# Entwicklung der Kontrastmittelechokardiografie am Rattenmodel zur Untersuchung des Einflusses von mesenchymalen Vorläuferzellen auf das Remodeling nach experimentellem Herzinfarkt

Dissertation
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Der unvergesslichen Kerstin Rabald, meiner lieben Mutti.

# Bibliographische Beschreibung

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Entwicklung der Kontrastmittelechokardiografie am Rattenmodel zur Untersuchung des Einflusses von mesenchymalen Vorläuferzellen auf das Remodeling nach experimentellem Herzinfarkt

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Referat:

Es werden in einer kumulativen Dissertationsschrift zwei wissenschaftliche Arbeiten zusammengefasst.
Die erste Arbeit beschreibt die Etablierung der Kontrastmittelechokardiografie zur Charakterisierung des Herzinfarktmodells an der Ratte im zeitlichen Verlauf. Es wird der Ablauf der geometrischen Änderungen am linken Herz nach Herzinfarkt gezeigt. Zusätzlich wird die Methode mit anderen etablierten echokardiografischen Methoden verglichen. Hier wird die Messung der linksventrikulären Querschnittsfläche der Volumenbestimmung nach der modifizierten Simpson-Methode gegenübergestellt. Es wird gezeigt, dass die Flächenmessungen, bei Nichtverfügbarkeit der Kontrastmittelechokardiografie eine valide Methode zur Verlaufsbeobachtung im Modell darstellt.
Die zweite Arbeit untersucht im Rattenversuch den Einfluss von mesenchymalen Vorläuferzellen aus Nabelschnurblut auf di e Entwicklung des Herzversagens nach Herzinfarkt. Die Injektion der Zellen erfolgt direkt in das Herzmuskelgewebe am Rand des Infarktareals. Zusätzlich zur Phänotypisierung mittels Echokardiografie wurden hämodynamische Messungen, sowie immunhistochemische und molekularbiologische Untersuchungen vorgenommen. Es konnte in einem Multigruppendesign gezeigt werden, dass im vorliegenden Versuch durch die Injektion von Vorläuferzellen kein Einfluss auf di e geometrischen und bi omechanischen Änderungen nach Herzinfarkt genommen werden konnte. Es konnten jedoch zusätzlich Differenzen zwischen den V ersuchsgruppen in der Genexpression von Signalmolekülen der extrazellulären Matrix gezeigt werden, welche Spekulationen über den Einfluss der Zellen auf parakrine Mechanismen im Herzgewebe zulassen.

# Inhaltsverzeichnis

# Vorbemerkung

Diese Arbeit umfasst wissenschaftliche Ergebnisse welche bereits unter den Titeln

- **Contrast enhanced echocardiographic follow-up of cardiac remodeling and function after myocardial infarction in rats**
  Rabald, S., Hagendorff, A., Pfeiffer, D., Zimmer, H. G. & Deten, A.
  Ultrasound in Medicine and Biology, (2007), 33, 1561-71

- **Cord blood cell therapy alters LV remodeling and cytokine expression but does not improve heart function after myocardial infarction in rats**
  Rabald, S., Marx, G., Mix, B., Stephani, C., Kamprad, M., Cross, M., Boltze, J., Briest, W., Zimmer, H. G. & Deten, A.
  Cellular Physiology and Biochemistry, (2008), 21, 395-408

in den genannten Fachzeitschriften erschienen sind. Im Sinne einer kumulativen Arbeit werden nachfolgend beide Arbeiten in Originalform mit entsprechenden einleitenden, verbindenden und diskutierenden Texten zusammengefasst dargestellt.

# 1 Einleitung

## 1.1 Der Herzinfarkt

### 1.1.1 Allgemeine Bemerkungen

Als Herzinfarkt bezeichnet man den durch plötzliche Blutmangelversorgung bedingten Untergang von Teilen des Herzmuskelgewebes.

Die Minderdurchblutung des Herzmuskels mit der damit verbundenen Herzleistungsschwäche ist eine der häufigsten Todesursachen weltweit (Weltgesundheitsorganisation, 2008). In Deutschland wurden 2003 insgesamt 820 874 Patienten mit der Hauptdiagnose „Ischämische Herzkrankheit“ in stationäre Behandlung aufgenommen. Davon starben im Krankenhaus 28 874 Patienten. Insgesamt starben in Deutschland im Jahr 2004 152 659 Menschen an einer ischämischen Herzkrankheit, die damit die wichtigste spezifische Todesursachengruppe darstellte (Statistisches Bundesamt, 2008).

Fast alle Patienten sind nach einem überlebten Herzinfarkt lebenslang arzneimittelpflichtig. 40 Prozent der Patienten klagen bereits im ersten halben Jahr nach dem Herzinfarkt über die Lebensqualität beeinträchtigende Beschwerden. Bei jedem zehnten Patienten entwickelt sich eine Herzinsuffizienz mit Abbau der körperlichen Leistungsfähigkeit, Müdigkeit und Atemnot. Die Sterblichkeit in dieser Gruppe ist insgesamt hoch. (Gesundheitsberichterstattung des Bundes, 2006)

1995 wurden 14 807 der insgesamt 186 368 (7,9 %) Frühberentungen bei Männern in Deutschland mit einer ischämischen Herzkrankheit begründet. Diese Patienten waren zum Zeitpunkt der Berentung durchschnittlich 54,9 Jahre alt. (Gesundheitsberichterstattung des Bundes, 2006)

### 1.1.2 Der kardiale Umbau - „Remodeling“

Nach einem akuten Herzinfarkt kommt es häufig zu Änderungen der Geometrie, sowohl im infarzierten Teil des Herzens, als auch im nicht infarzierten Teil der Herzmuskulatur. Vor allem nach großen, alle muskulären Wandschichten betreffenden Infarkten können diese Änderungen funktionell bedeutend sein (Hutchins and Bulkley 1978). Das Ausmaß dieser Umbauprozesse hat großen Einfluss auf die Herzfunktion und damit auch direkt auf Lebensqualität und Überleben des Patienten (Meizlish et al. 1984). Die früh nach dem akuten Infarkt eintretende Änderung der Herzgeometrie wurde als ein Dehnen der infarzierten

Gewebsteile des Herzen beschrieben. Diese Infarktdehnung („infarct expansion“) zeigt einen starken Zusammenhang mit der Wandspannung, der das zur Infarktnarbe heilende Gewebe ausgesetzt ist. Dem Gesetz von LaPlace

$$Wandspannung\ (\sigma) = \frac{Innendruck \cdot Radius}{2 \cdot Wanddicke}$$

folgend, ist zu diesem frühen Zeitpunkt nach dem Infarkt vor allem der Druck im Herzen die, die Wandspannung beeinflussende Größe. So konnte von Hammerman et al. (1985) gezeigt werden, dass in der frühen Phase nach Infarkt auch kurzzeitige Blutdruckanstiege deutliche Einflüsse auf die Dehnung des Infarktgebietes und damit auf die bleibende Vergrößerung der Herzhöhlen haben. Mit einer Vergrößerung der Herzhöhle und damit auch von deren Radius steigt auch die Wandspannung in den nicht vom Infarkt betroffenen Herzmuskelarealen. Da das überlebende Myokard die Gesamtleistung des Herzens tragen muss, finden dort rege Umbauprozesse zu Gunsten einer kompensatorischen Gewebsmassesteigerung statt. Da Herzmuskelzellen bei Erwachsenen nicht teilungsfähig sind, wird diese Gewebsmassesteigerung durch Volumenzunahme der einzelnen Muskelzellen erreicht. Während dieser Umbauprozesse kommt es auch zu einem Dehnen und damit zu einer Umfangsvergrößerung im gesunden Teil des Herzmuskels. Linzbach (1960) konnte zeigen, dass diese Vorgänge von Hypertrophie und Dehnung nicht mit einer Verlängerung der einzelnen Muskelzellen einhergeht. Der daraus zu ziehende Schluss, dass es zu einer Umordnung der an Dicke gewonnenen Muskelzellen kommt, lässt die Vermutung zu, dass es sich hierbei um Adaptationsprozesse handelt, mit deren Hilfe das Herz die regionale Spannung der Myozyten verschiedenen Erfordernissen anpassen kann. Nach einem ausgedehnten Herzinfarkt ist die initiale Vergrößerung des Herzens durch die Dehnung der heilenden Narbe so groß, dass die Umbauvorgänge in der nicht betroffenen Wand in einen sich selbst verstärkenden Kreislauf aus Wandspannung, Wanddehnung durch Umbau, Radiusvergrößerung und damit Wandspannungsvergrößerung eintreten. So kann es zu einer fortschreitenden Vergrößerung des Herzens kommen, lange nachdem die Infarktnarbe selbst zu einer dehnungsstabilen Bindegewebsnarbe geheilt ist. Mit vergrößerter Herzkammer steigt auch die Kraftanforderung an den einzelnen Myozyten zum Überwinden der Nachlast und damit zum Auswurf der Ventrikelfüllung. So ist bei begrenzter Maximalkraft des einzelnen Myozyten und fortschreitender Vergrößerung der Herzkammer, ein stetiger Verlust der Herzleistung und damit eine Herzschwäche unausweichlich. So konnte schon 1969

röntgenologisch gezeigt werden, dass die Größe des Ventrikels der stärkste Prognoseparameter für das Überleben nach einem Herzinfarkt ist (Shanoff et al. 1969; Kostuk et al. 1973). Für das endsystolische Volumen konnte im Verlauf nach einem akuten Herzinfarkt gezeigt werden, dass dessen Vergrößerung das Risiko an einer Herzinsuffizienz zu sterben, exponentiell steigert (Hammermeister et al. 1979; White et al. 1987).

Das Wissen um die Pathophysiologie der sich entwickelnden Herzinsuffizienz eröffnet prinzipiell mehrere therapeutische Angriffspunkte:

- Reduktion der Infarktausdehnung
- Stabilisierung der Infarktnarbe
- Reduzierung des intraventrikulären Druckes (oder der Druckdifferenz über der Wand)
- Blockade der die Dilatation begünstigenden Umbauvorgänge im überlebenden Gewebe.

### 1.1.3 Therapeutische Aspekte

Eine Reduktion der Infarktausdehnung reduziert auch das Ausmaß der Dilatation. Die effektivste therapeutische Maßnahme zur Infarktreduktion ist das schnelle Wiedereröffnen des verschlossenen Herzkranzgefäßes (White et al. 1987; Group N.H.F.o.A.C.T. 1988). Die Wiederherstellung der Gewebsdurchblutung innerhalb der Ischämietoleranzzeit des Herzmuskels verhindert den kritischen Substanz- und Funktionsverlust und damit auch die folgende Dehnung der Infarktregion. Die Ischämietoleranz kann durch präklinische Medikation mit Opiaten und Beta-Blockern verlängert werden. Andere Ansätze einer therapeutischen Infarktgrößenreduktion, wie medikamentöse Eingriffe im Fruchtzuckerstoffwechsel oder Apoptoseverminderung durch Manipulation des parakrinen Systems im Herz, sind Gegenstand wissenschaftlicher Bemühungen.

Die Maßnahmen, welche zur Infarktgrößenreduktion geeignet sind, eignen sich auch zur Stabilisierung der Infarktnarbe. So konnte von Braunwald (1989) und Califf et al. (1989) gezeigt werden, dass eine Wiederherstellung der Gewebsdurchblutung auch dann die Dilatation vermindert und damit das Überleben begünstigt, wenn dies bei zu langer Ischämiezeit nicht durch ein Überleben von Herzmuskelzellen zu erklären ist. Als Gründe hierfür werden die schnellere Narbenheilung im durchblutetem Gewebe und die längere Ischämietoleranz der neben den Myozyten im Herzmuskel beheimateten Zellfraktionen diskutiert. Die parakrinen und inflammatorischen

Vorgänge in der heilenden Infarktnarbe könnten in Zukunft auch Ansatzpunkt für Medikamente sein, welche dann eine schnellere Stabilisierung der Narbe begünstigen und damit eine Verminderung der Dilatation ermöglichen. Neben medikamentösen wurden auch chirurgische Alternativen zur Narbenstabilisierung gezeigt. So kann die Dehnung der Infarktnarbe effektiv verhindert oder aufgehoben werden, indem ein Transplantat aus Kunststoff oder Muskelgewebe über die Narbe genäht wird. Eine weitere wissenschaftliche Vision ist es, aus körpereigenen Zellen Transplantate aus Herzmuskelzellen zu züchten und diese funktionell in das Herzmuskelgewebe zu integrieren.

Die Zusammenhänge, welche auf histologischer und zellulärer Ebene zum Umbau und damit zur Dehnung des nichtinfarziertem Gewebes führen sind ebenfalls Gegenstand intensiver Forschung. Vor allem das parakrine System und das Umgehen der dogmatischen Teilungsunfähigkeit von Herzmuskelzellen scheinen hier Angriffspunkte neuer Therapien sein zu können.

### 1.1.4 Therapieoptionen mit Vorläuferzellen

Das Herzmuskelgewebe von Erwachsenen verfügt wegen der nicht vorhandenen Teilungsfähigkeit der Herzmuskelzellen nur über sehr begrenzte Fähigkeiten zur Regeneration. In der Regel können Gewebsschäden im Herz ausschließlich durch bindegewebigen Umbau geschlossen werden.

Nachdem in transplantierten Herzen Vorläuferzellen gefunden wurden, welche genetisches Material des Transplantatempfängers enthielten (Laflamme et al. 2002; Muller et al. 2002; Quaini et al. 2002), begann eine Diskussion um die Herkunft und Bedeutung dieser mutmaßlich eingewanderten Zellen. Es wurde diskutiert, dass es zirkulierende Vorläuferzellen gibt, welche in der Lage sind, zu Herzmuskelzellen zu transdifferenzieren und welche an einem sehr langsamen Zellersatz in der Muskelzellpopulation des Herzen beteiligt sind. Der Ursprung dieser Zellen wurde unter der Vorläuferzellen der Blutbildung im Knochenmark vermutet (Jackson et al. 2001). Verschiedene Gruppen zeigten in vitro, dass sich diese hämatopoetischen Vorläuferzellen in viele verschiedene Zelllinien, einschließlich Herzmuskelzellen, weiterentwickeln können (Toma et al. 2002; Deb et al. 2003). Die Hoffnungen, diese Zellen nutzen zu können, um den Verlust an Herzmuskelzellen nach einem Infarkt durch Regeneration auszugleichen, führten zu zahlreichen tierexperimentellen Studien. So zeigten Orlic et al. (2001) eine Regeneration von Herzmuskelgewebe nach der direkten Injektion von hämatopoetischen Stammzellen in die Wand

infarzierter Herzen an der Maus. Eine folgende Studie der gleichen Arbeitsgruppe zeigte eine Verbesserung von Funktion und Überleben nachdem in infarzierten Versuchstieren Vorläuferzellen des Knochenmarks mittels Wachstumsfaktoren mobilisiert wurden (Orlic et al. 2001). Diese ersten Befunde führten sehr schnell zu einer Anzahl an klinischen Studien (Assmus et al. 2002; Strauer et al. 2002; Perin et al. 2003).

Auf der anderen Seite wurde von verschiedenen Gruppen gezeigt, dass ins Herz injizierte Vorläuferzellen der Blutbildung ihre hämatopoetischen Eigenschaften beibehalten und zu Zellen ihrer eigenen Zelllinie transdifferenzieren (Murry et al. 2004). Es wurde gezeigt, dass es zwischen hämotopoetischen Zellen und Herzmuskelzellen zu einem Kerntransfer kommen kann (Nygren et al. 2004), was die Möglichkeit eröffnete, dass die in den Transplantatorganen gefundenen Zellen aus diesem Grund das Empfängergenom präsentierten.

In weiteren Versuchen am experimentellen Herzinfarkt wurde bei Mobilisierung und intravenöser Applikation von Vorläuferzellen keine Effekte auf die Funktion und keine Regeneration gesehen (Deten et al. 2005). Insgesamt ist die potentielle Rolle der Vorläuferzellen als therapeutische Alternative nach einem Herzinfarkt sehr umstritten. Neben echter Regeneration durch Transdifferenzierung werden auch alternative Mechanismen der Funktionsverbesserung durch Vorläuferzellen diskutiert. Die Injektion von neonatalen (Muller-Ehmsen et al. 2002) und adulten (Roell et al. 2002) Herzmuskelzellen in die Infarknarbe führte bei Versuchstieren zu einer Stabilisierung der Narbe und damit zu einem funktionellen Gewinn, ohne dass jedoch diese Zellen als pumpende Herzmuskelzellen funktioniert hätten. Der parakrine Einfluss injizierter Zellen auf Apoptose und Bindegewebsumbau wird in diesem Zusammenhang ebenfalls diskutiert.

Experimente zur Untersuchung der Wirkung von Vorläufer- und Stammzellen auf den Herzinfarkt bieten eine Vielzahl an manipulierbaren Parametern, welche die Inhomogenität der wissenschaftlichen Befunde erklären könnten. So sind zum Beispiel die Fragen nach der zu verwendenden Zelllinie, der Applikationsart, dem Applikationszeitpunkt und möglichen Kofaktoren noch Gegenstand der Forschung auf der Suche nach reproduzierbarer Funktionsverbesserung durch Vorläuferzellen im Tierversuch. Auch die Inhomogenität der verwendeten Tiermodelle, der untersuchten Parameter und deren Interpretation tragen zur derzeit kontroversen Situation in diesem Wissenschaftsfeld bei.

### 1.1.5 Das Tiermodell

Zur Erforschung der Mechanismen des Herzversagens nach einem Herzinfarkt und zur Erkundung von Interventionsmöglichkeiten ist der Versuch am lebenden Tier ein wichtiges Instrument zum Erkenntnisgewinn. Das komplexe Zusammenspiel von mechanischen und molekularbiologischen Einflüssen, welches den Umbau des Herzens bedingt, erlaubt es bis heute nicht, diese Zusammenhänge durch tierversuchsfreie Verfahren zu erkunden. Die chirurgische Ligatur einer Herzkranzarterie gilt hierfür seit langem als aussagekräftiges Modell für den Herzinfarkt.

Um den therapeutischen Wert oder funktionellen Einfluss einer Intervention am Versuchstier zu untersuchen, sind Messmethoden notwendig, welche Rückschlüsse auf die Leistungsfähigkeit und den Gesamtstatus des Herzkreislaufsystems erlauben. Da die primären Ziele der Humanmedizin, Lebensqualität und Lebenszeitverlängerung, an Versuchstieren sehr schwer zu erfassen sind, ist es von Vorteil, das Tiermodell um Techniken zu ergänzen, welche ähnlich auch in der Humanmedizin eingesetzt werden. Vor allem Methoden und Messvariablen, welche im Menschen bereits als aussagekräftige Parameter für die kardiale Funktion und Prognose validiert wurden tragen viel zur wichtigen Übertragbarkeit der Versuchsergebnisse auf die Humanmedizin bei.

## 1.2 Echokardiografie am Rattenmodel

Echokardiografie ist die speziell auf die Untersuchung des bewegten Organs Herz angepasste Ultraschalluntersuchung. Dabei werden per Ultraschalltechnik aufgezeichnete Schnittbilder des bewegten Herzens als Serien von Einzelbildern aufgezeichnet.

Auch können in Rahmen der echokardiografischen Untersuchung Blutflussprofile aufgezeichnet und gemessen werden. Hierfür wird der Doppler-Effekt ausgenutzt.

Die schnelle Entwicklung der Computertechnik und die folgende stetige Verbesserung der Herzultraschallgeräte führte dazu, dass die für die Humanmedizin entwickelten Geräte auch eingesetzt werden können, um die Herzen kleiner Versuchstiere zu untersuchen. So wurde die prinzipielle Anwendbarkeit von Ultraschall zur Beurteilung der Herzgeometrie bereits 1990 an Ratten gezeigt (Desimone et al. 1990; Pawlush et al. 1993). Litwin et al. (1994) beschrieben zuerst Änderungen am Rattenherz nach Herzinfarkt im zeitlichen Verlauf mittels Echokardiografie.

Die Echokardiografie ist ein prinzipiell nichtinvasives Verfahren, welches am selben Versuchstier wiederholte Untersuchungen erlaubt und damit die Dokumentation der Herzfunktion im zeitlichen Verlauf ermöglicht.
Die weite Verbreitung der Echokardiografie in der Humanmedizin und die genaue Validierung der Aussagekraft einzelner Parameter am Menschen machen die Echokardiografie auch im Hinblick der Übertragbarkeit von tierexperimentellen Daten in Richtung klinischer Versuche und medizinischer Anwendung lukrativ.

### 1.2.1 Messparameter der Rattenechokardiografie

Viele der zahlreichen, aus der Herzultraschalluntersuchung gewinnbaren Messgrößen wurden bereits aus der Humanmedizin auf die Ratte übertragen.
Die am häufigsten gemessenen Größen sind die systolische und diastolische Größe der linken Herzkammer (Backlund et al. 2003; Yoon et al. 2005). Meist werden diese zur Berechnung der relativen Auswurffraktion herangezogen. Die im klinischen Alltag häufig genannte Ejektionsfraktion oder die relativen Auswurffraktionen, welche man aus Flächen- oder Längenmessungen berechnet, sind die derzeit in der Wissenschaft am häufigsten benutzten Parameter, zur Beurteilung therapeutischer Effekte auf das Herzversagen nach einem Herzinfarkt (Backlund et al. 2003; Yoon et al. 2005).
Die Messung der Wandstärke an verschiedenen Abschnitten des linken Ventrikels aus ein- oder zweidimensionalen Ultraschallbildern wurde benutzt um das Herzgewicht nichtinvasiv vorherzusagen (Desimone et al. 1990).
Auch die unter Ausnutzung des Doppler-Effektes gewinnbaren Flussprofile aus verschiedenen Abschnitten des Herzens sind an Ratten in einer Qualität erhältlich, welche das Messen und Berechnen einer Vielzahl von Parametern und Indices erlaubt.
So wurden die aus dem Dopplerflussprofil der transmitralen Ventrikelfüllung gewonnenen Indices für das diastolische Versagen an der Ratte beschrieben (Prunier et al. 2002) und mit den hämodynamischen Parametern der Vorlast korreliert (Slama et al. 2005). Als weiterer Doppler-echokardiografischer Index wurde der „Myocardial performance index“ (Tei 1995), welcher sich aus den zeitlichen Verhältnissen zwischen den Flusskurven von Füllungs- und Austreibungsphase berechnet und ein Maß für die globale kardiale Funktion darstellen soll, an Ratten gezeigt (Salemi et al. 2004). Auch die Bestimmung des Schlag- und Herzzeitvolumens aus dem systolischen Flussmuster über der Aortenklappe konnte

bereits an der Ratte evaluiert werden (Slama et al. 2003). In weiteren Arbeiten wurde die Möglichkeit den pulmonalarteriellen Druck abzuschätzen (Jones et al. 2002) und die Untersuchbarkeit des linken (Shimizu et al. 2005) und (Boissiere et al. 2005) rechten Ventrikels mittels Gewebedopplers an Ratten gezeigt. Der größte Teil dieser aus Dopplerflussmessungen gewonnenen Parameter hat wegen der schwierigen Interpretierbarkeit der gewonnenen Werte keine breite tierexperimentelle Anwendung gefunden.

Auch für die Verwendung in klinischen Studien an Patienten mit Herzversagen nach Herzinfarkt empfiehlt die American Heart Association (Gottdiener et al. 2004) ausschließlich die Größe des linken Ventrikels als wissenschaftliche Prüfgröße zur Erkundung des Verlaufs dieser Erkrankung.

### 1.2.2 Größenstimmung am linken Herz

Die Bedeutung der geometrischen Größe des linken Ventrikels als Parameter für Remodeling und Herzversagen ist seit langem bekannt (Pfeffer und Braunwald 1990). Auch in den Empfehlungen des American Heart Association zur Verwendung der Echokardiografie in der klinischen Wissenschaft wird der Größe des linken Ventrikels bei der Untersuchung der Herzinsuffizienz nach Herzinfarkt die größte Bedeutung beigemessen (Gottdiener et al. 2004).

Im Rahmen der Echokardiografie an Ratten werden verschiedene Techniken benutzt um die Größe des linken Herzens zu bestimmen. Die ersten Größenmessungen am Rattenherz wurden mittels M-Mode-Echokardiografie durchgeführt (Desimone et al. 1990; Litwin et al. 1994). Der wichtigste Grund hierfür war, die mangelnde Zugänglichkeit des Rattenherzens für hochauflösende zweidimensionale Schichtbilddarstellungen mit den Geräten dieser Zeit. Es wurde in den bei Ratten verhältnismäßig einfach darstellbaren parasternalen Schnittachsen ein MMode aufgezeichnet und daraus der endsystolische und der enddiastolische Querdurchmesser des Herzens gemessen.

Allen Verfahren, welche die Größe und Funktion des linken Ventrikels aus Messungen in der parasternalen langen Achse oder einem von parasternal aufgenommenen MMode dokumentieren, können allerdings nur Aussagen über die Verhältnisse der schallkopfnahen Vorderwand und der schallkopffernen Hinterwand machen. Die freie Wand und das Ventrikelseptum entgehen vollständig der Beobachtung. Aus diesem Grund wurde in den Leitlinien der American Heart Association auch festgestellt, dass alle Verfahren, welche die Ventrikelgröße aus

einem einzelnen Längsschnitt des Herzen bestimmen, ausschließlich für normal proportionierte und nor mal große Herzen (wörtlich: „normally shaped and sized ventricles“) geeignet sind. Für Untersuchungen an größenveränderten Herzen mit geänderter Geometrie, wie sie nach einem Herzinfarkt die Regel sind, wird ausschließlich die Scheibchensummationsmethode nach Simpson aus zwei orthogonal zueinander aufgenommenen Längsschnitten empfohlen (Gottdiener et al. 2004).

Die Zugänglichkeit der hierfür notwendigen apikalen Schnittebenen des Herzens ist bei Ratten stark eingeschränkt. Die Herzspitze liegt bei Ratten wesentlich weiter dorsal als beim Menschen. Auch kann mit modernen Echokardiografiegeräten das Endokard in diesen Schnittebenen nur in Ausnahmefällen abgegrenzt werden. Geräte mit einer lateralen Auflösung, welche am kleinen Rattenherz und t rotz der hohen Herzfrequenz das Endokard abgrenzbar darstellen können, sind zurzeit noch nicht verfügbar. Am Menschen kann die ebenfalls gelegentliche schwierige Endokardabgrenzung mittels lungengängigen Ultraschallkontrastmittels erfolgen (Cohen et al. 1998). Die biplane Volumenbestimmung aus kontrastverstärkten apikalen Schnittebenen ist in der humanen Echokardiografie das genauste Verfahren zur Ermittlung der linksventrikulären Größe (Thomson et al. 2001; Hoffmann et al. 2005). Zur genauen Beobachtung des kardialen Umbaus bei Ratten sollte diese Methode an der Ratte etabliert werden.

## 1.3 Fragestellung der Arbeit

Im Rahmen dieser Arbeit sollte untersucht werden, welchen Einfluss nach einem Herzinfarkt die direkte Injektion von mesenchymalen Vorläuferzellen in das Herzmuskelgewebe auf den Verlust von kardialer Funktion und den Herzumbau hat. Um diese Fragestellung untersuchen zu können, sollte das etablierte Model des experimentellen Herzinfarktes an der Ratte weiterentwickelt und um die neu zu entwickelnde Methode der Kontrastmittelechokardiografie an Ratten ergänzt werden.

# 2 Originalartikel

In der zuerst aufgeführten Arbeit wird beschrieben, wie die echokardiografischen Untersuchungen der Herzgeometrie, nach Etablierung der Methode in der Arbeitsgruppe, an der, am Rattenmodell neu eingeführten Methode der Kontrastechokardiografie evaluiert werden konnten.

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● *Original Contribution*

# CONTRAST ENHANCED ECHOCARDIOGRAPHIC FOLLOW-UP OF CARDIAC REMODELING AND FUNCTION AFTER MYOCARDIAL INFARCTION IN RATS

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**Abstract—Echocardiography is a reliable and commonly used method to examine cardiac diseases. Recent employment of modern technologies provides new opportunities to study left ventricular (LV) remodeling after myocardial infarction (MI) also in small rodents. LV volumes as most important prognostic parameters can be estimated by noncontrast enhanced echocardiography in rats from M-mode or single cross sections only. In this study, contrast enhanced echocardiography and volume measurements by the biplane method of discs (Simpson's rule) were applied in rats to monitor remodeling and function after MI. MI was induced in female Sprague-Dawley rats ($n = 26$ for MI, and $n = 16$ for sham). LV remodeling and heart function were serially studied by contrast enhanced echocardiography for 12 to 16 wk. At the end of the observation periods hemodynamic data were additionally measured by left and right heart catheterization. LV end systolic volume (LVESV) measured by biplane method of discs correlated best with LV developed pressure as indicator for severely impaired heart function. Interestingly, LV end systolic area (LVESA) from native short axis view correlated well with LVESV ($R^2 = 0.93$) and was the second best predictor for depressed heart function. Moreover, left atrial size was a powerful indicator of severely impaired heart function whereas ejection fraction or fractional area change were primarily related to infarct size. In conclusion, contrast enhanced echocardiography in rats is feasible and an economical method to study time-dependent LV remodeling and deterioration of contractile function after MI.** **(E-mail: Alexander.Deten@vetphys.unizh.ch)** 

*Key Words:* **Echocardiography, Contrast medium, Myocardial infarction, Animal study, LV remodeling, Heart function.**

## INTRODUCTION

Echocardiography is one of the most commonly performed noninvasive diagnostic tests in patients with known or suspected cardiovascular diseases, including myocardial infarction (MI) and heart failure. It provides comprehensive evaluation of cardiovascular structure and function that characterize disease processes as well as therapeutic efficacy of treatment (for an overview see the recommendations of the American Society of Echocardiography [Gottdiener et al. 2004; Lang et al. 2005] and the European Association of Echocardiography [Lang et al. 2006] and references therein).

Left ventricular (LV) remodeling after MI is a well-known pathologic process that results in progressive dilation and distortion of the LV. It is mainly determined by the size and location of the ischemic area. This process can be recognized by many echocardiographic changes, such as an increase in LV size and volume, altered LV geometry, diastolic dysfunction and other hemodynamic changes. It has been shown that LV volume is one of the best prognostic parameters after acute myocardial infarction (Otterstad et al. 2001). Furthermore, therapeutic strategies that minimize the extent of LV remodeling and improve prognosis are echocardiographically characterized by an attenuated increase in LV dimensions. Therefore, it is recommended that characterization of LV remodeling includes accurate sequen-

Video Clips cited in this article can be found online at: http://www.umbjournal.org.

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tial assessment of LV size and volume and diastolic filling patterns (Gottdiener et al. 2004; Lang et al. 2006).

Recent technological developments in echocardiography facilitate the analysis of ventricular remodeling and heart function in small laboratory animals but there are limitations when compared with human echocardiography. Mainly, LV dimensions were estimated either from one-dimensional M-Mode tracings (Sjaastad et al. 2000), area measurements in parasternal views (Coatney 2001) or volume calculations from single cross sections (Francis et al. 2001). These methods have been validated in humans but it is generally recognized that the accuracy of the prolate-ellipse, area-length and truncated ellipsoid methods is limited to normally shaped and sized ventricles, whereas the biplane method of discs is accurate in abnormally shaped ventricles (Gottdiener et al. 2004; Lang et al. 2006).

Delineation of endocardial borders in rats is conventionally not possible in apical views. Therefore, the estimation of LV size in rats had to be modified to use parasternal sections only. These mathematical models require geometric assumptions that are probably not applicable since in rats, shape and size of the LV are also profoundly altered after MI in a time-dependent manner (Pfeffer and Braunwald 1990). In this study, contrast enhanced echocardiography (Cohen et al. 1998) and volume measurements by the biplane method of discs (Simpson's rule) were applied in rats to study time-dependent LV remodeling and deterioration of contractile function after MI. Furthermore, it was investigated whether area measurements from native short axis views also adequately reflect LV remodeling.

## METHODS

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85 to 23, revised 1996) and was approved by the appropriate State agency of Saxony.

### *Myocardial Infarction*

Myocardial infarction was induced in female Sprague-Dawley rats (Charles River, Sulzfeld, Germany) by ligation of the left coronary artery under isofluran anesthesia inhaled over a nose mask (Deten et al. 2002). Briefly, the fourth intercostal space was opened, the heart was exteriorized and the pericardium was cut. The left coronary artery was ligated between the left auricle and the pulmonary outflow tract with a monofil thread (Ethicon USP 6/0, Ethicon GmbH, Norderstedt, Germany) while holding the apex of the heart with forceps. Thereafter, the chest was closed. The electrocardiogram was monitored until the rats recovered from anesthesia. In case of ventricular fibrillation, the rats were resuscitated by heart compression and electrical defibrillation. Sham operated rats underwent the same procedure except for the ligature.

### *Study Design*

In a pilot study, 10 rats underwent surgery (six MI, four sham). Echocardiography was performed on the day before surgery and sequentially over 16 wk. Additionally, all rats were examined 6 h after surgery to verify the success of the ligature. Successful induction of MI was assumed when the entire left ventricular free wall appeared akinetic. One rat was excluded because of induction of MI failed. One rat died 8 wk after MI.

In a second study, 32 rats underwent surgery (20 MI, 12 sham). Echocardiography was performed on the day before surgery and 2, 4, 8 and 12 wk after surgery. Two rats were excluded in the echocardiographic selection due to failure of ligation. No rat died.

At the end of the observation period, heart function of all rats was additionally measured invasively by left and right heart catheterization. Infarct size was measured in the MI rats of the pilot study and seven additional MI rats that were used to establish echocardiography. Infarct size was measured in three to six slices after fixation, slicing and photographing as previously described (Pfeffer et al. 1979).

### *Echocardiography*

Echocardiographic measurements were done with a commercially available ultrasound system (GE Vivid 7 equipped with an 11.5 MHz sector scan probe, GE Healthcare, Technologies Norway AS, Oslo, Norway). Examinations were performed in spontaneously breathing animals, under 2% isofluran anesthesia, in left lateral decubitus position. Parasternal short axis view was recorded at the largest round diameter of the left ventricle (Fig. 1, supplementary video 1). For recording of the contrast enhanced loops, a tail vein was punctured and the canula was connected to a catheter (inner diameter 0.5 mm). The contrast medium (Optison®, GE Healthcare Amersham Health, Buckinghamshire, United Kingdom) consisted of a mixture of albumin and gas filled microbubbles. It was slightly shaken and filled into the catheter directly before the examination to prevent unmixing (phase-separation). After adjusting the apical view and reducing the ultrasound beam power, the contrast medium was injected from the prefilled catheter. The volume for the entire examination did not exceed 500 $\mu$l.

Criteria for all apical views were to display the mitral valve opening and the LV in its longest cross

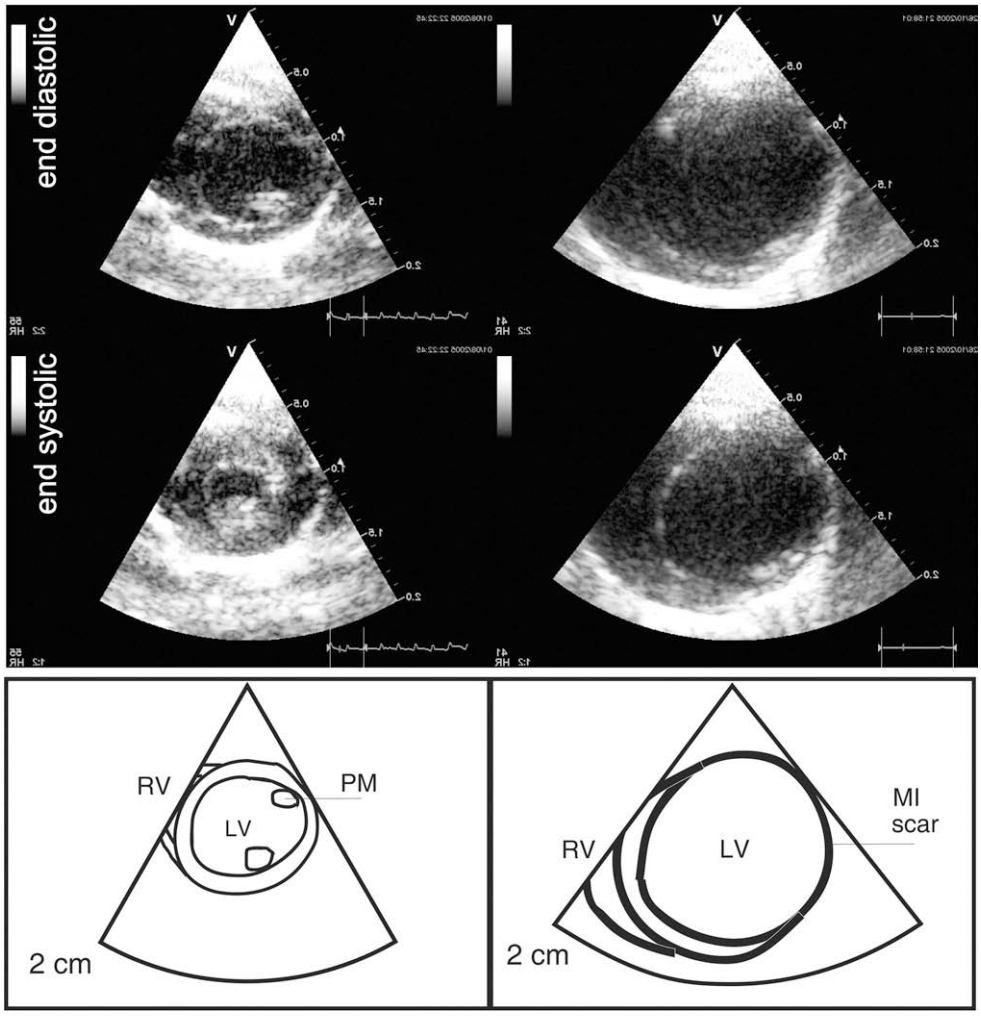


Fig. 1. Representative original recordings of native short axis views from a rat heart the day before (left) and 12 wk after (right) myocardial infarction.

section with minimal displacement effects on wall thickness (Fig. 2, supplementary video 2 and 3). Within these criteria, the four-chamber view was identified as the view with the largest cross-section of the right ventricle (RV). The two-chamber view was taken pragmatically orthogonal from the four-chamber view to calculate the most exact volume. Additionally, a standard record of established Doppler measurements and native recordings from four- and two-chamber view was taken.

Off-line analyses from the digital raw data were performed on a standard PC using ultrasound analysis software (EchoPac PC, GE Healthcare, Technologies Norway AS, Oslo, Norway). All measurements were done in three to five consecutive heartbeats. Left ventricular end systolic (LVESV) and end diastolic (LVEDV) volume were computed by the software's internal algorithm (Simpson, biplane) from the paired apical views. Ejection fraction (EF) was calculated by EF = (LVEDV-LVESV)/LVEDV. From short axis view, left ventricular end systolic (LVESA) and end diastolic (LVEDA) area were measured. Fractional area change (FAC) was calculated by FAC = (LVEDA-LVESA)/LVEDA. To determine the area of the left atrium (LA), the measured area from the native four-chamber view was added to the area from the two-chamber view. This best reflected the observed changes in LA size and was chosen since LA size could not be reliably measured according to Simpson's rule. The maximum early ($E_{max}$) and late ($A_{max}$) mitral inflow velocity, the velocity time integral of the outflow over the aortic valve ($VTI_{Ao}$) and the early diastolic velocity of the mitral annulus (e') were measured from Doppler records. Cardiac output (CO) was calculated from $VTI_{Ao}$ and heart rate (HR) measured from the same record by $CO = VTI_{Ao} \times HR \times$ [aortic valve opening area]. A constant aortic valve opening area of 0.045 $cm^2$ was assumed for all calculations, since it

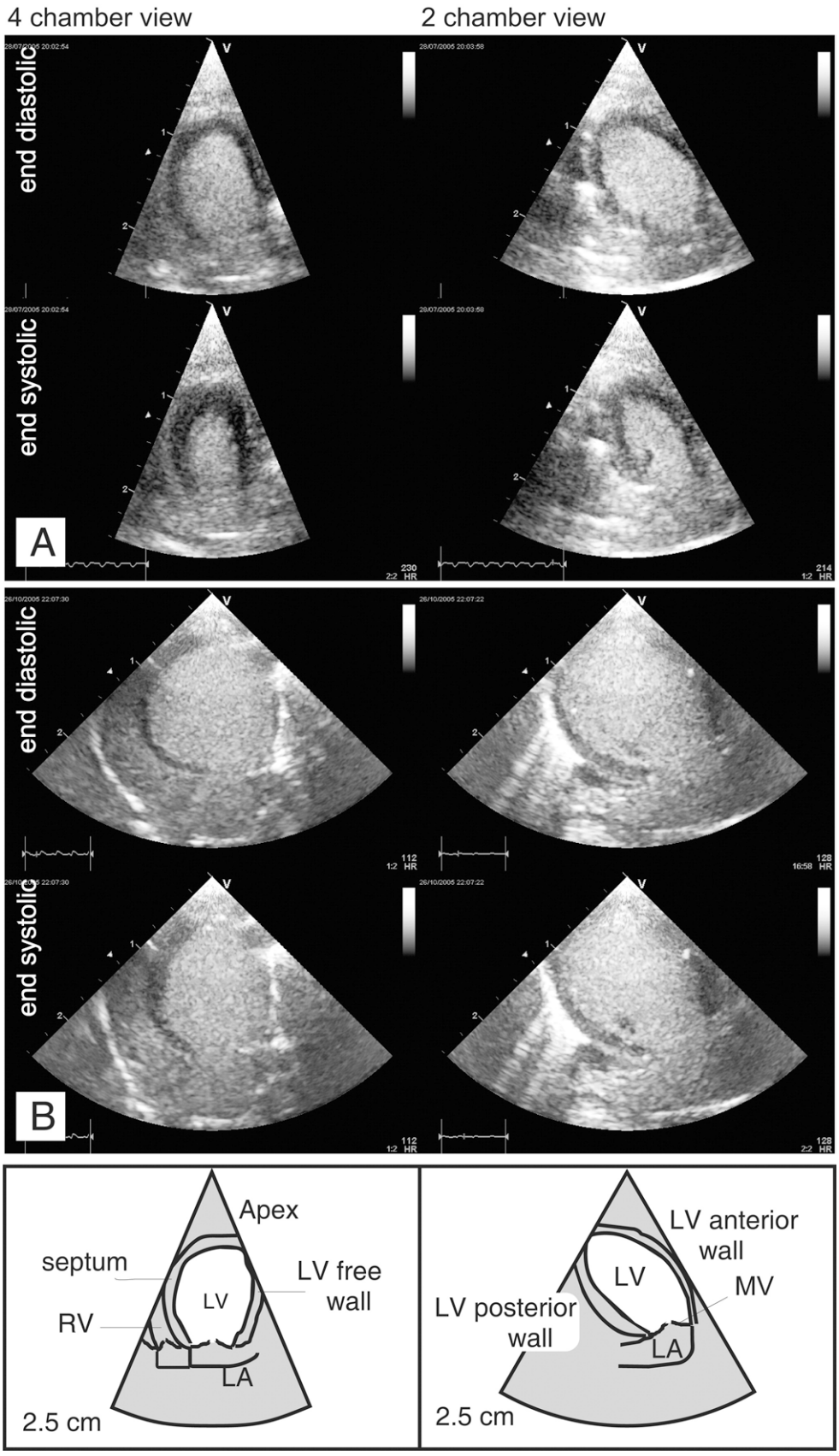


Fig. 2. Representative original recordings of contrast enhanced four- (left) and two-chamber views (right) from a rat heart the day before (A) and 12 wk after (B) myocardial infarction (same animal as in Fig. 1).

could not be measured with satisfying precision and reliability due to physical limitations.

*Hemodynamic Measurements*

At the end of the respective observation period, heart function was measured invasively in closed-chest spontaneously breathing rats anesthetized with thiopental sodium (Trapanal® 90 mg/kg IP, Byk Gulden, Konstanz, Germany) using ultraminiature catheter pressure-transducers (3 F, Millar Instruments Inc., Houston, TX, USA) (Deten et al. 2005; Zierhut and Zimmer 1989). Briefly, the right ventricular catheter (model SPR-291) was inserted into the right jugular vein and advanced into the RV *via* the right atrium. After collection of the RV data, the left ventricular catheter (model SPR-249) was placed in the right carotid artery and advanced upstream to the aorta and into the LV. Heart rate, RV and LV pressures and the rate in rise and fall of ventricular pressure (LV and RV d*P*/dt, respectively) were recorded continuously on a PC at a sampling rate of 2 kHz using DASYLab V7.00 software (National Instruments, München, Germany) for 10 to 15 min. After the hemodynamic data had been obtained, cardiac output was measured by the thermodilution method as described (Deten et al. 2005; Zierhut and Zimmer 1989).

*Statistics*

All data are shown as mean ± SD. The Mann-Whitney rank sum test was used to compare values in MI and sham-operated rats. A $p$ value $<0.05$ was considered statistically significant. Area and biplane values were compared by Pearson Product Moment linear correlation. The intraobserver variability was analyzed in three independent investigations in 16 rats as described by Bland and Altman (1986).

## RESULTS

Contrast enhanced echocardiography succeeded in 206 out of 215 examinations (95.8 %). Main reasons for failing were paravascular infusion or weak contrast. Short axis area measurements were always successful.

*Echocardiography*

Left ventricular dimensions increased over time after myocardial infarction (Fig. 3A and B). There was a pronounced increase of LV end diastolic dimensions within the first 2 wk after MI that continued further but less pronounced thereafter. Also the end systolic dimensions increased after MI. Since the pump function was severely impaired directly after coronary artery occlusion, end systolic enlargement occurred within the first day. Afterwards, end systolic dimensions increased in parallel to the end diastolic dimensions. Accordingly, the ejection fraction strongly decreased immediately after MI but declined only slightly thereafter over time (Fig. 3C).)

LV dilation was reflected by both, the volume as well as the area measurements. They were essentially comparable to each other (Fig. 3 and Fig. 4A). There was a very good correlation between LVEDV and LVEDA (LVEDV = 0.83 × LVEDA; $R^2 = 0.91$, $p < 0.001$) as well as between LVESV and LVESA (LVESV = 0.80 × LVESA; $R^2 = 0.93$, $p < 0.001$). Ejection fraction and fractional area change correlated also well (EF = FAC + 7; $R^2 = 0.90$, $p < 0.001$).

Also the size of the left atrium substantially increased within the first 2 wk after MI (Fig. 3D). Thereafter, the LA size essentially remained constant with only a slight increase at the end of the 16-wk pilot study.

The Doppler measurements of $E_{max}$, E/A and E/e' typically increased after MI with a maximum of about 1.27 m/s, 7.19 and 56, respectively, between 4 and 8 wk but declined thereafter. All values remained constant in the sham operated controls (mean 0.83 m/s, 1.25 and 23 for $E_{max}$, E/A and E/e', respectively). The $VTI_{Ao}$ and the derived cardiac output slightly declined over time after MI and were significantly reduced at the end of the observation period (Fig. 4 and Table 1).

All echocardiographic measurements were done and analyzed by the same investigator. The intraobserver variability of area size as well as of biplane volume measurements was analyzed according to Bland and Altman (1986). The bias (2SD) was 0.041 (0.061), 0.019 (0.026) and 3.9 (5.0) for LVEDV, LVESV and EF, respectively, and 0.045 (0.057), 0.031 (0.049) and 4.9 (7.3) for LVEDA, LVESA and FAC, respectively.

*Hemodynamics*

In accordance with previous studies (Pfeffer et al. 1979; Zimmer et al. 1990), the invasive hemodynamic measurements also showed severely impaired heart function after MI (Table 1). Importantly, LV developed pressure (LVDP) was dramatically reduced as well as LV end diastolic pressure (LVEDP) and RV systolic pressure (RVSP) increased. Additionally, the parameters for contractility and relaxation (LV $dP/dt_{max}$ and LV $dP/dt_{min}$, respectively) severely deteriorated compared with those in sham operated controls (Table 1).

Echocardiographic volume and area measurements were correlated to LVDP as established parameter for systolic function. There were very good linear correlations of LVDP with end systolic ($R^2 = 0.88$ and $R^2 = 0.81$ for LVESV and LVESA, respectively, all $p < 0.001$) as well as with end diastolic dimensions ($R^2 = 0.80$ and $R^2 = 0.73$ for LVEDV and LVEDA, respectively, all $p < 0.001$). In addition, the size of the

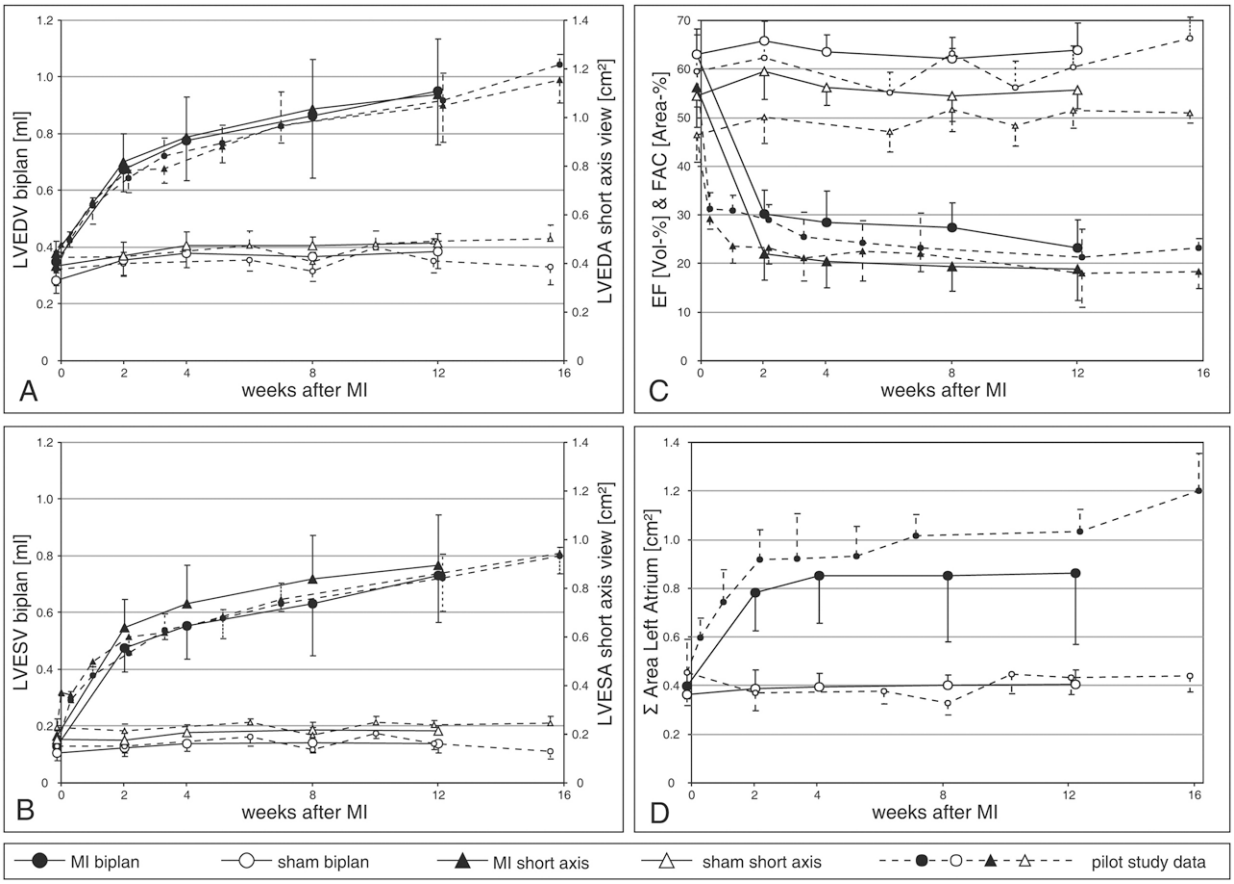


Fig. 3. Summary of echocardiographic measurements in the time course after myocardial infarction. In (A) and (B), biplane volume measurements (left scale) and short axis area measurements (right scale) are displayed for end diastole (A) and end systole (B). In (C), ejection fraction and fractional area change are shown on the same scale. In (D), left atrial size is shown. Data are mean ± SD.

left atrium and the LVEDP were correlated as parameters for diastolic function ($R^2 = 0.77$, $p < 0.001$). However, the size of left atrium also correlated well with LVDP ($R^2 = 0.88$, $p < 0.001$).

Since also the mitral Doppler measurements are recognized as predictive parameters for congestive heart failure, they were correlated with LVEDP. There were linear correlations with $E_{max}$ ($R^2 = 0.71$), E/A ($R^2 = 0.84$) and E/e' ($R^2 = 0.61$).

The infarct size measured 16 wk after MI (32%, ranging from 6% to 46%, $n = 11$) negatively correlated with the ejection fraction ($R^2 = 0.83$) and with fractional area change ($R^2 = 0.73$).

*Practical Aspects*

It took 20 min per rat to obtain a complete data set (including anesthesia). To acquire the recordings without contrast enhanced loops took about 10 to 15 min. One vial of Optison (3 mL) is sufficient for 15 to 20 examinations within about 2 wk, since the microbubbles are very sensitive to pressure changes and mechanical stress. Therefore, there is a loss in contrast intensity when using the same vial over days or if the vial is stored leaky or the cold chain is broken.

## DISCUSSION

Echocardiographic volume measurements from contrast enhanced apical cross sections represent a sensitive and noninvasive technique to study changes in ventricular remodeling and pump function over time after myocardial infarction in rats. By comparing these volume measurements with short axis area measurements, it could be shown for the first time in rats that the short axis area measurements also reflect well changes in geometry and function after MI.

In clinical echocardiography in patients, Simpson's method is routinely utilized to calculate the volume of the left ventricle from two orthogonal apical cross-sections. Importantly, this method involves

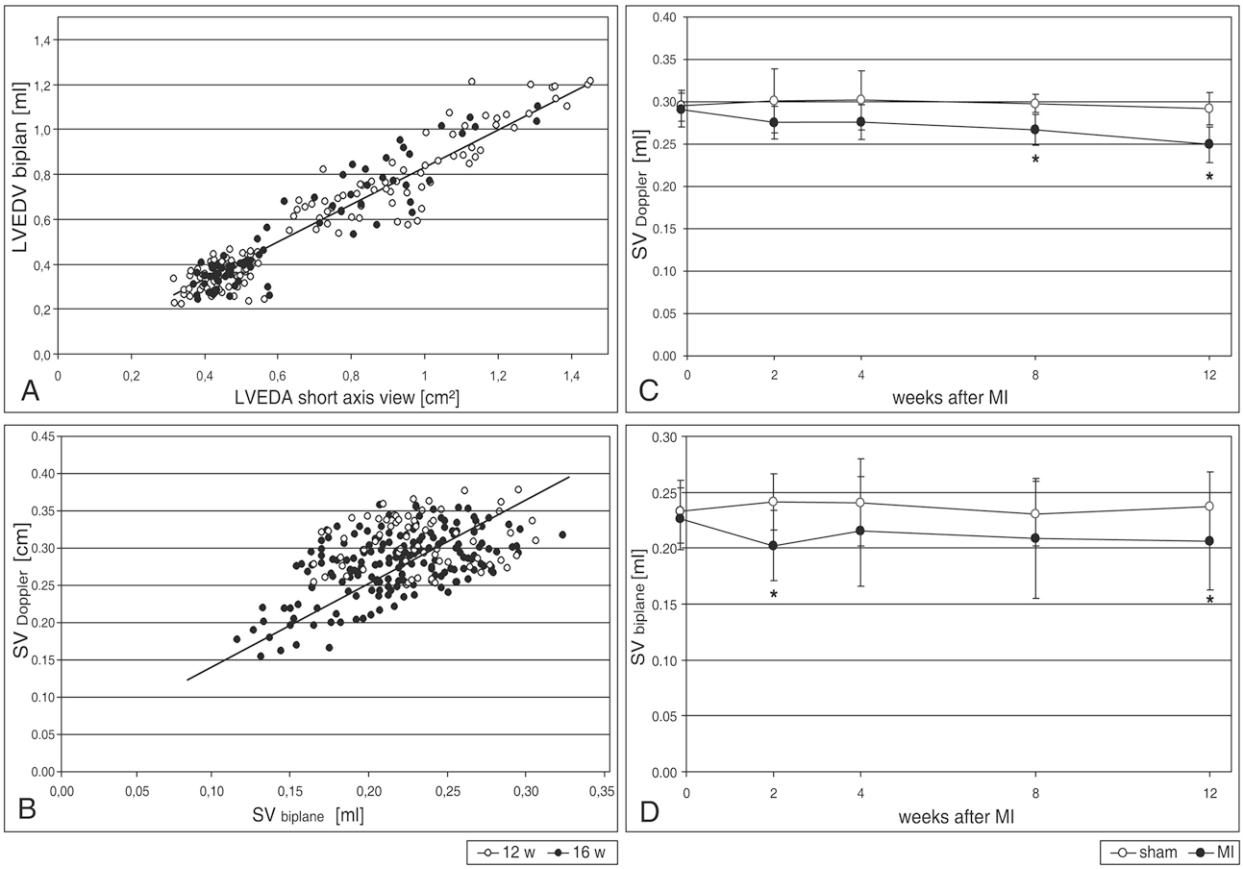


Fig. 4. Correlation analysis of LVEDV to LVEDA (A) and of stroke volume ($SV_{Doppler}$) determined by Doppler VTI to that measured by echo ($SV_{biplane}$) (B). Summary of $SV_{Doppler}$ (C) and $SV_{biplane}$ (D) in the time course after myocardial infarction. Data are mean ± SD. *$p < 0.05$ vs. sham-operated control.

only minimal assumptions regarding geometric proportions. Nonetheless, a clear endocardial delineation is required. Due to the small heart size, the high heart rate, as well as technical limitations, this is not possible in native apical cross-sections of rat hearts. The parasternal short axis view, on the other hand, allows a clear delineation of the LV cavity but does not allow volume measurements without geometrical assumptions. For this reason, the most reliable parameters from the short axis views are the end systolic and end diastolic areas. In this study, contrast enhanced echocardiography in the rat was applied. The contrast enhancement enabled cavity delineation in the apical views for volume estimations according to Simpson's method.

Left ventricular end systolic size has been shown to be the best independent parameter to predict development of congestive heart failure and clinical outcome after MI in human studies (White et al. 1987; Udelson et al. 2003). In the present study, LV size was correlated with the LV-developed pressure as parameter of depressed heart function. As in humans, LV end systolic volume turned out to be the best echocardiographic parameter for predicting severely impaired heart function ($R^2 = 0.88$). This further emphasizes the benefits of echocardiography to analyze noninvasively LV remodeling and deterioration of heart function as well as to predict the development of heart failure.

Reliable biplane volume measurements from the apical views require contrast enhancement for cavity delineation. Therefore, these volume calculations were compared with short axis area measurements, which do not require contrast enhancement and, thus, are easier to acquire. Interestingly, there was a good correlation between LV end systolic area and LVDP ($R^2 = 0.81$). This indicates that short axis area measurements are sufficient to estimate the global LV size, since they represent the entire LV circumference. Nevertheless, acquiring data from both, the volume and the short axis area measurements may considerably strengthen the data of echocardiographic characterization. The prognostic weight of different LV size measurements was also studied in humans (Otterstad et al. 2001), since the endocardial delineation in long axis

Table 1. Hemodynamic and echocardiographic measurements at the end of the observation period

| | 12-wk study | | 16-wk pilot study | |
|---|---|---|---|---|
| Hemodynamics | sham (n = 12) | MI (n = 18) | sham (n = 4) | MI (n = 4) |
| HR [bpm] | 377 ± 36 | 318 ± 36 | 349 ± 32 | 299 ± 36 |
| LVSP [mm Hg] | 167 ± 9 | 120 ± 24 | 155 ± 6 | 97 ± 10 |
| LVEDP [mm Hg] | 3 ± 1 | 16 ± 10 | 4 ± 1 | 21 ± 3 |
| LVDP [mm Hg] | 164 ± 9 | 103 ± 30 | 152 ± 8 | 77 ± 11 |
| LV dP/dt $_{max}$ [mm Hg/s] | 10014 ± 1024 | 5953 ± 1463 | 9944 ± 662 | 4469 ± 958 |
| LV dP/dt $_{min}$ [mm Hg/s] | -9336 ± 1106 | -3697 ± 1070 | -8414 ± 654 | -2804 ± 470 |
| LV $\tau_{(rel)}$ | 6.5 ± 0.2 | 10.1 ± 1.5 | 6.5 ± 0.5 | 9.2 ± 1.9 |
| RVSP [mm Hg] | 29 ± 2 | 50 ± 19 | 28 ± 2 | 48 ± 6 |
| CO [ml/min] | 89 ± 15 | 73 ± 14 | 92 ± 13 | 81 ± 11 |
| Echocardiography | | | | |
| HR [bpm] | 352 ± 40 | 320 ± 33 | 358 ± 29 | 321 ± 23 |
| LVESV [ml] | 0.14 ± 0.03 | 0.73 ± 0.17 | 0.11 ± 0.03 | 0.80 ± 0.03 |
| LVEDV [ml] | 0.38 ± 0.06 | 0.95 ± 0.19 | 0.33 ± 0.06 | 1.04 ± 0.04 |
| EF [Vol-%] | 63.9 ± 5.6 | 23.3 ± 5.7 | 66.4 ± 4.3 | 23.3 ± 2.0 |
| LVESA [cm$^2$] | 0.21 ± 0.02 | 0.89 ± 0.21 | 0.25 ± 0.03 | 0.94 ± 0.08 |
| LVEDA [cm$^2$] | 0.49 ± 0.04 | 1.10 ± 0.23 | 0.50 ± 0.06 | 1.15 ± 0.10 |
| FAC [Area-%] | 55.7 ± 3.8 | 18.8 ± 6.4 | 51.0 ± 2.1 | 18.3 ± 3.4 |
| CO $_{biplane}$ [ml/min] | 85 ± 11 | 67 ± 15 | 79 ± 11 | 72 ± 15 |
| CO $_{Doppler}$ [ml/min] | 87 ± 17 | 70 ± 17 | 93 ± 11 | 79 ± 15 |
| $E_{max}$ [m/s] | 0.80 ± 0.11 | 1.12 ± 0.22 | 0.76 ± 0.03 | 1.35 ± 0.10 |
| E/A | 1.2 ± 0.2 | 5.5 ± 4.0 | 1.2 ± 0.2 | 9.6 ± 2.0 |
| E/e' | 20 ± 6 | 38 ± 15 | 17 ± 2 | 44 ± 3 |
| MI size [%] from necropsy | | n.m. | | 32 ± 8 (ranging 6 – 46; n = 11) |

HR = heart rate; LVSP = left ventricular systolic pressure; LVEDP = left ventricular end diastolic pressure; LVDP = left ventricular developed pressure; LVdP/dt $_{max(min)}$ = maximal rate of rise (fall) in left ventricular pressure; LV$\tau_{(rel)}$ = time constant of early isovolumetric relaxation normalized to cardiac cycle length; RVSP = right ventricular systolic pressure; LVES(D)V(A) = left ventricular end-systolic (diastolic) volume (area); EF = ejection fraction; FAC = fractional area change; CO = cardiac output; $E_{max}$ = maximum velocity of early mitral inflow; E/A = ratio of $E_{max}$ and maximal late mitral inflow velocity; E/e' = ratio of $E_{max}$ and maximal early diastolic velocity of mitral ring tissue. Values are mean ± SD. $p < 0.05$ for all parameters after MI vs. corresponding sham-operated controls except for CO $_{biplane}$ in the 16-wk pilot study.

views is possible in about 60% to 70% of the patients only. In that study, the prognosis of patients after MI correlated best with biplane measured LVESV and second best with LVESA. Thus, LVESA should be measured in case that acceptable apical views are not accessible.

There were also good predictions for LVDP by the end diastolic LV size ($R^2 = 0.80$ for LVEDV and $R^2 = 0.73$ for LVEDA). Interestingly, the FAC as an often used parameter to describe global heart function in rats showed only a moderate correlation to LVDP ($R^2 = 0.61$ for FAC but $R^2 = 0.79$ for EF). Moreover, both EF and FAC changed only slightly over time after a severe reduction immediately after coronary artery occlusion (Fig. 3C). On the other hand, there was a good correlation between EF and MI size ($R^2 = 0.83$ for EF and $R^2 = 0.73$ for FAC). As a consequence, EF or FAC are informative early markers of impaired heart function but are not so useful to characterize the deterioration of heart function and remodeling after MI as well as to predict the occurrence of later heart failure. In human studies, it was also shown that EF and LVEDV do not improve the prognostic value if used in addition to LVESV (White et al. 1987).

The left atrial size has been established as a reliable independent prognostic parameter for congestive heart failure in human echocardiography (Modena et al. 1997). As demonstrated in humans (Lester et al. 1999), it is necessary to measure left atrial size in apical views to obtain reliable measurements. In contrast to humans, the geometry of the atrium differs especially in young rats. This did not allow to determinate a longitudinal axis and, thus, left atrial volume was not accessible by Simpson's method or area length calculation. For this reason, the LA size was given as the sum of left atrial areas measured in both, four- and two-chamber view. The good correlation of the LA size with LVEDP ($R^2 = 0.77$) in this study qualifies this measurement also in the rat as a good long-term predictor for diastolic failure. Furthermore, different measurements and indices from mitral valve flow have been demonstrated in humans (Sohn et al. 1997) and even in rats as good parameters for observing diastolic dysfunction (Bjornerheim et al. 2001; Slama et al. 2005). The correlations to LVEDP in this study

well reproduced the data reported by Slama et al. (2005) The interpretation of these Doppler measurements, however, is problematic because they all show a maximum at 4 to 8 wk and decrease thereafter. Moreover, all these mitral flow indices did not reach the correlation quality of left atrial size.

Previous studies in humans (Cohen et al. 1998) and also in mice (Denvir et al. 2005) report higher values in geometric measurements when contrast medium was used. This is likely caused by inclusion of the contrast medium within the endocardial trabecular network, which gives the appearance of an enlarged cavity compared with native records. This effect was not analyzed systematically in this study but in a few rats short axis view was also recorded after injection of contrast medium. The observations from these recordings agree with the literature. The difference in endocardial positioning between the two compared methods may explain the stable bias in the correlation of EF and FAC ($EF = FAC + 7$, $R^2 = 0.90$) in this study.

In previous studies, left ventricular volumes were also estimated from single long axis views or M-mode measurements (Coatney 2001; Francis et al. 2001; Sjaastad et al. 2000). These measurements were not considered in this study, since they do not include the full left ventricular circumference. Especially in inhomogeneous cardiac diseases such as after myocardial infarction, techniques that do not include the full circumference of left ventricle result in inaccurate data. Most importantly, movement and geometry of the hyperkinetic septal wall are not taken into account at all, because in rats the long axis view depicts the anterior and posterior LV wall only. The same applies for the M-mode in the short axis view. Interestingly, there was a better correlation of FAC than of FS to MI size ($R^2 = 0.71$ versus 0.26, respectively) in an acute model of myocardial infarction in mice as reported previously (Suehiro et al. 2001). Moreover, M-mode echocardiography does not offer any advantage, because the ultrasound machines today provide sufficient frame rates and longitudinal resolution for high quality two-dimensional records.

Contrast enhanced echocardiography in humans shows a comparable reproducibility as cardiac MRI (Malm et al. 2004; Hoffmann et al. 2005). There is, however, no gold standard to compare both methods. In rats, cardiac MRI has a number of limitations. Mainly, the equipment for cardiac MRI in rodents is very expensive and, therefore, the accessibility of the method is limited. The additionally high personnel expenditure and maintenance costs do not allow the use of cardiac MRI in large-scale studies without high consumption of economical recourses. Since, especially after MI, there is a large variability in heart function between the individual rats, a reasonably high number of animals is a prerequisite for powerful statistics and reliable results of a scientific study. Secondly, cardiac MRI requires a deep and long-lasting anesthesia that may profoundly influence heart function. For both reasons, echocardiography seems to be the method of choice to routinely study mechanisms and therapeutic interventions in cardiac diseases since it allows a high throughput at reasonable costs.

It was previously described that the ultrasound contrast medium possess the potency to induce tissue microlesions and capillary rupture (Miller and Quddus 2000; Miller et al. 2005). This property is used in animal research to facilitate gene transfer for gene therapeutic issues (Taniyama et al. 2002). None of the described effects on the heart tissue was found in this study. The main reason for this is, most likely, the used ultrasound power. In this study, the ultrasound power was reduced to about −8 dB to obtain the best contrast enhanced pictures. The resulting tissue index was about 0.1. In all studies where microlesions occurred, the ultrasound power used was higher than 0 dB (tissue index not reported). Importantly, it has been shown that there is no influence of diagnostic contrast echocardiography on the cardiac gene expression (Bekeredjian et al. 2004).

*Limitations of the Study*

There are several limitations of this study that should be addressed. First, the echocardiographic volume data were not compared with values obtained by a complementary method. MRI as presumed gold standard was not available to us. However, a comparison between $SV_{biplane}$ and $SV_{Doppler}$ as two independent echocardiographic volume estimations was done (Fig. 4B). This correlation analysis revealed a rather moderate correlation ($R^2 = 0.34$, $p < 0.001$). There may be several explanations. $SV_{biplane}$ is a calculated parameter on which even minor deviations of LVEDV and LVESV might have major impact. $SV_{Doppler}$, on the other hand, is calculated from VTI and the opening area of the aortic orifice. While the error of VTI measurements can be minimized by the use of sector scan probe, the opening area of the aortic orifice could in our hands not be measured with satisfying precision and reliability. Therefore, the moderate correlation between SV measured by echo to that determined by VTI might indicate that a constant opening area of the aortic orifice as assumed in our study does not accurately reflect the pathophysiological situation. However, both estimates decreased after MI. This decrease was slight but statistically significant indicating that SV is not the parameter of choice to study LV remodeling and function after MI in rats.

Second, biplane volume estimations according to Sipmson's rule minimize geometric assumptions but

still rely on only two planes that have to be properly oriented. Moreover, apical foreshortening of the LV cavity is a common source of underestimation of LV end-diastolic and end-systolic volumes. This problem was minimized by the use of contrast agent but can not completely be ruled out.

## CONCLUSION

Contrast enhanced echocardiography in rats is feasible and an economical method to study LV remodeling and deterioration of heart function after MI. In particular, the left ventricular end systolic volume is the most relevant parameter to characterize remodeling and predict heart failure after myocardial infarction. Also, area measurements from the short axis view may be applied in interchange when contrast medium is not used or in addition to volume measurements.

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## APPENDIX

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ultrasmedbio.2007.04.016.

Video Clips cited in this article can be found online at: http://www.umbjournal.org.

Nach Abschluss der methodischen Forschung wurden die echokardiografischen Methoden gemeinsam mit den in der Arbeitsgruppe bereits etablierten Techniken zur Phänotypisierung und histopatholgischen und molekularbiologischen Charakterisierung von Versuchstieren benutzt, um den Einfluss von mesenchymalen Vorläuferzellen auf den Herzumbau und die Funktion nach einem Herzinfarkt am Rattenmodell zu untersuchen.

**Original Paper**

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# Cord Blood Cell Therapy Alters LV Remodeling and Cytokine Expression but does not Improve Heart Function after Myocardial Infarction in Rats

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**Key Words**

Myocardial infarction • Heart function, remodeling • Cell therapy, animal model

**Abstract**

Objective: In this study the ability of unrestricted somatic stem cells (USSC) and mononuclear cord blood cells (MN-CBC) was tested to improve heart function and left ventricular (LV) remodeling after myocardial infarction (MI). Methods: The cells were delivered by i.v. or intramyocardial injections in rat models of MI by permanent coronary artery occlusion and by ischemia/reperfusion (I/R) injury. Heart function and remodeling was followed by recurrent echocardiography over 8 or 12 weeks after which catheterization was performed. Results: Although injected labeled cells could be observed within the myocardium for up to 6 d, there was no sign of cardiac regeneration 8 or 12 weeks after MI. However, the mRNA expression of components of the extracellular matrix was attenuated in the infarct scar 12 weeks after MI and cell injection. Additionally, the expression of interleukin (IL)-6 but not of IL-1$\beta$ increased at the site of injury and the adjacent border-zone 12 weeks after I/R and USSC-injection. However, these effects did not translate into improved heart function or attenuated LV dilatation. Conclusion: These data indicate that cord blood cell implantation after MI acts through paracrine mechanisms to modify remodeling rather than myocyte regeneration. The role of myofibroblasts and the optimal conditions of cell application need to be determined to translate these mechanisms into functional improvement.



## Introduction

After acute myocardial infarction (MI) had occurred, the basic pathology is characterized by irreversible and massive loss of the cardiomyocytes, which is eventually replaced by fibrous non-contractile cells to form scar tissues. Although the myocytes in the surviving myocar-

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dium undergo hypertrophy this is often not sufficient to preserve heart function. As a consequence, congestive heart failure develops even though the recent advances in the therapeutic approaches, including pharmacological and interventional therapies, and cardiovascular surgery provide some improvement. As a newly developed strategy, cellular cardiomyoplasty, which involves the implantation of healthy cells into the damaged myocardium, offers the promise to replace the lost cells.

Transplantation of cells, including cardiomyocytes [1, 2], skeletal myoblasts [3], bone marrow cells [4, 5], smooth muscle cells [6], and embryonic stem cells [7] has been reported to be of potential therapeutic value for the treatment of damaged myocardium in animal models. These transplanted cells may replace infarcted myocardium and increase the number of functional cardiomyocytes, limit the scar expansion, and reduce post-infarction heart failure [8]. Also, transplanted cells may contribute to the revascularization process and enhance myocardial angiogenesis [8]. They also may serve as a platform for therapeutic gene transfer to the myocardium [9]. The beneficial effect of cell transplantation on cardiac functions in preclinical studies has led to several clinical trials [10].

Mesenchymal stem cells (MSCs) isolated from adult bone marrow have shown a great potential for cell therapy because these cells possess pluripotent capabilities [11, 12], proliferate rapidly, can ´self-renew´, induce angiogenesis, and differentiate into myogenic cells [13-15]. MSCs can be isolated easily from a variety of sources, have genetic stability, and carry less immunological or ethical concerns. Hence, they were considered very suitable candidate donor cells for stem cell therapy and target cells for gene transfer [16, 17]. Recently, a pluripotent stem cell population with high proliferative potential, unrestricted somatic stem cell (USSC), was isolated from the endothelium/subendothelium layer of the human umbilical cord blood [18] that is morphologically and immunophenotypically similar to those MSCs isolated from bone marrow [19, 20]. Hence, USSCs have been suggested to be an earlier cell type than multipotent MSCs, possibly representing the precursor cells for MSCs as a comparison of the differentiation potentials of USSCs [18] and MSCs [21] has shown. In vitro and in vivo studies demonstrated that the USSCs have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, neurons, and myocytes [18]. Moreover, in a sheep model, the application of USSCs did not induce detectable tumors in a long-term study after transplantation [18]. Therefore, USSCs could be highly promising precursor cells for cardiac implantation after a myocardial infarction.

This study was performed to test the ability of USSCs to improve heart function and left ventricular (LV) remodeling after MI. The cells were delivered by intramyocardial injections in rat models of MI by permanent coronary artery occlusion and by ischemia/reperfusion (I/R) injury. Additionally, mononuclear cord blood (MN-CBCs) cells without further separation were tested and the effects compared.

## Materials and Methods

*Animal model*

Myocardial infarction was induced in male (3.5 months of age and 291±4 g of body weight at the beginning of the study) spontaneously hypertensive rats (SHR, Charles River) and female (3 month of age and 242±14 g of body weight at the beginning of the study) Sprague-Dawley rats (SD, Charles River) by ligation of the left anterior descending coronary artery (LAD) as previously described [22]. For the I/R experiments which were done only in the SD rats, a small ring (cord diameter 1 mm) was placed under the ligature. After 60 min of occlusion, the rats were again anesthetized, the chest was opened and both, the ring and the ligature were removed. Successful reperfusion was verified by Evans blue infusion in pilot experiments (not shown), but became also momentarily overt by the color change of the previously ischemic myocardium from pearl grey to pink.

After 24 h the surviving SHR (n=46) were randomly selected to receive either cells (MI+MN-CBC, $3x10^6$ cells in 500 µl medium, n=23) or medium (MI-CTRL, n=23) via a tail vein. The surviving SD rats (n=47) were also randomly selected after 24 h for direct intramyocardial injection of either cells (each $1x10^6$ cells, n=14 for MN-CBCs and n=15 for USSCs) or medium (n=18). In the I/R experiments, the rats were randomly selected to either receive cells ($1x10^6$ USSCs, n=9) or medium (n=9) by direct intramyocardial injection at the time of reperfusion (Tab. 1). The intramyocardial injections were performed at two sites (10 µl each) at the mid-anterior and mid-lateral left ventricular wall using a precision syringe (22G, Hamilton), the needle was equipped with additional tubing as retardant. All rats in the I/R sub-study received additional immune-suppressive therapy with Cyclosporine (Sandimmun Neoral®, Novartis, Germany, 15 mg/kg/d) beginning the day before surgery.

Twelve additional SHR were used to track injected CFSE-labeled cells after 6 and 24 h (n=4 for MN-CBCs and n=2 for medium at each time-point). Also 16 additional SD rats were analyzed for injected labeled cells immediately (MI, n=4), 1 d (MI, n=3) and 6 d (each n=3 for both, MI and I/R) after injection. Medium-treated MI hearts at 1 d after injection served as controls (n=3).

Sham-operated animals (n=24 for SHR and n=18 for SD) underwent the same procedure except that no ligation was performed. Also the groups of sham operated animals were divided into sub-groups to receive either cells or medium. These groups were combined for subsequent comparison with MI

**Table 1.** Study design and animal numbers. Total numbers of animals and in each group; in brackets: survived the first day after surgery; in parenthesis: survived observation period; MI: myocardial infarction; I/R ischemia/reperfusion; CTRL: injected with medium; MN-CBCs: injected with human mononuclear cord blood cells; USSCs: injected with human unrestricted somatic stem cells.

| | SHR (84) | SD (104) | |
|---|---|---|---|
| | MI (8 w) | MI (12 w ) | I/R (12 w) |
| sham | 24 | 18 | |
| all MI (I/R) [at day 1] | 60 [46] | 68 [47] | 18 [18] |
| - CTRL | 23 (14) | 18 (18) | 9 (9) |
| + MN-CBCs | 23 (13) | 14 (11) | |
| + USSCs | | 15 (14) | 9 (9) |

(I/R) rats, since there were no statistically significant differences between the differently treated sham groups. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the appropriate State agency of Saxony.

*Isolation of human cord blood cells*

Human cord blood was collected from umbilical cord vein of full-term pregnancies, after informed consent. The investigation conforms with the principles outlined in the Declaration of Helsinki. Mononuclear cord blood cells (MN-CBCs) were isolated by Ficoll-Hypaque density gradient separation followed by erythrocyte lysing in distilled water. MN-CBCs were cryopreserved in 8% dimethyl sulphoxide (DMSO) and 92% fetal calf serum (FCS) at –196°C (liquid nitrogen). After thawing by stepwise dilution in RPMI 1640 (PAA Laboratories) and washing by DNase-containing buffer (DNase I, Roche), the cells were analyzed by flow cytometry and used for transplantation. To isolate unrestricted somatic stem cells (USSCs), the mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient separation and plated at 4-6 $x10^6$ cells/ml and incubated at 1% O2 in cell culture flasks (Greiner Bioscience). Medium contained 70 % low glucose (5mM) DMEM (Sigma), 30 % FCS (Pan Biotech) and was changed every 7 days until formation of colonies of adherent cells was observed as previously described [18]. Cells were harvested after reaching confluency and further expanded with a lower concentration of Dexamethason at a density of $4x10^4$ cells/cm2. Only USSCs from passage 5-7 were used for experiments.

*Flow Cytometry*

MN-CBCs were analyzed for the cell surface antigens CD45, CD34, CD133 and the homing receptor CXCR4 compared to isotype controls in order to estimate the relative number of stem cell/progenitor population. CD45-PC7 and CD34-APC antibodies were purchased from Beckman Coulter (Immunotech) while the CD133-PE antibody was obtained from Miltenyi Biotech. The CXCR4 antibody was purchased from R&D Systems and the secondary goat-anti-mouse-FITC antibody from DAKO. Cells were incubated with antibodies 30 minutes at 4°C and washed twice. After fixation in 3% formaldehyde the cells were analyzed using FACSCalibur and CellquestPro software (Becton Dickinson) according to the manufactures protocol. The gating strategy for the hematopoietic stem cells was according to the ISHAGE guidelines [23].

USSCs were analyzed for the cell surface antigens CD13, CD34, CD90 (all Becton Dickinson), and CD105 (Serotec) compared to the isotype control. Cells were incubated with antibodies for 40 minutes at 4 °C and washed once with PBS. The cells were resuspended in PBS and immediately analyzed using FACSCalibur and CellquestPro software (Becton Dickinson).

*Cell labeling*

For a simple and rapid recovery the cells were labeled with 5 µM 5,6-Carboxyfluoresceindiacetat-succinimidylester (CFSE, Molecular Probes). The cells were incubated in RPMI with 1% CFSE for 10 minutes at 37°C and then washed twice with RPMI and 10% FCS. Cells were resuspended in RPMI 1640 / 1% FCS, analyzed by flow cytometry and used for transplantation.

*Echocardiography*

Echocardiographic measurements were performed with a commercially available ultrasound system (GE Vivid 7 equipped with an 11.5 MHz sector scan probe, GE Healthcare). Examinations were performed in spontaneously breathing animals, under 1.5 % isofluran anesthesia, in left lateral decubitus position. Parasternal short axis view was recorded at the largest round diameter of the left ventricle. For recording of the contrast enhanced loops, a tail vein was punctured, and contrast medium (Optison®, GE Healthcare) was injected after adjusting the apical view and reducing the ultrasound beam power. Criteria for all apical views were to display the mitral valve opening and the LV in its longest cross section with minimal displacement effects on wall thickness. Within these criteria the four chamber view was identified as the view with the largest cross-section of the right ventricle (RV). The two chamber view was taken pragmatically orthogonal from the four chamber view. Additionally, a standard record of established Doppler measurements was taken.

Offline analyses from the digital raw data were performed on a PC using ultrasound analysis software (EchoPac PC, GE Healthcare). All measurements were done in 3 to 5 consecutive heartbeats. Left ventricular end systolic (LVESV) and end diastolic (LVEDV) volume were measured by the software's internal algorithm (SIMPSON, biplane) from the paired apical views. Ejection fraction (EF) was calculated by EF=(LVEDV-LVESV)/LVEDV. From short axis view, left ventricular end systolic (LVESA) and end diastolic (LVEDA) area were measured. Fractional area change (FAC) was calculated by FAC=(LVEDA-LVESA)/LVEDA.

*Hemodynamic measurements*

At the end of the observation periods, left and right heart function was measured in closed-chest spontaneously breathing rats anesthetized with thiopental sodium (Trapanal® 80

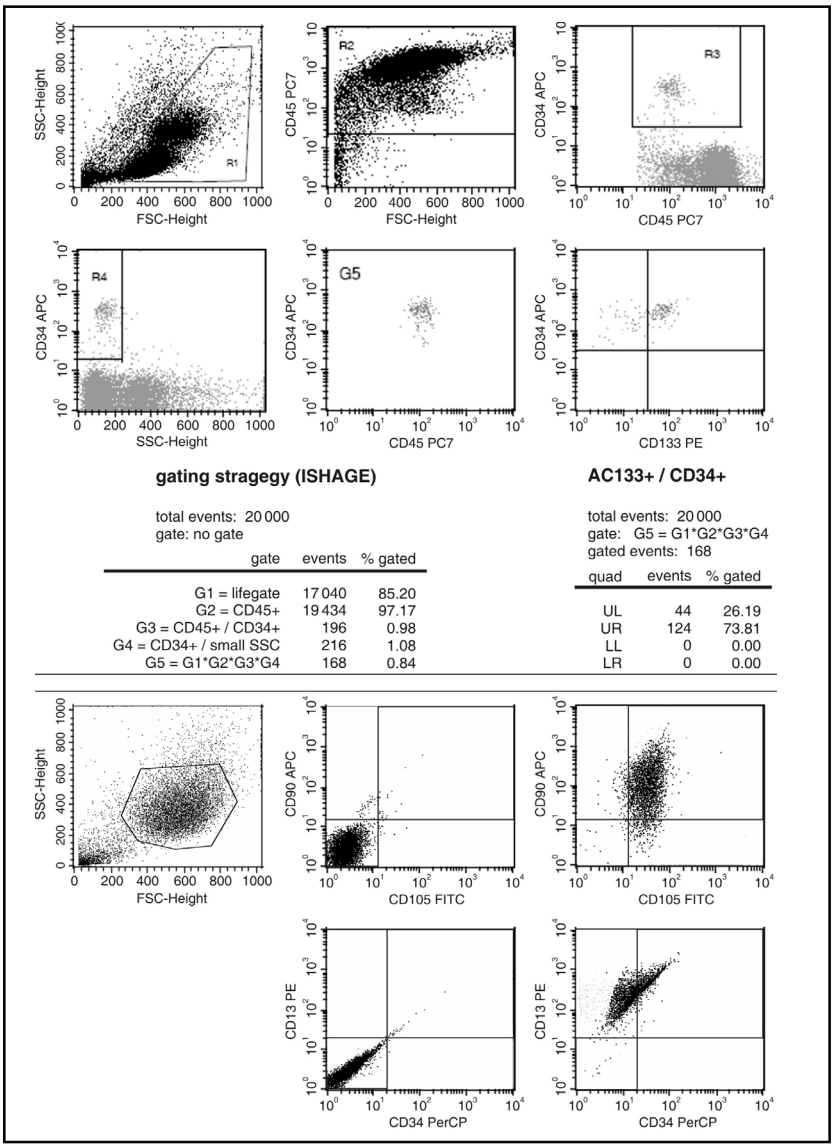


**Fig. 1.** FACS analysis of MN-CBCs for CD45, CD34, and CD133 (upper panels) and of USSCs for CD90, CD105, CD13, and CD34 (lower panels with isotype controls in the middle column) isolated from human cord blood. The gating strategy was according to the ISHAGE guidelines.

mg/kg i.p., Byk Gulden) using ultraminiature catheter pressure-transducers (3F, Millar Instruments Inc.) [22]. Cardiac output was measured by the thermodilution method (Cardiomax-IIR, Columbus Instruments) [24].

*Tissue collection and infarct size measurement*

After the hemodynamic measurements had been obtained, the hearts were arrested in diastole by KCl injection and rapidly excised. The hearts from the SHR sub-study were fixed in 4% paraformaldehyde, cut into 5 transversal sections and photographed. The MI size was calculated as ratio of the infarcted segment to the total LV perimeter averaged between endocardial and epicardial measurements using the ImageJ 1.33k software (NIH), and the average MI was expressed as a percentage of total LV perimeter [25, 26]. Additionally, segments at mid-papillary level were paraffin-embedded, and sectioned at 8 µm. The hearts from the SD rats were transversally cut in two halves approximately at mid-papillary level (site of largest infarct extension). The apical parts were used for histological analyses. From the basal part, the RV was trimmed away and the infarct scar was excised as well as a 2-3 mm border zone adjacent to the infarct scar. All tissue pieces were snap-frozen in liquid nitrogen for later analyses.

In additional experiments the hearts were harvested immediately as well as 6 h, 24 h and 6 d after injection of labeled cells or medium. The hearts were embedded in O.T.C. compound, cryopreserved in melting methylbutan, and subsequently sectioned at 7 µm. After DAPI-staining of the nuclei the sections were directly analyzed under a fluorescence-microscope (Zeiss Axioskop) and photographed.

*Immunohistochemical analysis*

The following antibodies were used: anti-smooth muscle actin (SMA; mouse monoclonal, dilution 1:1.000; Boehringer Mannheim), and desmin (rabbit polyclonal, dilution 1:500; PharMingen). Immunohistochemical analysis was performed according to the recommendations supplied by the manufacturer. Sections known to stain positively were included in each batch and negative controls were performed by replacing the primary antibody with serum (Sigma-Aldrich).

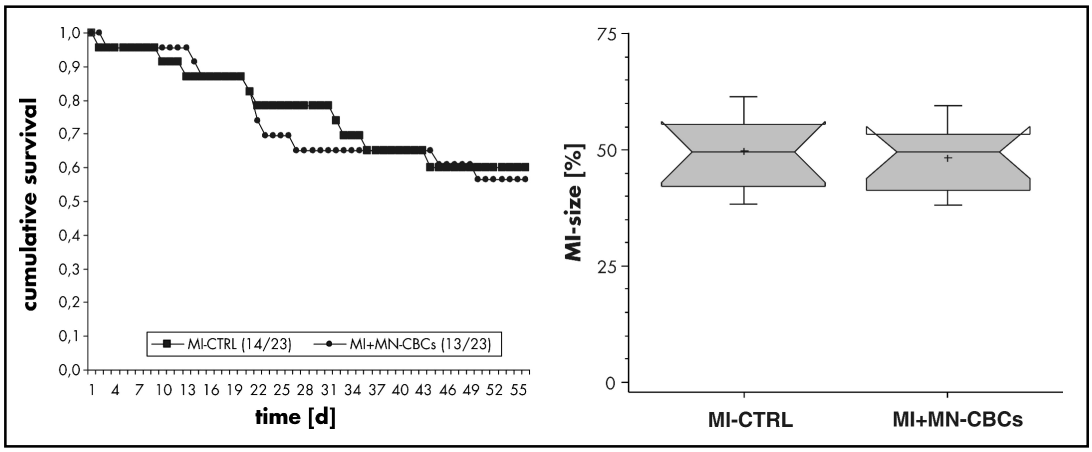


**Fig. 2.** Cumulative survival (left) and infarct-size (right) 8 weeks after myocardial infarction (MI) and medium (CTRL) or mononuclear cord blood cell injection (MN-CBC).

*RNase Protection Assay (RPA)*

Total RNA isolation and RNase protection assay (RPA) were performed as previously described [22, 27].

*Statistical Analysis*

The data are expressed as mean (SD). A Kruskall-Wallis ANOVA on ranks was used for multigroup comparison subsequently utilizing multiple comparison procedure according to Dunn's method (SigmaStat 3.10, SPSS Corp.). Cumulative survival was analyzed by Kaplan-Meier-Plot including overall comparison according to Mantel-Cox, Breslow, and Tarone-Ware (SPSS 13.0 for Windows, SPSS Corp.) A value of $p < 0.05$ was considered statistically significant.

## Results

*Cell characterization*

Nearly all of the alive, isolated mononuclear cord blood cells were positive for CD45. Only 0.7±0.09 % of those (medium intense CD45+) cells were also positive for CD34, but 79.4±2.2 % of the CD45+/CD34+ cells stained for CD133 (Fig. 1). Moreover, after cryopreservation 30.6±16.2 % of the CD34+ cells also expressed the stem cell receptor CXCR4. Nearly all of the cultured USSCs were positive for CD105 and CD13 surface antigens and, also for CD90. However, staining for CD34 was weak (Fig. 1).

*Survival and infarct size*

The mortality within the first 24 h after coronary artery occlusion in the SHR sub-study was 19% (14/72). The animals which had survived for 24 h, were randomly selected to receive either MN-CBCs or medium. Twelve rats were used to track the injected cells after 6 and 24 h while the remaining 46 rats were followed for 8 weeks. During this observation period the cumulative survival of the MI+MN-CBC group (13/23) was not significantly different from that of the medium-treated MI-CTRL group (14/23) (Fig. 2, left). There was also no difference in the infarct size (Fig. 2, right). None of the sham-operated rats died.

The mortality within the first 24 h after coronary artery occlusion in the SD MI sub-study was 27% (21/78). The surviving animals were also randomly selected to receive either cells or medium. Ten rats were used to track the injected cells while the remaining 47 rats were followed for 12 weeks (Tab. 1). During this observation period 2 of the MN-CBC-treated, but none of the medium-treated rats died. Two more rats (each 1 of the MN-CBC-treated and 1 of the USSC-treated rats) died during the night after last echocardiography, but before final hemodynamic measurements. Statistics of cumulative survival was not done due to the small numbers. Also, MI size was not measured, since a part of the hearts was used for molecular biological analyses. None of the I/R rats or of the sham-operated rats died.

*Echocardiography*

Echocardiography was performed in the SD sub-studies only. Successful induction of MI was confirmed by an echocardiographically akinetic LV free wall in all animals a few hours after surgery (details not shown). Severely impaired heart function became overt by greatly reduced EF (and FAC) 2 weeks after MI (Fig. 3). Thereafter, it only slightly decreased over time. LV dimensions at both, end-diastole and end-systole, increased dramatically 2 weeks after coronary artery occlusion. Thereafter, they further increased, but less pronouncedly. Reduced LV pump function was also indicated by an

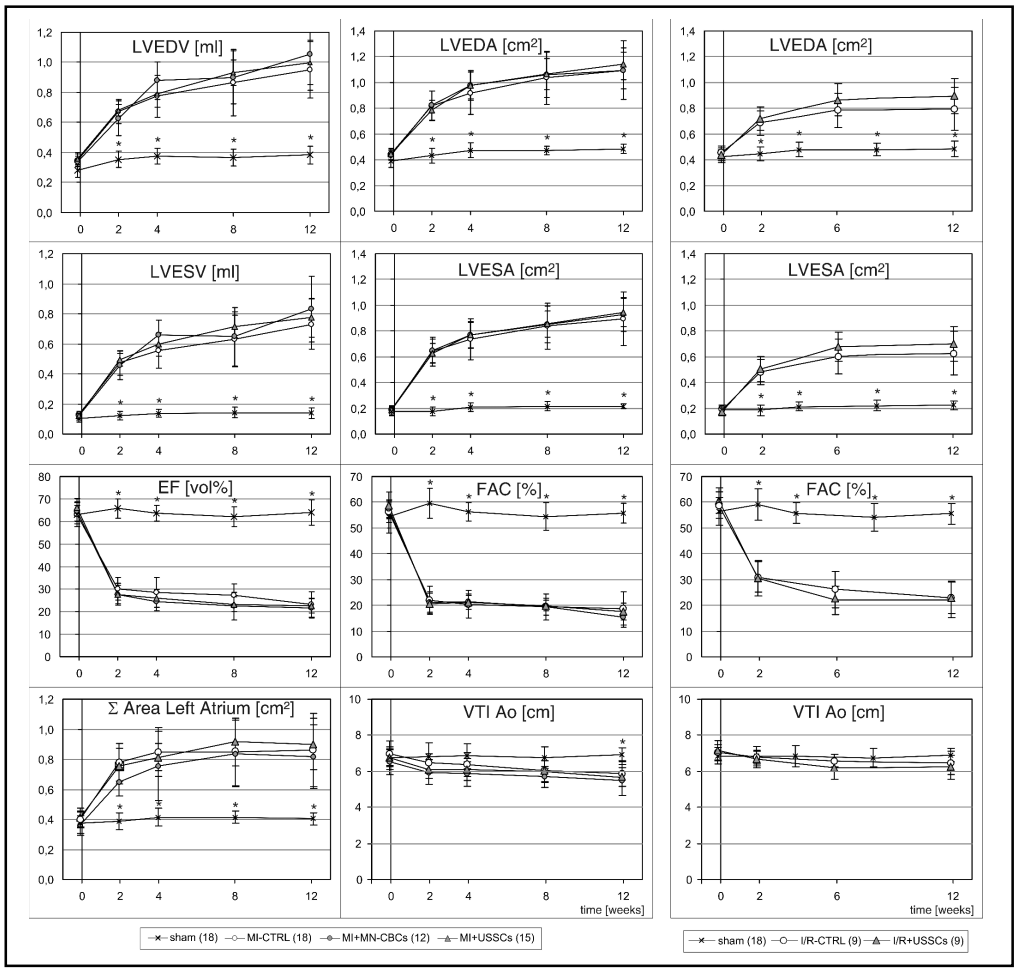


**Fig. 3.** Summary of echocardiographic data over 12 weeks after permanent coronary artery occlusion (MI, left and middle column) or ischemia/reperfusion (I/R, right column). LVED(S)V(A) left ventricular end-diastolic (-systolic) volume (area); EF ejection fraction; FAC fractional area change; VTI velocity time integral. Data are mean±SD; * $p<0.05$ for all MI-groups vs sham (data for 6 weeks I/R were calculated vs the mean of 4 and 8 weeks sham).

increased size of the LA. VTIAo, on the other hand, decreased only slightly and this decrease was statistically significantly different from sham-operated rats 12 weeks after surgery (Fig. 3). Most importantly, these changes were observed in all MI rats, the cell-injected rats being indistinguishable from the medium-injected MI-CTRLs.

FAC decreased severely 2 weeks after I/R and further deteriorated over time thereafter (Fig. 3, right column). Moreover, LV dimensions increased 2 weeks after I/R, but this increase was less pronounced compared to MI. VTIAo was not statistically significantly reduced after I/R. Importantly again, these changes were not different between USSC-injected and medium-injected I/R rats.

*Hemodynamic measurements*

Heart function could not be measured in all rats, since in all MI-groups 2 rats died shortly after thiopental injection for anesthesia. Hemodynamic data revealed severely depressed heart function after MI. The LV systolic pressure (LVSP), LVdP/dtmax and LVdP/dtmin severely decreased after MI while LV end-diastolic pressure (LVEDP) and τ increased (Tab. 2). Total peripheral resistance (TPR) also declined, but mainly due to the reduced mean arterial pressure, since cardiac output decreased significantly only in the SHR after MI. Impaired pump function of the LV became also overt by increased parameters of the RV. Importantly, all these parameters of deteriorated LV and elevated RV function were not significantly different between medium-injected and cell-injected MI-rats, except for TPR in the SD MI-sub-study.

The hemodynamic consequences were less profound 12 weeks after I/R (Tab. 2). LVSP remained normal while LVEDP increased only slightly. Consequently, RV function was comparable to sham-operated rats. The parameters of LV contractility and relaxation, on the other hand, were significantly impaired after I/R. This, however, was not different between USSC- and medium-injected rats.

*Cell tracking*

CFSE-labeled cells were detected by green fluorescence (Fig. 4). They were observed after i.v. injection in paravasal regions of the border zone adjacent to

| | | SHR (8 weeks) | | | SD (12 weeks) | | | |
|---|---|---|---|---|---|---|---|---|
| | sham (24) | MI-CTRL (12) | MI+MN-CBCs (11) | sham (18) | MI-CTRL (16) | MI+MN-CBCs (9) | MI+USSCs (12) | I/R-CTRL (9) | I/R+USSCs (9) |
| weight [g] | 339 (16) | 346 (17) | 343 (24) | 296 (19) | 293 (17) | 305 (17) | 297 (15) | 279 (18) | 281 (32) |
| HR [bpm] | 412 (21) | 369 (33) * | 378 (39) * | 374 (35) | 311 (29) * | 326 (35) * | 312 (24) * | 332 (24) * | 317 (38) * |
| MAP [mmHg] | 205 (13) | 157 (22) * | 163 (20) * | 146 (8) | 100 (16) * | 111 (16) * | 105 (12) * | 139 (11) | 132 (21) |
| AoPP [mmHg] | 47 (8) | 36 (7) * | 39 (4) * | 38 (4) | 25 (5) * | 27 (5) * | 25 (5) * | 37 (4) | 36 (3) |
| CO [ml/min] | 83 (8) | 76 (9) * | 74 (9) * | 85 (12) | 82 (10) | 76 (6) | 73 (8) * | 81 (10) | 83 (16) |
| CI [ml/(min*kg)] | 206 (24) | 201 (27) | 191 (31) | 297 (48) | 278 (21) | 253 (39) * | 235 (23) * † | 292 (27) | 292 (49) |
| SV [µl] | 243 (18) | 216 (22) * | 212 (28) * | 247 (45) | 252 (35) | 245 (42) | 239 (34) | 247 (35) | 256 (40) |
| TPR [mmHg*min*kg/ml] | 0,87 (0,21) | 0,74 (0,09) | 0,82 (0,18) | 0,53 (0,07) | 0,38 (0,09) * | 0,47 (0,11) † | 0,46 (0,09) † | 0,48 (0,06) | 0,46 (0,09) |
| systemic | | | | | | | | | |
| SP [mmHg] | 231 (18) | 175 (25) * | 186 (24) * | 166 (10) | 118 (24) * | 129 (23) * | 121 (17) * | 159 (13) | 151 (21) |
| EDP [mmHg] | 4 (2) | 23 (6) * | 26 (7) * | 3 (1) | 18 (9) * | 19 (11) * | 23 (7) * | 8 (3) | 9 (4) * |
| $dP/dt_{max}$ [mmHg] | 11.102 (1.028) | 8.221 (1.101) * | 8.859 (1.143) * | 10.140 (640) | 5.887 (1.485) * | 6.547 (1.792) * | 5.447 (893) * | 7.816 (895) * | 7.016 (1.322) * |
| $dP/dt_{min}$ [mmHg] | -8.422 (1.248) | -3.969 (597) * | -4.502 (582) * | -9.052 (1.203) | -3.612 (1.043)* | -3.871 (1.267) * | -3.232 (516) * | -5.295 (1.118) * | -5.004 (1.292) * |
| τ [ms] | 14 (2) | 20 (3) * | 20 (2) * | 11 (1) | 18 (2) * | 20 (3) * | 20 (2) * | 19 (2) * | 18 (2) * |
| τ (rel) [%] | 9 (1) | 13 (2) * | 13 (2) * | 7 (1) | 9 (1) * | 11 (1) * | 10 (1) * | 11 (2) * | 10 (2) * |
| LV $MVO_2$ [$*10^6$ mmHg$^2$/s$^2$] | 1.061 (106) | 505 (148) * | 618 (135) * † | 589 (65) | 172 (75) * | 212 (123) * † | 173 (57) * | 423 (64) * | 413 (103) * |
| SP [mmHg] | 32 (2) | 66 (24) * | 65 (26) * | 28 (2) | 60 (13) * | 65 (19) * | 63 (12) * | 29 (4) | 32 (7) |
| EDP [mmHg] | 3 (0) | 4 (1) | 4 (1) | 2 (0) | 3 (2) | 4 (2) | 4 (2) | 2 (0) | 3 (1) |
| $dP/dt_{max}$ [mmHg] | 1.707 (160) | 2.628 (618) * | 2.952 (789) * | 1.752 (180) | 2.676 (276) * | 2.753 (639) * | 2.813 (301) * | 1.842 (150) | 2.126 (262) |
| $dP/dt_{min}$ [mmHg] | -1.316 (164) | -2.186 (681) * | -2.433 (740) * | -1.306 (137) | -2.153 (241) * | -1.990 (536) * | -2.189 (255)* | -1.412 (164) | -1.528 (303) |
| τ [ms] | 10 (1) | 12 (1) * | 12 (1) * | 10 (1) | 13 (1) * | 13 (3) * | 14 (1) * | 8 (2) | 9 (2) |
| τ (rel) [%] | 6 (1) | 7 (1) | 7 (0) | 6 (1) | 7 (1) | 8 (2) | 8 (1) | 7 (3) | 7 (2) |
| RV $MVO_2$ [$*10^6$ mmHg$^2$/s$^2$] | 21 (3) | 34 (16) * | 41 (27) * | 19 (3) | 51 (10) * | 41 (18) * | 53 (10) * | 17 (3) | 23 (10) |

**Table 2.** Hemodynamic characterization 8 (SHR) or 12 weeks (SD) after sham-operation, myocardial infarction, ischemia/reperfusion or MI (I/R) and intramyocardial cell injection. Data are mean (SD); MI myocardial infarction; I/R ischemia/reperfusion; MI(I/R)-CTRL MI(I/R) sham-treated with medium; MI+MN-CBCs; MI treated with human mononuclear cord blood cells; MI(I/R)+USSCs MI(I/R) treated with human unrestricted somatic stem cells; HR heart rate; SAoP and DAoP aortic systolic and diastolic pressure, respectively; MAP mean arterial pressure; AoPP aortic pulse pressure; CO cardiac output; CI cardiac index; SV stroke volume; TPR total peripheral resistance; SP systolic pressure; EDP end-diastolic pressure; d*P* /dt max/min maximal rate of rise/fall in ventricular pressure; τ time constant of isovolumetric relaxation; τ (rel) τ normalized to cardiac cycles length; MVO2 triple product of SP*dP/dt max*HR indicative for myocardial. oxygen consumption; * $p < 0.05$ *vs.* corresponding sham; † $p < 0.05$ *vs.* corresponding MI-CTRL.

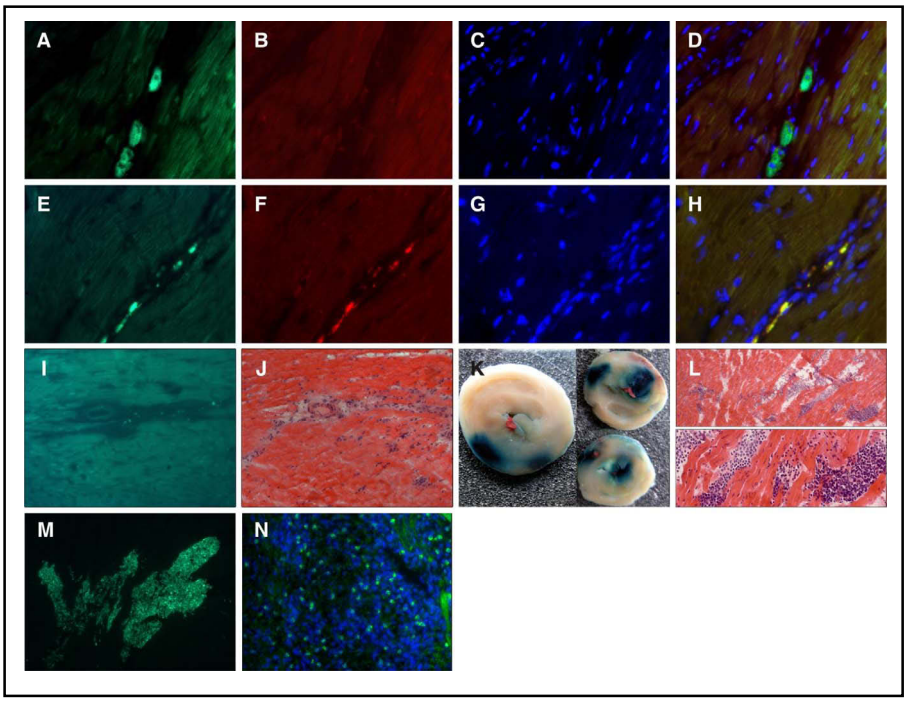


**Fig. 4.** Tracking of CFSE-labeled cells. Green, red, blue fluorescence, and merge in the border-zone 48(24) h after MI and i.v. cell (A-D) or medium (E-F) injection. I and J Green fluorescence and HE staining of a consecutive section from the border-zone 48(24) h after MI and i.v. cell injection. K Amidoblack-injections to verify intramyocardial delivery. HE staining and green fluorescence immediately (L and M) or 6 d (N) after I/R and intramyocardial injections.

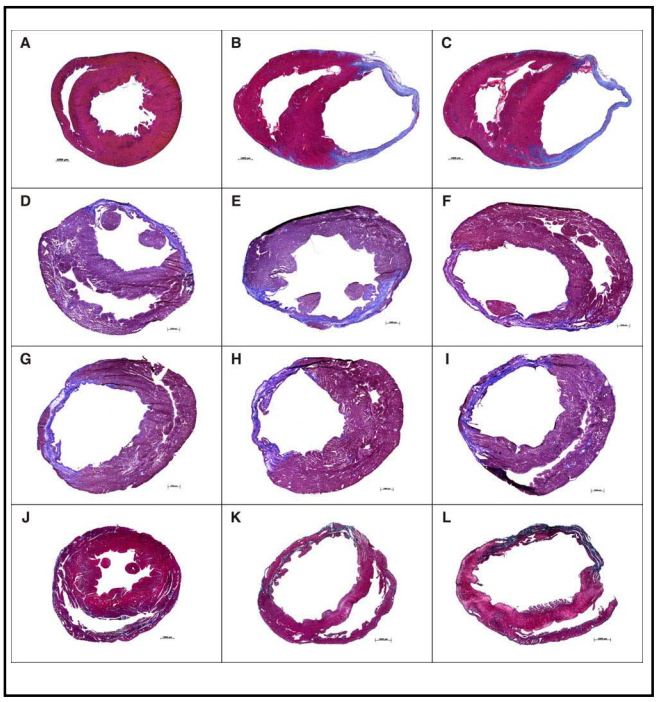


**Fig. 5.** Histological overviews (Mason´s trichrome) after MI and I/R. A SHR sham; B SHR MI-CTRL; C MI+MN-CBCs; D/G SD MI-CTRL; E/H SD MI-MC-CBCs; F/I SD MI+USSCs; J SD sham; K SD I/R-CTRL; L SD I/R+USSCs.

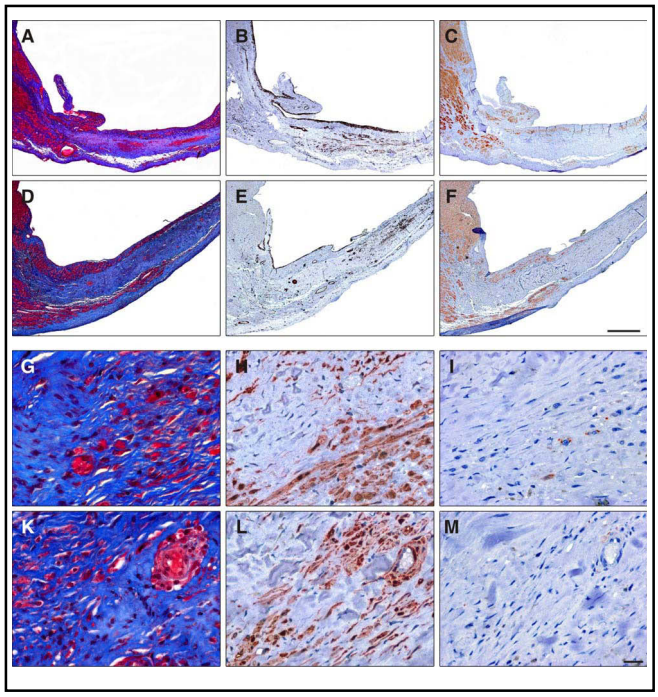


**Fig. 6.** Overviews (2 upper rows) and higher magnification (2 lower rows) of Mason´s trichrome (left) and consecutive anti-SMA (middle) and anti-desmin (right) staining 12 weeks after MI and medium (A-C and G-I) or MN-CBC (D-F and K-M) injection.

the infarct area (identified as infiltration zone in HE stained serial sections) 6 and 24 h after cell injection (Fig. 4A-J). Green spots were also detected in medium-injected MI-CTRL-hearts (Fig. 4E-H). However, these spots showed also red fluorescence and could not be assigned to a nucleus. Therefore, they represent non-specific auto-fluo-

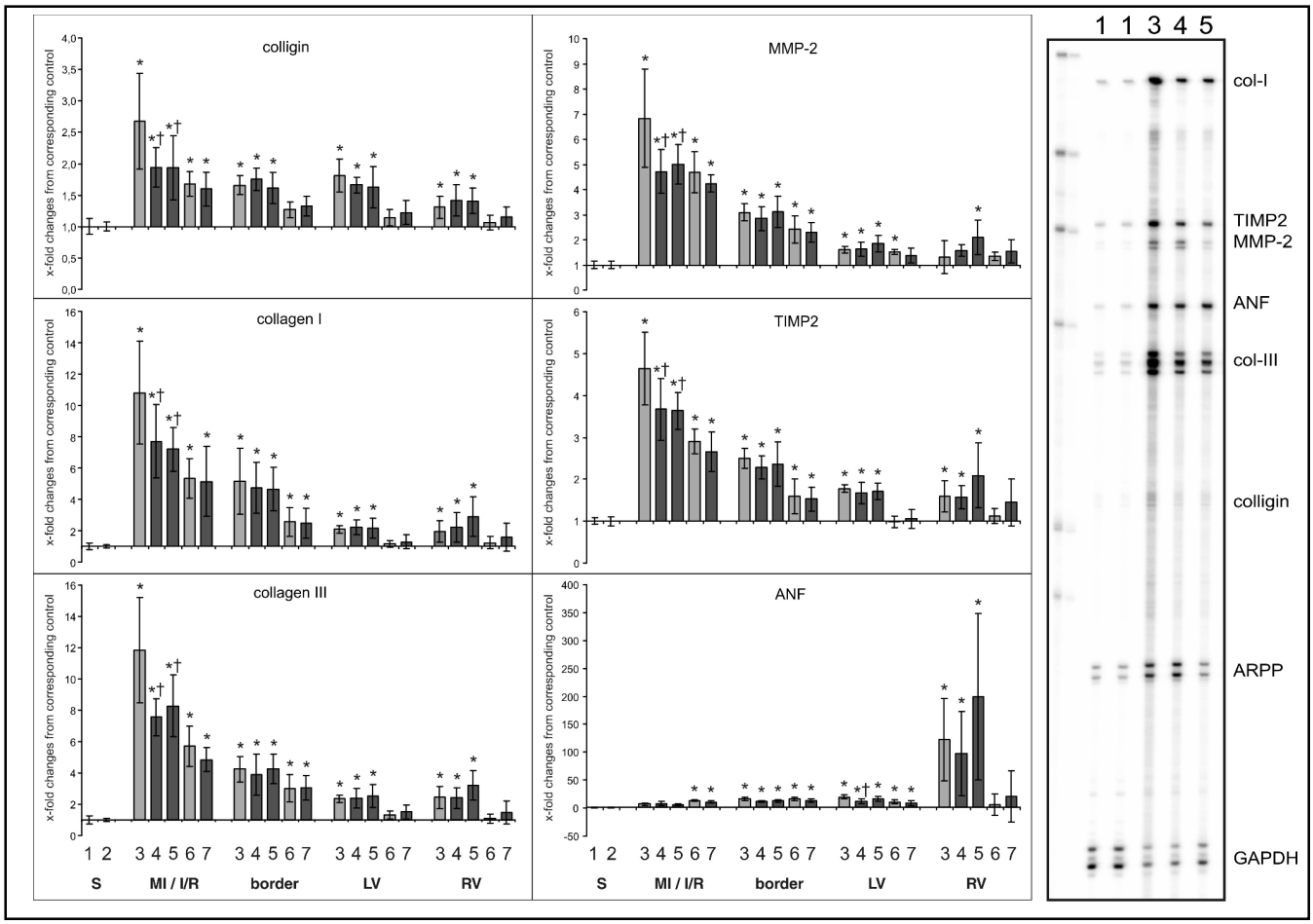


**Fig. 7.** Summary of mRNA expression of extracellular matrix components (left) and representative RPA (right). 1 sham LV; 2 sham RV; 3 MI-CTRL; 4 MI+MN-CBCs; 5 MI+USSCs; 6 I/R-CTRL; 7 I/R+USSCs. Data are normalized to the acidic ribosomal phosphoprotein (ARPP) and shown as mean±SD. * $p<0.05$ vs corresponding sham; † $p<0.05$ vs MI-CTRL.

rescence. Successful intramyocardial cell injection was verified immediately after injection (Fig. 4K-M). Also 6 d after injection, labeled cells could be detected in the hearts, but apparently in smaller numbers (Fig. 4N).

*Histological and Immunohistochemical analyses*

All sections of the MI-hearts revealed typical changes for old infarcts with a subendocardial layer as well as patchy subepicardial amounts of surviving myocytes (Fig. 5). Despite similar infarct-sizes and no obvious signs of regeneration at the site of former injury, Mason's trichrome overview staining showed islets of red-stained cell bodies within the blue-stained collagenous scar (Fig. 6, left column). Immunohistochemical analysis revealed that those cells were positive for α-SMA but negative for desmin (Fig. 6, middle and right column). Moreover, neither occurrence nor characteristics of those cell islets were different between cell-injected and medium-injected hearts.

*Expression of components of the extracellular matrix and of cytokines*

The mRNA-expression of some major components of the extracellular matrix (ECM) namely collagen I, collagen III, the collagen chaperon colligin (also known as Hsp47), matrix-metalloproteinase (MMP)-2 and tissue inhibitor of matrix-metalloproteinases (TIMP) 2 substantially increased after MI (Fig. 7). This increase was most pronounced in the infarct area, but was also observed in the border-zone adjacent to the MI as well as in the non-infarcted LV and RV. Interestingly, these changes were significantly attenuated in both, the MN-CBC and the USSC-treated hearts. However, this was observed only in the infarct area in which the ECM expression in the cell-treated hearts after MI was comparable to that after I/R. In general, ECM expression also increased after I/R, but to a lesser extent and only in the area of injury and in the adjacent border-zone. However, the increase in ECM expression 12 weeks after I/R was not different

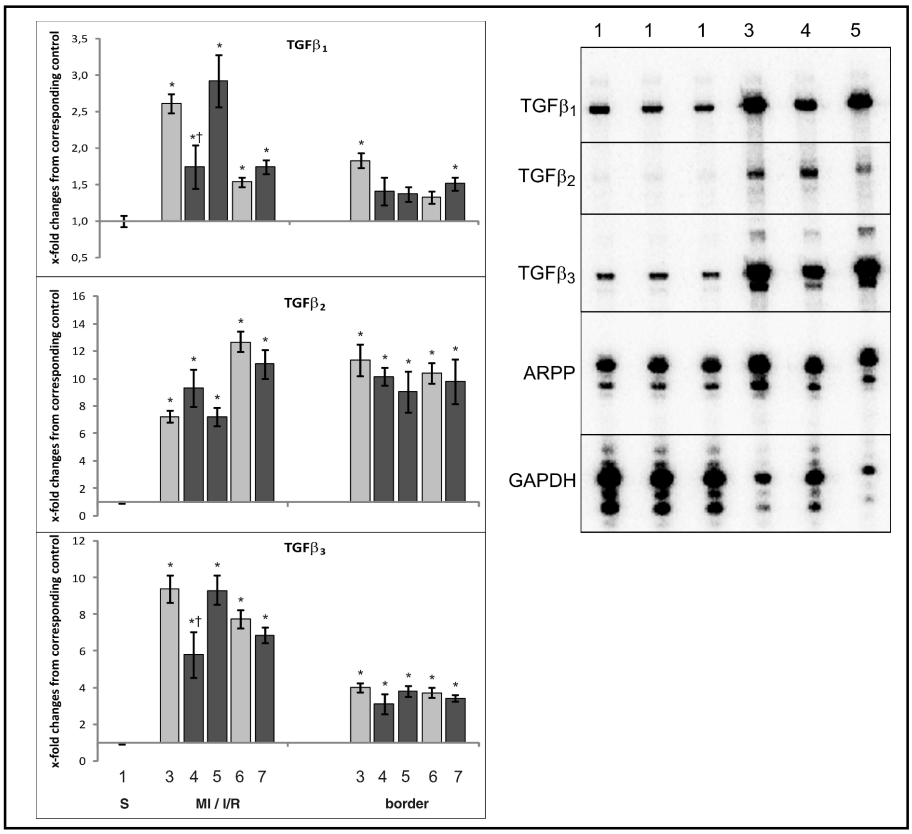


**Fig. 8.** Summary of mRNA expression of TGF-β-isoforms (left) and representative RPA (right). 1 sham LV; 3 MI-CTRL; 4 MI+MN-CBCs; 5 MI+USSCs; 6 I/R-CTRL; 7 I/R+USSCs. Data are normalized to the acidic ribosomal phosphoprotein (ARPP) and shown as mean±SD; * $p<0.05$ vs corresponding sham; † $p<0.05$ vs MI-CTRL.

between the USSC-treated and the medium-treated hearts.

Also ANF expression was induced after MI and I/R (Fig. 7). The most pronounced increase was observed after MI in the RV, in which it was absent after I/R. However, ANF expression was not influenced by cell-treatment, except for a slight but statistically significant reduction in the non-infarcted LV of the MN-CBS-treated rats.

The mRNA-expression of the TGF-β isoforms was induced after MI (Fig. 8). TGF-β1 expression increased predominantly in the infarct area after permanent coronary artery occlusion. This increase was attenuated in the rats treated with MN-CBCs, but not in the rats treated with USSCs. The induction in TGF-β3 was similar to that of TGF-β1, but generally more marked. Again, the most pronounced increase occurred in the infarct area after permanent coronary artery occlusion and was attenuated in the MN-CBC treated rats, but not after treatment with USSCs. Also TGF-β2 mRNA expression was induced after injury. In contrast to the other isoforms, the increase in TGF-β2 was not reduced after MN-CBC therapy but slightly more pronounced, although this did not reach statistical significance. Moreover, the induction of TGF-β2 was more pronounced after I/R and in the border zone adjacent to the infarct area after permanent coronary artery occlusion (Fig. 8).

The mRNA-expression of interleukin (IL)-1β and IL-6, but not of tumor necrosis factor-α was induced in the area of injury and in the adjacent border-zone, but not in the non-infarcted LV and RV (Fig. 9). Moreover, the increase in IL-1β was more pronounced after MI compared to I/R, but not influenced by cell application. In contrast, the increase in IL-6 expression was comparable after MI and I/R. Moreover, it was significantly pronounced in both, the injured area and the adjacent border-zone of the USSC-treated hearts. However, this was observed after I/R but not MI.

## Discussion

After intramyocardial injection of both, MN-CBCs and USSCs, into the site of injury 24 h after permanent coronary artery occlusion, the mRNA expression of components of the ECM was attenuated in the scar after 12 weeks (Fig. 7). This was accompanied by a reduced induction of TGF-β1 and TGF-β3 expression after MN-CBC, but not after USSC treatment (Fig. 8). Additionally, the expression of IL-6 but not of IL-1β was increased

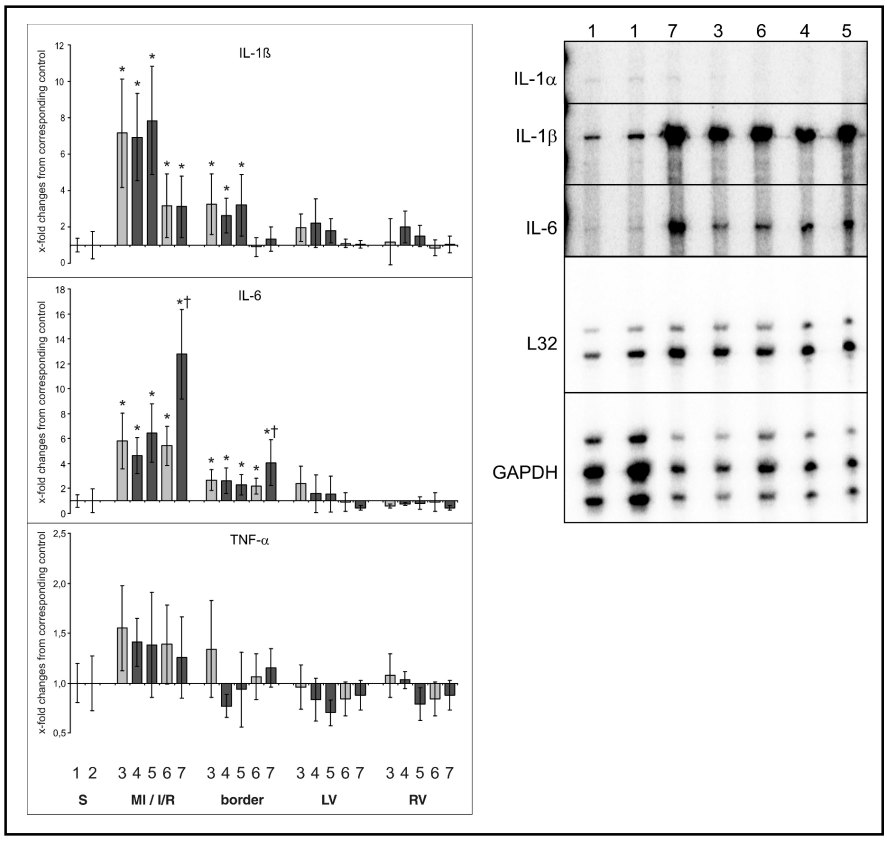


**Fig. 9.** Summary of mRNA expression of cytokines (left) and representative RPA (right). 1 sham LV; 2 sham RV; 3 MI-CTRL; 4 MI+MN-CBCs; 5 MI+USSCs; 6 I/R-CTRL; 7 I/R+USSCs. Data are normalized to the mRNA of the ribosomal protein L32 and shown as mean±SD; * $p<0.05$ vs corresponding sham; † $p<0.05$ vs I/R-CTRL.

at the site of injury and the adjacent border-zone 12 weeks after I/R and USSC-injection (Fig. 9). However, these effects did not translate into improved heart function or attenuated LV dilatation (Tab. 2 and Fig. 3). Moreover, there was no sign of regeneration (Fig. 5 and 6).

The effect of stem and progenitor cells after MI is still controversial. In this study, USSCs as precursors of MSCs with remarkable pluripotent capabilities [18, 28] and, therefore, as promising candidates were compared with MN-CBCs in regard to their effectiveness. The latter have recently been shown to exert beneficial functional effects in a very similar setup of experimental stroke in SHR [29, 30]. The lack of any obvious myocardial regeneration is in line with previous reports showing that myocyte transdifferentiation of various progenitor cells originating from the bone marrow (BM) is a very rare event, if any [31-35]. Moreover, intracoronary injection of USSCs 7 d after I/R in a porcine model did not attenuate MI-induced LV remodeling or ameliorate global and regional LV dysfunction [36]. An earlier study, however, reported that direct injection of USSCs into the border zone of the infarct area 4 weeks post-MI in a porcine model of permanent occlusion resulted in significant improvement of ejection fraction compared to medium-treated controls at 8 weeks post-MI [37]. Also in rats direct injection of MN-CBCs at 1 week after permanent coronary artery occlusion significantly improved LV function 4 weeks after treatment [38]. Additionally, intravenous injection of MN-CBCs 1 day after permanent coronary artery ligation in mice was reported to reduce infarct size [39].

It is, however, difficult to reconcile the data of the present study with the findings of the previous reports. A number of factors like pre-selection and pre-treatment of the cells, time and route of application, isolation and storage method, and the experimental setup might be of influence. The timing of cell injection cannot explain the observed lack of benefit on LV remodeling, function or infarct size, since previous studies showed beneficial effects of cord blood cell injection as early as 20 min, 60 min, or 1 day [39-41] after coronary ligation, but also as late as 1 or 4 weeks after ligation [37, 38]. The results of these previous studies were observed 3-4 weeks after MI, also indicating that the 8-12 week follow-up period was sufficient to allow detection of an effect of cell transplantation.

Cord blood cells are assumed to possess immune-privileges and are supposed to be hypo-immunogenic [42]. Furthermore, in a previous study labeled USSCs were detected in the infarct zone 4 days after injection with

and without immunesuppression, suggesting the absence of hyper-acute rejection [36]. Since hypoimmunogenicity of the umbilical cord blood derived cells is still questionable and, also, may change in case differentiation takes place, an additional immunosuppressive therapy was applied in the I/R sub-study to exclude the possibility that the lack of functional effects was due to rejection.

For the experiments in hypertensive rats, the cells were injected intravenously after permanent coronary occlusion. The low level of engraftment of these cells into the injured adult heart after tail vein infusion may not be surprising, because the occluded artery makes it difficult for the injected cells to reach the infarcted myocardium. This problem is compounded by the fact that intravenously injected cells are likely to be sequestered in various organs. However, labeled cells were clearly detected in paravasal regions of the border zone adjacent to the infarct area (Fig. 4), but not quantified. A much higher number of cells was detected after direct cell injection, but considerably decreased after 6 d (Fig. 4). Only freshly cultured USSCs were used in this study, but cryopreserved MN-CBCs. Although a small portion of the cryopreserved cells is not viable after thawing, the majority of these cells can unrestrainedly be cultured (details not shown). To generally minimize cell damage during injection, a canula with a large diameter was used (22G). It can also not completely be ruled out that CFSE labeling impairs the capabilities of the cells. In pilot studies, however, it did not affect proliferation and viability of cultured MN-CBCs (details not shown). Nonetheless, unlabeled cells were used for the long-term experiments, since after that time also other factors like fusion or loss of CFSE during proliferation might complicate possible interpretations. Since the histological examination revealed typical changes for old infarcts and, more importantly, the cell-treated hearts were indistinguishable from the medium-treated controls, further staining for typical cardiac transcription factors (GATA4, MEF2) was not performed. Therefore, it can not completely be ruled out that a very small number of the injected cells could be able to differentiate and remained at the site of injury. This would be in line with a previous study in pigs reporting only a few of the injected USSCs after 4 weeks [36]. Moreover, the injected USSCs did not transdifferentiate into a cardiomyocyte or endothelial phenotype since they were negative for Troponin and vWF, but were still CD45 positive.

It has also been hypothesized that enhanced neoangiogenesis after BM cell application might contribute to improved ventricular function after MI. From recent studies, however, involvement of BM derived cells in peri-infarct or hypoxia-induced angiogenesis has been questioned [34, 43], although this is very likely for endothelial progenitor cells. Neoangiogenesis in scar tissue might be important for remodeling, but was not analyzed in this study, since myocardial functional improvement directly caused by enhanced neoangiogenesis cannot be expected, given the absence of contractile cells in the scar.

On the other hand, paracrine mechanisms have been suggested to contribute to the beneficial effects of progenitor cell application after MI [44, 45]. Interestingly, the application of both, MN-CBS as well as of USSCs after MI attenuated the expression of ECM components (Fig. 7) pointing to such indirect mechanisms even in the absence of regeneration and even 12 weeks after the injury. Notably, this was observed in the infarct scar only and only after permanent coronary artery occlusion. Moreover, the induction of ECM expression after MI was attenuated to levels comparable to that after I/R, but did not translate into functional improvement. This might designate the critical importance of the time of cell injection after injury. Since labeled injected cells were detected at 6 d after I/R (Fig. 4), it might be speculated that the substantial differences in the pathomechanisms after permanent occlusion and I/R may also require different strategies for cell therapy. The fact that USSCs administered after I/R led to increased expression of IL-6, but not of IL-1β in the infarct and the peri-infarct (Fig. 9), further suggests paracrine effects after cell therapy.

It has now been accepted that the infarct scar is a highly dynamic tissue for months and even years after MI in rodents and men [27, 46, 47]. Myofibroblasts are the main mediators of fibrogenesis and remodeling after myocardial injury [48], but may also serve as precursor cells necessary for angiogenesis [49] and, therefore, are essential for the capacity of the infarcted heart to heal. Recently, a high number of eGFP positive fibroblasts and myofibroblasts were observed in the infarct and peri-infarct in a mouse model of MI after BM replacement by eGFP expressing cells. This suggests that BM derived myofibroblasts may be of particular importance for the benefit of BM cells in myocardial healing processes [34]. A recent report, on the other hand, did not show differentiation of BM derived cells into myofibroblasts [50]. Myofibroblast-like cells were observed in the infarct area of both, cell and medium treated MI hearts (Fig. 6), but not quantified. Since the injected cells were not labeled and also screening for human chromosome by FISH was not done, it remains to be elucidated, if injected cells di-

rectly take part in remodeling by transformation into myofibroblasts or rather indirectly modulate the remodeling by paracrine mechanisms like the expression of cytokines and growth factors. It should be pointed out that rats injected with inactive cells after MI would be the more appropriate controls to test for released factors or the induction of active processes within the injured tissue. However, the data on TGF-β expression indicate differential effects induced by MN-CBCs and USSCs (Fig. 8). This also indicates that additional factors different from TGF-β seem to be important for cardiac remodeling after MI.

In summary, this study indicates that cord blood cell implantation after myocardial infarction acts trough paracrine mechanisms to modify remodeling rather than myocyte regeneration. The role of myofibroblasts and the optimal conditions of cell application need to be determined to translate these mechanisms into functional improvement.

## Acknowledgements

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# 3 Zusammenfassende Diskussion

## 3.1 Kontrastmittelechokardiografische Verlaufsuntersuchungen an Ratten mit experimentellem Herzinfarkt

Es konnte gezeigt werden, dass die in der Humanmedizin etablierte Methode der Kontrastmittelechokardiografie prinzipiell auf das Rattenmodel übertragbar ist. Es konnte weiterhin gezeigt werden, dass es möglich ist, mittels Kontrastmittelechokardiografie Verlaufsuntersuchungen an Gruppen von Versuchstieren durchzuführen. Darüber hinaus konnte gezeigt werden, dass beim verwendeten Infarktmodell Flächenmessungen in der kurzen Achse im Vergleich zu den biplan bestimmten Ventrikelvolumina ebenfalls die Größenänderungen des linken Ventrikels adäquat wiedergeben.

Für die echokardiografische Volumenbestimmung am linken Herzen wird in der Humanmedizin die Bestimmung nach der modifizierten Methode nach Simpson mittels zweier rechtwinklig zueinander stehender apikaler Ultraschallachsen empfohlen (Gottdiener et al. 2004). Gerade die inhomogene Änderung der Ventrikelgeometrie nach Herzinfarkt machte es notwendig diese Methode am Rattenmodell zu etablieren. Auch mit den v erwendeten hochmodernen Ultraschallgeräten war es nur bei einer sehr kleinen Zahl der Ratten möglich, die parallel zur Schallachse stehenden Endokardlinien valide abzugrenzen. Aus diesem Grund erfolgte bei allen Tieren der Einsatz von lungengängigem Ultraschallkontrastmittel.

Aus den einleitenden Bemerkungen zur Dimensionsbestimmung in der kurzen Achse des linken Ventrikels geht die nach Literaturrecherche bestandene prinzipielle Skepsis dieser Methode gegenüber hervor. Es konnte jedoch gezeigt werden, dass am verwendeten Infarktmodell eine sehr gute Korrelation zwischen dieser an der Ratte deutlich einfacher aufzunehmenden Messung und der aufwendigeren biplan bestimmten Ventrikelgröße besteht. Mittels Bland-Altmann-Analyse konnte die Austauschbarkeit der beiden Methoden im wissenschaftlichen Versuch gezeigt werden. Es muss jedoch darauf hingewiesen werden, dass dieser Beleg nur für ein Modell gezeigt wurde, in welchem durch Ligatur des gleichen Gefäßes die Herzinfarkte bei allen Tieren sehr ähnlich waren. Man kann annehmen, dass die Korrelation sich auf homogen, das gesamte Myokard betreffende Krankheitsmodelle und topisch inhomogene Myokardschädigungen, welche im Versuch stets gleich lokalisiert sind, übertragen lässt.

Äquivalent zu Arbeiten aus der Humanmedizin (Otterstad et al. 2001), wo verschiedene Dimensionsmessungen mit der Prognose korreliert wurden, gelang es uns vor allem, das endsystolische Ventrikelvolumen und die endsystolische Ventrikelquerschnittsfläche mit dem etabliertem hämodynamischen Parameter des linksventrikulär enddiastolischen Blutdrucks zu korrelieren. Die Ergebnisse deckten sich mit Beobachtungen aus der Humanmedizin (Otterstad et al. 2001), weswegen man ebenfalls die Empfehlung ableiten kann, dass die Größenbestimmung des linken Ventrikels bei apikal ungenügender Endokardabgrenzung und Nichtverfügbarkeit von Kontrastmittel auch als Flächenmessung in der kurzen Achse vorgenommen werden kann.

Für die Größenbestimmung am linken Herz mittels Kontrastechokardiografie und mittels kardialer Magnetresonanztomografie konnten in humanmedizinischen Untersuchungen vergleichbar gute Reproduzierbarkeiten gezeigt werden (Hoffmann et al. 2005). Ein Vergleich von Ultaschall- und MR-Untersuchung am Rattenmodell steht noch aus. Zeitlich und räumlich ausreichend auflösende MRT-Geräte stehen derzeit nur sehr begrenzt zur Verfügung. Die begrenzte Verfügbarkeit der Methode, sowie lange Untersuchungs- und Narkosezeiten stellen die Eignung des kardialen MRT zur Phänotypisierung im Rattenversuch in Frage. Eine begrenzte Serie zur Vergleichsuntersuchung zur Kontrastechokardiografie erscheint auch aus diesem Grund erstrebenswert.

Limitierend für den Einsatz der Kontrastmittelechokardiografie ist neben den Kosten für Kontrastmittel und der längeren Untersuchungszeit pro Tier vor allem eine im Vergleich zur Aufnahme anderer Parameter flache Lernkurve beim Untersucher zu nennen. Es wurden keine Versuche zum Vergleich verschiedener Untersucher durchgeführt. Bis zur Durchführung solcher Versuche sollte die Methode im Versuch bei einem Untersucher bleiben.

Zusammenfassend konnte gezeigt werden, dass die Kontrastmittelechokardiografie zur linksventrikulären Volumenbestimmung eine in Versuchsserien unproblematisch durchführbare Methode ist und dass Flächenmessungen in der kurzen Achse ähnlich aussagekräftig sind, um die Geometrieänderungen nach Herzinfarkt zu beobachten. Die parallele Nutzung beider Methoden und Verlaufsuntersuchungen an Versuchstieren versprechen eine höhere Validität der erhobenen Daten und damit zuverlässigere wissenschaftliche Aussagen.

### 3.2 Einfluss der hämatopoetischen Vorläuferzellen nach Herzinfarkt

In der oben genannten Arbeit (Rabald et al. 2008) wird beschrieben wie Ratten nach experimentellem Herzinfarkt durch direkte Injektion zwei verschiedener Vorläuferzellen aus humanem Nabelschnurblut in den Herzmuskel behandelt wurden. Zusätzlich wurden Tiere nach operativ induziertem Herzinfarkt und anschließender Reperfusion auf den Einfluss von myokardial injizierten Vorläuferzellen untersucht.

Die Tiere wurden im Verlauf von 8-12 Wochen mit wiederholten echokardiografischen Untersuchungen und am Ende der Untersuchung mittels invasiver hämodynamischer Messung phänotypisiert.

Nach Tötung der Tiere erfolgte bei einem Teil der Tiere eine Infarktgrößenbestimmung. Die Herzen der übrigen Tiere wurden geteilt. Es erfolgte die immunhistochemische Untersuchung auf Zeichen von Regeneration. Ebenfalls wurde die RNA-Expression von Proteinen der extrazellulären Matrix quantitativ in verschiedenen Gewebebezirken untersucht.

In Vorversuchen konnte mit Hilfe von Farbstoffen und fluoreszenzmarkierten Zellen der Erfolg der Zellinjektionstechnik, sowie die Nachweisbarkeit der injizierten Zellen sechs Tage nach Injektion gezeigt werden.

Für die oben beschriebene Injektion von verschiedenen Vorläuferzellen der Blutbildung in das Myokard von Rattenherzen 24 S tunden nach Myokardinfarkt konnte kein Effekt auf das Überleben (Fig. 2 in (Rabald et al. 2008)), sowie auf die funktionellen und ge ometrischen Veränderungen nach dem Infarkt beobachtet werden (Tab. 2 und Fig. 3 in (Rabald et al. 2008)).

Zwölf Wochen nach Infarkt und Zellbehandlung war jedoch zwischen den Therapie- und Kontrollgruppen ein Unterschied in der mRNA-Expression von Komponenten der extrazellulären Matrix zu verzeichnen. Die RNA-Expression für TGF-β1 und TGF-β3 war nach Behandlung mit den MN-CBC (mononuclear cord blood cells), jedoch nicht nach Behandlung mit USSC (unrestricted somatic stem cells) im Bereich der Infarktnarbe reduziert. Zusätzlich war in beiden Therapiegruppen der Ischämie-Reperfusionsversuche nach zwölf Wochen die Expression von IL6, jedoch nicht von IL1-β im Bereich der Infarktnarbe gesteigert.

Die Ergebnisse der vorgestellten Versuche reihen sich insgesamt in eine noch sehr widersprüchliche Datenlage ein.

In einem Schweinemodell konnte acht Wochen nach Herzinfarkt eine Verbesserung der Ejektionsfraktion beobachtet werden, wenn in der vierten Woche USSCs in das

infarzierte Myokard gespritzt wurden (Kim et al. 2005). An der Ratte führte die direkte Injektion von MN-CBC eine Woche nach Herzinfarkt zu einer Besserung der linksventrikulären Funktion nach insgesamt fünf Wochen (Leor et al. 2006). An der Maus konnte nach intravenöser Injektion von MN-CBC am ersten postoperativen Tag eine Reduktion der Infarktgröße gezeigt werden (Ma et al. 2005).

Auf der anderen Seite wurde in jüngerer Vergangenheit in einer Reihe von Arbeiten kein regenerativer Effekt von Vorläuferzellen der Blutbildung auf den experimentellen Herzinfarkt gesehen (Balsam et al. 2004; Murry et al. 2004; Nygren et al. 2004; Deten et al. 2005; Mollmann et al. 2006).

Die Unterschiede der bislang veröffentlichten Ergebnisse sind insgesamt schwierig zu erklären. Argumente für die großen Differenzen werden häufig in den zahlreichen veränderbaren Faktoren, die die Ergebnisse beeinflussen können, gesucht. Die hohe Zahl der sich gegenüberstehenden Befunde und die Inhomogenität bezüglich Tiermodell, Zellisolationsverfahren, Zellapplikationsweg und Behandlungszeitpunkt entkräftet allerdings die Bedeutung dieser Details für das prinzipielle Ergebnis der Versuche.

Eine ebenfalls schwierig zu beantwortende Frage, ist die nach der Notwendigkeit einer Immunsuppression. Die verwendeten Vorläuferzellen exprimieren selbst keine immunogenen Oberflächenmoleküle, was als stärkstes Argument gegen ei ne Immunsuppression herangezogen wird. Andererseits ist es wahrscheinlich, dass nach einer Transdifferenzierung zu Herzmuskelzellen, die Zellen dem Wirtsimmunsystem durchaus MH C-Moleküle präsentieren. Da diese potenziell abgestoßen werden könnten, liegt hier ein Argument für eine Immunsupression. Diesem Gedanken entgegen steht, dass eine Zahl der vorgenannten Positivbefunde ebenfalls ohne Immunsuppression produziert wurden.

Inwiefern die verschiedenen wissenschaftlichen Befunde durch unterschiedliche experimentelle Techniken beeinflusst werden, kann nicht beurteilt werden.

Zusammenschauend muss festgehalten werden, dass es trotz der veröffentlichten erfolgreichen Versuche zur Regeneration von Herzmuskelgewebe durch adulte Vorläuferzellen bei ebenfalls zahlreichen Negativbefunden weiterhin große Zweifel am Konzept der Geweberegeneration und der Funktionsverbesserung durch einfache Injektion weitestgehend unbehandelter Zellen nach Herzinfarkt bestehen.

Die gezeigte Beeinflussung der extrazellulären Matrix durch die Zelltherapie weist allerdings auf die prinzipielle Möglichkeit hin, durch das Einbringen von Zellen mittelfristig Einfluss auf die parakrinen Vorgänge im Herzgewebe zu nehmen.

Obwohl die gezeigten Veränderungen bisher keinen Effekt auf die Funktion des Herzens haben, lässt sich vermuten, dass eine positive Beeinflussung des Herzumbaus durch eine gezielte Manipulation von Vorgängen, wie parakriner Apoptosesteuerung oder Neoangiogenese, möglich ist. Weitere Experimente zur parakrinen Funktionsweise des Herzgewebes nach einem Infarkt, zur Rolle der nichtmuskulösen Zellfraktionen des Herzens und z um Einfluss verschiedener Faktoren sind notwendig.

# Zusammenfassung der Arbeit

Dissertation zur Erlangung des akademischen Grades Dr. med.

Titel:
Entwicklung der Kontrastmittelechokardiografie am Rattenmodel zur Untersuchung des Einflusses von mesenchymalen Vorläuferzellen auf das Remodeling nach experimentellem Herzinfarkt

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Gelingt nach einem abgelaufenen Herzinfarkt nicht die frühzeitige Rekanalisierung des verschlossenen Gefäßes, kommt es in Folge des Gewebsunterganges zu geometrischen Änderungen am Herz, welche eine dramatische Verschlechterung der kardialen Leistungsfähigkeit nach sich ziehen. Nachdem im Herzmuskelgewebe knochenmarkstransplantierter Patienten Spendergenom nachgewiesen wurde, wurde über eine sehr langsame Regeneration von Herzmuskelzellen aus Vorläuferzellen der Blutbildung spekuliert. In der Folge wurden widersprüchliche Befunde, welche für oder gegen die Regeneration von Herzmuskel durch diese Vorläuferzellen sprechen. Zur Charakterisierung des Modells und vor Durchführung der Experimente wurde die echokardiografische Phänotypisierung am Rattenmodel einer methodischen Untersuchung unterzogen. Hierbei sollte die in der Humanmedizin als Goldstandart der Echokardiografie geltende Volumenbestimmung aus biplan, kontrastmittelgestützt gewonnenen Bildern nach der modifizierten Methode nach Simpson untersucht werden.

Der Arbeit liegen im Sinne einer kumulativen Dissertationsschrift zwei wissenschaftliche Originalarbeiten zu Grunde.

In der ersten Arbeit wird Etablierung und Untersuchung der Kontrastmittelechokardiografie am Infarktmodell an der Ratte beschrieben. Es wurden 26 weibliche Sprague-Dawley-Ratten einer operativen Ligatur der linken Herzkranzarterie und 16 Tiere einer entsprechenden Scheinoperation unterzogen. Es wurden im Verlauf von 12 Wochen vier echokardiografische Untersuchungen durchgeführt. Es zeigte sich der nach Herzinfarkt typische Verlauf mit Dilatation der Herzhöhlen im Verlauf, und plötzlichem Abfall der Auswurffraktion nach Herzinfarkt. Aus der Humanmedizin bekannte Korrelation von geometrischen Parametern zur Prognose, konnten mit Korrelation der im Versuch für das linksventrikuläre Volumen und für die linksventrikuläre Querschnittsfläche in Korrelation zum linksventrikulären entdiastolischen Druck als etabliertem hämodynamischen Prognoseparameter nachvollzogen werden. Es konnte eine gute Korrelation zwischen Volumen und Querschnittsfläche der linken Herzkammer gezeigt werden. Eine Bland-Altmann-Analyse zeigte die Austauschbarkeit der beiden Methoden für die wissenschaftliche Praxis für das untersuchte Modell. Es konnte gezeigt werden, dass die Kontrastechokardiografie eine auch für größere Tierserien durchführbare Verlaufsuntersuchung zur Phänotypisierung ist. Der Vergleich zur echokardiografischen Querschnittflächenmessung am linken Ventrikel ohne Einsatz von Kontrastmittel zeigte, dass diese einen sinnvollen Ersatz zur Verlaufsbeobachtung nach Herzinfarkt darstellt, wenn der Einsatz von Kontrastmittel nicht möglich ist.

In der zweiten Veröffentlichung wurde der Einfluss mesenchymaler Stammzellen auf den Herzumbau nach Myokardinfarkt untersucht. Es wurden nach entsprechenden methodischen Versuchen insgesamt 128 Ratten einer operativen Infarktinduktion unterzogen. Bei insgesamt 42 Ratten wurde eine entsprechende Scheinoperation durchgeführt. Zusätzliche 18 Ratten wurden in einer adaptierten Operationstechnik nach 60 Minuten Herzinfarkt einer Reperfusionsbehandlung unterzogen. Sowohl die scheinoperierten, als auch die Tiere mit Myokardinfarkt wurden nach Randomisierung in jeweils drei Gruppen durch die Injektion von mononukleären Nabelschnurblutzellen, USSC-Zellen oder Medium in das Herzmuskelgewebe behandelt. Die Tiere wurden über acht bis zwölf Wochen mittels Echokardiografie im Verlauf untersucht, anschließend einer invasiven hämodynamischen Messung unterzogen und getötet. Das gewonnene Herzmuskelgewebe wurde

immunhistochemisch auf Zeichen von Regeneration und molekularbiologisch auf die Genexpression für Signalmoleküle der extrazellulären Matrix untersucht. Die Phänotypisierung im Experiment zeigte keine Unterschiede in den Änderungen von Herzgeometrie und Funktion nach Herzinfarkt durch die Injektion der Zellen. Alle Infarktgruppen zeigten den für einen unbehandelten Myokardinfarkt typischen Verlauf der gemessenen Parameter. Die immunhistochemische Untersuchung zeigte eine zwischen den Gruppen identisch niedrige Dichte von muskelgewebstypischen Proteinen im Bereich der Infarktnarbe und des Infarktrandes. In der molekularbiologischen Aufarbeitung des Gewebes fielen differente Expressionsmuster der mRNA für Interleukin 6, nicht aber für Interleukin 1 im Randsaum des Infarktes auf. Zu diesem Befund gab es jedoch kein funktionelles Korrelat. In Zusammenschau der Befunde konnte im Experiment kein Einfluss von intramyokardial injizierten adulten Vorläuferzellen auf den U mbau und Funktionsverlust des Herzmuskels nach Infarkt gezeigt werden. Die beobachteten Änderungen der Genexpression lassen Raum für Spekulationen über einen möglichen Einfluss der injizierten Zellen auf das parakrine System des Herzmuskelgewebes, welche sich allerdings nicht in funktionellen Änderungen niederschlägt. Die großen Unterschiede der berichteten Effekte von mesenchymalen Vorläuferzellen auf den Herzinfarkt sind bei der großen Fülle an t eilweise sehr verschieden durchgeführten Experimenten, allein durch unterschiedliche Rahmenbedingungen nicht zu begründen. Gemeinsam mit diesem Experiment mehren eine Anzahl ähnlicher Befunde starke Zweifel am Modell der Myokardregeneration durch mesenchymale Vorläuferzellen. Die Erprobung der Potenz embryonaler Stammzellen, sowie die Erforschung der Funktionsweise und Pathophysiologie der extrazellulären Matrix und der nichtmuskulären Zelllinien des Herzmuskelgewebes, versprechen neue wissenschaftliche Ansätze zur gezielten Beeinflussung des Herzumbaus nach Herzinfarkt.

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## Erklärung über die eigenständige Abfassung der Arbeit

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