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Immunhistochemischer Nachweis des akut letalen Myokardinfarkts: neue Marker und potenzielle Einflussfaktoren

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Inhaltsverzeichnis

1 Abkürzungsverzeichnis

Cx43	-	Connexin 43
cTnI	-	*cardiac troponin I*
cTnT	-	*cardiac troponin T*
DNS	-	Desoxyribonukleinsäure
eM	-	extrazelluläre Matrix
ET-1	-	Endothelin-1
GDF-15	-	growth differentiation factor 15
HE	-	Hämatoxylin-Eosin
IHC	-	Immunhistochemie
MMP	-	Matrixmetalloproteinase
ROS	-	*reactive oxygen species*
TIMP	-	*tissue inhibitor of matrixmetalloproteinases*
CK	-	Creatinkinase
FABP	-	*fatty acid binding protein*

2 Übersicht der beitragenden Originalarbeiten

Mayer F, Pröpper S, Ritz-Timme S, Dityrosine, a protein product of oxidative stress, as a possible marker of acute myocardial infarctions, Int J Legal Med, 2014, 128: 787-794

Mayer F, Falk M, Huhn R, Ritz-Timme S, Dityrosine as a marker of acute myocardial infarction? Experiments with the isolated Langendorff heart, Int J Legal Med, 2016, 130: 1053-1060

Mayer F, Falk M, Huhn R, Behmenburg F, Ritz-Timme S, Matrixmetalloproteinases and tissue inhibitors of metalloproteinases: Immunhistochemical markers in the diagnosis of lethal myocardial infarctions? For Sci Int, 2018, 288: 181-188

Falk M, Huhn R, Behmenburg F, Ritz-Timme S, **Mayer F**, Biomechanical stress in myocardial infarctions: can endothelin-1 and growth differentiation factor 15 serve as mmunohistochemical markers? Int J Legal Med, 2018, 132: 509-518

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3 Zusammenfassung

Der Nachweis eines letalen Myokardinfarkts spielt bei der Untersuchung von plötzlichen und unerwarteten Todesfällen in der rechtsmedizinischen Routinearbeit eine große Rolle. Ein wichtiges Werkzeug bei der postmortalen Diagnostik stellen immunhistologische Untersuchungen feingeweblicher Schnitte auf Marker einer myokardialen Ischämie dar. In jedem Fall zuverlässige Marker oder Markersets konnten bislang nicht etabliert werden, insbesondere bei einem sehr akuten Todeseintritt mit nur kurzer Ischämiedauer besteht nach wie vor ein großer Bedarf an neuen Markern. An humanem Herzmuskelgewebe aus Autopsiefällen sowie an Rattenherzen aus Versuchen mit dem Langendorff-Modell mit jeweils unterschiedlichen Ischämiezeiten wurden neue, potentielle Marker ausgetestet: Dityrosin, Matrixmetalloproteinasen 2 und 9, *tissue inhibitor of matrixmetalloproteinases 1*, Endothelin-1 und *growth differentiation factor 15*. Unter den untersuchten Markern erwies sich Dityrosin als geeignet für einen sehr schnellen Nachweis nach Einsetzen der Ischämie sowohl in menschlichem als auch in Ratten-Gewebe, so dass ein Einsatz in der postmortalen Diagnostik empfohlen werden konnte. Die anderen Marker konnten in dieser Hinsicht nicht überzeugen, allerdings ergab sich - sozusagen als Nebenbefund - für die Matrixmetalloproteinasen und ihren Inhibitor die Möglichkeit eines Einsatzes in der forensischen Wundaltersschätzung. Weitere Untersuchungen wurden zur Frage eines potenziellen Einflusses von Geschlecht und ischämischer Präkonditionierung auf den Nachweis von immunhistochemischen Myokardinfarkt-Markern durchgeführt. Die Versuche ergaben keinen relevanten Einfluss auf den Nachweis von Dityrosin. Im Falle einer ischämischen Präkonditionierung war demgegenüber die Anfärbbarkeit von Connexin 43 deutlich reduziert und die Depletion von *cardiac troponin T und C* erhöht.

4 Einleitung

4.1 Postmortale Myokardinfarktdiagnostik

Unter einem Myokardinfarkt versteht man eine ischämische Schädigung der Herzmuskulatur, welche innerhalb etwa einer Minute zunächst zu einem relevanten Verlust der Kontraktilität und binnen weiterer etwa 20 bis 40 Minuten über zahlreiche ineinandergreifende Prozesse zur irreversiblen Zellschädigung durch Apoptose, Onkose und Autophagie führt [1, 2]. Die pathophysiologischen Konsequenzen der Ischämie sind ausgesprochen komplex und laufen unter Beteiligung zahlreicher unterschiedlicher Mediatoren, Enzyme und Zellen ab. Typische Ursache eines Myokardinfarkts ist eine Unterbrechung bzw. maßgebliche Verminderung der Durchblutung von Herzmuskelgewebe, z.B. durch eine lichtungseinengende Sklerose der Koronargefäße. Obgleich durch eine Reperfusion ischämischen Myokards zwar grundsätzlich eine Rettung betroffener Myozyten möglich ist, birgt sie auch große Risiken und kann ihrerseits über das sog. Reperfusionssyndrom maßgeblich zur erlittenen Schädigung beitragen [1-4]. Als Krankheitsentität, die nicht nur sehr plötzlich, sondern häufig auch unerwartet und nicht zwingend mit einschlägigen, vorbestehenden Symptomen zum Tod führen kann, spielt der akute Myokardinfarkt in der rechtsmedizinischen Routine eine große Rolle. Ungeklärte Todesfälle, bei welchen die Möglichkeit eines Myokardinfarkts zwar besteht, aufgrund der gegebenen Umstände aber nicht sicher festgestellt werden kann, gelangen regelmäßig zur rechtsmedizinischen Untersuchung. Die zweifelsfreie Diagnose eines letalen Myokardinfarkts und die damit einhergehende Einordnung des Sachverhalts als natürlichen Tod stellt eine wesentliche Erkenntnis für ein Todesermittlungsverfahren dar.

Die postmortale Diagnostik eines Myokardinfakts stützt sich in erster Linie auf morphologische Verfahren. Diese umfassen nicht nur die Beurteilung makroskopischer, sondern insbesondere auch feingeweblicher Veränderungen, da die makromorphologische Diagnostik gerade im typischen rechtsmedizinischen Fallgut mit sehr akutem Todeseintritt häufig an ihre Grenzen stößt. Allerdings erfordert auch das Auftreten einschlägiger Gewebsveränderungen, welche mit konventionell-histologischen Färbeverfahren dargestellt werden können, zumindest eine Überlebenszeit von wenigen Stunden. Hier konnte die histomorphologische Diagnostik durch die Einführung enzym- und immunhistochemischer Färbeverfahren gegen Marker des akuten Myokardinfarkts maßgeblich verbessert werden [5, 6]. Letztlich blieb beziehungsweise bleibt aber nach wie vor bei Fällen mit sehr kurzen Überlebenszeiten von maximal ca. 20 Minuten die postmortale Diagnostik eines tödlichen Herzinfarkts ausgesprochen schwierig und es besteht nach wie vor Bedarf an neuen Methoden bzw. neuen Markern, die helfen, auch diese letzte diagnostische Lücke zu schließen. Die rechtsmedizinische Forschung hat hier neben den etablierten Verfahren auch neue Ansätze verfolgt, die aber letztlich nicht uneingeschränkt anwendbar sind. Postmortale biochemische Untersuchungen zeigen zwar einen Zusammenhang zwischen erhöhten Konzentrationen von Infarktmarkern, beispielsweise in der Perikardflüssigkeit, aufgrund vieler möglicher Einflussfaktoren und einer großen Streuung der bestimmten Werte bleiben aber relevante Unsicherheiten (zum Beispiel [7, 8]). Postmortale magnetresonanztomographische Untersuchungen sind ebenfalls in der Lage, perakute ischämische Läsionen darzustellen, allerdings bestehen auch hier Schwierigkeiten im Falle einer sehr kurzen Überlebenszeit, zudem ist ein hoher apparativer Aufwand erforderlich [9-11]. Vor diesem Hintergrund haben immunhistochemische Untersuchungen nach wie vor einen hohen Stellenwert in der rechtsmedizinischen Diagnostik des akuten Myokardinfarkts. Potenzielle

neue Marker, bei welchen eine Untersuchung ihrer Nutzbarkeit vielversprechend erscheint, finden sich in der klinischen Forschung bzw. der Grundlagenforschung zu den komplexen Mechanismen der zugrundeliegenden Pathophysiologie.

4.2 Das isoliert perfundierte Langendorff-Herz

Bei der Untersuchung neuer, potenzieller immunhistochemischer Marker für die postmortale Myokardinfarktdiagnostik spielt insbesondere die Frage nach der Geschwindigkeit des Nachweises relevanter Veränderungen nach Einsetzen der Ischämie eine entscheidende Rolle. Studien an humanen Gewebeproben gehen hier typischerweise mit dem Nachteil einher, dass der genaue Zeitpunkt des Beginns der Ischämie und damit die Zeitspanne bis zur Probenentnahme, bzw. in rechtsmedizinischen Fällen bis zum Todeseintritt, nicht bekannt ist. Tiermodelle bieten die Möglichkeit, gezielt Infarkte bzw. Ischämien zu provozieren und nach definierten Zeitspannen Gewebeproben zu entnehmen. Das Modell des isoliert perfundierten Säugetierherzens wurde 1895 von Oscar Langendorff entwickelt [12]. Die Methode umfasst die direkt postmortale Entnahme eines Herzens von z.B. einer Ratte, welches ohne Zeitverzug in eine Anlage verbracht und dort durch retrograde Perfusion der Koronargefäße über die Aorta „am Leben“ erhalten wird. Weiterentwicklungen des Ansatzes von Oscar Langendorff umfassten u.a. den Ersatz von Blut durch eine Perfusionslösung, sowie die Etablierung eines *working modes* [13]. Das in der Langendorff-Anlage für einige Stunden funktionsfähige Herz bietet die Möglichkeit grundlegender (patho-)physiologischer und funktioneller Untersuchungen [14]. Dazu gehört auch die für die vorliegende Arbeit relevante Möglichkeit der Induktion generalisierter oder lokaler Ischämien: eine Reduzie-

rung / ein kompletter Stopp der Perfusion führt zu einem allgemeinen Sauerstoffmangel im untersuchten Herz, wohingegen die Ligatur eines Koronargefäßes lokale myokardiale Ischämien verursacht [14, 15]. Durch Lösen einer Ligatur kann eine Reperfusion nach ischämischen Ereignissen simuliert werden. Die Fixierung der so manipulierten Herzen nach definierten Zeitintervallen in Formalin stellt das Äquivalent zur Überlebenszeit bei letalen Myokardinfarkten dar. Durch eine kombinierte Untersuchung von „Langendorff-Herzen“ und humanen Gewebeproben ist eine umfassende Evaluation potenzieller neuer Infarktmarker möglich.

5 Eigene Untersuchungen und Ergebnisse

5.1 Probenkollektiv

Die Untersuchung potenzieller neuer, immunhistochemischer Marker für die postmortale Myokardinfarktdiagnostik erfolgte sowohl an humanen Gewebeproben als auch an Rattenherzen unter Verwendung des Langendorff-Modells.

Humanes Herzmuskelgewebe wurde im Rahmen von Sektionen im Institut für Rechtsmedizin in Düsseldorf asserviert. Ausgewählt wurden Fälle mit einem konventionell-histologisch sicher nachgewiesenen, oder aufgrund der vorhandenen Informationen zur Vorgeschichte (Krankengeschichte der verstorbenen Person, etwaige typische Symptome / Beschwerden) zumindest höchstwahrscheinlich zu vermutenden, letalen Myokardinfarkt. Auf der Basis der Ergebnisse konventionell-histologischer Färbungen mit Hämatoxylin-Eosin (HE) wurde eine genauere Einschätzung des Alters der Myokardinfarkte und eine Gruppeneinteilung, angelehnt an die Stadien-Einteilung nach Cummings et al. [16], vorgenommen (Tabelle 1). Die Zahl der in die Studiengruppe

aufgenommenen Fälle variierte in den Arbeiten zwischen 32 und 61 und in der Kontrollgruppe zwischen 9 und 11. Als Kontrollfälle wurden solche ausgewählt, bei welchen sicher eine andere Todesursache als ein letaler Myokardinfarkt vorlag. Diese Todesursachen umfassten verschiedene Formen des Erstickens (Erhängen, Erdrosseln, Erwürgen, Thoraxkompression, Kohlenmonoxidintoxikation), Polytrauma und zentrales Regulationsversagen bei Kopfschuss.

Tabelle 1: Einteilung des Infarktalters bei humanen Gewebeproben anhand konventionell histologischer Befunde nach Cummings et al. [16]. (eigene Tabelle)

Gruppe	Infarktalter	konventionell histologische Befunde
A	0 - 4 Stunden	keine
B	4 - 24 Stunden	Kontraktionsbandnekrosen, hypereosinophile Zellen, Verlust der Kernfärbbarkeit, Hämorrhagien
C	1 - 7 Tage	vorwiegend granulozytäres Entzündungszellinfiltrat
D	8 - 14 Tage	Granulationsgewebe, beginnende Vaskularisierung
E	2 Wochen - 2 Monate	Kollagenablagerung, abnehmende Vaskularisierung
F	über 2 Monate	kollagene Narbe

Im Langendorff-Modell wurden Rattenherzen durch eine Ligatur der linken Koronararterie lokale Ischämien / Myokardinfarkte zugefügt. Die „Überlebenszeit“, also die Zeit nach Beginn der Ischämie, wurde über einen unterschiedlich langen Verbleib der Herzen an der Anlage, bevor eine sofortige Fixierung in Formalin erfolgte, variiert. Mit Blick auf die Frage des zeitlichen Nachweises potenzieller Myokardinfarktmarker wurden 24 Herzen Infarkte ohne Reperfusion mit Überlebenszeiten von 5 – 60 Minuten zugefügt. Bei 5 weiteren Herzen wurde die Gefäßligatur nach 20 Minuten gelöst und dadurch eine Reperfusion des Infarktareals ermöglicht. Die anschließenden Reperfusionszeiten betrugen zwischen 10 und 40 Minuten, entsprechend variierte auch die Gesamtzeit nach Anlegen der Ligatur. Als Kontrollgruppe dienten 7 Herzen, davon wurden 2 Herzen für 60 Minuten ohne weitere Manipulation in der Langendorff-Anlage belassen, bei

3 Herzen wurden durch Elektroschocks mehrere Sekunden andauernde Arrhythmien provoziert und 2 Herzen wurden nach postmortaler Entnahme direkt in Formalin fixiert. Zur Untersuchung möglicher Einflüsse des Geschlechts und einer ischämischen Präkonditionierung auf die untersuchten Marker wurden jeweils 12 Herzen männlicher und 12 Herzen weiblicher Ratten durch Ligatur der linken Koronararterie Myokardinfarkte ohne Reperfusion mit Überlebenszeiten zwischen 10 Minuten und 60 Minuten zugefügt. Weitere 12 Herzen männlicher Ratten erfuhren zunächst eine ischämische Präkonditionierung durch Unterbrechung der Perfusion des gesamten Herzens über eine Zeit von 30 Minuten. Danach erfolgte eine reguläre Perfusion über 60 Minuten, bevor die Ligatur der linken Koronararterie erfolgte. Die Überlebenszeiten in diesen Fällen variierten ebenfalls zwischen 10 Minuten und 60 Minuten. Eine Übersicht über die Versuche mit dem Langendorff-Modell findet sich in Tabelle 2.

Tabelle 2: Überblick über die Versuche mit dem Langendorff-Modell. IP = ischämische Präkonditionierung. (eigene Tabelle)

	Ischämiedauer	Reperfusionsdauer	Anzahl Herzen
Etablierung neuer Marker	5 bis 60 Minuten	keine	24
	20 Minuten	10 bis 40 Minuten	5
Kontrollen	keine, 60 Minuten in Anlage	keine	2
	keine, Elektroschocks, 20 bis 30 Minuten in Anlage	keine	3
	keine, nach Entnahme direkt fixiert	keine	2

	Geschlecht	Ischämiedauer	Anzahl Herzen
Einfluss Geschlecht	männliche Ratten	10 bis 60 Minuten	12
	weibliche Ratten	10 bis 60 Minuten	12

	IP	Ischämiedauer	Anzahl Herzen
Einfluss IP	30 Minuten Ischämie, 60 Minuten Perfusion	10 bis 60 Minuten	12
	ohne	10 bis 60 Minuten	12

5.2 Oxidativer Stress: Dityrosin

Relevante Arbeiten:

[17] **Mayer F**, Pröpper S, Ritz-Timme S, Dityrosine, a protein product of oxidative stress, as a possible marker of acute myocardial infarctions, Int J Legal Med, 2014, 128: 787-794

[18] **Mayer F**, Falk M, Huhn R, Ritz-Timme S, Dityrosine as a marker of acute myocardial infarction? Experiments with the isolated Langendorff heart, Int J Legal Med, 2016, 130: 1053-1060

Auch im gesunden Myokard werden während physiologischer Prozesse sogenannte *reactive oxygen species* (ROS), das heiß hochreaktive Sauerstoffradikale, gebildet, welche allerdings durch zell- und gewebeeigene Schutzmechanismen, zum Beispiel die Superoxiddismutasen, abgefangen werden [19]. Im Rahmen eines Myokardinfarkts entsteht ein Milieu oxidativen Stresses, welches verursacht wird durch ein Ungleichgewicht der oxidativen ROS und der antioxidativen Schutzsubstanzen [19-22]. In Folge einer Ischämie und einer eventuellen Reperfusion werden vermehrt ROS gebildet, welche die Kapazitäten des antioxidativen Systems übersteigen und als Konsequenz Proteine, unter anderem auch an der Energiegewinnung beteiligte Enzyme, Lipide und Desoxyribonukleinsäure (DNS) oxidieren und schädigen und dadurch Anteil haben am ischämiebedingten Energiedefizit und an Zellschäden, wodurch Apoptose und Nekrose beschleunigt werden [19, 20, 23]. Die Entstehung von ROS stimuliert außerdem die Bildung von Cytokinen und führt zur Aktivierung von Matrixmetalloproteinasen (MMPs) [24]. Die Hauptquelle für die frühe Entstehung von ROS im ischämischen Myokard sind die Mitochondrien, in welchen es zu einem vermehrten *electron leakage*

aus den Komplexen der Atmungskette kommt [19-23, 25, 26]. Weiteren Anteil an der Bildung von ROS haben beispielsweise eine vermehrte Umsetzung von Xanthin und Hypoxanthin aufgrund einer hohen Konzentration an Xanthinoxidase [19, 20, 23, 24], eine vermehrte Produktion von Stickoxid [19, 21, 23, 25], sowie im weiteren Verlauf des Infarktes die Zellen des Immunsystems [23, 24]. Nicht überraschend wurde eine Unterstützung des körpereigenen, antioxidativen Systems als Therapieoption nach Myokardinfarkten vorgeschlagen [22-24].

Mit Blick auf den schädigenden Effekt an Proteinen attackieren ROS unter anderem Tyrosin-Reste in Proteinen und provozieren damit die Entstehung von Tyrosyl-Radikalen; über inter-molekulares *crosslinking* zweier solcher Tyrosyl-Radikale entsteht das Dimer Dityrosin [23, 27-29]. Obgleich Dityrosin auch natürlicherweise in bestimmten Geweben vorkommt, wird es als Biomarker für eine oxidative Proteinschädigung angesehen [29]. Im Rahmen von Erkrankungen wird die Bestimmung von Dityrosin als Nachweis erhöhten oxidativen Stresses genutzt (zum Beispiel [30]). Da Dityrosin im Gewebe auch immunhistologisch detektiert werden kann [31] war die Nutzung bei der postmortalen Myokardinfarktdiagnostik überprüfenswert.

Die Untersuchungen von Rattenherzen, welchen im Langendorff-Modell lokale Infarkte ohne Reperfusion zugefügt wurden, zeigten erste positive, immunhistochemische Färbeergebnisse für Dityrosin bereits nach einer sehr kurzen Überlebenszeit von 5 Minuten. Überhaupt wiesen die Herzen der Studiengruppe nur einzelne, vollkommen negative Färbeergebnisse auf (Beispiel in Abbildung 1). Positive Färbeergebnisse ergaben sich auch für die Herzen mit Reperfusion, ohne, dass sich wesentliche Unterschiede zu nicht-reperfundierten Infarkten gezeigt hätten. Auch die feingeweblichen Schnitte der Kontrollherzen zeigten einige positive Färbebefunde, allerdings waren diese aufgrund offensichtlicher Unterschiede im Färbemuster und der Lokalisation problemlos von den Herzen der Studiengruppe zu unterscheiden.

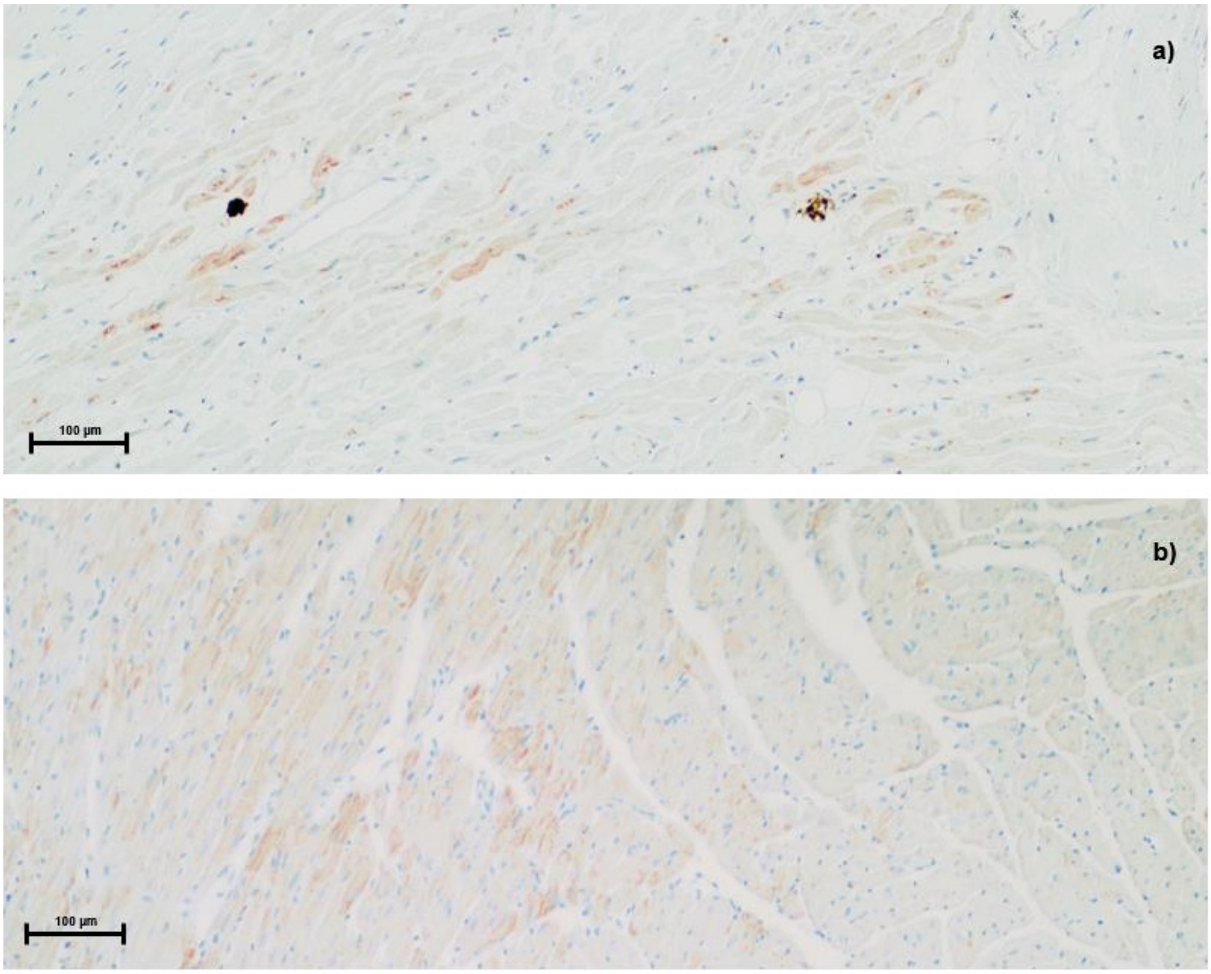

Abbildung 1: Dityrosin, Beispiele für positive Färbeergebnisse, jeweils 100-fache Vergrößerung a) humanes Myokard der Gruppe A mit einer anzunehmenden Überlebenszeit von 0 – 4 Stunden b) Rattenherz mit einer Überlebenszeit von 20 Minuten. (eigene Abbildung)

Die Studie an menschlichem Herzmuskelgewebe mit insgesamt 61 Fällen ergab in der Gruppe mit einer sehr kurzen Überlebenszeit, bei welcher ein Myokardinfarkt aufgrund einer typischen Klinik / Symptomatik sehr wahrscheinlich war, einzelne positive Ergebnisse (Beispiel ebenfalls in Abbildung 1). Die Vergleichsfärbungen gegen die etablierten Infarktmarker $C_{5b\text{-}9}$ und Fibronektin zeigten ebenfalls einzelne positive Befunde, allerdings in anderen Fällen. In der Gruppe mit Infarkten und Überlebenszeiten von etwa 4 bis 24 Stunden wiesen nahezu alle Fälle positive Färbeergebnisse für alle drei Marker auf, lediglich vereinzelt blieb eine Färbung negativ. Ab Überlebenszeiten von

etwa 2 Wochen nahm die Zahl der positiven Färbeergebnisse für alle Marker deutlich ab und es zeigte sich wieder ein Überwiegen negativer Färbeergebnisse.

5.3 Degradation der extrazellulären Matrix, cardiac remodelling: Matrixmetalloproteinasen und tissue inhibitors of matrixmetalloproteinases

Relevante Arbeit:

[32] **Mayer F**, Falk M, Huhn R, Behmenburg F, Ritz-Timme S, Matrixmetalloproteinases and tissue inhibitors of metalloproteinases: Immunhistochemical markers in the diagnosis of lethal myocardial infarctions? For Sci Int, 2018, 288: 181-188

Bei MMPs und ihren Inhibitoren den *tissue inhibitors of metalloproteinases* (TIMPs) handelt es sich um eine Familie von Enzymen, welche in zahlreichen Geweben vorkommen. MMPs sind beteiligt an der Degradation von Peptiden, insbesondere der extrazellulären Matrix, wobei sich die einzelnen Enzyme bezüglich ihrer präferierten Substrate unterscheiden [33-35]. Eine Untergruppe der MMPs bilden die sogenannten Gelatinasen, hierzu zählen MMP-2 und MMP-9, welche neben Gelatin auch Kollagene degradieren [36]. Nahezu alle MMPs werden als inaktive Form, sogenannte proMMP, synthetisiert, welche in erster Linie proteolytisch aktiviert werden [37]. Reguliert werden MMPs durch ihre natürlichen Inhibitoren, die sogenannten TIMPs. Eine reguläre Expression von TIMPs leistet einen wesentlichen Beitrag zur Erhaltung einer normalen Myokardstruktur [36]. MMPs spielen eine wichtige Rolle bei zahlreichen physiologischen (Gewebeentwicklung und -differenzierung, Wachstum) und pathologischen Prozessen. Mit Blick auf das kardiovaskuläre System ist MMP-2 beispielsweise an der Entwicklung von Gefäßen und Herzklappen beteiligt [33], MMPs werden aber auch zu den Biomarkern des Herzversagens gezählt [38]. Im Rahmen von Myokardinfarkten

kommt es frühzeitig zu einer Aktivierung und einer vermehrten Synthese verschiedener MMPs und TIMPs in ischämischem Herzmuskelgewebe [36, 39, 40]. Für MMP-2 weist eine Studie an einem Rattenmodell beispielsweise erhöhte Aktivitäten ab etwa eine Stunde nach Infarkt aus, für MMP-9 konnte nach etwa 2 Stunden eine erhöhte Aktivität nachgewiesen werden [41]; in einem Mausmodell fand sich eine Erhöhung von zirkulierendem MMP-9 binnen eines Tages nach Myokardinfarkt [42], im gleichen Zeitraum wurde eine erhöhte MMP-9 Aktivität in einem Kaninchenmodell festgestellt [43]. In einer Studie mit Schweinen konnte eine erhöhte MMP-Aktivität in ischämischem Myokard bereits nach 10 Minuten gefunden werden [44]. Im Plasma von Patienten mit akutem Myokardinfarkt wurden innerhalb eines Tages insbesondere eine erhöhte Konzentration vom MMP-9, aber auch von TIMP-1 detektiert [45]. Eine erhöhte Expression von TIMP-1 in infarziertem Myokard weist eine Studie mit Ratten nach [46]. Verschiedene Arbeiten konnten darüber hinaus zeigen, dass MMPs im Rahmen einer Ischämie des Herzens wohl auch am Abbau intrazellulärer Proteine beteiligt sind, und das offenbar bereits kurze Zeit nach Beginn des Sauerstoffmangels im Moment des sogenannten *myocardial stunning*, ausgelöst durch oxidativen Stress, wobei hier auch eine nicht-proteolytische Aktivierung möglich ist [35, 47, 48]. Ischämien des Myokards und dadurch entstehender oxidativer Stress können außerdem zu einer Imbalance von MMPs und TIMPs führen [20, 24, 49], wodurch das genannte *myocardial stunning* begünstigt wird [33]. Im weiteren Zeitverlauf nach einem Infarkt sind MMPs an der Zellkommunikation [39] und vor allem am *cardiac remodelling* beteiligt [50, 51]. Klinisch wurde eine Nutzung von MMPs und TIMPs als prognostische Marker diskutiert [42, 52], außerdem wurden therapeutische Ansätze zur Reduzierung des *cardiac remodelling* nach Infarkten, beispielsweise über eine Hemmung von MMPs durch Gabe von

TIMPs oder anderer, die Wirkung von MMPs blockierender Medikamente, darunter unter anderem Tetrazyklin-Antibiotika, vorgeschlagen [37, 47, 53].

Vor dem Hintergrund einer Nutzung als Marker in der postmortalen Myokardinfarktdiagnostik wurden immunhistochemische Färbungen an einem Probenset wie oben dargestellt durchgeführt. Die humanen Proben umfassten in dieser Studie Myokard aus insgesamt 32 Fällen. In der Studiengruppe der Rattenherzen zeigten sich positive Färbebefunde ab einer Überlebenszeit von 20 Minuten (TIMP) beziehungsweise 25 Minuten (MMP-9) und 30 Minuten (MMP-2); dies galt sowohl für Herzen mit, als auch ohne Reperfusion. Herzen mit längeren Überlebenszeiten zeigten allerdings nicht durchweg positive Färbeergebnisse, auch bei Überlebenszeiten von 60 Minuten wurden noch negative Befunde (TIMP-1) festgestellt. Die Kontrollgruppe wies durchweg negative Färbeergebnisse auf. Nebenbefundlich ergaben die Versuche mit dem Langendorff-Modell positive Färbeergebnisse für die untersuchten Marker in Umgebung mechanischer Myokardverletzungen (Einstichstellen von Gefäßligaturen und EKG-Elektroden). In sieben Fällen kamen derartige Verletzungen auf den Schnitten deutlich zur Darstellung, sechs davon wiesen positive Färbeergebnisse für die untersuchten Marker auf: MMP-9 war in allen sechs Fällen positiv, MMP-2 und TIMP-1 jeweils in einem Fall (Beispiele zeigt Abbildung 2).

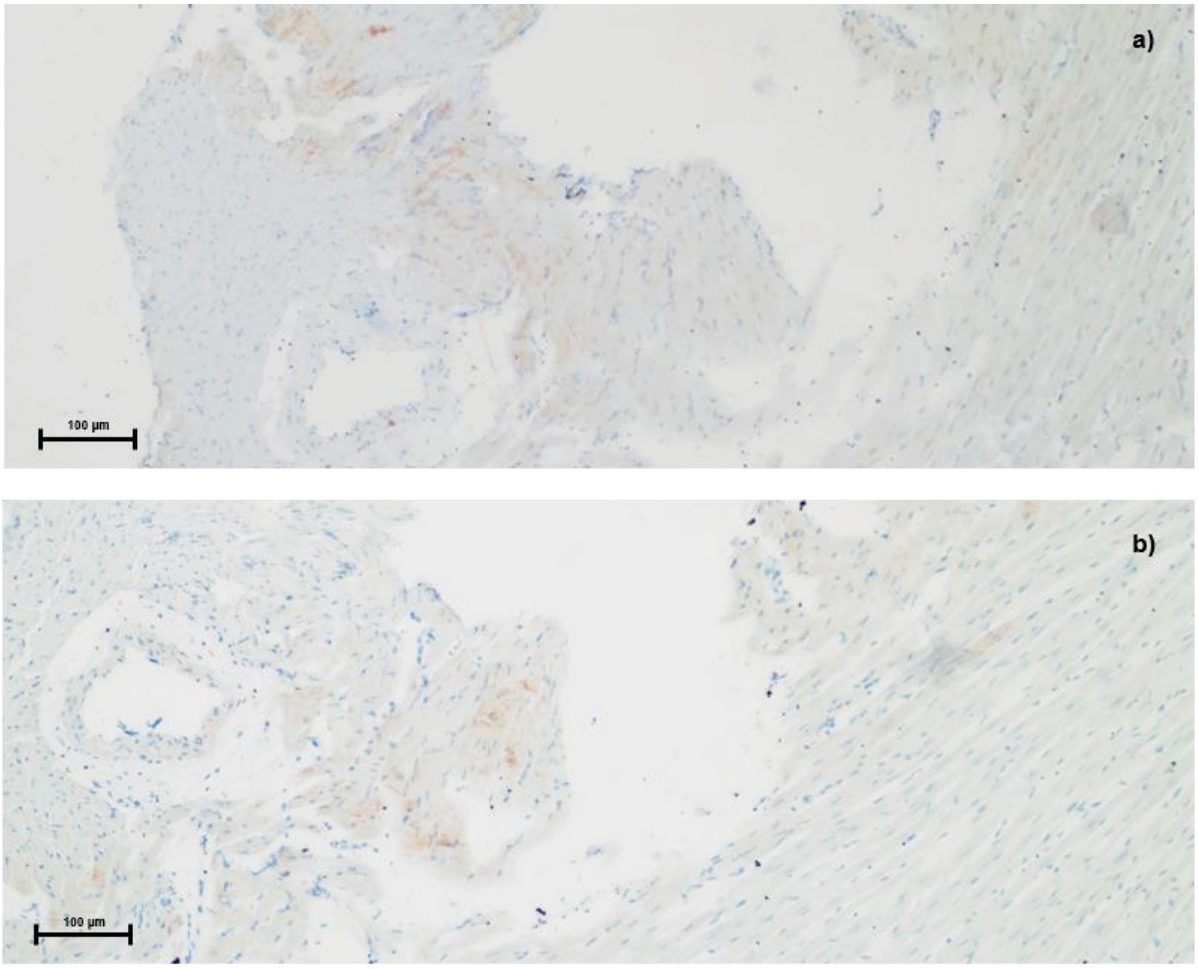

Abbildung 2: Beispiele für positive Färbebefunde in Umgebung mechanischer Muskelverletzungen (hier Gefäßligatur) bei Versuchen mit dem Langendorff-Modell, Herz mit einer Überlebenszeit von 60 Minuten, jeweils 100-fache Vergrößerung a) MMP-2 b) MMP-9. (eigene Abbildung)

Demgegenüber waren die Ergebnisse des menschlichen Probenkollektivs deutlich heterogener. Eine vermehrte Aktivität bzw. Synthese konnte für alle drei untersuchten Marker lediglich in Fällen mit einer längeren Überlebenszeit (ab etwa einem Tag) festgestellt werden. Die für die forensische Fragestellung besonders relevante Gruppe mit einer geringen Überlebenszeit im Rahmen weniger Stunden zeigte lediglich einen positiven Befund für TIMP-1. Die Kontrollgruppe zeigte positive Ergebnisse für MMP-9 im Myokard des rechten Ventrikels in drei Fällen, wobei in zwei der Fälle kardiopulmonale Wiederbelebungsmaßnahmen durchgeführt wurden und in einem Fall eine vermehrte Rechtsherzbelastung bei Lungenthrombembolie zu diskutieren war.

5.4 Biomechanischer Stress: Endothelin-1, growth differentiation factor-15

Relevante Arbeit:

[54] Falk M, Huhn R, Behmenburg F, Ritz-Timme S, **Mayer F**, Biomechanical stress in myocardial infarctions: can endothelin-1 and growth differentiation factor 15 serve as immunohistochemical markers? Int J Legal Med, 2018, 132: 509-518

Die ischämisch bedingte Schädigung von Myozyten äußert sich unter anderem in einem Verlust interzellulärer Verbindungen. In der Folge kommt es zu einer vermehrten Dehnung der Myozyten, welche auch als biomechanischer Stress bezeichnet wurde [55]. Der vermehrten Dehnung der Herzmuskelzellen wird eine erhöhte Sekretion und Synthese verschiedener Markermoleküle, darunter Endothelin-1 (ET-1) und *growth differentiation factor-15* (GDF-15), zugeschrieben [56-58].

Das Zytokin GDF-15 gehört zur *transforming growth factor-ß* (TGF-ß) Familie und erfüllt wichtige Funktionen im Rahmen der Entwicklung, Differenzierung und Reparatur von Gewebe [59]. In gesundem Gewebe wird GDF-15 stark in Plazenta und Prostata exprimiert, in anderen Geweben, darunter auch das Myokard, erfolgt allenfalls eine geringgradige Expression [59]. Im Rahmen einer Ischämie, beziehungsweise eines Infarkts, kommt es zu einer gesteigerten Synthese von GDF-15 in der Herzmuskulatur, welche durch Verminderung der Rekrutierung von Entzündungszellen und dadurch bedingter Senkung des Rupturrisikos letztlich einen kardioprotektiven Effekt hat [59, 60]. Im Tiermodell konnte eine erhöhte Expression von GDF-15 binnen einer Stunde nach Einsetzen der Ischämie festgestellt werden [61]. Im weiteren Verlauf nach einem Infarkt verhindert beziehungsweise reduziert GDF-15 die myokardiale Hypertrophie [59]. Verschiedene Studien ergaben einen erhöhten Nachweis von GDF-15, zum Beispiel im Sinne einer erhöhten Konzentration im Serum, bei Patienten mit schweren

Komplikationen nach Myokardinfarkten, weshalb die Nutzung als Prognosemarker vorgeschlagen wurde [62-67].

Das Endothelin-System umfasst die vier Isoformen ET-1, ET-2, ET-3 und ET-4, wobei ET-1 die wichtigste Isoform darstellt und der stärkste bekannte Vasokonstriktor ist [68, 69]. ET-1 wird nicht nur in den Zellen der Herzgefäße, sondern auch in den Kardiomyozyten selbst synthetisiert [69]. Normale Konzentrationen von ET-1 sind wichtig für einen physiologischen Gefäßtonus, im Rahmen von Herzinfarkten finden sich aber erhöhte Konzentrationen – besonders lang andauernd nach schwerwiegenden und komplikationsreichen Infarkten – welche eine Rolle spielen bei der Pathophysiologie des Infarkts selbst (Myokardnekrose, Arrhythmien), bei der Narbenbildung nach Infarkt (chemotaktischer Effekt auf Makrophagen und Stimulation von neutrophilen Granulozyten) und beim *remodelling* (Proliferation von Fibroblasten und Bildung der eM) [68, 69]. Indirekt bewirken erhöhte Konzentration an ET-1 auch eine gesteigerte Aktivität von MMPs [70]. Erhöhte Konzentrationen von ET-1 gehen zum einen auf eine gesteigerte Synthese, zum anderen insbesondere zu Beginn des Infarkts auf eine Freisetzung aus intrazellulären Speichern zurück [69]. Neben der oben genannten Dehnung von Kardiomyozyten, dem sogenannten biomechanischen Stress, wird ET-1 auch vermehrt infolge des Sauerstoffmangels im Rahmen einer Ischämie exprimiert: Untersuchungen an Zellkulturen glatter Muskelzellen konnten zeigen, dass die Expression von ET-1 durch oxidativen Stress gesteigert wird [71-73]. Ein Studie an ischämischen Schweineherzen ergab nicht nur, dass bereits eine alleinige Ischämie zu einer gesteigerten ET-1 Synthese führt, sondern auch, dass diese vorwiegend in den Myozyten stattfindet [74]. Klinische Studien zeigten eine erhöhte Langzeit-Mortalität bei Patienten mit Herzinfarkt und erhöhten Serumkonzentrationen von ET-1 [68, 75].

Die immunhistochemischen Färbungen der Rattenherzen der Studiengruppe sowohl mit als auch ohne Reperfusion, ergaben für beide Marker positive Befunde im Stromgebiet des ligierten Gefäßes in allen Fällen, also ab einer Überlebenszeit von 5 Minuten. Die Färbeergebnisse für ET-1 fielen im Vergleich etwas diskreter aus als für GDF-15. Die Kontrollherzen ohne Manipulation zeigten positive Färbebefunde lediglich im Sinne typischer Artefakte. Im Falle der Kontrollherzen mit Arrhythmien fanden sich positive Färbebefunde zirkulär in der Wand der linken Kammer.

In humanem Gewebe konnte ein Nachweis von ET-1 zuverlässig erst bei Fällen mit Überlebenszeiten von wenigstens etwa einer Woche, bei welchen bereits konventionell-histologisch typische, infarktbedingte Veränderungen nachweisbar waren, gelingen. Bei Fällen mit geringeren Überlebenszeiten fanden sich nur einzelne positive Färbebefunde. Demgegenüber war GDF-15 in nahezu allen Fällen positiv, wobei die Färbebefunde bereits bei geringen Überlebenszeit zum Teil sehr kräftig ausfielen. GDF-15 war auch in allen humanen Kontroll-Fällen positiv. Für ET-1 fanden sich hier ebenfalls positive Befunde, allerdings betont in der Muskulatur der rechten Herzkammer, und zwar in Fällen mit einer anzunehmenden, erhöhten Rechtsherzbelastung (Lungenembolie, Erhängen, Ersticken).

5.5 Geschlecht und ischämische Präkonditionierung: Auswirkung auf den Nachweis immunhistochemischer Marker des akuten Myokardinfarkts

Relevante Arbeit:

[76] Scholl K, Huhn R, Ritz-Timme S, **Mayer F**, The impact of sex and myocardial ischemic preconditioning on immunohistochemical markers of acute myocardial infarction, Int J Legal Med, 2018, 133: 529-538

Zahlreiche Faktoren können einen Einfluss auf Schwere und Verlauf eines Myokardinfarkts haben, darunter auch das Geschlecht der betroffenen Person, sowie eine potenziell stattgehabte, ischämische Präkonditionierung.

Obgleich die exakten, zugrundeliegenden Mechanismen noch nicht vollständig geklärt sind und Studienergebnisse kein einheitliches Bild ergeben, muss davon ausgegangen werden, dass das Geschlecht einen relevanten Einfluss auf Häufigkeit, Schwere und *outcome* von Herzinfarkten hat [77, 78]. In einigen Tierversuchen zeigten Herzen weiblicher Individuen eine größere Resistenz gegenüber Ischämie- / Reperfusionsschäden mit geringeren Infarktgrößen und geringer eingeschränkter Pumpfunktion [79-84]. Gleichsam existieren Studien, die in dieser Hinsicht keine relevanten Unterschiede feststellen konnten [85, 86]. Humanes Myokard von Patient*innen mit Herzversagen und Herztransplantation zeigte bei Frauen ein deutlich geringeres Ausmaß von Myozytenapoptose und -nekrose [87]. Klinische Studien weisen eine höhere Inzidenz von Herzinfarkten bei Männern aus, wohingegen bei Frauen, insbesondere bei jüngeren, eine höhere Mortalität beobachtet wird [80, 88-90]; allerdings ist zu bedenken, dass in den Studienpopulationen neben dem Geschlecht auch weitere Einfluss-

faktoren existieren (z.B. Alter, prä- / post-Menopause, Risikofaktoren, weitere Vorerkrankungen, klinische Symptomatik) und die Unterschiede im Laufe der letzten Jahrzehnte zumindest teilweise geringer geworden sind.

Unter ischämischer Präkonditionierung versteht man kurze Episoden vorübergehender, sowohl lokaler, als auch globaler Ischämien, welche einen protektiven Effekt hinsichtlich folgender ischämischer Ereignisse des Herzens (aber auch anderer Organe / Gewebe) haben [4]. Obgleich noch nicht im Detail verstanden, löst eine ischämische Präkonditionierung eine komplexe Signalkaskade aus, welche letztlich zur Aktivierung mehrerer *pro-survival pathways* führt [23, 91, 92]. Es konnte gezeigt werden, dass der Effekt der ischämischen Präkonditionierung in zwei Zeitintervallen, einmal nach ein bis zwei Stunden und dann erneut in einer *second window of* protection nach etwa 24 Stunden, eintritt [1, 4, 91]. Im experimentellen Setting bewirkt die ischämische Präkonditionierung an Herzen eine Verminderung der Infarktgröße [1, 2, 93-96]. Der protektive Effekt wirkt sich aber nicht nur auf die Zell- / Gewebeintegrität, beispielsweise durch Verringerung der Mitochondrien-Schädigung [26, 97], sondern offenbar auch auf die Herzunktion aus [98], zum Beispiel durch Schutz vor dem *myocardial stunning* [99] oder vor Rhythmusstörungen [95]. Als klinisches Korrelat der ischämischen Präkonditionierung wird die prä-Infarkt Angina angesehen [100-102].

Für die Studie zur möglichen Relevanz bei der postmortalen Myokardinfarkt-Diagnostik wurden insgesamt 44 Rattenherzen wie oben dargestellt mit Hilfe des Langendorf Modells untersucht. Es wurden immunhistochemische Färbungen gegen die bereits etablierten Infarktmarker *cardiac troponin T* (cTnT) und *cardiac troponin I* (CTnI), sowie das in vorangegangenen Studien untersuchte Dityrosin (siehe 5.1) angefertigt. Außerdem wurde als weiterer Marker dephosphoryliertes Connexin 43 (Cx43) ausgewählt, welches in Versuchen anderer Arbeitsgruppen vielversprechende Ergebnisse gezeigt hatte [103]. Für Cx43 konnte außerdem bereits nachgewiesen werden, dass es nach

erfolgter ischämischer Präkonditionierung zu einer verminderten Dephosphorylierung und einer verminderten Verlagerung im Laufe der Ischämie zunächst in den Bereich der *gap junctions* und schließlich in intrazellulären Pools kommt [4, 104].

Die Färbeergebnisse zeigten für den Marker Dityrosin keine Unterschiede bezüglich des Geschlechts oder einer erfolgten ischämischen Präkonditionierung. Unter den Kontrollherzen fanden sich positive Färbebefunde nach direkter Fixierung in Formalin (entsprechend den Ergebnissen der vorangehenden Studien), sowie bei den Herzen, welche lediglich eine ischämische Präkonditionierung erfahren hatten.

Für Cx43 ergaben sich keine wesentlichen Unterschiede mit Blick auf das Geschlecht, die Intensität der Färbebefunde hing vielmehr von der Überlebenszeit nach Gefäßligatur ab. Demgegenüber waren die Färbebefunde im Myokard von Herzen mit ischämischer Präkonditionierung deutlich diskreter ausgeprägt (Beispiele in Abbildung 3). Einzelne Kontrollherzen (ein Herz in der Langendorff-Anlage für 60 Minuten, ein direkt fixiertes Herz und ein Herz mit alleiniger ischämischer Präkonditionierung) zeigten ebenfalls diskrete, positive Färbeergebnisse.

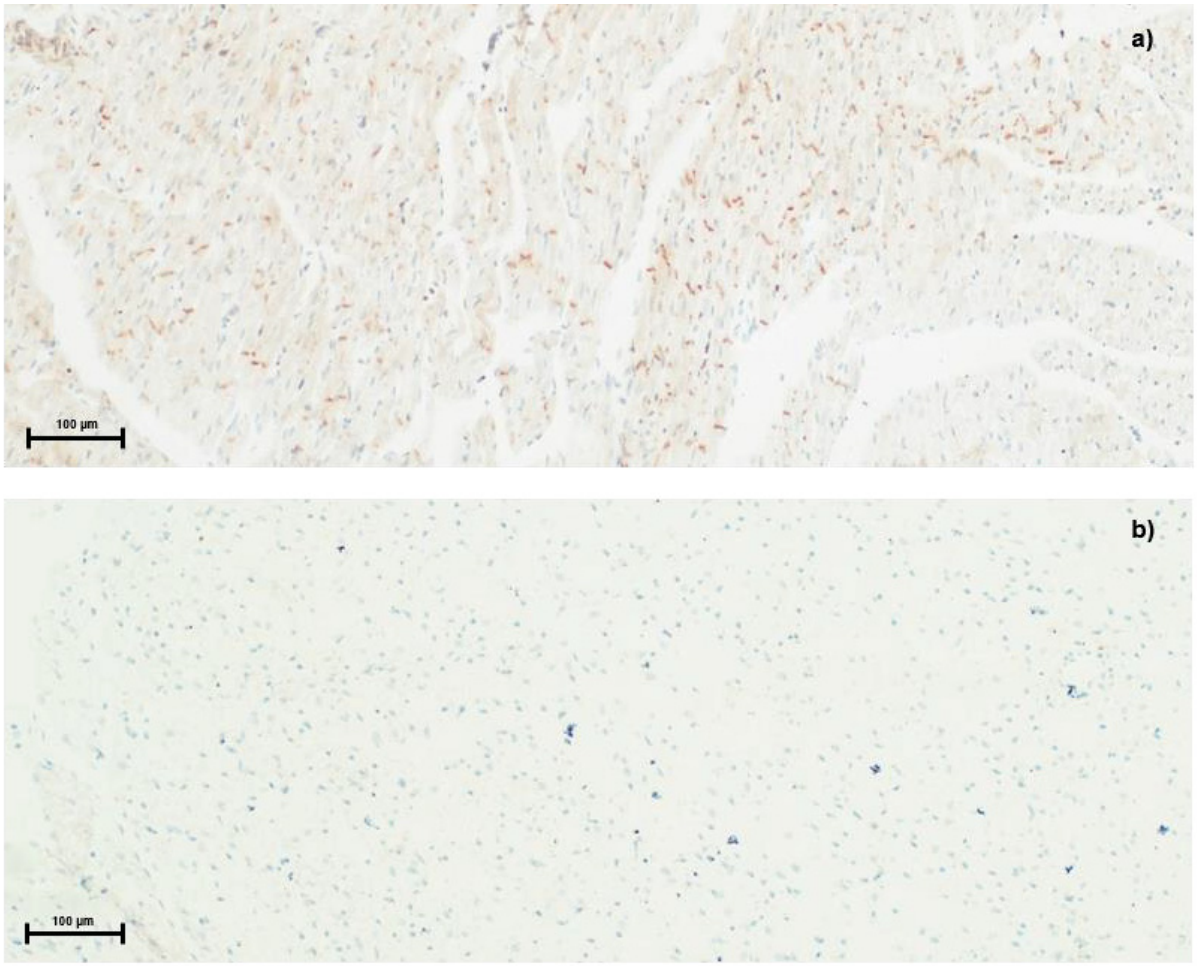

Abbildung 3: Connexin 43, Beispiele für Ergebnisse von Untersuchungen mit dem Langendorff-Modell, jeweils 100-fache Vergrößerung a) 50 Minuten Überlebenszeit ohne ischämische Präkonditionierung b) 60 Minuten Überlebenszeit mit ischämischer Präkonditionierung. (eigene Abbildung)

Die Ergebnisse für cTnT und cTnI zeigten generell sehr deutliche, positive Färbebefunde im Sinne einer Depletion, mit Blick auf cTnT schien diese bei Herzen männlicher Ratten etwas ausgeprägter auszufallen. In Herzen mit ischämischer Präkonditionierung war die Depletion noch ausgeprägter als in Fällen ohne eine solche (Beispiele in Abbildung 4).

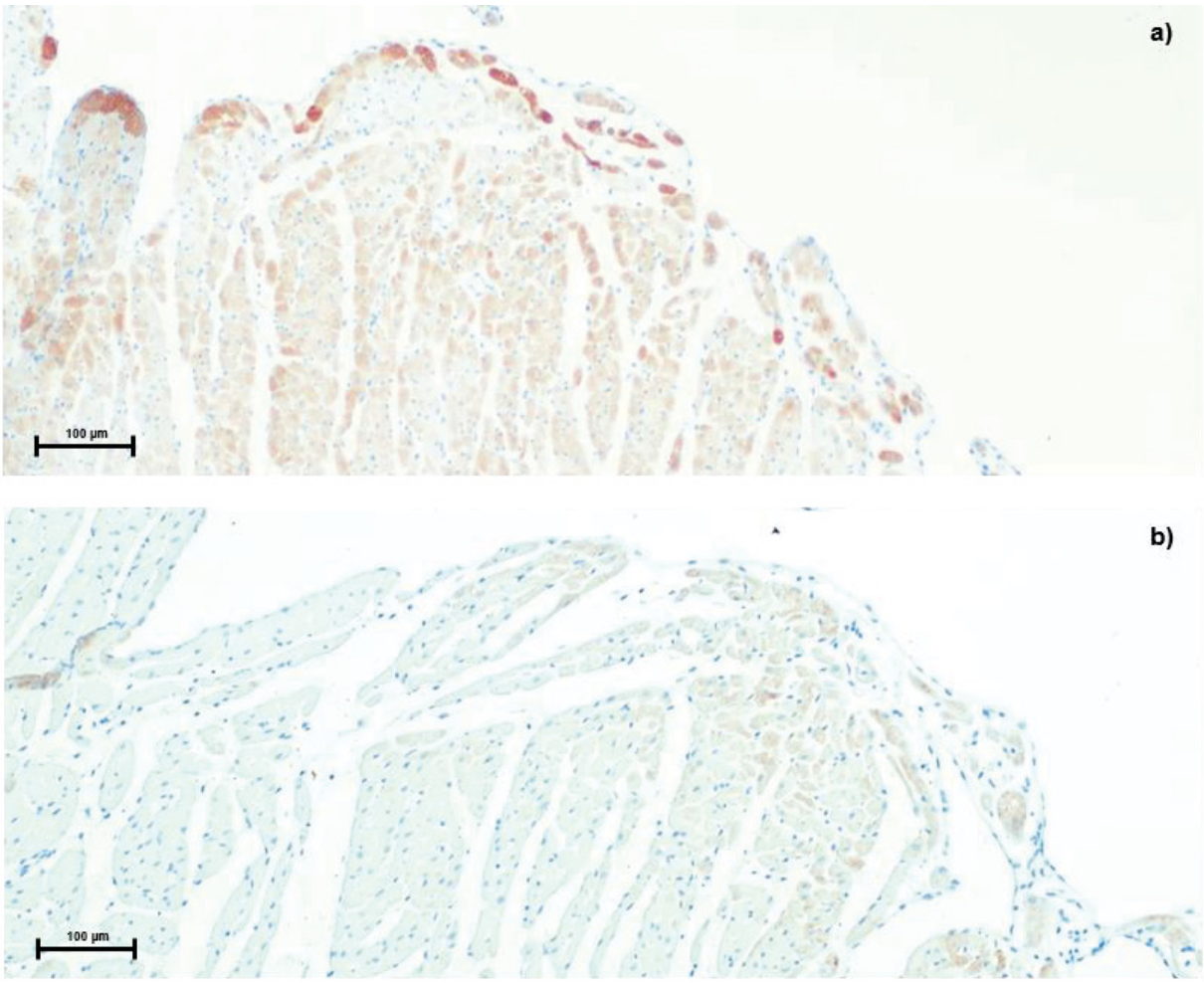

Abbildung 4: *Cardiac troponin T*, Beispiele für Färbeergebnisse bei Untersuchungen mit dem Langendorff-Modell, Überlebenszeit in beiden Versuchen 30 Minuten, jeweils 100-fache Vergrößerung a) ohne ischämische Präkonditionierung b) mit ischämischer Präkonditionierung. (eigene Abbildung)

Auch die Kontrollherzen zeigten einen deutlichen Verlust der Troponine. Auffallend waren hier vollkommen ausbleibende Färbungen für cTnI bei allen Kontrollherzen abgesehen von einem Herz mit ischämischer Präkonditionierung. Diese Auffälligkeit konnte methodisch nicht erklärt werden: auch bei Wiederholung der Färbungen blieb das völlige Fehlen einer Anfärbbarkeit bestehen, zeitgleich gefärbte Studienherzen wiesen aber normale Färbebefunde auf.

6 Diskussion

Im Vergleich zu einer rein makromorphologischen Beurteilung und zur Aussagekraft konventionell histologischer Färbungen gelingt der postmortale Nachweis eines Myokardinfarkts über immunhistochemische Methoden, im Sinne eines (vermehrten) Auftretens oder einer (vermehrten) Depletion bestimmter Marker infolge einer Zellschädigung [105-132], auch bei verhältnismäßig akutem Todeseintritt. Letztlich setzen aber auch die bislang etablierten Marker eine gewisse Überlebenszeit nach Einsetzen der Ischämie voraus, wobei Studienergebnisse hinsichtlich der mindestens zu veranschlagenden Überlebenszeiten unterschiedliche Ergebnisse ausweisen. So gelang der immunhistochemische Nachweis des Komplementkomplex C_{5b-9} frühestens ab etwa einer Überlebenszeit von 30 Minuten [133] beziehungsweise von etwa 2 Stunden [103]. Für die Myozytenproteine cTnT, cTnI, Creatinkinase (CK), *fatty acid binding protein* (FABP) und Desmin werden als früheste Nachweiszeiten 15 bis 30 Minuten [122, 127, 133] und etwa 1 Stunde genannt [103]. Ebenfalls ab etwa 30 Minuten Überlebenszeit kann der Nachweis der Glykoproteine Fibrinogen und Fibronektin gelingen [133], wobei auch hier in einer Studie längere Zeitintervalle von etwa 1 Stunden beschrieben wurden [103]. Die immunhistochemische Färbung gegen dephosphoryliertes Cx43 gelang in einzelnen Fällen bereits nach einer Überlebenszeit von 15 Minuten, für den Transkriptionsfaktor JunB ab etwa 30 Minuten [103]. Ebenfalls ab etwa 15 Minuten nach Einsetzen der Ischämie ergab sich immunhistochemisch eine Depletion des Ca^{2+}-bindenden Myozytenproteins S100A1 [119]. Die Varianz des frühesten Auftretens der Marker könnte zumindest in Teilen in der Methodik der Studien (Tiermodell vs. humane Gewebeproben) begründet sein, letztlich muss aber auch mit individuellen Unterschieden im Einzelfall gerechnet werden. Da der Nachweis der einzelnen Marker

insofern vor allem bei kurzen Überlebenszeiten zwangsläufig mit Unsicherheiten einhergeht, wurde bereits festgestellt, dass eine zuverlässige, immunhistologische Diagnostik eines akut letalen Myokardinfarkts niemals über einen Marker allein, sondern nur über ein Markerset erfolgen kann [134]. Dahingehend existiert aber noch kein abschließender Konsens, zudem erfolgt die Nutzung der einzelnen Marker in der Praxis uneinheitlich, obgleich C_{5b-9} und Fibronektin wahrscheinlich am häufigsten verwendet werden dürften.

Die hier dargestellten Untersuchungen ergaben für Dityrosin, ein Proteinprodukt von oxidativem Stress, dass ein immunhistochemischer Nachweis bereits sehr kurze Zeit nach Einsetzen einer Ischämie im Myokard gelingen kann. Die Untersuchungen an humanem Gewebe zeigten, dass ein Nachweis in Einzelfällen früher möglich ist als bei den bereits seit längerer Zeit etablierten Markern C_{5b-9} und Fibronektin. Insofern stellt Dityrosin eine wertvolle Ergänzung des Markerspektrums für die postmortale Myokardinfarktdiagnostik dar. Vorauszusetzen ist allerdings eine sorgfältige Beurteilung der Färbeergebnisse, auch mit Blick auf Lokalisation und Muster der Befunde. Die Ergebnisse der humanen und der Ratten-Kontrollgruppen zeigen, dass es nicht nur bei Myokardinfarkten, sondern auch unter anderen Umständen zu oxidativem Stress und der Entstehung nachweisbarer Mengen von Dityrosin kommt. Allerdings unterscheidet sich das Erscheinungsbild dieser Befunde maßgeblich von den untersuchten Infarktfällen: während bei Infarkten positive Färbeergebnisse in umschriebenen Zellgruppen (welche bei den Rattenherzen im Stromgebiet des ligierten Gefäßes lokalisiert waren) festgestellt werden konnten, waren die positiven Befunde in den Kontrollfällen eher diffus und im Bereich der „letzten Wiese“ (subendokardial, Papillarmuskeln) verteilt. Dies dürfte dem Umstand geschuldet sein, dass bei diesen Fällen weniger eine lokale

als vielmehr eine gewisse generelle Hypoxie, mit Blick auf die Rattenherzen beispielsweise begründet durch das experimentelle Setting, bestanden haben dürfte. Besonders eindrückliche, positive Färbeergebnisse zeigten solche Rattenherzen, die direkt nach der Entnahme in Formalin fixiert wurden: hier bestand eine kräftige, „gürtelartige" Färbung der äußersten Zellschichten, welche am ehesten auf die Fixierung des noch schlagenden Herzens in Formalin beim Vorhandensein von Blut zurückzuführen sein dürfte – Herzen, die aus der Langendorff-Anlage heraus fixiert wurden, zeigten diese Auffälligkeiten nicht. Die Ergebnisse der Untersuchungen mit dem Langendorff-Modell ergaben außerdem, dass es bei Infarkten mit und ohne Reperfusion gleichermaßen zur Bildung von Dityrosin kommt. Damit belegen unsere Untersuchungen auch, dass eine reperfusionsunabhängige Schädigung von Mitochondrien im Rahmen oxidativen Stresses relevante Mengen reaktiver Spezies generiert und die folgende Entstehung von Dityrosin nicht an eine Reperfusion gebunden ist. Dieser Umstand kann als weiteres Argument für die Verwendung von Dityrosin als postmortalen Myokardinfarkt-Marker und als Vorteil insbesondere gegenüber dem etablierten Markern C_{5b-9} gewertet werden, da dessen Nachweis als Teil des Komplementsystems das Vorhandensein zumindest einer gewissen Perfusion des infarzierten Gewebes voraussetzt.

Demgegenüber war aufgrund der Studienergebnisse eine Anwendung von GDF-15 und ET-1 als Myokardinfarktmarker in der postmortalen Diagnostik nicht zu empfehlen. Obwohl die Ergebnisse der Versuche mit dem isoliert perfundierten Langendorff-Herz vielversprechend waren, ergab sich bei der Färbung von humanem Gewebe für ET-1 ein Nachweis erst bei Fällen mit größeren Überlebenszeiten und bereits vorhandenen, einschlägigen, konventionell-histologisch erkennbaren Gewebealterationen. Für GDF-15 zeigte sich auf der anderen Seite, dass für dessen Nachweis offenbar die im Rahmen einer, wenn auch nur kurzen, Agonie zwangsläufig auftretende Gewebehy-

poxie ausreicht. Interessanter waren eher die Färbeergebnisse der humanen und Ratten-Kontrollfälle für ET-1, bei welchen sich positive Ergebnisse nach einer provozierten Arrhythmie (Ratten) und mit einer anzunehmend erhöhten Belastung des rechten Ventrikels (humane Fälle mit Todesursache Lungenembolie, Erhängen und Ersticken) ergaben. Auf dieser Basis war eine mögliche Nutzbarkeit von ET-1 im Rahmen der Diagnostik dieser Todesursachen zu diskutieren, mit Blick auf die geringe Fallzahl in dem darauf nicht fokussierten Probenkollektiv aber nicht weiter zu überprüfen.

Die Untersuchung der Marker MMP-2, MMP-9 und TIMP-1 konnte zeigen, dass auch hier ein sehr früher Nachweis nach Einsetzen einer Ischämie, wie er für die forensische Praxis wünschenswert wäre, grundsätzlich möglich ist – allerdings lediglich unter den idealisierten Bedingungen des Langendorff-Modells. Demgegenüber konnte unter „realen“ Bedingungen in humanem Gewebe eine vermehrte Aktivierung beziehungsweise vermehrte Synthese zuverlässig lediglich in Fällen mit längeren Überlebenszeiten, bei welchen eine Diagnose auch durch konventionell histologische Untersuchungen problemlos möglich wäre, festgestellt werden. Eine Nutzung für die postmortale Myokardinfarktdiagnostik erscheint für diese Marker insofern nicht sinnvoll. Vor dem Hintergrund der positiven Färbeergebnisse im Bereich mechanischer Myokardverletzungen war allerdings die Frage nach einer Anwendung zur forensischen Wundaltersschätzung zu stellen. Die Überlebenszeiten der Rattenherzen, welche derartige Befunde aufwiesen, betrug mindestens 30 Minuten. Da allerdings einige der betreffenden Befunde durch die Anlage der EKG-Elektroden knapp 20 Minuten vor Anbringung der Gefäßligatur entstanden sein dürften, war diese zeitliche „Untergrenze“ kritisch zu bewerten. Da MMP-2 und MMP-9 hinsichtlich ihrer Anwendung zur forensischen Wundaltersschätzung von einer anderen Arbeitsgruppe bereits an Hautwunden ausgetestet

worden waren [135], führten wir eine Untersuchung bzgl. der Nutzung bei Verletzungen von Muskelgewebe in einer gesonderten Arbeit durch [136].

Trotz einer eher geringen Fallzahl konnten die Versuche zu möglichen Einflussfaktoren auf den Nachweis immunhistochemischer Marker des akuten Myokardinfarkts zeigen, dass ein relevanter Effekt insbesondere durch eine ischämische Präkonditionierung berücksichtigt werden muss. Mit Blick auf die Untersuchung von Herzen von männlichen und weiblichen Ratten zeigten sich lediglich für die kardialen Troponine diskrete Unterschiede mit einer minimal erhöhten Depletion in Herzen männlicher Ratten. Hinsichtlich einer ischämischen Präkonditionierung ergaben sich demgegenüber deutliche Unterschiede für Cx43, welches bei Herzen mit einer solchen in geringerem Maße immunhistochemisch nachgewiesen wurde, sowie für die kardialen Troponine, bei welchen die Depletion sehr viel kräftiger ausgeprägt war. Entsprechende Erkenntnisse konnten für Cx43 bereits in einer anderen Studie gewonnen werden, allerdings erst ab einer Überlebenszeit von 1 Stunde [137]. In den Kontrollfällen unserer eigenen Arbeit mit einer „reinen“ ischämischen Präkonditionierung waren außerdem eine Depletion der kardialen Troponine, beziehungsweise positive Färbebefunde für Dityrosin zu beobachten. Für die praktische Anwendung bedeutet dies, dass der Nachweis einer Depletion von cTnT und / oder cTnI nicht zwingend auf einen letalen Infarkt, sondern gegebenenfalls auf eine transiente, myokardiale Ischämie zurückzuführen ist. Gleiches gilt für Dityrosin, so dass für alle drei Marker nicht nur das reine Vorhandensein einer Färbung / einer Depletion entscheidend, sondern auch eine sorgsame Beurteilung des konkreten Färbemusters zu fordern ist. Cx43 könnte andererseits mit Blick auf die Ergebnisse der Untersuchung bei einem letalen Myokardinfarkt, dem eine ischämische Präkonditionierung vorausgegangen ist, nicht oder zumindest weniger nachweisbar sein, wodurch die postmortale Diagnostik erschwert würde. Bezüglich der

auffälligen, vollkommen negativen Färbebefunde für cTnI in der Mehrzahl der Kontrollherzen konnte keine schlüssige Erklärung gefunden werden, zu diskutieren war allenfalls ein möglicher Verlust durch die Fixierung in Formalin. Für Myoglobin konnte bereits nachgewiesen werden, dass es hier durch Formalinfixierung zu einer Art „Auswaschen“ kommen kann [138]. Letztlich waren die Ergebnisse aber gerade aufgrund einer fehlenden Erklärung als Warnung hinsichtlich der Anwendung von cTnI als immunhistochemischem Myokardinfarktmarker in der Praxis zu werten.

Neben den konkreten Ergebnissen für die einzelnen Marker stellen die Resultate der präsentierten Studien zudem eine deutliche Aufforderung dar, bei der zukünftigen Evaluation von möglichen immunhistochemischen Markern des akuten Myokardinfarkts potenzielle Einflussfaktoren (Geschlecht und ischämische Präkonditionierung, aber auch andere, bislang nicht berücksichtigte Aspekte wie zum Beispiel das Alter) mitzubedenken und gezielt zu untersuchen. Außerdem muss auch bei dieser forensischen Fragestellung die Übertragung von Studienresultaten an Tiermodellen auf den Menschen kritisch und vorsichtig erfolgen, wie auch schon von anderen Autoren angemerkt wurde [139]. Da Vorteile sowohl für Tierexperimente (kontrollierte Bedingungen, genaue Kenntnis bzgl. der Überlebenszeit) als auch für menschliches Gewebe (bildet potenzielle Einflussfaktoren des Individuums und des Gesamtorganismus ab) bestehen, sollten Studien für neue Marker idealerweise beide methodischen Ansätze umfassen. Zudem sollte zukünftige Forschung an frühen Markern einer myokardialen Zellschädigung auch Ischämien mit einem anderen pathophysiologischen Hintergrund (zum Beispiel Strangulation, anoxisches Ersticken, Herzrhythmusstörungen) vermehrt berücksichtigen. Es ist bereits bekannt, dass auch hier ein immunhistochemischer Nachweis dieser Marker gelingen kann [139], wodurch zwar deren Spezifität für den

Infarktnachweis verringert und der Bedarf einer differenzierteren Auswertung von Färbeergebnissen nötig, letztlich aber auch eine neue Möglichkeiten für die postmortale Diagnostik eröffnet wird.

7 Abbildungen und Tabellen

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9 Eidesstattliche Erklärung

Ich versichere an Eides statt, dass ich die vorliegende Habilitationsschrift ohne unerlaubte Hilfe angefertigt habe und, dass das benutzte Schrifttum vollständig erwähnt wurde. Ethische Grundsätze sowie die Empfehlungen zur guten wissenschaftlichen Praxis wurden eingehalten. Diese Habilitationsschrift wurde bislang keiner anderen Fakultät vorgelegt.

Dr. med. Felix Mayer

Düsseldorf, Mai 2022

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11 Anhang: Originalarbeiten (mit Erlaubnis der veröffentlichenden Verlage)

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ORIGINAL ARTICLE

Dityrosine, a protein product of oxidative stress, as a possible marker of acute myocardial infarctions

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Abstract The verification of acute and lethal myocardial infarctions remains a crucial problem in the daily routine work of legal medicine. In order to enhance the possibilities in micromorphologic diagnostics, we investigated if dityrosine as a protein product of oxidative stress can be detected in myocardial tissue after an infarction and, if so, if it occurs early enough to be used in the diagnosis of infarctions with a short survival time. We examined tissue samples from 61 autopsy cases (37 male, 24 female) with verified or suspected infarctions as well as 11 control cases (7 male, 4 female). Immunohistochemical staining was performed for dityrosine and the established markers fibronectin and $C_{5b\text{-}9}$. Positive staining for dityrosine was obtained in nearly all cases with infarctions aged 4 h to 2 weeks. Single positive results were obtained in cases with older (up to 2 months) or assumedly very fresh (up to 4 h) infarctions. Furthermore, single positive results with a different staining pattern were obtained in the control group. We concluded that dityrosine as a marker of oxidative stress can be detected after infarctions and might occur early enough to be helpful in the diagnosis of infarctions with a short survival time. Though dityrosine does not seem to be specific for infarctions, the different staining patterns enable a differentiation.

Keywords Myocardial infarction · Histology · Immunohistochemistry · Oxidative stress

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Introduction

The diagnosis of an acute and lethal myocardial infarction is a common problem in the daily routine work of legal medicine. Ischemia of the heart muscle may lead to an unexpected and sudden death of a person, possibly as the first manifestation of a coronary heart disease. Though cases like these are often being examined in institutes for legal medicine and have been object of intensive research, it is still challenging to diagnose an acute myocardial infarction with only little time of survival. Micromorphologic changes of the myocardium (e.g. hypereosinophilic cells and contraction bands) can be detected not before 4 to 6 h after an infarction. Immunohistochemical markers like fatty acid-binding protein (FABP), myoglobin, fibronectin and $C_{5b\text{-}9}$ can be detected earlier, up to minutes after an infarction [12]. Comparisons between these markers indicated that cellular markers like FABP or myoglobin react faster than serum markers like $C_{5b\text{-}9}$ and fibronectin. However, there is no absolutely reliable marker that gives proof of a myocardial infarction in any case [12]. Therefore, at least a combination of different markers is recommended. The identification of new markers might extend the available morphological tools for the diagnosis of an acute myocardial infarction.

With regard to the pathophysiology of myocardial infarctions, products of oxidative stress might be interesting candidates as markers for an acute infarction. Ischemia of the myocardium goes along with oxidative stress affecting all types of molecules, including proteins. Since now, it has not been evaluated if products of oxidative stress can be detected morphologically in heart muscle tissue after a myocardial infarction. Since reactive species are produced in the damaged muscle tissue itself, interaction with fatty acids, proteins or DNA occurs rather fast, and products of oxidative stress should be detectable early after an ischemic event. The attack

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of reactive species on proteins can result in the formation of amino acid radicals. One of these is the tyrosyl radical. Tyrosyl radicals can interact with each other and form a binding that leads to the formation of dityrosine [8], which may be a useful marker of acute myocardial infarctions.

In search of new markers for the diagnosis of myocardial infarction, we addressed the following questions:

1. Can dityrosine (as protein product of oxidative stress) be detected immunohistochemically in ischemic heart muscle tissue?
2. If so, does dityrosine as a marker of oxidative stress appear early enough to be suitable for the diagnosis of myocardial infarctions even after a short survival time?

Material and methods

Immunohistological detection of dityrosine was performed in the myocardium of deceased persons with suspected myocardial infarctions and in control cases with varying duration of agony. In parallel, the well-established markers fibronectin and $C_{5b\text{-}9}$ were detected. The project was approved by the ethical committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf.

Tissue samples

Tissue samples of myocardium from 72 autopsy cases were taken in the Institute of Legal Medicine at the University Hospital of Düsseldorf. In cases with macromorphologic signs of a myocardial infarction, samples of the affected muscle area were drawn; otherwise, samples were taken from the right ventricle, the left ventricle (anterior, posterior and papillary muscle) and the septum. The collected samples were fixed in formalin and embedded in paraffin.

Study group

For the study group, 61 cases were selected. Cases were included if symptoms, clinical findings and/or autopsy findings led at least to the suspicion of a myocardial infarction. Samples of 37 male and 24 female individuals were selected, their age ranged from 27 to 96 years. We included cases with and without cardiopulmonary resuscitation (CPR). Cases with obvious signs of decomposition were excluded.

Control group

The control group consists of 11 cases with a cause of death different from myocardial infarctions and with varying periods of agony. Death was caused by hanging, strangulation, choking in combination with compression of thorax, drowning, carbon monoxide intoxication, polytrauma and headshot. Seven male and four female deceased were selected, their age ranged from 5 to 90 years. All selected cases did not show signs of relevant cardiac diseases.

Graduation of the "age" of an infarction

To estimate the "age" of infarctions in the study group, samples were stained with H&E and categorised into six groups, labelled A–F, using the system presented in Table 1 [3]. Table 1 also gives an overview of the distribution of the study cases into the six age groups.

Immunohistochemistry

Tissue sections were deparaffinised and washed in distilled water three times for 5 min, then washed twice in Tris-buffered saline (TBS)-buffer with 0.5 % Tween 20 for 5 min.

Dityrosine The primary antibody against dityrosine (Acris, mouse, AM 20243PU-S) was used in a concentration of 1:600, slides were incubated overnight at +4 °C. Slides were then washed in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Endogenous peroxidase was blocked with 0.03 % H2O2 for 15 min; afterwards, slides were washed in distilled water twice for 5 min and in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Then slides were incubated with a peroxidase-marked polymer (Medac, Histofine® Simple Stain MAX PO against mouse, 413132) for 30 min.

Fibronectin Slides were treated with proteinase K (Dako, S3020) for 7 min and washed twice in TBS-buffer with 0.5 % Tween 20 for 5 min. The primary antibody against fibronectin (Biozol, rabbit, DAK-A024502-2) was used in a concentration of 1:2,000, slides were incubated overnight at + 4 °C. Slides were then washed in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Endogenous peroxidase was blocked with 0.03 % H2O2 for 5 min. Afterwards, slides were washed in distilled water twice for 5 min and in TBS-buffer with 0.5 % Tween 20 twice for 5 min before being incubated with a peroxidase-marked polymer (Medac, Histofine® Simple Stain MAX PO against rabbit, 414142) for 30 min.

$C_{5b\text{-}9}$ The primary antibody against $C_{5b\text{-}9}$ (Abcam, rabbit, ab 55811) was used in a concentration of 1:150, slides were incubated overnight at +4 °C. Slides were then washed in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Endogenous peroxidase was blocked with 0.03 % H2O2 for 30 min; afterwards, slides were washed in distilled water twice for 5 min and in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Then slides were incubated with a peroxidase-marked polymer (medac, Histofine® Simple Stain MAX PO against rabbit, 414142) for 30 min.

Table 1 Histological classification of infarction age according to Cummings et al. [3]

Infarction age	Microscopically visible alterations	Cases
A: 0–4 h	No microscopically visible alterations	Case 1–13
B: 4–24 h	Contraction bands, hypereosinophilic cells, loss of nucleus, haemorrhages	Case 14–34
C: 1–7 days	Inflammation cells, esp. neutrophils	Case 35
D: 8–14 days	Granulation tissue, beginning of vascularisation	Case 36
E: 2 weeks–2 months	Collagen deposit, tissue less vascularised	Case 37–50
F: over 2 months	Collagen scar	Case 51–61

Tissue samples were washed in TBS-buffer with 0.5 % Tween 20 twice for 5 min and were then stained with substrate-chromogen AEC (MEDAC, AEC + (3-amino-9-ethylcarbazole) Substrate-Chromogen, K3469). Afterwards, the slides were washed with distilled water for 5 min, counterstained with Mayers Hematoxylin (Merck, Mayers hemalum solution, 1092492500) and washed again in distilled water and tap water. Sections were mounted with Aquatex® (Merck, 1.05862.0050).

Evaluation

Evaluation of staining results was performed following a semi-quantitative classification system:

O No positive staining
1 Positive staining of single cells
2 Positive staining of groups of cells
3 Positive staining of cells in large tissue areas

Results

Study group

Table 2 shows the staining results of the cases of groups A and B with estimated survival times of either 0–4 h or 4–24 h, including available information about CPR.

In group A (estimated survival time 0–4 h), 5 of 13 cases stained positive only for dityrosine, 1 stained positive only for fibronectin and 2 stained positive only for $C_{5b\text{-}9}$. One case showed positive staining for all three markers, 5 cases showed no positive staining at all. Positive staining for dityrosine was rather delicate.

In group B (estimated survival time 4–24 h), 18 of 21 cases stained positive for dityrosine, 20 stained positive for fibronectin and 19 stained positive for $C_{5b\text{-}9}$. One case showed positive staining only for $C_{5b\text{-}9}$ and dityrosine, and 1 case showed positive staining only for fibronectin. In those 3 cases negative for dityrosine, staining for the other markers as well as the detected contraction band necrosis was very discreet. There was no case that did not show any positive staining at all.

Table 3 shows the staining results of the cases of groups C, D, E and F, including available information about CPR.

Case 35, the only case in group C (estimated survival time 1–7 days) stained positive for all three markers.

Case 36, the only case in group D (estimated survival time 1–2 weeks) stained also positive for all three markers.

In group E (estimated survival time 2 weeks–2 months) 6 of 14 cases stained positive for dityrosine, 5 stained positive for fibronectin and 7 stained positive for $C_{5b\text{-}9}$. Positive staining for all three markers occurred in 1 case, positive staining only for dityrosine and fibronectin was found in 1 case and positive staining only for dityrosine and $C_{5b\text{-}9}$ was seen in 2 cases. There were 2 cases with positive staining only for dityrosine. In 3 cases, there was no positive staining at all.

In group F (estimated survival time over 2 months) 1 of 11 cases stained positive only for dityrosine, 2 stained positive for fibronectin and 3 stained positive for $C_{5b\text{-}9}$. Eight cases showed no positive staining at all.

In groups A–F, there were no obvious differences between cases with and without CPR. In both groups, positive and negative staining results for all three markers were found. Because the information about CPR was very inhomogeneous (whether CPR was performed at all and, if so, for how long) there was no basis for a further evaluation or statistical analysis.

In general, the staining pattern for dityrosine resembled the one for $C_{5b\text{-}9}$: staining was found in the intracellular space, and it was restricted to the damaged muscle area. Staining for fibronectin, on the other hand, could be found in the intracellular space as well as in the extracellular matrix, and it covered a larger area (Fig. 1).

Control group

Table 4 shows the staining results of the control group, including available information about CPR.

Two of the 11 cases showed positive staining only for dityrosine, 4 cases only for fibronectin and one showed

Table 2 Staining results of groups A and B (o=no staining; + =positive staining of single cells; ++=positive staining of cell groups; +++=positive staining of cells in larger tissue areas) and available information about CPR

Cases graded in estimated survival time		$C_{5b\text{-}9}$	Fibronectin	Dityrosine	CPR?
Group A (0–4 h)	Case 1	o	o	o	Yes, duration unknown
	Case 2	o	o	++	Yes, duration unknown
	Case 3	o	o	o	Yes, duration unknown
	Case 4	+	o	o	No
	Case 5	o	+	o	No
	Case 6	o	o	o	Unknown
	Case 7	o	o	+	30 min
	Case 8	o	o	o	No
	Case 9	o	o	+	No
	Case 10	o	o	+	Yes, duration unknown
	Case 11	o	o	o	No
	Case 12	+	o	+	Unknown
	Case 13	+	++	+	40 min
Group B (4–24 h)	Case 14	++	++	+	No
	Case 15	+++	+++	+	Yes, duration unknown
	Case 16	+	+	o	60 min
	Case 17	+	++	++	60 min
	Case 18	o	+	+	No
	Case 19	++	+++	+	30 min
	Case 20	++	++	+	No
	Case 21	+++	+++	++	Yes, duration unknown
	Case 22	+++	+++	++	Yes, duration unknown
	Case 23	+	+	++	Unknown
	Case 24	+	+	+	Yes, duration unknown
	Case 25	+	o	+	Yes, duration unknown
	Case 26	+++	+++	+++	No
	Case 27	+++	+++	+++	No
	Case 28	+++	+++	+++	No
	Case 29	+	+	o	90 min
	Case 30	o	+	o	105 min
	Case 31	++	+++	+++	80 min
	Case 32	+++	+++	++	No
	Case 33	++	+++	+++	Yes, duration unknown
	Case 34	+	+	+	45 min

positive staining only for $C_{5b\text{-}9}$. Two cases showed positive staining for dityrosine and fibronectin.

In cases with a presumably shorter agony (polytrauma, head shot, decapitation), only discreet positive staining for dityrosine was found, located subendocardial in the papillary muscle. Case 63 with a presumably longer agony on the other hand (hanging) showed a somewhat stronger and diffuse staining, especially in samples drawn from the septum (Fig. 2). In general, staining for fibronectin was stronger and was found in various regions, also in tissue drawn from the right heart. Positive staining for $C_{5b\text{-}9}$ in case 67 was very discreet.

Discussion

In the study groups B, C and D (estimated survival time 4–24 h, 1–7 days and 1–2 weeks), all but three cases (of group B) showed positive staining for dityrosine. Positive staining was found in the intracellular space, and it was sharply restricted to the infarction area. Since conventional histological findings as well as positive staining results for the established immunohistochemical markers fibronectin and $C_{5b\text{-}9}$ verified a myocardial infarction, the results prove clearly that dityrosine, as a protein product of oxidative stress, emerges after myocardial infarctions and can be detected immunohistochemically.

Table 3 Staining results of groups C, D, E and F (o=no staining; +=positive staining of single cells; ++=positive staining of cell groups; +++=positive staining of cells in larger tissue areas) and available information about CPR

Cases graded in estimated survival time		$C_{5b\text{-}9}$	Fibronectin	Dityrosine	CPR?
Group C (1–7 days)	Case 35	+	+	+	40 min
Goup D (1–2 weeks)	Case 36	+++	+++	++	No
Group E (2 weeks–2 months)	Case 37	+	o	o	No
	Case 38	+	o	+	45 min
	Case 39	o	o	o	Yes, duration unknown
	Case 40	++	+	o	Yes, duration unknown
	Case 41	++	o	o	No
	Case 42	o	o	o	Unknown
	Case 43	o	++	o	No
	Case 44	o	+	++	No
	Case 45	+++	+++	o	No
	Case 46	+	++	+++	Yes, duration unknown
	Case 47	o	o	+++	120 min
	Case 48	o	o	++	No
	Case 49	+	o	+	Unknown
	Case 50	o	o	o	No
Group F (over 2 months)	Case 51	o	o	o	No
	Case 52	o	o	++	No
	Case 53	o	o	o	50 min
	Case 54	++	+	o	Unknown
	Case 55	+	o	o	No
	Case 56	+	+	o	70 min
	Case 57	o	o	o	Yes, duration unknown
	Case 58	o	o	o	No
	Case 59	o	o	o	No
	Case 60	o	o	o	Yes, duration unknown
	Case 61	o	o	o	No

Results and staining patterns of fibronectin and $C_{5b\text{-}9}$ matched the results from previous studies [13, 16–18]. The staining pattern of dityrosine resembled the one of $C_{5b\text{-}9}$; as far as can be judged from our results, staining appears later and is slightly weaker than staining for fibronectin. The three cases of group B that stained negative for dityrosine showed only discreet positive staining for the other markers and very discreet contraction band necrosis. Hence, it has to be discussed if either the amount of oxidative damage or the timeframe after the infarction was not sufficient for dityrosine to occur.

In cases with a histologically verified myocardial infarction and a longer estimated survival time, 2 weeks to 2 months (group E) and over 2 months (group F), positive staining for dityrosine was found in 7 out of 25 cases. It has been assumed that most oxidative damage of proteins is not being repaired but leads to catabolism of the affected molecule; this also counts for dityrosine which may serve as a trigger for protein degradation [4, 7]. However, there is no reliable data available on how long it takes for myocardial fibres with oxidative damage to be eliminated. Therefore, we do not know how long dityrosine can be detected in damaged tissue, especially myocardium, and we do not know for sure if the positive results of groups E and F are still a residue of the old infarction or consequence of another acute ischemic event. With a view to the results of the established markers, especially fibronectin, the somehow inconsistent staining results of group E might still be a result of the initial ischemic event, with markers starting to vanish. However, this assumption seems unlikely in those cases showing only a collagenous scar (group F).

Staining for dityrosine was also positive in 4 cases of the control group. In 2 cases, it was the only detectable marker. Positive staining was found in cases with a presumably longer agony (hanging) as well as in cases with a presumably shorter agony (e.g. head shot). Staining in cases with a shorter agony was rather delicate and located subendocardial in the papillary muscle. The case with a supposed longer agony, however, showed a somewhat stronger and diffuse staining, especially in one sample from the septum.

The staining results for fibronectin in the control group again matched those of previous studies [6, 11]. Positive

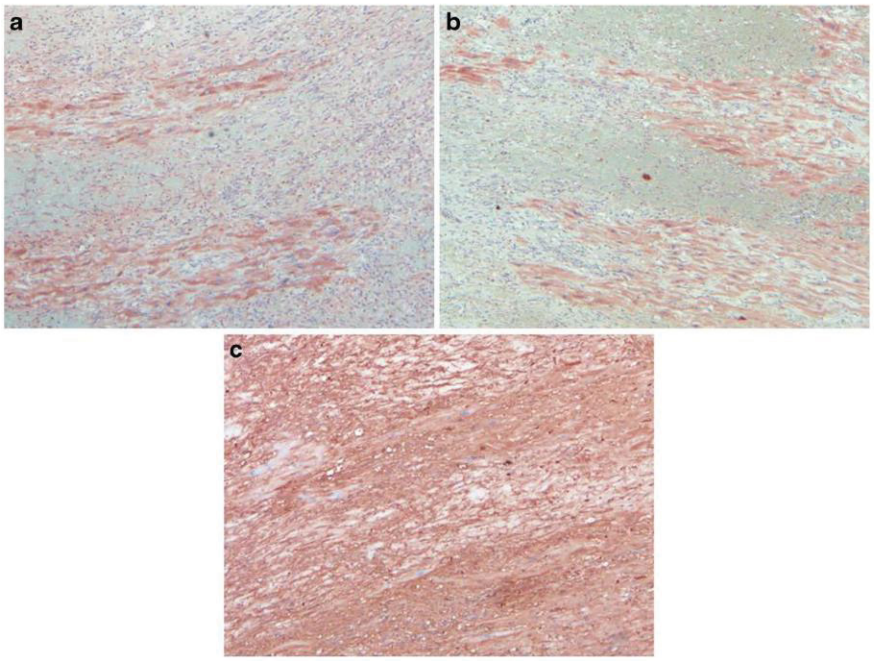

Fig. 1 Staining results of case 22 group B: **a** C_{5b-9}, **b** dityrosine, **c** fibronectin. Staining for fibronectin was stronger and covered a larger area; staining for C_{5b-9} and dityrosine was weaker but restricted to the damaged muscles cells

staining was particularly evident in cases with a presumably longer agony; in those cases, positive results were also obtained in samples from the right chamber. Surprisingly, there was also one case only positive for C_{5b-9}. This seems contradictory to results from previous studies [5, 15]; however, positive staining was very discreet.

Our results suggest that even in cases with a short agony myocardium might still suffer from at least minimal oxidative damage in its most vulnerable area, the papillary muscle. The positive staining for dityrosine in one case with a presumably longer agony might be considered as a hint that oxidative stress makes a contribution to the fresh cardiac damage that occurs in such a case [6].

Although the results show that the appearance of dityrosine is not specific for myocardial infarctions, especially the staining pattern of the cases with a presumably shorter agony can

Table 4 Staining results of control group (o=no staining; += positive staining of single cells; ++=positive staining of cell groups; +++=positive staining of cells in larger tissue areas) and available information about CPR

	Cause of death	C_{5b-9}	Fibronectin	Dityrosine	CPR?
Case 62	Drowning	o	o	o	No
Case 63	Hanging	o	+	++	No
Case 64	Hanging	o	++	o	Unknown
Case 65	Choking and compression of thorax	o	++	o	No
Case 66	Carbon monoxide intoxication	o	++	o	No
Case 67	Polytrauma	+	o	o	No
Case 68	Polytrauma	o	+	+	No
Case 69	Polytrauma	o	+	o	No
Case 70	Decapitation	o	o	+	No
Case 71	Head shot	o	o	+	No
Case 72	Polytrauma	o	o	o	No

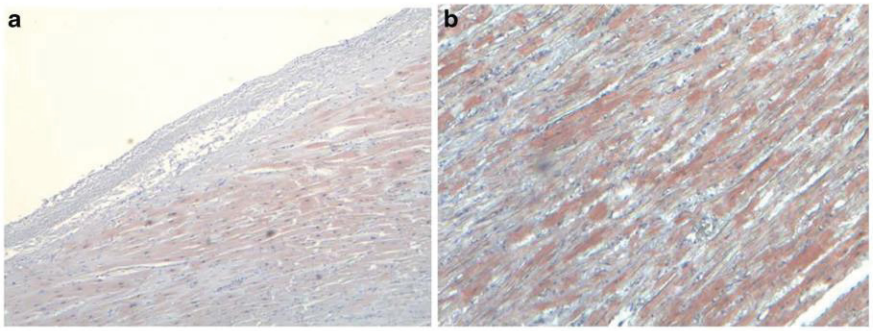

Fig. 2 Staining results of the control group for dityrosine: **a** papilary muscle of case 70 with a presumably shorter agony, **b** septum of case 63 with a presumably longer agony

easily be differentiated: the discreet positive staining in the subendocardial region of the papillary muscles does not resemble the staining pattern of the cases of the study group. A longer agony may result in a somewhat stronger staining, however, this staining exhibits a diffuse pattern and is not restricted to a defined area as in the case of a myocardial infarction.

Research on autopsy material goes along with specific uncertainties due to missing informations. Concerning myocardial infarctions, the problem is that the precise time of the infarction and the survival time are not exactly known in most cases. They can only be estimated by the evaluation of clinical symptoms or, if present, by morphological findings. In many cases, it is also difficult to evaluate if or to which extent CPR has been performed. With regard to this lack of information, the testing of new markers can hardly sum up in exact time spans of occurrence and disappearance. The material is also not suitable for a statistical evaluation, e.g. we cannot say for sure if all cases of group A actually suffered from a myocardial infarction. The gathered tissue samples can only be used for a first attempt to find out about the potential of a possible new marker for forensic routine work. In order to gain more information about dityrosine as a marker for myocardial infarctions, we decided to compare it to established immunohistochemical markers fibronectin [9, 17] and $C_{5b\text{-}9}$ [13, 15, 16] that might, in theory, be detectable in approximately the same time interval.

Dityrosine formation begins with the generation of tyrosyl radicals. In an oxidative environment, reactive species might attack tyrosine residues in proteins, leading to the generation of tyrosyl radicals. By forming an inter-molecular crosslinking, two of those tyrosyl radicals can form a dimer called dityrosine [4, 7]. With a view to myocardial infarctions, it is not yet clear if the absence of oxygen alone leads to the generation of sufficient amounts of reactive species to inflict oxidative damage on the myocardium and to generate measurable amounts of dityrosine. Some authors propose that major oxidative damage occurs only after reperfusion of damaged tissue, which causes a burst of reactive species production. During ischemia, xanthine dehydrogenase can be transformed into xanthine oxidase. Simultaneously, the depletion of ATP causes the accumulation of hypoxanthine. When the tissue is reoxygenated, xanthine oxidase oxidises hypoxanthine, leading to a rapid generation of reactive species [1, 2]. Other authors, however, point out that during ischemia damage is also done to the mitochondria of the myocardium. This damage causes an increase of reactive species production in the mitochondria and makes them more "leaky", releasing the reactive species [10, 14]. From a forensic point of view, the question whether or not oxidative stress relies on reperfusion of ischemic myocardium is interesting with regard to the time of occurrence of the products of oxidative stress that might be used as infarction markers. For fibronectin and $C_{5b\text{-}9}$, it is well-known that their occurrence is bound to a reperfusion of damaged or wounded myocardium [11].

In our study, dityrosine was detected in six cases of a suspected myocardial infarction with a survival time of less than 4 h. The staining in those cases was rather delicate. In other cases of this group, single positive results for fibronectin and $C_{5b\text{-}9}$ were obtained. Previous studies were able to show that $C_{5b\text{-}9}$ can be detected earliest at 30–40 min after an infarction, fibronectin seems to appear even earlier [12]. Our results imply that dityrosine emerges after at least a similar time interval. Since there are four cases with positive staining only for dityrosine, it might, under certain circumstances, appear even earlier than the other two markers. Additionally, the cases of the control group show that even in cases with a short agony positive staining results for dityrosine can be obtained. Overall, our findings suggest that dityrosine occurs rather fast after the infliction of oxidative damage to the myocardium. Therefore, dityrosine might also be a suitable marker for myocardial infarctions with a very short survival time.

In those cases of group A (estimated survival time 0–4 h) with no positive staining results, it cannot be differentiated if either no myocardial infarction took place or if the survival time was too short for the markers to appear. As we already outlined, this is a common problem when working with material from autopsy cases: there is a lack of information that complicates the interpretation of results. In order to determine a chronological bottom line for the occurrence of dityrosine, we therefore are currently working on the establishment of an animal model that delivers the information needed for a comprehensive assessment of dityrosine as a marker for an acute myocardial infarction.

In general, staining for fibronectin was stronger and covered a larger area than staining for $C_{5b\text{-}9}$ and dityrosine. Single cases of the study groups, however, showed stronger staining for dityrosine and/or $C_{5b\text{-}9}$, e.g. case 23. There were even some cases that showed positive staining only for dityrosine, e.g. cases 47 and 48. The reason for these findings remains unclear since neither morphological findings of the tissue sections nor anamnestic data of the cases provided any explanations. There seem to be circumstances that either promote the emergence or prevent the reduction of dityrosine and that are not yet understood. At least in part, it might be associated with the question if the emergence of dityrosine depends on reperfusion or not. Due to the fact that the appearance of fibronectin and $C_{5b\text{-}9}$ depends on a reperfusion of the muscle's damaged tissue, our results might be a clue that oxidative stress and its products are also mostly a consequence of reperfusion of ischemic myocardial tissue. However, considering the results of the control group, our study does not allow to rule out the assumption that maybe little amounts of dityrosine can be produced during ischemia alone. This is another question that might be answered by an animal model for acute myocardial infarctions.

Conclusion

We were able to demonstrate that dityrosine, as a protein product of oxidative stress, can be detected immunohistochemically in myocardium after an infarction. In healthy heart muscle tissue positive staining might appear due to final ischemia; however, the resulting staining patterns can easily be differentiated from the staining pattern after a myocardial infarction. Compared to the established markers fibronectin and $C_{5b\text{-}9}$, dityrosine is assumed to appear also quite early after an ischemic event, though it might depend in a large part on a reperfusion of the damaged tissue. However, since our tests on healthy myocardium propose that there is production of small amounts of dityrosine solely during ischemia, we think that a further evaluation of this marker is promising since it might help to detect myocardial infarctions with a very short time of survival; our animal model will also address this assumption. Another reason why dityrosine might constitute a valuable methodical addition in diagnosing an acute myocardial infarction is the fact that it refers to a pathophysiological aspect that has not been considered yet: oxidative stress.

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ORIGINAL ARTICLE

Dityrosine as a marker of acute myocardial infarction? Experiments with the isolated Langendorff heart

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Abstract The isolated Langendorff heart was used to evaluate dityrosine as a marker of acute myocardial infarctions. The animal model allowed the generation of local infarctions with defined survival times, as well as infarctions with and without reperfusion. The results showed that dityrosine, at least under the conditions of the animal model, occurs very shortly after early ischemia and infarctions, since positive staining results were already obtained after a survival time of only 5 min. Furthermore, it could be proved that the occurrence of dityrosine does not depend on a reperfusion of the ischemic muscle area and that there are no differences in the staining patterns of infarctions with and without reperfusion. Positive staining results for dityrosine in control hearts without infarctions had to be considered when evaluating the tissue samples of the study hearts. In part, the positive staining results of the control hearts seemed to be an artefact of the Langendorff system, easily identifiable by a distinctive staining pattern. Positive staining results in tissue samples of hearts that suffered from arrhythmia on the other hand implied that the occurrence of dityrosine is not specific for myocardial infarctions. Taking into account the results of previous works on human tissue samples, however, these findings did not question the use of dityrosine as a diagnostic tool; they simply showed that myocardial damage due to oxidative stress might occur under various pathologic conditions.

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Introduction

In the routine work of legal medicine, diagnosing a lethal myocardial infarction is a frequent task. Since macromorphological changes are visible for the earliest 4 h after an infarction, histological examinations, including immunohistochemistry, are often performed. However, the most common immunohistological markers demand a survival time of at least 20–30 min to appear [16], so there still is a diagnostic gap of about half an hour. Because of that, new markers that can be detected shortly after an infarction are still needed. In a previous study, we were able to show that dityrosine, a protein product of oxidative stress, is usable as a marker of acute myocardial infarctions [14]. Positive results were obtained in at least the same time range as with established markers $C_{5b\text{-}9}$ and fibronectin. Unfortunately, a complete evaluation of dityrosine was limited by the fact that the study used human tissue samples drawn during autopsies. Working with such material typically goes along with a lack of certain information, especially concerning the exact "age" of the infarction. Therefore, a specific baseline for the appearance of dityrosine after ischemia could not be determined. Thus, we chose an animal model for further investigations regarding the new marker.

With the isolated Langendorff heart, post mortem harvested hearts of rats can be kept "alive" and beating for several hours. During this time, it is possible to induce myocardial infarctions and vary the survival time afterwards. By doing so, myocardial infarctions with a known "age" can be generated and evaluated immunohistochemically. Electrocardiograms (ECG), measurement of coronary flow

(CF), and the visible changes of color of the myocardium (pallor) serve as objective proof that an infarction had been induced. It is also possible to induce infarctions with and without reperfusion. Furthermore, electrical stimulation of a heart allows inducing arrhythmias as examples for other cardiac pathologies besides an infarction.

With this study we aimed on answering the following questions:

1. How early can dityrosine be detected after a myocardial infarction?
2. Does the appearance of dityrosine depend on reperfusion or is ischemia alone sufficient for it?
3. Is dityrosine specific for myocardial infarctions or does it also appear as a consequence of other pathophysiological disturbances?

Material and methods

All experiments were performed in accordance with the German legislation on protection of animals and 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). The protocol for the Langendorff system was approved by the local Animal Ethics Committee.

Isolated Langendorff heart

Male Wistlar rats were used for this study. The animals were kept on a 12:12 light/dark schedule (lights on at 0600 h) with food and water ad libitum. The rats were anesthetized by intraperitoneal injection of Pentobarbital 90 $mg \cdot kg^{-1}$ and 0.2 ml Heparin. Deep sedation was verified by the absence of reactions to pain. In this condition, rats were decapitated, an immediate thoracotomy was conducted, and hearts were excised and mounted on a Langendorff system. Hearts were perfused at constant pressure (80 mmHG) with modified Krebs-Hensleit solution: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_2 + 7\ H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA, 2.25 mM $CaCl_2$, 11 mM D(+)-glucose, and 1 mM Lactat. All hearts were allowed a stabilization period of at least 20 min. Electric activity (ECG) was measured by placing one electrode in the myocardium of the left chamber close to the apex and one electrode in the myocardium of the right atrium close to the auricle and one indifference electrode on the aorta. Heart rate was measured by ECG and coronary flow was measured continuously by a pressure transducer (8–20 ml/min). The data were digitalized using an analog to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle hill, Australia) at a sampling rate of 500 Hz, and they were recorded continuously on a personal computer using Chart for Windows v5.0 (ADInstruments).

Study group

The study group comprised 29 hearts.

In 24 hearts, local early ischemia/myocardial infarctions without reperfusion were induced by ligating the left coronary artery close to its origin. As objective proof of an ischemia/infarction ECG-alterations, changes of color of the myocardium (pallor) and decrease of coronary flow were documented. After a defined survival time after ligation—varied from 5 min up to 60 min—hearts were removed from the Langendorff system and directly fixed in formalin.

In five hearts, myocardial infarctions with reperfusion were induced. Reperfusion of the ischemic myocardium was realized by removal of the ligation of the left coronary artery. The ischemic period lasted for 20 min; the reperfusion period varied from 10 min up to 40 min. The survival time after ligation varied respectively from 30 min up to 60 min.

Control group

Seven hearts without a myocardial infarction served as a control group.

Two hearts were harvested post mortem and mounted on the Langendorff system without any manipulation. They were removed after 60 min and directly fixed in formalin.

Three hearts were harvested post mortem, mounted on the Langendorff system, and treated with 26 V electro shocks with a frequency of 10 ms^{-1}. Electric stimulation provoked arrhythmias. The hearts remained in the Langendorff system for 20, 25, and 30 min after the electric stimulation.

Another two hearts were excised and directly fixed in formalin.

Figure 1 presents an overview of the experimental protocol.

Dityrosine immunohistochemistry

Tissue sections were deparaffinized and washed in aqua-dest three times for 5 min, then washed twice in TBS-buffer with 0.5 % Tween 20 for 5 min. The primary antibody against dityrosine (Acris, mouse, AM 20243PU-S) was used in a concentration of 1:600; slides were incubated over night at +4 °C. Endogenous peroxidase was blocked with 0.03 % H_2O_2 for 15 min; afterwards, slides were washed in aqua-dest twice for 5 min and in TBS-buffer with 0.5 % Tween 20 twice for 5 min. Then, slides were incubated with a peroxidase-marked polymer (Medac, Histofine® Simple Stain MAX PO against mouse, 413132) for 30 min. Again tissue samples were washed in TBS-buffer with 0.5 % Tween 20 twice for 5 min and were then stained with substrate chromogen AEC (Dako, AEC+ (3-amino-9ethylcarbazole) Substrate-Chromogen).

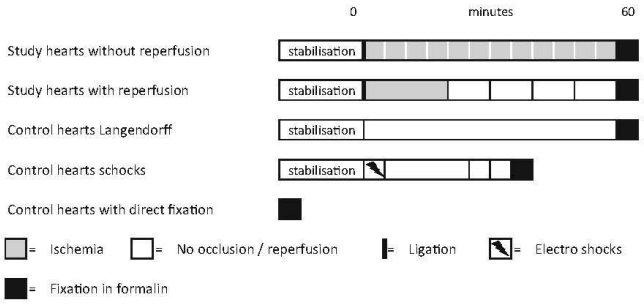

Fig. 1 Experimental protocol. All hearts in the Langendorff system were allowed a stabilization period. Survival times after ligation (study hearts without reperfusion) and after electro schocks (control hearts schocks), as well as reperfusion times, (study hearts with reperfusion) were varied

Afterwards, the slides were washed with aqua-dest for 5 min, counterstained with Mayers Hematoxylin (Merck, Mayers hemalum solution, 1092492500), and washed again in aqua-dest and tap water. Sections were mounted with Aquatex® (Merck, 1.05862.0050).

Evaluation

Evaluation of staining results was performed following a semi-quantitative classification system:

o = no positive staining
x = positive staining of single cells
xx = positive staining of groups of cells
xxx = positive staining of cells in large tissue areas

Results

Study group

Table 1 and Fig. 2 present the staining results of hearts with an early ischemia/myocardial infarction without reperfusion (no 1 to no 24). Positive staining results for dityrosine were detected in all but two cases: one heart with a survival time of 5 min (no 2) and one heart with a survival time of 50 min (no 20) did not present positive results. Staining was a bit more delicate in hearts with a very short survival time. However, one heart with a survival time of 5 min showed surprisingly intense staining results. From a survival time of 15 min upwards, no significant differences in staining intensity could be observed. Nevertheless, staining intensity still varied slightly from case to case. Positive staining results were located deep in the myocardium of the wall of the left chamber and in the septum, in the area of supply of the ligated artery. Positive staining in the subepicardial regions, especially in the wall of the right chamber, was suspicious for artefacts (see below) and not taken into account. Few cases (no 1, no 3, no 19) showed strikingly intense staining.

Table 2 summarizes the staining results of hearts with a myocardial infarction and reperfusion (no 25 to no 29). Positive staining results for dityrosine were visible in all cases. The staining pattern did not differ from the pattern of hearts without reperfusion. As in the cases without reperfusion, staining was found deep in the myocardium of the wall of the left chamber and the septum.

Control group

Table 3 gives an overview over the staining results for the hearts of the control group.

Two hearts were mounted on the Langendorff system for 60 min without any manipulation (no c1 and no c2). These hearts showed positive staining results circular in the subepicardial muscle cells. Staining was rather intense, especially in one case in which nearly the whole wall of the right chamber was affected. However, deeper in the myocardium of the wall of the left chamber or the septum, only single cells showed positive staining, and only if there were positive staining results in the correspondent subepicardial cells (Fig. 3a).

Three hearts that were treated with electro shocks (no c3–c5) showed discreet positive staining in the subepicardial regions of the muscle tissue. Furthermore, these hearts also presented discreet positive staining deeper in the myocardium. Different to the study hearts, positive staining did not seem to be strictly localized but was found in various regions of the myocardium.

The two hearts that were fixed directly in formalin after excision (no c6 and no c7) showed very impressive positive staining of the subepicardial myocardium all around the heart. Staining was even more intense than in the other control hearts and had a "belt-like" appearance (Fig. 3b). There was no positive staining deeper in the myocardium.

Discussion

In our previous study on dityrosine with human, autopsy-drawn tissue samples [14], we encountered some typical problems: we lacked information on the exact survival time after an infarction; it was often unsure, if there had been a

Table 1 Study hearts with early ischemia/ myocardial infarction without reperfusion. Staining pattern describes location of positive staining

No.	Survival time	Signs of infarction	Staining results	Staining pattern
1	5	Decrease of CF of 3.5 ml/min	xxx	Very intense, including subepicardial cells
2	5	Decrease of CF of 5.3 ml/min	o	Deep in left chamber wall/septum
3	10	ST-elevation, decrease of CF of 3.9 ml/min	xxx	Very intense, including subepicardial cells
4	10	Pallor, ECG-alterations, decrease of CF of 3.9 ml/min	x	Deep in left chamber wall/septum
5	15	Pallor, ECG-alterations, decrease of CF of 3.5 ml/min	xx	Deep in left chamber wall/septum
6	15	Pallor, decrease of CF of 6.0 ml/min	x	Deep in left chamber wall/septum
7	20	Pallor, decrease of CF of 3.6 ml/min	xx	Deep in left chamber wall/septum
8	20	Pallor, decrease of CF 8.0 ml/min	xxx	Deep in left chamber wall/septum
9	25	Pallor, ECG-alterations, decrease of CF of 5.1 ml/min	xx	Deep in left chamber wall/septum
10	25	Pallor, decrease of CF of 6.1 ml/min	xx	Deep in left chamber wall/septum
11	30	Discreet ECG-alterations, decrease of CF of 3.6 ml/min	xxx	Deep in left chamber wall/septum
12	30	Pallor, decrease of CF of 7.9 ml/min	xx	Deep in left chamber wall/septum
13	35	Pallor, decrease of CF of 2.4 ml/min	xx	Deep in left chamber wall/septum
14	35	Pallor, ECG-alterations, decrease of CF of 11.2 ml/min	xxx	Deep in left chamber wall/septum
15	40	ECG-alterations, decrease of CF of 1 ml/min	xx	Deep in left chamber wall/septum
16	40	Discreet pallor, ECG-alterations, decrease of CF of 1.2 ml/min	xx	Deep in left chamber wall/septum
17	45	Pallor, decrease of CF of 5.0 ml/min	xx	Deep in left chamber wall/septum
18	45	Pallor, decrease of CF of 4.6 ml/min	xx	Deep in left chamber wall/septum
19	50	Pallor, decrease of CF of 2.7 ml/min	xxx	Very intense, including subepicardial cells
20	50	Pallor, ECG-alterations, decrease of CF of 5.8 ml/min	o	Deep in left chamber wall/septum
21	55	Pallor, decrease of CF of 3.7 ml/min	xxx	Deep in left chamber wall/septum
22	55	Pallor, decrease of CF of 3.4 ml/min	xx	Deep in left chamber wall/septum
23	60	Decrease of CF of 7.7 ml/min	xxx	Deep in left chamber wall/septum
24	60	Pallor, decrease of CF of 3.8 ml/min.	xxx	Deep in left chamber wall/septum

Survival time time between ligation and fixation in formalin. *Signs of infarction* including pallor of myocardium, typical electrocardiogram (ECG) -alterations and decrease of cardiac flow (CF). *Staining results o* no positive staining, *x* positive staining of single cells, *xx* positive staining of groups of cells, *xxx* positive staining of large tissue areas

reperfusion of the ischemic myocardium, in some cases that were highly suspicious for an infarction, e.g. due to typical clinical symptoms, neither conventional histology nor established immunohistochemical markers showed positive results so that we were unsure if an infarction actually had occurred. As a consequence, some questions concerning the use of dityrosine as an early marker of acute myocardial infarctions remained unanswered. The isolated Langendorff heart offered a functional and easy way to address these remaining questions and to evaluate possible markers of myocardial infarctions with an animal model.

Since now, only few studies with animal models for the establishment of immunohistochemical markers of acute myocardial infarctions for forensic use have been conducted. Most of them included living animals and rather complex methods [2, 6, 10, 15]. The isolated Langendorff heart is an easier approach to address the important questions mentioned above [1, 8]. It offers the opportunity to generate local early ischemia and myocardial infarctions with and without reperfusion and with a defined survival time. Since these aspects have an important impact on the occurrence of some markers of myocardial infarctions, control of those parameters is a precious tool for the establishment of new markers. However, the method is limited to markers that are generated in myocardial tissue itself. Since blood is replaced by a buffer solution, markers that derive from blood cannot be examined.

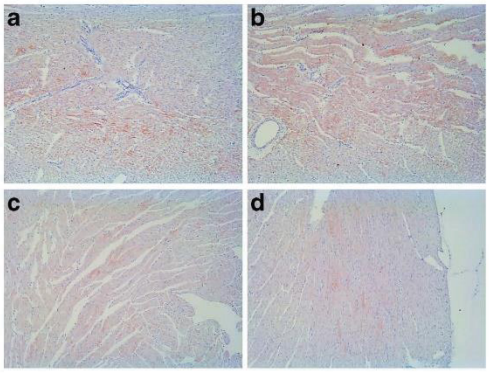

Fig. 2 Examples of study hearts with different survival times, fivefold magnification: **a** no 24, survival time 60 min; **b** no 15, survival time 40 min; **c** no 7, survival time 20 min; **d** no 4, survival time 10 min. Positive staining results are located deep in the myocardium of the wall of the left chamber and the septum

All but two of the 29 study hearts, one with a survival time of 5 min (no 2) and one with a survival time of 50 min (no 20), presented positive staining for dityrosine (Table 1). We were able to detect dityrosine earliest after a survival time of approximately 5 min. Though the maximum of staining intensity already seemed to be reached at a survival time of 15 min, one heart with a survival time of 5 min (no 1) also showed surprisingly strong staining results. Most of the tissue samples clearly showed positive staining results in the area of supply of the ligated artery (myocardium of the wall of the left chamber and/or the septum), according to the anatomy of rats' hearts [12]. Examples are shown in Fig. 2. Few hearts presented very intense staining (no 1, no 3, no 19), including the subepicardial myocardium so that a superimposition of artefacts had to be discussed. However, staining located deep in the myocardium of the left chamber/the septum was still most probably the consequence of an infarction. The slight differences in staining intensity in cases with survival times longer than 15 min were caused presumably by different qualities of the ligations. Although the procedure of ligation was the same for all study hearts, the exact location and the degree of compression of the coronary vessels might have differed to some extent from heart to heart. Incomplete compression of the ligated blood vessel is also most likely the reason for the negative staining results of hearts no 2 and no 20. It seems obvious that in case of a survival time of only 5 min a total inhibition of perfusion is necessary to generate enough oxidative stress to provoke a detectable formation of dityrosine. The protocol of heart no 20 furthermore revealed that a fast normalization of the ECG had been observed. This might also be a clue for an incomplete ligation resulting in only a moderate ischemia, insufficient for an actual infarction.

In order to check the method for possible artefacts caused by the Langendorff system or the rather unusual fixation of a still beating heart in formalin, we prepared seven control hearts without infarctions (Table 3). Two of those hearts were mounted on the Langendorff system for 60 min without any manipulation (no c1 and no c2), two hearts were fixed in formalin directly after excision (no c6 and no c7), and three were mounted on the Langendorff system and treated with electro shocks to provoke arrhythmias (no c3–c5). Positive staining results were found in tissue samples of all of those

Table 2 Study hearts with myocardial infarction with reperfusion. Staining pattern describes location of positive staining

No.	Ischemic period (min)	Reperfusion period (min)	Survival time in total (min)	Signs of infarction	Staining results	Staining pattern
25	20	40	60	discreet ECG-alterations, decrease of CF of 6.8 ml/min	x	deep in left chamber wall / septum
26	20	10	30	ECG-alterations, decrease of CF of 3.1 ml/min	xx	deep in left chamber wall / septum
27	20	20	40	ECG-alterations, decrease of CF of 1,8 ml/min	xx	deep in left chamber wall / septum
28	20	30	50	ECG-alterations, decrease of CF of 12.1 ml/min	xx	deep in left chamber wall / septum
29	20	40	60	pallor, ECG-alterations, decrease of CF of 9.8 ml/min	xx	deep in left chamber wall / septum

Ischemic period duration of ligation, *reperfusion period* time between re-opening of ligation and fixation in formalin. *Survival time in total* time between ligation and fixation in formalin. *Signs of infarction* including pallor of myocardium, typical electrocardiogram (ECG)-alterations and decrease of cardiac flow (CF). *Staining results o* no positive staining, *i* positive staining of single cells, *xx* positive staining of groups of cells, *xxx* positive staining of large tissue areas

Table 3 Control hearts without myocardial infarctions. Staining pattern describes location and "appearance" of positive staining results

No.	Description	Staining results	Staining pattern
c1	Mounted on Langendorff system for 60 min, no manipulation	x	Subepicardial
c2	Mounted on Langendorff system for 60 min, no manipulation	xxx	Subepicardial
c3	Mounted on Langendorff system, treated with electro shocks (2 times), survival time after electro shocks 30 min	xx	Subepicardial and deeper in the myocardium
c4	Mounted on Langendorff system, treated with electro shocks (1 time), survival time after electro shocks 25 min	xx	Subepicardial and deeper in the myocardium
c5	Mounted on Langendorff system, treated with electro shocks (1 time), survival time after electro shocks 20 min	x	Subepicardial and deeper in the myocardium
c6	Fixed in formalin directly after excision	xxx	Subepicardial, like a "belt"
c7	Fixed in formalin directly after excision	xx	Subepicardial, like a "belt"

Description specific treatment of the control hearts. *Staining results* o no positive staining, *x* positive staining of single cells, *xx* positive staining of groups of cells, *xxx* positive staining of large tissue areas

control hearts. These staining results showed a very distinctive pattern: dityrosine could be detected in muscle cells located subepicardial. Especially the hearts fixed in formalin directly after excision showed very intense staining that had the look of a "belt" surrounding the heart (Fig. 3). Only the hearts treated with electro shocks exhibited positive staining deep in the myocardium too; in contrast to the study hearts, the pattern seemed to be more scattered, rather than localized. To avoid misinterpretation when evaluating the study hearts, positive staining of subepicardial tissue or of the wall of the right chamber was not taken into account, since the control hearts had shown that these findings could be an artefact.

Our results also show clearly that the occurrence of dityrosine does not depend on reperfusion of the ischemic myocardium. Dityrosine formation is caused by reactive species, attacking tyrosine residues in proteins and, by doing so, generating tyrosyl radicals. Two tyrosyl radicals can form inter-molecular cross-links producing a dimer called dityrosine [7, 11]. Since now, it has been unclear if the lack of oxygen alone generates enough reactive species to trigger the generation of a measurable amount of dityrosine. Some publications suggested that only the reperfusion of ischemic tissue and the oxidation of hypoxanthine by xanthine oxidase lead to a relevant generation of reactive species [3, 5]. Other authors point out that mitochondria take serious damage during ischemia alone. This damage might not only lead to the generation of reactive species but might also cause leakage of their membranes releasing the reactive species [13, 17, 20]. A recent review of literature data suggested that xanthine oxidase and inflammatory mediators might contribute to the generation of reactive species but damage of mitochondria seems to play the leading role [18]. Since we found positive staining results for dityrosine in nearly all tissue samples from hearts with early ischemia/infarctions without reperfusion, even after only a short survival time, our results support this hypothesis. Ischemia alone obviously leads to a detectable oxidative damage of proteins, at least concerning the formation of dityrosine.

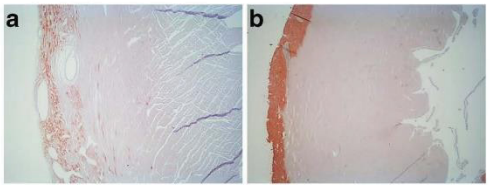

Fig. 3 Staining morphology of control hearts with a 2.5 magnification: **a** no c2, mounted on the Langedorff-system for 60 min without any manipulation; **b** no c6, immediate fixation in formalin after excision. Staining results are very characteristic with positive results in the subepicardial region of the myocardium. Staining results of no c6 has a "belt-like" appearance

Nevertheless, in order to check for possible differences between infarctions with and without reperfusion, we reopened the ligation of the left coronary artery in five study hearts. For infarct size measurements with TTC (Triphenyltetrazolium chloride) staining, protocols for the durations of ischemic period and reperfusion period have been published in the past [9, 19]. According to those publications, an ischemic period of at least 40 min and a reperfusion period of at least 60 min were recommended. However, in order to have a better comparison between the study hearts with infarctions without reperfusion and the ones with reperfusion, we decided to reduce the duration of the ischemic phase to 20 min and varied the reperfusion time from 10 min up to 40 min. All tissue samples of hearts with infarctions and reperfusion showed strong positive staining for dityrosine. There were

no differences in staining patterns compared to the hearts with infarctions without reperfusion. Therefore, we refrained from preparing more hearts with infarctions with reperfusion.

Altogether, the results of the present study demonstrate that dityrosine appears only minutes after ischemia, even faster than some established immunohistochemical markers. Furthermore, ischemia alone triggers the formation of dityrosine. Plasma derived antigens $C_{5b\text{-}9}$ and fibronectine need at least approximately 30 min of survival time after an infarction and reperfusion of the affected muscle tissue before they can be detected [16]. A look on the results of other studies on markers of myocardial infarctions conducted with animal models proves that dityrosine is expressed really fast after ischemia. Meng et al. ligated the left anterior descending artery (LAD) of living rats [15]. After various ischemic intervals, the animals were killed and tissue samples were drawn. Immunohistochemical staining revealed a depletion of heart fatty acid binding protein earliest 15 min after ischemia. Bi et al. investigated the depletion of S100A1 in rats' hearts after surgical LAD occlusion [2]. Depletion in few subendocardial cells was seen also earliest 15 min after ischemia. Fischbein et al. used tissue samples from various animal experiments including dogs, pigs, and rats to evaluate the depletion of troponin T and I [10]. All the selected cases had undergone coronary occlusion. Depletion of troponin T and I, especially troponin T, was observed after 0.5 h of occlusion. However, since there was no case with a shorter time of occlusion, depletion might start earlier.

Though very promising, to this point, the results of our study only apply for the animal model used for the experiment. Conclusions concerning the survival time necessary to detect dityrosine in human myocardial tissue can only be drawn by taking the results of our previous study [14] into account, too. In some autopsy cases that were highly suspicious for a myocardial infarction, positive staining for dityrosine, but not for $C_{5b\text{-}9}$ and fibronectin was seen. With our newly gained knowledge, it can be assumed that in those cases, we detected either an infarction without reperfusion or an infarction with very severe oxidative stress that led to the formation of dityrosine before $C_{5b\text{-}9}$ or fibronectin occurred in the infarction zone. In both cases, dityrosine seems to have advantages compared to the other two markers of myocardial infarctions.

Concerning the specificity of dityrosine as a marker of myocardial infarctions, we compared the results of the study hearts to the results of the control hearts treated with electro shocks. The staining results were quite similar though staining in the control hearts was found in various areas of the myocardium and not as localized as after an infarction. This implies that the appearance of dityrosine is not strictly limited to myocardial infarctions. However, this is not really surprising considering that the cause for the generation of dityrosine is oxidative stress which might occur under many pathologic conditions, e.g., arrythmias. Our study with human tissue samples even suggested that dityrosine also occurs in cases with a prolonged agony and a general decrease of oxygen supply [14]. Taking all these findings into account, the assumption that different pathologic conditions of the heart are caused by completely solitary mechanisms is an illusion. It always has to be considered that there are certain common pathways that trigger the appearance of markers under various conditions.

Though this might sound discouraging, it does not question the use of markers of acute myocardial infarctions, in this case dityrosine, as other authors already pointed out [4]. The various reasons for positive staining results and the different staining patterns have simply to be kept in mind when evaluating tissue samples in routine case work: positive staining for dityrosine in a circumscribed area is therefore most likely caused by local ischemia, i.e. a myocardial infarction; on the other hand, diffuse positive staining in numerous locations might rather be the consequence of general oxidative stress, for example caused by an arrhythmia. Either way, the detection of dityrosine is a solid argument that the heart has experienced some kind of abnormal condition going along with oxidative stress that might be the cause of death of a person.

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Matrixmetalloproteinases and tissue inhibitors of metalloproteinases: Immunhistochemical markers in the diagnosis of lethal myocardial infarctions?

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ABSTRACT

Matrixmetalloproteinases (MMP) 2 and 9 as well as tissue inhibitor of metalloproteinases (TIMP) 1 were tested as markers of myocardial early ischemia/infarctions. Experiments with an animal model, the isolated Langendorff heart, and analysis of human tissue samples drawn during autopsies were performed. Results of the experiments with the Langendorff model implied that the detectable amount of the markers might increase early after the onset of ischemia, in less than one hour, under ideal conditions. The results of the examined human cases showed that MMP-2 is constantly detectable in human myocardial tissue with an increased amount in case of an infarction with longer survival times. MMP-9 and TIMP-1 were negative in control cases, distinct positive staining results were obtained mainly in cases of infarctions with longer survival times and only rarely in those with a short survival time. According to these results MMPs and TIMPs do not qualify as first choice markers of myocardial infarctions. As an interesting side finding in the Langendorff experiments, positive staining results for all three markers were seen in myocardial areas that were mechanically traumatized by ECG-electrodes or ligation of blood vessels. These findings make the markers interesting for forensic wound age estimation.

1. Introduction

Diagnosing a lethal myocardial infarction is a common task in forensic routine work, since such cases often occur sudden and unexpected. Because survival times of myocardial infarctions can be very short, macroscopically visible alterations of the myocardial tissue may not be present. Therefore, immunohistochemical examinations have been established in forensic medicine. An ideal marker for the detection of myocardial infarctions should appear early after the onset of ischemia and should be specific for this pathological event. In the past, evaluation of possible new markers has been difficult since they have mainly been tested on human myocardial tissue drawn during autopsies. In such cases, there typically is a lack of certain information, especially the exact survival time after the infarction or a possible reperfusion of the ischemic myocardium. Because of this, defining the precise chronological occurrence of new markers has been challenging. We have been able to adapt an animal model, the isolated Langendorff heart, so that it solves this problem and allows the investigation of possible new markers of myocardial infarctions especially with a view to the time of their appearance [15]. Furthermore, the method allows the generation of infarctions with and without reperfusion, as well as the preparation of different kinds of "control hearts". By combining experiments with the Langendorff-model and the examination of human tissue samples, an extensive evaluation of possible markers for myocardial infarctions is possible.

In the present study we tested two matrix metalloproteinases (MMP), MMP-2 and MMP-9, as well as one tissue inhibitor of metalloproteinases (TIMP), TIMP-1, for their use as immunohistochemical markers of acute myocardial infarctions. MMPs and TIMPs can be found in many tissues and organs. MMPs are important for the degradation of peptides, mainly of the extracellular matrix (eM), and therefore take part in many physiological and pathophysiological processes. Their function in the heart has been subject of extensive research and is especially

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well known for MMP-2 [5,12]. This enzyme is not only involved in the development of the heart and vessels, it is also activated under pathological conditions like infarctions or chronic heart failure. The activation can be triggered by various stimuli like oxidative stress, ischemia/reperfusion injury or inflammatory cytokines. As a consequence, MMP-2 activity can not only be found in the eM but also in the cardiomyocytes. Intracellular MMP-2 is responsible for the degradation of several proteins, amongst them troponin. In the eM, MMPs take part in developmental processes, as described above, and the remodelling due to injuries and diseases.

With a view to ischemia and infarctions not only MMP-2, but also other MMPs and TIMPs have been studied [3,6,7,9,10,16,18,20,21]. Degradation of collagen fibres starts only a short time after the onset of ischemia and is in a large part attributed to an increased activity of MMPs already present in muscle tissue, though animal models have also detected an increased expression of the enzymes during the post-ischemic time course. Herzog et al. detected an increased activity of MMP-2 approximately one hour after an infarction and an increased activity of MMP-9 approximately two hours after an infarction in a rat model with infarctions without reperfusion [9]. The alterations, however, were not as distinct as they were one day after the infarction. The study of Cleutjens et al. presents results of a rat model with myocardial infarctions without reperfusion that show increased amounts of TIMP-1 mRNA in infarcted myocardial tissue about six hours after the onset of ischemia [3]. Romanic et al. detected a markedly increased expression of the active form of MMP-9 and a slightly increased expression of the active form of MMP-2 within one day after infarction in a rabbit model with myocardial infarctions without reperfusion [18]. Furthermore, their study revealed a decreased expression of the active form of TIMP-1 within the first four days after an infarction.

In clinical studies it has been discussed that the concentration of MMPs and TIMPS in a patient's serum might be usable as a prognostic marker after an infarction [13,19]. The inhibition of MMPs, for example by the application of TIMPs or doxycycline, is also being suggested as a therapy after myocardial infarction, since it inhibits extensive cardiac remodelling which is known for increasing the risk of fatal (short or long term) complications [2,8].

These experimental and clinical findings make MMPs and TIMPs also interesting for forensic use. Since no studies regarding the immunohistochemical detection MMPs/TIMPs after myocardial ischemia in post mortem drawn tissue have been published to date, our project aimed on answering the following questions:

1. Can MMP-2, MMP-9 and TIMP-1 be detected immunohistochemically in myocardial tissue after early ischemia/infarction?
2. If yes, how early do MMP-2, MMP-9 and TIMP-1 appear/increase in the detectable amount after the onset of ischemia.
3. Does the detectable amount of MMP-2, MMP-9 and TIMP-1 depend on a reperfusion of the ischemic myocardium?
4. Can alterations of the detectable amount of MMP-2, MMP-9 and TIMP-1 only be seen after myocardial infarctions or can they also be found under other pathophysiological conditions?

2. Material and methods

Animal experiments were performed in compliance with the German legislation on protection of animals, as well as 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). The protocol for the Langendorff-system was approved by the local Animal Ethics Committee (project no. O 27/11).

The examination of human myocardium drawn during autopsies was approved by the ethical committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf (project no. 3527).

2.1. Isolated Langendorff heart

For this study, we used Male Wistar rats. The rats weighed between 230 g and 340 g and were kept on a 12:12 light/dark schedule (lights on at 0600 h) with food and water ad libitum. The animals were anesthetised by intraperitoneal injection of Pentobarbital (90 mg kg^{-1}) and Heparin (0.2 ml). The depth of sedation was verified by the absence of reactions to pain. In this state, rats were decapitated, a thoracotomy was conducted immediately and hearts were excised and mounted onto a Langendorff-system. Treatment in the Langendorff-system and measurement of electric activity (ECG), heart rate and coronary flow (CF) were performed as described before [15].

2.2. Study hearts rat

The study group contained 29 hearts.

We prepared 24 hearts with local early ischemia/myocardial infarction without a reperfusion by ligating the main stem of the left coronary artery close to the aorta. As objective proof of an ischemia/infarction, changes in the ECG, alterations of colour of the myocardium (pallor) and lessening of coronary flow were documented. After a predefined time of ischemia – duration of ligation was varied in steps of 5 min and ranged from a total of 5 min up to a total of 60 min – the hearts were removed from the Langendorff-system and fixed in formalin. Two hearts were prepared for each predefined time of ischemia.

Five hearts were prepared with myocardial infarctions with reperfusion. Reperfusion of the ischemic myocardium was enabled by loosening the ligation of the left coronary artery. The hearts suffered from a constant 20 min ischemic period, the total time after ligation was 30 min, 40 min, 50 min and 60 min caused by a variation of the reperfusion period that lasted 10 min, 20 min, 30 min and 40 min. For each total time after ligation one heart was prepared except for 60 min, for which we prepared two hearts.

2.3. Control hearts rat

The rats' control group consisted of seven hearts without a myocardial infarction.

Two hearts were excised after decapitation of the rats, mounted on the Langendorff-system, removed after 60 min and fixed in formalin without any further manipulation.

Three hearts that were excised after decapitation of the rats and mounted on the Langendorff-system were treated with 26 V electro shocks with a frequency of 10 ms^{-1}. These shocks resulted in tachycardic arrhythmias. The self-limiting arrhythmias lasted for several seconds. The hearts were removed 20 min, 25 min and 30 min after the electric stimulation.

Two hearts were excised after decapitation of the rats and fixed in formalin without being mounted on the Langendorff-system.

2.4. Study group human

We selected 32 cases that were either highly suspicious for or had actually suffered from a myocardial infarction due to clinical symptoms and/or autopsy findings. The cases comprised 24 men and 8 women aged 31–96. All cases showed no signs of decomposition, post mortem interval ranged between 0.5 and 6 days. Cases with and without cardiopulmonary resuscitation (CPR) were included.

Table 1
Histological classification of infarction age according to Cummings et al. [4].

Group/infarction-age	Microscopically visible alterations
A: 0–4 h	No microscopically visible alterations
B: 4–24 h	Contraction bands, hypereosinophilic cells, loss of nucleus, hemorrhages
C: 1–7 days	Inflammation cells, esp. neutrophils
D: 8–14 days	Granulation tissue, beginning of vascularization
E: 2 weeks–2 months	Collagen deposit, tissue less vascularized
F: Over 2 months	Collagen scar

2.5. Control group human

The control group consisted of nine cases with different causes of death: pulmonary embolism, severe traumatic brain injury, anoxic asphyxia, hanging, haemorrhagic shock, carbon-monoxide intoxication, polytrauma and decapitation. Six men and three women were selected, their age ranged from 14 to 75 years. All cases showed no signs of decomposition.

2.6. Graduation of the "age" of infarction in human myocardial tissue

To estimate the "age" of infarctions, i.e. the survival time after the infarction, human myocardial tissue samples were stained with H&E and categorized into six groups, labelled A – F, using the system presented in Table 1 [4].

2.7. Immunohistochemistry

Staining procedures were the same for human and rat tissue samples. These staining procedures were similar to the methods used in previous studies [14,15], however, a specific protocol for each marker was developed. We chose antibodies that detected MMPs/TIMP in rat tissue as well as in human tissue. According to information provided by the producers, the antibodies were not tested for their specifity for the active form of the enzymes.

Tissue sections were deparaffinised, washed in distilled water three times for 5 min and washed in TBS-buffer with 0.5% Tween 20 two times for 5 min.

MMP-2: Slides were boiled in citrate buffer pH 6.0 for 10–15 min, cooled and then washed in distilled water two times for 5 min. Primary antibody against MMP-2 (Medac, rabbit, E 18012) was used in a concentration of 1:200, the slides were incubated over night at +4 °C. Slides were washed in TBS-buffer with 0.5% Tween 20 two times for 5 min. Endogenous peroxidase was blocked with 0.03%H_2O_2 for 20–25 min.

MMP-9: Slides were treated with proteinase K (Dako, S3020) for 7 min and washed two times in TBS-buffer with 0.5% Tween 20 for 5 min. Primary antibody against MMP-9 (biorbyt orb, rabbit, 13583) was used in a concentration of 1:300, the slides were incubates over night at +4 °C. Slides were washed in TBS-buffer with 0.5% Tween 20 two times for 5 min. Endogenous peroxidase was blocked with 0.03%H_2O_2 for 10–15 min.

TIMP-1: Primary antibody against TIMP-1 (biorbyt orb, rabbit, 195994) was used in a concentration of 1:300, the slides were incubated over night at +4 °C. Slides were then washed in TBS-buffer with 0.5% Tween 20 two times for 5 min. Endogenous peroxidase was blocked with 0.03%H2O2 for 10–15 min.

After blocking of endogenous peroxidase all slides were washed in distilled water two times for 5 min and then in TBS-buffer with 0.5% Tween 20 two times for 5 min. Afterwards all slides were incubated with a peroxidase-marked polymer (Medac, Histofine® Simple Stain MAX PO against rabbit, 414142) for 30 min. Staining with AEC and counterstaining of the tissue samples with Mayers Hematoxylin were performed as described before [14,15].

2.8. Evaluation

Evaluation of the staining results was accomplished following two semi-quantitative staging systems, derived from a staging system we used in previous studies [14,15].

MMP-2:
- o = no positive staining.
- x = positive, perivascular and strand-like staining of eM between muscle fibres.
- xx = positive, net-like staining of eM in large tissue areas.
- xxx = positive staining of eM and cardiomyocytes.

MMP-9 and TIMP-1:
- o = no positive staining.
- x = positive staining of single cells.
- xx = positive staining of groups of cells.
- xxx = positive staining of cells in large tissue areas.

3. Results

3.1. General staining pattern

Positive staining for MMP-2 was found mainly in the eM. Only few cases of the human study group showed increased amounts of MMP-2 in the cardiomyocytes. This fact was taken into account in the staging system. Positive staining for MMP-9 and TIMP-1 was found exclusively in the intracellular space.

3.2. Study hearts rat

As an unexpected secondary finding, the markers could also be detected in myocardium surrounding mechanical lesions of the tissue caused by the ligation and the ECG-electrodes. Although this was not our primary interest, we decided to evaluate those findings separately. Therefore, staining results have to be distinguished between positive staining in these areas and in the presumed area of early ischemia/infarction (myocardium of the wall of the septum and the left chamber).

Table 2 presents the results of rats' hearts with an early ischemia/infarction without reperfusion. For MMP-2 delicate positive staining in the area of early ischemia/infarction was found earliest in one heart with a time after ligation of 30 min. Hearts with a longer time of ischemia also showed discreet, positive staining, though not constantly. Even one heart with an ischemic period of 55 min was negative for MMP-2. Slightly positive staining for MMP-9 was found in one heart with a time of ischemia of 25 min. However, similar to MMP-2 not all hearts with a longer ischemic period showed positive results, the longest ischemic period with negative staining results was also 55 min. TIMP-1 showed the least positive results. Only three hearts, with a minimum ischemic period of 20 min, were positive for this marker.

Table 3 presents the results of the rats' study hearts that suffered from early ischemia/infarction with reperfusion. MMP-2 showed discreet, positive staining in all but one case with a time after ligation of 50 min in total. Staining against MMP-9 was

Table 2
Rats' hearts of the study group with early ischemia/myocardial infarction without reperfusion. This table only lists the findings in the ischemic areas of the myocardium; findings in the surrounding of traumatic lesions are excluded. Time after ligation = time between ligation of the left cornary artery and fixation of the hearts in formalin. Signs of infarction including typical ECG-changes, lessening of coronary flow (CF) and pallor of myocardium. Evaluation of staining results based on a staging system used in previous studies [14,15]: MMP-2: o = no positive staining, x = positive, perivascular staining of eM, xx = positive, strand-like staining of eM between muscle fibres, xxx = positive, net-like staining of eM in large tissue areas. MMP-9 and TIMP-1: o = no positive staining, x = positive staining of single cells, xx = positive staining of groups of cells, xxx = positive staining of large tissue areas.

No.	Time after ligation (min)	Signs of infarction	Staining results MMP-2	Staining results MMP-9	Staining results TIMP-1
r1	5	Lessening of CF of 3.5 ml/min	o	o	o
r2	5	Lessening of CF of 5.3 ml/min	o	o	o
r3	10	ECG-changes lessening of CF of 3.9 ml/min	o	o	o
r4	10	Pallor ECG-changes lessening of CF of 3.9 ml/min	o	o	o
r5	15	Pallor ECG-changes lessening of CF of 3.5 ml/min	o	o	o
r6	15	Pallor lessening of CF of 6.0 ml/min	o	o	o
r7	20	Pallor lessening of CF of 3.6 ml/min	o	o	o
r8	20	Pallor lessening of CF 8.0 ml/min	o	o	xx
r9	25	Pallor ECG-changes lessening of CF of 5.1 ml/min	o	o	o
r10	25	Pallor lessening of CF of 6.1 ml/min	o	x	o
r11	30	Discreet ECG-changes lessening of CF of 3.6 ml/min	o	o	o
r12	30	Pallor lessening of CF of 7.9 ml/min	x	o	xx
r13	35	Pallor lessening of CF of 2.4 ml/min	x	xx	o
r14	35	Pallor ECG-changes lessening of CF of 11.3 ml/min	o	o	xx
r15	40	ECG-changes lessening of CF of 1 ml/min	xx	xx	o
r16	40	Discreet pallor ECG-changes lessening of CF of 1.2 ml/min	o	x	o
r17	45	Pallor lessening of CF of 5.0 ml/min	o	o	o
r18	45	Pallor lessening of CF of 4.6 ml/min	o	x	o
r19	50	Pallor lessening of CF of 2.7 ml/min	x	x	o
r20	50	Pallor ECG-changes lessening of CF of 5.8 ml/min	x	o	o
r21	55	Pallor lessening of CF of 3.7 ml/min	o	o	o
r22	55	Pallor lessening of CF of 3.4 ml/min	xx	x	o
r23	60	Lessening of CF of 7.7 ml/min	x	x	o
r24	60	Pallor lessening of CF of 3.8 ml/min	x	xx	o

Table 3
Rats' hearts of the study group with early ischemia/myocardial infarction with reperfusion. This table only lists the findings in the ischemic areas of the myocardium; findings in the surrounding of traumatic lesions are excluded. Ischemic period = length of time of ligation. Reperfusion period = length of time between re-opening of ligation and fixation in formalin. Total time after ligation = length of time between ligation of the left coronary artery and fixation of the hearts in formalin. Signs of infarction including typical ECG-changes, lessening of coronary flow (CF) and pallor of myocardium. Evaluation of staining results based on a staging system used in previous studies [14,15]: MMP-2: o = no positive staining, x = positive, perivascular staining of eM, xx = positive, strand-like staining of eM between muscle fibres, xxx = positive, net-like staining of eM in large tissue areas. MMP-9 and TIMP-1: o = no positive staining, x = positive staining of single cells, xx = positive staining of groups of cells, xxx = positive staining of large tissue areas.

No.	Ischemic period (min)	Reperfusion period (min)	Total time after ligation (min)	Signs of infarction	Staining results MMP-2	Staining results MMP-9	Staining results TIMP-1
r25	20	10	30	ECG-alterations decrease of CF of 3.1 ml/min	x	x	o
r26	20	20	40	ECG-alterations decrease of CF of 12.1 ml/min	x	o	o
r27	20	30	50	ECG-alterations decrease of CF of 1.8 ml/min	o	o	o
r28	20	40	60	Discreet ECG-alterations decrease of CF of 6.8 ml/min	x	xx	o
r29	20	40	60	Pallor ECG-alterations decrease of CF of 9.8 ml/min	x	xx	o

positive in four of the six cases. TIMP-1 was negative in all cases with reperfusion.

Table 4 summarizes those cases, in which the slides showed tissue lesions caused by the ligation or ECG-electrodes (one case with reperfusion and five cases without reperfusion): MMP-2 was positive in two cases with a minimum time after ligation of 45 min. MMP-9 showed positive staining in six hearts, one with a minimum time after ligation of 30 min. TIMP-1 was positive in two cases, the minimum time after ligation was 50 min. One heart with a time after ligation of 20 min showed a definite traumatic lesion but staining was negative for all three markers.

An example for the staining results in the ischemic area of rats' study hearts is shown in Fig. 1. Examples for the staining results in the area of mechanically traumatized tissue can be found in Fig. 2.

3.3. Control hearts rat

All rats' hearts that served as a control group showed no positive staining results for the three markers.

3.4. Study group human

Results of the staining of human tissue samples are presented in Table 5. The cases are graded according to the estimated "age" of the infarction as determined in conventional histology.

Staining for MMP-2 was positive in all cases. Throughout all groups most cases showed staining results according to stages x and xx. Only five cases, one in group C, two in group D and two in group E presented positive staining of cardiomyocytes according to stage xxx.

Table 4
Rats' hearts of the study group with lesions due to traumatic injury (ligation, ECG-electrodes). This table only lists the findings in the surrounding of injuries; findings in ischemic myocardium are excluded. Time after ligation = time between ligation of the left cornary artery and fixation of the hearts in formalin. Evaluation of staining results based on a staging system used in previous studies [14,15]: MMP-2: o = no positive staining, x = positive, perivascular staining of eM, xx = positive, strand-like staining of eM between muscle fibres, xxx = positive, net-like staining of eM in large tissue areas. MMP-9 and TIMP-1: o = no positive staining, x = positive staining of single cells, xx = positive staining of groups of cells, xxx = positive staining of large tissue areas.

No.	Time after ligation (min)	Staining results MMP-2	Staining results MMP-9	Staining results TIMP-1
r8	20	o	o	o
r11	30	o	x	o
r17	45	x	x	o
r19	50	o	x	xx
r21	55	o	xx	o
r23	60	xx	xx	xxx
r26	40 (20 ischemia + 20 reperfusion)	o	xx	o

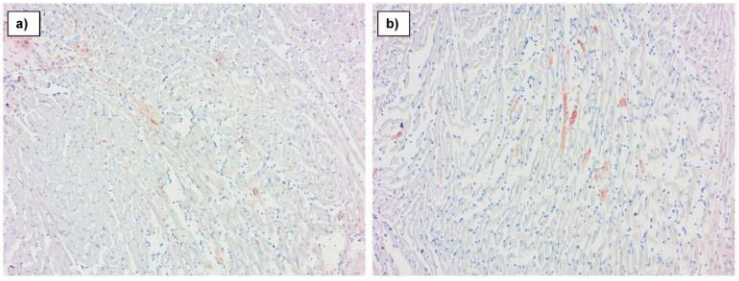

Fig. 1. Staining results of case r23 in the area of ischemic myocardium, infarction without reperfusion and with a time after ligation of 60 min, 100-fold magnification: (a) MMP-2; (b) MMP-9.

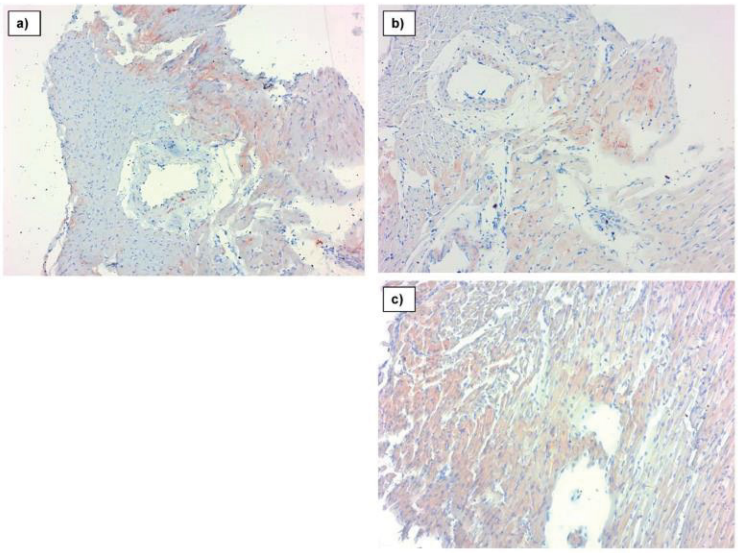

Fig. 2. Staining results of case r23 in the area of the ligation, 100-fold magnification: (a) MMP-2; (b) MMP-9; (c) TIMP-1.

Positive staining for MMP-9 was found in none of the cases of group A. One out of five cases of group B, as well as all cases of group C and D showed positive staining. In group E three cases out of six and in group F one case out of six were found positive for MMP-9. Positive staining was most intense in cases of group D and E.

For TIMP-1, one case out of six was positive in group A and two cases out of five in group B. From the three cases in group C two

Table 5
Staining results of the study group of the human tissue samples. Evaluation of staining results based on a staging system used in previous studies [14,15]: MMP-2: o = no positive staining, x = positive, perivascular staining of eM, xx = positive, strand-like staining of eM between muscle fibres, xxx = positive, net-like staining of eM in large tissue areas. MMP-9 and TIMP-1: o = no positive staining, x = positive staining of single cells, xx = positive staining of groups of cells, xxx = positive staining of large tissue areas. CPR: available information on resuscitation.

Cases graded according to estimated survival time		MMP-2	MMP-9	TIMP-1	CPR
Group A (0–4 h)	h1	xx	o	o	30 min
	h2	xx	o	o	No
	h3	xx	o	o	No
	h4	xx	o	xx	No
	h5	xx	o	o	Yes, duration unknown
	h6	xx	o	o	Yes, duration unknown
Group B (4–24 h)	h7	x	o	x	60 min
	h8	x	o	o	No
	h9	xx	xx	xx	No
	h10	xx	o	o	90 min
	h11	xx	o	o	No
Group C (1–7 days)	h13	x	xx	o	40 min
	h14	xxx	x	xxx	No
	h14	xx	xx	xxx	30 min
Group D (1–2 weeks)	h15	x	x	xxx	No
	h16	xx	xxx	xxx	45 min
	h17	x	xx	xxx	No
	h18	xxx	x	xx	70 min
	h19	xxx	x	xxx	No
	h20	xx	x	xx	40 min
Group E (2 weeks–2 months)	h21	xxx	xx	xx	No
	h22	xx	x	xxx	No
	h23	x	xx	xx	Yes, duration unknown
	h24	xx	o	xxx	35 min
	h25	xxx	o	xxx	No
	h26	xx	o	o	Yes, duration unknown
Group F (over 2 months)	h27	xx	o	o	Yes, duration unknown
	h28	xx	xx	xxx	No
	h29	xx	o	xx	No
	h30	xx	o	xx	45 min
	h31	xx	o	xx	No
	h32	xx	o	o	No

were positive. In group D all cases, in group E all cases but one presented positive staining for TIMP-1 and in group F four cases out of six. The highest staining intensity was found in groups D and E.

Fig. 3 presents examples of the staining results of human study hearts.

3.5. Control group human

Table 6 shows an overview of the staining results of the human control cases.

There was positive staining for MMP-2 in all slides of all cases. Staining was exclusively found in the eM, corresponding with stages x and xx. Three cases presented positive staining for MMP-9, one of them with a presumably increased load of the right ventricle (pulmonary thromboembolism) and two with cardiac resuscitation. There was no positive staining of cardiomyocytes. MMP-9 and TIMP-1 showed no positive staining.

4. Discussion

In clinical research, new markers have been discovered for the diagnosis or the prognosis of myocardial infarctions. Those markers are also interesting for forensic case work. In two previous studies we were able to demonstrate that dityrosine, a protein product of oxidative stress, can be used as a marker for lethal myocardial infarctions with only a short survival time [14,15].

In the present study we combined experiments performed with the isolated Langendorff heart and examinations of human tissue samples to test, if matrix metalloproteinases and tissue inhibitors of metalloproteinases, MMP-2, MMP-9 and TIMP-1, might also be useful in the post mortem diagnosis of lethal myocardial infarctions. The study and its results were mainly meant as a proof of principle to test if and how fast those markers appear after ischemia. This was mainly accomplished by performing experiments with the Langendorff model, whilst the examination of tissue drawn during autopsies was meant to clarify, in how far this also accounts for human tissue.

Rats' study hearts presented positive staining for MMP-9 after a minimum ischemic period of 25 min. MMP-2 was positive after 30 min and one heart presented positive staining for TIMP-1 after 20 min. However, hearts with a longer time of ischemia did not uniformly show positive staining; only single hearts presented positive results for one or more markers. This accounted for early ischemia/infarctions without and with reperfusion. No positive staining result was detected in the rats' control hearts. These findings suggest that under the ideal conditions in the animal model, the markers tested in this study might be detectable after a similar survival time like other infarction markers, e.g. $C_{5b\text{-}9}$, fibronectin and dityrosine [1,14,15,17]. Considering the lack of staining in the rats' control group, the results also at first implied a high specificity of the markers for ischemia/infarctions.

However, the results of the staining of human tissue samples did not meet the expectations. There were obvious differences in the

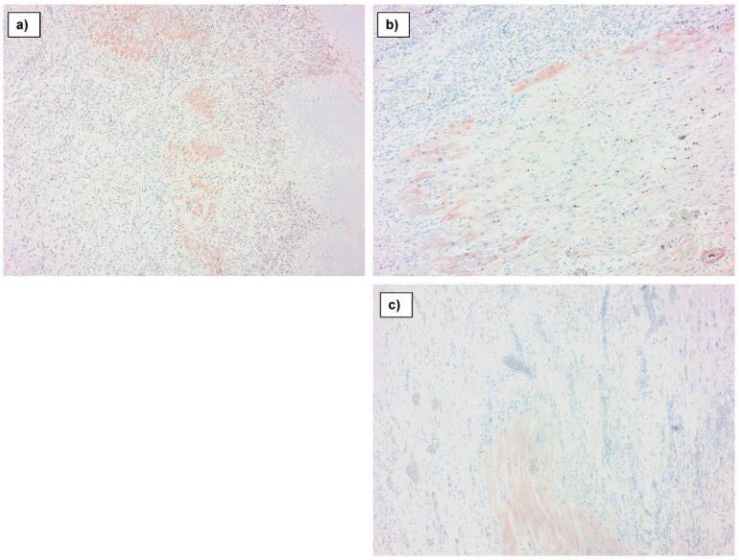

Fig. 3. Staining results of hearts of the human study group, 40-fold magnification: (a) case h21, infarction group E, MMP-2; (b) case h17, infarction group D, MMP-9; (c) case h19, infarction group D, TIMP-1.

Table 6
Staining results of human tissue samples of the control group. Evaluation of staining results based on a staging system used in previous studies [14,15]: MMP-2: o = no positive staining, x = positive, perivascular staining of eM, xx = positive, strand-like staining of eM between muscle fibres, xxx = positive, net-like staining of eM in large tissue areas. MMP-9 and TIMP-1: o = no positive staining, x = positive staining of single cells, xx = positive staining of groups of cells, xxx = positive staining of large tissue areas. CPR: available information on resuscitation.

No.	Cause of death	MMP-2	MMP-9	TIMP-1	CPR
ch1	Pulmonary thrombembolism	xx	o	o	No
ch2	Severe traumatic brain injury	xx	o	o	No
ch3	Anoxic asphyxia	x	o	o	No
ch4	Hanging	xx	x (right ventricle)	o	No
ch5	Haemorrhagic shock	xx	o	o	No
ch6	Pulmonary thrombembolism	xx	xx (right ventricle)	o	45 min
ch7	Carbon-monoxide intoxication	xx	xx (right ventricle)	o	Yes, duration unknown
ch8	Polytrauma	xx	o	o	
ch9	Decapitation	xx	o	o	No

occurrence of MMPs and TIMPs, either caused by the different species (rat – human) or by the different "origin" of the infarctions (model – in vivo). Regardless of the distinct cause, these differences make it difficult to transfer the findings of the Langendorff experiment to forensic cases. For MMP-2 our results imply that a certain amount of the enzyme is constantly present in the eM of human myocardial tissue independent from any pathologies, which is in accordance with the previous research findings, for example by Herzog et al. [9]. Nevertheless, increased amounts of MMP-2 were found in some cases of the infarction groups C, D and E. Increased amounts were indicated by positive staining of myocytes. In contrast, staining for MMP-9 and TIMP-1 was negative in the human control cases as well as in most study cases of infarction groups A and B. Distinctive and strong positive staining for those two markers was found in infarction groups C, D and E and, a bit less intense, in group F. Although those results were quite impressive, the results of the human tissue samples certainly question the use of MMPs and TIMPs as markers of early ischemia/myocardial infarctions. Obviously, under real life conditions, there are factors that delay the increase of the detectable amount of MMP-2, MMP-9 and TIMP-1. Only rarely the circumstances seem to be appropriate for quick alterations after the onset of ischemia as indicated by positive staining results against TIMP-1 in case no h4. Therefore, in cases suspicious for an acute myocardial infarction, in which staining for other makers like C5b-9 or dityrosine is negative, MMP-9 and especially TIMP-1 might be considered for a second attempt, but they will certainly not be the first choice.

The assessment of our results is slightly limited by the fact that the antibodies we used were not tested for their specifity for the active forms of the enzymes. However, our findings imply that only the antibody against MMP-2 might detect both forms since the results show certain amounts of MMP-2 also in healthy myocardium, presumably in the enzymes latent form. Using an antibody strictly specific for the active form might lead to some different findings, but, regarding the data published since now, there is little hope that it would change the overall conclusion. Though Herzog et al. [9] described a beginning increase of the activity of MMP-2 already one hour after an infarction, it substantially increased not before one day after the onset of ischemia.

Besides these sobering results, the evaluation of the Langendorff hearts came up with some surprising secondary findings that were not the main interest of this study but that are nevertheless interesting for forensic routine work. In seven hearts of the rats'

study group the tissue sections clearly showed lesions caused by the ligation of the coronary vessels or by the ECG-electrodes. Six of these seven cases presented positive staining for one or more markers in the adjacent myocardial tissue. With a view to the human control cases, a "mechanical traumatization" caused by right ventricular overload or cardiac resuscitation might also explain the positive staining for MMP-9. Especially MMP-9 seems to be expressed very fast after mechanical trauma since it was found positive in all of these six rats' cases with a minimum ischemic period of 30 min. However, the time designations have to be handled with care, since they refer to the time after the onset of ischemia/infarction, meaning the moment of the ligation of the coronary vessels. Some of the lesions we found, however, have more likely been caused by the insertion of the ECG-electrodes which happened before the ligation. Therefore, in some of the cases with positive staining around a tissue lesion the time between trauma and occurrence of the marker was longer than the documented ischemic period — a time span of about 20 min has to be added, meaning the duration of the stabilization period that usually started after the insertion of the ECG-electrodes. This means that we are not able to define a distinct chronological bottom line for the occurrence of the markers after a mechanical injury of the myocardium, but with regard to heart r23 it might be assumed that it takes less than one hour: the lesion in this sample was definitely caused by the ligation (Fig. 2) and the ischemic period of 60 min was sufficient for strong, positive staining against all three markers. Considering these findings, positive staining in the rats' control hearts would also have been possible since those hearts also were injured by the ECG-electrodes. However, though several sections were prepared we were not able to securely identify the respective lesions (a macroscopic detection of the very tiny lesions was not possible at all) and there were no positive staining results suspicious for a consequence of traumatic injury.

MMP-2 and MMP-9 have already been tested as possible markers for wound age estimation of the skin [11]. The results of the study revealed an increase of MMP-9-positive cells only in skin wounds older than 1 day. For MMP-2, a definite increase was seen after 9 days. Our findings suggest, that an increased expression in injured myocardium may appear much faster. In order to find out more about the use of MMP-2, MMP-9 and TIMP-1 for wound age estimation of mechanical muscle traumata, we are currently planning a study with the isolated Langendorff heart and human tissue samples focussing on this particular question.

5. Summary

Our experiments with the Langendorff-model and post mortem drawn, human tissue samples showed that immunohistochemical staining of myocardial tissue after an infarction/ischemia indeed presents changes in the detectable amounts of MMP-2, MMP-9 and TIMP-1. However, in human cases these changes were mainly seen after a longer survival time, questioning the use of the markers for the detection of acutely lethal infarctions. They might rather be used as a second attempt when other, established markers do not come up with satisfying results. As a side finding, positive staining in areas of mechanically damaged myocardium suggest that the markers might be used for age estimation of muscle wounds.

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Declarations of interest

None.

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ORIGINAL ARTICLE

Biomechanical stress in myocardial infarctions: can endothelin-1 and growth differentiation factor 15 serve as immunohistochemical markers?

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Abstract Myocardial infarctions go along with biomechanical stress, i.e. stretching of muscle fibres, and the expression of certain marker molecules. We tested if two of those markers, endothelin-1 (ET-1) and growth differentiation factor 15 (GDF-15), can be used as immunohistochemical markers for myocardial ischaemia/infarctions. The study included experiments with an animal model, the isolated perfused Langendorff heart, as well as the investigation of human tissue samples drawn during autopsies. The overall picture of our results showed that GDF-15 is very sensitive and expressed very fast, not only as a consequence of ischaemia/infarctions, but also under other circumstances. Even an expression only caused by agony had to be discussed. ET-1, on the other hand, was less sensitive but only positive in those human cases with ischaemia/infarction that also showed typical alterations in conventional histology. Therefore, both markers did not proof to be a suitable diagnostic tool for myocardial infarctions. However, positive staining for ET-1 was also seen in rats' hearts that suffered from arrhythmias after electric shock and in the myocardium of the right ventricle in human control cases in which a right heart failure has to be discussed. Thus, especially ET-1 should be subject of further studies that focus on these pathologies.

Keywords Immunohistochemistry · Myocardial infarctions · Right heart failure · Animal model

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Introduction

The investigation of a sudden and unexpected death is a frequent routine task in the daily work of legal medicine. Quite often, an acute and lethal myocardial infarction has to be considered as a possible cause of death. Although myocardial infarctions go along with morphological changes of the heart's muscle tissue, these alterations are often of limited value because they need a certain time to appear. Macroscopically visible tissue alterations demand a survival time of at least 24 h. In conventional histology, an infarction can only be detected after a period of at least about 4 h. With immunohistochemical examinations, certain markers of myocardial infarctions can be detected considerably earlier. Some of the established markers appear after a survival time of approximately 20–30 min [1]. However, there still is a diagnostic gap of about 20 min, and the use of some markers is limited to cases of infarctions with a reperfusion of the ischemic tissue [1]. In previous clinical studies, immunohistochemical markers for biomechanical stress, which means an elevation of systolic and diastolic wall stress as a consequence of the loss of intermyocyte connections [2], e.g. due to a degradation of collagen [3], were tested as possible new detectors for an acute myocardial infarction. Those studies were able to show that in myocardial infarctions, biomechanical stress provokes the synthesis of endothelin-1 (ET-1) [4] and growth differentiation factor-15 (GDF-15) [5, 6]. From a forensic point of view, the synthesis of those markers is also interesting since they could be of use in the post mortem diagnosis of a lethal myocardial infarction. A complete evaluation of possible new markers for a forensic application is always problematic if it is only done on human tissue samples, because important information, e.g. regarding the survival time, is typically missing. Therefore, we decided to investigate ET-1 and GDF-15 not only on post mortem drawn, human tissue samples, but also in

an animal model. The isolated perfused Langendorff heart allows inducing objectively verifiable, local myocardial infarctions with a defined survival time as well as with or without a reperfusion. It also allows generating other pathophysiologic conditions like arrhythmias that help to evaluate the specificity of possible new infarction markers.

With this study, we aimed on answering the following questions:

1. Can ET-1 and GDF-15 be detected after early ischaemia/infarction of myocardial tissue?
2. How fast do the markers appear after the onset of ischaemia?
3. Can the markers also be detected in healthy myocardium or under other pathophysiologic conditions?

Material and methods

All experiments were accomplished in accordance with the German legislation on protection of animals and 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). The protocol for the Langendorff system was approved by the local Animal Ethics Committee.

An overview of the study protocol for the animal model is given in Fig. 1.

Experiments with post mortem drawn, human tissue samples were approved by the local ethics committee.

The isolated perfused Langendorff heart

For our experiments with the Langendorff system, male Wistar rats with a weight between 230 and 340 g were used. As blood substitute, we used the modified "Krebs-Henseleit solution" to perfuse the isolated hearts. This solution was made of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_2$ + $7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA, 2.25 mM $CaCl_2$, 11 mM D(+)-glucose and 1 mM Lactat. The oxygenation was accomplished with a carbogen mixture (95% O_2, 5% CO_2). To verify the correct composition and a sufficient oxygenation of the solution, an arterial blood gas test was made before the experiments started.

The rats were anaesthetised by an intraperitoneal application of 2 ml pentobarbital (1:10) and 0.2 ml heparin. The sedation was checked by inducing a pain stimulus and the absence of reactions to that verified the adequate depth of the anaesthesia. Rats were then decapitated and an immediate thoracotomy was performed. The still beating heart was removed and mounted on the Langendorff system. All hearts underwent a stabilisation period of 20 min.

Monitoring of cardiac function was done with an electrocardiogram (ECG), measuring the electric activity and the heart rate. Therefore, one electrode was placed in the myocardium of the left ventricle near the apex. The second electrode was placed in the myocardium of the right atrium in the immediate vicinity of the auricle and the third, as an indifferent electrode, on the aorta. Furthermore, coronary flow was measured by a pressure transducer (8–20 ml/min).

Digitalisation of the data was accomplished with an analog to digital converter (PowerLab/8SP, ADInstruments Pty Ltd., Castle Hill, Australia) at a sampling rate of 500 Hz. The data were recorded continuously on a personal computer using Chart for Windows v5.0 (SDInstruments).

Study group rats

The study group contained 29 hearts. In 24 hearts, a local ischaemia/myocardial infarction without reperfusion was induced by ligating the left coronary artery with a tourniquet next to the left auricle. Success of the ligation was verified by typical ECG alterations, decrease of coronary flow and macroscopically visible colour changes of the myocardium (pallor) in the expected area of early ischaemia/infarction. The survival time after the ligation was varied from 5 to 60 min with an interval of 5 min. Afterwards, the hearts were

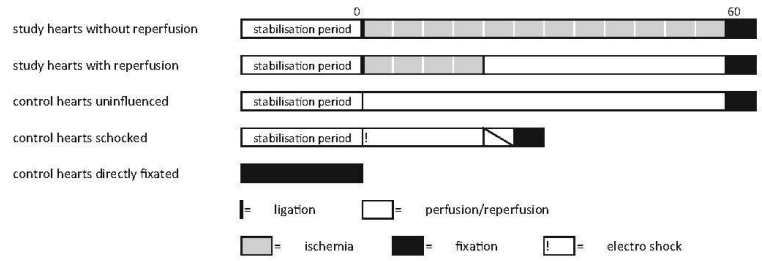

Fig. 1 Study protocol for rat's hearts: every heart on the Langendorff system underwent a stabilisation period of 20 min. Study hearts varied with regard to survival times after ligation and reperfusion. Control hearts were treated with electro shocks or remained in the Langendorff system without any manipulation for 60 min

removed from the Langendorff system and immediately fixed in formalin.

The remaining five hearts received a reperfusion after a constant infarction period of 20 min. Reperfusion time was varied from 10 up to 40 min with an interval of 10 min. Reperfusion was realised by loosening the torniquet on the left coronary artery. After a total survival time of 30 to 60 min after the ligation, the hearts were removed from the system and immediately fixed in formalin.

Control group rats

The control group comprised seven hearts without ischaemia or myocardial infarction.

Two hearts were mounted on the Langendorff system for 60 min, after a stabilisation period of 20 min, without any further manipulation. They were then directly fixed in formalin.

Three hearts were treated with electro shocks while being mounted on the Langendorff system. The shocks were induced with a voltage of 26 V and a frequency of 10 ms^{-1} and provoked tachycardic arrhythmias that lasted for several seconds and were followed by very short periods of an irregular heartbeat. Twenty, 25 and 30 min after shocking, the hearts were removed and fixed in formalin.

The last two hearts were immediately fixed in formalin after thoracotomy and excision without being mounted on the Langendorff system.

Human group

In this group, 41 cases with different causes of death were examined. The 32 cases of the study group suffered from a (suspected) myocardial infarction, based on histological findings and/or clinical information and symptoms. These hearts were divided into six groups, named A–F, based on the histological classification of infarction age according to Cummings et al. [7]. Criteria for the classification were no visible signs of hypoxia—group A; contraction bands, loss of cell nucleus, hypereosinophilia of cells and haemorrhages—group B; infiltrate of inflammation cells—group C; vascularisation and granulation tissue—group D; collagen formation and decrease of vascularisation—group E; and collagen scar—group F. Cases were labelled "suspicious for an infarction" when clinical information came up with typical signs for myocardial ischaemia (retrosternal pain, dyspnoea, etc.), but no corresponding tissue alterations in conventional histology were found (group A).

The control group comprised cases with causes of death other than myocardial infarctions: traumatic brain injury, carbon-monoxide intoxication, lung embolism, anoxic asphyxia, hanging, haemorrhagic shock and polytrauma.

All tissue samples were drawn during autopsies in the Institute for Legal Medicine in Düsseldorf, Germany. Both sexes, as well as cases with and without cardiopulmonary resuscitation (CPR) were included. Also, only cases with a post mortem interval not longer than 2 days were taken into account. Cases with either macroscopically or microscopically visible signs of putrefaction were excluded.

GDF-15 immunohistochemistry

Tissue sections were deparaffinised and washed with aqua dest and TBS (Tris-buffered saline) buffer, pH 7.6. After that, proteinase K was applied for 10 min and slides were washed again twice with TBS buffer for 5 min. Polyclonal primary antibody against GDF-15/MIC-1 (ABIN AK-online Gmbh (Bioss), bs-3818R-HR, rabbit) was used in a dilution of 1:300. Slides were incubated with the primary antibody overnight at a temperature of 4 °C. Afterwards, 0.03% H_2O_2 was used for 10–15 min to inhibit endogenous peroxidase. The samples were washed again with aqua dest and TBS buffer 0.5% Tween 20 for 2 × 5 min before they were treated with Histofine Simple Stain Max PO (Medac 414 142 F) for 30 min and washed again with TBS buffer two times for 5 min. Slides were stained with AEC (2,3-amino-9-ethylcarbazol) (Medac TA-125-) for 2–5 min before being rinsed with aqua dest for 5 min. Finally, the samples were counterstained with Mayers Haemalaun (Fa. Merck 1.09249.250) for 7–10 s, washed with tab water, brought into effect for 20 min and fixed with aquatex (Fa. Merck 1.08562).

ET-1 immunohistochemistry

Tissue sections were deparaffinised and washed with aqua dest and TBS buffer pH 7.6. Afterwards, they were boiled in acitrate buffer of pH 6 for 15 min, before being washed again with tap water, aqua dest and TBS buffer 0.5% Tween 20 for 2 × 5 min. Polyclonal primary antibody against ET-1 (ABIN AK-online GmbH (Bioss) bs-0188R, rabbit) was applied in a dilution of 1:150. Slides were incubated with the primary antibody overnight at a temperature of 4 °C. To inhibit the endogenous peroxidase. 0.03% H_2O_2 was used for 10–15 min. Subsequently, the slides were washed with aqua dest and TBS buffer for 2 × 5 min each. Simple Stain Max PO (Medac 414 142 F) was used for 30 min and afterwards washed with TBS buffer 2 × 5 min, followed by AEC (Medac TA-125-) for 2–5 min before being rinsed with aqua dest for 5 min. Finally, the samples were counterstained with Mayers Haemalaun (Fa. Merck 1.09249.250) for 7–10 s, washed with tab water, brought into effect for 20 min and fixed with aquatex (Fa. Merck 1.08562).

Evaluation

For the classification of the microscopically visible staining results, a semi-quantitative classification system was used:

0 = no positive staining
X = discreet positive staining in scattered cells
XX = intensive staining of groups of cells
XXX = very intensive positive staining in large areas

Results

Study group rats

The hearts of this group showed a specific staining pattern after early ischaemia/infarction for both markers. Positive results could be detected deep in the myocardium of the left ventricle, in the area supplied by the ligated artery. Positive staining in other areas was classified as artefacts as revealed by control hearts (see below).

Table 1 presents the staining results for ET-1 and GDF-15 for all 24 Langendorff hearts with early ischaemia/myocardial infarction without reperfusion and with a survival time from 5 up to 60 min (nos. R1 to R24).

ET-1 staining was generally more discreet compared to GDF-15. The intensity of ET-1 staining also varied from case to case; however, no relation to the survival time could be detected. Seven hearts showed discreet results, from a survival time of 15 up to 60 min. Intensive results could be detected in 15 hearts. The survival time of these hearts ranged from 5 to 60 min. The two remaining hearts, with survival times of 20 and 30 min, showed very intensive results.

All hearts showed positive staining results for GDF-15. Three hearts, with a survival time of 5, 15 and 50 min, showed only discreet results. Sixteen hearts, with a survival time of 10 up to 60 min, showed intensive results, and five hearts with a short survival time, e.g. heart no. 1, as well as a long survival time, e.g. heart no. 24, showed very intensive results. There was no obvious difference between the hearts with a short or long survival time in terms of staining intensity.

Table 2 shows the staining results for Langendorff hearts with early ischaemia/myocardial infarction and a subsequent reperfusion (nos. R25 to R29). The results matched the findings in cases without a reperfusion; however, positive staining for ET-1 seemed to be slightly less intense. Here, we found three hearts with discreet positive staining and two hearts with intensive staining. Very intensive results could not be detected in these hearts. In the case of GDF-15, the intensity varied, for example, in both hearts with a reperfusion period of 60 min, from very intensive to very discreet (nos. R25 and R29).

Table 1 Staining results of rats' study hearts without reperfusion

No.	Survival time (min)	infarction signs	ET-1	GDF-15
R1	5	Decrease CF of 3,5 ml/min	XX	XXX
R2	5	Decrease CF of 5,3 ml/min	XX	X
R3	10	Decrease CF of 3,1 ml/min, typical electrocardiogram-alterationsDecrease CF of 3,1 ml/min, typical electrocardiogram-alterations	XX	XX
R4	10	Decrease CF 3,9 ml/min, typical electrocardiogram-alterations, pallorDecrease CF 3,9 ml/min, typical electrocardiogram-alterations, pallor	XX	XXX
R5	15	Decrease CF of 3,5 ml/min, typical electrocardiogram-alterations, pallorDecrease CF of 3,5 ml/min, typical electrocardiogram-alterations, pallor	XX	X
R6	15	Decrease CF of 6 ml/min, pallor	X	XXX
R7	20	Decrease CF of 3,7 ml/min, pallor	XX	XX
R8	20	Decrease CF of 8 ml/min, pallor	XXX	XXX
R9	25	Decrease CF of 5,1 ml/min, pallor	XX	XX
R10	25	Decrease CF of 6,1 ml/min, pallor	X	XX
R11	30	Decrease CF of 4,6 ml/min, pallor	XX	XX
R12	30	Decrease CF of 7,9 ml/min, pallor	XXX	XX
R13	35	Decrease CF of 2,4 ml/min, pallor	XX	XX
R14	35	Decrease CF of 11,3 ml/min, typical electrocardiogram-alterations, pallorDecrease CF of 11,3 ml/min, typical electrocardiogram-alterations, pallor	XX	XX
R15	40	Decrease CF of 1 ml/min, typical electrocardiogram-alterations, moderate pallor	XX	XX
R16	40	Decrease CF of 1,2 mil/min, typical electrocardiogram-alterations, moderate pallor	X	XX
R17	45	Decrease CF of 5 ml/min, pallor	XX	XX
R18	45	Decrease CF of 4,6 ml/min, pallor	XX	XX
R19	50	Decrease CF of 2,7 ml/min, pallor	XX	XX
R20	50	Decrease CF of 5,8 m/min, typical electrocardiogram-alterations, pallor	X	X
R21	55	Decrease CF of 3,7 ml/min, pallor	X	XX
R22	55	Decrease CF of 3,4 ml/min, intense pallor	X	XX
R23	60	Decrease CF of 7,7 ml/min	X	XX
R24	60	Decrease CF of 3,8 ml/min, intense pallor	XX	XXX

Staining results: O = no positive staining; X = discreet positive staining in scattered cells; XX = intensive staining of groups of cells; XXX = very intensive positive staining in large areas. Survival time = time between ligation and fixation of the heart in formalin. CF = coronary flow.

Control group rats

Table 3 lists up the staining results for the hearts of the rats' control group.

Table 2 Staining results of rats' study hearts with reperfusion

No.	Ischemic period (min)	Reperfusion period (min)	Survival time total (min)	Infarction signs	ET-1	GDF-15
R25	20	40	60	Decrease CF of 4.8 ml/min, typical electrocardiogram alterations	X	X
R26	20	10	30	Decrease CF of 3.7 ml/min, typical electrocardiogram alterations	X	XX
R27	20	30	50	Decrease CF of 1.8 ml/min	X	XX
R28	20	20	40	Decrease CF of 12.1 ml/min, typical electrocardiogram alterations	XX	XX
R29	20	40	60	Decrease CF of 9.8 ml/min, pallor, typical electrocardiogram alterations	XX	XXX

Staining results: no positive staining (O), discreet positive staining in scattered cells (X), intensive staining of groups of cells (XX), very intensive positive staining in large areas (XXX). Ischemic period = time between ligation and reopening of the artery. Reperfusion period = time between the reopening of the artery and fixation of the heart in formalin. Survival time = time between the ligation of the artery and fixation of the heart in formalin

CF coronary flow

The two hearts mounted on the Langendorff system for 60 min without any manipulation presented positive staining in some subendocardial cells and in the wall of the right chamber. These results were considered to be artefacts caused by the Langendorff system and therefore ignored when assessing the study hearts.

The three hearts which were treated with electroshocks showed positive staining results in a nearly circular pattern, including the wall of the left chamber and the septum. GDF-15 staining was more intense than ET-1 staining. It seemed as if the staining intensity increased with a longer survival time for both markers.

The two hearts that were directly fixed in formalin after excision showed very intensive staining for GDF-15 and for ET-1 in subendocardial cells all around the heart. This very distinct staining pattern did not appear in any of the other hearts and was assigned to the immediate fixation.

Figure 2 shows an example for staining results of rats' control group.

Study group human

A detailed listing of the staining results of the tissue samples of the human study group is shown in Table 4; examples for staining results are given in Fig. 3.

Five out of six hearts of cases without visible signs of ischaemia/infarction in conventional histology showed positive staining for GDF-15. Of the six cases of this group, only one presented positive staining results for ET-1.

Twenty five out of 26 cases that presented typical, microscopic alterations due to early ischaemia/infarction presented positive GDF-15 staining, and 17 also presented positive ET-1 staining. GDF-15 staining seemed to be most intense in areas adjacent to obvious infarctions; however, the differences were mostly marginal. Positive ET-1 staining was again more discreet than positive GDF-15 staining. The most intense staining results for ET-1 were found in cases with infarctions of groups C, D and E.

No differences were found between cases with and without CPR.

Control group human

Staining results of the human control group are shown in Table 5.

GDF-15 was positive in all nine control cases. Furthermore, positive staining was very intense and found in various regions of the control hearts. In some cases, the intensity and the size of the results were similar in every examined part of the heart; in other cases, the intensity varied.

Positive ET-1 staining was also found in six of the nine cases. The results for ET-1 were quite similar to the ones of GDF-15. However, it seemed that the most intense results could be found in the right ventricle, especially in cases with a supposedly increased workload of the right ventricle. Examples for this phenomenon are shown in Fig. 2.

No differences were found between cases with and without CPR.

Discussion

Though intensive research has been carried out in the past, the detection of an acute and lethal myocardial infarction in daily routine work of legal medicine is still difficult. Several immunohistochemical markers have been established to help coping with this challenge. However, their applicability might be limited: $C_{5b\text{-}9}$, for example, needs a survival time of about 20–30 min after an infarction [1, 8] and a reperfusion of the infarcted area needs to occur [1]. Ca^{2+}-binding protein S100A1 shows depletion in few cardiomyocytes 15 min after ischaemia, whereas larger areas without staining were seen at about 1 h [9]. Other markers like JunB, connexin 43 and cytochrome c are not yet fully established and still under evaluation; however, though first research results are promising, they also

Table 3 Staining results of rats' control hearts

No.	Survival time (min)	Manipulation	ET-1	GDF-15
R30	60	No infarction induced	X	XX
R31	60	No infarction induced	XX	X
R32	30	Treated with electric shock	XX	XX
R33	25	Treated with electric shock	X	XX
R34	20	Treated with electric shock	X	X
R35	0	Directly fixed in formalin	O	O
R36	0	Directly fixed in formalin	O	O

Staining results: no positive staining (O), discreet positive staining in scattered cells (X), intensive staining of groups of cells (XX), very intensive positive staining in large areas (XXX). Survival time = time between manipulation and fixation of the heart in formalin

suggest a detection of ischaemia-induced alterations at about 30 min after its onset [10].

In a previous study we were able to prove that dityrosine, as a marker of oxidative stress, is helpful in diagnosing an acute myocardial infarction as a cause of death. In this context, we described the difficulty of evaluating new immunohistochemical markers with post mortem drawn, human tissue samples because important information, needed for a profound evaluation, is typically missing [11]. This accounts mainly for the exact survival time after the infarction and a possible reperfusion. Therefore, we chose an animal model to conduct experiments for the evaluation of new markers for myocardial infarctions under controlled conditions: the isolated perfused Langendorff heart. In this system, an isolated heart of a rat can be kept "alive and beating" for several hours. The hearts are harvested post mortem, mounted onto the system and the blood is replaced by a buffer solution. The model allows the induction of infarctions with a defined survival time and with or without reperfusion. Ischaemia/infarctions can be objectified by typical ECG alterations, pallor of the myocardium due to a lack of perfusion and a reduced coronary flow. Since we were successful in establishing dityrosine as a marker for early ischaemia/myocardial infarction, we used this method to test more possible markers.

The cytokine GDF-15 is related to the TGF-β family [12]. These molecules are very important for regulating development, differentiation and tissue repair [13]. The expression of GDF-15 in myocardial tissue and other tissues is very low under physiological, "healthy" circumstances [14, 15]. Under pathologic conditions, such as ischaemia, proinflammatory cytokine stimulation or oxidative stress, an increased expression can be detected [5, 6]. Furthermore, an "unnatural" stretching of muscle fibres, i.e. biomechanical stress, and a low ejection fraction lead to high plasma concentrations of GDF-15 and vice versa [16]. With a view to infarctions, this increased expression mainly takes place in the border zone adjacent to healthy myocardium [5]. Tibor et al. described the importance of GDF-15 for the repairing process after a myocardial infarction. They ascertained that the absence of GDF-15 leads to an increased rate of fatal complications such as heart rupture [17]. This allows the conclusion that GDF-15 plays a major role in the healing process after infarctions [17]. GDF-15 as a marker

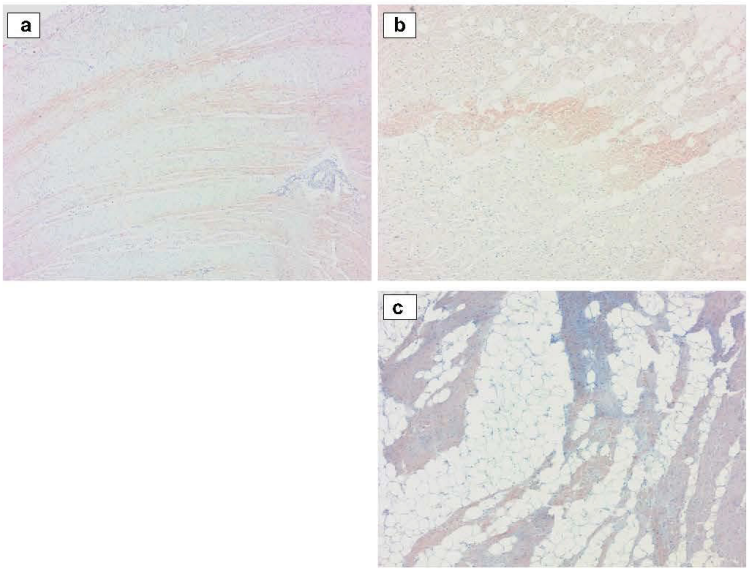

Fig. 2 Examples for staining results of ET-1 in the cases of arrythmia (control hearts of rat) and supposed right heart failure (control hearts of human), fourfold magnification. **a** R33, septum. **b** H35, right ventricle. **c** H36, right ventricle

Table 4 Staining results of cases of the human study group

No.	conv. age	ET-1	GDF-15	CPR
H01	A	O	XX	Yes (duration unknown)
H02	A	O	O	30 min
H03	A	O	XXX	No
H04	A	O	XXX	No
H05	A	O	XX	Yes (duration unknown)
H06	A	XXX	XX	No
H07	B	X	XXX	No
H08	B	O	XXX	No
H09	B	O	O	60 min
H10	B	O	X	No
H11	B	X	XX	No
H12	B	O	X	90 min
H13	C	O	XX	40 min
H14	C	XXX	XX	30 min
H15	D	XX	XXX	No
H16	D	X	XX	45 min
H17	D	XXX	XX	No
H18	D	XX	XXX	No
H19	D	X	XX	70 min
H20	D	XXX	XX	No
H21	D	XXX	XXX	40 min
H22	E	X	XX	No
H23	E	X	XXX	No
H24	E	X	X	Yes (duration unknown)
H25	E	X	XX	No
H26	E	O	XX	Yes (duration unknown)
H27	E	XXX	XXX	35 min
H28	F	X	X	45 min
H29	F	O	XX	Yes (duration unknown)
H30	F	X	XX	No
H31	F	O	XXX	No
H32	F	O	XXX	No

Staining results: O = no positive staining; X = discreet positive staining in scattered cells; XX = intensive staining of groups of cells; XXX = very intensive positive staining in large areas. Conv. Age = conventional age of infarction according to Cummings et al.: A = no microscopically visible alterations; B = contraction bands, hypereosinophilic cells, loss of nucleus, hemorrhages; C = inflammation cells, esp. neutrophils; D = granulation tissue, beginning of vascularization ; E = collagen deposit, tissue less vascularized; F = collagen scar. CPR = information on cardiopulmonary resuscitation if known

is also of use in clinical settings: it has been shown that the levels of GDF-15, "as an independent predictor of future cardiovascular events in patients with suspected myocardial infarction", are able to "identify and risk-stratify" patients who need a coronary intervention [18].

ET-1 is a paracrine/autocrine hormone [19] and the most potent vasoconstrictor peptide that is known yet [20]. Its expression in macrophages, fibroblasts, endothelial cells and cardiomyocytes [21–23] is induced by infiltration of inflammatory cells [24, 25], TNFα, IL-1 and TGF-β [25, 26]. ET-1 is the most important signal substance of acute and chronic heart failure [27, 28] and promotes proliferation of smooth muscle cells [29] and fibroblasts [30] and induces hypertrophy of cardiomyocytes [31] by stimulating specific receptors distributed in the myocardial tissue [32]. Former studies showed that the increase of ET-1 is related to myocardial ischaemia and infarction [4], as well as stretching of the myocardium, e.g. during the diastole [21]. The heart reacts to ischaemia with a de novo synthesis of ET-1 [4] in the cardiomyocytes themselves [4, 33, 34]. In this context, it has been shown that a significant increase of ET-1 occurs in patients with poor coronary collateral circulation by over 95% coronary chronic occlusion [35], which in turn indicates a relationship between ET-1 elevation and cardiac ischaemia. The highest expression of ET-1 could be detected directly in the granulation tissue of the infarcted area, in the border zone and the adjacent myocardium [21–23]. Other studies were able to show that the expression of ET-1 in the myocardium and in the walls of the coronary arteries increases after ischaemia and reperfusion. The maximum expression of ET-1 is reached 3 h after ligation of a coronary artery [36]. Very similar results have been obtained in studies which described an increase of ET-1 levels in the coronary arteries of an infarcted heart at early stages following a percutaneous coronary intervention [37]. ET-1 could thus play an important role in the reparation process of the myocardium after ischaemia or an infarction [4].

The rats' study hearts with and without reperfusion showed positive staining results for both markers. Positive results after early ischaemia/infarction were located deep in the myocardium of the left chamber. Since the two control hearts that were mounted on the Langendorff system for 60 min without any manipulation showed positive staining results in the tissue of the right chamber and subepicardial in the left chamber, those findings were defined as an artefact and not taken into account when evaluating the study hearts. Positive staining results for both markers were found in nearly all hearts with early ischaemia/myocardial infarction from a survival time of 5 min up to a survival time of 60 min. Staining results for GDF-15 were very intense; the positive results of ET-1 were slightly more delicate. Variation of the intensity of the staining and the size of the stained area of the myocardium might be explained by a varying quality of the ligation. This could also explain the variation of the ECG alterations, the coronary flow and the pallor of the affected myocardium.

Though the results of the rats' hearts were very promising, the results of the experiments with human tissue samples did not meet the expectations. We found positive GDF-15 staining results in nearly all cases, in the study hearts as well as in the control hearts. It has to be assumed that the expression of GDF-15 in cardiomyocytes is triggered by various circumstances and that GDF-15 is very sensitive to pathologic

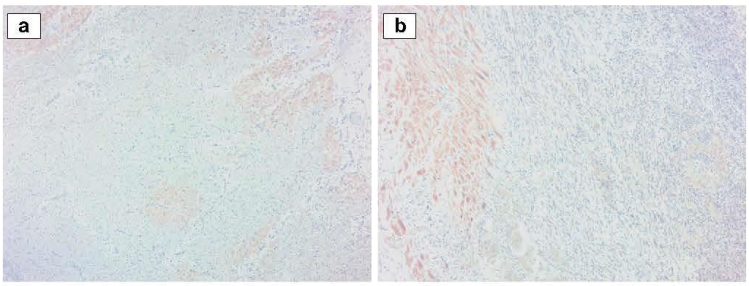

Fig. 3 Examples for staining results of ET-1 and GDF-15 in human study cases, fourfold magnification. **a** H14, ET-1. **b** H16, GDF15

conditions but not specific for early ischaemia/infarction. With a view to the control cases with very short survival times (polytrauma, TBI), it even seems to be possible that cardiac failure during agony alone might lead to the expression of GDF-15. This is a general problem of control cases that has to be kept in mind when working with sensitive markers of myocardial infarctions: although there is no primary cardiac problem, in the end, all cells suffer from a lack of oxygen, possibly leading to respective alterations—at least to a certain degree. This idea of a "final hypoxia" also makes it necessary to compare cases with and without resuscitation. Although our results imply that CPR does not have an effect on the investigated markers, the observation of GDF-15 being especially sensitive to hypoxia at least opens up the possibility that this marker might also be expressed not only due to agony but also due to resuscitation. To distinguish positive staining in such cases from an actual infarction, the staining pattern has to be taken into account as proposed by Ortmann et al. [1]: a rather diffuse staining pattern or positive staining only in very "vulnerable" regions (subendocardial cells, papillary muscles) is typical for general hypoxia rather than a myocardial infarction. In terms of ET-1, we found positive staining results mainly in tissue samples with ischemic/infarcted myocardium. The marker appeared to be less sensitive and slightly more specific than GDF-15. However, positive results only appeared in cases in which alterations of the myocardium due to a lack of oxygen were also visible in conventional staining. In cases with a supposedly very short survival time, positive staining results for ET-1 were not obtained.

Though both markers did not prove to be useful for the diagnosis of myocardial infarctions with short survival times, our study came up with some interesting side findings. The three hearts of the rats' control group that were treated with electroshocks also showed positive staining results in the myocardium of the left chamber. However, in these cases, the tissue of the septum was also affected (see Fig. 2), thus giving the staining pattern a more circular appearance. Since the expression of GDF-15 and ET-1 is triggered by stretching of cardiomyocytes [4–6], these findings are probably caused by the irregular contraction during the provoked arrhythmias. Staining seemed more intense the longer the survival time. This implies that at least ET-1, since it is less sensitive than GDF-15, could be a helpful diagnostic tool in cases with lethal cardiac arrhythmias.

Furthermore, there were also some positive staining results for ET-1 in the hearts of the human control group. However, in contrast to GDF-15, not all tissue samples were affected, but mainly the ones of the right ventricle in the cases in which an increased burden of this part of the heart can be assumed: lung embolism, hanging and suffocating (see also Fig. 2). In these

Table 5 Staining results of cases of the human control group

No.	ET-1	GDF-15	CPR	COD
H33	XXX (especially right ventricle)	X	No	lung embolism
H34	XXX (especially right ventricle)	XX	45 min	lung embolism
H35	XX (especially right ventricle)	XXX	No	anoxic asphyxia
H36	XX (especially right ventricle)	XX	No	hanging
H37	XX	XX	Yes (duration unknown)	carbon-monoxide intoxication
H38	X	X	No	traumatic brain injury
H39	O	XXX	No	haemorrhagic shock
H40	O	XXX	No	polytrauma
H41	O	XXX	No	traumatic brain injury

Staining results: O = no positive staining; X = discreet positive staining in scattered cells; XX = intensive staining of groups of cells; XXX = very intensive positive staining in large areas. CPR = information on cardiopulmonary resuscitation if known. COD = cause of death.

cases, the high workload of the right ventricle might have led to a stretching of the muscle cells, triggering the expression of the marker protein.

In summary, our results show that the tested markers, GDF-15 and ET-1, are not suitable for the diagnosis of a myocardial infarction with a short survival time. With a view to our key questions, we were able to detect GDF-15 and ET-1 in myocardial tissue after early ischaemia/infarction [1]. GDF-15 appeared very fast after the onset of hypoxia while ET-1 only showed up with positive staining results in the cases in which a diagnosis of the infarction was also possible by conventional staining [2]. Furthermore, GDF-15 seems very sensitive to a lack of oxygen and is obviously triggered by a vast variety of cardiac pathologies—maybe even during agony alone [3]. However, the fact that the expression of the evaluated markers is triggered by stretching of cardiomyocytes (biomechanical stress) might open up new possibilities for the diagnosis of lethal arrhythmias, or failure of the right ventricle. This primarily accounts for ET-1 which showed interesting results in the respective hearts/cases. Though these results seem promising, our study did not focus on these pathologies or questions so that more/other experiments should be conducted to verify the proposed application of the markers.

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ORIGINAL ARTICLE

The impact of sex and myocardial ischemic preconditioning on immunohistochemical markers of acute myocardial infarction

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Abstract
The immunohistochemical detection of dityrosine, troponins I (cTNI) and T (cTnT), and connexin 43 has been proposed as a tool for the diagnosis of myocardial infarction with short survival times. Results of clinical and experimental studies reveal that gender and/or ischemic preconditioning of the heart may have an influence on severity and magnitude of myocardial infarction. To clarify the question, if the above-mentioned markers are influenced by sex or ischemic preconditioning, experiments on isolated rat hearts using the Langendorff technique were performed. Using the hearts of 12 male and 12 female Wistar rats a local ischemia was induced through ligation of the left coronary artery. Furthermore, 12 male rat hearts underwent ischemic preconditioning of the heart by stopping the perfusion of the whole heart for 30 min and subsequently reperfusing the heart for another 60 min, before inducing local ischemia. The perfusion time after ligation varied from 10 to 60 min. A control group was comprised out of 6 male and 2 female rat hearts. These were placed in the Langendorff system for 60 min without further manipulation or received ischemic preconditioning without subsequent local ischemia or were excised without being mounted on the Langendorff system at all. All hearts were fixed in formalin and stained immunohistochemically. Depletion of the marker cTnT appeared to be less in females when compared to male hearts, for all other markers tested, no apparent difference in staining results were seen when comparing male and female rat hearts. Male rat hearts with ischemic preconditioning showed no difference compared to male rat hearts without ischemic preconditioning when stained fort dityrosine. Connexin 43 staining was less pronounced in hearts with ischemic preconditioning, whereas cTnI as well as cTnT depletion was more pronounced in preconditioned hearts. The presented findings indicate to some extent the vulnerability of the investigated markers for the influencing factors tested.

Keywords Myocardial infarction · Sex · Ischemic preconditioning · Immunohistochemistry · Animal model

Introduction

The post-mortem diagnosis of an acute myocardial ischemia with only a short survival time may be difficult since morphological findings might not be detectable by autopsy and conventional histological methods in the early interval after ischemia. Certain immunohistochemical markers have been established that have been shown to either appear or be depleted within the affected area. One marker recently tested for the post-mortem detection of early myocardial ischemia is dityrosine [1, 2]. Immunohistochemical staining of dityrosine has been shown to be positive in nearly all examined cases of myocardial infarction aged 4 h to 2 weeks [1]. In an experimental model using rat hearts, dityrosine appeared only minutes after ischemia [2]. Troponins T (cTnT) and I (cTnI) are widely used as diagnostic markers of myocardial infarction in clinical medicine, therefore suggesting their validity as immunohistochemical markers of early cardiac ischemia [3]. The gap junctional protein connexin 43 (Cx43) in its dephosphorylated form has also recently been tested as a marker for early ischemia [4].

Sex and ischemic preconditioning are known to have an impact on severity and development of an acute myocardial infarction. The incidence of cardiac diseases in general is lower in women when compared to men [5]. Guerra et al. [6] found that necrosis as well as apoptosis in cases of heart failure were markedly reduced in females. In a study using a model of mice, Wang et al. [7] have demonstrated that female

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hearts are less susceptible to cell death as a result of oxidative stress, which suggests a cardioprotective effect of the female sex. A study by Bae and Zhang [8] using the Langendorff technique found that female rat hearts regained a better left ventricular function and had a reduced infarction size when compared to male rat hearts.

Ischemic preconditioning consists of short episodes of transient ischemia, applied to the heart either locally or globally, which may protect the heart from subsequent ischemic insults. According to the results of clinical studies involving patients with known pre-infarction angina, ischemic preconditioning may account for significant reduction of infarction size and protection of left ventricular function [9, 10]. A number of experimental studies on various animal models testing the effect of regionally applied ischemia (i.e. the occlusion of single coronary arteries) revealed that these episodes tend to have a protective effect on the myocardium in relation to the severity of myocardial infarction [11–14]. Global ischemia, meaning a depletion of oxygen applied to the whole heart rather than a single region, showed to be effective for protection from myocardial infarction when applied for 15 to 45 min [15, 16]. It has been shown that preconditioning only provides protection for myocardial tissue within a certain window of time. Initially, the heart is protected for 2 h, then followed by a so-called second window of protection 24 h after preconditioning [17, 18].

Considering these findings, it is of utmost interest for postmortem diagnostics to know whether the detectability of immunohistochemical markers is influenced by sex or ischemic preconditioning. If this would be the case, the choice of markers, as well as the interpretation of diagnostic findings would have to ensue with due consideration of the respective indications in each individual case. This means that the sex of the deceased person and a possible history of angina pectoris would have to be taken into account when performing immunohistochemical examinations with a view to a possibly lethal myocardial infarction.

On the basis of experiments on rat hearts with the Langendorff technique [19], we therefore addressed the following questions:

1. Does sex have an impact on the detectability of immunohistochemical markers of early myocardial ischemia?
2. Does global ischemic preconditioning of the myocardium have an effect on the appearance of immunohistochemical markers of early myocardial ischemia?

Materials and methods

The experiments in this study were conducted in accordance with the German legislation on protection of animals and 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). The protocol for the Langendorff experiments was approved by the local Animal Ethics Committee (Project No. O 27/11). The project was designed as an observational study and its results are mainly meant as a proof of principle regarding the key questions.

Isolated Langendorff heart

Hearts from male and female Wistar rats were used in this study, ranging from 226 to 308 g in weight. The same protocol for isolated Langendorff hearts as described in the study by Mayer et al. [2] was used for this experiment.

Study group

Impact of sex

In 12 male and 12 female rat hearts, local ischemia without reperfusion was induced by ligation of the left coronary artery (LAD). Electrocardiogram-alterations (ECG) as well as changes in colour of the myocardium (pallor) and a decrease in coronary flow (CF) were documented as an indicator of infarction/ischemia. The defined times after ligation varied from 10 to 60 min, after which the heart was removed from the Langendorff system and fixed in formalin.

Impact of preconditioning

In 12 male rat hearts, myocardial infarction after ischemic preconditioning was induced. Ischemic preconditioning was achieved by stopping the perfusion of the whole heart for 30 min, then reperfusing the heart for another 60 min, before ligation of the LAD was performed. The time after ligation varied from 10 to 60 min.

Control group

Six male rat hearts and two female rat hearts served as a control group. Two male rat hearts and one female rat heart were mounted on the Langendorff system without any manipulation and removed after 60 min and subsequently fixed in formalin. Two male rat hearts and one female rat heart were excised and directly fixed in formalin. Two male rat hearts received ischemic preconditioning without subsequent ligation of the LAD.

Immunohistochemistry

Formalin-fixed tissue sample sections were deparaffinised and washed first in distilled water three times for 5 min, then in Tris-buffered saline (TBS) buffer with 0.5% Tween 20 twice for 5 min.

Dityrosine The primary monoclonal antibody against dityrosine (Acris, mouse, AM20243PU-S) was applied in a 1:600 dilution to the sections and the slides left to incubate over night at 4 °C. The slides were then washed in TBS buffer with 0.5% Tween 20 twice for 5 min. Endogenous peroxidase was blocked using 0.03% H2O2 for 10–15 min, the slides were subsequently washed in distilled water twice for 5 min and TBS buffer with 0.5% Tween 20, also twice for 5 minutes. Afterwards, the slides were left to incubate with peroxidase-marked polymer (Medac, Histofine Simple Stain MAX PO against mouse, 414 132) for 30 min.

Troponin T The primary monoclonal antibody to troponin T (Biorbyt, mouse, orb-89032) was applied in a 1:75 dilution to the sections. The slides were treated equally to the staining protocol of dityrosine.

Troponin I The primary monoclonal antibody to troponin I (Novus, mouse, N3100-91537) was applied in a 1:200 dilution to the sections. The slides were treated equally to the staining protocol of dityrosine.

Connexin 43 The primary polyclonal antibody to dephosphorylated connexin 43 (Biorbyt, rabbit, orb-193245) was applied in a 1:100 dilution to the sections. The slides were treated equally to the staining protocol of dityrosine. The slides were incubated with peroxidase-marked polymer (Medac, Histofine, 414 142) for 30 min.

All slides were washed in TBS buffer with 0.5% Tween 20 twice for 5 min before being stained using substrate-chromogen AEC (Dako, AEC+ (3-amino-9ethylcarbazole) substrate Chromogen) and then washed with distilled water for 5 min. Subsequently, the slides were counterstained using Mayers Hematoxylin (Merck, Mayers hemalum solution, 1.09249.2500) and washed with distilled water for 5 min. Sections were ultimately covered with Aquatex® (Merck, 1.08562.0050).

Evaluation

Evaluation of immunohistochemical staining was conducted using a semi-quantitative classification system as previously established by Mayer et al. [1, 2] for the staining of *dityrosine*:

- o No positive staining
- x Positive staining of single cells
- xx Positive staining of groups of cells
- xxx Positive staining of cells in large tissue areas

For the evaluation of *connexin 43*, the classification system was adapted as follows:

- o No positive staining
- x Positive staining located in the intercalated discs only
- xx Lateralisation of staining to cell membranes and positive staining of intracellular space of single cells
- xxx Positive intracellular staining of cells in large tissue areas

For the evaluation of troponins I and T, the classification system was adapted as follows:

- o No depletion
- x Depletion of single cells
- xx Depletion of cells in large tissue areas
- xxx Depletion of the majority of tissue

Results

Study group

Positive results in this study were defined as staining/loss of staining deep in the myocardium of the left chamber as well as the septum, being the area of supply affected by ligature of the LAD. Staining in the subepicardial regions and the myocardium of the right chamber is most likely due to artefacts and not a consequence of the interruption of the blood/buffer flow, as it has been shown before [2], and was therefore not taken into account.

Table 1 summarises the staining results of male hearts with an early myocardial ischemia (nos. 1–12); Table 2 gives an overview of the staining results of female hearts with an early myocardial ischemia (nos. 13–24). Comparing the results of both sexes leads to the following findings, examples are also shown in Fig. 1:

- Positive staining results for dityrosine were detected in all cases, with no apparent difference in male or female hearts.
- Staining for Cx43 was positive in all but two cases of male rat hearts, one with a time after ligation of 10 min and one with a time after ligation of 20 min. Otherwise, no differences were seen with a view to sex. However, Cx43 showed considerable difference in occurrence and pattern depending on the times after ligation.
- Depletion of cTnI and cTnT was found in all cases, whereas the depletion of cTnT seemed more pronounced in male hearts.

Table 3 summarises the staining results of male rat hearts with global ischemic preconditioning and early myocardial ischemia (nos. 25–36). Dityrosine staining results showed no apparent difference when compared to male rat hearts without ischemic preconditioning (see Table 1). Staining of Cx43 appeared to be less pronounced in hearts with ischemic preconditioning. Overall, three cases showed negative staining for Cx43,

Table 1 Staining results of study hearts of male rats with early ischemia/myocardial infarction using the following evaluation system: Dityrosine: o, no positive staining; x, positive staining of single cells; xx, positive staining of groups of cells; xxx, positive staining of cells in large tissue areas. Connexin 43: o, no positive staining; x, positive staining located in the intercalated discs only; xx, lateralisation of staining to cell membranes and positive staining of intracellular space of single cells; xxx, positive intracellular staining of cells in large tissue areas. Troponins I and T: o, no depletion; x, depletion of single cells; xx, depletion of cells in large tissue areas; xxx, depletion of the majority of tissue. Signs of infarction including pallor of myocardium, typical electrocardiogram (ECG)-alterations and decrease of cardiac flow (CF)

No.	Time after ligation	Signs of infarction	Dityrosine	Cx43	cTnI	cTnT
1	10	Pallor, ECG-alterations, decrease of CF	xxx	o	xxx	x
2	10	Pallor, decrease of CF	xx	x	xx	xxx
3	20	Pallor, ECG-alterations, decrease of CF	x	x	xx	xx
4	20	Pallor, decrease of CF	xxx	xx	xxx	xx
5	30	Pallor, ECG-alterations, decrease of CF	xxx	x	xx	xx
6	30	Pallor, ECG-alterations, decrease of CF	x	x	xx	x
7	40	Pallor, ECG-alterations, decrease of CF	xxx	xx	xx	xx
8	40	Pallor, decrease of CF	xxx	xx	xxx	xx
9	50	Pallor, ECG-alterations, decrease of CF	xxx	xx	xx	xx
10	50	Pallor, ECG-alterations, decrease of CF	x	o	xx	xx
11	60	Pallor, decrease of CF	xxx	xx	xx	xx
12	60	Pallor, ECG-alterations, decrease of CF	xxx	xx	xx	xx

with times after ligation of 10 and 20 min, respectively. Both troponins showed to be more widely depleted if the hearts received ischemic preconditioning. Representative pictures of these findings are shown in Fig. 2.

Control group

Table 4 presents an overview of the staining results for the controls:

- Whereas dityrosine did not stain positive in the control hearts of male as well as female hearts that were mounted on the Langendorff system without manipulation, it showed intense positive staining when the hearts were excised and directly fixed in formalin, the very distinct pattern resembling the one already described by Mayer et al. [2]. The control hearts having received ischemic preconditioning with no other manipulation also stained positive.

Table 2 Staining results of study hearts of female rats with early ischemia/myocardial infarction using the following evaluation system: Dityrosine: o, no positive staining; x, positive staining of single cells; xx, positive staining of groups of cells; xxx, positive staining of cells in large tissue areas. Connexin 43: o, no positive staining; x, positive staining located in the intercalated discs only; xx, lateralisation of staining to cell membranes and positive staining of intracellular space of single cells; xxx, positive intracellular staining of cells in large tissue areas. Troponins I and T: o, no depletion; x, depletion of single cells; xx, depletion of cells in large tissue areas; xxx, depletion of the majority of tissue. Signs of infarction including pallor of myocardium, typical electrocardiogram (ECG)-alterations and decrease of cardiac flow (CF).

No.	Time after ligation	Signs of infarction	Dityrosine	Cx43	cTnI	cTnT
13	10	Pallor, ECG-alterations, decrease of CF	x	xx	xx	x
14	10	Pallor, ECG-alterations, decrease of CF	xx	x	xx	xxx
15	20	Pallor, ECG-alterations, decrease of CF	xxx	x	xxx	x
16	20	Pallor, ECG-alterations, decrease of CF	x	xxx	xx	x
17	30	Pallor, ECG-alterations, decrease of CF	xxx	xxx	xx	x
18	30	Pallor, ECG-alterations	xxx	xxx	xx	x
19	40	Pallor, decrease of CF	xxx	xx	xxx	xxx
20	40	Pallor, ECG-alterations	xxx	o	xxx	xx
21	50	Pallor, ECG-alterations, decrease of CF	xxx	xxx	xx	x
22	50	Pallor, ECG-alterations, decrease of CF	xxx	xxx	xxx	xx
23	60	Pallor, decrease of CF	x	x	xxx	xx
24	60	Pallor, ECG-alterations, decrease of CF	xxx	xxx	xx	xx

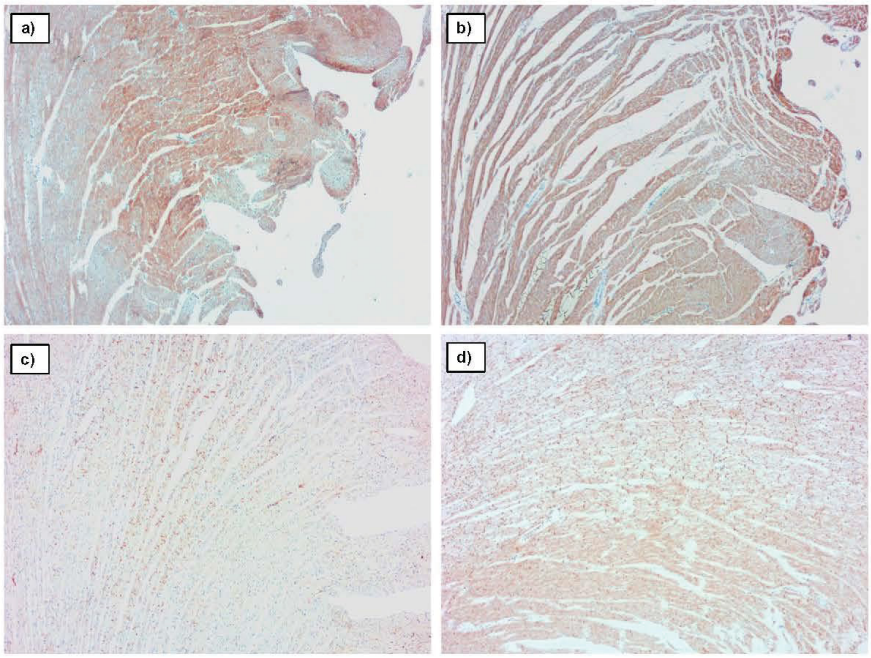

Fig. 1 Examples for staining results of study hearts from male and female rats, 100-fold magnification. Sex-related staining differences of cTnT: **a** no. 10, male, time after ligation 50 min; **b** no. 21, female, time after ligation 50 min. Staining differences of Cx43 depending on time after ligation: **c** no. 14, female, time after ligation 10 min; **d** no. 24, female, time after ligation 60 min

- Cx43 showed positive staining in three cases, one of which being a male rat heart fixed in formalin directly after excision, whereas a second male rat heart fixed in formalin directly after excision showed no positive staining.

Table 3 Staining results of study hearts of male rats with ischaemic preconditioning and early ischemia/myocardial infarction using the following evaluation system: Dityrosine: o, no positive staining; x, positive staining of single cells; xx, positive staining of groups of cells; xxx, positive staining of cells in large tissue areas. Connexin 43: o, no positive staining; x, positive staining located in the intercalated discs only; xx, lateralisation of staining to cell membranes and positive staining of intracellular space of single cells; xxx, positive intracellular staining of cells in large tissue areas. Troponins I and T: o, no depletion; x, depletion of single cells; xx, depletion of cells in large tissue areas; xxx, depletion of the majority of tissue. Signs of infarction including pallor of myocardium, typical electrocardiogram (ECG)-alterations and decrease of cardiac flow (CF)

No.	Time after ligation	Signs of infarction	Dityrosine	Cx43	cTnI	cTnT
25	10	Pallor	xx	x	xxx	xx
26	10	Pallor, decrease of CF	x	o	xxx	xx
27	20	Pallor, decrease of CF	x	o	xxx	xxx
28	20	Pallor, decrease of CF	x	o	xx	xxx
29	30	Pallor, decrease of CF	xx	xx	xx	xxx
30	30	Pallor	xx	o	xxx	xxx
31	40	Pallor, decrease of CF	xxx	x	xxx	xxx
32	40	Pallor, decrease of CF	xxx	xx	xxx	xxx
33	50	Pallor, ECG-alterations, decrease of CF	xxx	xx	xxx	xxx
34	50	Pallor, ECG-alterations, decrease of CF	xx	o	xxx	xxx
35	60	Pallor, decrease of CF	xxx	o	xxx	xxx
36	60	Pallor, decrease of CF	xxx	x	xxx	xxx

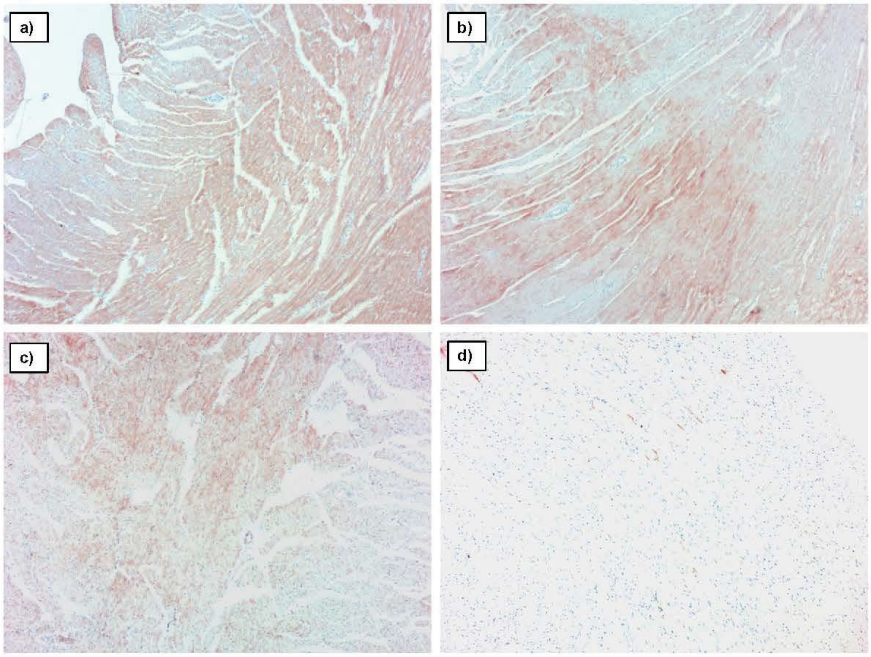

Fig. 2 Examples of staining results depending on ischemic preconditioning, 100-fold magnification: **a** no. 6, male, without ischemic preconditioning, time after ligation 30 min, cTnT; **b** no. 30, male, with ischemic preconditioning, time after ligation 30 min, cTnT; **c** no. 9, male, without ischemic preconditioning, time after ligation 50 min, Cx43; **d** no. 34, male, with ischemic preconditioning, time after ligation 50 min, Cx43

- The staining for cTnT showed depletion in all control hearts. All but one control case showed largely no staining for cTnI. Even after a second repetition of the staining, cTnI could not be detected at all in the examined tissue. Only in one male heart which was subjected to ischemic preconditioning did cTnI show a staining result.

Table 4 Staining results of control hearts without early ischemia/myocardial infarction using the following evaluation system: Dityrosine: o, no positive staining; x, positive staining of single cells; xx, positive staining of groups of cells; xxx, positive staining of cells in large tissue areas. Connexin 43: o, no positive staining; x, positive staining located in the intercalated discs only; xx, lateralisation of staining to cell membranes and positive staining of intracellular space of single cells; xxx, positive intracellular staining of cells in large tissue areas. Troponins I and T: o, no depletion; x, depletion of single cells; xx, depletion of cells in large tissue areas; xxx, depletion of the majority of tissue

No.	Description	Dityrosine	Cx43	cTnI	cTnT
c1	Male heart, mounted on Langendorff system without manipulation for 60 min.	o	o	–	xx
c2	Male heart, mounted on Langendorff system without manipulation for 60 min.	o	o	–	xxx
c3	Female heart, mounted on Langendorff system without manipulation for 60 min.	o	x	–	xx
c4	Male heart, fixed in formalin directly after excision	xxx	o	–	xx
c5	Male heart, fixed in formalin directly after excision	xxx	xx	–	xx
c6	Female heart, fixed in formalin directly after excision	xx	o	–	xx
c7	Male heart, mounted on Langendorff system and received ischaemic preconditioning protocol	xx	x	–	xx
c8	Male heart, mounted on Langendorff system and received ischaemic preconditioning protocol	xxx	o	xxx	x

Typical examples for the staining results can be found in Fig. 3.

Discussion

Previous studies revealed that dityrosine, as well as cTnI and cTnT can be used as immunohistochemical markers for early myocardial ischemia [1–4]. The use of Cx43 has not been thoroughly investigated so far, but first results are promising [4]. Though the impact of sex and ischemic preconditioning on the severity and outcome of myocardial infarction has already been described, there are no studies regarding the impact of sex and ischemic preconditioning on the immunohistochemical markers for the diagnosis of early myocardial ischemia so far. Whereas studies based on human cases will suffer from the lack of clinical information, standardised experiments are possible with the isolated Langendorff heart model.

Ischemic preconditioning in the animal model

With the Langendorff-system, global ischemia as well as localised ischemic events of a defined time can be generated and later evaluated with regard to immunohistochemical staining of the markers mentioned above. One difficulty of establishing ischemic preconditioning in the animal model is the variety of protocols for such. Research on global ischemia suggests that periods of 15 to 45 min of global ischemic preconditioning account for a visible reduction in infarction size [15, 16]. Ischemic intervals of 2 to 15 min have been used before in canine, pig, and rabbit models to achieve the effect of myocardial preconditioning [13, 14, 20–22]. Ischemic preconditioning in rat hearts was achieved by Liu and Downey [12] by inducing three serial 5-min coronary arterial occlusions, establishing that the threshold for protection in rat hearts is above a single 5-min occlusion. However, this protocol employed left main coronary occlusion, rather than introducing a global ischemia to the heart. We decided to employ a protocol of global ischemia of one interval of 30 min to achieve a maximum effect of pre-infarction ischemia, so as to make sure that the global ischemia lasted long enough to potentially elicit the markers we tested. Considering that the first window of protection after classical ischemic preconditioning is defined as 2 h [17, 18], we decided on a reperfusion time of 60 min to allow for sufficient functional recovery before LAD occlusion took place. The duration of LAD

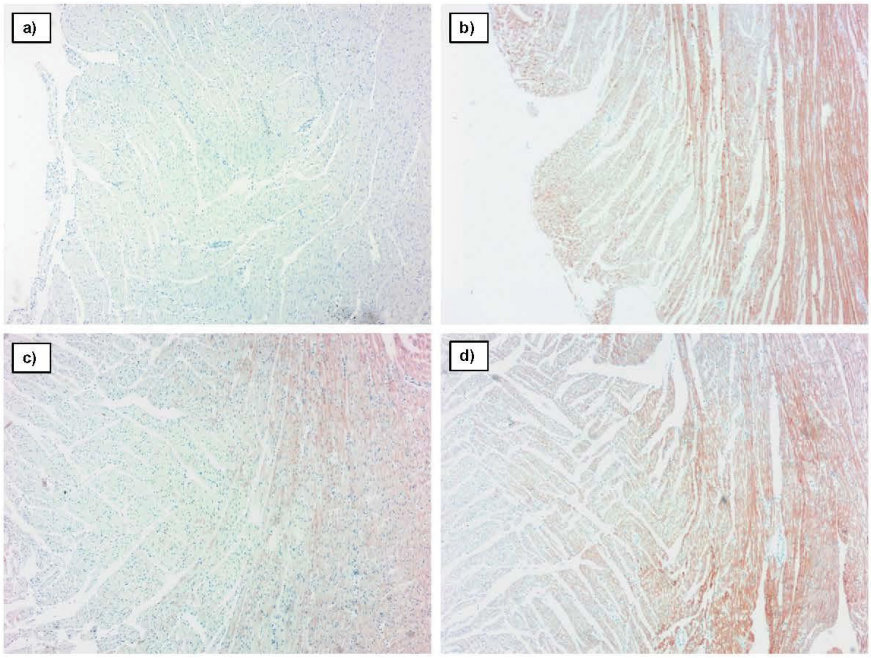

Fig. 3 Examples for staining results of control hearts compared to study hearts, 100-fold magnification: **a** no. c1, male, 60 min in Langendorff system without manipulation, cTnI; **b** no. 11, male, time after ligation 60 min, cTnI; **c** no. c2, male, 60 min in Langendorff system without manipulation, cTnT; **d** no. 12, male, time after ligation 60 min, cTnT

occlusion varied between 10 and 60 min, therefore well within the time frame of a 2-h protection period after ischemic preconditioning took place.

When considering a protective effect of ischemic preconditioning, it needs to be taken into account that a difference exists between acute preconditioning and delayed preconditioning. In terms of the classical or acute preconditioning, it is important for the preconditioning and a subsequent ischemia to take place less than approximately 2 h apart, or the effect of protection through preconditioning will be lost [17, 18]. Another, so-called second window of protection occurs 24 h after preconditioning [17, 18]. In the experimental animal model, these time frames can be easily adapted and applied to achieve a protective effect. However, the resulting implications are that in routine case work, where the time interval between transient global ischaemia and myocardial infarction cannot be safely established, markers that appear to be sensitive to the protective effect of ischemic preconditioning might only be reliable to a certain extent.

The impact of sex and ischemic preconditioning on the detection of the markers dityrosine, Cx43, and troponins T and I

As a protein product occurring only after an event of oxidative stress, previous research has established that dityrosine can be considered a selective marker for tissue damage [23]. No differences in the intensity or the staining pattern of dityrosine were detected when comparing hearts of both sexes, or hearts with ischemic preconditioning.

In myocardial ischemia, Cx43 undergoes progressive dephosphorylation with dephosphorylated Cx43 being primarily located in the intercalated discs. With ongoing ischemia, dephosphorylated Cx43 accumulates and translocates into intracellular pools [24]. Sabatasso et al. [4] described an expression of dephosphorylated Cx43 after 15 min of ischemia, with the accumulation of dephosphorylated Cx43 becoming more pronounced with increased duration of ischemia. In the current study, dephosphorylated Cx43 staining was positive as early as 10 min after the occurrence of ischemia, starting with localised staining mostly in the intercalated discs, and becoming more pronounced with longer survival times. This marker, just like dityrosine, exhibited no significant difference when comparing male and female hearts. However, the occurrence of dephosphorylated Cx43 was less pronounced when hearts had received ischemic preconditioning. One possible explanation for these findings might be a cardioprotective effect of preconditioning, which has been shown to reduce the severity of myocardial infarction in experimental settings [11–14]. Preconditioning reduces necrosis as well as apoptosis [16]. This finding suggests that the products of cell damage such as dephosphorylated Cx43 may also be reduced when infarction occurs after a preconditioning situation. An experimental study on the rat heart in vivo was also able to show that remote ischemic preconditioning decreased the level of ischemia-induced dephosphorylation of Cx43 [25], supporting the assumption that ischemic preconditioning as such has a inhibiting effect on the occurance of dephosphorylated Cx43 in myocardial tissue.

cTnT and cTnI have previously shown to be sensitive immunohistochemical markers of cardiac ischemia of several hours [3, 26]. Furthermore, cTnT and cTnI were visibly depleted after 1 h of ischemia in an experimental setting using rat hearts [4]. A study using post-mortem pericardial fluid as well as serum revealed a correlation between the presence of cTnI and previous myocardial infarction, which indicated a detectable depletion of cTnI from cardiac tissue [27]. In the current study, cTnT was depleted as early as 10 min after ligation, in both male and female hearts. When looking at hearts with ischemic preconditioning, the depletion of cTnT was more pronounced in comparison to hearts without preconditioning, whereas both troponins seemed to be somewhat less depleted in female hearts when compared to males. However, the overall depletion of both troponins was relatively pronounced, which makes it difficult to capture the described differences. All control hearts that only underwent ischemic preconditioning and no subsequent myocardial infarction also showed depletion of cTnT. A more pronounced depletion of cTnT in myocardial tissue which has been exposed to ischemic preconditioning is what might be expected, considering that serum troponin elevation is a common diagnostic marker for ischemic heart conditions. In this study, also mounting the hearts on the Langendorff system and leaving them there for 60 min without any manipulation led to a depletion of cTnT. The results of staining for cTnI in the study groups were overall similar to those obtained by cTnT. However, when looking at the controls, most of the hearts tested presented no staining at all for cTnI. Since the identical hearts showed staining results when using the other three markers, we do not suspect an underlying inadequacy of the Langendorff experiment or the staining protocol; even more, since we repeated the staining procedure with new tissue sections and new antibodies. Still the control hearts in question showed absolutely no staining at all while tissue sections from other hearts presented convincing staining results. The marker cTnI may have been affected by such circumstances as the fixation in formalin. A study by Kobayashi et al. [28] for example showed that myoglobin, which also serves for a marker of myocardial infarction, may appear depleted due to exudation of myoglobin during formalin fixation. This may also hold true for other immunohistochemical markers. However, this is just an explanation attempt because one might raise the objection that in this case similar staining results for cTnI in the study groups had to be expected as well, because all hearts we examined were fixed in formalin for a period of time before being stained. But since cTnI exhibited very promising results in the study groups and a

methodological error had been excluded, we cannot explain why a large part of the control group yielded no positive staining at all. Although these findings are puzzling, we think it is important to present them in this context because they call for further investigation and should be taken into account when using cTnI as an immunohistochemical marker for research on early myocardial ischemia. In general, considering the very pronounced depletion of both troponins even when looking at the control group, both markers appear to be too sensitive to any myocardial damage for their use as markers of early myocardial ischemia in a daily routine.

Conclusion

According to the presented results of experimental data, sex does not have a relevant impact on the detection of the investigated markers, only troponins seem to be (slightly) affected by it. In contrast, effects of ischemic preconditioning on pattern and occurrence of an immunohistochemical marker were evident at least for the markers Cx43, cTnI, and cTnT. Troponins were markedly depleted even in controls with ischemic preconditioning only (and no subsequent myocardial infarction). If this finding from the animal model can be transferred into human cases, it would implicate that a lethal infarction cannot be clearly separated from a non-lethal period of ischemia by the immunohistochemical detection of troponins. Dephosphorylation of Cx43, on the other hand, might be inhibited by non-lethal ischemic events and the detectability of dephosphorylated Cx43 after a following infarction markedly reduced. This is in line with clinical data revealing a protective effect of ischemic preconditioning [9, 10]. The findings of this study—though collected with a limited number of cases and in an animal model—strongly suggest that some of the markers investigated are indeed sensitive for influences such as ischemic preconditioning. This has to be taken into account when further experimentally evaluating the tested markers for a use in everyday routine.

Compliance with ethical standards

The experiments in this study were conducted in accordance with the German legislation on protection of animals and 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). The protocol for the Langendorff experiments was approved by the local Animal Ethics Committee (Project No. O 27/11).

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