Future of Animal Modeling for Poststroke Tissue Repair

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Numerous preclinical animal studies have shown beneficial effects of cell therapies after stroke, including reduction of functional deficits and lesion size. Early stage clinical studies currently aim to confirm this therapeutic potential. Despite the progress in translating cell therapy for stroke, true cell replacement and stem cell-based tissue regrowth have not been achieved yet. Multimodal regeneration improving effects, such as immunomodulation or paracrine growth factor support, are considered the primary mechanisms of action in cell therapies.[1] This is not surprising for systemically administered adult progenitor or mixed populations, which typically do not enter brain tissue. However, even brain tissue-derived cells that, in principle, have the ability to give rise to neurons and glia are thought to exert their therapeutic benefits mainly via multimodal regeneration improving effects.[2] Current clinical trials are designed to reflect this supportive role of cell therapy rather than tissue reconstruction.[3]

Our increasing understanding of brain development on the one hand and poststroke pathophysiology on the other illustrate the challenges in true tissue restoration. First, tissue replacement requires a perfect synchronization between participating cells and the host tissue in spatial, temporal, and functional dimensions.[1] Second, anatomic cues being decisive for brain tissue growth during embryo/fetogenesis rapidly decline in postnatal brain maturation.[4] Third, major brain lesions cause a hostile local environment that detrimentally impacts graft survival and integration. Fourth, restoring brain tissue requires an adequate blood supply, posing a major challenge in larger lesions and bigger brains.[5] Fifth, lack of adequate functional interaction between host tissue and the graft leads to tissue restoration failure.[6]

To go beyond multimodal regeneration improving effects, a careful orchestration of therapeutic approaches relying on and promoting endogenous (eg, neurogenesis-based) or exogenous (eg, stem cell transplantation-based) tissue restoration need to be established. Selection of appropriate, restoration-permissive target lesions will also be required to enhance chances for successful tissue regeneration. Herein, we propose a 4-component in vivo research strategy with the potential to foster tissue repair and replacement in stroke.

Component 1: Selection of Restoration-Permissive Stroke Models

Large Territorial Ischemic Lesions

Preclinical research and clinical studies focus on large territorial lesions. These represent a major proportion of strokes in patients and exhibit salvageable tissue for acute neuroprotective interventions. However, exactly this lesion type may be the hardest to repopulate because of the large volume, a hostile intra- and perilesional microenvironment, and massively disturbed local blood supply. Consequently, tissue restoration was hardly observed in animal models of large territorial lesions, even when graft differentiation and integration were initially successful.[6] More specialized stroke models may mimic clinical stroke subtypes and cofactor profiles being less challenging or even supportive of tissue restoration.

Lacunar Infarcts and White Matter Injuries

Small, lacunar-like lesions involving white matter represent 20% to 25% of all strokes.[7] Lacunar infarcts can be induced by potent vasoconstrictors such as ET-1 (endothelin-1) or photothrombosis. They are interesting for tissue repair strategies because the tissue volume to restore is small and anatomic structures are preserved. Endogenous or graft-borne cells repopulating the lesion have easier access to blood supply from the surrounding, unaffected areas. Lacunar infarcts often affect white matter areas.

However, experimental models of lacunar lesions are rarely used because of some challenges. First, the white matter content in rodents is smaller than in humans.[8] Second, inducing small, reproducible lesions in deep brain structures is technically demanding. Third, behavioral deficits are mild, so animals often recover rapidly even without a therapeutic intervention. Thus, there is a need for reliable subcortical white matter injury models with substantial functional impairment. Some existing models come close to this demand.

Global white matter injury models, such as bilateral common carotid artery occlusion, produce chronic, but diffuse and extended, damage which may be hard to repopulate.[9]

Received September 8, 2017; final revision received December 13, 2017; accepted December 14, 2017.

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(*Stroke*. 2018;49:1099-1106. DOI: 10.1161/STROKEAHA.117.018293.)

Hypertensive rats spontaneously develop both punctuate and diffuse white matter lesions, and cognitive impairment.[10] Focal white matter injury can be induced by ET-1. The vasoconstrictor is injected stereotactically into the corpus callosum to damage axons projecting to the contralateral cortex, or into the posterior internal capsule, which contains corticospinal tract and thalamic sensory projections. ET-1 injections into the corpus callosum induce impairments on grid walking in aged mice.[11] The model also causes long-term cognitive impairment in novel object recognition tasks, but no apparent deficit in the cylinder or grid-walking tests, locomotor activity, water maze or Y-maze in mice.[12] Intact fibers in the corpus callosum compensate the damage, explaining minor behavioral deficits. ET-1 injections into the internal capsule produce a more robust behavioral deficit. Importantly, behavioral impairment severity varies according to the injection site. Only animals with lesions in the most posterior location show a long-term deficit in adhesive removal, cylinder, and foot-fault test. Selection of vasoconstrictor and angle of the injection is critical.[13] ET-1–induced white matter lesions potentially facilitate targeted restorative therapy developments.[11]

Cerebral Hemorrhages

Intracerebral hemorrhage (ICH) and subarachnoid hemorrhage are the main subtypes of hemorrhagic stroke, accounting for 10% to 15% and 6% to 8% of all strokes, respectively. They share important comorbidities with ischemic stroke, occur unaccompanied or as an important complication of ischemic stroke treatment and prevention.[14] Recent experimental evidence suggests that the pathophysiological mechanisms underlying ischemic and hemorrhagic stroke are different. Specifically, the hematoma and blood breakdown products released from the bleeding cause secondary, delayed damage leading to neuronal cell death.[15] Therapeutic strategies being different from those used for ischemic stroke are needed to promote tissue repair after hemorrhagic stroke.

Most preclinical ICH studies use 1 of 2 large hemorrhage rodent models: intracerebral injection of either autologous blood or bacterial collagenase that degrades the endothelial basement membrane. Replacing brain tissue in a large hematoma cavity poses similar challenges as for large ischemic lesions and may require biomaterial support (see below). Moreover, these models do not mimic spontaneous occurrence of hemorrhage. On the other hand, using models with spontaneously developing hemorrhages is challenging because of the difficulty to detect their occurrence and variable lesion sizes and locations. In a large study in stroke-prone spontaneously hypertensive rats, 7.4% showed bleeding and 17.9% hemorrhagic transformation in various locations.[16]

Some genetic models have been developed to mimic cerebral microbleeds.[17,18] Cerebral microbleeds cause small lesions that, theoretically, may foster tissue repair approaches. However, they are often clinically silent[19] and are hard to access, particularly when situated in subcortical regions. Therefore, they are currently no target for therapeutic approaches of tissue restoration.

Large Animal Models of Lacunar Infarcts, White Matter Injury, and Hemorrhagic Stroke

Gyrencephalic large animals exhibit a higher percentage of white matter,[8] and some specialized large animal ischemia models feature focal white matter lesion.[20,21] However, those are not yet widely applied in preclinical research. Moreover, only a few studies investigate white matter changes after hemorrhagic stroke, although the underlying mechanisms may facilitate cell-based tissue repair. White matter injury can be investigated in a piglet model of autologous blood injection into the frontal lobe.[22] Some preclinical studies demonstrated that white matter damage also occurs in the rodent corticospinal tract after ICH[23] and cortical projections are lost early after ICH in mice.[24] Understanding of how brain hemorrhage leads to white matter changes, preferably in models with comparable brain white matter content, should be a research priority, and will help develop strategies for white matter repair and regeneration.

Hematoma resolution in humans takes much longer than in rodents because of bigger absolute hematoma volumes, which can be modeled in larger animals. There are models of autologous blood injection into the ventricle or frontal lobe of piglets.[25] Large animal models also provide the opportunity to investigate tissue repair strategies after hematoma evacuation, especially combinatorial treatments involving biomaterials and pharmacological agents.

Component 2: Use of Supportive Biomaterials

Biomaterials offer novel avenues to treat brain damage and support tissue regeneration. A major advantage of biomaterials is the potential for controlled local release of growth factors, anti-inflammatory agents, and guidance cues at a specific dose over a given time.[26] Many synthetic (eg, polyethylene glycol) and natural biomaterials (eg, hyaluronic acid) have favorable biocompatibility and do not invoke an adverse host response. However, invasive intracerebral delivery is required to deposit materials at the appropriate site.[27] This approach avoids systemic effects of compounds and does not interfere with other brain regions. It can also circumvent the challenge of delivering large molecules across the blood–brain barrier (Figure 1).

Controlled release of factors is achieved either by incorporation or by conjugation of a factor into microspheres/particles or hydrogels. Microspheres will typically not change shape on injection, whereas hydrogel will more easily permeate through tissue. Microparticle incorporation of fenofibrate, brain-derived growth factor, and vascular endothelial growth factor have been used in stroke.[28] Efficacy of these approaches is dependent on the distribution of microparticles and their sphere of influence on damaged tissue. Hydrogels can permeate more extensively and potentially exert more significant functional effects, such as polarizing microglia/macrophages toward M2 phenotype that supports tissue repair.[29] A continued stimulation of the endogenous stem cell pool only requires an epicortical placement of a hydrogel to deliver epidermal growth factor.[30] The time-controlled sequential release of multiple factors promoting tissue repair is an opportunity to increase the complexity of approaches that promote tissue repair (Figure 1).

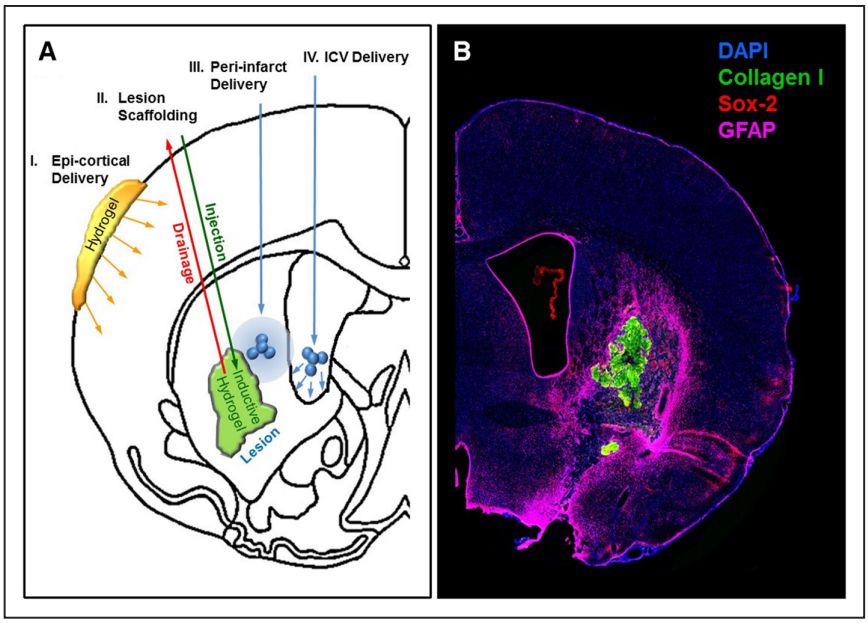

Figure 1. Application of biomaterials in repairing stroke brain. **A**, Biomaterials enable local release of factors and provide structural support in case of tissue loss. The least invasive means to deliver factors to the stroke damage brain is by the epicortical placement of biomaterials, such as a hydrogel, releasing factors into the surrounding cortex (I). Hydrogels can also serve as a scaffolding material in the lesion cavity (II). An injection-drainage approach is preferable to ensure homogenous filling of the volume. Repair response in the peri-infarct area can be influenced by injecting microparticles that release growth factors, and that would not easily penetrate across the blood–brain barrier (III). Injection of microparticles into the lateral ventricle (IV) can be used to influence the subependymal zone to increase neurogenesis. **B**, Exemplary overview immunofluorescent staining of brain slice showing extracellular matrix hydrogel (detected by collagen I in green) in the lesion cavity.

Apart from tissue damage in stroke, there is a complete regional loss of tissue integrity that leads to cavitation in which the structural component of tissue, the extracellular matrix (ECM), is cleared through macrophages. A lack of structural support in this region prevents the invasion of endogenous cells to replace lost tissue, including axonal regrowth. Providing a scaffold within the cavity hence can provide a means for de novo tissue to form. This may be of particular value when targeting larger ischemic or hemorrhagic lesions. However, to fill the tissue cavity completely with biomaterial, superfluous extracellular fluid needs to be drained simultaneously during the injection process.[31]

To harness the endogenous response, an inductive material that induces structural remodeling of the scaffold is required. ECM is widely used to promote soft tissue repair. Although brain ECM can be formulated as a hydrogel, urinary bladder ECM is more abundant while inducing greater neuronal differentiation and neurite extensions at therapeutically relevant concentrations.[32] Implantation of ECM hydrogel into the stroke-damaged brain induces a strong endogenous response of cells, including neuronal progenitors.[33] Axonal regrowth can also be promoted using anisotropic scaffolds. Glial scarring surrounding the stroke cavity is hence not an insurmountable barrier to tissue regeneration in the brain, and neural progenitors can respond to cues beyond the tissue border.

However, long-term remodeling of the implanted hydrogel and its potential to support functional neuronal network restoration remains unknown.[34] In the absence of regional cues providing a positional specification of invading cells, topological encoding of guidance and specification cues will need to be delivered to form site-appropriate de novo tissue.[35] Vascularization and formation of functional synapses will require additional strategies if this new tissue is to support behavioral functions.[5] It is also unclear if principles developed in small rodent models will eventually translate to larger brains.

Large Animal Models in Biomaterial Assessments

Targeted, individual administration of biomaterials and cells is feasible in large animals as stereotaxic techniques are established, and related brain atlases were published for relevant species.[36] Precision is higher than in comparable rodent models, which also allows targeting microlesions more precisely. Moreover, the intervention can be tailored to lesion confirmation, for instance using magnetic resonance imaging-based lesion cartography in each individual subject.[37] Similar to rodents, large animal species can exhibit significant differences to the human immune system, requiring careful configuration of immunosuppression protocols where needed.[38]

Component 3: Application of Neurorehabilitation

Neurorehabilitation fosters adaptive brain plasticity after stroke, including circuit reorganization[39] and activation of endogenous stem cell reservoirs. Recent data suggest that both migration and survival of newborn cells from the subventricular zone can be enhanced by forced limb use even in aged subjects.[40] In addition, neurogenesis in the perilesional cortex is involved in motor map reorganization and improved behavioral performance induced by skilled forelimb training, and causality between neurogenesis and functional recovery was proven. In turn, lack of physical activity limits endogenous cell-based repair mechanisms after stroke.[41]

Although maladaptation can occur if inappropriate task integration is provided,[40] neurorehabilitation is a promising restoration-supporting strategy after stroke. However, rehabilitation is rarely included in experimental settings, perhaps because of complex study design and methodological uncertainties. Indeed, rehabilitative training is fundamentally different in rodents and stroke patients. For instance, a therapist kindly guides and assists patients while training of rodents is based on testing apparatus, reward and aversive stimuli, which may at worst impose extra stress and mask treatment effects. Speed and completeness of spontaneous recovery in stroke rodents differ

compared with that in stroke patients.[42] Hence, selection of the appropriate experimental rehabilitation therapy is important.

Mimicking Neurorehabilitation in Animal Models

Various approaches such as voluntary or forced physical training, special rehabilitative training devices, forced use of a forelimb, skilled reaching task, and aerobic training have been introduced.[43] Housing in an enriched environment, resembling the more diverse patient environment during rehabilitation, has been used to provide multiple sensory, motor, social, and visual stimuli, and to support exogenous cell grafts.[44] Miniature robotic and electric devices enable intensive, controllable, and repeatable training approaches in rodents.[45,46] The Table summarizes experimental neurorehabilitation protocols and stroke model settings in which they have been applied successfully.

Ranking of training strategies is challenging given the variety of approaches, experimental models, and outcomes. Recent meta-analyses have addressed this problem. Forced physical training (eg, treadmill) and skilled forelimb training might be the most effective in stroke animals, whereas task-oriented motor training seems to generalize to other motor functions. Constraint-induced movement therapy was not efficient in animal models.[47]

Importantly, rehabilitative and restorative strategies effective in improving motor function are different from those inducing cognitive recovery. Further research should therefore also focus on repair strategies enhancing recovery of cognitive function and common psychological complications such as poststroke depression.

Combination of Neurorehabilitation Strategies

Individual neurorehabilitative paradigms can be combined to improve outcome and tissue restoration. Rehabilitative training of the impaired forelimb together with environmental stimulation both increased dentate neurogenesis in rats subjected to cortical infarcts, correlating with improved water-maze performance.[48] Combining enriched environment with running wheel training increases survival of transplanted cells.[59] Constraint-induced movement therapy enhanced functional recovery, dendritic arborization, and neuronal plasticity in a rat model of ICH, whereas forced impaired limb use starting 1 day after ICH led to better functional recovery, restoration of forelimb representation in the motor cortex, and axonal sprouting.[57]

Timing of Neurorehabilitation

Timing of rehabilitative training is critical. There seems to be a therapeutic time window for neurorehabilitative training. Early training (24 hours) may exacerbate brain damage after focal brain ischemia in rats possibly through excitotoxic mechanisms.[60] Such mechanisms may also affect cell-based restorative attempts in the subacute stage. However, poststroke brain displays increased sensitivity to rehabilitative experience in early chronic stages after stroke (5–14 days), which declines with time.[61] This was confirmed in rats being trained in a reaching task starting 4 or 25 days postlesion.[58] Reaching performance improved only in the early training group. Recent meta-analysis is in line with this showing reduced infarct volume and improved cognitive and motor functions when training is initiated between days 1 to 5 after stroke. Similarly, rehabilitation started between 1 and 7 days after hemorrhagic stroke enhanced functional recovery and plasticity.[50] There is no clear relationship between treatment frequency and treatment effects, but the improved performance by training is lost if the training is suspended.[62]

Large Animal Models and Neurorehabilitation

Functional readout protocols are established for large animal species ranging from simple scoring systems[63] to highly sophisticated cognitive[20] and fine motor/sensor tests.[64] A potential problem in using large animal models for assessing functional end points is the higher variability as compared with rodents. Although this reflects the situation seen in human stroke patients, it limits study power. Applying neurorehabilitation treatment also requires highly specialized equipment and experimenters. This can be critical because of higher experimental costs and thus lower sample sizes are common.[65] Hence, assessing the functional impact of complex neurorehabilitation strategies is not a primary domain of large animal models. It should be limited to confirmative studies when an effect was clearly observed in rodent models. However, large animal models feature larger brains and can, therefore, be used to indirectly demonstrate neurorehabilitation effects, for instance using structural, functional, and diffusion tensor imaging. Magnetic resonance imaging may even be combined with a noninvasive assessment of brain metabolism using positron emission tomography.[66]

Component 4: Supportive Pharmacotherapy

It is well-known that various drugs, growth factors, and bioactive molecules positively influence poststroke neurogenesis, repair, and plasticity processes. A tempting idea, therefore, is to combine strategies supporting restoration and regeneration with pharmacological interventions for additive or synergistic benefits.[67] Complement C3a stimulates neurogenesis and controls neural progenitor migration, accelerating functional recovery when applied intranasally in stroke mice.[68] Fluoxetine, a serotonin uptake inhibitor, showed efficacy in human stroke patients.[69] Recent approaches include noncoding RNAs including microRNA,[67] cell-derived bioactive microvesicles,[70] and antibodies such as anti-Nogo-A.

However, the sequence of the different therapies is crucial. For instance, a wrong temporal combination of a cell-therapeutic approach and granulocyte colony-stimulating factor-induced detrimental effects.[71] The same holds true for plasticity-enhancing approaches: sequential therapy with first anti-Nogo-A and then skilled forelimb training resulted in enhanced recovery.[72] Concurrent application was not effective, suggesting that postlesionally formed connections should first be stabilized before plasticity is enhanced further.[73] Although pharmacological measures can be applied after ischemic and hemorrhagic stroke, potential species-specific pharmacodynamics and species-specific kinetics should be taken into consideration. A great advantage of the pharmacological approach is that it can be easily combined with hydrogel applications allowing controlled spatial and temporal release from within the brain.[74]

Table. Experimental Neurorehabilitation, Endogenous Neurogenesis, and Behavioral Outcome in Stroke Animals

Rehabilitation Paradigm	Model	Training				Lesion Size	Neurogenesis			Outcome		Comments	Reference
		Category	Onset	Intensity	Duration		SVZ	DG	Perilesional	Sensorimotor	Cognitive		
Enriched environment	Photothrombosis	EE+skilled forelimb training	24 h	50–100 pellets/d	42 d	±	+	n.a.	n.a.	n.a.	+(water maze)		48
	tMCAO	EE+atipamezole	48 h	Atipamezole 1 mg/kg per d	10 d	±	±(28 d)	n.a.	n.a.	Transient improvement (sticky label test)	n.a.	No correlation between neurogenesis and behavior	49
	Collagenase in striatum	EE+skilled reach training	7 d	2×/daily for 5 d/wk	2 wk	±	±(32 d)	n.a.	±(32 d)	+skilled reaching test +walking ability ±cylinder test	n.a.	Enhanced dendritic complexity after rehab	50
Voluntary exercise	tMCAO	Running wheel vs swimming	7 d	Swimming 2×1 min/d	42 d	n.a.	n.a.	Increased cell survival (49 d)	n.a.	n.a.	+(water maze in running group)	Upregulation of CREB signaling	51
	ET-1	Activity box+task-specific training	3 d	30 min/d	30 d	n.a.	+(30 d)	n.a.	n.a.	+(forelimb placing) ±(Montoya's stair case) ±(ladder rung)	n.a.	Perilesional, BDNF-expressing cells	52
Forced exercise	tMCAO	Treadmill	2 d	20 m/min, 30 min/d	7 or 28 d	–(7 d)	n.a.	+(7 d)	+(7 and 28 d)	+(Longa score)	n.a.	Involvement of caveolin-1/ VEGF pathway	53
	tMCAO	Rotarod	3 d	12 m/min, 40 min/d	14 d	±	+(17 d)	+(17 d)	n.a.	n.a.	n.a.		54
CIMT	ET-1	Plast	7 d	Permanent	3 wk	±	n.a.	±	n.a.	+(tapered ledged beam)	n.a.	Aged rats	39
	ET-1	Plast	7 d	Permanent	3 wk	±	+(33 d)	+(33 d)		+(tapered ledged beam)	+(water maze)		55
	Collagenase in globus pallidus	Plast	1 vs 17 d	Permanent	7 d	±	n.a.	n.a.	+(in early group)	+(skilled reaching test) +(ladder stepping test)	n.a.		56
Forced limb use	Collagenase in internal capsule	+intracortical microstimulation	1 d	Permanent	7 d	±	n.a.	n.a.	+	+(skilled reaching test) +(ladder stepping test)	n.a.		57
Skilled training	Photothrombosis	Single pellet reaching	4 d	2 sessions/5 d per 1 wk	4 wk	±	+(28 d)	n.a	n.a	+(single pellet reaching)	n.a.	Motor map reorganization	58

+ indicates increased/improved; –, reduced; ±, unaffected; BDNF, brain-derived neurotrophic factor; CIMT, constraint-induced movement therapy; DG, dentate gyrus; EE, enriched environment; ET-1, endothelin-1; ICH, intracerebral hemorrhage; n.a., not available/addressed; SVZ, subventricular zone; tMCAO, transient middle cerebral artery occlusion; and VEGF, vascular endothelial growth factor.

Recommendations for a Translational Roadmap

Common rodent stroke models are homogenous in stroke outcome, fostering the investigation of therapeutic approaches using relatively small sample sizes. However, they insufficiently reflect patient heterogeneity about age, sex, comorbidity, and confounding comedication.[8] Although it may be scientifically valuable to mirror the complexity and diversity of human stroke in animal models, it stresses development time lines and resources to a critical level and does not provide an obvious advantage for the investigation of basic principles of tissue repair approaches. An alternative strategy is to enhance specificity of the translational strategy by targeting restoration-permissive lesion configurations (Figure 2).

In this scenario, an animal model reflecting selected human patient populations is chosen to test the restorative treatment approach, for example, the use of stem cells. Next, application of potential supportive measures is decided. For instance, the use of functionalized biomaterials is recommended when targeting larger, single lesions cavities as seen after ICH or focal ischemia, but may be of limited use when addressing diffuse tissue damage. In turn, neurorehabilitation and pharmacological support may be particularly efficient in the latter because the ECM and other structural cues fostering cell replacement are preserved to some extent.

Timing of supportive measure application is also critical. For instance, the benefit of neurorehabilitation and pharmacological support of neurogenesis and plasticity is limited to a subacute time window, whereas application of functionalized biomaterials may be most efficient after lesion maturation in the chronic stage. The combination of both approaches must, therefore, be carefully planned considering the particular lesion type. Proving an additional benefit of double or even triple restoration-supportive strategies is also challenging statistically.

If the efficacy of a tissue-restorative paradigm was explored and proven, subsequent confirmative experiments challenging

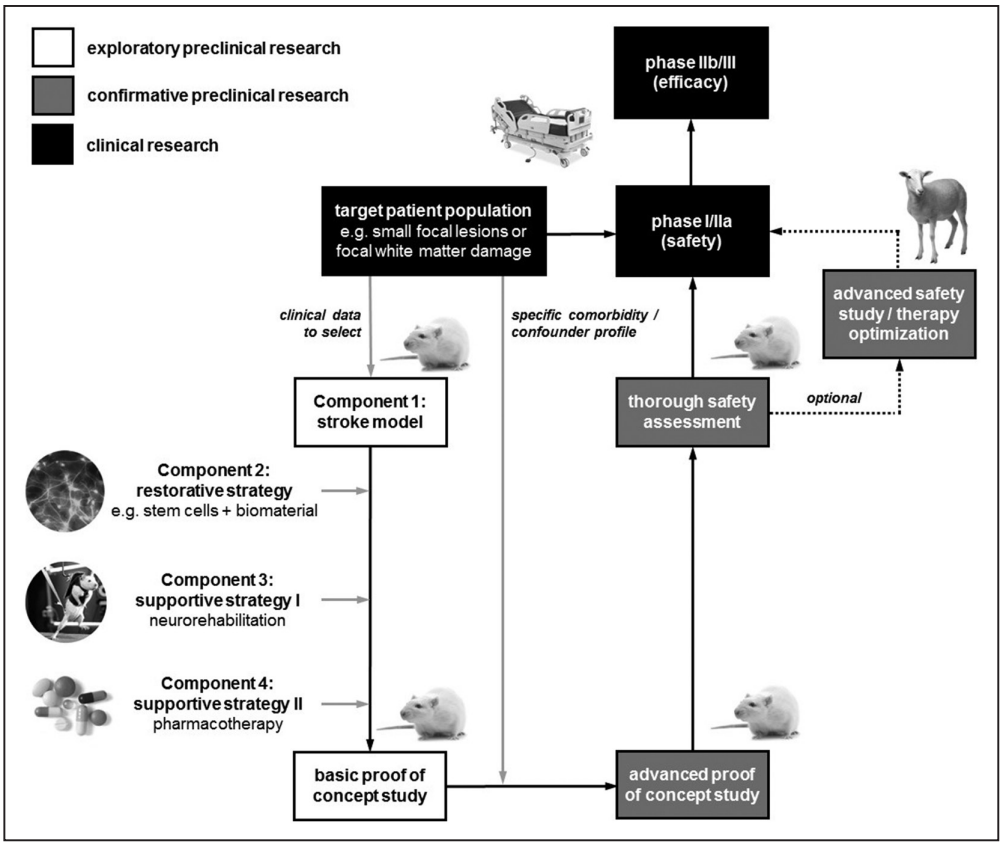

Figure 2. Research strategy draft for clinical translation of tissue restoration in stroke. After identification of the target patient population, a stroke model well reflecting this population is selected. The tissue restoration strategy, ideally combined with a supportive strategy, is then applied to this model in a basic (exploratory) efficacy trial. In the second step, the typical comorbidity and confounder profile of the target patient population is simulated in the stroke model for an advanced (confirmative) efficacy test, which should also include a thorough safety end point. If required, large animal modeling can be used for advanced safety assessments or therapy optimization before moving on to clinical investigations.

the paradigm can be limited to comorbidities and confounders in patients presenting the target lesions. Confirmative research should also address safety. For instance, a successful restoration strategy may require transplantation of biomaterials with or without cellular components into the lesioned brain, but multiple stereotaxic transplantations can be a safety issue.[75] Large animal models are suitable to simulate repeated transplantation, provide benefits about transplantation accuracy, or individualized therapeutic approaches. Sophisticated imaging protocols and detailed histological assessments may partly compensate for smaller sample sizes. Investigating these elements in large animal models can also help in clinical trial design.

A general limitation of the proposed strategy is that not all stroke patients benefit equally from emerging developments, and clinical trial study populations must be carefully selected. On the other hand, treating every patient with the same strategy is unlikely to be successful, so the selection of patient populations being sensitive to a restorative therapy is a valid strategy. A similar concept, that is, selecting patients with a salvageable penumbra before thrombectomy, has contributed to the recent success of mechanical recanalization.[76] Knowledge gained from successful tissue restoration experiments may also inform neighboring research and disease areas in need of true cerebral tissue restoration including myelodegenerative diseases and storage disorders, or diseases involving loss of selected neuronal populations such as Parkinson disease or amyotrophic lateral sclerosis.

Sources of Funding

This work was partly supported by NIH grants R01NS08226 and R01EB016629.

Disclosures

None.

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KEY WORDS: graft survival ■ immunomodulation ■ neurogenesis ■ regeneration ■ stem cell transplantation

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Stroke. 2018;49:1099-1106; originally published online April 18, 2018;
doi: 10.1161/STROKEAHA.117.018293
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628