristics of patients in the post-mortem substudy of STEMS-2.
NB Control patients were not part of the randomised STEMS-2 trial.

Patient N°	Age	Sex	Treatment allocation	Mode of death	Time of death from onset of neurological symptoms	Time from death to autopsy
1	35	M	Control	Cerebral infarct secondary to hydrocephalus and colloid cyst	Unknown	2 days
2	81	F	Control	MCA infarct & aspiration pneumonia	10 days	1 day
3	77	F	Control	MCA infarct	5 days	7 days
4	76	F	G-CSF	MCA infarct	29 days	3 days
5	70	F	G-CSF	Pneumonia	92 days	3 days
6	81	M	G-CSF	Ischaemic bowel	37 days	2 days

G-CSF, granulocyte colony stimulating factor; MCA, middle cerebral artery

Table 28. Distribution of endothelial, neuronal, microglial and astrocytic CD34, G-CSF and G-CSF receptor immunoreactivity in ischaemic stroke brain in subjects treated with G-CSF and controls.

N°	Brain region	Group	Infarct age	Immunoreactivity											
				CD34				G-CSF protein				G-CSF receptor			
				V	N	M	A	V	N	M	A	V	N	M	A
1	Hippocampus	Control	Acute	+	-	-	-	(+)	-	-	-	-	+++	-	+++
2	Cortex	Control	Chronic	+	-	-	-	+	-	-	-	-		+	-
3	Cerebellum	Control	Chronic	(+)	-	-	-	+	-	-	-	-	-	++	-
4	Cortex	G-CSF	Chronic	+	-	-	-	+	-	-	-	-	-	++	+
5	Cortex	G-CSF	Chronic	+	-	-	-	+	-	(+)	(+)	-	+ß	++	++
6	Basal ganglia	G-CSF	Chronic	++	-	-	-	+++	-	-	-	-	-	++	(+)

(+), patchy immunoreactivity; +, weak immunoreactivity; ++, good immunoreactivity; +++, strong immunoreactivity; V, vascular cells (endothelium); N, neurons; M, microglia; A, astrocytes; ß, potential cross reactivity with chromatolytic neurones

Figure 38. Recruitment flow in to the post-mortem substudy of ‘The Stem cell Trial of recovery EnhanceMent after Stroke 2’ (STEMS-2).

(G-CSF, granulocyte colony stimulating factor)

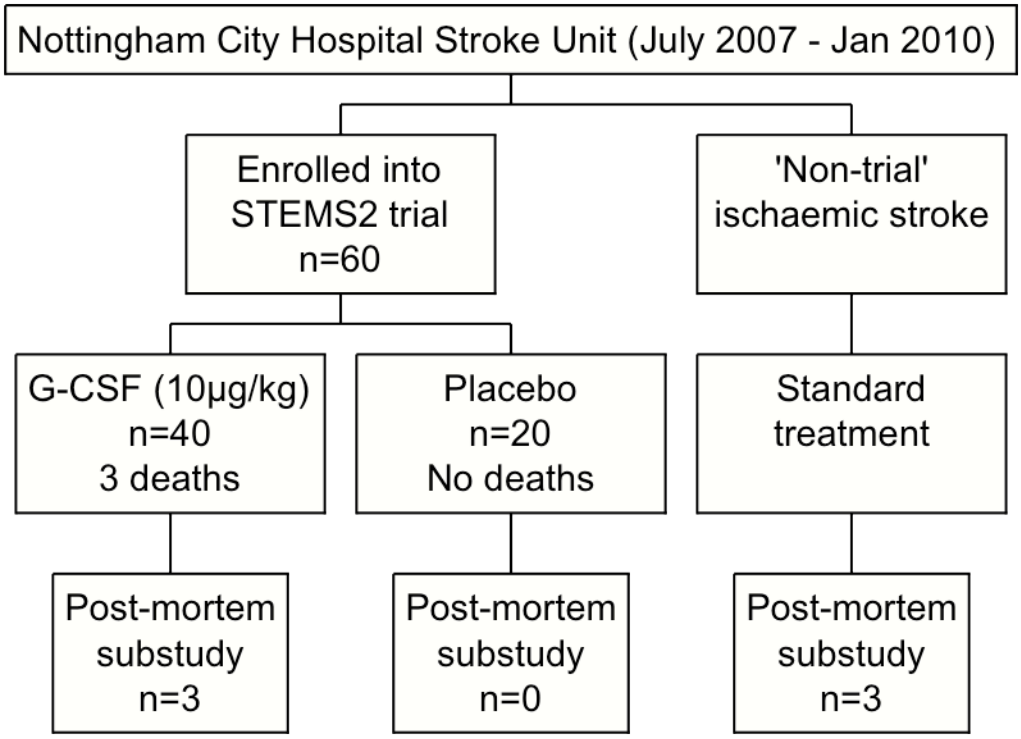


Figure 39. GFAP immunostaining in a region of cortical infarction.

(A) Discrete areas of infarct are apparent with reactive gliosis and dissociation of GFAP within the infarct. (B) There is preservation of GFAP matrix in the adjacent gyrus, in particular in peri-vascular regions. (C) Compared to the ‘normal’ H&E section (not shown), there is widespread loss of astrocytic architecture producing a washed out appearance in areas of global hypoxia.

Figures 40-46. Cases correspond to recruits 1-6 in tables 27 and 28. Immunostaining for each case including haematoxylin and eosin (H&E), glial fibrillary acid protein (GFAP) (staining astrocytes), CD68 (microglia/macrophages), CD34, G-CSF protein and its receptor (G-CSFR). Magnification x4 unless otherwise stated.

Figure 40. Case 1 (control group): acute infarct in the hippocampus.

CD34 and G-CSF protein stains endothelium and neurons are immunopositive for G-CSFR.

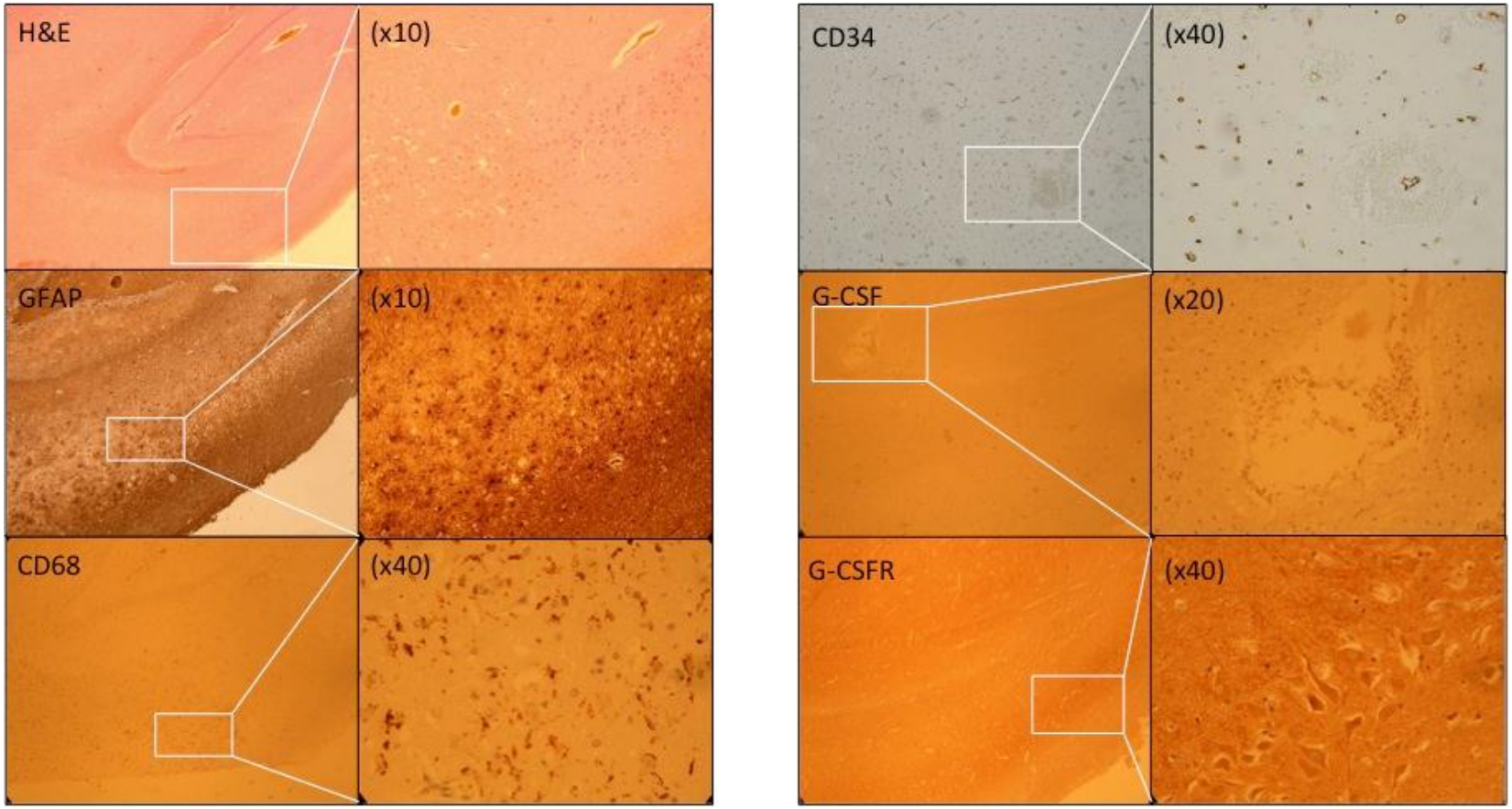


Figure 41. Case 2 (control group): chronic infarct in the cortex.

There is peri-vascular preservation of astrocytes (GFAP) and endothelium is weakly stained by CD34 and G-CSF. Macrophages are positive for G-CSFR.

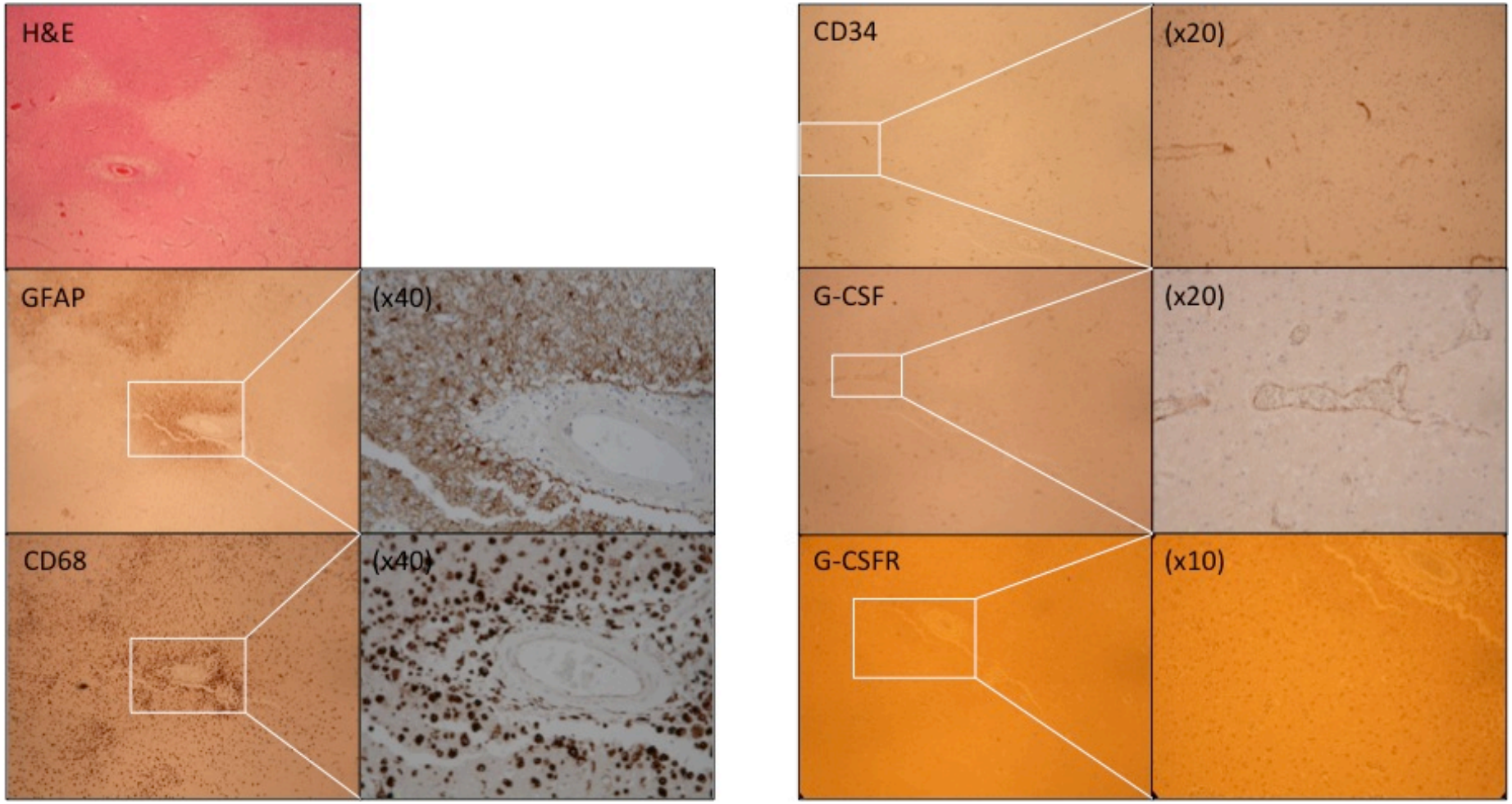


Figure 42. Case 3 (control group): chronic infarct in the cerebellum with astrocytic gliosis.

The CD34 stain is negative whilst G-CSF protein is weakly immunopositive. Microglia/macrophages are positive for G-CSFR within the chronic infarct.

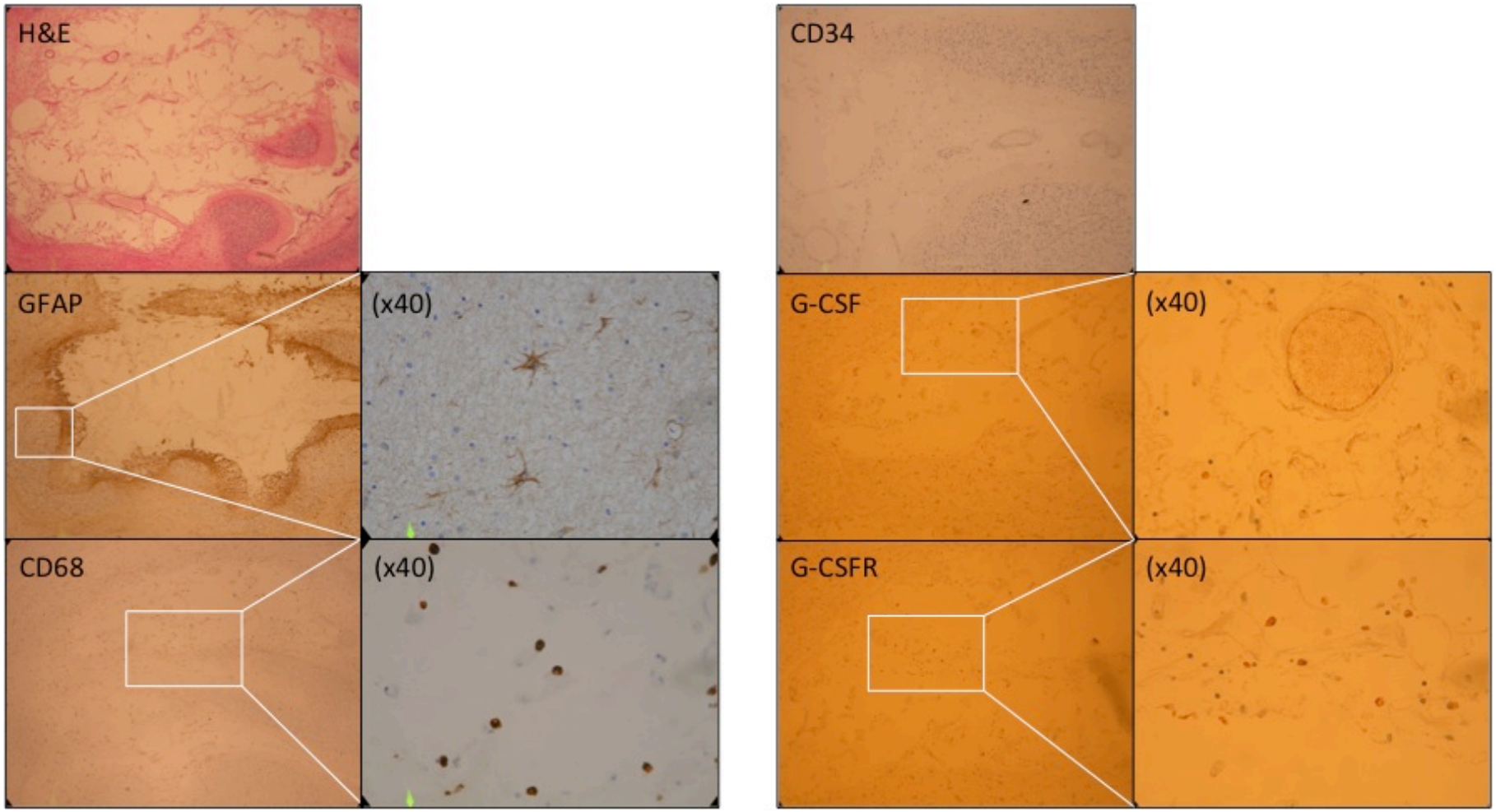


Figure 43. Case 4 (G-CSF group): chronic cortical infarct.

Similar to case 2 (control) - endothelium is weakly stained by CD34. Macrophages are positive for G-CSFR (no stain for G-CSF protein performed).

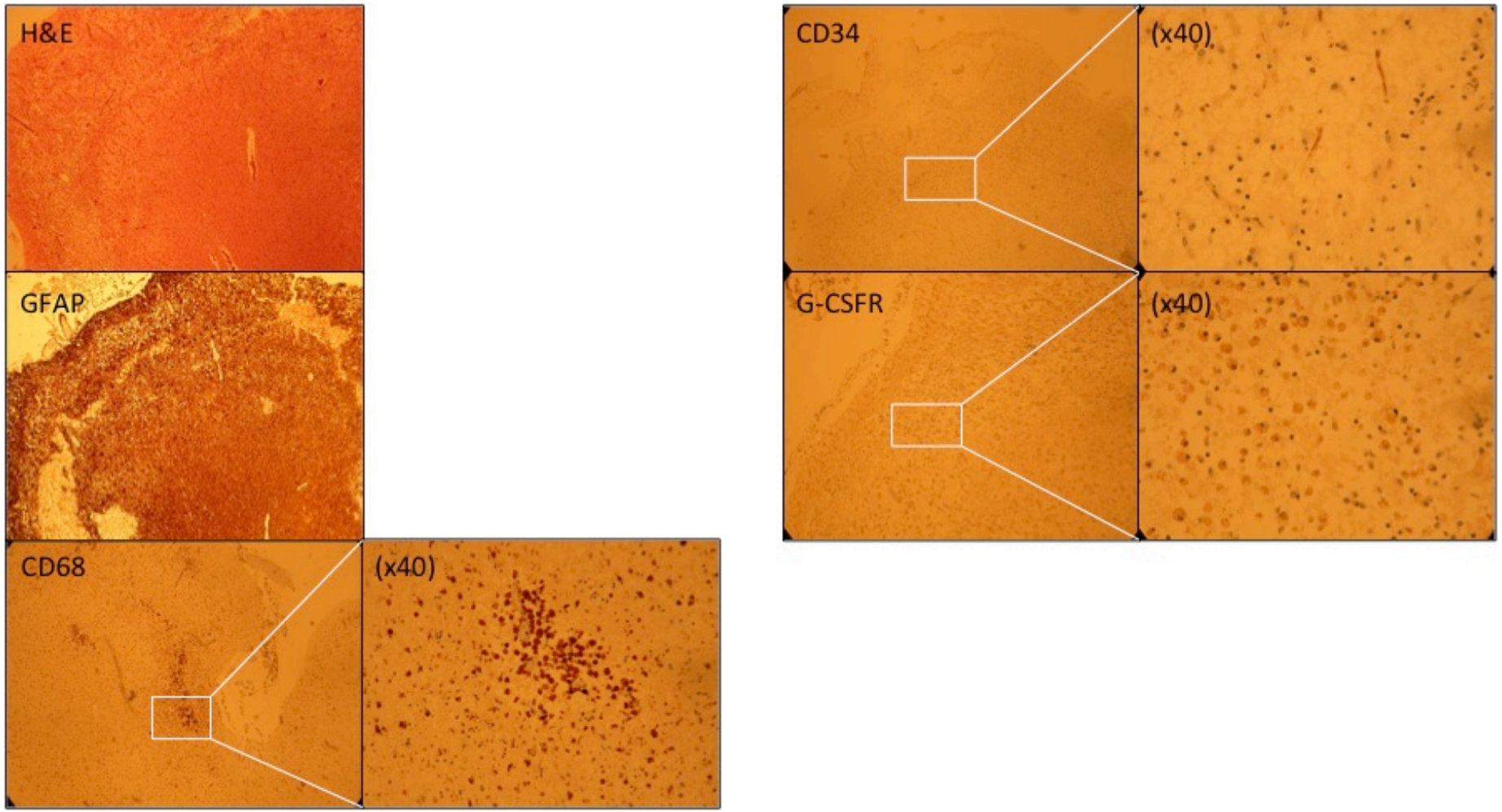


Figure 44. Case 5 (G-CSF group), established cortical infarct.

Again, endothelial cells stain positively for CD34 and G-CSF protein. Interpretation of G-CSFR profile is confounded by cross reactivity with lipofuscin (cytoplasmic lipids, protein and carbohydrates).

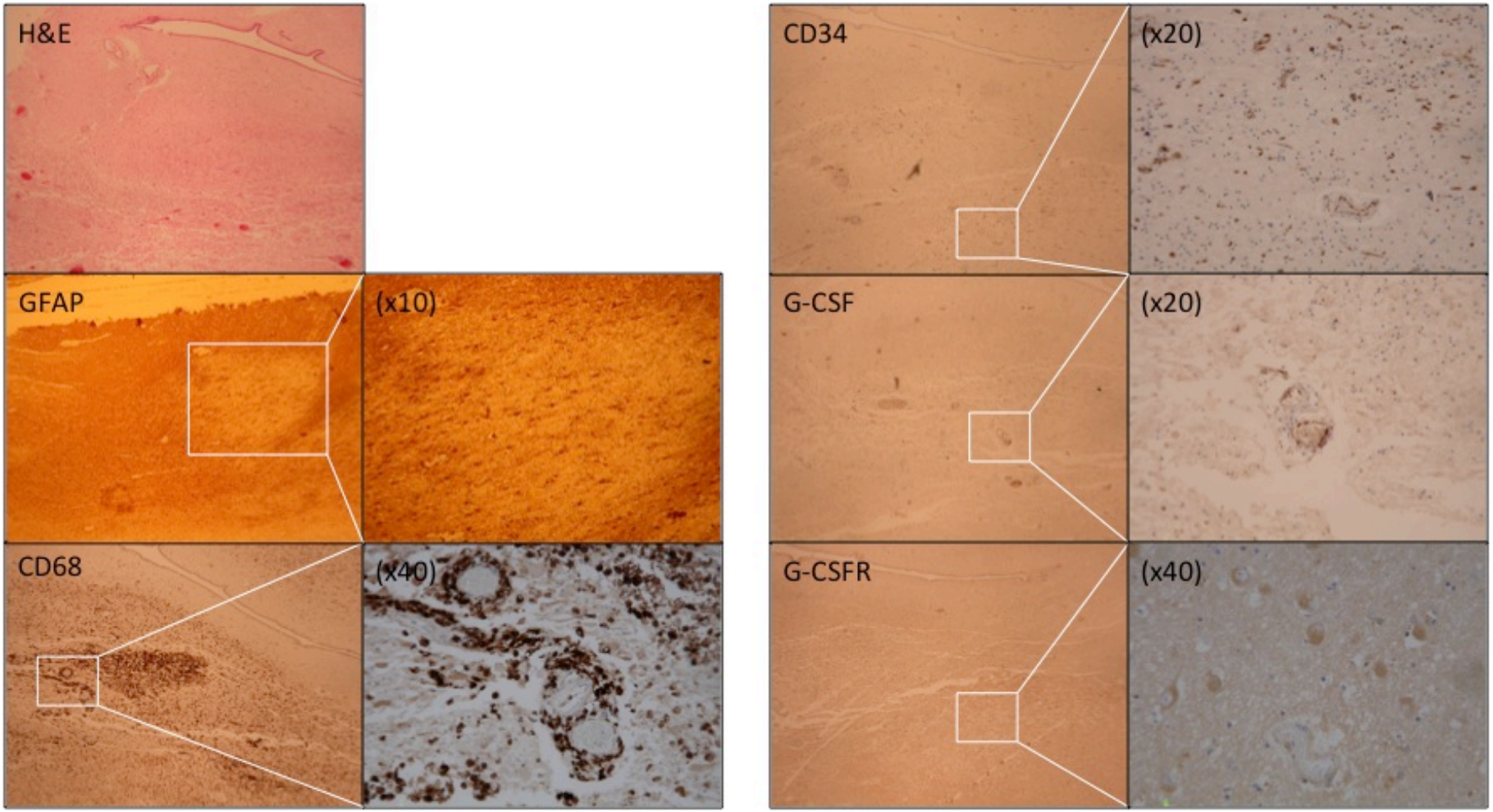


Figure 45. Case 6 (G-CSF group): chronic infarct in the basal ganglia.

Intensity of staining for G-CSF protein was much greater in this case in comparison to all other cases.

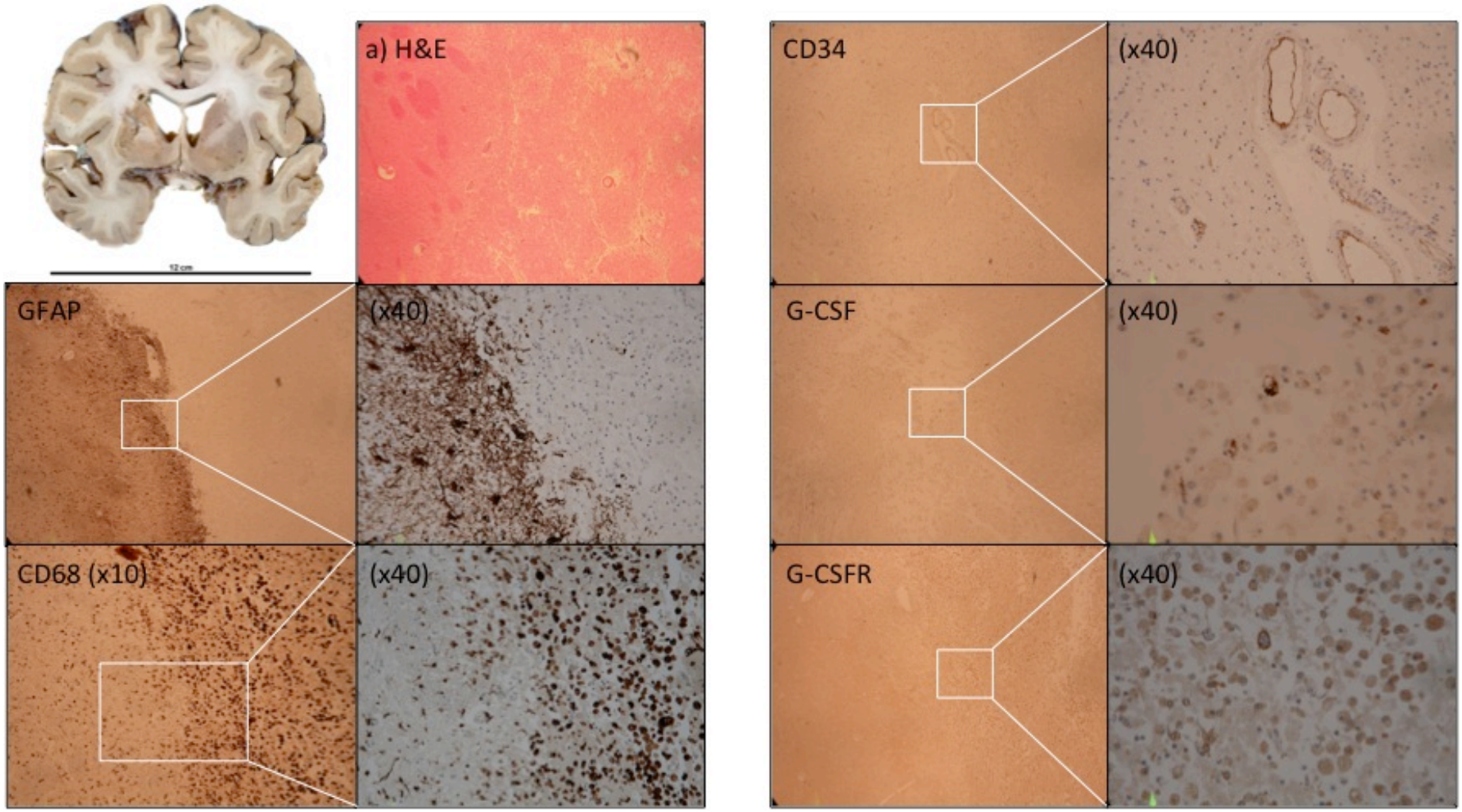
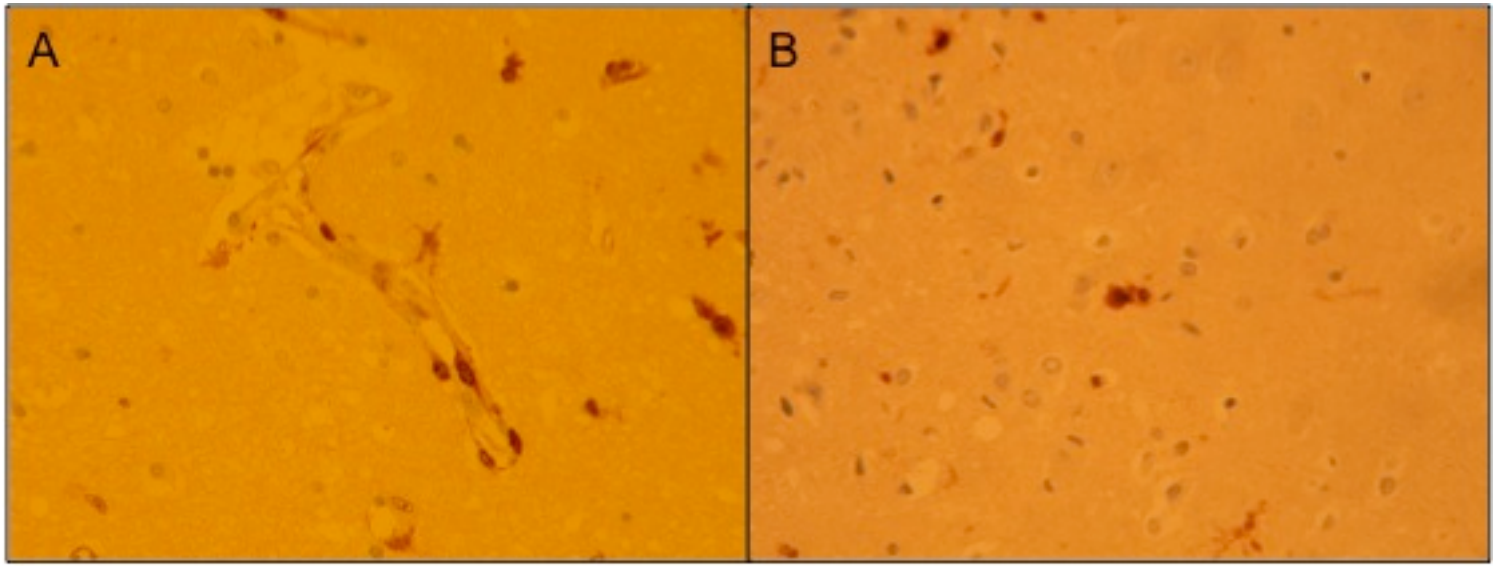


Figure 46. Mib-1 immunopositive stain in (A) endothelium within acute stroke indicating ongoing angiogenesis and (B) indeterminate cell types in the ischaemic border zone of acute infarction.

Magnification x40.



DISCUSSION

Post-mortem analysis of the brains of patients recruited into STEMS-2 provides information about the cellular profile within areas of cerebral infarction following treatment with G-CSF. Our findings are consistent with an earlier autopsy series of 21 stroke patients which has described G-CSF protein expression in endothelial cells in acute cerebral infarction and observation of G-CSF receptor expression in neurons (acute infarction), microglia (subacute) and reactive astrocytes (chronic).[366] In the present study, however, G-CSF receptor was consistently immunopositive in CD68+ macrophages and microglia in chronic infarction.

There were no obvious differences between the brains of subjects in our treatment and control groups with the exception of one patient treated with G-CSF who had increased intensity of endothelial G-CSF protein immunoreactivity. It is tempting to speculate that this was secondary to treatment, though it could also have been due to heterogeneity in case mix such as mode of death or time to autopsy; the sample size is too small to draw any conclusions. Of note, the absence of G-CSF protein expression in cerebral, non-vascular, chronically ischaemic cells does not support the concept of endogenous G-CSF production as reported by experimental (acute) ischaemia where the protein is up regulated more than 100-fold.[137]

Nonetheless, the expression of G-CSF receptor in subacute and chronic infarction suggests a potential mechanism underlying improvements in functional outcome as seen in experimental models.[235] Neuronal G-CSF receptor stimulation in acute stroke may reduce infarct volume via the anti-

apoptotic P13K/Akt pathway;[137] the receptor's role in subacute and chronic infarction has not been investigated and is much less clear. Recovery from experimental subacute and chronic stroke following administration of G-CSF (in combination with stem cell factor, SCF) was shown in one study to augment functional recovery via mobilisation and migration of bone marrow derived stem cells,[441] promoting a favourable microenvironment for neurogenesis and angiogenesis through up regulation of IL-10.[346] Interestingly, in the same study, no significant changes in G-CSF and SCF expression were observed following co-administration of G-CSF and SCF 11 to 20 days post stroke.[346]

Evidence of increased peripheral blood CD34+ cells post stroke,[213] expression of G-CSF and EPO receptors in the CNS,[137, 254] and expression of neurotransmitter receptors on CD34+ cells [442] highlight the importance of understanding the 'brain-bone-blood' axis.[443] Migrating CD34+ haematopoietic stem cells may play a key role in orchestrating neurorepair post stroke and perhaps the G-CSF receptor is involved in chemotaxis, similar to other stem cell hormones and their receptors, for example, stromal cell derived factor (SDF-1) and CXCR4.[444]

CD34 antigen has been shown to be present in CNS endothelium,[445] reduced in inflammation and demyelination [445] and expressed in microglia on days 3 to 5 subsequent to acute neural injury.[446] Microglial cell populations expand following neuronal ischemia. This could be due to proliferation of resident microglia, migration of microglia from adjacent brain areas or, relevantly, by recruitment of migrating microglial progenitors from the blood.[447] However, our results revealed no evidence of CD34 expression in cell types other than

endothelium (in small to medium sized vessels). Nonetheless, evidence of viable endothelial tissue within infarcted brain and proliferating Mib-1 immunopositive angiogenic cells in acutely ischaemic areas suggest there are areas within the ischaemic brain for drugs to target and augment recovery.

G-CSF and other colony stimulating factors involved in microglial proliferation and activation (such as macrophage colony stimulating factor (M-CSF [448]) and granulocyte macrophage colony stimulating factor (GM-CSF [232])) are potential drug candidates for the treatment of stroke. The majority of work assessing G-CSF in experimental stroke supports its use as a therapeutic agent through infarct volume reduction [376] and suppression of proinflammatory cytokines (e.g. IL-1B,[138, 357] neuronal and glial tumour necrosis factor,[356, 357] matrix metalloproteinase-9 [244]). However, negative effects have also been observed;[167] in one study, a murine model of permanent stroke resulted in impaired behavioural function following treatment with G-CSF in association with an increase in inflammatory infiltrate of F4/80+ macrophages/microglia. This concern has not been apparent in clinical safety trials of G-CSF to date and further trials are ongoing.[299]

Our study must be read with a number of caveats in mind. First, although all suitable patients within the STEMS-2 trial were approached, fewer subjects were recruited than expected. The total number of recruits who died within 90 days was 3, all of whom were included. In order to increase recruitment, the follow-up period of the trial would have to be extended beyond the original 90 days. The unpredictable nature of mortality within a trial leads to practical recruitment difficulties and as a consequence interpretation of data from such a

small sample size should be interpreted with ‘healthy scepticism’. Second, there was a natural selection bias since it was considered clinically not appropriate to approach some patients and families. Third, the delay between death and post mortem significantly affects tissue viability and antigen expression in humans, a phenomenon not encountered in experimental stroke. Thus we may have underestimated any effects G-CSF treatment could have on the cerebral cellular profile. Fourth, the results are confounded by heterogeneity in case mix, with substantial variation in time to death post stroke onset in the G-CSF group. This did, however, allow different ages of stroke to be analysed. In addition, the control cases were not age- and sex-matched (instead selected at random), which has led to further confounding of the results. Finally, interpretation of antigen expression may have been influenced by cross-reactivity with other proteins, such as with cells containing lipofuscin (accumulation of lipids, proteins and carbohydrate granules, a common feature in aging) or chromatolytic neurons (ballooned, swollen neurons reacting to axonal damage) producing potential false positive results.

Despite the above limitations, this is the first clinical study exploring the effects of G-CSF treatment on the human ischaemic brain and cellular expression of CD34, G-CSF and its receptor.

CHAPTER 9

DISCUSSION

Safety of G-CSF for treatment of stroke

The primary outcome of STEMS-2, from which the body of this thesis is derived, has suggested that subacute administration of G-CSF in ischaemic stroke is safe when compared to placebo. This is also supported by evidence aggregated from all randomised controlled trials addressing the use of colony stimulating factors in stroke (see chapter 5); in 5 small trials (n=167) G-CSF had a neutral effect on death and dependency and there was evidence of a trend towards improvement in early impairment. G-CSF was well tolerated with no significant differences in serious adverse events when compared to placebo. However, future phase III efficacy trials must make safety analysis a key feature, particularly in light of the recent negative outcome of a trial assessing erythropoietin in hyperacute ischaemic stroke.[256] Although this revealed a higher death rate in EPO treated subjects, the trial was potentially confounded by the unusually large number of participants who were thrombolysed. This should not be an issue when administering G-CSF subacutely since it is given at a time point beyond the wash-out period of rtPA; the subgroup of thrombolysed recruits in STEMS-2 (7%) did not highlight any specific safety concerns, though the sample size is obviously very small.

The failure of numerous experimental drug therapies that could be used for stroke to be translated successfully into clinical research is an ongoing concern, and is a focus for the STAIR and STEPS committees who have produced a

series of recommendations and guidelines suggesting how to address the matter.[152, 275, 276] Chapter 2, a systematic review of G-CSF in experimental stroke, highlights that pre-clinical studies assessing G-CSF have fulfilled only a proportion of these recommendations and that further evidence in permanent models of ischaemia, which are an additional representation of human stroke, are required. In addition, experimental evidence to date reports just one study evaluating the effects of G-CSF and thrombolysis given together - early combination with rtPA showed no additional benefit compared to rtPA or G-CSF alone and did not lead to side effects.[246] Producing representative embolic animal models for evaluating rt-PA are technically difficult but further animal studies with G-CSF are still required in this area. Pre-clinical experiments assessing EPO in stroke also fulfilled a large proportion of the recommendations [449] but did not include any studies assessing an EPO/rtPA combination before the negative phase III trial. A subsequent experiment has demonstrated a hitherto unknown interaction of rtPA with EPO, in which vascular permeability is promoted and there is extracellular matrix breakdown at the blood-brain interface.[415]

Tracking haematopoietic stem cells post stroke

Understanding the capacity of a stem cell to migrate to its therapeutic target is a key determinant of how potential cell therapies can be delivered in future care. Following administration via intravascular or intracerebral routes, cells have been tracked with a variety of techniques in animal models that clearly have limited application in human clinical trials. The majority of previous human cell tracking studies use radiolabelling (17 studies, mostly in acute or chronic

myocardial infarction [450]) compared with 3 assessing iron labelling studies in spinal cord injury,[424] brain trauma [422] and diabetes.[423]

We have concentrated on iron-labelling techniques in Chapter 3, a systematic review of the pre-clinical literature assessing paramagnetic labelling of stem cells in ischaemic stroke. This draws attention to the heterogeneity between experiments; including differences in stroke model (transient versus permanent ischaemia), timing and route of cell transplantation, stem cell type and dose, and inconsistent use of a transfection agent as part of the labelling process. Our experiment (Chapter 7) demonstrated that compounds already licensed in humans for other indications (ferumoxide and protamine sulphate) show promise for use in future cell-labelling studies.[385, 389, 391, 392] The presence of foreign material within a cell may lead to cellular dysfunction; intracellular iron concentration therefore needs to be kept to a minimum in order not to interfere with cell dynamics (e.g. the ability of a cell to migrate to another site) but should be high enough for the cells to be visualised on MRI scans. However, altering the intrinsic components of a cell by adding intracellular compounds could not only have detrimental effects on cell viability but also on the potential therapeutic target. This has been observed in animal models of stroke using GRID labelled cells over a 1 year follow up; animals receiving labeled cells had a poorer outcome in the form of increased stroke lesion volume.[397]

It was not apparent from the existing medical literature that undifferentiated CD34+ HSCs would internalise SPIO without a transfection agent. We therefore addressed this (Chapter 7) and established that CD34+ cell uptake of ferumoxide is enhanced by but is not dependent upon the transfection agent

protamine sulphate. The techniques used did not demonstrably affect CD34+ cell viability or differentiation capacity when compared to unlabelled cells in the short term but we have not addressed the effects of labelling on long term cell function. Before prospective mechanistic trials using iron-labelling techniques are used to assess cell dynamics in stroke, further research assessing long term follow up in cell labelling experiments is therefore justified.

In Chapter 6, we have demonstrated that labelling CD34+ haematopoietic stem cells (HSC) with an extracellular paramagnetic iron oxide nano-particle and re-administering them intravenously in the subacute phase of ischaemic stroke is safe and feasible. We noted a new hypodensity within an infarct on gradient echo (T2*) imaging subsequent to administration of labelled cells in one patient and suggest this may be evidence of CD34+ cells migrating to the stroke lesion. Interpretation of cell migration is restricted by the presence of blood (iron) in infarcted brain tissue, present in a high proportion of ischaemic strokes on MR imaging.[400] Future trials tracking stem cells in stroke using iron labels should therefore be more highly selective and exclude patients with baseline haemorrhagic transformation of infarction. It would also be interesting to assess CD34+ cells labelled with intracellular iron (such as ferumoxide, a superparamagnetic iron oxide particle, SPIO), to trial higher cell doses or alternative routes and times of administration. If it were possible to recruit a sufficient number of patients after HTI was excluded in order to explore these parameters further, interpretation of subsequent MR images will always be qualitative and under question: is the new hypodensity *new* blood or label? There will also be questions over cell specificity (i.e. another cell type can phagocytose the label) and cell viability. With these significant limitations in

mind, pursuing iron labeled techniques in stroke is unlikely to be successful in providing clear evidence of cell migration in human stroke.

Limitations of the current work

STEMS-2 was a robust randomised placebo-controlled safety trial but, in addition to the limitations of the labelling substudy described above, there are other caveats to consider. First, the total sample size of 60 does not allow any conclusions about treatment efficacy to be reached. Our updated Cochrane review (of five small trials) does not indicate that treating stroke with G-CSF improves death or dependency when compared to placebo. Encouragingly, however, the review revealed a trend towards improvement in early impairment (as measured by NIHSS) in subjects treated with a colony-stimulating factor (weighted mean difference -0.4, 95% confidence interval -1.82 to 1.01, p=0.58). Furthermore, in STEMS-2, the median value in some efficacy parameters (NIHSS, grip strength, motricicty index, NEADL, MMSE) was non-significantly better when compared to placebo. This did not hold true for the mRS or Barthel index, in which the median values were the same in each group. Progression to a larger phase III efficacy trial would be necessary in order to confirm the presence of any possible treatment effect. Further criticism can be made regarding the choice of the mood and cognitive assessment tools; these were both affected by the presence of dysphasia and more appropriate and validated tools could have been used, such as the SADQ-H10 (10-item Hospital version of the Stroke Aphasic Depression Questionnaire), which is valid and reliable in measuring depressive symptoms in stroke patients with aphasia.[328]

Other restrictions, by nature of the trial design, are introduced when subsets of patients were excluded from STEMS-2 and its substudies (a selection bias). The trial required subjects to be hospital inpatients for a minimum of 8 days (recruited on day 3 post-stroke, followed by 5 days of treatment); hence patients with mild stroke and short lengths of hospital stay were not included. In addition, selection by presence of limb weakness excluded those with isolated dysphasia and posterior stroke syndromes, an important group of patients with functional loss who could also benefit from the proposed treatment. The wide time window of inclusion (3 to 30 days) should also be refined in future trials; considerable changes in neurological status can occur over this period and this confounds comparison of the 2 groups (for example, a patient with an NIHSS of 8 three days after a stroke has a greater chance of improvement than a patient with the same NIHSS score at 30 days). All measures taken will be influenced similarly by the wide time window of inclusion. Nonetheless, mean time to randomisation (one of the minimisation factors) in both groups was similar (8.0 days in the G-CSF group, 8.3 days in the placebo group).

Finally, the CD34+ cell labelling and post mortem substudies suffered from small sample sizes (n=8 and n=3 respectively) and consequential selection bias. Bias was introduced, for example, by clinical judgement that in some cases would have been inappropriate or insensitive to approach patients and families to discuss brain donation in the event of death so soon after a major stroke. Nonetheless, both these facets of the trial represent novel scientific work and progress our understanding of the relationship between G-CSF, CD34+ cells and stroke. In chapter 8, the cellular profile of infarcted brain tissue subsequent to treatment with G-CSF was explored. Although no differences in

expression of CD34 antigen, G-CSF protein and its receptor were found when compared to control, expression of the G-CSF receptor on macrophages / microglia, neurons and astrocytes suggest that it is important to continue to evaluate the effects of G-CSF as a potential therapy for stroke.

The future for clinical trials of G-CSF and stroke

3 paradigms are currently being investigated: the effects of G-CSF on hyperacute, subacute and chronic stroke. AXIS-1 reported a small safety study of hyperacute administration in 44 patients.[368] The full results of AXIS-2 (n=328) are awaited. [302] AXIS-2 enrolled patients with ischaemic stroke in the MCA territory within 9 hours of onset and baseline NIHSS scores between 6 and 22. Patients were recruited if rtPA had been received provided the NIHSS score was still greater than 6. Initial data presented at the recent international stroke conference (February 2012, New Orleans) did not show a difference in efficacy between treatment and placebo groups: G-CSF mean mRS 3.31 (95% confidence interval 3.06-3.56) vs placebo mRS 3.12 (95% CI 2.87-3.37). There were no significant differences in safety or mortality between groups though the absolute number of deaths was higher in the treatment group (22% vs 18%, p=0.4). These are not very encouraging results and assessment of G-CSF in the hyperacute phase of stroke is unlikely to continue.

Evaluation of G-CSF in stroke in its subacute and chronic phases has not yet been sufficiently tested in order to comment on efficacy. STEMS-1,[294] STEMS-2 [304] and other smaller safety trials [295, 296, 303] administered G-CSF in the subacute phase (between 3 and 30 days post stroke) assessing the treatment concept of neurorepair with the assumption that mobilised haematopoietic progenitor cells

influence neuronal and vascular regeneration.[161] Most pre-clinical data have been developed in models that administer the drug in the hyperacute phase,[376] but positive benefits on outcome with drug administration in the subacute phase (between 1 day and 1 week post ictus) have also been seen.[235, 236] One negative preclinical experiment has demonstrated, however, an exacerbation of inflammation within the stroke lesion when G-CSF was given 24, 48, and 72 hours after induction of stroke.[167] No other negative pre-clinical trials in this area have been reported, suggesting the presence of publication bias. Therefore, the optimum time window of administration is not clear but it seems sensible to adopt a time when the inflammatory reaction has started to settle (beyond three days), to target a period 1-2 weeks post stroke, or in the chronic phase when scar tissue has formed. One ongoing trial, Stem cell Trial of recovery EnhanceMent after Stroke 3 (STEMS-3), is enrolling patients 90 days after ischaemic or haemorrhagic stroke using a total G-CSF dose of 10mcg/kg for 5 days.

Stroke is a heterogeneous disease, and selecting the most appropriate stroke population for future G-CSF trials is also unclear. The vast majority of pre-clinical evidence uses rodents with transient MCA ischaemia. This is a good representation of a proportion of human stroke but there are no models that will consistently represent other forms of stroke that behave differently, for example, a lacunar stroke or those with specific cortical signs such as dysphasia. Further experimental work may help to derive the most suitable population. Though rodent stroke models with other co-morbidities (diabetes,[359] age [245]) have shown benefit from G-CSF, there are fewer experiments using permanent models of ischaemia.[376] There are also no experiments published using larger

animal models (e.g. primates), which would help in determining validity across species with gyrencephalic brains. Despite this uncertainty, it would be reasonable to include all stroke subtypes in a phase II/III trial as they may all benefit from a strategy underpinned either by cell replacement (if migrated cells reach the lesion) or by enhancing endogenous regeneration (via secretion of chemokines from mobilised cells).

In ischaemic stroke, it is thought that the neuroprotective effect of G-CSF is mediated by inhibiting apoptosis. This mechanism may be of benefit also in patients with intracerebral haemorrhage (ICH) since compression effects of blood also cause ischaemia in neighboring cells. In rodent models of haemorrhagic stroke, G-CSF given 2 hours after ICH (50μg/kg, intra-peritoneal), then daily for 3 days, improved sensori-motor behavioural tests when compared to placebo measured up to 5 weeks after the stroke;[247] there was also evidence of reduced inflammation, brain oedema and peri-haematomal cell death. In another study, G-CSF was given to male rats 24 hours after ICH (15μg/kg, intra-peritoneal) then daily for 5 days. Compared to control, functional restoration was significantly better in the G-CSF treated animals;[451] circulating CD34+ HSCs were identified in peri-lesional tissue and not in control animals. There was also evidence of neurogenesis with expression of GFAP (astrocytes) and nestin (neurons) in the G-CSF group.[451] Furthermore, high serum levels of growth factors, such as G-CSF are associated with a good outcome following intracerebral haemorrhage.[452] More recently, the same authors evaluated CD34+ cell counts and outcome in 32 patients with ICH;[453] day 7 CD34 levels were independently associated with a good functional outcome (modified Rankin score <2), suggesting that CD34+ haematopoietic progenitor cells may

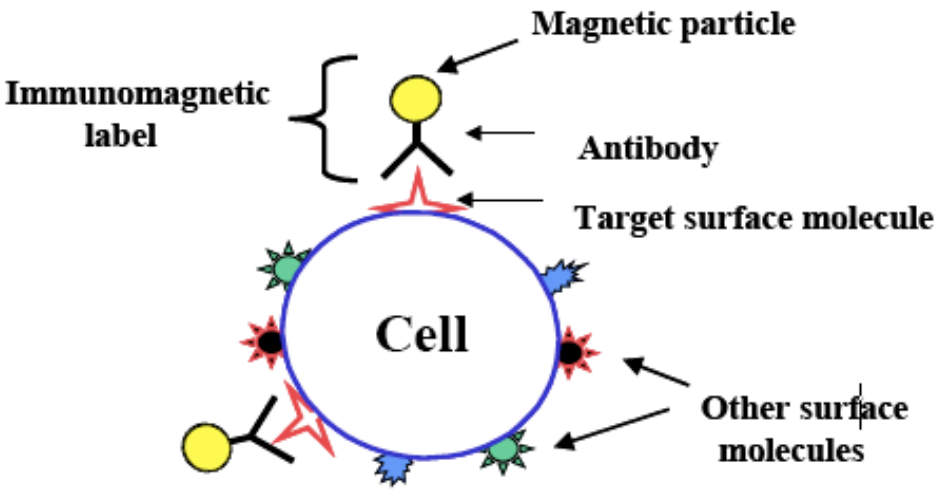
contribute to functional recovery in ICH. A stage II safety trial assessing G-CSF in patients with ICH is therefore warranted. It is supported by positive outcomes in preclinical experiments of ICH,[247, 451] evidence of good neurological outcomes in patients with higher CD34 counts and serum G-CSF levels [452, 453] and initial safety data from the subset of patients from the STEMS-2 trial.[304]

Although G-CSF may not be considered directly as 'stem cell treatment', endogenous CD34+ cells can be mobilised into peripheral blood using G-CSF in a dose dependent manner following a stroke.[294] G-CSF also demonstrates a multimodal mechanism of action, including neuroprotective and neuroregenerative qualities. G-CSF already possesses a good safety record and pharmacological profile in humans and, though it is essential that that thorough pre-clinical work precedes human clinical trials, the use of G-CSF in the hyperacute phase has rightly progressed onto phase III clinical trials. In light of the results of STEMS-2, it is now reasonable to consider evaluating the efficacy of G-CSF in the subacute phase of stroke.

Appendix A

Immunoaffinity-based cell separation

This method relies on a specific ligand (e.g. monoclonal antibody) binding to a cell surface protein (e.g. CD34 or CD133).[454]



Methods in chapter 6 employ immunoaffinity-based cell separation using a monoclonal anti-CD34 antibody conjugated to iron oxide nanobeads. After incubation of the stem cell product with the antibody, the cells are passed through a magnetic column which attracts the antigen-antibody complex whilst the remainder of the cells are washed away (the negative fraction). Removing the magnet subsequently allows collection of the CD34+ cells (the positive fraction). The iron-oxide nanoparticle attached to the selected cells will act as a contrast agent for T2* weighted MR imaging when injected into a patient. The above method uses a CliniMACS system (Miltenyi Biotec).

The volume of blood taken from the patient for CD34+ cell selection and labelling is approximately 150mls and is collected in a blood bag containing anti-coagulant (Baxter PL146-CPDA-1-35mls ref R8107). The CliniMACS system will not process whole blood effectively as other cell types interfere with immunomagnetic selection. It is therefore vital to minimise interruption from these cells by removing them first. Several methods of cell separation could achieve this aim but each has its drawbacks. Density gradient centrifugation with Ficoll would enable selection of the mononuclear cells but the procedure, which ideally requires a closed system to reduce contamination risk, involves too many 'open' steps for the volume of blood required here. Furthermore, no clinical grade Ficoll (or equivalent) is available so should not be used if the final product is going to be used in humans. Overall, it was felt that red blood cell sedimentation using Dextran 70 followed by separation of the 'buffy-coat' (containing the leukocytes of interest) was the most appropriate initial step. This method and the steps involved in selecting the CD34+ cells are described below. The procedure took place in a clean room (Clinical Tissues Laboratory, Queens Medical Centre, Nottingham), an environment necessary to ensure sterility of the final product. It complied with 'Good Manufacturing Practice' (GMP) and held a license with the Human Tissue Authority. The air was filtered to remove air-borne contaminants and there was a full sample tracking system in place.

Harvest of CD34+ Progenitor Cells

Materials

1. Blood collection bag (Baxter PL146 – CPDA-1 – 35mls, Ref R8107)
2. Transfer bags
    a. 600mls Teruflex Transfer bag (Ref BB*T060CM)
    b. 600mls Baxter (PL1813/1 Ref R4R2022)
    c. 150mls Teruflex Transfer bag (Ref BB* T015CM)
3. Luer spike connector (EMC1401)
4. Flexible Luer spike connector (RMC 3476)
5. Plasma transfer tubing set with coupler and Luer adapter (Baxter Ref VMC2240)
6. Plasma Expressor
7. Heat Sealer (Hematron III)
8. Vibrax shaking mixer / orbital rotator
9. Top pan balance (S-2002)
10. Preparation room centrifuge: 3-16k
11. CliniMACS CD34 complete kit including:
    a. CliniMACS CD34 reagent, 7.5mls
    b. CliniMACS tubing set
    c. CliniMACS PBS/EDTA buffer, 3 x 1 litre
    d. Flexible Luer spike connector (RMC 3476)
    e. Pall filter, 40µm
12. 50ml syringes.
13. 10ml syringes.

14. 19g long form hypodermic needles.
15. Plastic tubing clamps ('hemostats')
16. Dextran 70 for infusion (Baxter, 500ml, code B5013)
17. Normal saline 500mls
18. CD34 antibody for FACS analysis (CD34-PE, human)
19. CD45 antibody for FACS analysis (CD45-FITC, human)
20. Human serum albumin (HSA), 20% solution – BPL.

Procedure

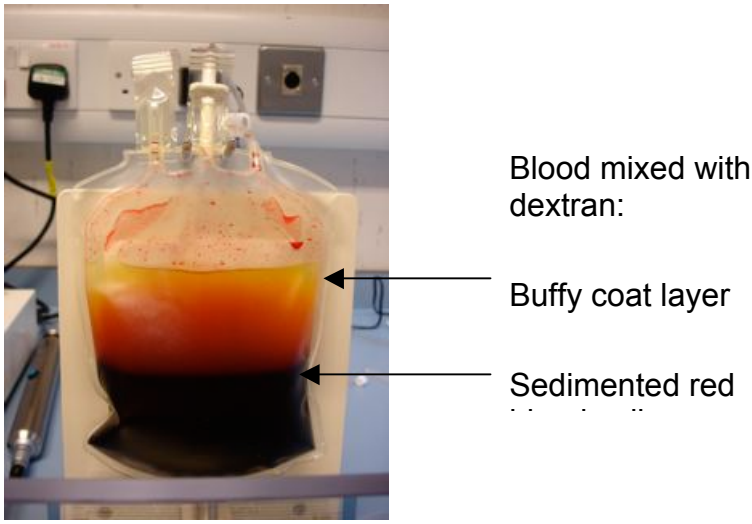
1. Weigh the blood collection bag in order to accurately measure the total volume collected (empty bag is approx 75g). Total volume will be approximately 150mls.

2. Using the heat sealer unit, seal the collection port on the blood bag about 2cm from the bag. Transfer the bag to the clean room.

3. [CLEAN ROOM]. Add 20%v/v dextran 70 (approximately 30mls) using a 50ml syringe into a Luer spike connector (EMC1401) inserted into the right hand port.

4. Mix the dextran with the contents of the blood collection bag by hand. Leave the bag and contents on the manual expressor device and allow the cells to sediment (figure 47). This process takes approximately one hour.

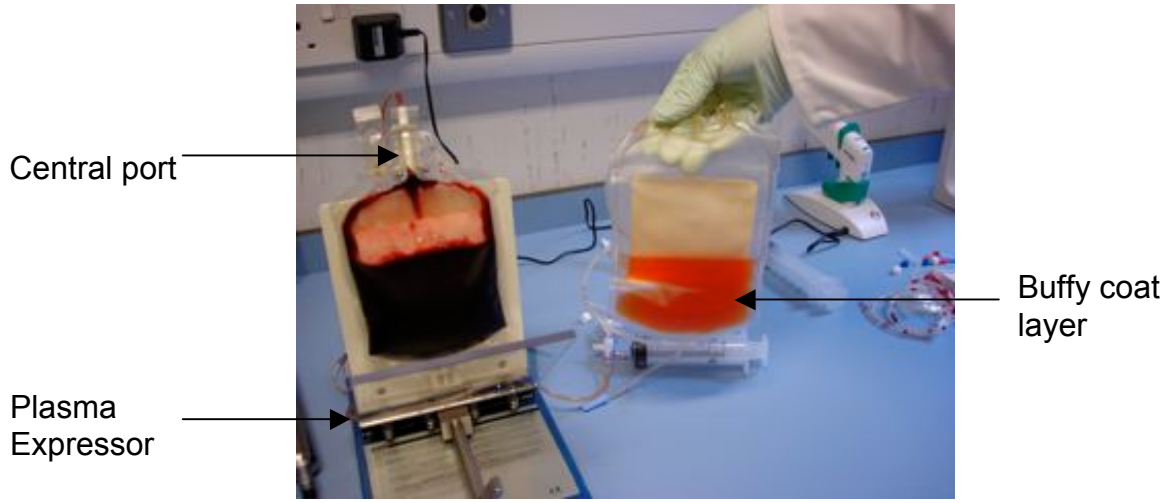
Figure 47. Blood collection bag.



5. Whilst waiting for RBC sedimentation, make up the centrifuge balance bucket:
    a. [CLEAN ROOM] Using a transfer bag (T060CM), insert a flexible Luer spike connector (RMC3476). Attach to this a 500ml bag of normal saline using the plasma transfer tubing set (VMC2240). Close the two existing clamps and pass to the preparation room.
    b. Open the clamps and transfer the saline into the transfer bag up to a total weight of 400g using the S-2002 balance. Stop the transfer at 390g and use the tubing clamp to control the final weight. Place the transfer bag in the centrifuge bucket. Close the bucket using a safety lid and place in the centrifuge (3-17k)

6. [CLEAN ROOM]. After approximately one hour, express plasma/buffy layer into a 600ml transfer bag (T060CM): the spike from the transfer bag is inserted into the central port on the blood collection bag (figure 48). Use a haemostat to clamp the tubing about 6cm from the bag.

Figure 48. Transferring buffy layer from blood collection bag.



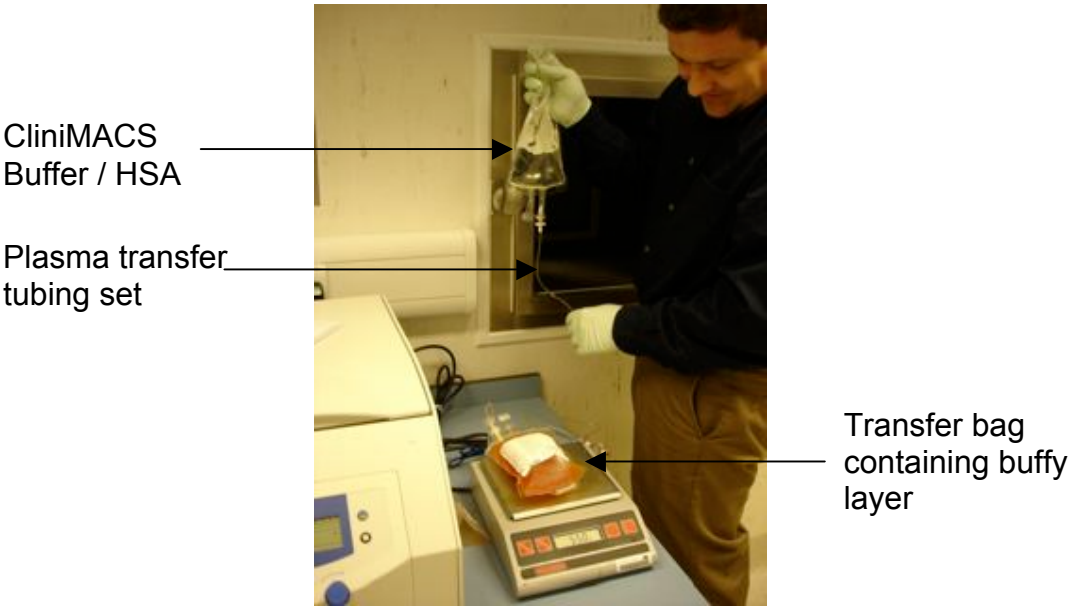
7. Transfer the bag set to the preparation room. Heat-seal the transfer tubing about 4cm from the bag and remove the clamp. Dispose of the initial blood collection bag and pass the transfer bag to the clean room.

8. [CLEAN ROOM]. Add HSA to the CliniMACS buffer to a final concentration of 0.5% v/v albumin (using 20% HSA, add 25ml in total). Use the right needle port in the CliniMACS buffer pack and a 19g (long) hypodermic needle fitted to a 50ml syringe.

9. [CLEAN ROOM]. Connect the buffer pack to the transfer bag (containing the separated buffy layer) via a flexible Luer spike connector (RMC3476) inserted into the left port of the transfer bag and unused port on the buffer pack using a plasma transfer tubing set (VMC2240). Attach the CliniMACS buffer pack and clamp the tubing using both the (attached) tubing clamps. Transfer to the preparation room.

10. Transfer the buffer/HSA manually: make up to 400g using the S-2002 balance (this is sufficient to fit into a centrifuge bucket) – see figure 49. Stop the transfer at 390g and use the tubing clamp to control the final weight. Finally, ensure the clamps on both the tubing set and on the transfer bag are closed. Pass the connected set back to the clean room.

Figure 49. Transferring CliniMACS buffer / HSA to the transfer bag



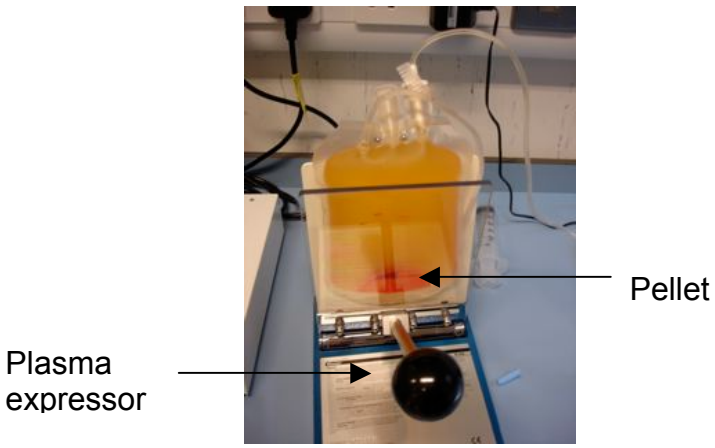
11. [CLEAN ROOM]. Separate the buffer bag and the transfer bag and replace both caps (buffer bag and transfer bag). Place the transfer bag in the centrifuge bucket. Close the bucket using a safety lid. Transfer to the preparation room.

12. Place the bucket and contents in the rotor of the 3-16k centrifuge. All four buckets in the centrifuge must balance (otherwise the centrifuge will cut out). Put the bucket inserts in the empty balance buckets to increase the weight.

Ensure that safety lids are placed on all buckets. Centrifuge the bag at 300g for 10 minutes using soft start/soft stop settings. When centrifugation is complete, pass the unopened centrifuge bucket (containing the transfer bag) to the clean room.

13. [CLEAN ROOM]. Place the centrifuged bag in the expressor. Attach a waste bag (R4R2022) using the existing Luer spike (RMC 3476). Let the expressor spring exert the necessary pressure on the bag contents, in order to effect the transfer to the attached bag (i.e., do not force expression of the supernatant by hand, see figure 50). Removal of the supernatant is complete when the bag is 'flat'. Disconnect the waste bag and discard. Resuspend the pellet within the transfer bag. Attach the CliniMACS buffer/HSA bag using the existing tubing set and Luer spike (RMC3476). Return this connected set to the preparation room.

Figure 50. Using the plasma expressor



14. Using buffer/HAS (from the attached CliniMACS buffer bag) fill the transfer bag to a final weight of 400g, using the S-2002 balance. Clamp the tubing

using both (attached) clamps. Transfer the connected bag set to the clean room.

15. [CLEAN ROOM]. Disconnect the buffer bag and replace both caps (buffer bag and transfer bag). Place the transfer bag in a centrifuge bucket, close the safety lid and pass the bucket plus contents to the preparation room.

16. Place the bucket and contents in the rotor of the 3-16k centrifuge. All four buckets in the centrifuge must balance (otherwise the centrifuge will cut out). Put the bucket inserts in the empty balance buckets to increase the weight. Ensure that safety lids are placed on all buckets. Centrifuge the bag at 200g for 10 minutes using soft start/soft stop settings. When centrifugation is complete, pass the unopened centrifuge bucket (containing the transfer bag) to the clean room.

17. [CLEAN ROOM]. Place the transfer bag in the expressor and attach a waste bag (R4R2022) via the existing Luer spike (RMC3476). Remove the supernatant, letting the expressor spring exert the necessary pressure on the bag contents (i.e. do not force expression of the supernatant by hand). Detach the waste bag and discard. Resuspend the pellet. Reattach the CliniMACS buffer bag using the existing tubing set and Luer spike (RMC3476). Close both (attached) clamps. Transfer the bag set to the preparation room.

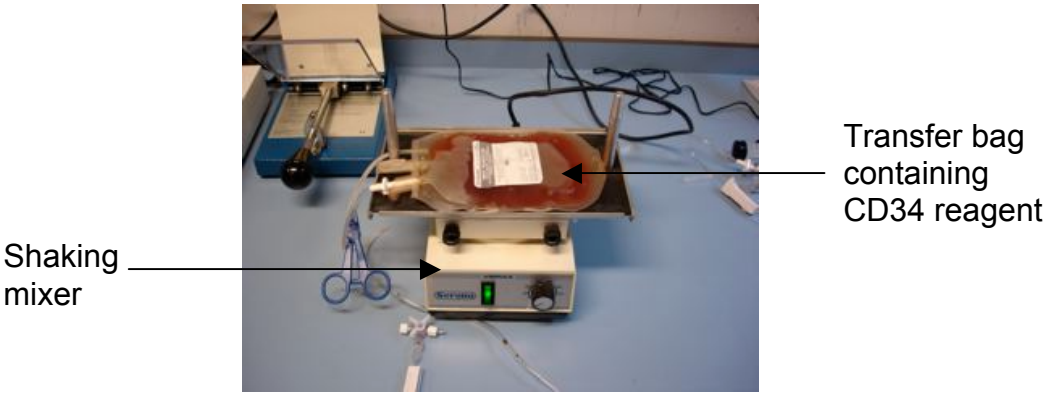
18. Add CliniMACS buffer/HSA after releasing the tubing clamps to a final weight of 129g; i.e. 95g buffer + 34g (the weight of the transfer bag), using

the S-2002 balance. Close both (attached) tubing clamps. Transfer the bag set to the clean room.

19. [CLEAN ROOM]. Detach the buffer bag and replace the caps. Add CliniMACS CD34 reagent to the transfer bag: withdraw 7.5 ml of the reagent from the vial with a 10ml syringe and add to the bag via the transfer tubing. Finish with 50ml of air (via a 50ml syringe) – to gently inflate the bag (this ensures easier mixing of the contents). Clamp the transfer bag tubing using the clamp on the Luer spike connector. Transfer to the preparation room.

20. Place the transfer bag plus contents on the Vibrax shaking mixer at 100rpm. Allow mixing for 30 minutes (no need to clamp the bag in place). Ensure that bubbles within the bag are moving to ensure that mixing is effective (see figure 51). Transfer the bag plus contents to the clean room.

Figure 51. Mixing CD34 reagent within the transfer bag



21. [CLEAN ROOM]. Add HSA to a new pack of CliniMACS buffer to a final concentration of 0.5% v/v albumin (using 20% HSA, add 25ml in total). Use

the right needle port in the CliniMACS buffer pack and a 19g (long) hypodermic needle fitted to a 50ml syringe.

22. [CLEAN ROOM]. Attach the (new) CliniMACS/HAS buffer bag to the transfer bag using a plasma transfer set (VMC2240) and the existing Luer spike. Clamp both bags using the (attached) tubing clamps. Pass the bag set to the preparation room.

23. Release both clamps and transfer the buffer/HSA manually: make the transfer bag up to 400g using the S-2002 balance. Finally, ensure the clamps on both the tubing set and on the transfer bag are closed. Pass the bag set to the clean room.

24. [CLEAN ROOM]. Detach the CliniMACS buffer bag and replace the caps. Place the transfer bag in a centrifuge bucket and close the safety lid. Return the centrifuge bucket and contents to the preparation room.

25. Place the bucket and contents in the rotor of the 3-16k centrifuge. All four buckets in the centrifuge must balance (otherwise the centrifuge will cut out). Put the bucket inserts in the empty balance buckets to increase the weight. Ensure that safety lids are placed on all buckets. Centrifuge the bag at 300g for 10 minutes using soft start/soft stop settings. When centrifugation is complete, pass the unopened centrifuge bucket (containing the transfer bag) to the clean room.

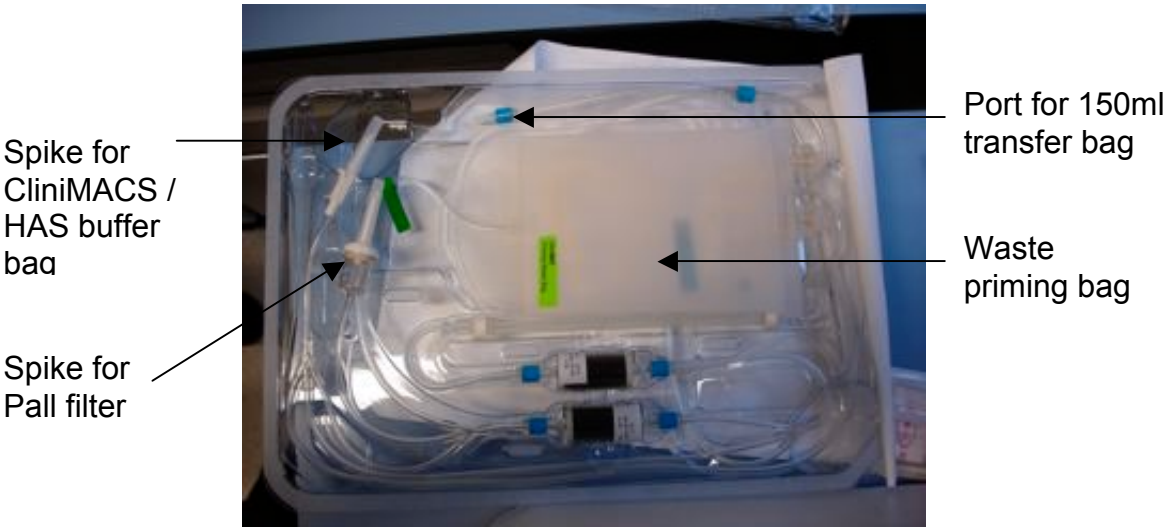
26. [CLEAN ROOM]. Remove the transfer bag from the centrifuge bucket. Place the transfer bag in the expressor and attach a waste bag (R4R2022) via the existing Luer spike (RMC3476). Remove the supernatant, letting the expressor spring exert the necessary pressure on the bag contents (i.e. do not force expression of the supernatant by hand). Detach the waste bag and discard. Resuspend the pellet. Reconnect CliniMACS buffer pack number 1 (this should have the smaller quantity of the 2 buffer packs). Close both tubing clamps and pass the bag set to the preparation room.

27. Release the tubing clamps and add CliniMACS buffer/HSA to a final weight of 94g; i.e. 60g buffer + 34g (the weight of the transfer bag), using the S-2002 balance. Close both (attached) tubing clamps. Transfer the bag set to the clean room.

28. [CLEAN ROOM]. Detach the CliniMACS buffer bag and replace the caps. Clamp the transfer bag. Take a 1ml sample from the transfer bag, using a 2ml syringe and 19g needle. Transfer this sample into a ‘bijou’ vial. This sample will be used to obtain a CD34+ cell count (before harvest).

29. [CLEAN ROOM]. Open a CliniMACS tubing set (figure 52).

Figure 52. CliniMACS tubing set



Three connections within this to set up in the clean room:

a. Spike in the central port (this is the spike with the microbead filter attached) into the Pall filter (spike into the top of the filter). Clamp below the Pall filter using a plastic tubing clamp (haemostat) and then spike the Pall Filter into the transfer bag containing the product.

b. Spike into the CliniMACS/HSA buffer bag (the 2nd pack containing the most buffer) and clamp with a haemostat.

c. Spike the 150ml transfer bag (T015CM) (this will be the positive fraction bag) with a Luer connector (EMC1401) and attach this to the final available port in the CliniMACS tubing set.

Do not clamp the waste priming bag tubing.

Pass the prepared ClinMACS tubing set to the preparation room.

30. Prepare the CliniMACS machine:

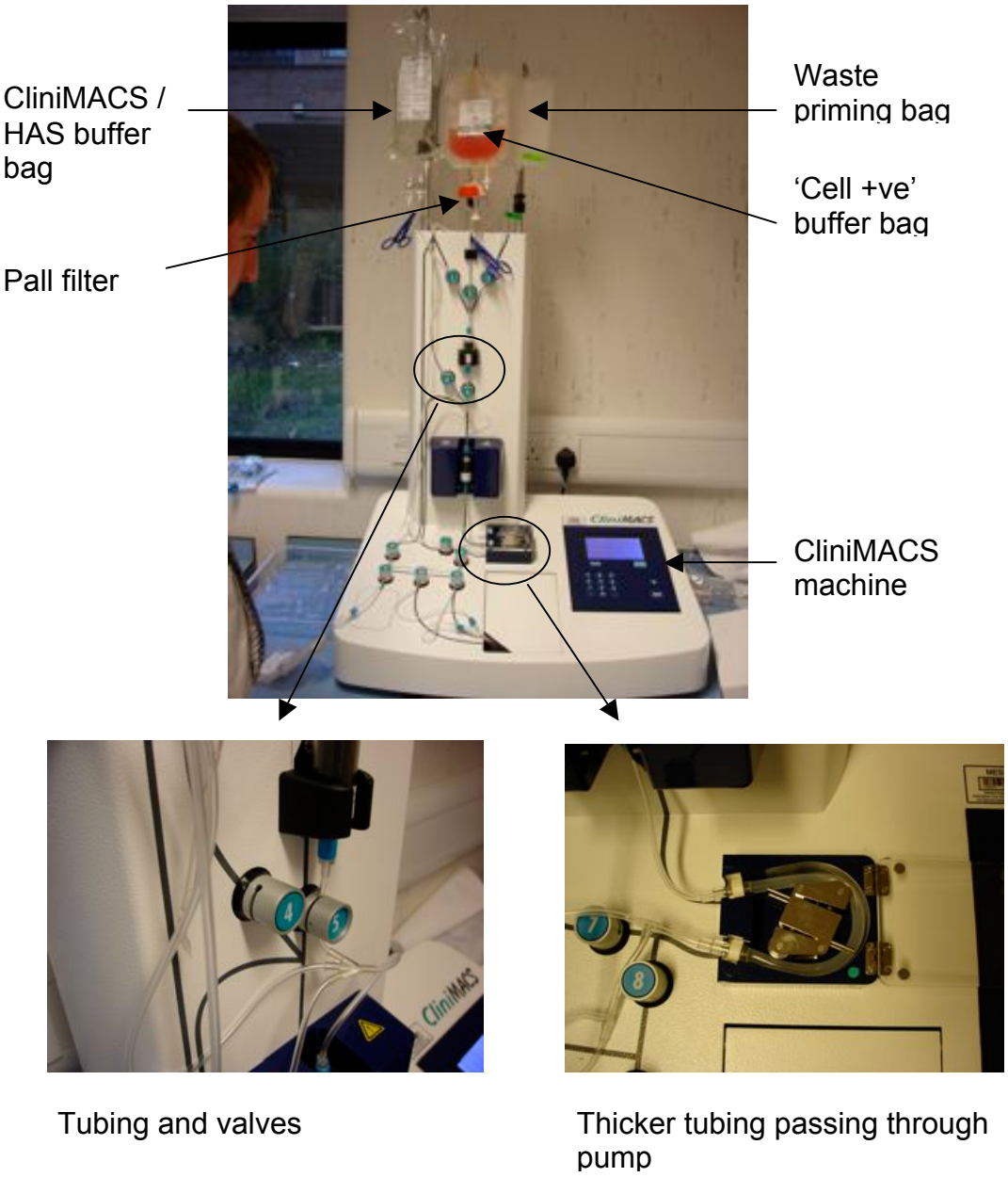
    a. Programme the CliniMACS harvester. Follow the onscreen instructions.

    b. Input the tubing set reference number.

    c. Input the CD34 reagent reference number.

31. Take both this ‘cell +ve’ buffer bag and then the waste priming bag and hang from the top of the CliniMACS machine. The pre-column is inserted with the ‘fins’ facing outward. Adjust the height of the bags. Attach the selection columns (fins outward). Attach the tubing through the valves as per the onscreen instructions. When the onscreen instructions indicate ‘attach buffer bag’, remove the clamp. The valves can be adjusted manually if necessary (push button, pull tubing). The thicker of the tubing goes through the pump. The contents of the buffer waste bag and negative fraction bag are directed into the waste container. Check that all tubing is seated correctly (e.g. check for twists etc.).

Figure 53. The CliniMACS machine and tubing set



32. Start the run as per the onscreen instructions.

33. The screen prompts for an 'integrity test'. This is an extra priming step (for FDA regulations) and takes 30 minutes. Omit this step as the original priming step tests the system sufficiently.

34. When prompted to ‘connect bag’, remove the clamp (hemostat). Collect the harvest – this should be 42g ± 7g (plus the weight of the empty bag = 22g). The negative fraction bag = 30g. Negative fraction = 230g (total = 260g).

35. Analyse the following three samples for FACS analysis:
    a. Pre-harvest CD34+ cell separation (already taken).
    b. CD34 positive fraction - withdraw 1ml via the Luer spike connector.
    c. CD34 negative fraction – withdraw 1ml.

36. Record the process code at the end of the harvest run.

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