Cover title: STEPS 4: accelerating preclinical research

Stem cells as an emerging paradigm in stroke (STEPS) 4: advancing and accelerating preclinical research

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Introduction

The scientific community has continuously advanced promising treatment concepts for cell-based therapies in stroke. These approaches principally aim to modulate post-ischemic immune responses and augment endogenous repair. Another aim currently under study at the bench level is to transplant new tissue and restore neural circuits. Many stem and non-stem cell populations have shown encouraging efficacy in preclinical models, and selected types of cell therapies are currently undergoing testing in clinical trials.[1-4]

Recent mechanistic studies have tremendously advanced our understanding of the different parameters that influence experimental stroke therapies. While cell therapies offer unprecedented therapeutic time window expansions of days to weeks (and possibly even months to years after stroke), there are several potential factors that may affect their impact. These include age[5], comorbidities[6,7], concurrent medications[8], and even chronobiological mechanisms.[9] In theory, thorough preclinical research should take into account all of these factors or at least their most relevant combinations. However, budgetary constraints, the lack of adequate *in vitro* and *in vivo* models, and the enormous amount of time required to address the multitude of relevant factors severely impairs such attempts in research practice. This dilemma affects current and future translational work and thus requires careful consideration.

The Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) meetings have regularly brought together academic and industry leaders and experts from regulatory authorities to discuss the latest developments in cell therapies for stroke and to publish recommendations for preclinical and clinical research.[10-12] The fourth STEPS meeting aimed to update previous preclinical guidelines with respect to novel stroke models, biomaterials, and advanced approaches combining cell therapies with biomaterials, drugs, or neurorehabilitation. STEPS delegates further provide new recommendation on preclinical study designs including multi-center preclinical trials (MCPTs) and suggest a strategy to accelerate and improve clinical translation of cell therapies for stroke without sacrificing scientific rigor and patient safety. This

can be achieved by a close interlink of preclinical and clinical studies while targeting particular stroke patient subpopulations. Main recommendations are summarized at the end of the STEPS 4 report.

Part I: Updated preclinical guidelines

Stroke model selection in the era of recanalization therapies

We recommend selecting models that best represent the clinical population targeted with a particular cell therapy. The recent advent of mechanical thrombectomy has changed the clinical landscape, and the application of cell therapies are discussed directly after recanalization.[13] Transient models should be selected when investigating this scenario. The filament model is widely used to represent mechanical recanalization[14]; however, its use for long-term studies poses some limitations due to large infarcts associated with high mortality.[15] Thromboembolization followed by thrombolysis is a clinically important model for testing cell therapies in the context of thrombolysis.[16] Moreover, reperfusion is often incomplete in patients undergoing thrombolytic therapy or spontaneous recanalization. This is also observed in spontaneously hypertensive rats that can serve as a model for these conditions[17] while also exhibiting other important stroke comorbidities. Total reperfusion failure or persistent occlusion can be modelled by permanent MCAO.

Large animal models

The gyrencephalic brain featured by large animal modes (LAMs) is bigger than the rodent brain and more suitable for sophisticated clinical imaging approaches.[18,19] Grey-to-white-matter ratio approximates that of humans.[9] LAMs allow more realistic and precise testing of cell delivery techniques including stereotaxic and intra-arterial cell administration, and dose translation to human clinical trials. Cell migration and paracrine effects, as in the human brain, are challenged by larger anatomic distances. LAMs are also suitable to investigate stroke

sequelae such as cognitive impairment and decline[20], and are further recommended to assess the value of potential biomarkers indicating cell therapy safety and efficacy.

On the other hand, LAM studies typically involve smaller sample sizes as they are more expensive and require dedicated infrastructure. Major endpoints including functional outcome and lesion size tend to be more variable than in standardized rodent studies. Although resembling the situation in human patient cohorts, these issues can significantly reduce study power.[21] LAMs are therefore of limited use in exploratory cell therapy studies. Meaningful LAM experiments require a precise understanding of the addressed endpoint(s), as well as of sample and effect sizes. Nevertheless, LAMs are highly valuable translational tools when considering their limitations and employing them in well-planned confirmative studies.[11] Funding bodies are encouraged to support research using LAMs in such scenarios, particularly when critical information on patient safety and delivery route efficiency can be obtained.

Sex differences, age, and comorbidities

In line with previous recommendations[11,12], the STEPS group recommends testing cell therapies in animal models of different age, sex, and comorbidities. However, we also recognize that modeling these variables, especially comorbidities, has limitations due their multitude and complexity. The impact of these factors might be better investigated in large phase III clinical trials allowing for sub-hoc analyses of patient populations with respective comorbidity profiles, or in MCPTs combining the capacities of many labs. An alternative approach (outlined in part III) is to focus on stroke patient subpopulations with particular stroke configuration and comorbidity profiles, and to design preclinical studies accordingly.

Dose escalation studies: novel implications

In line with previous recommendations[10,11] and in light of the neutral results from the MASTERS (multipotent adult progenitor cells given intravenously, NCT01436487) and

ACTISSIMA (SB623 administered intracerebrally, NCT02448641) trials that may partially be related to dosing issues, the STEPS group continues to recommend efficacy-focused preclinical dose escalation studies for all routes of administration. Intra-arterial administration of cells may cause microvascular obstruction under certain circumstances.[22] Hence, dose escalation studies are not only important for preclinical efficacy assessments, but are highly recommended when assessing safety aspects. This particularly accounts for intra-arterial or more invasive application routes. Methods capable of predicting the target territory of cell infusions may help to optimize the safety profile. LAMs may be suitable to simulate clinical transplantation scenarios regarding vessel dimensions and imaging-based surveillance.[23]

Drug-cell interactions

It is likely in clinical scenarios that patients receiving cell therapy also receive medications for stroke comorbidities and for secondary prevention. Cell therapies may further be combined with pharmacological treatments to enhance their therapeutic impact.[24] Given the paracrine effects of many cell therapies, interactions between drugs and cells cannot be excluded. This important aspect requires careful consideration when moving towards the clinic, but little is known about these potential interactions. Detrimental effects were seen when combining granulocyte-colony stimulating factor and bone marrow mononuclear cells, each of which is effective as a stand-alone treatment in rodents.[25,26] On the other hand, synergistic effects have been reported for the combination of cell therapies with other commonly prescribed medication such as statins.[27]

The STEPS 4 group recommends more research on potential drug-cell interactions in appropriate *in vitro* and in *in vivo* test systems. Drug classes being predominantly used in stroke patients, such as antiplatelets, anti-hypertensive, and statins, should be the main focus. We further suggest testing on autologous cell preparations when applied in patients receiving multiple medications. These tests can be tailored to the medication profile of individual patients.

Biomaterials

Biomaterials are increasingly being incorporated for the delivery of cells to reduce shear stress induced by needle injections[28,29] but also to provide factors that improve post-transplantation cell survival.[30,31] Scaffolds can support transplanted cells inside the lesion cavity[32] by providing structural cues and biochemical signals.[33,34] Post-stroke tissue restoration[35], and a guided neuronal differentiation[36] can be achieved using biomaterials engineered to release growth factors, mediators of angiogenesis, or immunomodulators in a temporal sequence and without exerting systemic side effects.[37-39] A systematic optimization of a hydrogel, for instance, improved the survival of human neural stem cells implanted into the stroke-damaged brain and controlled their differentiation. However, it remains unclear if the combined use of biomaterials and cells will transfer to further improvements in functional recovery. To date, most studies combining biomaterials and cells for transplantation are of an exploratory rather than definitive/confirmative nature. We therefore recommend long-term studies to investigate the safety and efficacy profile of biomaterial applications once a basic therapeutic benefit has been shown. LAMs may help to optimize application procedures. Early involvement of regulatory authorities, ideally already during early-stage preclinical research, is recommended, as biomaterial-cell combinations are challenging from a regulatory perspective.

Neurorehabilitation

Most stroke survivors receive some form of rehabilitation. Thus, neurorehabilitation is important to consider when developing cellular therapies for stroke. Indeed, treadmill running and intravenous delivery of mesenchymal stem cells together improve behavioral recovery in animals with ischemic stroke.[40,41] Timing of such combination therapy is crucial when targeting stroke recovery as there is a sensitive phase for neurorehabilitation (Fig. 1A). It is possible that some cell therapies might re-open a plasticity time window in chronic stroke, and

neurorehabilitation may be beneficial in such scenarios by stabilizing the recovered functions. The recent Stroke Recovery and Rehabilitation Roundtable (SRRR)-1[15] and SRRR-2[42] recommendations are valuable in designing preclinical rehabilitation studies and in improving clinical translation. However, as in the case of comorbidities, including rehabilitation renders study designs complex and difficult to implement. Also, the effects of add-on neurorehabilitation should be discriminated from stand-alone cell therapies, which may be challenging as shown recently with adipose tissue-derived stem cells and enriched environments.[43] Routine investigation of cell therapy in combination with neurorehabilitation is recommended when significant additional therapeutic effects are expected from this combination, or when the combination is a central mode of action.

Part II: New considerations on preclinical study designs

Potential new models and targets: lacunar, white matter, and hemorrhagic strokes

Most preclinical studies model large territorial infarcts. However, other important clinical target populations are patients with smaller infarcts in the subcortical grey and white matter. Importantly, the smaller volume of the infarct and the preservation of some anatomical tissue structures may foster repair.[44] Small deep white matter infarcts may be particularly suitable for cells (e.g. glial progenitors) capable of or intended for tissue restoration[45] and might be responsive to cell-borne local paracrine mechanisms. We recommend to consider such stroke types (see supplementary table) as alternative targets to large territorial infarcts and/or when working on tissue-restorative cell therapies.

Intracerebral hemorrhage (ICH)[46] involves pathogenic mechanisms that may provide novel cell therapy targets. Hemoglobin breakdown products (HBPs), such as hemin, damage axons and induce ferroptosis and necroptosis in distant, primarily intact neuronal somata.[47] These processes might be mitigated or reversed by factors released from therapeutic cells. Smaller hemorrhagic lesions or damage caused by HBPs may also be promising targets for

tissue regeneration approaches. Furthermore, peripheral and central inflammatory processes also contribute to further brain injury after ICH and these mechanisms might make excellent targets for some cell-based therapies.

Preconditioning of cell transplants

Long-term survival of transplanted cells is an important aspect for approaches that target long-term engraftment of neural stem cells to support or repair damaged neuronal circuits, or for which long-term trophic support is required. While cell survival has been poor in most previous studies, recent advantages were made in the field of cell preconditioning.[48,49] These techniques can significantly enhance and/or prolong survival of transplanted cells and should be considered for approaches that may benefit thereof.

Behavioral readout parameter selection

Functional tests should be sensitive to detect long-term impairment and treatments effects, but not be affected by repeated testing or compensation.[20] Various reaching tasks, foot fault, cylinder and adhesive tests provide quantitative and objective assessment in efficacy studies.[15] Simpler tasks can overestimate treatment effects but are valuable for exclusion of stroke animals with no/minor impairment, stratification regarding impairment severity, and treatment assessment during the acute phase. Appropriate tests should be selected for the respective stroke model, species, scenario, and study duration (Fig. 1B).

Smaller lesions require particularly sensitive and precise behavioral outcome measures. These lesions are more sensitive for functional compensation/spontaneous recovery and impairments may be masked. Automated readout systems carry high specificity and sensitivity and are being increasingly used in neurodegenerative disorders with initial subtle motor deficits.[50] The supplementary table summarizes information on specific deficits and their

measurement in lacunar lesions. Lastly, cognitive impairment and depression are common stroke complications, but at present there is no consensus on which tests to use in animals.

Safety assessments as a focus

Definitive demonstration of safety across multiple preclinical endpoints will be an invaluable resource when advancing cellular therapies for stroke treatment. The cell administration site should be evaluated for signs of inflammation or edema as well as acute respiratory problems for intravenous delivery to ensure the cell therapy is not inducing local or systemic immune responses. This may include animals with a humanized immune system. When performing repetitive administration of a cells, recipient sensitization (e.g., by lymphocyte proliferation assays), indicating adaptive immune system activation, should be contemplated.

Short- and long-term biodistribution and possible cell engraftment should be evaluated to determine cell persistence, particularly if the intended goal is engraftment. However, cell types exerting paracrine and immunomodulatory mechanisms, or exogenous cells may not persist which is viewed as an attractive component of approaches for which cell survival is not necessarily required. Complete endpoint evaluations of tissues and organ systems should be performed to definitively demonstrate that the cell administration does not have any off-target effects. Abnormal tissue growth, tumorigenesis or aberrant ectopic fiber sprouting should be excluded when using pluripotent stem cells or other cell types with high proliferation, differentiation, and fiber projection capabilities.

Multicenter trials

Innovative preclinical study designs including MCPTs have been proposed since the last STEPS recommendations. MCPTs mimic the design of large scale, efficacy-centered clinical trials with rigorous implementation of quality assurance measures as performed in clinical research.[51] MCPTs are believed to enhance predictive value and statistical power in preclinical

research, and to provide a close-to-practice assessment of the potential treatment. They may be of particular value when assessing cell therapies with mild to moderate impact on stroke (i.e. improvements of 10 to 20%)[52] or when assessing the impact of multiple therapy-influencing factors. MCPTs can also help to verify the benefit of combination therapies. This requires greatly enhanced statistical power to discriminate the effect of the combination from the impact of the individual therapies (e.g., rehabilitation plus cell therapy). The MCPT concept has been well received throughout the stroke community[53,54], and first MCPTs revealed effect sizes being considerably lower than what would have been expected from standard single center preclinical studies.[55]

However, MCPTs are more challenging to harmonize and carry much higher costs than standard study designs. Industry may benefit from MCPTs prior to initiating a clinical study.[56] The STEPS 4 consortium recommends considering MCPTs as an option when planning a translational research program in cell therapy for stroke. Importantly, NIH recently supported the creation of MCPTs and has launched the Stroke Preclinical Assessment Network (SPAN) program currently focused on multicenter evaluations of acute neuroprotectants as a complementary treatment to recanalization. Industry participation is highly encouraged in SPAN. Experience from the program will be invaluable to learn how MCPTs can be organized best to fully benefit from the enhanced power in assessing complex treatments, and how the complex logistics of MCPTs can be mastered. Ideally, successful SPAN activities will serve as a role model for MCPTs in cell therapies.

Potency assay development and qualification

A new recommendation from the STEPS group is the development of surrogate potency assays. Demonstrating a direct measurable correlation between a cell therapy and a biomarker or another quantifiable biological process with a beneficial outcome is critical to monitor the hypothesized mechanism of action. Biomarkers for putative mechanisms of action are also

critical to regulators for late stage clinical trial authorization. Biomarkers might be used to develop potency assays that should be robust, specific, informative, and reproducible in describing a fundamental biological effect of the expected benefit. Qualified potency assays are "locked down" as part of phase III clinical testing. They need to be transferred and performed under Good Manufacturing Practice (GMP) conditions before officially filing for product approval with the Food and Drug Administration in the United States. The development of potency assays during preclinical animal testing is therefore paramount prior to moving cellular therapies into advanced stages of clinical trials. As hypotheses change to reflect advances in the fundamental understanding of how cellular therapies provide benefit, new potency assays should be developed to parallel our understanding of cell-mediated benefits. For example, given increasing studies showing how many cell therapies target immune responses after stroke, immunomodulation may be an important potency assay for some cell therapies.[57]

Part III: Concepts for accelerating and improving preclinical research

Rethinking content and sequence of preclinical and clinical trials

State of the art preclinical research on cell therapy safety and efficacy takes significant time and resources. The broad and increasing spectrum of potential confounders is expected to engender additional budgetary and temporal demands that may severely hamper clinical translation. STEPS 4 discussed options to accelerate preclinical research while giving consideration to the complexity of potential confounding factors. A promising concept is to more clearly discriminate exploratory and confirmatory preclinical research[58], and to rigorously distinguish the primary goals of phase I/II clinical trials (safety) from later phases (efficacy). This allows a well-orchestrated sequence of preclinical and clinical tests with partially parallel workflows (Fig. 2).

Once a cell therapeutic paradigm is identified in initial exploratory studies, research activities are divided into two parallel tracks. First, exploratory research in standard rodent

stroke models confirms basic efficacy. Second, confirmative research investigates safety. This should also consider the most important comorbidities in the expected patient population, risks exhibited by the approach and the intended route of administration.[59] Thorough confirmation of safety and basic efficacy then allows proceeding to a phase I/IIa clinical trial which should not have a major focus on efficacy endpoints, but would be powered to confirm safety. Moreover, it should identify predominant profile characteristics of the targeted patient population such as type and frequency of comorbidities, infarct location and size, and co-medications.

This information is used to design advanced preclinical efficacy tests tailored to the target patient population profile. Ideally, these efficacy studies would be conducted in parallel to the phase I/IIa study. They may also be designed to identify subgroups with a pronounced benefit from the particular cell therapy which can be considered in a subsequent phase IIb/III clinical trial.

This approach has three major advantages: First, basic and enhanced preclinical efficacy studies can be organized in parallel to preclinical or clinical safety tests, saving valuable time. Second, the sequence of investigations in animal models and patients yields important data that will help to identify the most suitable patient populations for efficacy-driven clinical trials. Third, more thorough preclinical efficacy data can be used to design GMP potency assays with a higher predictive value than commonly applied ones.

Cell therapy responders versus non-responders

The STEPS 4 working group recommends storage of tissues and samples from animals that both respond and do not respond to cell therapy. As we learn more about the mechanisms of action through which cell therapies provide benefit, we may be able to retrieve stored samples from previous experiments to compare if preclinical responders and non-responders differ regarding newly identified or proposed biomarkers or pathways. This enables to refine our

clinical understanding of "responders" or "non-responders" and to better identify patients who can optimally benefit.

Preclinical data sharing platforms

A complementary opportunity to handle the increasing complexity of preclinical data are (open) sharing platforms. STEPS 4 participants unanimously agreed that such platforms, also including information from cell therapy cases in patients, are beneficial. Data would be available for benchmarking against other research programs, enhance study power, and facilitate meta-analyses. A central registry and predefinition of common preclinical data elements are required, but can be informed by existing clinical registries. The Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMRADES) database is an excellent role model, although a cell therapy registry for stroke must reflect the specific requirements of the community in detail.

Original data may be sensitive when related to pending intellectual property or commercial interests. Industry leaders among the STEPS 4 group stressed that such data should enjoy special protection, but is not necessarily excluded from sharing. For instance, the identity of a sensitive cell product could be concealed, but cell-treated subjects as well as all insensitive information on the cell product can be disclosed. Contributors using highly sensitive cell products may at least provide control cases.

Options to motivate contribution to data sharing platforms may be to allow access only to those who contribute and/or a general requirement that publically funded cell therapy research for stroke shall be publically. The STEPS 4 consortium suggests that decision makers at the NIH or the European Commission should consider funding schemes that help realizing data platforms tailored to cell therapies. Ideally, open data registries are organized internationally and provide connection hubs for industry and clinical cell therapy data.

Novel collaboration formats and the role of industry

The increasing complexity of preclinical stroke research and the parallel need for acceleration without sacrificing specificity and accuracy may not only require novel research strategies but also novel research alliances. Providing methodological knowhow, flexibility, and sufficient funds is required to meet the increasing need for rigor in preclinical research, raising the need for academic-industry alliances. Such alliances should not be restricted to sponsored contract research but true collaboration.[56] Academic-industry collaborations are also pivotal to sustainably utilize MCPTs. Finally, the experience of industry in meeting regulatory demands, technical aspects of cell therapies, and related logistics as well as clinical trial design is invaluable to inform preclinical research in order to advance the field. The STEPS 4 group recommends long-term academic-industry partnerships to thoroughly develop cell therapeutics from bench to bedside through closer collaborations.

Recommendation summary

1. A stronger focus on safety rather than confirming efficacy in early preclinical research, followed by early, safety-oriented clinical research has the potential to accelerate translational research without sacrificing quality.

2. We recommend thorough and advanced safety assessments and sufficient (standard stroke model) efficacy testing to support phase I/II safety trials. Advanced preclinical efficacy testing should be tailored to match targeted patient populations. This approach addresses the increasing complexity of potential confounding factors in a reasonable time. Appropriate primary readout parameters should be chosen for subsequent phase IIb/III trials.

3. Specific stroke models should best mimic the targeted patient population. LAMs are recommended if they provide additional, crucial information for clinical translation.

4. High priority should be given to developing specific and validated potency assays. Investigating drug-cell interactions and identifying cell therapy responders versus non-responders is recommended.

5. Sharing preclinical and clinical data will help the community tackle more complex research questions (e.g., whether comorbidities affect efficacy or safety).

6. Confirmative MCPTs are a valuable confirmative research format, but larger research consortia including industry joint ventures are required for successful implementation. MCPTs are preferred prior to definitive efficacy trials

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Figure Legends

Figure 1. Functional improvement by neurorehabilitation and recommended readout parameters.

(A) Schematic time course of spontaneous functional recuperation (light grey line), functional improvement with cell therapy alone (grey line), and with additional, appropriately timed supportive rehabilitation (black line). The relatively small differences between the therapy groups may require large sample sizes. (B) Behavioral tests differ with respect to sensitivity and specificity. Simple tests detect relatively large deficits in the acute and subacute stage. More sensitive tests address particular sensory and motor functions. Elaborated, often highly automated tests reveal very fine motor and sensory differences, or mental/cognitive impairment following stroke.

Figure 2. Proposed concept for accelerated clinical translation.

The basic suggestion of the concept is to initially focus on thorough and advanced safety assessments. Exploratory (basic) efficacy results warrant entering an early stage, safety-oriented clinical trial (phase I/IIa). This trial should also retrieve important characteristics of the target patient population, directly informing the design of more advanced, confirmative preclinical efficacy study (optionally followed by a multicenter preclinical trial) and of tailored potency assays. Those allow moving forward to clinical efficacy studies (phase IIb/III) tailored to the expected patient population, but in less time as would be required by sequential research programs. Regulatory authorities should be consulted regularly to ensure adequate planning of each parallel step.

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