Transfusion Medicine and Hemotherapy

Review Article

Transfus Med Hemother 2016;43:256–267
DOI: 10.1159/000447458

Received: April 19, 2016
Accepted: June 1, 2016
Published online: July 20, 2016

Mesenchymal Stem/Stromal Cells in Regenerative Medicine: Can Preconditioning Strategies Improve Therapeutic Efficacy?

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Keywords
Bone marrow · Stem cell transplantation · Stem cells · Stroke

Summary
Mesenchymal stem/stromal cells (MSCs) are becoming increasingly important for the development of cell therapeutics in regenerative medicine. Featuring immunomodulatory potential as well as secreting a variety of trophic factors, MSCs showed remarkable therapeutic effects in numerous preclinical disease models. However, sustainable translation of MSC therapies to the clinic is hampered by heterogeneity of MSCs and non-standardized in vitro culture technologies. Moreover, potent MSC therapeutics require MSCs with maximum regenerative capacity. There is growing evidence that in vitro preconditioning strategies of MSCs can optimize their therapeutic potential. In the following we will discuss achievements and challenges of the development of MSC therapies in regenerative medicine highlighting specific in vitro preconditioning strategies prior to cell transplantation to increase their therapeutic efficacy.

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Introduction

More than four decades ago Alexander Friedenstein identified bone marrow(BM)-derived fibroblastoid clonogenic cells that supported hematopoiesis and showed multipotent differentiation capacity [1, 2]. In the following years, being depicted as ‘mesenchymal stem/stromal cells’ (MSCs), in particular their differentiation potential and ‘stemnesss’ suggested the potential applicability of MSCs in a field that later became regenerative medicine. To date, MSCs have been isolated and characterized from a great variety of postnatal organs such as bone, BM, periosteum, synovial membrane/fluid, adipose tissue (AT), skeletal muscle, skin, periodontium and pancreatic islets, as well as from prenatal tissues like umbilical cord (blood) (UC(B)), amniotic fluid (AF) and placenta [3–7].

Numerous studies investigating in the multipotentiality of MSCs emphasized the capability of MSCs to differentiate not only in vitro and in vivo into adipogenic, osteogenic, chondrogenic, endothelial and myogenic lineages [8–14], but also into epithelial [15, 16] and neural cell types [17, 18]. There is growing scepticism about functional differentiation capacities of MSCs beyond adipocytes, osteocytes, and chondrocytes [19–21]. Yet, evidence of therapeutic capacities of MSCs in animal studies, modeling diseases such as stroke, myocardial ischemia or diabetes [22–24], raised great expectations that MSCs could be used as ‘multi-talent cell source’ for cell therapeutics with broad clinical applicability. Promising early clinical proof-of-concept studies for treatment of graft-versus-host disease [25–28] and regenerative applications [23, 29] appear to point towards this direction yet. Nevertheless, we are still waiting for the sustainable translation of MSC therapies to the clinic.

In the following, we will discuss achievements and challenges of the development of MSC therapies in regenerative medicine, highlighting specific in vitro preconditioning strategies prior to cell transplantation to increase their therapeutic efficacy.

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1660–3796/16/0434–0256$39.50/0

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Table 1. Most frequent conditions for MSCs therapies clinical trials

Source of MSCs	Condition	Total number of clinical trials	Autologous	Allogeneic
BM-MSCs	Ischemia*	28	21	8
	Bone regeneration	18, thereof 9 TE#	14	3
	Graft rejection	12	4	9
	Degenerative diseases§	12	9	2
	Lung diseases†	9	3	6
	Multiple sclerosis	9	9	0
	Amyotrophic lateral sclerosis	3	3	0
AT-MSCs	Ischemia*	10	6	2
	Degenerative diseases§	8	7	1
	Crohn's disease	3	1	2
	Spinal cord injury	2	2	0
	Liver cirrhosis	2	2	0
	Multiple sclerosis	2	2	0
	Amyotrophic lateral sclerosis	2	1	1
BM-MSCs	GvHD	23	1	20
AT-MSCs	GvHD	3	1	2

*Including myocardial infarction, stroke, and critical limb ischemia.
#Tissue engineering, e.g. MSCs seeded on bioceramic plates or immobilized in allogeneic bone prior to implantation.
§e.g. osteoarthritis, degenerative disc disease.
†e.g. emphysema, respiratory distress syndrome.

The Heterogeneity of MSCs and Non-Standardized ex vivo Culture Technologies

In the human bone and BM, which are currently a major source for MSC therapeutics (table 1), MSC subpopulations, such as fibroblastoid reticular stromal cells, adipose stromal cells, round stromal cells and bone lining cells, reside in distinct microanatomical localizations [30]. In addition, the perivascular niche harbors cells featuring MSC phenotypes as shown for various organs [4, 30, 31]. Due to the low frequency of MSCs in the BM (below 0.1% of nucleated cells [32]), manufacturing substantial numbers of MSC doses for therapeutic applications requires isolation/enrichment of MSCs as well as their in vitro expansion.

Currently, it is unclear how in detail, most importantly on functional level, the in vivo heterogeneity of MSCs is reflected by their in vitro culture, i.e. if, and if yes to which extent, different MSC phenotypes feature different functional properties. The most widely used MSC isolation and expansion technique is sub-culturing adherent cells from BM mononuclear cell fraction, enriched by density gradient [33], or from the stromal vascular fraction (SVF), obtained by enzymatic treatment of lipoaspirate [6]. These procedures, or alternative isolation techniques such as collagen and fibrin matrices or specific culture conditions (e.g. low oxygen, media enriched with growth factors), may select for certain MSC subpopulations and/or promote their in vitro expansion [34, 35]. In addition to considerable donor-donor variations, BM- and AT-MSC preparations feature significant heterogeneity in vitro as shown by a highly variable expression profile of marker sets potentially defining MSC subpopulations, e.g. CD140a, CD146, CD200, CD201, or CD271 [33, 36]. In addition, CD106 and CD271 expression is dynamic (decreasing) during the course of MSC in vitro culturing [35]. In contrast, CD73, CD90, and CD105 feature high and stable expression of MSCs in vitro, and therefore, together with a tri-lineage differentiation capacity and absence of hematopoietic markers, were elected by the International Society of Cell Therapy (ISCT) to characterize human MSC preparations [8].

Another variable to be considered for the development of MSC therapies is the influence of so far only poorly defined blood components such as human platelet lysate (HPL) used for media supplementation. Compared to fetal bovine serum, media supplemented with HPL or other human blood components are superior for the growth promotion and differentiation potential of MSCs [37–39]. Further studies are required to assess a possible influence of human blood components on the therapeutic efficacy of MSCs but also their potential immunogenicity.

Possible Implications of MSC Heterogeneity on Their Regenerative Potential

To address the question whether or not, and if yes to what extent, the heterogeneity of MSCs might affect their therapeutic potential in regenerative medicine,in the following, we will discuss the variables source and subpopulations with respect to the MSC function.

Given their multipotentiality, it might be obvious to focus on the differentiation potential of MSCs in order to assess its function

pertinent to tissue regeneration. However, this approach poses several challenges: first, how to assess MSC differentiation on a functional level, and second, how to translate observations of in vitro differentiation to the ultimately relevant regenerative setting in vivo.

Detection of markers such as glial fibrillary acidic protein, nestin, synaptophysin or β tubulin III/Tuj1 by 'neurogenic' differentiated MSCs [17, 18, 40] without evidence of neurotransmitter-modulated neuron-specific electrophysiological properties might not be sufficient to prove the neurogenic differentiation capacity of MSCs [19]. The limited applicability of 'lineage-specific markers' to assess MSC differentiation also refers to the numerously claimed cardiomyogenic differentiation potential of MSCs. Specifically native MSCs have been shown to express 'cardiomyogenic markers' (e.g. troponin I or atrial natriuretic protein), as well as MSCs after treatment with differentiation media, without featuring functional myogenic properties (e.g. formation of contractile cell structures) [21, 41]. Yet, given the heterogeneity of MSCs and our still limited understanding of the biology of MSCs, the existence of probably rare MSC subpopulations featuring true transdifferentiation potential could not completely be ruled out at this point, in particular as MSC differentiation is a clonal event [21, 42].

Regarding influence of source, UCB-MSCs had impaired adipogenic differentiation potential compared to AT-MSCs, BM-MSCs [6, 43], and BM-MSCs showed greater osteogenic differentiation potential compared to UCB-MSCs, AT-MSCs, or placenta-derived MSCs [43, 44]. Interestingly, BM-MSCs showed variable osteogenic differentiation potential depending on the harvest technique (reamer/irrigator/aspirator, spoon, fine-needle aspiration) and the anatomical localizations (femur, iliac crest) [45]. Amongst postnatal sources, BM-MSCs appear highly applicable for regeneration of cartilage defects featuring acceptable graft integration and biological similarities to cartilage tissue [46]. Comparing MSCs only from fetal sources, UCB-MSCs showed greater chondrogenic differentiation capacity than MSCs from amniotic fluid [47]. A recent study compared two cell types from the same source (UCB), i.e. UCB-mononuclear cells and UCB-MSCs, with cord matrix(CM)-MSCs regarding their therapeutic potential in a rat stroke model. All tested cell types showed therapeutic efficacy; however, more severe complications could be detected in the CM-MSC group [48].

Identification and quantification and/or enrichment of potent MSC subpopulations could mark a significant step towards optimized and standardized MSC therapies for regenerative medicine. Regarding possible differences of regenerative potential between MSC subpopulations, several studies tackled this complex and technically challenging problem. To date, different strategies have been developed to functionally characterize MSC subpopulations and to assign MSC subpopulation phenotypes to function.

First, investigating a substantial number of BM-MSC preparations in vitro, correlation analyses suggested an association of surface markers (CD10, CD71, CD106, CD119, CD146, CD166, and CD271) in order to assess the differentiation and clonogenic potential of BM-MSCs [33]. Another study applied a broad antibody panel (>200 markers) to compare AT-MSC surface after osteogenic or adipogenic differentiation to undifferentiated AT-MSCs. Here, an increased expression of CD164 was associated with an osteogenic differentiation, whereas CD36, CD40, CD146, CD164, and CD271 were higher expressed after adipogenic differentiation [49].

Another correlation concept identified, in addition to gene expression modulation experiments, the transcription factor TWIST1 as being significantly involved in BM-MSC function such as differentiation, support of angiogenesis and immunomodulation. Based on these observations, a 'clinical indication prediction scale' was developed assigning a more pro-angiogenic potential at higher TWIST1 expression to BM-MSCs, whereas a lower TWIST1 expression indicated a more pronounced immunomodulatory function of BM-MSCs [50].

Second, positive selections and subsequent functional analyses of MSC subpopulations could mark a further step forward. However, with the limitations of a potentially decreasing purity during sub-culturing and/or producing 'culture artifacts', as interplay of subpopulations might be relevant for their function, the CD271+ MSC subpopulation is abundant in intramedullary cavities of the long bones, and CD271+ sorted BM-MSCs featured greater osteogenic differentiation potential compared to non-sorted BM-MSCs [32, 51]. Meanwhile, first steps have been taken towards clinical-grade GMP production of CD271+ sorted BM-MSCs for bone regeneration [52]. Amongst placenta-derived MSCs, a greater osteogenic differentiation potential could be assigned to the CD146+ subpopulation, whereas, after sorting, CD146– MSCs could not form mineralized extracellular matrix, an indicator for functional osteogenic differentiation [53].

Third, MSC subpopulations may be defined by their 'performance' during in vitro culture, e.g. proliferation potential. A recent study compared BM-MSCs with long-term growth potential ('high growth') with BM-MSCs featuring lower cumulative population doublings and cumulative cell numbers ('low growth'). The study detected differences with respect to their osteogenic differentiation potential in vitro and in vivo. Specifically, 'low growth' BM-MSCs appeared to be more potent in osteogenic differentiation in vitro, whereas in vivo application of 'high growth' BM-MSCs induced larger volumes of ectopic bone in a rodent model [54].

Fourth, novel technologies, such as high-resolution microfluidic single-cell transcriptional profiling, allow clustering of subpopulations within MSC preparations [55]. Applying a combined library of surface markers with targets that are regarded as being indicative for MSC regenerative function, a recent study identified a provascular phenotype within the BM-MSC preparation that might be applicable for regeneration of the brain (stroke), the heart (myocardial infarction) or other clinical indications where improvement of vascularization is needed [56].

MSCs Secrete Trophic Factors and Produce Extracellular Vesicles

The aforementioned limited evidence of the functional differentiation capacity of MSCs together with the observation that MSCs

exert regenerative potential even without long-term survival after in vivo transplantation [57] led to the conclusion that trophic factors, released by MSCs, mainly mediate their therapeutic effects [58]. Indeed, various studies confirmed that MSCs produce and secrete a great variety of factors that are supposed to exert regenerative effects [33, 59, 60]:

- Angiopoietin-1: induction of angiogenesis, promotion of myocytes survival in myocardial infarction, increased survival of implanted MSCs, reduction of infarct size and fibrosis
- Brain-derived neurotrophic factor (BDNF):reduction of infarct size, promotion of neuronal tissue survival and differentiation
- Erythropoietin: angiogenesis, anti-apoptotic effects
- Fibroblast growth factor 1/2/4/7/9 (FGF-1/2/4/7/9): induction of angiogenesis, anti-apoptotic and anti-fibrotic effects, proliferative effects
- Glial cell-derived neurotrophic factor (GDNF): reduction of infarct size and axonal growth, promotion of dopaminergic neurons, motoneurons survival and morphological differentiation
- Granulocyte/macrophage-colony stimulating factor (G/M-CSF): progenitor cell mobilization, anti-apoptotic effects
- Hepatocyte growth factor (HGF): progenitor cell mobilization, induction of angiogenesis, promotion of cell growth, anti-apoptotic and anti-fibrotic effects
- Insulin-like growth factor 1/2 (IGF-1/2): progenitor mobilization, induction of renal tubular cells proliferation, anti-apoptotic effects
- Leukemia inhibitory factor (LIF): progenitor cell mobilization
- Nerve growth factor (NGF): neuroprotective effects
- Platelet-derived growth factor (PDGF): proliferative effects
- Stromal cell-derived factor 1 (SDF-1): progenitor cell mobilization
- Secreted frizzled-related protein 2 (Sfrp-2): myocardial survival and repair after ischemic injury
- Stanniocalcin 1 (STC-1): anti-apoptotic effects
- Transforming growth factor β1/2/3 (TGF-β1/2/3): stem cell differentiation and protection, tubulogenesis in kidney, anti-apoptotic effects
- Vascular endothelial growth factor (VEGF): induction of angiogenesis, stimulation of peritubular capillaries proliferation, progenitor cell mobilization, anti-apoptotic effects.

Interestingly, the secreted factor profile of AT-MSCs appears to be different compared to BM-MSCs [61]. To date, only few studies identified specific trophic factors of MSCs as relevant for regeneration of distinct pathologies (e.g. BDNF for stroke [62–64] and VEGF for myocardial infarction [65]).

There is growing evidence that extracellular vesicles (EVs) released by MSCs contribute to the therapeutic repertoire of the trophic factors of MSCs [66, 67]. Application of MSC-derived EVs has shown efficacy in various animal models such as myocardial infarction, limb ischemia, wound healing, or kidney, liver and lung injury [68, 69]. Although promising, before a sustainable translation into the clinic as possible alternative to MSC-containing therapeutics, MSC-EVs need to be defined more clearly and to be proven similar or superior with regard to efficacy and safety compared to engrafted MSCs.

The most frequent conditions in regenerative medicine for MSC therapies in clinical trials include ischemia (myocardial infarction, stroke, critical limb ischemia), degenerative diseases (osteoarthritis, degenerative disc disease), and bone regeneration with BM-MSCs as the most frequently used source (table 1). Interestingly, in total more MSC clinical trials have been conducted on regenerative medicine therapies than on GvHD.

Concepts of MSC Preconditioning

As pointed out in the previous section, MSC therapies are promising options to support organ and tissue regeneration. The protective effects of MSCs, their conditioned medium (CM), or MSC-EVs have been shown to support regeneration after various organ and tissue injuries. Yet, transplantation of MSCs or application of MSC-CM or MSC-EVs require MSCs with maximum regenerative capacity. Therefore, the development of new strategies to improve the regenerative efficiency of MSCs is urgently needed. In vitro pretreatment ('preconditioning') strategies can enhance survival, engraftment, and paracrine properties of MSCs and, therefore, optimize their reparative and regenerative capacity. In the following section, we summarize different MSC preconditioning technologies that have been developed in the last decade. Specifically, we focus on preconditioning regimens tested in in vivo disease models, and not only in vitro studies with appropriate animals for control, i.e. animals treated with non-preconditioned cells.

To date, various in vitro preconditioning strategies have been applied to enhance the regenerative capacity of MSCs [59]. MSC preconditioning includes modulation of culture atmosphere (hypoxic or anoxic), 3D culture, addition of trophic factors (growth factors, cytokines, or hormones), lipopolysaccharides, and pharmacological agents. The factors secreted by MSCs in response to the preconditioning regime are manifold and exert immunomodulatory or immunosuppressive anti-apoptotic, pro-angiogenic, and trophic effects [59]. The secretome (or the paracrine profile) of pretreated MSCs varies according to the preconditioning regimen used. Different preconditioning methods either activate or suppress different molecular signals and signal transduction cascades. The cellular responses are complex, and, in most cases, the pretreatment procedure affects a great number of factors, and not only a single, specific molecule or protein. In contrast, it should be mentioned that another approach to enhance the release of a specific regenerative factor is to overexpress a single factor in MSCs [70–74]. Nevertheless, these genetically engineered MSCs are not reviewed in this article.

Preconditioning by Environmental Variations

Several studies showed that preconditioning by hypoxia or anoxia substantially enhanced the regenerative potential of MSCs (table 2) [75–82]. Exposure of MSCs to reduced oxygen partial pressure induced the expression of genes involved in migration

Table 2. Selected recent studies using preconditioned MSCs in in vivo models[a]

Preconditioning regimen	Source of MSCs/species	In vivo model/species	Main findings of cell transplantation (versus non-preconditioned MSCs)	Year of publication [reference]
Hypoxia	BM-MSCs, human	hind limb ischemia, mouse	increased restoration of blood flow	2008 [75]
Hypoxia	BM-MSCs, mouse	myocardial infarction, mouse	increased angiogenesis, reduced cell death and apoptosis of implanted cells	2008 [76]
Hypoxia	AT-MSCs, human	acute kidney injury, rat	improved renal function, improved vascularization and histological injury	2014 [77]
Hypoxia	AT-MSCs, mouse	acute kidney injury, mouse	ameliorated renal function, lower levels of pro-inflammatory cytokines	2016 [78]
Anoxia	BM-MSCs, mouse	myocardial infarction, mouse	increased left ventricular ejection, reduced apoptotic cardiomyocytes	2006 [87]
Anoxia	BM-MSCs, rat	diabetic cardiomyopathy, rat	increased capillary density, attenuated myocardial fibrosis, increased fractional shortening of diabetic heart	2008 [79]
3D spheroid culture	BM-MSCs, human	peritonitis, mouse	better lung trafficking, more effective in suppressing inflammatory responses	2010 [88]
EGF	BM-MSCs, mouse	hind limb ischemia, mouse	recovery of blood flow and angiogenesis	2010 [118]
PDGF-BB	BM-MSCs, human	myocardial infarction, mouse	enhanced functional recovery	2015 [96]
TGF-β	BM-MSCs, mouse	acute myocardial injury, rat	enhanced myocardial functional recovery	2010 [92]
GDNF	AF-MSCs, human	acute kidney injury, mouse	ameliorated renal function and tubular injury, increased MSC homing to the tubulointerstitial compartment	2012 [93]
IGF-1	BM-MSCs, mouse	acute kidney injury, mouse	improved cell migration capacity, reduction in tubular necrosis, restored renal function	2013 [94]
FGF-2, IGF-1, BMP-2	BM-MSCs, rat	myocardial infarction, rat	smaller infarct size, better cardiac function, enhanced gap junction formation	2008 [91]
TNF-α	AT-MSCs, human	cutaneous wound-healing model, rat	accelerated wound closure, angiogenesis, proliferation, improved wound repair	2011 [95]
SDF-1	BM-MSCs, rat	myocardial infarction, rat	reduction in infarct size and fibrosis, significant improvement in cardiac function, enhanced cell survival, engraftment, and vascular density	2008 [90]
Angiotensin-II	BM-MSCs, rat	myocardial infarction, rat	better cardiac function, less cardiac fibrosis, smaller infarct size, higher expression of VEGF in ischemic myocardium	2015 [103]
Melatonin	BM-MSCs, rat	focal cerebral ischemia, rat	reduced apoptosis, reduced brain infarction and improved neurobehavioral outcomes	2014 [104]
Melatonin	BM-MSCs, rat	acute kidney injury, rat	increased MSC survival, proliferation of renal cells, accelerated renal recovery	2008 [105]
Oxytocin	UC-MSCs, human	myocardial infarction, rat	increased ejection fraction, lower cardiac fibrosis and macrophage infiltration	2012 [119]
LPS	BM-MSCs, mouse	myocardial infarction, rat	enhanced survival of engrafted MSCs and neovascularization, stimulated expression of VEGF, enhanced recovery of cardiac function	2009 [108]
TLR3 activation (Poly(I:C))	BM-MSCs, porcine	cardiomyopathy, hamster	improved cardiac function, decreased inflammatory cells and cytokines	2012 [120]
Hydrogen peroxide	WJ-MSCs, human	myocardial infarction, mouse	improvement in left ventricular contractility, increased neovascularization and reduced myocardial fibrosis	2012 [121]
Deferoxamine	BM-MSCs, rat	streptozotocin-induced diabetes, rat	increased homing of MSCs in pancreas	2013 [111]

[a]We applied a PubMed search using the terms 'preconditioning' and 'mesenchymal stem'. In addition, we only focus on preconditioning regimens tested in in vivo disease models and not simply in vitro studies with appropriate control animals, i.e., animals treated with non-preconditioned cells.
AF-MSCs = Amnion fluid-derived MSCs; AT-MSCs = adipose tissue-derived MSCs; BM-MSCs = bone marrow-derived MSCs; BMP = bone morphogenetic protein; EGF = epidermal growth factor; FGF = fibroblast growth factor; GDNF = glial cell-derived growth factor; IGF-1 = insulin-like growth factor-1; LPS = lipopolysaccharide; PDGF = platelet-derived growth factor; Poly(I:C) = polyinosinic:polycytidylic acid; SDF-1 = stromal cell-derived factor-1; ROS = reactive oxygen species; TGF-β = transforming growth factor-β; TLR = Toll-like receptor; TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor; WJ-MSCs = Wharton`s jelly-derived MSCs.

and homing (e.g. *CXCR4* and *SDF-1*), mainly regulated by the activity of hypoxia-inducible factor 1α (HIF-1α) [83]. Moreover, the culture of MSCs in a hypoxic environment appears to be more similar to the real in situ setting of MSCs than 'artificial' standard culture conditions (21% O_2) [40]. Notably, the cellular responses to hypoxia in vitro seem to vary between different oxygen concentrations (0.1–5% O_2) (reviewed in [82]).

In vitro preconditioning by hypoxia was shown to stimulate the secretion of growth factors, cytokines, and other proteins and the release of EVs (exosomes and microvesicles) from MSCs [84]. In this case, EVs in turn were also shown to carry a variety of biomolecules such as growth factors, receptors, enzymes, transcription factors, signaling and immunomodulatory molecules, DNA, RNA transcripts, and noncoding RNA including retrotransposons, vault RNA, long non-coding RNAs and microRNAs [85, 86].

Hypoxia preconditioning has also been shown to enhance cell survival and proliferation [77, 80–82]. Also, hypoxic pre-incubation of MSCs induced metabolic changes that resulted in higher in vivo cell survival after transplantation [81]. It was also shown that culture in hypoxia enhanced the angiogenic potential of MSCs and improved their survival in both in vitro and in vivo studies [80, 81]. The involved downstream signaling pathways are the translocation of HIF-1α to the nucleus with the activation of gene expression (e.g. VEGF), but also the generation of reactive oxygen species (ROS) and phosphorylation of Akt and MAPK ERK1/2 [82].

The positive in vivo effects of hypoxic preconditioned and systemically applied (i.v.) MSCs have been shown in a rat model of ischemic acute kidney injury [77]. In this study, vascularization, apoptosis, renal injury, and levels of serum creatinine as well as blood urea nitrogen were significantly improved in the group that received preconditioned MSCs compared with the non-pretreated control groups [77]. Also, anoxic preconditioning has been shown to enhance MSC survival and to promote their regenerative capacity [79]. Mechanistically, anoxia-induced increased phosphorylation of cell survival factors such as Akt and endothelial nitric oxide synthase are discussed [87].

A report by Bartosh and coworkers [88] showed that MSCs cultured as 3D spheroids had increased therapeutic potential. Specifically, the investigators used a hanging drop protocol to induce the spheroid formation of MSCs and found that these MSCs expressed the anti-inflammatory protein TNF-α-stimulated gene/protein 6 (*TSG-6*) at very high levels in vitro. Another very interesting result of this study was that larger numbers of the cells trafficked through the lung after infusion and were recovered in spleen, liver, kidney, and heart. The 3D spheroid MSCs were about one-fourth the volume of MSCs from adherent standard cultures, which may explain the enhanced trafficking through the lung [88]. Another study from this group demonstrated that cell activation in 3D culture also depends critically on the culture medium used [89].

Specifically, the authors described that only chemically defined, xeno-free media supplemented with human serum albumin resulted in compact spheres with high cell viability and high expression of anti-inflammatory (PGE2, TSG-6) and anti-cancer molecules (TRAIL, IL-24) [89].

Growth Factors, Cytokines, Chemokines, Hormones

In addition to the culture environment, several growth factors or other small molecules were shown to enhance the regenerative capacity of MSCs in vitro. Indeed, the growth factors EGF, GDNF and IGF-1, the pro-inflammatory cytokine TNF-α, the chemokine SDF-1 (CXCL12), or hormones such as angiotensin-II have been shown to enhance regenerative capacity or the paracrine functions of MSCs (table 2) [90–98].

Previously, a functional EGF receptor was identified on MSCs with evidence of an active EGF signal transduction [99]. EGF promoted in vitro expansion of MSCs without altering their multipotency [97, 98, 100], and the effects of EGF on MSC cell motility and migration are also well described [98–100]. Pretreatment of MSCs with EGF enhanced the release of factors such as VEGF, HGF, HB-EGF, IL-6, and IL-11, but not FGF-2 [97, 101]. VEGF and HGF play a pivotal role in MSC-mediated accelerated wound healing through inducing angiogenesis and improving oxygen supplies to the ischemic tissues [97]. EGF treatment has also been shown to enhance MSC motility, which is required for repopulation of MSCs within the wound bed [97]. Furthermore, the functional outcome of hind limb ischemia has been shown, most likely due to the delivery of pro-angiogenic factors by MSCs [97].

Herrmann et al. [92] have shown that TGF-β increased the VEGF production of MSCs in vitro and, to a greater extent, in combination with TNF-α or hypoxia. VEGF production was upregulated by a p38 MAPK-dependent mechanism and could be suppressed by p38 MAPK inhibition. Furthermore, the investigators infused TGF-β-preconditioned MSCs immediately before myocardial ischemia/reperfusion injury and could show that the post-ischemic myocardial functional recovery was improved in hearts infused with preconditioned MSCs compared with untreated MSCs or vehicle.

Treatment with CM derived from TNF-α-preconditioned MSCs accelerated wound healing and angiogenesis in vivo [95]. In addition, TNF-α-pretreated MSCs increased the release of cytokines, chemokines, and proteases, as shown by proteomic analysis. This study identified the enhanced secretion of 118 proteins into the culture medium upon TNF-α incubation [102]. Specifically, the TNF-α-induced secretome of MSCs included many molecules known to be critically involved in inflammatory processes (e.g. IL-6, IL-8, and MCP-1). Inflammation is a key response to tissue injury and is critical for regeneration, with many cytokines being associated with this process. Enhanced expression of IL-6, IL-8, or MCP-1 goes along with enhanced migration of monocytes to the site of injury, hereby promoting a pro-inflammatory response.

Hormones such as angiotensin-II or melatonin were used to pretreat MSCs before their use in in vivo models. Pretreatment of MSCs with angiotensin-II resulted in an improved cardiac function and a reduced occurrence of cardiac fibrosis, a smaller infarct size, and a higher expression of VEGF and von Willebrand factor in ischemic myocardium [103]. Another study examined the effect of melatonin pretreatment of MSCs in vivo after transplantation into the ischemic brain [104]. MSCs preconditioned with melatonin re-

duced brain infarction, improved neurobehavioral outcomes, and showed increased angiogenesis and VEGF expression [104]. In addition, MSCs treated with melatonin displayed an increased expression of HGF and FGF-2 [105]. CM from melatonin-treated MSCs stimulated tube formation of endothelial progenitor cells and proliferation of renal proximal tubular cells in vitro. Moreover, the study showed that melatonin pretreatment strongly increased MSC survival after transplantation in a model of acute kidney injury. This effect was concomitant with an increased angiogenesis, proliferation of renal cells, and accelerated recovery of renal function [105].

Preconditioning Regimens Using Pharmacological or Chemical Agents

Alternative preconditioning concepts to prime MSCs in vitro prior to their use in in vivo models include pretreatment with atorvastatin, curcumin, and several other pharmacological or chemical agents.

Atorvastatin, a statin used as a lipid-lowering agent and for prevention of events associated with cardiovascular disease, enhanced the expression of CXC chemokine receptor 4 (CXCR4) on MSCs and stimulated MSC migration in vitro [106]. The significant role of SDF-1 and its receptor CXCR4 in mobilization and migration of MSCs to sites of injury has been elucidated [90, 107]. Therefore, MSC pretreatment with atorvastatin was tested in an in vivo model of myocardial infarction [106]. Li et al. [106] found an increased migration and homing of MSCs toward the infarcted myocardium compared to non-pretreated MSCs and suggested that atorvastatin pretreatment is an effective preconditioning regimen to promote the cell-therapeutic potential of MSCs. In addition, enhanced regenerative efficacy of MSC transplantation has also been shown after MSC preconditioning with LPS [108]. LPS, an endotoxin of Gram-negative bacteria, is known as the ligand of Toll-like receptor-4, which is expressed by MSCs. In a model of myocardial infarction, LPS pretreatment ameliorated the cardiac function, reduced fibrosis, stimulated expression of VEGF, and activated the PI3K/Akt pathway [108].

Curcumin, an agent extracted from the spice turmeric, has been reported to show potent antioxidant and anti-inflammatory properties and free radical-scavenging activity [109]. A study by Liu et al. [109] showed that pretreatment of MSCs with curcumin improved the tolerance to oxidative stress injury and resulted in an enhancement of the therapeutic potential of MSCs in myocardial repair after infarction.

Pharmacological MSC preconditioning with diazoxide, a mitochondrial ATP-sensitive potassium channel opener, protected cells from oxidative stress injury by upregulating the expression of FGF-2 and HGF [110]. In an in vivo model of myocardial infarction, diazoxide preconditioning improved the survival rate of the infused MSCs, reduced the infarct size, and increased left ventricular function compared to the transplantation of non-pretreated MSCs [110].

Preconditioning with deferoxamine, an iron-chelating drug, stabilized HIF-1α under normoxic conditions as well as the activity of two metalloproteases [111]. Notably, the stabilization of HIF-1α resulted in an increased activity as well as in increased transcription of genes involved in cell migration [112]. Deferoxamine preconditioning prior to transplantation also increased homing of MSCs through affecting chemokine receptors as well as metalloproteases [111].

ROS and reactive nitrogen species are biologically active oxidants and are regarded as important physiological signaling molecules. Various reports indicate the role of ROS as second messengers in the O_2 sensing [113, 114]. In a previous study, ROS preconditioning has been shown to enhance the pro-angiogenic properties of MSCs [114]. Applying a pharmacological preconditioning strategy with the mitochondrial inhibitors antimycin and rotenone to modulate ROS generation in MSCs, the authors found that this regimen strongly improved revascularization and the number of MSC-derived CD31+ cells in the ischemic area. Furthermore, ROS generation increased MSC secretion of the pro-angiogenic and anti-apoptotic factors VEGF and HGF, but did not affect the ability of MSCs to differentiate into cells with endothelial phenotype in vitro [114].

Another study showed that MSC preconditioning with valproat and lithium chloride promoted functional recovery, increased angiogenesis, and reduced the infarcted zone in the brain in a rat cerebral artery occlusion model [115]. Additionally, increased migration and homing of MSCs towards the ischemic site, possibly mediated by an increased CXCR4 expression, was observed [115].

And What about Current Clinical Studies on MSC Preconditioning Therapies?

As mentioned above, recent data clearly indicate that a functional improvement of the regenerative capacities of MSCs could be obtained by applying different MSC preconditioning regimens in vitro and after MSC infusion in different in vivo models. Clinical trials using MSCs have been expanding quickly in the last decade. However, although various MSCs preconditioning strategies have been developed and have been already evaluated in animal models, currently only three clinical trials are registered on *www.clinicaltrials.gov* (table 3). All three clinical studies use a comparable (but not equal) preconditioning regimen (hypoxia, ischemic preconditioning) for in vitro pretreatment of BM-MSCs; three different pathologies have been investigated (table 3). The purpose of the first study is to evaluate the efficacy of hypoxia-preconditioned autologous BM-MSCs for patients with ischemic heart diseases. The second study examines the regeneration of the lung in patients suffering from pulmonary emphysema after transplantation of hypoxia-preconditioned autologous BM-MSCs. Currently, only these two studies are listed on *www.clinicaltrials.gov*, and the study protocol of the third study was published in a scientific journal [116]. The objective of this study is to evaluate the efficacy of preconditioned MSCs in patients with ischemic stroke. The chosen pretreatment ('ischemic

Table 3. Current clinical trials using MSCs after preconditioning to enhance their therapeutic efficacy (*www.clinicaltrials.gov*).

Study	Identifier
The STem Cell Application Researches and Trials In NeuroloGy-2 (STARTING-2) Study [116] Condition: Stroke, ischemic Intervention: Transplantation of autologous BM-MSCs preconditioned with autologous serum obtained at acute phase of stroke ('ischemic preconditioning') Study start date: November 2012	NCT01716481
Clinical Study of the Efficacy and Safety of the Application of Allogeneic Mesenchymal (Stromal) Cells of Bone Marrow, Cultured Under the Hypoxia in the Treatment of Patients With Severe Pulmonary Emphysema Condition: Pulmonary emphysema Intervention: Infusion of allogeneic BM-MSCs, in vitro preconditioned under 1% hypoxia Study start date: March 2014	NCT01849159
Therapy of Preconditioned Autologous BMMSCs for Patients with Ischemic Heart Disease Conditions: Acute myocardial infarction; ischemic cardiomyopathy Intervention: Transplantation of autologous BM-MSCs with hypoxia precondition and endothelial preinduction Study start date: November 2015	NCT02504437

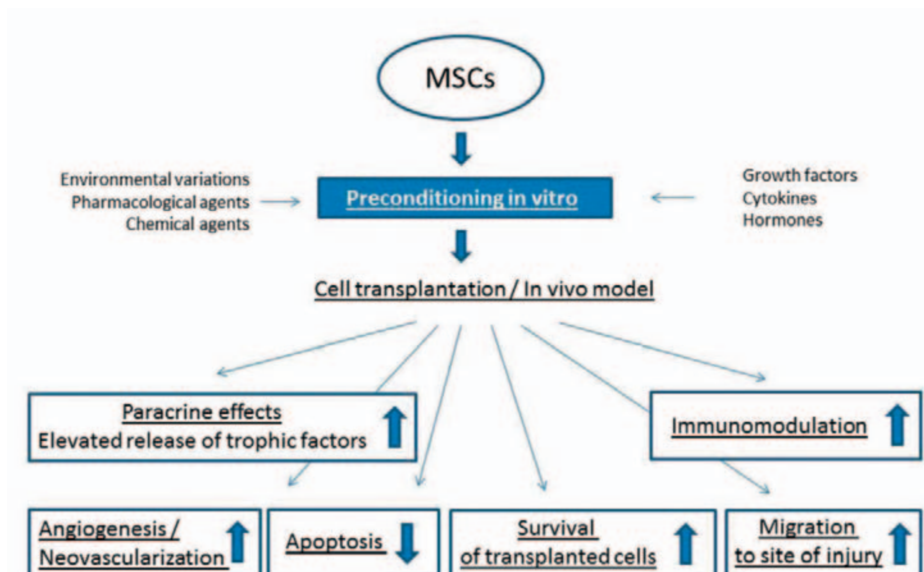

Fig. 1. Mechanisms involved in the enhanced therapeutic potential of preconditioned MSCs.

preconditioning') is in vitro culture of MSCs in media supplemented with autologous serum that is obtained at the acute phase of stroke from patients. A previous study from this group with rat MSCs cultured in media supplemented with serum obtained from a rat stroke model showed an increased expansion rate of MSCs with decreased cell death, increased trophic factor secretion, and increased migration capacity compared to MSCs cultured in media supplemented with fetal bovine serum. In addition, another study showed recently that stroke serum priming of MSCs upregulated the expression of miRNA-20a, which promoted MSC proliferation by regulating the cell cycle inhibitor p21 CDKN1A [117].

In summary, due to the limited number (and to date not published results) of clinical trials using preconditioning strategies to optimize the regenerative capacity of MSCs (or their CM), more clinical trials investigating the effects of different preconditioning regimens in varying pathological situations are urgently needed.

Final Remarks

In summary, transplantation of preconditioned MSCs has shown promising results. Whereas not finally proven, it seems clear that manifold mechanisms are involved in the increased benefit of cell therapy using preconditioned MSCs (fig. 1). As shown by numerous experimental studies reviewed in this article, the enhancement of the therapeutic potential of MSCs by precondition-

ing is mediated by a great variety of mechanisms at which enhancement of paracrine factors release by pretreated MSCs appears as highly relevant mechanism. Nevertheless, other events are likely involved, such as upregulation of different surface proteins/receptors or enhanced survival of transplanted cells. The complete effects and the whole secretome of MSCs after different preconditioning regimens have not been investigated in a comprehensive manner yet. Advances in high-throughput technologies, protein and RNA arrays, and bioinformatics have already facilitated analysis of the secretome including EVs and will continue to help identifying the factors released by MSCs under different precondition regimens [81]. In addition, data from different in vivo models are often conflicting and hampered by varying MSC isolation protocols, culture or proliferation methods, preconditioning regimen and schedule, application sites, and numbers of transplanted MSCs [59]. To date, methods for in vitro pretreatment or preconditioning, possibly by combination of factors, have not been optimized to improve MSCs or their conditioned medium-based therapies, and, therefore, need to be substantially improved in future works.

A huge gap between experimental approaches and their application is observed in the clinic. To date, clinical studies confirming the preclinical results are missing. Thus, additional research using in vivo studies to determine the exact underlying mechanisms and, in particular, clinical trials to show the regenerative efficacy and the benefit of preconditioned MSCs are required and are expected in future years. Nevertheless, encouraging preclinical studies fuel the hope that preconditioning regimens can enhance the regenerative capacities of MSC therapies.

Disclosure Statement

The authors declare no conflict of interest.

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Imprint

ISSN Print Edition: 1660–3796
ISSN Online Edition: 1660–3818

Journal Homepage: http://www.karger.com/tmh

Publication Data: Volume 43, 2016 of 'Transfusion Medicine and Therapy' appears with 6 issues.

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Price list No. 30 of January 1, 2016 is effective.

Publisher: S. Karger Verlag für Medizin und Naturwissenschaften GmbH
Wilhelmstr. 20A, 79098 Freiburg (Germany)
www.karger.de, information@karger.com

V.i.S.d.P. (Person responsible according to the German Press Law):
Sibylle Gross

Type setting and printing: Kraft Druck GmbH, 76275 Ettlingen, Germany.
Printed on acid-free and non-aging paper (ISO 9706).

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