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Untersuchungen Implantat-assoziierter Infektionen im Tiermodell

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Abkürzungsverzeichnis

AP	Alkalische Phosphatase
CAS/HA	Calciumsulfat/Hydroxyapatit
CAS/HA-G	Calciumsulfat/Hydroxyapatit-Gentamycin
CAS/HA-V	Calciumsulfat/Hydroxyapatit-Vancomycin
CDC/NHSN	Centre for Disease Control and Prevention/National Healthcare Safety Network
cDNA	Complementary DNA
cfDNA	cell-free DNA/zirkulierende freie DNA
CFU	Colony forming units/Koloniebildende Einheiten
CRP	C-reaktives Protein
CSD	Critical size defect/großer Knochendefekt
FRI	Fracture-related Infection/Implantat-assoziierte Infektion nach Fraktur
HBO	Hyperbare Sauerstofftherapie
HPF	High Power Fields
IL-6	Interleukin-6
MRSA	Methicillin-resistenter Staphylococcus aureus
ODS	Osteomyelitis Diagnose Score
OM	Osteomyelitis
PINP	Prokollagen Typ I N-Propeptid
PJI	Periprosthetic Joint Infection/Periprothetische Gelenkinfektion
PMN	Polymorphkernige Neutrophile
SA	Staphylococcus aureus
SE	Staphylococcus epidermidis

Übersicht der beitragenden Originalarbeiten

Büren C, Hambüchen M, Windolf J, Lögters T, Windolf CD. Histological score for degrees of severity in an implant-associated infection model in mice. Arch Orthop Trauma Surg. **2019**; 139(9):1235-1244. doi: 10.1007/s00402-019-03188-6 [1].

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Zusammenfassung

Implantat-assoziierte Infektionen nach Frakturstabilisierung stellen im Fachgebiet der Orthopädie und Unfallchirurgie eine relevante und herausfordernde Komplikation dar. Die Behandlung ist in aller Regel langandauernd und stellt hohe Anforderungen sowohl an die betroffenen Patienten als auch das behandelnde Team. Neben den klassischen chirurgischen und antimikrobiellen Konzepten stehen auch adjuvante Verfahren als therapeutische Maßnahmen zur Verfügung. Allerdings machen die Pathophysiologie des Krankheitsbildes und die verhältnismäßig geringen Inzidenzen eine standardisierte Untersuchung in klinischen Studien schwierig. Hingegen erlauben *in-vivo* Tiermodelle, die Pathogenese der Infektion weiter zu untersuchen und die Entwicklung neuer prophylaktischer und therapeutischer Behandlungsstrategien zu entwickeln.

Auf Grundlage eines in unserer Arbeitsgruppe entwickelten murinen Modells, welches eine akute, lokale durch *Staphylococcus aureus* induzierte Infektion bei einliegender Plattenosteosynthese beschreibt, schließen sich in dieser Arbeit nun weitere Studien zur Untersuchung Implantat-assoziierter Infektionen im Tiermodell an. Ziel war es zunächst, diagnostische und therapeutische Ableitungen im Mausmodell zu generieren und im Folgenden aus den Erkenntnissen des murinen Modells ein Großtiermodell zu etablieren.

Die erste in dieser Habilitationsschrift zusammengefasste Arbeit beschreibt die Entwicklung eines klinischen Scores, welcher unter Berücksichtigung von radiologischen, histopathologischen und mikrobiologischen Aspekten eine Diagnose und Quantifizierung des Schweregrads von Implantat-assoziierten Infektionen im murinen Modell erlaubt [1]. Der beschriebene Score war zusammengefasst gut durchführbar und mit einer hohen Sensitivität versehen. Daher könnte dieser Score zukünftig ein nützliches Instrument sein, um infektionsbedingte Veränderungen nach einer Fraktur in weiteren Studien zu quantifizieren und miteinander zu vergleichen.

In zwei weiteren Arbeiten wurden potentiell adjuvante Therapiestrategien in der Behandlung von Implantat-assoziierten Infektion im Mausmodell analysiert. In dem standardisierten Mausmodell wurde das linke Femur der Mäuse osteotomiert und durch eine Titan-Verriegelungsplattenosteosynthese fixiert. Die lokale Osteitis wurde durch die Gabe einer definierten Menge an *Staphylococcus aureus* in den Bruchspalt induziert. Neben der Standardtherapie aus chirurgischem Debridement wurde zum einen der Effekt einer hyperbaren Sauerstofftherapie und zum anderen der Einfluss von Antibiotika-versetztem

Knochenersatzmaterial untersucht [2,3]. Als Zielgrößen wurde das lokale Bakterienwachstum, sowie die Immunreaktion bestimmt. Des Weiteren erfolgten radiologische Aufnahmen zur Beurteilung der Knochenheilung. Zusammengefasst konnte in allen Fällen eine erfolgreiche Etablierung der lokalen Osteitis nachgewiesen werden. Es konnte jedoch weder für die hyperbare Sauerstofftherapie noch für das Antibiotika-versetzte Knochenersatzmaterial ein Vorteil gegenüber der Standardtherapie beschrieben werden.

In einem weiteren Schritt wurde das nun gut etablierte und für mehrere Versuchsreihen genutzte Mausmodell genutzt, um ein Großtiermodell zu etablieren. Aufgrund einer entsprechenden Ähnlichkeit von Anatomie, Physiologie und Immunologie wurde sich für ein Mini Pig Modell entschieden. Hier gelang es, eine durch einen Methicillin-resistenten *Staphylococcus aureus* induzierte lokale Osteitis mit Knochenlyse zu generieren. Aufgrund der anatomischen Gegebenheiten konnte auch ein Wechsel des einliegenden Osteosynthesematerials im Zuge der Debridement Operation durchgeführt werden, um sich der klinischen Praxis weiter zu nähern [4]. In der Folge kann nun dieses standardisierte Modell in zukünftigen Studien verwendet werden, um weitere therapeutische Maßnahmen in der Behandlung von Implantat-assoziierten Infektionen zu untersuchen.

Einleitung

Implantat-assoziierte Infektionen – Fakten und aktueller Stand der Forschung

Die Osteomyelitis (OM) bzw. Osteitis beschreibt die akute oder chronische infektiöse Erkrankung des Knochens und des Knochenmarks, welche mit einer schrittweisen Destruktion des Knochens einhergeht [5–7]. Hierbei kann der entzündliche Prozess sowohl nur eine einzige Region des Knochens betreffen, als auch verbreitet, wie zum Beispiel im Knochenmark, dem knöchernen Kortex, dem Periost und den umgebenden Weichteilen vorkommen [5]. Zur Diagnose der OM und der Identifizierung des zugrundeliegenden Erregers können mehrere Knochenbiopsien erforderlich sein, da die Heterogenität der Knochenkolonisation auch zu falsch-negativen Ergebnissen führen kann [5,8]. *Staphylococcus aureus* (SA) ist mit Abstand der häufigste Erreger in Bezug auf knöcherne Infektionen und für 30 – 60 % der Fälle beim Menschen verantwortlich [9,10]. Alle Staphylokokken Spezies zusammen betrachtet verursachen sogar circa 75 % der Osteomyelitiden [11]. Die Entzündung des Knochens kann entweder durch eine indirekte, sogenannte hämatogene Ausbreitung über den Blutstrom oder, wesentlich häufiger, durch eine direkte Kontamination des Knochens im Rahmen von offenen Frakturen oder operativen Eingriffen herbeigeführt werden [5–8,12]. Klassischerweise beschreibt die OM hierbei den sogenannten zentrifugalen Infektionsweg (von innen nach außen) und somit die hämatogene Ausbreitungsform, die zunächst das Myelon befällt [7]. Die Osteitis bezeichnet den zentripetalen Infektionsweg (von außen nach innen), wobei die Keime von außen in Richtung Knochenmark vordringen [7]. Für diese Arbeit ist vor allem die Betrachtung der direkten Kontamination wichtig. Pathophysiologisch stellen sowohl die Haut als auch die Schleimhäute eine natürliche Schutzbarriere dar, sind allerdings mit einer Vielzahl von opportunistischen Krankheitserregern, wie insbesondere dem SA und dem *Staphylococcus epidermidis* (SE), kontaminiert [13,14]. Ist nun diese Schutzschicht gestört, können Keime in die Wunde eintreten und möglicherweise das umliegende Gewebe und den Knochen infizieren. Somit besteht bei allen offenen Frakturen, als auch bei chirurgischen Eingriffen das Risiko eine OM zu entwickeln. Häufig steht die OM in einem direkten Zusammenhang mit einem orthopädischen Implantat (Prothese, Platte, Nagel etc.). In diesem Zusammenhang spricht man dann von einer sogenannten Implantat-assoziierten Infektion. Diese stellen auf orthopädisch-unfallchirurgischem Fachgebiet eine der größten Herausforderungen dar. Zwar ist die Gesamtzahl an Implantat-assoziierten Infektionen sowohl nach operativer Frakturversorgung als auch nach Implantation von Endoprothesen

vergleichbar gering, jedoch führt das Krankheitsbild in der Folge häufig zu langandauernden Krankheitsverläufen mit ausgesprochen hoher Morbidität und Mortalität [15–17]. Bezüglich dem operativen Gelenkersatz liegt die Häufigkeit von periprothetischen Gelenkinfektionen, im Englischen *Periprosthetic Joint Infection* (PJI) Infektionen, je nach Studie bei bis zu 2 % [15,18–21]. Bei den frakturbezogenen, Implantat-assoziierten Infektionen, im englischen *Fracture-related Infection* (FRI), zeigt sich in Abhängigkeit vom jeweiligen Frakturtyp, patientenspezifischen Faktoren und der Expertise des behandelnden Ärzteteams ein Infektionsrisiko von 1 – 2 % für geschlossene und bis zu 30 % für offene Frakturen [22–24]. Die wahrhaftige Inzidenz wird jedoch noch wesentlich höher eingeschätzt, da insbesondere sogenannte Low-Grade-Infektionen schwer zu diagnostizieren sind und daher häufig unerkannt bleiben [24]. Die Entwicklung einer FRI wird hierbei sowohl von systemischen (Diabetes mellitus, pAVK), als auch lokalen (Gewebeschädigung, Durchblutung) Faktoren des Wirtes, als auch der Pathogenität des vorliegenden Keimspektrums beeinflusst. Bezüglich des Keimspektrums werden Zweidrittel aller Implantat-assoziierten Infektionen durch SA (34%) und SE (32%) verursacht [25,26]. Die opportunistischen Staphylokokken Spezies dringen durch die Wunde ein und haften am Knochen an. Das Vorhandensein der Fremdoberflächen von Implantaten und Prothesen fördert hierbei die bakterielle Anheftung. Insbesondere die Bildung des sogenannten Biofilms führt zur dauerhaften Keimbesiedlung der Fremdoberflächen [27]. Trotz der vielen möglichen Definitionen kann dieser Biofilm vereinfacht als ein strukturiertes Konsortium von Bakterien in einer selbsterstellten Matrix beschrieben werden [28]. Hierbei werden durch die Bakterien Adhäsine exprimiert, welche in Interaktion mit extrazellulären Matrixproteinen, wie beispielsweise Fibronektin und Kollagen, des Wirtes treten [13,29,30]. Durch direkten Kontakt mit den Fremdmaterialien können die Bakterien ebenfalls durch hydrophobe oder elektrostatische Wechselwirkungen an dem Implantaten anheften [13,31]. Innerhalb eines solchen Biofilms sind die pathogenen Keime in der Lage, sowohl Antibiotika, Desinfektionsmitteln, der Phagozytose als auch anderen Komponenten des angeborenen und adaptiven Immunsystems des Wirts zu entgehen [28,29,32,33]. Häufig treten die Bakterien innerhalb dieses Biofilms auch in eine Art Ruhezustand ein [34].

Therapeutische Optionen in der Behandlung von Implantat-assoziierten Infektionen nach Frakturen

Die Behandlung von FRIs ist herausfordernd und stellt hohe Anforderungen sowohl an die betroffenen Patienten als auch das behandelnde Team und das Gesundheitssystem [8,35]. Die Therapie ist in der Regel langandauernd und vielseitig. Auch ein Rezidiv der Infektion während oder nach der Behandlung ist keine Seltenheit [36,37]. Insbesondere durch SA verursachte Infektionen sind mit hohen Rezidivraten assoziiert, selbst wenn der Keim in den zu Beginn der Therapie entnommenen Gewebeproben nicht nachgewiesen werden konnte [38].

Ein erster wichtiger Schritt, um eine angemessene Therapie einer FRI einzuleiten, ist die sichere Diagnose der Erkrankung [39]. 2018 wurde von einer Expertengruppe eine entsprechende international anerkannte Definition zur Diagnosestellung veröffentlicht und zwei Jahre später weiter angepasst [40,41]. Der Algorithmus in Abbildung 1 fasst die Schritte zur sicheren Diagnostik einer FRI zusammen.

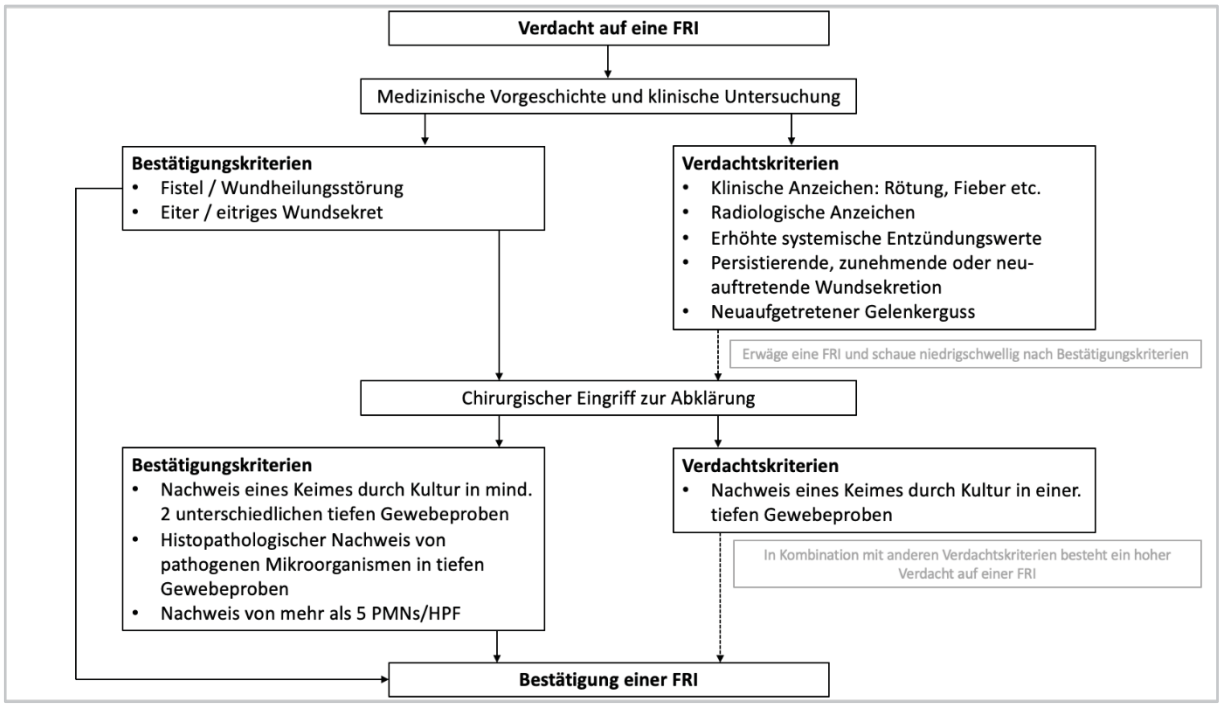

Abbildung 1. Algorithmus zur Diagnosestellung einer Implantat-assoziierten Infektion nach Fraktur / *fracture-related infection* (FRI) nach Govaert GAM 2020 [40]. PMN= Polymorphkernige Neutrophile, HPF= High Power Fields. Mit freundlicher Genehmigung des Springer-Verlags.

Neben der reinen Diagnostik spielt auch die Klassifikation der vorliegenden FRI eine Rolle in der therapeutischen Entscheidungsfindung [39]. Fang et al. nimmt demnach eine Unterscheidung bezüglich der Zeit bis zum Einsetzen der Symptome nach

Frakturstabilisierung, der Dynamik der Erkrankung, der Schwere der Erkrankung, dem jeweiligen Infektionsweg, der Lokalisation der Infektion, der Frakturstabilität, dem Wirt samt seiner Begleiterkrankungen, dem begleitenden Weichteilschaden sowie den infektionsverursachenden Erregern vor [42]. Leider sind standardisierte Behandlungsstrategien aufgrund eben dieser Heterogenität der Erkrankung schwierig zu definieren und aktuell fehlend [43]. Die Expertengruppe fordert deshalb schon bald, weitere Faktoren in ihren Algorithmus zu integrieren, um an Hand der Diagnose und Klassifikation gezieltere therapeutische Entscheidungen treffen zu können [39].

Primäres Ziel in der Behandlung von FRIs sollte die Konsolidierung der Fraktur unter Vermeidung einer chronischen OM sein [44]. Eine sorgfältige und individuelle Planung der therapeutischen Strategie ist daher essenziell. Hierbei muss eine vollständige Infektsanierung nicht immer zwingend notwendig sein. Die Suppressionstherapie kann bei vitalem Knochen und Nachweis von niedrigvirulenten Erregern auch gute Ergebnisse darstellen [22,23,45]. Zu Beginn der Therapie sollte daher festgelegt werden, ob eine Implantatentfernung möglich ist, eine Infekteradikation bei einliegendem Implantat angestrebt wird oder ggf. die Infektsuppression bis zur knöchernen Konsolidierung mit anschließender Implantatentfernung und Ausheilung des Infektes, eine Option darstellt. In der Behandlung von FRIs stellt die chirurgische Therapie samt ihren verschiedenen Aspekten den Grundpfeiler dar:

(1) Frakturstabilisierung
(2) Chirurgisches Wunddebridement
(3) Probengewinnung zum Keimnachweis

Die Stabilisierung der vorliegenden Fraktur ist von größter Bedeutung. Neben der eigentlichen Frakturheilung nimmt die Frakturversorgung auch einen positiven Einfluss auf das Infektgeschehen [46,47]. Im Gegensatz zur Therapie bei periprothetischen Frakturen haben FRIs den Vorteil, dass das Implantat nach der Frakturkonsolidierung entfernt werden kann, um das Fremdmaterial samt Biofilm zu eliminieren und eine chronische OM zu umgehen. Da insbesondere der oben beschriebene Biofilm auf Implantaten zur Unterhaltung der Infektion führt und dieser therapeutisch kaum adressiert werden kann, sollte bei vorliegendem Infekt und noch nicht konsolidierter Fraktur das einliegende Implantat entfernt und durch ein anderes, (sauberes) ausgetauscht werden. In allen Fällen ist auf eine ausreichende Weichteildeckung zu achten [39]. Das chirurgische Wunddebridement ist ebenfalls essentiell. Es umfasst im Allgemeinen die Exzision von nekrotischem und schlecht durchblutetem

Gewebe (es trägt nicht zur Wundheilung bei und kann keine Antibiotika aufnehmen) sowie die Entfernung aller nicht erforderlichen Fremdmaterialien (gebrochene oder gelockerte Schrauben / Drähte, nicht-resorbierbares Fadenmaterial etc.). Im Rahmen eines jeden Debridements ist die Entnahme von Gewebeproben erforderlich [40]. Hierdurch kann neben der Bestätigung der Infektion auch der direkte Keimnachweis erfolgen. Die Kombination aus sowohl mikrobiologischer und histopathologischer Aufarbeitung der Proben verbessert die Diagnostik und ermöglicht somit eine spezifischere antiinfektive Therapie [48–50].

Neben der chirurgischen Therapie ist sowohl die lokale als auch systemische antimikrobielle Therapie zu berücksichtigen. Bezogen auf die lokale Gabe gibt es weder für die Art des Antibiotikums noch für die jeweilige Applikation und Dosierung klare Empfehlungen, so dass die Anwendung im klinischen Alltag höchst variabel ausfällt [51,52]. Die Verabreichung systemischer Antibiotika ist abhängig vom Keimspektrum, der lokal epidemiologischen Resistenzrate, als auch von patientenspezifischen Faktoren. Nur bei Vorliegen einer Sepsis sollte eine sofortige Therapie nach Entnahme von Blutkulturen erfolgen. In allen anderen Fällen kann eine empirische Therapie nach Entnahme der Gewebeproben begonnen werden. Nach Erhalt des Keimspektrums und Einholung mikrobiologischer Expertise kann auf eine gezielte Therapie umgestellt werden. Das anschließende Therapieregime ist abhängig vom Ausmaß des Infektes, dem Keimspektrum, dem Vorhandensein eines Implantats, der Frakturkonsolidierung und wiederrum patientenspezifischer Faktoren, wie bspw. Begleiterkrankungen.

Die o.g. chirurgischen und antibiotischen Konzepte gehören zu den klassischen Behandlungsstrategien in Bezug auf FRIs. Aufgrund des heterogenen Erscheinungsbilds der Erkrankung und der hohen Rate an Rezidiven werden auch immer wieder neue, sogenannte adjuvante Therapien entwickelt und untersucht. Diese sollen im Folgenden kurz aufgegriffen werden. Die hyperbare Sauerstofftherapie (HBO) wirkt sich beispielweise positiv auf die Heilung diabetischer Wundsyndrome aus. Durch die Erhöhung der lokalen Sauerstoffkonzentration um das 10-15-fache hat diese Therapie nachgewiesener Weise einen antiödematösen, antibakteriellen und neovaskularisierenden Effekt auf das umliegende Gewebe [53,54]. Hierdurch wird ebenfalls eine erhöhte Fibroblastenproliferation, Kollagenproduktion und Epithelisierung erzielt [54,55]. Aufgrund dieser Eigenschaften wurde die HBO schon als mögliche Behandlungsoption in der Therapie der OM erörtert [56,57]. Auch bestimmte Wachstumsfaktoren werden in der additiven Therapie von knöchernen

Infektionen diskutiert [58]. Beispielsweise konnte in Tiermodellen und Fallstudien eine positive Auswirkung von Blutplättchen- und Leukozyten-angereichertem Plasma durch eine schnellere Ausheilung der Infektion durch antimikrobielle Effekte gezeigt werden [59–61]. Elektro- und Nanotechnologische Verfahren werden insbesondere durch die Auflösung des Biofilms als mögliche Therapien vorgeschlagen. Kasimanickam fasst hierzu unter anderem die Bedeutung des elektrischen Stroms, des Ultraschalls und der photodynamischen Therapie zusammen [62].

Durch das radikale Debridement von infiziertem und nekrotischem Gewebe im Zuge der chirurgischen Therapie entsteht häufig eine größere Defektzone von sowohl Weichteilgewebe als auch Knochen, dem sogenannten Totraum. In den letzten Jahren ist vor allem auch die Bedeutung und Behandlung dieses Totraums im Zusammenhang mit FRIs in den Vordergrund gerückt, insbesondere da dieser einen optimalen Platz für die Ansiedlung von Bakterien bietet. Lokale antibiotische Therapien stellen daher eine mögliche Strategie dar [63,64]. Metsemakers et al. haben erst kürzlich ihre evidenzbasierten Empfehlungen zu diesem Thema zusammengefasst [63].

Antimikrobiell-beschichtete Materialien werden ebenfalls seit einigen Jahren in der Therapie Implantat-assoziierter Infektionen diskutiert. Neben gut definierten Strategien, Materialien zu entwickeln, welche entweder die Proteinadsorption und die frühe bakterielle Adhäsion am Implantat verhindern oder die mikrobielle Besiedlung samt Aufbau des Biofilms stören oder die Zerstörung des Biofilms begünstigen [65]. Campoccia et al. unterscheidet hierbei zwischen bakteriostatischen, bakteriziden und Biofilm-aktiven Substanzen, welche in der Therapie von Implantat-bezogenen Entzündungen Anwendung finden können [65].

Tiermodelle als Grundlage der experimentellen Forschung der Osteomyelitis / Osteitis

Die Vielfalt des Krankheitsbildes und der Pathophysiologie der OM als auch die verhältnismäßig geringen Inzidenzen machen eine standardisierte Untersuchung in klinischen Studien schwierig [66]. Diese beschränken sich in aller Regel auf retrospektive Analysen oder Fallstudien. Aufgrund dessen erlauben *in-vivo* Tiermodelle, die Pathogenese der Infektion zu untersuchen und die Entwicklung neuer prophylaktischer und therapeutischer Behandlungsstrategien zu entwickeln [67,68]. In den Anfängen dieser Modellentwicklungen standen Kaninchenstudien. Hier wurden abgeschwächte Staphylokokken intravenös injiziert, jedoch festgestellt, dass durch die alleinige intravenöse Injektion keine progressive

intramedulläre Entzündung generiert werden konnte [69]. In nachfolgenden Studien wurden die Staphylokokken sowohl intravenös als auch direkt auf den Knochen injiziert. Auf diese Weise konnten zwar osteomyelitische Läsionen erzeugt werden, diese waren allerdings kaum fortschreitend und nicht vergleichbar mit der im Menschen vorkommenden Pathophysiologie. Inzwischen liegen zahlreiche Studien vor, die in verschiedensten Spezies Untersuchungen zur OM aufzeigen. Reizner et al. geben in ihrer systematischen Übersichtsarbeit von 2015 einen Überblick über die bis dato vorliegenden Tiermodelle zur Untersuchung einer SA-assoziierten OM [70]. Zusammengefasst konnten die Autoren in den eingeschlossenen Studien neun verschiedenen Spezies zur Untersuchung der *in-vivo* SA-OM identifizieren (Maus:7, Ratte:29, Meerschweinchen:1, Hamster:1, Kaninchen:36, Hund:1, Schaf:7, Ziege:4, Schwein:2). Die wenigsten dieser Studien untersuchen eine OM in Bezug auf eine vorliegende Fraktur und / oder einer Osteosynthese [70]. Die Suche nach dem idealen Modell, welches die klinische Situation einer akuten OM bei einliegendem Implantat nach Frakturversorgung wiederspiegelt dauert jedoch weiter an. Modelle zur Therapie einer Implantat-assoziierten Infektion erfordern die Fähigkeit, aktuellen chirurgischen, antibiotischen und additiven Therapieverfahren standzuhalten. Aus diesem Grund begann unsere Arbeitsgruppe 2013 ein in-vivo Tiermodell zu entwickeln, um weitere therapeutische Maßnahmen anzuschließen. Ziel war es zunächst, in einem Maus-Modell eine Implantat-assoziierte Infektion mit dem am häufigsten vorkommenden Keim, dem biofilmbildenden SA, bei vorliegender Fraktur und einliegender Plattenosteosynthese zu genieren [71]. Nach der erfolgreichen Etablierung ermöglichte nun dieses Modell, weitere therapeutische und prophylaktische Ansätze in der Behandlung der Biofilm-assoziierten SA-OM bei einliegendem Implantat zu analysieren. In der Folge konnte gezeigt werden, dass eine Beschichtung der zur Frakturstabilisierung genutzten Titanplatten mittels Lysostaphin einen positiven Einfluss sowohl auf das Infektgeschehen als auch der Frakturheilung hat [72]. Die eigenen wissenschaftlichen Arbeiten knüpfen nun an dieser Stelle an, um zum einen das vorhandene Modell weiter zu entwickeln und weitere therapeutische Ansätze zu beurteilen.

Eigene Studien und Ergebnisse

Ziel der eigenen Studien ist es, an Hand von Tiermodellen (Maus als Kleintiermodell, Schwein als Großtiermodell) diagnostische und therapeutische Optionen bezogen auf Implantatassoziierte Infektionen zu untersuchen. Zunächst wurde ein Score entwickelt, um die Frakturheilung im Osteitis Mausmodell unter Berücksichtigung histopathologischer, mikrobiologischer und radiographischer Parameter standardisiert quantifizieren zu können. Des Weiteren wurden die hyperbare Sauerstofftherapie (HBO) als auch antibiotisch versetzte Knochenersatzmaterialen als adjuvante Therapie in der Behandlung einer Implantat-assoziierten Infektion im Mausmodell untersucht. Die neueste Studie beschäftigt sich nach der mehrfach erfolgreich induzierten Osteitis im Kleintiermodell mit dem Versuch und dem Gelingen der Etablierung einer Implantat-assoziierten Infektion im Großtiermodell in Form von Mini Pigs.

1. Histologischer Score zur Beurteilung des Schweregrads in einem Implantat-assoziierten Infektionsmodell in Mäusen

Histological score for degrees of severity in an implant-associated infection model in mice.
Büren C et al. Arch Orthop Trauma Surg, 2019.

Zur Diagnosestellung und Klassifikation von Knocheninfektionen wurden in der Literatur bereits verschiedene Scores beschrieben [73,74]. Diese beziehen sich jedoch in der Regel auf OM ohne einliegendem Fremdmaterial oder auf periprothetische Knocheninfektionen [75]. Interessanterweise berücksichtigen Studien dieser Art allerdings nie das Vorliegen einer Fraktur bzw. einer Frakturkonsolidierung im Verlauf. Dies erscheint überraschend, da die Frakturstabilisierung, also ein einliegendes Implantat, einer der größten Risikofaktoren für die Entwicklung einer OM ist. 2018 entwickelte die Expertengruppe um Metsemakers einen Algorithmus zur Diagnosestellung einer FRI [41]. Hier wurden bereits viele Parameter mit einbezogen, allerdings unter Nichtbeachtung histopathologischer Aspekte, wie bspw. der Anzahl von Polymorphkernigen Neutrophilen (PMN). Ziel der vorliegenden Studie war es daher, einen Score zu entwickeln, der sowohl die Diagnose als auch die Quantifizierung einer FRI im Mausmodell unter Berücksichtigung radiologischer, mikrobiologischer und histopathologischer Gesichtspunkte ermöglicht.

Zur Bestimmung des Gesamtscores wurden insgesamt vier Parameter berücksichtigt: (1) Kallusbildung des Knochens, (2) Konsolidierung der Fraktur, (3) strukturelle Veränderungen

der Markhöhle auf histopathologischer Ebene und (4) Anzahl der Bakterien. Die Gesamtpunktzahl wurde durch Addition der Punkte der einzelnen Parameter beschrieben. Tabelle 1 fasst die Definition und Gewichtung der einzelnen Parameter zusammen.

Tabelle 1. Definition und Gewichtung der Parameter Kallusbildung, Knochenheilung, Struktur des Markraums und Anzahl an Bakterien zur Bestimmung einer Implantat-assoziierten Infektion im Mausmodell.

Parameter	Punkte	Beschreibung	Definition
Kallusbildung des Knochens	6	viel Kallusbildung	Quotient: Kallus / knöcherner Kortex > 1.6
	3	Kallusbildung	Quotient: Kallus / knöcherner Kortex 1.1 - 1.6
	1	keine Kallusbildung	Quotient: Kallus / knöcherner Kortex < 1.1
Konsolidierung der Fraktur im Röntgen	12	Konsolidierung	vollständige Konsolidierung der Osteotomie
	6	unvollständige Konsolidierung	unvollständige Konsolidierung der Osteotomie
	1	keine Konsolidierung	Atrophie des knöchernen Kortex
Strukturelle Änderung des Markraums	6	physiologische Zellstruktur	gut strukturierte Struktur
	3	unphysiologische Zellstruktur	unstrukturierte Struktur, akkumulierte Immunzellen
	1	Nekrose	avitales Gewebe
Anzahl der Bakterien	3	niedrige Anzahl	< 4 / 20 Gesichstfelder bei 100-facher Vergrößerung
	2	moderate Anzahl	4-10 / 20 Gesichstfelder bei 100-facher Vergrößerung
	1	hohe Anzahl	< 10 / 20 Gesichstfelder bei 100-facher Vergrößerung

Anschließend wurden diese Parameter in einem bereits etablierten, standardisierten Implantat-assoziierten Mausmodell in 35 BALB/c-Mäusen angewendet. In diesem Modell wurde das linke Femur osteotomiert, mit einer Titan-Verriegelungsplattenosteosynthese stabilisiert und anschließend eine lokale Infektion durch Impfung von SA in den Frakturspalt induziert. In der Kontrollgruppe wurde lediglich eine Osteotomie mit anschließender Osteosynthese ohne Induktion eines SA Infektes durchgeführt. Der entwickelte Score wurde an den Tagen 7, 14 und 28 postoperativ bestimmt. Wir gingen davon aus, dass ein niedrigerer Gesamtscore mit einer höheren Wahrscheinlichkeit für eine postoperative bestehenden Implantat-assoziierte Osteitis korreliert.

Jeder einzelne der vier Parameter zeigte niedrigere Werte für die Infektionsgruppe im Vergleich zu den Kontrollen nach vier Wochen. Unabhängig vom bewerteten Zeitpunkt war der Gesamtscore in der Infektionsgruppe niedriger im Vergleich zur Kontrollgruppe (Abb. 2). Für die Kontrollgruppe lag der mediane Gesamtscore bei 18 ± 1,8 Punkten eine Woche postoperativ und bei 21 ± 1,3 Punkten vier Wochen postoperativ. Die höhere Punktzahl nach vier Wochen spiegelt hier den physiologischen Wiederaufbau des Knochens nach Osteotomie ohne Infekt wider. In der Osteitis-Gruppe betrug der Gesamtscore 13 ± 2,3 Punkte nach einer Woche postoperativ (vs. Kontrollgruppe: $p = 0,03$), 10,5 ± 4 Punkte nach zwei Wochen

postoperativ und 8,5 ± 4,8 Punkte nach vier Wochen postoperativ (vs. Kontrollgruppe: p = 0,002).

Die Analyse ergab eine Sensitivität von 0,85, eine Spezifität von 1,0, einen negativen Vorhersagewert von 0,67 und einen positiven Vorhersagewert von 1,0.

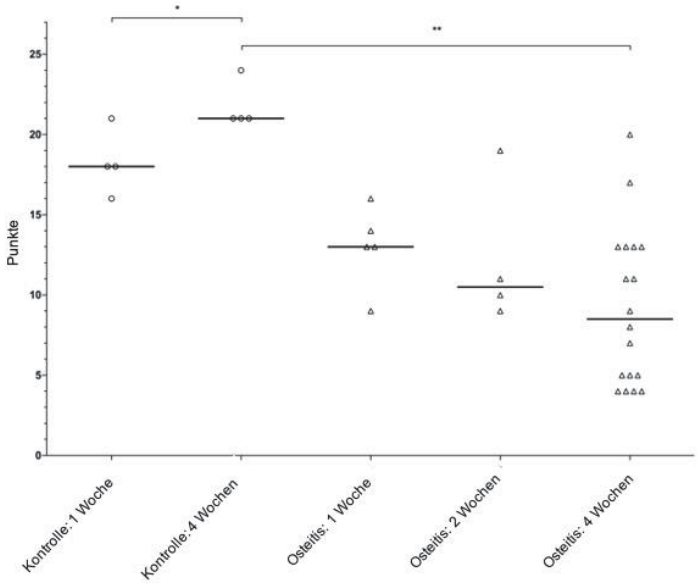

Abbildung 2. Darstellung des Gesamtscores. Der Gesamtscore in der Infektionsgruppe (Osteitis) war zu allen untersuchten Zeitpunkten niedriger im Vergleich zur Kontrollgruppe (Kontrolle). Angepasst nach [1]. Mit freundlicher Genehmigung des Springer-Verlags.

Der entwickelte Score zur Beurteilung des Schweregrads einer Implantat-assoziierten Infektion im Mausmodell unter Beachtung radiologischer, mikrobiologischer und histopathologischer Gesichtspunkte war gut durchführbar und mit einer hohen Sensitivität versehen. Daher könnte dieser Score zukünftig ein nützliches Instrument sein, um infektionsbedingte Veränderungen nach einer Fraktur in weiteren Studien zu quantifizieren und miteinander vergleichen zu können.

2. Einfluss der hyperbaren Sauerstofftherapie (HBO) auf Implantat-assoziierte Infektionen am Femur im Maus-Modell

Effect of hyperbaric oxygen therapy (HBO) on implant-associated osteitis in a femur fracture model in mice. **Büren C** et al. PlosOne, 2018.

Die hyperbare Sauerstofftherapie (HBO) besteht aus der intermittierenden Verabreichung von 100% Sauerstoff bei Drücken von mehr als einer absoluten Atmosphäre (ATA). Sie wird bereits sehr erfolgreich in der Behandlung verschiedener orthopädisch-unfallchirurgischer Krankheitsbilder, wie beispielsweise in der adjuvanten Therapie chronischer Wunden

eingesetzt [76,77]. Im Tiermodell konnte ebenfalls eine positive Wirkung der HBO auf den Knochenmetabolismus und die Frakturheilung aufgezeigt werden [78]. Weitere Studien legen eine vorteilhafte Wirkung der HBO in der Behandlung von bakteriellen Infektionen des Knochens nahe [79–83]. Aufgrund dieser immun- und frakturmodelierenden Effekte wurde sie auch bereits als mögliche Option in der erweiterten Behandlung für FRIs diskutiert. Ziel dieser Studie war es, die Bedeutung der HBO im Rahmen einer Implantat-assoziierten lokalisierten Osteitis durch SA als weitere Maßnahme neben der chirurgischen Behandlung zu bewerten.

In einem zuvor etablierten, standardisierten Mausmodell wurde das linke Femur von insgesamt 120 BALB/c-Mäusen osteotomiert und durch eine Titan-Verriegelungsplattenosteosynthese fixiert. Die lokale Osteitis wurde durch die Gabe einer definierten Menge an SA in den Bruchspalt induziert. Ein radikales Debridement mit Spülung wurde im Sinne der chirurgischen Standardtherapie an den Tagen 7, 14, 28 und 56 durchgeführt. Um die Wirkung der HBO-Therapie zu bewerten, wurden die in Tabelle 2 zusammengefassten Gruppen analysiert.

Tabelle 2. Gruppeneinteilung zur Beurteilung der HBO-Therapie in einer Implantat-assoziierten Infektion. SA=Staphylococcus aureus.

Gruppe	Anzahl (n)	Beschreibung
1. Kontrolle	12	Kontrollgruppe: keine Fraktur, kein Infekt, keine HBO Therapie
2. Kontrolle - HBO	12	Kontrollgruppe: keine Fraktur, kein Infekt, HBO Therapie
3. Osteotomie	24	Osteotomiegruppe: Fraktur, kein Infekt, keine HBO Therapie
4. Osteotomie - HBO	24	Osteotomiegruppe: Fraktur, kein Infekt, HBO Therapie
5. Osteotomie - Infekt	24	Infektgruppe: Fraktur, SA-Infekt, keine HBO Therapie
6. Osteotomie – Infekt - HBO	24	Infektgruppe: Fraktur, SA-Infekt, HBO Therapie

Die Gruppen, welche eine HBO-Therapie erhielten, wurden einmal täglich an den Tagen 7-21 postoperativ für 90 Minuten einer Überdrucksauerstofftherapie (2 ATA, 90% O_2) ausgesetzt (Abb. 3). Das lokale Bakterienwachstum wurde durch die Bestimmung der *Colony forming units* (CFU) in den im Zuge der Debridement Operationen entnommenen Lavageproben bestimmt. Die lokale Immunreaktion durch Analyse von Polymorphkernigen Neutrophilen (PMN), Interleukin (IL)-6 und der zirkulierenden freien DNA (cfDNA) wurden ebenfalls untersucht. Die Frakturheilung wurde an den Tagen 7, 14, 28 und 56 in a.p. Röntgenbildern, als auch systemisch mittels Alkalischer Phosphatase (AP) und Prokollagen Typ I N-Propeptid (PINP) aus dem Serum quantifiziert.

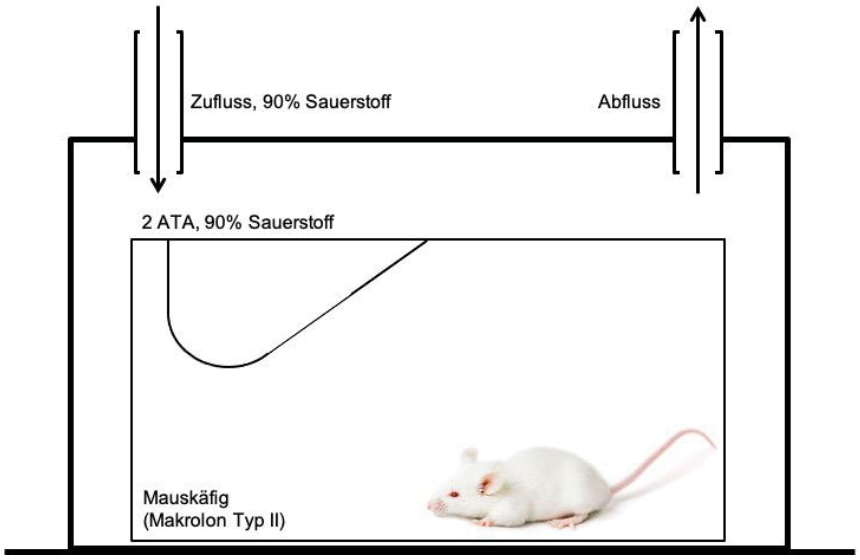

Abbildung 3. Darstellung der hyperbaren Sauerstofftherapie (HBO) bei Mäusen. Die Standardkäfige (Makrolon Typ II) mit BALB/c-Mäusen wurden in eine geschlossene Kammer gestellt. 90% Sauerstoff wurde über einen Zufluss bereitgestellt. Anschließend wurden die Mäuse für 90 Minuten einem Überdruck (2 ATA) ausgesetzt. Angepasst nach [2]. Mit freundlicher Genehmigung des Public Library of Science Verlags.

In den Osteotomie-SA-Infektionsgruppen konnte die Etablierung einer lokalen Osteitis im Vergleich zu den Kontrollgruppen mit und ohne Plattenosteosynthese durch signifikant erhöhter IL-6-, cfDNA- und PMN-Spiegel in den Lavageproben (an Tag 7 und 14 jeweils $p < 0.05$) nachgewiesen werden. Jedoch zeigte die additive HBO-Therapie keinen signifikanten Einfluss auf die CFU und die Immunantwort im Vergleich zu der chirurgischen Standardtherapie (jeweils $p > 0.05$). Gleichzeitig war die HBO-Therapie mit einer verzögerten Frakturheilung und einer höheren Rate an Pseudarthrosen bis Tag 28 assoziiert (Abb. 4).

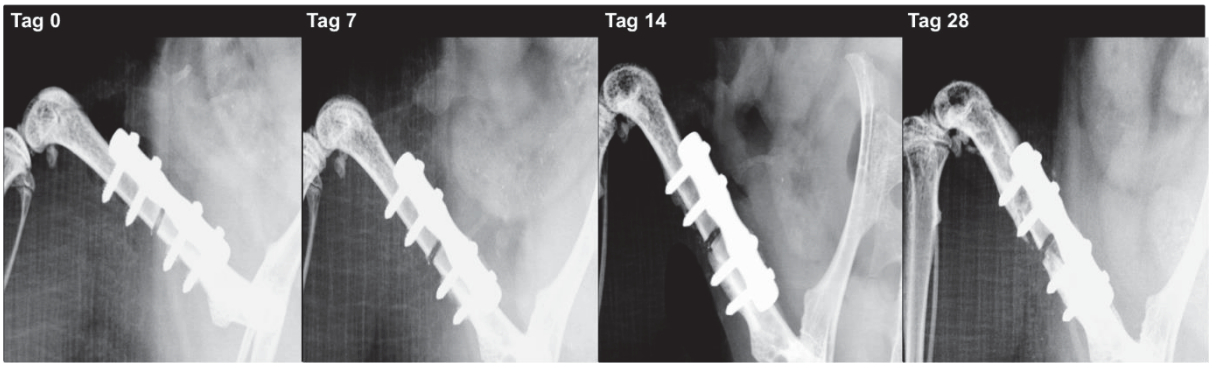

Abbildung 4. Radiologische Aufnahmen zur Beurteilung der Frakturheilung bei infizierten Mäusen. Im Gegensatz zu den nicht infizierten Mäusen zeigten Mäuse, die beiden Osteitis-Gruppen (mit und ohne HBO) zugeteilt wurden, häufiger ein Ausbleiben der Frakturkonsolidierung. Dieses Beispiel (serielle Röntgenaufnahmen einer Maus an den Tagen 0, 7, 14 und 28) aus der der Osteitis/HBO-Gruppe zeigt die Entwicklung einer Pseudarthrose. Angepasst nach [2]. Mit freundlicher Genehmigung des Public Library of Science Verlags.

Zusammenfassend konnte eine lokale Implantat-assoziierte Knocheninfektion im Mausmodell induziert werden, bei der allerdings die HBO weder einen positiven Einfluss auf die lokale Infektion und Immunantwort noch auf die Frakturheilung im Vergleich zur chirurgischen Standardtherapie darstellte. Somit kann die HBO in dem beschriebenen Modell nicht als adjuvante Therapie zur Behandlung von Implantat-assoziierten Knocheninfektion empfohlen werden.

3. Wirkung von antibiotisch versetzten Calciumsulfat/Hydroxyapatit (CAS/HA)- Einsätzen auf Implantat-assoziierte Infektionen am Femur im Maus-Modell

Effect of antibiotic infused calcium sulfate/ hydroxyapatite (CAS/HA) insets on implant-associated osteitis in a femur fracture mode in mice. Oezel L, **Büren C** et al. PLoS One, 2019.

Cerament® (Bonesupport Holding, Lund, Schweden) ist ein bioresorbierbares, synthetisches Knochenersatzmaterial, bestehend aus 60% Calciumsulfat und 40% Hydroxyapatit (CAS/HA) [84]. Calciumsulfat fungiert hierbei als resorbierbarer Träger für Hydroxyapatit. Hydroxyapatit selbst ist stark osteokonduktiv und fördert das Knocheneinwachsen. Cerament® kann somit als Transplantat bei Knochendefekten, wie bspw. Pseudarthrosen verwendet werden [84].Des Weiteren haben mehrere klinische Studien die Wirksamkeit von antibiotika-versetztem CAS/HA in der Behandlung von infizierten Knochendefekten nachweisen können [64,85–87]. Diese Arbeiten konzentrierten sich entweder auf eine primäre Infektionsprävention oder auf die Behandlung einer chronischen Osteitis und können somit keine Aussage über die Bedeutung des antibiotika-versetzen CAS/HA in der Behandlung einer akuten Osteitis nach Osteosynthese treffen. Darüber hinaus wurden bislang die immunologischen Hintergründe nur unzureichend untersucht. Ziel der Studie war es daher, erstmals die Wirkung von antibiotisch infundiertem CAS/HA auf die Knochenheilung, das lokale Infektgeschehen und der systemischen Immunantwort in einem bereits gut untersuchten Implantat-assoziierten Osteitis-Mausmodell, nun mit einem *critical size defect* (CSD) zu beurteilen.

Das linke Femur von 72 BALB/c-Mäusen wurde zunächst osteotomiert und eine kritische Defektzone von 2,5mm generiert. Anschließend erfolgte eine Stabilisierung des Knochens mittels einer 6-Loch Titan-Verriegelungsplatte. Die Osteitis wurde durch Impfung von SA in die Defektzone induziert. Anschließend wurde die Defektzone mittels Cerament® aufgefüllt. Hierbei wurde die Wirkung von CAS/HA im Vergleich zu antibiotikaversetzen CAS/HA verglichen. Hierzu wurden folgende Gruppen untersucht: (1) CAS/HA, (2) CAS/HA mit

Gentamycin (CAS/HA-G) und (3) CAS/HA mit Vancomycin (CAS/HA-V). An Tag 7 und 42 erfolgten Revisionsoperationen mit lokalem Debridement und Lavage. Es wurden Proben zur Beurteilung des lokalen Bakterienwachstums mittels CFU und der Immunreaktion (IL-6, PMN) entnommen. Die Frakturheilung wurde ebenfalls an Tag 7 und 42 in Form von Röntgenaufnahmen und Knochenheilungsmarker (AP, PINP) aus Blutproben evaluiert.

Eine erfolgreich induzierte Osteitis konnte an Tag 7 mit signifikant erhöhten CFU-, IL-6- und PMN-Spiegeln in den Lavageproben nachgewiesen werden. Die Parameter zeigten eine Reduktion in allen Gruppen an Tag 42. CAS/HA-V zeigte eine signifikante Reduktion von CFU und PMNs in Lavageproben am 42 im Vergleich zur Infektgruppe ohne Knochenersatzmaterial und im Vergleich zum nicht antibiotikaversetztem Knochenersatzmaterial. Eine positive Wirkung auf die Knochenheilung konnte nur bei den nicht infizierten Kontrollgruppen nachgewiesen werden. Während die Anwendung von bloßem CAS/HA und auch dem antibiotikaversetzten CAS/HA-G und CAS/HA-V Tendenzen zur Lyse des Knochens zeigten (Abb. 5 und 6).

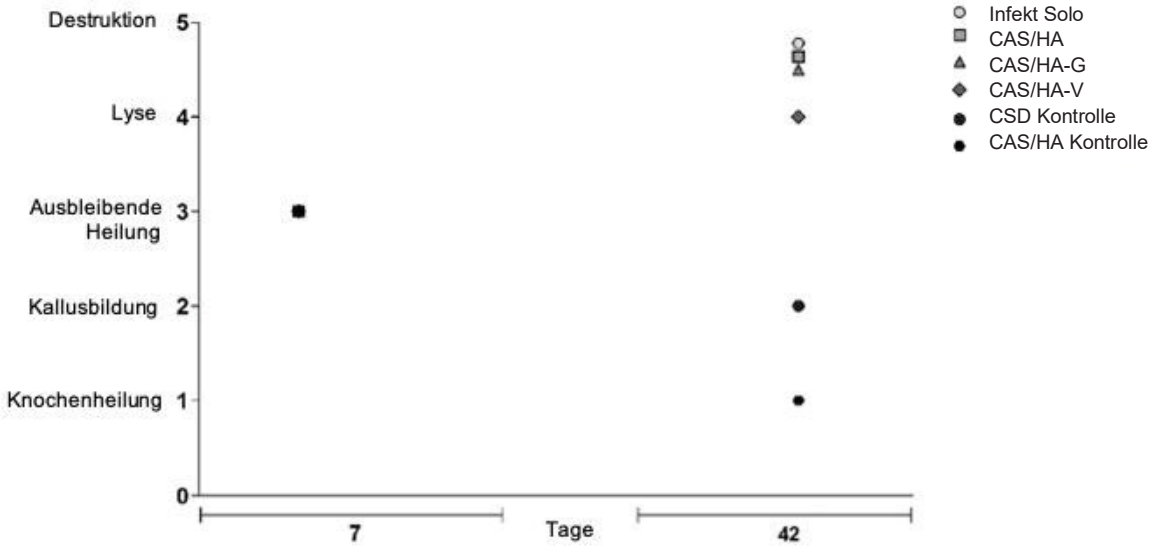

Abbildung 5. Frakturheilung an Tag 7 und 42 in Abhängigkeit des jeweiligen verwendeten Knochersatzmaterials. Es wurden die Mittelwerte der einzelnen Gruppen zusammengefasst. Eine höhere Häufigkeit von Pseudarthrosen, Lyse und Zerstörung lag bei allen infizierten Mäusen mit oder ohne CAS/HA-Einsätze (Infekt Solo, CAS/HA, CAS/HA-G und CAS/HA-V) vor. Knochenheilungstendenzen konnten nur in den Kontrollgruppen (CSD Kontrolle, CAS/HA Kontrolle) nachgewiesen werden. Angepasst nach [3]. Mit freundlicher Genehmigung des Public Library of Science Verlags.

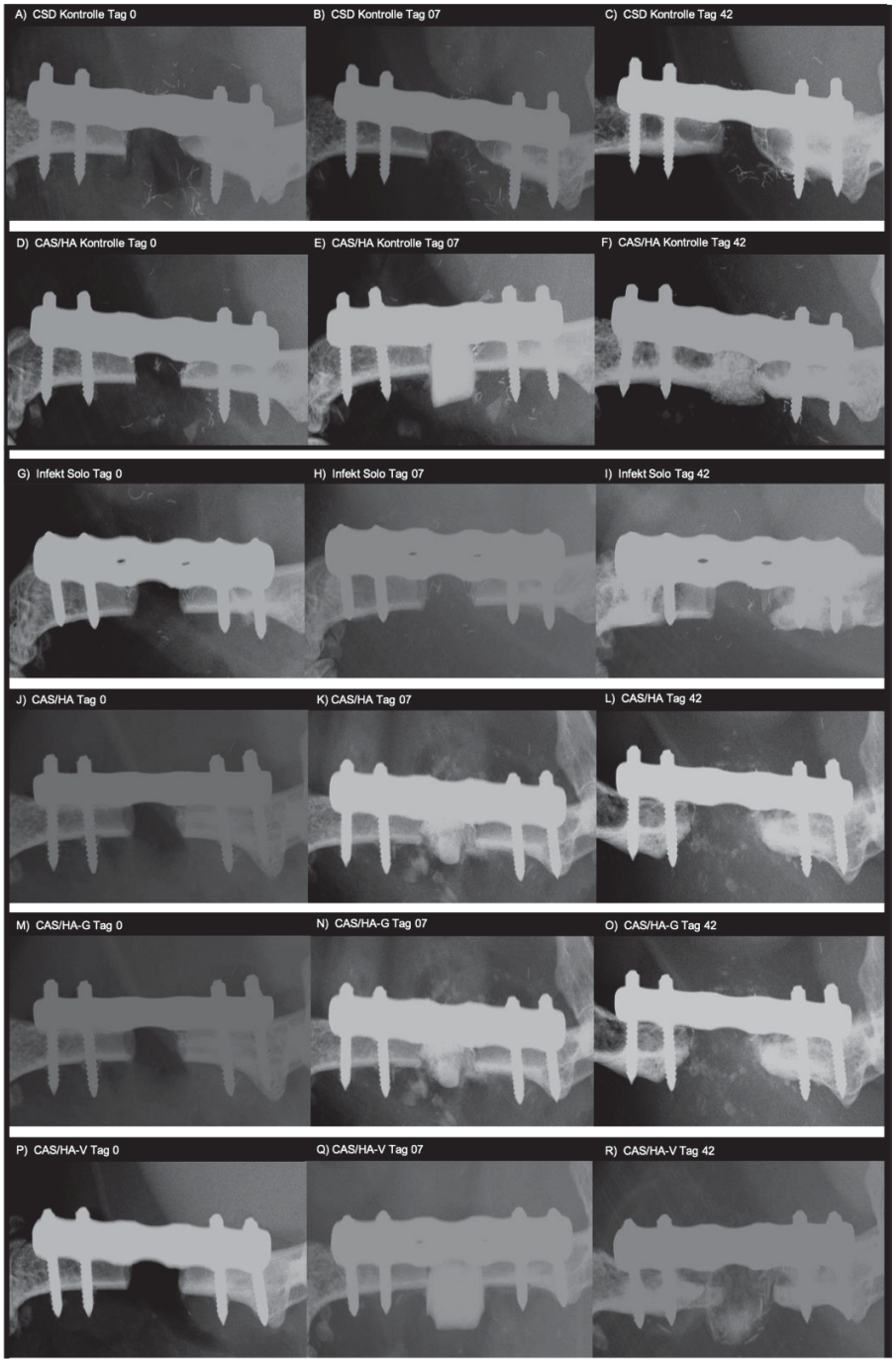

Abbildung 6. Radiologische Aufnahmen zur Beurteilung der knöchernen Heilung. Je Gruppe sind von links nach rechts immer Tag 0, 7 und 42 dargestellt. A-C) Röntgenbilder einer der Kontrollgruppe zugeordneten Maus ohne Infektion des Frakturspalts und ohne Implantation eines Spacers (CSD Kontrolle). D-F) Röntgenbilder einer der Kontrollgruppe zugeordneten Maus ohne Infektion des Frakturspalts, jedoch mit Implantation eines CAS/HA-Spacers (CAS/HA Kontrolle). Eine Integration des Spacers sowie fortschreitende Frakturheilung ist sichtbar. G-I) Röntgenbilder einer infizierten Maus ohne Implantation eines Spacers (Infekt Solo). An Tag 42 zeigt sich eine fortgeschrittene Lyse des Knochens. J-L) Röntgenbilder einer infizierten Maus mit Implantation eines CAS/HA-Spacers ohne Antibiotikazusatz (CAS/HA). Hier zeigt sich ebenfalls eine Lyse an Tag 42 mit einer Auflösung des CAS/HA-Spacers. M-O) Röntgenbilder einer infizierten Maus mit Implantation eines CAS/HA-Spacers mit Gentamycin (CAS/HA-G). Hier zeigt sich ebenfalls eine Lyse an Tag 42 mit einer Auflösung des CAS/HA-G-Spacers. P-R) Röntgenbilder einer infizierten Maus mit Implantation eines CAS/HA-Spacers mit Vancomaycin (CAS/HA-V). Hier zeigt sich lediglich eine beginnende Lyse an Tag 42 mit einer nur leichten Auflösung des CAS/HA-V-Spacers. An Tag 42 kann eine Abnahme der Größe des CSD verzeichnet werden. Angepasst nach [3]. Mit freundlicher Genehmigung des Public Library of Science Verlags.

Somit können die Ergebnisse die Anwendung von CAS/HA bei akuten Implantat-assoziierten Infektionen nicht empfehlen. Bei nicht infektiösen knöchernen Defekten oder nach Infekt-Rekonvaleszenz könnte CAS/HA jedoch als additive Therapie in der Unfallchirurgie und orthopädischen Chirurgie dienen.

4. Etablierung einer lokalen Implantat-assoziierten Infektion durch den biofilmbildenden Methicillin-resistenter Staphylococcus aureus (MRSA) im Mini Pig Modell

Plate-associated localized osteitis in mini-pig by biofilm forming Methicillin-resistant Staphylococcus aureus (MRSA): Establishment of a novel experimental model. **Jaekel C** et al. Eur J of Trauma, 2022.

Die zunehmende Zahl an Implantat-assoziierten Infektionen im orthopädisch-unfallchirurgischen Fachgebiet durch biofilmbildende SA in Kombination mit einer zunehmenden Resistenz gegen konventionelle Antibiotika erfordert neue Therapiestrategien. Unsere Arbeitsgruppe konnte bereits die Entwicklung eines standardisierten Implantat-assoziierten Infektmodells am Femur der Maus aufzeigen und konnte an eben diesem Modell verschiedenste therapeutische Optionen untersuchen [71,72]. Leider war es aufgrund der geringen Größe des murinen Femurs nicht möglich, bei einer bestehenden Infektion einen Plattenwechsel im Rahmen des chirurgischen Debridements durchzuführen. Ziel dieser Studie war es daher, ein neuartiges Modell einer standardisierten Implantat-assoziierten MRSA-Infektion im Femur bei Mini Pigs zu etablieren, um die klinische Situation einer einzeitigen Revision mit Plattenwechsel zu simulieren.

Am Femur von sieben Aachener Mini Pigs wurde ein Knochendefekt gesetzt und anschließend mit Methicillin-resistentem Staphylococcus aureus (MRSA ATCC 33592) infiziert. Die Defektzone wurde mit einer 5-Loch Titanplattenosteosynthese stabilisiert. Die Tiere erhielten sowohl eine orale (Meloxicam und Metamizol) als auch intramuskuläre (Buprenorphin) analgetische Behandlung. Vom ersten bis zum dritten postoperativen Tag wurde eine systemische Antibiotikatherapie mit Enrofloxacin appliziert, mit dem Ziel, eine systemische Immunrekation zu verhindern. Nach 14 Tagen wurden ein lokales Wunddebridement mit Lavage und Plattenwechsel (7-Loch Titanplattenosteosynthese) durchgeführt. Schließlich wurden die Tiere nach 42 Tagen erneut debridiert und lavagiert, gefolgt von einer Euthanasie (Abb. 7). Die lokale Infektion wurde durch die Bestimmung der CFU in der Lavage quantifiziert. Die Frakturheilung wurde sowohl radiologisch als auch histologisch beurteilt.

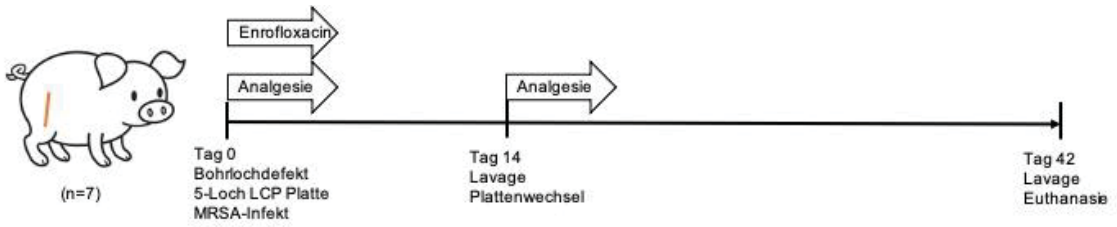

Abbildung 7. Versuchsaufbau des Implantat-assoziierten Infektmodells am Femur von Mini Pigs. Tag 0: Primäroperation mit setzen eines Knochendefektes, Implantation einer 5-Loch Plattenosteosynthese und Infekt Etablierung mittels MRSA. Tag 14: Revisionsoperation mit Plattenwechsel auf eine 7-Loch Plattenosteosynthese, sowie Debridement mit Lavage und Probengewinnung. Tag 42: Finaloperation mit erneuter Lavage und Probengewinnung sowie anschließender Euthanasie der Mini Pigs. LCP=locking compression plate, MRSA=Methicillin-resistenter Staphylococcus aureus. Mit freundlicher Genehmigung des Springer-Verlags.

Es konnte eine lokale Osteitis mit radiologisch sichtbarer Knochenlyse etabliert werden (Abb. 8). Die unverändert hohen CFU in der Lavage, die signifikanten Unterschiede von IL-6 im Blut im Vergleich zur Lavage und der fehlende Anstieg der Alkalischen Phosphate (ALP) im Serum über den gesamten Beobachtungszeitraum bestätigen den lokalen Infekt.

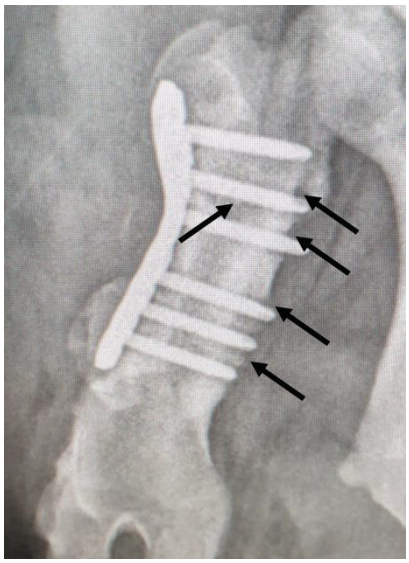

Abbildung 8. Röntgenaufnahme des Femurs vom Mini Pig an Tag 42 nach Infektetablierung. Die schwarzen Pfeile zeigen die Lysen des Knochens, betont um die einliegenden Schrauben. Angepasst nach [4]. Mit freundlicher Genehmigung des Springer-Verlags.

Die Studie zeigt die erfolgreiche Etablierung einer Implantat-assoziierten Infektion am Femur von Mini Pigs. Die erfolgreiche Induktion einer Osteitis mit nachgewiesener Knochenlyse, dem Fehlen einer enzymatischen Aktivität zur Mineralisierung des Knochens, bei jedoch nicht systemischer Immunreaktion, bestätigt das lokale Geschehen des Infektes. Daher kann dieses standardisierte Modell nun in weiteren Studien verwendet werden, um verschiedene beschichtete Implantate sowie weitere adujvante Therapiemaßnahmen zu untersuchen.

Diskussion

Implantat-assoziierte Infektionen nach Frakturversorgung (FRI) sind im Fachbereich der Orthopädie und Unfallchirurgie eine immer wieder auftretende und ernstzunehmende Komplikation. Sowohl die einwandfreie Diagnostik als auch die sich anschließende Therapie sind für das behandelnde Team eine große Herausforderung. Aufgrund der Pathophysiologie und der Komplexität sind prospektiv-klinische Untersuchungen diesbezüglich schwierig und eine wissenschaftliche Rarität. Dementsprechend sind die Forscher dazu angehalten, Modelle zu entwickeln, welche die klinische Situation weitestgehend widerspiegeln und eine differenzierte Untersuchung des Krankheitsbildes weiter voranbringen.

Im Zuge der vorliegenden Arbeiten wurde ein Score entwickelt, der zur besseren Quantifizierung der Frakturheilung dienen soll, um damit eine bessere numerische Vergleichbarkeit der Diagnostik und Therapie in Bezug auf FRIs im Mausmodell zu ermöglichen. Infektionen nach Frakturen führen im Allgemeinen zu einer Beeinträchtigung der Frakturkonsolidierung und Kallusbildung sowie zur Invasion immunkompetenter Zellen in den Markraum [5,7]. Diese strukturellen Veränderungen werden in der klinischen Praxis zur Diagnose und Einordnung von Knocheninfektionen herangezogen und sind beispielsweise integraler Bestandteil des Klassifikationsscores der Osteomyelitis von Schmidt *et al.* [74]. Für murine FRI-Modelle wurden solche Werte jedoch noch nicht festgelegt. Eine wichtige Frage bei einem neu entwickelten Score ist seine Aussagekraft: "Was bedeutet ein hoher Score bzw. was bedeutet ein niedriger Score?" Bei dem hier vorgestellten Score, welcher in Anlehnung an Schmidt *et al.* aufgebaut wurde, bedeutet ein hoher Wert, dass der Zustand nach der operativen Therapie relativ nah am präoperativen Status liegt, heißt, die Anzahl an operativ induzierten Läsionen also minimal ist und die Abheilung optimal erfolgt [74]. Idealerweise müsste ein solcher histologisch-mikrobiologisch-radiologischer Score nach seiner Etablierung mit weiteren Markern für die Schwere der manifesten Infektion weiter korreliert werden. In der klinischen Praxis werden hier zum einen weitere Serummarker wie zum Beispiel Leukozyten, C-reaktives Protein (CRP) genutzt, aber auch lokale (Rötung, Schwellung, Wundsekret), wie auch systemische (Fieber) körperliche Folgeerscheinungen herangezogen. Leider ist diese Umsetzung im Tiermodell mit starken Einschränkungen behaftet, da bei einer überschießenden Infektion mit starker systemischer Reaktion der Exitus letalis der Versuchstiere vorprogrammiert wäre und sich damit ein längerer Beobachtungszeitraum ausschließen würde. Auch wenn solche Untersuchungen wünschenswert wären, sind sie

sowohl auf Grund des Tierschutzes als auch aus eigenen ethischen Erwägungen nicht umsetzbar, so dass sich die hier vorliegenden Infektionen auf die lokale-induzierte Osteitis begrenzen. Ein wichtiger Aspekt bei der Erstellung des Scores war es, dass er die gleichen Parameter quantifiziert, die auch bei einer möglichen Beurteilung der OM im Menschen genutzt werden können. Unser Score für die Beurteilung im experimentalen Modell war eng an die Gewichtung angelehnt, die im klinischen Alltag genutzt wird [74]. Der Osteomyelitis Diagnose Score (ODS) beruht auf fünf diagnostischen Parametern: (1) Klinischer Hintergrund und Risikofaktoren, (2) klinische Untersuchung und Laborwerte, (3) Diagnostische Bildgebung (Ultraschall, Radiologie, CT, MRI, Nuklearmedizin und Hybridmethoden), (4) Mikrobiologie, und (5) Histopathologie. Unser Score umfasst, wie in Tabelle 1 gezeigt, die Kallusbildung des Knochens (Histopathologie), Konsolidierung der Fraktur im Röntgen (diagnostische Bildgebung), strukturelle Änderung des Markraums (Histopathologie) sowie Anzahl der Bakterien (Mikrobiologie). In der Literatur werden eine Reihe unterschiedlicher Bewertungen bei verschiedenen Tiermodellen verwendet. Gemäß der US-amerikanischen Diagnoseleitlinien für Knocheninfektionen des *Centre for Disease Control and Prevention/National Healthcare Safety Network* (CDC/NHSN) wurden Patienten mit OM anhand von vier Hauptkriterien klinisch identifiziert: (1) mikrobiologische Untersuchung von Blut und Knochen, (2) anatomische und histologische Bewertung, (3) lokalisierte Anzeichen und Symptome und vor allem (4) eine radiologische Untersuchung, die durch eine Korrelation mit dem klinischen Bild unterstützt wird [88]. Um die klinische Diagnose einer Osteomyelitis im Tiermodell möglichst genau nachzubilden, wird empfohlen, eine Bewertung in Anlehnung an die o.a. Kriterien des CDC zu verwenden, bei dem eine radiologische Untersuchung und die Bewertung mindestens eines der drei anderen Kriterien durchgeführt werden sollte. Zu den in der Literatur beschriebenen Bewertungssystemen im Tiermodell, neben dem von uns vorgeschlagenen Punktesystem, welche selbst eine hohe Sensitivität und Spezifität für die Bewertung des Schweregrads erreicht, gehören der radiographische Union Score für die Tibia und der Lane- und Sandhu-Score [89–91]. Einige in der Vergangenheit publizierte Studien verwendeten eigene Bewertungsschemata, die zum damaligen Zeitpunkt nicht validiert wurden. Hierzu zählen neben den eigenen Vorarbeiten unserer Arbeitsgruppe noch die Arbeiten von Chen *et al.* und Southwood *et al.* [58,71,72,92]. Ein Grund für die mangelnde Kohärenz der in der Literatur verwendeten Bewertungsschemata könnte darin liegen, dass Komponenten wie Sequestrierung, Periostreaktionen und Implantatinstabilität fehlen. Die Einführung einer

allgemein verwendeten Bewertungsskala, die eigens für präklinische Studien zu Knocheninfektionen entwickelt wurde, könnte den Forschern in Zukunft Konsistenz und Genauigkeit bei der Beurteilung von FRIs liefern.

Das durch unsere Arbeitsgruppe etablierte Mausmodell bietet die Möglichkeit, verschiedene therapeutische Optionen in der Behandlung der FRI zu untersuchen. Einer der Arbeiten beurteilt diesbezüglich den Einfluss der HBO auf das Infektgeschehen und die Frakturheilung. Im Gegensatz zu einigen bis dahin publizierten Studien konnten wir in unserer Untersuchung keinen signifikanten Einfluss der HBO auf die lokale Bakterienbesiedlung als auch der Frakturheilung aufzeigen [79,81]. Wiederum andere Arbeitsgruppen konnten ähnliche Ergebnisse, sprich keinen positiven Effekt der HBO, feststellen [93,94]. Eine entscheidende Frage bezüglich der Effizienz einer Sauerstofftherapie ist: "Welche positiven Effekte können über welche Mechanismen erzielt werden?" Ein entscheidender und in der Einleitung bereits aufgegriffener Mechanismus für die Pathogenese einer SA induzierten OM ist die Bildung von Biofilmen auf Implantatoberflächen. Diese Biofilme sind aufgrund ihrer Struktur besonders schwer zu therapieren, da diese die Diffusion von Antibiotika zu den Bakterienzellen einschränken, die Durchdringung von Immunzellen hemmen und auch einer mechanischen Zerstörung widerstehen. Darüber hinaus sind die Bakterienzellen innerhalb eines Biofilms aufgrund der Gradienten der Nährstoff- und Sauerstoffverfügbarkeit metabolisch vielfältig, und könnten damit in unterschiedlichem Maße den direkten und indirekten Effekten der HBO widerstehen. Eine weitere Ursache für die negativen Ergebnisse unserer Studie mag in der Infektion mit SA liegen. SA zählt zu den sogenannten aeroben Baktierenspezies, bei der Sauerstoff wohlmöglich keinen negativen Effekt bewirkt. Somit könnten die positiven Effekte in anderen Studien wohlmöglich durch kompetitiv inhibitorische Effekte der HBO auf nicht-SA Bakterien begründet sein: Durch die Stimulation mit Sauerstoff könnte es bei Infektionen mit multiplen unterschiedlichen Bakterienspezies zur kompetitiven Ersetzung anaerober Keime durch aerobe Bakterien kommen, die möglicherweise vom Immunsystem besser erkannt und in Schach gehalten werden können. Die Präsenz anaerober Bakterien, wie beispielsweise *Propionibacterium acnes*, stellt bei Implantat-assoziierten Infektionen im humanen Bereich eine zusätzliche Herausforderung dar, da die klinischen Diagnosekriterien in hohem Maße von den Ergebnissen der Gewebekultur abhängen. Dieser Organismus lässt sich nur schwer kultivieren und erfordert zum Nachweis wiederholte Tests und längere Inkubationszeiten. *In-vivo* Studien deuten darauf hin, dass die durch *Propionibacterium acnes* verursachte

Osteomyelitis vom Vorhandensein eines Implantats abhängig ist [95]. Shiono *et al.* unterstreichen durch ihre Studie damit nochmals die entscheidende Rolle der Biofilmbildung in der Pathogenese der FRIs und damit möglichweiser auch den Schutz vor Sauerstoff der Bakterien innerhalb dieses Biofilms.

Die Bildung von Biofilmen auf Implantaten verhindert ebenfalls die Diffusion von Antibiotika und somit auch die umfängliche Wirksamkeit einer Intervention durch oral oder intravenös applizierte Antibiotika [28]. Daher überprüften wir in unserem Mausmodell die Hypothese, ob die lokale Applikation von Antibiotika über osteokonduktive Hydroxyapatit-Spacer zu einer verbesserten Penetration und damit Reduktion der Keimzahl führt und eine Knochenheilung im FRI Modell fördert. Frühere Studien hatte eine Wirksamkeit dieser Vorgehensweise bei primärer Infektionsprävention oder der Behandlung einer chronischen Osteitis nachgewiesen [60,80–82]. Wir untersuchten hierbei erneut verschiedene Parameter der Entzündung und Knochenheilung. Während wir keine signifikanten Unterschiede zwischen unbehandelten, infizierten Tieren und denen, die mit nicht-versetzten oder Gentamycin-versetzten Knochenersatzmaterial behandelt wurden, nachweisen konnten, zeigte die Behandlung mit Vancomycin-versehenen Spacern signifikante Effekte, insbesondere bei den AP- und PINP-Serumlevels PINP an Tag 42 postoperativ. Ob der beobachtete Effekt der Vancomycin-versehenen Spacer größer ist, als er bei systemischer Vancomycin-Therapie gewesen wäre, konnten wir in dem vorliegenden Versuchsaufbau nicht ermitteln. Eine Diskussion, ob und inwiefern lokale Antibiotikatherapie das Risiko der Ausbildung von multiplen Resistenzen stärker oder weniger stark erhöht als systemische Anwendung, geht über den Rahmen dieser Arbeit hinaus. Im humanen Bereich sind bislang lokale Behandlungen in der Therapie von Knocheninfektionen mit unterschiedlichem Erfolg erprobt worden und die lokale Antibiotikatherapie in der Behandlung weiterhin umstritten. Traditionell wurden antibiotikafreisetzende Kügelchen verwendet, die je nach untersuchter anatomischer Position und klinischem Setting unterschiedliche Auswirkungen auf die Behandlungsergebnisse hatten [96,97]. Eine aktuelle retrospektive Studie im Gebiet der PJI untersuchte zwei gematchte Kohorten von je 20 Patienten, die bei manifester Infektion entweder Standardtherapie durch (1) Debridement plus Antibiotika zur Implantaterhaltung oder (2) zusätzlich lokale, antibiotikagetränkte Calcium-Sulfat Perlen erhalten hatten [98]. Die Autoren schlussfolgerten, dass auflösbare, mit Antibiotika beladene Kalziumsulfatkügelchen die Inzidenz von rezidivierenden PJIs nach 2 Jahren oder 90 Tagen postoperativ nicht verringerten. In

Anbetracht der Kosten der lokalen Antibiotikabehandlung konnten sie die zusätzliche lokale Antibiotikagabe schließlich nicht empfehlen. Die Schlussfolgerung stimmt mit unseren Ergebnissen am Mausmodell überein, so dass man im weitesten Sinne von einer Validierung unserer Ergebnisse reden kann, wobei man immer bedenken muss, dass die genauen Umstände bei Mensch- und Tiermodell, oder auch bei verschiedenen Tiermodellen, selbst bei identischen Spezies, nie gleich und selten auch nur ähnlich sind, was die Vergleichbarkeit stark beeinträchtigt.

Schweine werden inzwischen häufiger als Versuchstiere bestimmter Studien verwendet, insbesondere, weil einige ihrer Hauptmerkmale wie Größe, Anatomie und auch Physiologie denen der Menschen ähneln [99,100]. Da das komplette Genom des Hausschweins sequenziert wurde, stehen viele Labormaterialen (beispielsweise Antikörper) für Untersuchungen am Schwein und dessen Gewebe zur Verfügung [99,101]. Hierbei finden sowohl konventionelle Schweine als auch Mini Pigs Anwendung, allerdings kann mit dem Mini Pig, welches in aller Regel ein Körpergewicht zwischen 40 - 80kg erreicht, ein besseres Modell zur Vergleichbarkeit des erwachsenen Patienten erreicht werden [102,103]. Verschiedene histopathologische Studien konnten des Weiteren zeigen, dass auch die Struktur des Schweineknochens dem vom Menschen am ähnlichsten ist, im Vergleich zu Ziegen, Schafen und Hunden [104–108]. Zwar ist die trabekuläre Struktur des spongiösen Anteils des Knochens insgesamt dichter als beim Menschen, allerdings die mineralische Zusammensetzung sowie der Remodellingprozess nahezu identisch. Bezogen auf die Immunantwort eignen sich Schweine ebenfalls als gutes Modell, da viele immunologische Schlüsselproteine von Menschen und Schweinen strukturell und funktionell vergleichbar sind [109]. Auf Grundlage dessen stellt das Mini Pig eine Spezies dar, welche näher an der Realität der klinischen Frakturversorgung bei Menschen angesiedelt ist und hierdurch sowohl von der experimentellen Durchführung als auch von den biochemischen, histologischen und anatomischen Aspekten her wesentlich direktere Rückschlüsse auf die Therapie einer FRI zulässt. Entsprechend ist aber auch der erforderliche räumliche und finanzielle Rahmen zur Durchführung einer solchen Studie größer. Durch die Verwendung eines solchen Großtiermodells können wir jetzt die Operationen von der physischen Durchführung wie auch dem Zeitrahmen her dem Ablauf anpassen, wie er bei einer FRI im klinischen Setting für einen humanen Patienten vorkommt. In unserer Pilotstudie im Großtier nutzten wir die gleichen Parameter für Infektion und Analysezeitpunkte, wie wir sie in den vorherigen Experimenten

im Mausmodell etablierten. Der Vorteil gegenüber dem Mausmodell war nun, dass wir 14 Tage nach der ersten Operartion die kontaminierte Platte durch ein sauberes und längeres Implantat ersetzen konnten. Allerdings wechselten wir den Keim von einem sensiblen SA auf einen MRSA, ein Setting, das im klinischen Alltag wesentlich gefürchteter und in der Therapie komplexer ist. Ein solches Modell zur Untersuchung einer FRI am Großtier ist nach unserem Wissenstand einzigartig. In der Literatur sind bislang nur fünf weitere Modelle zur Untersuchung knöcherner Infektionen im traumatologischen Setting beschrieben [102,110–113]. In vier der fünf Modelle wird ein sensibler SA Stamm und in einer der Studie ein *Staphylococcus epidermidis* zur Infektetablierung genutzt. Lediglich zwei der Studien sind als Implanatassoziierte Infektion zur verstehen, von der wiederum nur eine zu unseren Versuchen vergleichbare Plattenosteosynthese durchführte [102,111]. Allerdings lag das Ziel dieser Studie nicht in der Untersuchung infektiologischer, klinischer und radiologischer Parameter, sondern in der Kartierung von *complemantry* DNA (cDNA) [111]. Somit können weder Vergleiche noch Rückschlüsse zu unserer Arbeit gezogen werden.

Zusammengefasst ist die in dieser Arbeit beschriebene Mini Pig Studie die erste Untersuchung zur erfolgreichen Induktion einer lokalen Implantat-assoziiertem Osteitis im Schweinemodell, welches in weiteren klinischen Studien eingesetzt werden kann, um Knochenheilung, Biofilmbildung, Immunantwort und therapeutische Optionen weiter analysieren zu können.

Schlussfolgerung und Ausblick

Die hier dargestellten und diskutierten Arbeiten geben einen Einblick über die Möglichkeiten, aber auch Einschränkungen von Tiermodellen als Grundlage zur Untersuchung Implantat-assoziierter Infektionen im Fachgebiet der Orthopädie und Unfallchirurgie. Mit der lokalen Osteitis im Bereich der Fraktur am Femur der Maus bei einliegender Plattenosteosynthese stand ein reproduzierbares Modell zur Untersuchung von FRIs zur Verfügung. An Hand dieses Modells konnten verschiedene therapeutische Optionen untersucht als auch ein Score unter Berücksichtigung von histologischen-mikrobiologischen und radiologischen Gesichtspunkten entwickelt werden. Aufbauend auf den Versuchen in der Maus und mit den daraus gemachten Erkenntnissen wurde ein Großtiermodell im Mini Pig entworfen. Mit der Etablierung eines Großtiermodells zur spezifischen Untersuchung von FRIs sind wir nun in der Lage, das operative Vorgehen, insbesondere einen Wechsel des Osteosynthesematerials nach Induktion einer Infektion, in einem System zu testen, welches die klinische Situation beim Menschen wesentlich besser widerspiegelt als das bis dahin genutzte Osteitis Mausmodell. Darüber hinaus haben wir begonnen, das hochaktuelle Problem der Infektion mit resistenten oder sogar multiresistenten Keimen, in diesem Fall MRSA, anzugehen, was in Anbetracht der steigenden Prävalenz solcher Infektionen im nosokomialen Bereich dringend erforderlich ist. Zukünftig wollen wir den Einfluss Lysostaphin-beschichteter Implantate auf die MRSA-induzierte Osteitis im Mini Pig Modell weiter untersuchen. Im Rahmen dieser Versuchsreihe ist ebenfalls geplant, weitere Parameter zur Erfassung der Frakturheilung, als auch die Untersuchung weiterer immunologischer Parameter mit einzubeziehen. Sollten die Untersuchungen im Großtiermodell eine Bestätigung der im Mausmodell gemachten Erkenntnisse untermauern, also einen positiven Einfluss der Lysostaphin-beschichten Implantate aufzeigen, gilt es, klinische Studien im humanen Setting anzuschließen.

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Düsseldorf, den 20.04.2022 Dr. med. Carina Jaekel

Anlagen

Beitragende Originalarbeiten

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TRAUMA SURGERY

Histological score for degrees of severity in an implant-associated infection model in mice

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Abstract

Introduction Several scores were introduced to diagnose and to classify osteomyelitis in practice. Mouse models are often used to study the pathophysiology of bone infection and to test therapeutic strategies. Aim of the present study was to design a score to diagnose and quantify implant-associated infection in a murine experimental model.

Materials and methods Four independent parameters were developed: existence of callus, consolidation of the fracture, structural changes of the medullary cavity and number of bacteria. The score was assessed in a standardized implant-associated mouse model with 35 BALB/c-mice. The left femur was osteotomized, fixed by a titanium locking plate and infection was induced by inoculation of *Staphylococcus aureus* into the fracture gap. For the sham group, the procedure was performed without inoculation of bacteria. The score was assessed on days 7, 14 and 28. Each item of the score showed lower values for the infection group compared to the controls after 4 weeks.

Results Regardless of the assessed time point, the overall total score was significantly higher in the control group compared to the infection group ($p < 0.0001$). Analysis revealed a sensitivity of 0.85, specificity of 1.0, negative predictive value of 0.67 and positive predictive value of 1.0.

Conclusion The proposed score assessing severity of fracture-related infection in an implant-associated murine model was easy to access, feasible to diagnose and estimate bone healing and infection in a murine bone infection with a high sensitivity. Therefore, this score might be a useful tool to quantify infection-related changes after fracture in further future preclinical studies.

Keywords Mouse model · Fracture-related infection · Score · *Staphylococcus aureus*

Introduction

Fracture-related infections (FRI) are one of the major challenges in orthopedic and trauma surgery. Long-term antibiotic uses, multiple surgical treatments, including debridement and implant removal, as well as high healthcare costs and poor functional outcome for the concerned patients are often associated with implant-associated infections [1–3]. However, FRI is a severe complication following musculoskeletal trauma with a prevalence of 0.5–3% for closed and up to 25–30% for open fractures. However, posttraumatic infection is a severe complication following bone fracture with a prevalence of 0.5–3% for closed fractures and up to 25–30% after open extremity fractures [1, 2, 4, 5]. Reconstruction of fractures using implants is an additional risk factor for the development of FRI [6–8]. About 10–30% might chronify [9]. *Staphylococcus aureus* (SA) is the most common bacteria proven in posttraumatic infections and responsible for 50–70% of posttraumatic bone infections [10, 11]. Several clinical studies tried to establish scores for a definition of osteomyelitis [12, 13]. Interestingly, most of these studies take into account neither the presence of a fracture nor the application of an implant. This seems surprising

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because fracture stabilization is one of the most risk factors for developing a FRI [6–8]. Studies concerning diagnosis and therapy of FRI are various, but a definition is still missing. A universally accepted definition of implant-associated infections after fracture fixation is yet not established. There is still a lack of clear definition [2, 14, 15]. For this reason, an expert group recently developed a consensus definition for FRI [15]. Furthermore, histopathology studies in FRI are limited. Followed up to the consensus definition, Morgenstern et al. investigate the value of histological analysis in the diagnosis of FRI by measuring number of neutrophil granulocytes [16]. Scores, taking into account fracture consolidation as well as number of bacteria and histopathological alterations, are not established yet. Most insights into the pathophysiological mechanism of bone infections are gained from animal experiments. Animal models have not only been shown to be useful in studies on drug safety but transgenic models have also allowed for the study of complex pathophysiological pathways in great detail. Animal models are used to resemble the inflammatory pathophysiological conditions occurring in humans during infection. Therefore, similar FRI classification systems may simplify comparability of experimental and clinical conditions and improve transfer "from bench to bedside" in future studies. Therefore, similar classification systems in experimental and clinical FRI may simplify comparability of the experimental and the clinical condition and simplify the transfer "from bench to bedside" in further studies. However, such scores cannot not always be used in experimental settings as features such as "clinical history and risk factors" are not available for experimental animals. On the other hand, other features of the human classifications, such as microbiological or histopathological examinations, are routinely assessed in experimental bone infection models. However, this well-established scores assessing severity of infection cannot be used in the experimental situation as features such as "clinical history and risk factors" are not feasible for experimental animals. However, other features of the human classifications, such as microbiological or histopathological examinations, are routinely assessed in experimental bone infection models. Aim of the present study was to design a score to diagnose and quantify infection in a murine experimental implant-associated infection model. Following further clinical scores, the new classification system should involve diagnostic imaging, microbiology and histopathology.

Materials and methods

Ethical statement

The present animal experiment was approved by the local institutional committee on animal care ("Landesamt für Naturschutz, Umwelt und Verbraucherschutz" of the federal state of North Rhine–Westphalia, Germany—file number: 87-51.04.2010.A375) and was in line with the European Communities Council Directive (86/609/EEC). Specific effort was made to minimize the number of animals. Reporting of the results of the present study adheres to the "Animals in Research: Reporting in vivo Experiments" criteria (ARRIVE criteria).

Animals

Female non-transgenic BALB/c-mice with an average weight of 21 g were used for the present study. The age ranged between 10 and 12 weeks. Mice were kept in the local animal research institution in standard polycarbonate (Makrolon type II) cages under a conventional 12-h light–dark cycle (7:00 a.m./p.m.). A total of six mice were kept in one cage. Mice had free access to food and water.

Animal surgery/the implant-associated osteitis model

A well-characterized and standardized implant-associated infection model in mice was used as described before [17, 18]. In detail, general anesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg bodyweight) and xylazine (2%, 5 mg/kg body weight). Osteotomy of the left femur was performed in its middle third with a Gigli saw (0.22 mm in diameter) after sterile preparation. The femur was reconstructed by a 4-hole stable-angle plate and screw combination (length 7.75 mm, width 1.5 mm, thickness 0.7 mm and each 2 two self-cutting screws at the proximal and distal fragments; AO Foundation, Research Implants Systems, Davos, Suisse). The femur osteotomy and position of the implants were controlled by X-ray radiography on days 7, 14 and 28. For the infection group, infection was induced by inoculation of the osteotomy gap with $1x10^3$ SA in 1 µl PBS (type ATCC 29213; 10^3 colony-forming units, CFU). Mice were kept on a heating pad during surgery and the postoperative period to maintain a body temperature of 37 °C. Meloxicam (5 mg/kg s.c., directly after surgery and five more days every 24 h) was used for postoperative analgesia. Mice were re-anesthetized on the 7th, 14th and 28th days and killed by cervical dislocation on day 28. Radiographic examinations were done on the 7th, 14th and 28th days. Mice were visited and checked once a day. The right femur was sterilely removed and fixated by formalin solution. In total, 35 BALB/c-mice were randomly allocated to treatment groups. The infection group included 27 mice (day 7:5 mice, day 14:4 mice and day 28:18 mice) and the control group included 8 mice (4 mice each on day 7 and day 28). All experiments were performed in the local animal research institution. Mice were euthanized by cervical dislocation

when the following termination criteria were existing: (1) pain within 1 week (no use of the impeller), (2) refusal to eat with weight loss of overall 20% or 15% in 12 h, (3) unsuccessful fracture stabilization and (4) infections, which are not under control with the surgical treatment.

Experimental outcomes

Femurs were decalcified with EDTA solution for 7 days and irrigated for additional 12 h at 36° C. Decalcified samples were embedded in paraffin, cut by a microtome and stained using hematoxylin–eosin (HE) or Giemsa. Microscopy was performed at 10-, 40- and 100-fold magnification.

Score

Based on our clinical experience, we suggested a score assessing severity of FRI, which involved four independent histological parameters:

1. Existence of callus;
2. Consolidation of the osteotomy;
3. Structural changes of the medullary cavity;
4. Number of bacteria.

The definition of each parameter and its weighting are summarized in Table 1.

Existence of callus was evaluated in the HE staining of the femur. Therefore, the quotient between callus and bony cortex was estimated. A quotient > 1.6 was considered as good (Fig. 1a), a quotient between 1.1 and 1.6 as poor (Fig. 1b) and quotient < 1.1 as no callus formation (Fig. 1c). Consolidation of the osteotomy was also evaluated in the histological examinations: a complete healing (complete radiological healing of the osteotomy site, Fig. 2a) was distinguished from an incomplete (Fig. 2b) healing and destruction (atrophy of the bony cortex, Fig. 2c). Physiological structural changes of the medullary cavity were defined as an organized cell structure and the absence of polymorphonuclear neutrophil granulocytes (PMN) as the standard of immunocompetent cells (Fig. 3a). Further, a disorganized cell structure with an accumulation of PMN (Fig. 3b) or—more severe—necrosis with devitalized tissue within the medullary cavity was considered as changes due to osteomyelitis (Fig. 3c). Quantification of bacteria was evaluated by Giemsa staining. The number of bacteria was assessed by the hotspot method in 20 visual fields at 100-fold magnification [19]. Low number of bacteria was defined as less than 4 bacterial colonies (Fig. 4a), a moderate number as 4–10 bacterial colonies (Fig. 4b), and a high number of bacteria as > 10 bacterial colonies per 20 visual fields (Fig. 4c). Using this score, maximally 27 and minimally 4 points could be achieved. A lower total score was suggested to correlate with a higher probability for a post-procedural FRI.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA). Data are presented as the median ± standard deviation (SD). *P* values of 0.05 and below were considered significant. The Mann–Whitney *U* test was used to assess significance. To analyze the diagnostic value of the evaluated score, a receiver operating characteristics (ROC) curve was generated and analyzed. The area under the curve (AUC), the optimal sensitivity, specificity, negative (NPV) and positive predictive value (PPV) were assessed.

Table 1 A score to assess severity of osteitis in mice—definition of each parameter

Parameter	Score points	Description	Definition
Existence of callus	6	Large callus formation	Ratio callus/bony cortex > 1.6
	3	Large callus formation	Ratio callus/bony cortex 1.1–1.6
	1	No callus formation	Ratio callus/bony cortex < 1.1
Consolidation of the fracture	12	Consolidation	Complete radiological healing of the osteotomy
	6	Incomplete consolidation	Incomplete radiological healing of the osteotomy
	1	Destruction	Atrophy of the bony cortex
Structural changes of the medullary cavity	6	Physiological conditions	Well-organized cell structure
	3	Disorganized cell structure	Disorganized cell structure with accumulation of immune cells
	1	Necrosis	Devitalized tissue
Number of bacteria	3	Low number	<4/20 visual fields at 100-fold magnification
	2	Moderate number	4–10/20 visual fields at 100-fold magnification
	1	High number	<10/20 visual fields at 100-fold magnification

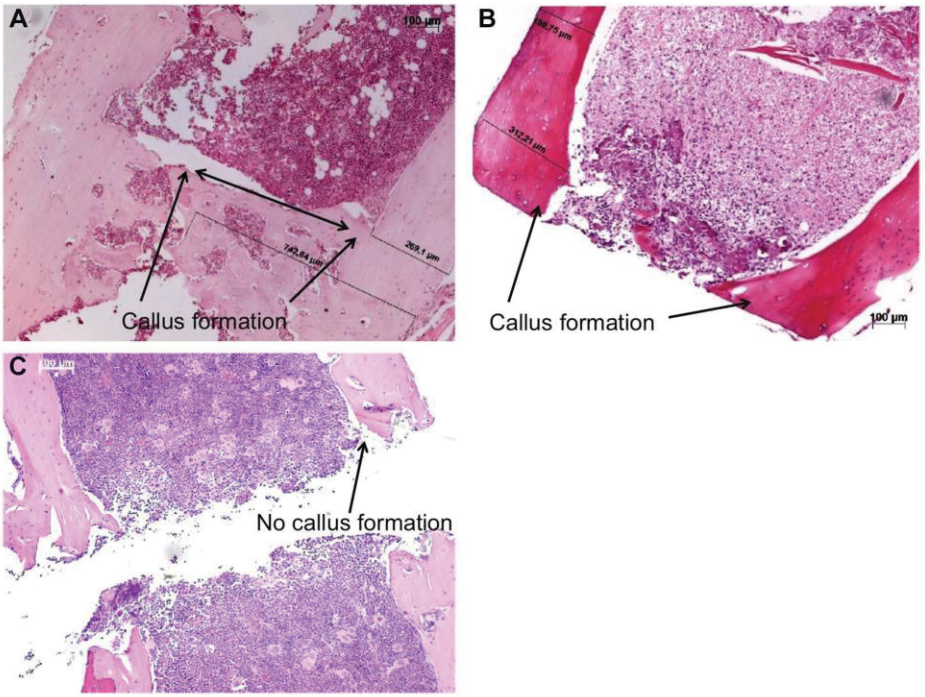

Fig. 1 Callus formation. A quotient > 1.6 was considered as good (**a**) a quotient between 1.1 and 1.6 as poor (**b**) and a quotient < 1.1 as no callus formation (**c**)

Results

Baseline data

Osteotomy and reconstruction of the femur were performed on 35 mice (27 in the osteitis model and 8 in the control group) as described above. We observed no case fatalities due to surgery or anesthesia (mortality rate 0/35).

Callus formation

Callus formation was observed in the control group 1 week after osteotomy. According to the proposed score, mice in the control group reached a median of 3 ± 1.8 pts. 1 week and 6 ± 1.3 pts. 4 weeks after osteotomy (Fig. 5a). Mice assigned to the infection group showed comparable callus formation within the first week (3 ± 1 pts.) but not thereafter (1 ± 1.2 pts. 4 weeks after osteotomy).

Bone consolidation

Bone consolidation is a sign of a physiological fracture healing and should be distinguished from callus formation. Complete osteotomy consolidation was only observed in one mouse in the control group after 4 weeks (1/4 mice, 25%, Fig. 5b). All control mice in the 1-week group and 3/4 mice in the 4-week group showed a partial osteotomy consolidation with a persisting definable fracture gap (score 6 ± 0 pts. at 1 week; 6 ± 2.6 at 4 weeks). Comparably, mice designated to the implant-associated infection model showed no complete osteotomy consolidation. Infection mice showed a progressive destruction of the fracture gap and the cortical bone. The median score for the infection mice was 6 ± 0 pts. after 1 week, 6 ± 2.2 after 2 weeks and 1 ± 3.2 after 4 weeks.

Structural changes of the medullary cavity

All control mice showed neither a disorganized cell structure nor an accumulation of PMN within the medullary cavity after 1 or 4 weeks (median score: 6 ± 0 pts., each 1 and 4 weeks after fracture). In contrast, infection mice showed changes of the medullary cavity with a disorganized cell structure at 1 and 2 weeks after fracture (median score: 3 ± 2.2 after 1 week and 2.2 ± 2 after 2 weeks, Fig. 5c). Infection mice additionally showed an invasion of PMN

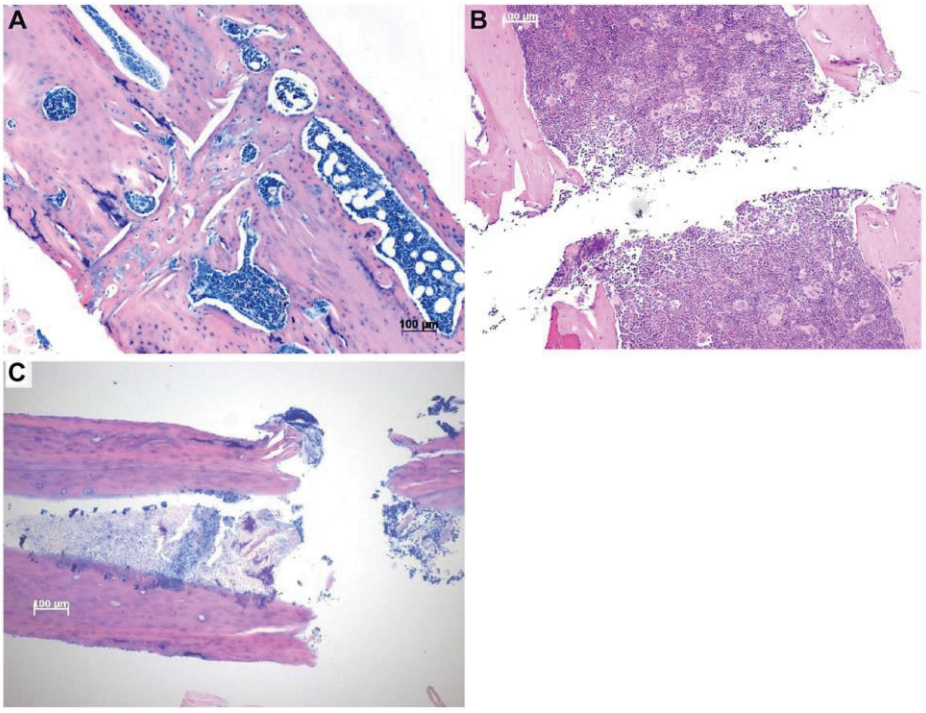

Fig. 2 Osteotomy consolidation. A complete healing osteotomy (**a**) was distinguished from an incomplete (**b**) healing osteotomy and a destruction/atrophy of the bony cortex (**c**)

within the medullary cavity after 4 weeks (median score: 3 ± 1.4).

Bacteria

As a high number of bacteria within the fracture gap indicate an infection, number of bacteria in the histopathological slices was quantified using the hotspot method in 20 visual fields at 100-fold magnification [19]. As expected, only single bacteria were found in control mice at 1 and 4 weeks after initial surgery (median score: 3 ± 0 pts., each at week one and four, Fig. 5d). The infection group showed with 4/5 mice (80%) a high number of bacteria at 1 week (> 10 bacterial colonies per 20 visual fields; median score: 2 ± 0.8 pts.). A high number of bacteria in the fracture gap were also observed 2 (score: 2 ± 1 pts.) and 4 weeks (score: 2 ± 0.8 pts.) after osteotomy.

Total score

The total score was calculated by addition of the scores based on the analyzed four independent histological parameters. We assumed that a lower total score correlates with a higher probability for a post-procedural FRI. For the control group, median total score was 18 ± 1.8 pts. at one and 21 ± 1.3 pts. at 4 weeks (Fig. 6). The higher score at 4 weeks might reflect the physiological re-constitution of the bone. For mice assigned to the osteitis group, the total score was 13 ± 2.3 pts. at 1 (vs. control group: $p = 0.03$), 10.5 ± 4 pts. at 2 and 8.5 ± 4.8 pts. at 4 weeks (vs. control group: $p = 0.002$).

Regardless of the assessed time point, the overall total score was significantly higher in the control group (21 ± 2.4 pts.) compared to the osteitis group (11 ± 4.6 pts.; $p < 0.0001$). For a further prediction of the diagnostic value of the proposed score, we performed a ROC analysis. Using the ROC, the cut-off value for detecting an osteitis was 15 pts. Using 15 pts. as cutoff value, ROC analysis revealed an AUC of 0.97, sensitivity of 0.85, specificity of 1.0, NPV of 0.67 and PPV of 1.0 for the group of 35 studied animals (Fig. 7).

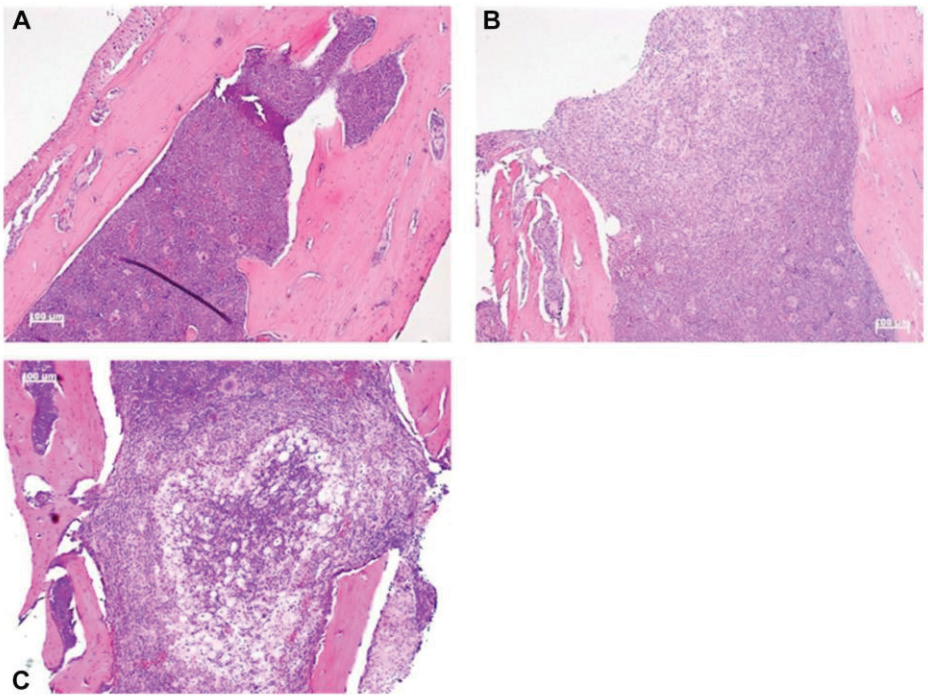

Fig. 3 Structural changes of the medullary cavity. This figure demonstrates the structural changes of the medullary cavity following bone infection. A physiological configuration (**a**) has to be differentiated from a disorganized cell structure (**b**) and necrotic tissue (**c**)

Discussion

The present study aimed to design a score to diagnose and quantify infection in murine experimental FRI. The proposed score involving diagnostic imaging, microbiology and histopathology was (1) easy to access, (2) feasible to estimate bone healing and infection with a high sensitivity and (3) might, therefore, be a useful tool to quantify infection-related changes after FRI in further studies.

In humans, FRI leads to an impairment of fracture consolidation and callus formation as well as invasion of immune-competent cells into the medullary cavity [20–22]. These structural changes are used to diagnose and classify bone infections in clinical practice and are, e.g. integral parts of the classification score of osteomyelitis by Schmidt et al. [13]. However, such scores were not yet established for murine FRI models. Introduction of comparable scores in a murine model might facilitate the comparability in the human situation. The presently proposed score involving imaging, microbiological and histopathological features was based on a previous clinical score [13]. Evaluation of a well-established implant-associated infection model revealed an impaired callus formation and consolidation of the fracture, a disorganized cell structure of the medullary cavity with invasion of PMN and a high number of bacteria in osteitis animals compared to sham animals. These changes are well known from the human situation after osteitis. Recent studies tried to fulfill the lack of a clear definition concerning FRI [15, 16]. Experts developed a consensus definition. Therefore, they determined two different levels of certainty: first, confirmatory items and second suggestive items. Infection is confirmed if a sinus fistula or a wound infection is present, there is intraoperative purulence, cultures identify phenotypically indistinguishable pathogens from at least two separate deep-tissue or implant specimens, or microorganisms are identified in deep-tissue specimens on specific staining. This definition has a high impact on clinical diagnosis of FRI, but the expert group was unable to include histology as a criterion for the diagnosis of FRI. Morgenstern et al. recognized this missing part in definition and investigated the role of quantitative histological analysis in the diagnosis of FRI [16]. This study confirms our own

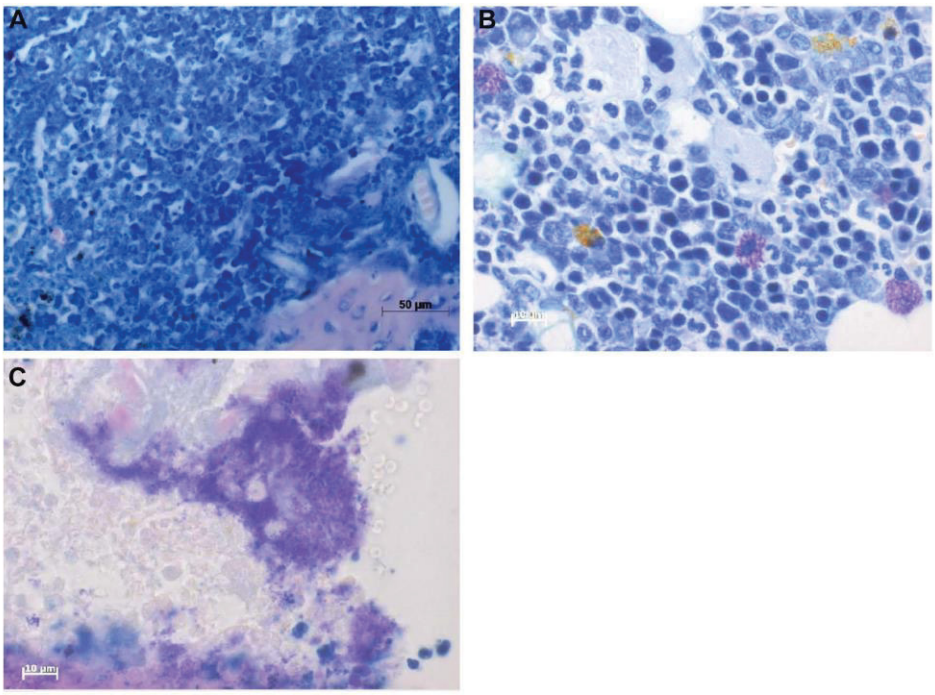

Fig. 4 Quantification of bacteria. Low number of bacteria was defined as less than 4 bacterial colonies (**a**), moderate number as 4–10 bacterial colonies (**b**), and high number of bacteria as > 10 bacterial colonies per 20 visual fields (**c**)

results: histology can be used as a diagnostic tool for FRI. Therefore, we established a score including diagnostic imaging, microbiology as well as histopathology. Combining all four items and calculating an overall score allowed diagnosing FRI with high sensitivity and specificity. The proposed score enables to estimate severity of induced infection, to compare infection in different studies and might, therefore, be a useful tool for further studies addressing pathomechanisms and new therapeutic approaches to FRI.

Limitations

Finally, we acknowledge different limitations of the present study: (1) recently, it was questioned if and how animal models reflect the pathophysiological and molecular changes occurring in humans after infection. Genomic responses after acute inflammatory stress were mentioned to poorly mimic the human inflammatory condition [23]. However, similar genomic responses in mouse models and human inflammatory diseases were found in a later study using the same data set [24]. Analysis of genomic or molecular responses of osteitis was beyond the scope of the present study. However, the imaging, microbiological and histopathological changes observed after murine osteitis are well known from the human situation. (2) Our present score was evaluated in a limited number of mice allocated to osteitis and sham group. However, higher numbers of animals might not be reasonable for animal protection reasons. (3) The diagnostic reliability of newly introduced tests is usually assessed by comparing the new test with gold standard method. To our best knowledge, this is the first test to diagnose osteitis in mice by combining imaging, microbiological and histopathological features. Therefore, a comparison to the gold standard method was not possible.

Conclusion

In the present study, we designed a score to diagnose and quantify FRI in an implant-associated murine model by combining diagnostic imaging, microbiological and histopathological features. The proposed score was easy to

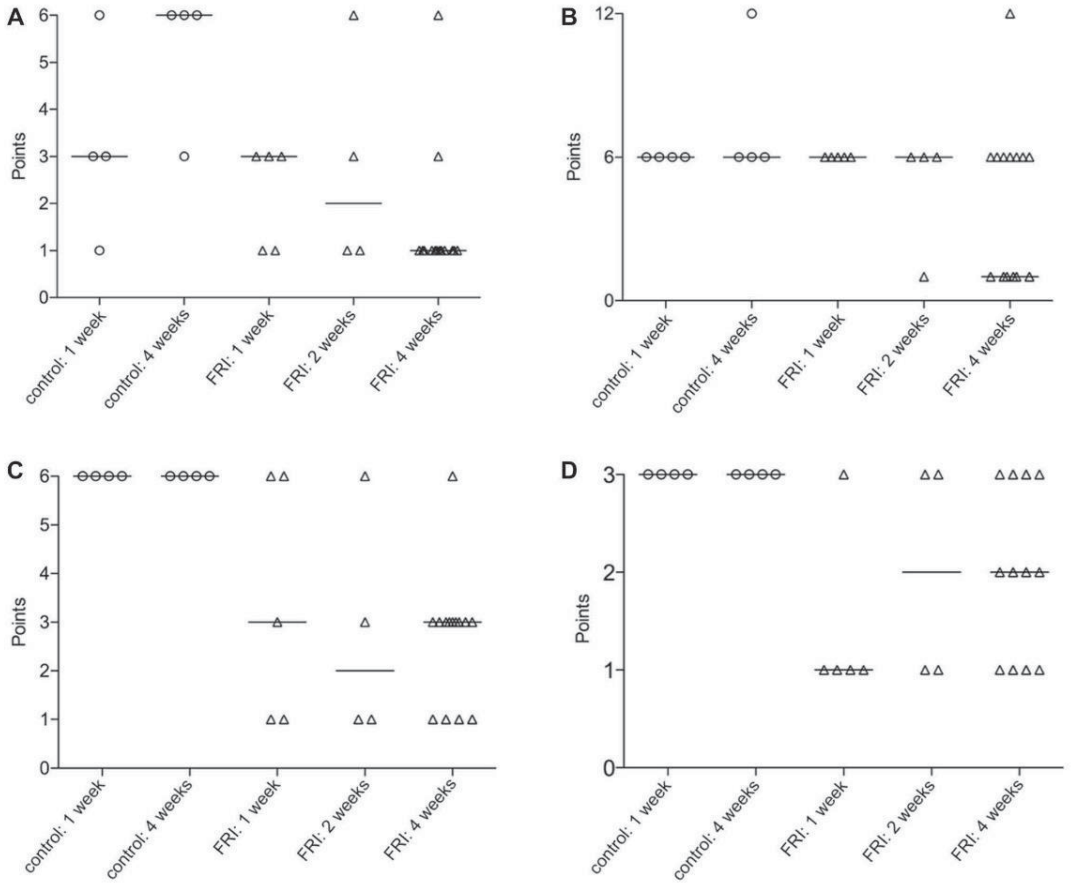

Fig. 5 Distribution of values for each parameter of the score assessing osteitis. This figure shows the assessed values and median values (black lines) in the control and FRI (fracture-related infection) groups for callus formation (**a**), consolidation of the fracture (**b**), structural changes of the medullary cavity (**c**) and number of bacteria (**d**)

access, feasible to diagnose and estimate bone healing and infection with a high sensitivity. Therefore, this score might be a useful tool to quantify infection-related changes after fracture fixation in future preclinical studies. In the present study, we design a score to diagnose and quantify osteitis and osteomyelitis in murine experimental osteitis by combining diagnostic imaging, microbiological and histopathological features. The score was easy to access; feasible to diagnose and estimate bone healing and infection in a murine osteitis with a high sensitivity. Therefore, this score might be a useful tool to quantify infection-related changes after osteitis in further studies and to compare the results of different studies.

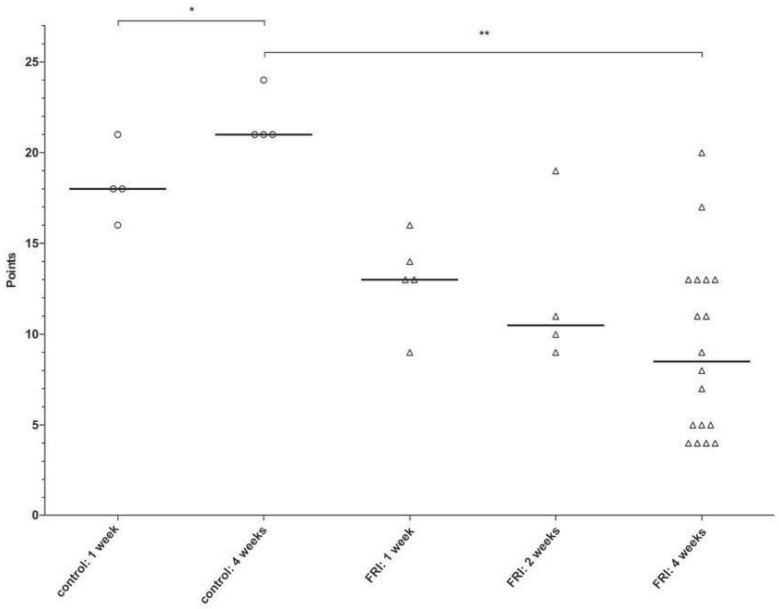

Fig. 6 Total score. This figure summarizes the values of the total score for each group. The higher score in the control group at 4 weeks might reflect the physiological re-constitution of the bone. Mice assigned to the FRI (fracture-related infection) group showed significantly lower median score values at one and 4 weeks after induction of the infection

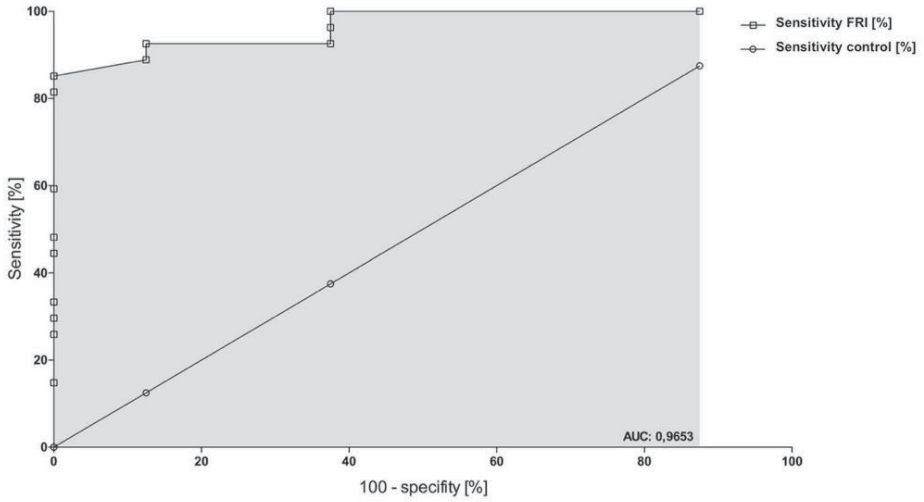

Fig. 7 Receiver operating characteristics (ROC) for the total score. *FRI* fracture-related infection

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Author contributions All authors have read and approved the final submitted manuscript. CB: literature search, study design, data collection,

data analysis, data interpretation and writing. MH: study design, data collection, data analysis, data interpretation. TL and CDW: study design, data collection, data analysis, data interpretation and critical revision. JW: critical revision.

Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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RESEARCH ARTICLE

Effect of hyperbaric oxygen therapy (HBO) on implant-associated osteitis in a femur fracture model in mice

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Abstract

Hyperbaric oxygen therapy (HBO) is applied very successfully in treatment of various diseases such as chronic wounds. It has been already suggested as adjunctive treatment option for osteitis by immune- and fracture modulating effects. This study evaluates the importance of HBO in an early implant-associated localized osteitis caused by *Staphylococcus aureus* (SA) compared to the standard therapy. In a standardized murine model the left femur of 120 BALB/c mice were osteotomized and fixed by a titanium locking plate. Osteitis has been induced with a defined amount of SA into the fracture gap. Debridément and lavages were progressed on day 7, 14, 28 and 56 to determine the local bacterial growth and the immune reaction. Hyperbaric oxygen (2 ATA, 90%) was applied for 90 minutes on day 7 to 21 for those mice allocated to HBO therapy. To evaluate the effect of HBO therapy the following groups were analyzed: Two sham-groups (12 mice / group) with and without HBO therapy, two osteotomy groups (24 mice / group) with plate osteosynthesis of the femur with and without HBO therapy, and two osteotomy SA infection groups (24 mice / group) with and without HBO therapy. Fracture healing was also quantified on day 7, 14, 28 and 56 by a.p. x-ray and bone healing markers from blood samples. Progression of infection was assessed by estimation of colony-forming units (CFU) and immune response was analyzed by determination of polymorphonuclear neutrophils (PMN), Interleukin (IL) - 6, and the circulating free DNA (cfDNA) in lavage samples. Osteitis induced significantly higher IL-6, cfDNA- and PMN-levels in the lavage samples (on day 7 and 14, each $p < 0.05$). HBO-therapy did not have a significant influence on the CFU and immune response compared to the standard therapy (each $p > 0.05$). At the same time HBO-therapy was associated with a delayed bone healing assessed by x-ray radiography and a higher rate of non-union until day 28. In conclusion, osteitis led to significantly higher bacterial count and infection parameters. HBO-therapy neither had a beneficial influence on local infection nor on immune response or fracture healing compared to the standard therapy in an osteitis mouse model.

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Introduction

Induction of anti-sepsis and evolution of perioperative standards as well as surgical techniques have significantly reduced the risk of incorporation of bacteria during surgery. Despite these advances, the overall incidence of early infections after a fracture stabilization ranges between 0.1% and 1.7% [1]. In open fractures incidence of infection significantly increases from 2.7% up to 43%, depending on the degree of soft tissue damage and fracture region [1]. Thus, perioperative infections are one of the major challenges in orthopedic and trauma surgery. Some authors designate perioperative infections involving the entire bone including its cortex as "osteitis" and distinguish those infections from infections of the bone marrow (e.g. by hematogenous dissemination) designated as "osteomyelitis" [2,3]. Perioperative infection can result in an osteitis related to a direct contamination of bacteria in open fractures or / and bone stabilization with orthopedic implants. Several infection predisposing factors were identified including the degree of the primary or secondary local tissue damage and systemic host factors such as underlying diseases like diabetes and local vascularity [4,5]. Moreover, the presence of foreign surfaces like plates or prosthesis significantly increases the risk for the development of an infection [6]. In principle, various types of bacteria may cause perioperative osteitis. *Staphylococcus aureus* (SA) and *Staphylococcus epidermidis* (SE) are most commonly responsible for osteitis in pyogenic and medical-device associated osteitis [7,8]. Radical surgical debridement and antibiotic therapy are the main treatment columns of early implant-associated infections. However, bacteria have developed several mechanisms, which allow for growth and evasion of host defense. SA forms a biofilm on foreign surfaces. This biofilm consists of a hydrated matrix of extracellular components including several proteins, in which a multilayer cell cluster of sessile bacteria is embedded [6,8]. Biofilms protect bacteria from the host´s defenses and are resistant against most antibiotics, both enabling SA to cause a chronic infection after fracture stabilization. Biofilm production allows SA to escape from host defense. Next to these mechanisms, SA was considered to directly invade host cells (in particular immune cells such as macrophages and neutrophils) and therefore to undermine the host´s defense [7,9]. Furthermore, fracture healing is impeded by infection progress as well as by a frustrating activation of the innate immune system [10]. Activation of polymorphonuclear neutrophils represents an important mechanism of bacterial defense. However, the release of high levels of potentially cytotoxic molecules like proteases has negative effects on fracture healing [11]. Last, SA develop resistance against antibiotic therapy which poses a serious clinical obstacle to the treatment of osteitis [7]. Despite a radical surgical debridement and antibiotic therapy, recovery from biofilm-associated infections frequently necessitates complete implant removal. Newly developed therapeutic approaches intend to destroy or prevent biofilm formation on implant surfaces. Although with promising results, these approaches have not resulted in a breakthrough regarding the therapy of implant-associated infection [7,12–14]. Moreover, the fracture healing process itself is not addressed by these strategies.

Hyperbaric oxygen therapy (HBO) consists of intermittently administering 100% oxygen at pressures greater than one atmosphere absolute (ATA) in a pressure vessel. This technology has been used to treat a variety of diseases. Positive effects are described particularly in patients suffering from chronic wounds or delayed- and non-unions after a fracture [15]. HBO has antimicrobial effects and accelerates fracture healing *in vitro* and *in vivo* [16]. Moreover, HBO reduces inflammatory response in an ischemic wound model [17]. Some recent animal studies suggested a beneficial effect of HBO in the treatment of bacterial infections of the bone marrow (osteomyelitis) [18–22]. In these studies, the effect of HBO therapy was more related to direct antibacterial activity of HBO than to a better phagocytosis of SA with rising intramedullary oxygen tensions during HBO therapy [17], [18]. Despite a great number of established

animal models addressing bacterial bone infections, differences of pathophysiological mechanisms of bacterial infections of the bone marrow (osteomyelitis) and perioperative implant-associated infections (osteitis) are poorly understood. As osteomyelitis was induced by an intramedullary bacteria injection in the recent studies analyzing the effect of HBO, results from these studies can probably not be translated to implant-associated osteitis. Therefore, it remains unresolved whether HBO presents an immune- and fracture healing-modulating therapeutic approach for early implant-associated osteitis. A potentially beneficial effect of HBO therapy in implant-associated osteitis models might have a great impact on clinical practice. As osteitis animal models are considered to resemble the human situation, results might be translated "from bench to bedside" and HBO therapy might be an additive therapeutic option next to surgical and antibiotic therapy of osteitis.

The aim of the present study was to evaluate the effect of HBO on fracture healing, infection progress and immune response in an early implant-associated localized osteitis caused by SA in a murine femur fracture model.

Material and methods

Ethical statement

The present animal experiments were approved by the local institutional committee on animal care ("Landesamt für Naturschutz, Umwelt und Verbraucherschutz" of the federal state of North Rhine-Westphalia, Germany—file number: 87–5104.2010.A375) and are in line with the European Communities Council Directive (86/609/EEC). Specific effort was made to minimize the number of animals. Reporting of the results of the present study adheres to the "Animals in Research: Reporting in vivo Experiments"criteria (ARRIVE criteria) [23].

Animals

Female wild-type BALB/c-mice were used for the study. The age ranged between 10 to 12 weeks with an average weight of 22 g. Mice were kept in the local animal research institution (animal facility of the Heinrich-Heine-University Düsseldorf, Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben, ZETT, Germany) in standard polycarbonate (makrolon type II) cages under a conventional 12 h light–dark cycle (7:00 a.m. / p.m.). Mice had free access to food and water.

The implant-associated osteitis model

A well-characterized and standardized implant-associated osteitis model in mice was used as described before [24,25]. In detail, mice were anesthetized by i.p. injection of xylazine (5 mg / kg body weight) and ketamine (100 mg / kg body weight). The thigh was gently shaved and cleaned with betadine and alcohol swabs. After a 2 cm skin incision along the left lateral thigh, the fascia was opened and the muscles were gently dissected to expose the femur. Afterwards, a 4-hole titanium locking plate with locking self-tapping micro-screws (MouseFix plate, RISystem, Davos, Switzerland) was applied to the femur. After plate fixation, an osteotomy using a Gigly saw (diam. 0.22 mm) was performed in midshaft of the femur. For the mice allocated to an osteitis group, implant-associated infection was induced by inoculation of the fracture gap with 1 μl of SA solution (strain ATCC 29213, averaged 1.94 x 10^3 colony forming units / μl) [24]. All groups were re-anaesthetized 7 and 14 days after primary surgery and a standardized lavage with 250 μl phosphate buffered saline (PBS) twice and debridement of infected tissue was performed. Local surgical debridement was implemented with a sharp curette without involving the periosteum. The lavage fluid was recovered and PBS added to a total volume of 1

ml. The lavage fluid was further analyzed for the number of SA colony-forming units (CFU), polymorphonuclear neutrophils (PMN), Interleukin (IL) - 6, and the circulating free DNA (cfDNA). Parallel to the surgical lavage, blood serum was obtained from the tail vein on day 7, 14 and 28 and from heart puncture on day 56 for further analysis of serum bone healing markers: alkaline phosphatase (AP) and amino-terminal propeptide of type I collagen (PINP). Mice were euthanized by cervical dislocation on day 28 or 56.

The hyperbaric oxygen therapy (HBO)

The optimal setup for a HBO therapy in mice was evaluated in a series of pre-tests, as an HBO mouse model was not yet established. As a ventilation of 90% oxygen via air mask was not feasible in mice, the cages with mice allocated to the HBO group were put inside a closed chamber (Fig 1). First, 90% oxygen was applied over an in- and outflow for 2 minutes, and then mice were exposed to HBO 90 Min at 2 ATA. Mice were awake during the HBO therapy. Mice allocated to an HBO group received HBO therapy for 3 weeks (5 days per week) with the beginning on day 7.

Allocating animals to experimental groups and sample size

In total, 120 BALB/c-mice were included in the experiments. To study physiological responses in reaction to an implant-associated infection and to evaluate fracture healing, infection

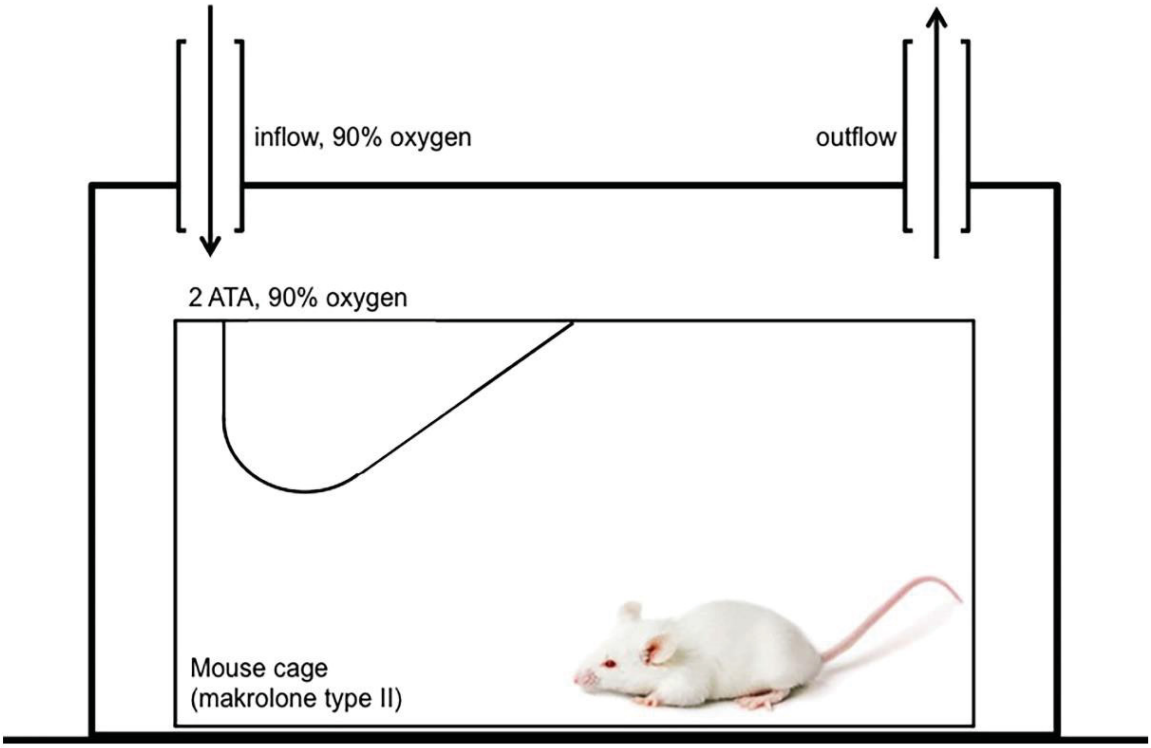

Fig 1. Setup for hyperbaric oxygen therapy in mice. Standard cages (Makrolon type II) with BALB/c mice were placed inside a closed chamber. 90% oxygen was applied over an in- and outflow for 2 minutes. Then mice were exposed to hyperbaric oxygen therapy for 90 Min at 2 absolute atmospheres (ATA).

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recovery, and immune response after implant-associated infection of a femoral fracture in reaction to HBO therapy, mice were allocated to the following six experimental groups: In two sham-groups (12 mice / group) only a skin incision was performed. One of these sham-groups had HBO therapy as described above (90% oxygen, 2 ATA, 3 weeks). The other group had no further therapy (room air and conditions at all times). In two osteotomy groups one with and one without HBO therapy, osteotomy and plate osteosynthesis of the femur was performed (24 mice / group). In addition, infection was induced by inoculation of the fracture gap with SA in two osteitis groups one with and one without HBO therapy (24 mice / group).

Experimental setup and outcome measures

Fracture healing was examined by x-ray analysis on day 0, 7, 28 and 56. Progress of infection in the wound was monitored by estimation of the counts of SA in lavage on day 7, 14, 28 and 56. The local inflammatory response was characterized by measuring the quantification of PMN, cfDNA and IL-6-levels in the lavage on day 7, 14, 28 and 56 after osteotomy. The serum AP and PINP were evaluated on day 7, 14, 28 and 56 in 12 mice per group.

X-ray analysis

Standard anteroposterior radiographic images (MX20 Faxitron, Tucson, Arizona, USA; 40 kV, 16 mA) of the femora were taken under anesthesia on days 0, 7, 14, 28 and 56 after plate fixation. The fracture gap size was measured at the plate opposing cortical bone. The MouseFix plate has a length of 8 mm. This internal standard allows an exact calculation of the distance of the fracture gap. The fracture gap was classified by using a modification of a previously established score [25]: 1 point was considered a completely healed fracture gap. Decreasing diameters of fracture gaps representing fracture healing were rated with 2 points. A constant fracture gap meaning no healing was rated with 3 points, increasing facture gap was rated with 4 points, obvious osteolysis with 5 points and destruction of the femur with 6 points.

Counts of colony-forming units (CFU)

The number of CFU was attained and determined from the lavage on day 7, 14 and 28 each. 200 ml lavage were serially diluted in PBS and four replicates of 10 μl of each dilution plated on Columbia Agar plates with 5% sheep blood and incubated under aerobic conditions at 37˚C. Bacterial colonies were counted after 24 h. Results were specified as CFU per 1 ml.

Polymorphonuclear neutrophils (PMN). The local inflammatory response was characterized by measuring the PMNs in the lavage using flow cytometry (FACSCanto II; BD Biosciences, Heidelberg, Germany) with the following antibody (FITC Rat Anti-Mouse Ly-6G; BD Pharmingen).

Quantification of Interleukin (IL)-6

IL-6 levels in the lavage were determined using a commercially available IL-6 ELISA kit according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The lower detection limit for IL-6 was 16 pg / ml.

Quantification of neutrophil extracellular traps (NETs)

NET's quantification in the lavage was performed by detecting cfDNA using the Quant-iT Pico green dsDNA assay (Invitrogen GmbH, Darmstadt, Germany). This assay is already used and described by our group [26,27]. The fluorescence intensity reflects the amount of DNA and was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively in a

microplate reader (Victor3, PerkinElmer, Waltham, USA). A standard calibration curve by means of defined calf thymus DNA (Sigma, St. Louis, USA) amounts ranging from 0 to 2 μg / ml has been used in all analyses.

Blood alkaline phosphatase levels (AP)

AP activity as a non-specific marker for bone healing was determined in serum. We used an AP Assay Kit measuring the AP activity directly without pretreatment (Abnova, Taipei, Taiwan). This method utilizes p-nitrophenyl phosphate that is hydrolyzed by ALP into a yellow colored product. The rate of the reaction is directly proportional to the enzyme activity and was measured at wavelengths of 405 nm 0 and 4 minutes after reaction (Victor3, PerkinElmer, Waltham, USA).

Amino-terminal propeptide of type I collagen (PINP)

PINP concentration in serum was measured by a competitive ELISA assay for human N-terminal propeptide of collagen type I (Cloud-Clone Corp., Katy, USA). We followed the manufacture's instruction while using this commercially available ELISA kit. The intensity of color was read in a microplate reader (Victor3, PerkinElmer, Waltham, USA) and is inversely proportional to the concentration of PINP in the sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software, San Diego, CA). Real-valued data was first tested for normality using D´Agostino and Pearson normality test. If the variables themselves were normally distributed, the t-test was applied directly to the data to check whether the distribution means significantly differ. Not-normally distributed data was tested for statistical significance with two-tailed Mann–Whitney-test. P-values of 0.05 and below were considered significant. A trend towards significance was defined by a p-value between 0.1 and 0.05.

Results

Baseline data

Surgery was performed on 120 female wild-type BALB/c mice in the age range 10 – 12 weeks and a weight range of 18 – 27 g (mean 22 g). 18 mice died during the experimental procedures (overall mortality rate of 15%). There were no significant differences between the experimental groups. 102 mice were considered for further analysis.

Fracture healing

On days 0, 7, 14, 28 and 56 after plate fixation anteroposterior radiographic images of the femora were taken under anesthesia. All mice with osteosynthesis without infection showed a healing fracture gap (Fig 2). In the animals of the osteotomy and osteotomy / HBO group, the fracture completely healed within the observation period. This was reflected by median bone healing score of 3 after 7 days, of 2 after 14 days and 1 after 4 weeks each. Mice with infection showed different results (Fig 3). Animals of the osteotomy / infection group had the same median values as the controls but showed a greater individual heterogeneity and nonunion in individual animals. In contrast, median bone healing score increased in the osteotomy / infection / HBO group till day 28. On day 56 after fracture, the mean score value in all experimental groups suggested a sufficient fracture healing in the majority of animals of all groups (Fig 4). Analysis of AP and PINP in the blood serum revealed only significant differences for both

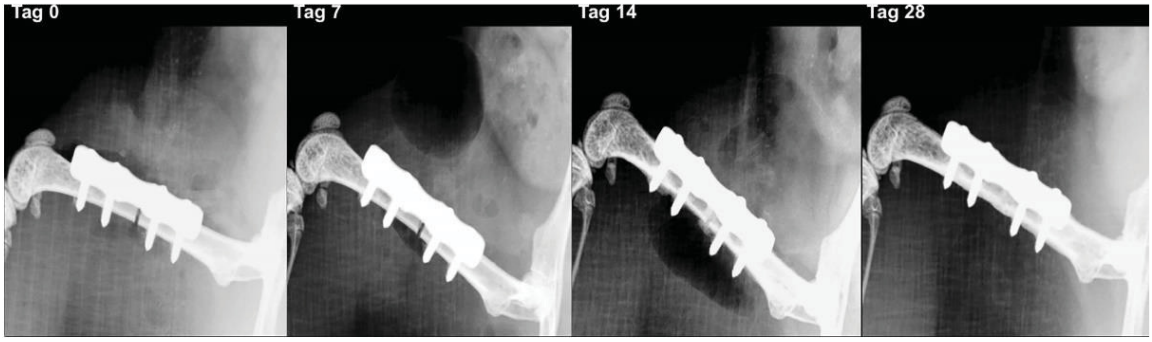

Fig 2. Radiographic analysis of fracture healing in non-infected mice. X-ray scans of the left femur in a mouse allocated to the osteotomy group after day 0, 7, 14 and 28. The fracture completely healed within the observation time.

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parameters between the osteotomy and the osteitis alone on day 7 and between osteotomy / HBO and the osteitis / HBO group on day 14 (Figs 5 and 6).

Infection progress

Infection progress was verified by estimation of the numbers of CFU gained from the lavage on day 7, 14, 28 and 56. The sham-groups and the groups with osteosynthesis without infection showed no evidence for SA in all lavages (sham, sham / HBO, osteotomy and osteotomy / HBO: each 0 ± 0). In contrast, SA was verified in all infection groups. HBO therapy did not significantly influence SA CFU at any point in time (each $p > 0.05$, Fig 7).

Inflammatory response

The inflammatory response was analyzed by quantification of the PMN, NETs and IL-6 (Figs 8, 9 and 10) levels in the lavage samples. A local infection induced a significant inflammatory response with an increase of local PMNs, NETs and IL-6 on day 7 and 14 (osteotomy vs. osteitis and osteotomy / HBO vs. osteitis / HBO), respectively. The osteitis / HBO but not the osteitis only group showed a significant increase of these outcome measures compared to the

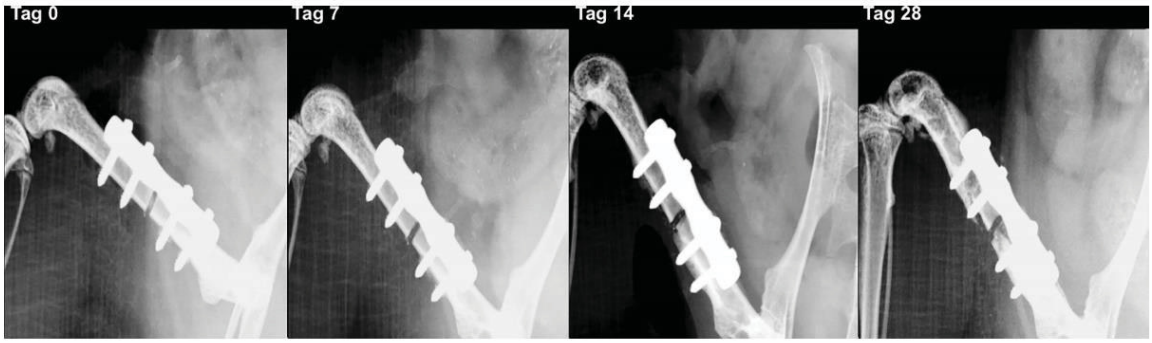

Fig 3. Radiographic analysis of fracture healing in infected mice. In contrast to the non-infected mice, mice allocated to both osteitis groups showed a higher frequency of nonunion. This mouse was allocated to the osteitis / HBO group and serial X-ray on day 0, 7, 14, and 28 shows development of a nonunion.

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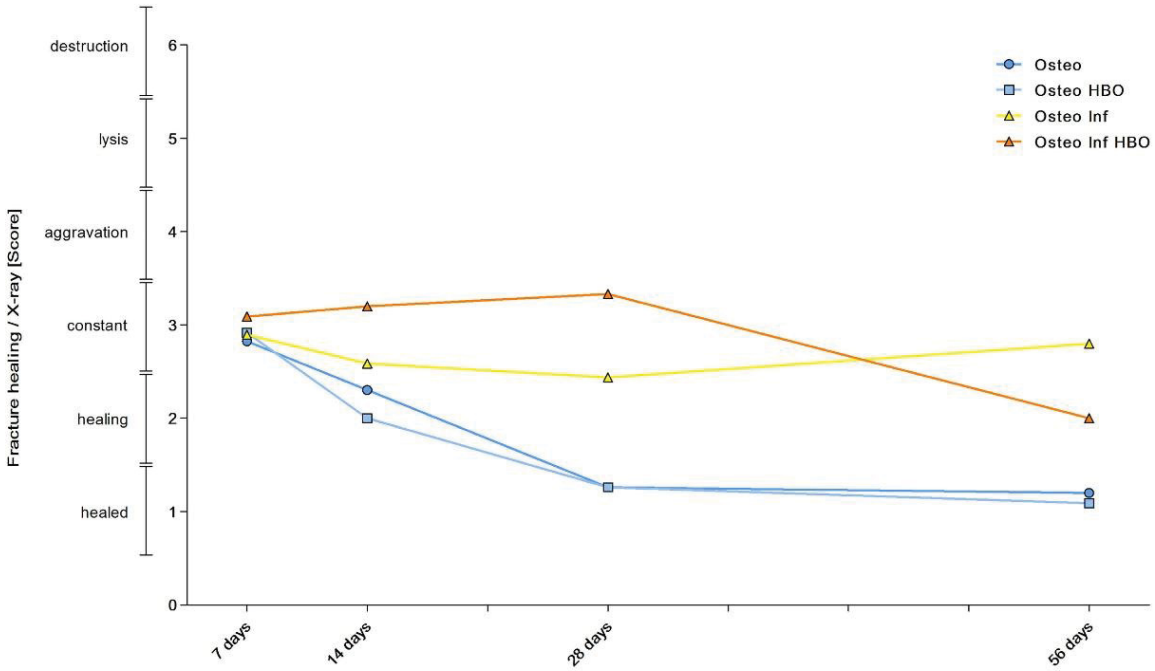

Fig 4. Mean score values of fracture healing. The fracture gap was measured on a.-p. X-ray scans and fracture healing was classified by a score. Individual and mean values of the score are summarized. Animals of the osteotomy and osteotomy / HBO group showed a sufficient healing of the fracture within the observation period and subsequently decreasing mean values as assessed by the score. Mean score values also decreased for the osteitis group over time but the score showed a greater individual heterogeneity and nonunion in individual animals. In contrast, median bone healing score increased for the osteitis HBO group till day 28 and improved on day 56.

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corresponding control groups on day 28 and 56. Next to infection, osteotomy alone, in comparison to the sham mice, results in an increase of proportional PMNs and NET's on day 7 and 14. HBO therapy after osteitis was associated with lower NETs levels on day 28 but did not have further significant influence on the following immune response during osteitis.

Discussion

The main findings of this retrospective analysis of the present study evaluating the effect of HBO on fracture healing, infection progress and immune response in a mouse osteitis model is that HBO did not significantly influence bone healing and local infection in our osteitis mouse model and that mice exposed to the bone infection showed significant higher colony forming units and infection parameters.

HBO therapy was previously evaluated in several murine osteitis models: Hamblen induced osteomyelitis by injection of SA into the intramedullary cavity of rat tibia and described similar infection rates but an increased bone healing after established osteitis in the HBO groups as compared to the controls [18]. A similar model was used in rabbits: Here, HBO therapy led to a significant reduction of the intramedullary bacterial load but did not influence the radiographically assessed severity of the bone infection [20]. The therapeutic effectiveness of HBO therapy was more related to direct antibacterial activity of HBO than to a better phagocytosis of SA with rising intramedullary oxygen tensions during HBO therapy [19,20]. Recently,

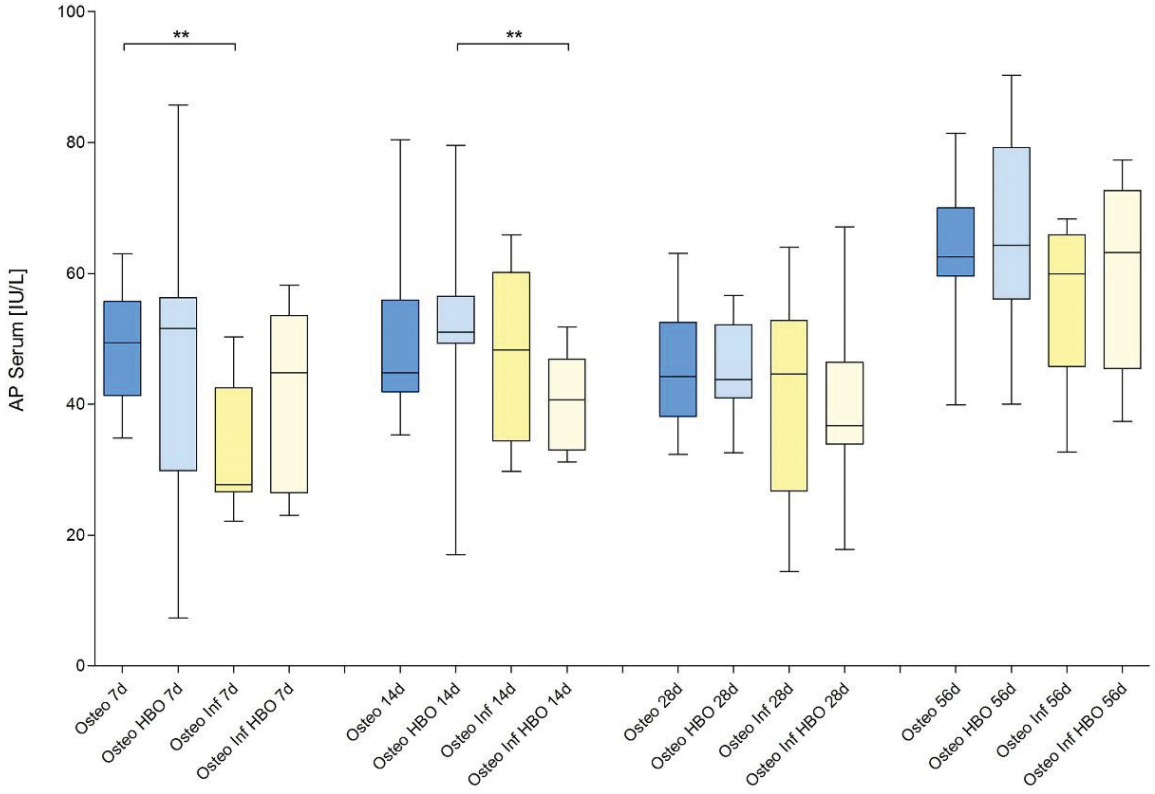

Fig 5. Blood alkaline phosphatase levels. Analysis of alkaline phosphatase (AP) in the blood serum revealed only significant differences between the osteotomy and the osteitis alone on day 7 and between osteotomy / HBO and the osteitis / HBO group on day 14.

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effects of HBO after induction of chronic osteomyelitis by intramedullary injection of SA into the right tibia were reevaluated in 6-month old Wistar rats: In comparison to the control groups, 2-weeks of HBO therapy led to a reduction of the mean SA CFU from mean 3.6 to 1.2 x 10^6 CFU/g tibial bone and a 4-week HBO therapy led to a SA CFU reduction from 2.9 x 10^6 to 6.2 x 10^5 CFU/g tibial bone [22]. Accordingly, osteitis related radiographic changes improved as measured by a score [22]. However, it remains unclear if the improvements are significant. A later study of the group led to similar results for the HBO therapy alone and control groups [21].

In contrast to these results, the present results reveal neither a beneficial effect of HBO on bone healing nor on infection progress or inflammatory response in a mouse implant-associated osteitis model of the femur. This observation is in line with the two previous study evaluating. HBO therapy in a mouse osteitis model: Shandley and coworkers analyzed the impact of HBO in methicillin-resistant SA (MRSA), *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* induced implant-associated osteitis of the tibia in C57BL/6 mice. In this model, HBO accelerated the growth of MRSA and resulted in more severe lesion scores [28]. Interestingly, only MRSA and *Pseudomonas aeruginosa* sufficiently induced a local osteitis but not *Klebsiella*

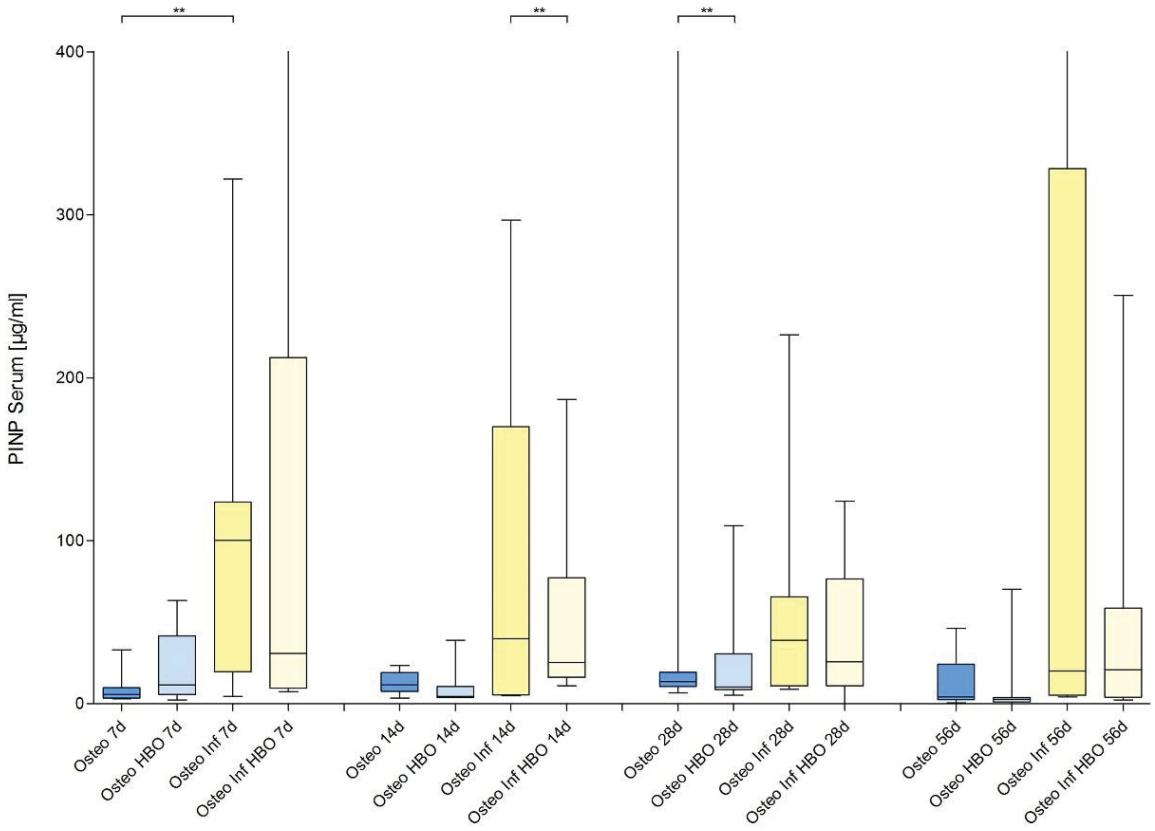

Fig 6. Blood amino-terminal propeptide of type I collagen levels. Analysis of amino-terminal propeptide of type I collagen (PINP) levels in blood samples revealed only a significant influence of HBO therapy after osteitis 14 days after infection.

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pneumonia. The authors concluded, "HBO does not appear to be a useful treatment for osteitis in this model" [28]. More recently, additional effect of HBO combined with conventional antibiotic treatment (daptomycin and rifampicin) was compared to antibiotic standard regime in a mouse model of implant-associated osteomyelitis [29]. HBO (3 ATA, 100% for 60 min) was applied from day 11 to 14 after induction of osteitis by inoculation of a transcortical tibia implant with SA. In this setup, HBO therapy in combination with daptomycin and rifampicin did not significantly improve the cure rate or the bacterial load on the implants compared to antibiotic therapy alone. In contrast to our results, HBO induced a significantly elevated bone turnover as assessed by estimation of serum PINP and Tartrate-resistant acid phosphatase 5b concentration. Subsequently, the authors conclude that efficacy of antibiotic therapy cannot be improved by adjuvant HBO [29]. The effect of HBO was further evaluated in a different implant-associated model. Here, a Kirschner wire was introduced in the femoral cavity of Sprague-Dawley rats and osteitis induced by intramedullary injection of SA. The combination of vancomycin treatment and HBO therapy did not lead to a significant reduction of the bacterial load, the histo-pathologically evaluated degree of osteitis and IL-1b, IL-10, and TNF-a levels, in comparison to vancomycin treatment alone.

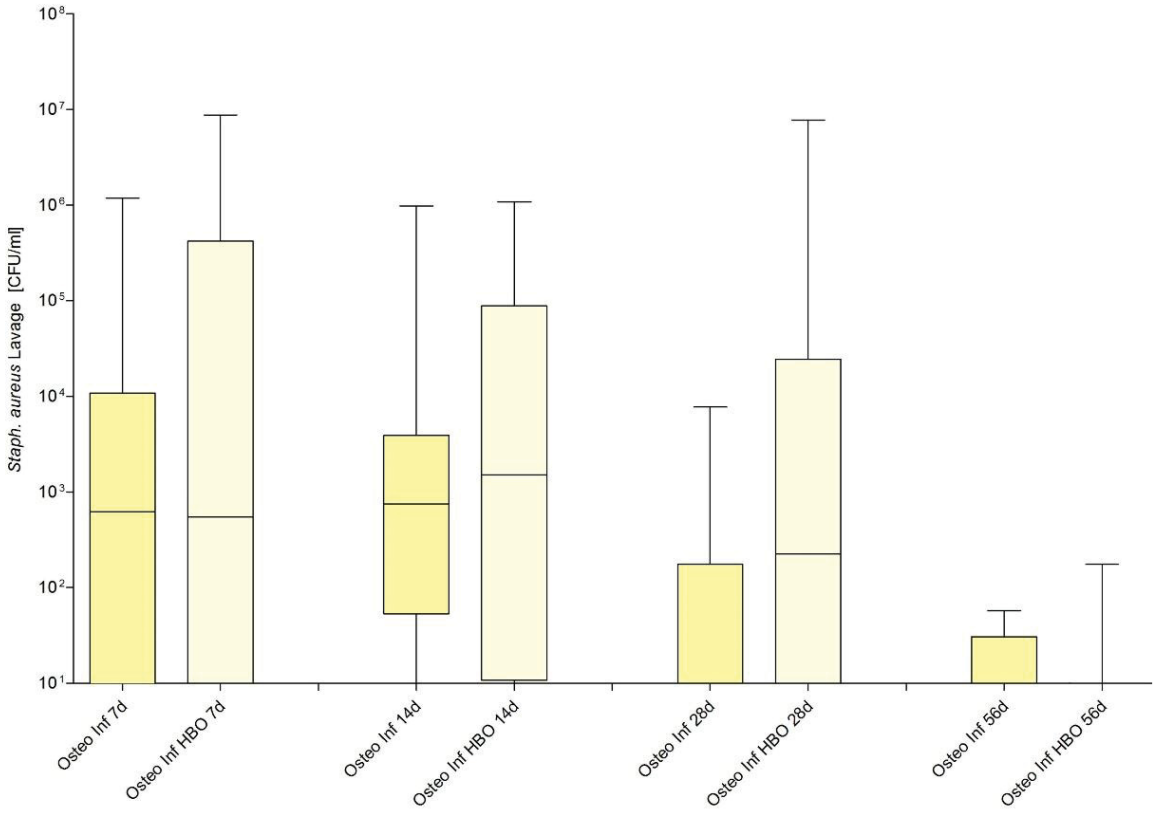

Fig 7. Detection of counts of Staphylococcus aureus (SA) around the facture side. Colony-forming units (CFU) in the lavage samples obtained on day 7, 14, 28 and 56.

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Therefore, in contrast to those studies, in which osteomyelitis was induced by intramedullary SA injection, models of implant-associated osteitis reveals no additional benefit of an HBO therapy. This observation might be due to several reasons or a combination of them: First, orthopedic implants might interfere with a potential effect of HBO therapy and might enable bacteria to escape from HBO-supported host defenses. Second, we performed stabilization of the femur with a plate and osteotomy. Injury of the bony cortex and the periosteum is therefore unique characteristic of our model and might be another explanation for the differences in regard to the previously mentioned injection models. Injury of the bony cortex and the periosteum might not be accessible for the benefits of HBO therapy. Again, Mader and coworkers attributes the therapeutic effectiveness of the HBO therapy not to a direct antibacterial activity but to a better phagocytosis of SA with rising intramedullary oxygen tensions during HBO therapy [19,20]. Rising intramedullary oxygen pressure was not evaluated in the present study, but elevation of the intramedullary oxygen tension might not necessarily promote phagocytosis in the fracture gap and the adjacent soft-tissue.

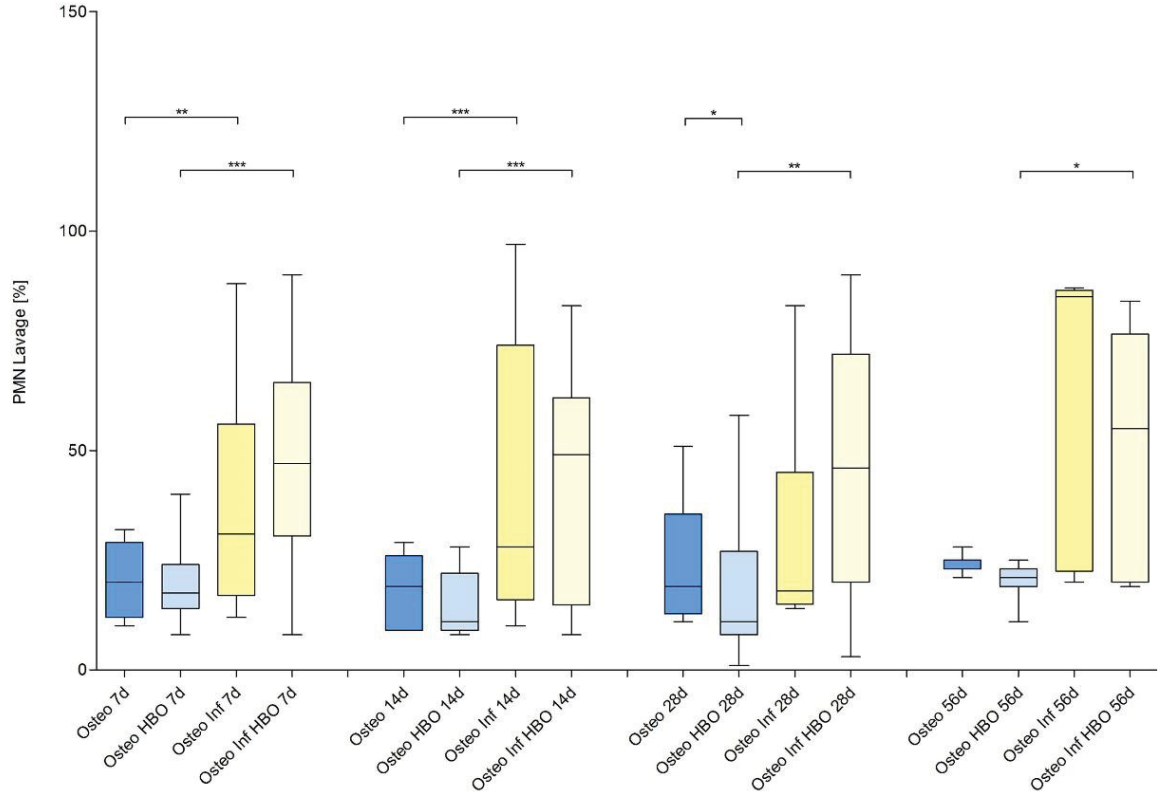

Fig 8. Quantification of polymorphonuclear neutrophils (PMNs).

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Limitations

We acknowledge several limitations of our present study: First, in principle, the lack of influence of the HBO therapy on the osteitis might be due to a lack of induction of an infection constellation in the present model. However, we could rule this explanation out as our previous experiments and our controls clearly show that our model is sufficient to induce an osteitis constellation with a significant bacterial burden, an impairment of the natural bone healing and an inflammatory reaction. Second, not all possible parameters, such as intramedullary oxygen pressure or medullary perfusion, were analyzed in the present study. To minimize the number of experimental animals, the numbers of analysis are limited due to the small size of mice and researches have to focus on the most interesting parameters. Furthermore, we analyzed representative outcome measures for fracture healing, infection progress and immune response. Third, we cannot rule out that HBO might have an effect on other bacteria or polymicrobial infections. Especially poly-microbial infection and antibiotic resistance (in particular MRSA and multi-resistant *Enterobacteriaceae*) clinically might be problem as their rates increase [7,30]. Interestingly, Shandley and coworkers reported a HBO-mediated acceleration of the growth of MRSA in osteitis mice and subsequently an impaired bone healing. Fourth, other HBO protocols with variation of the HBO therapy, oxygen concentration and chamber

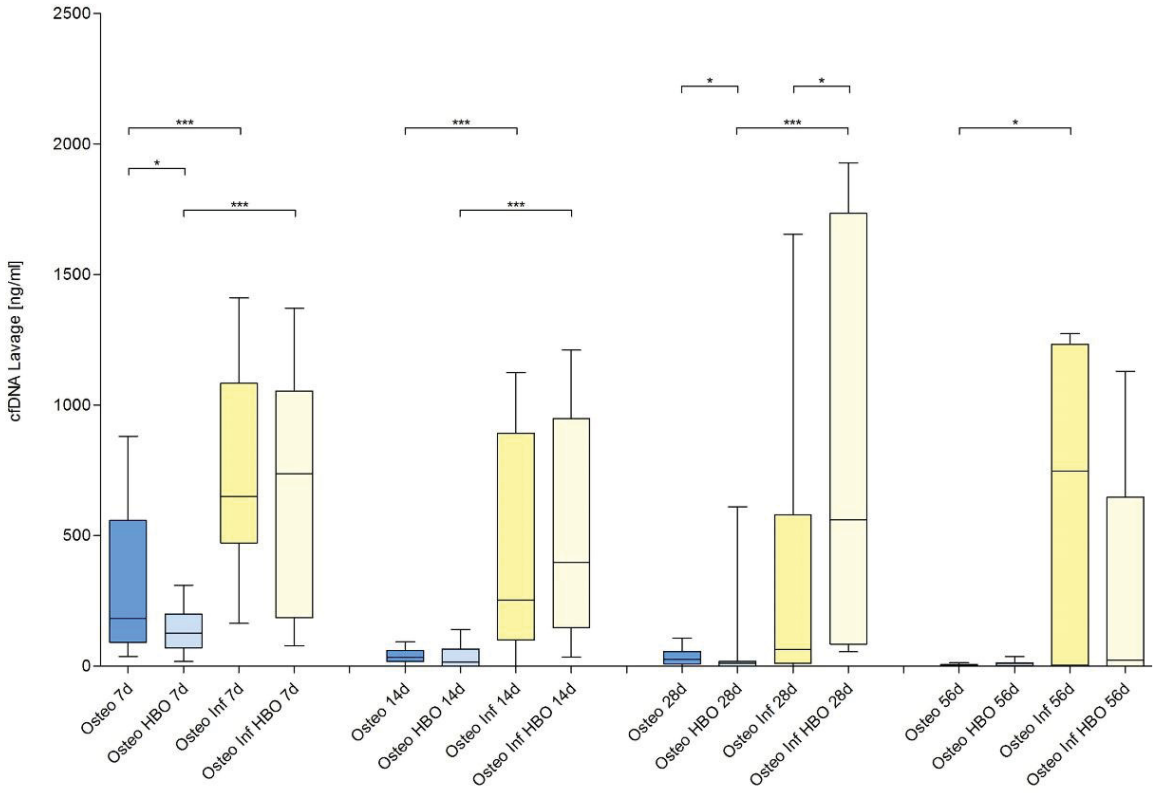

Fig 9. Quantification of circulating free DNA (cfDNA).

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pressure might lead to other results. Finally, our model might not necessarily resemble the human condition in every detail. Previously, it was questioned how far sepsis models resemble the human condition on a molecular level [31]. Again, our osteitis model shows a significant bacterial load, an impairment of the natural bone healing and an inflammatory reaction. These are important features of the human condition [7]. Finally, we used the term "osteitis" for description of the *SA* induced bone infection in the present model. A universally accepted definition of the terms "osteomyelitis" and "osteitis" is not yet established [2,3,32,33]. Especially in the Anglo-American literature, "osteomyelitis" is the preferred term to describe all kinds of bone infections [2,3]. In contrast, in particular in the German literature, the term "osteitis" is distinguished from the term "osteomyelitis": First, different ways of infections were considered in osteitis and osteomyelitis [34]. In osteitis, way of infection occurs from outside to inside (centripetal way of infection) and in osteomyelitis way of infection appears from inside to outside (centrifugal way of infection). Infection progress in osteomyelitis was considered to be caused by hematogenous dissemination of pathogens and to firstly affect the bony marrow [34]. In contrast, in osteitis pathogens intrude from outside to inside in open fractures, perisurgery and in our model. Second, the term "osteomyelitis" refers to infection of the bone mar row as the term "osteitis" is used to describe an involvement of the entire organ including the

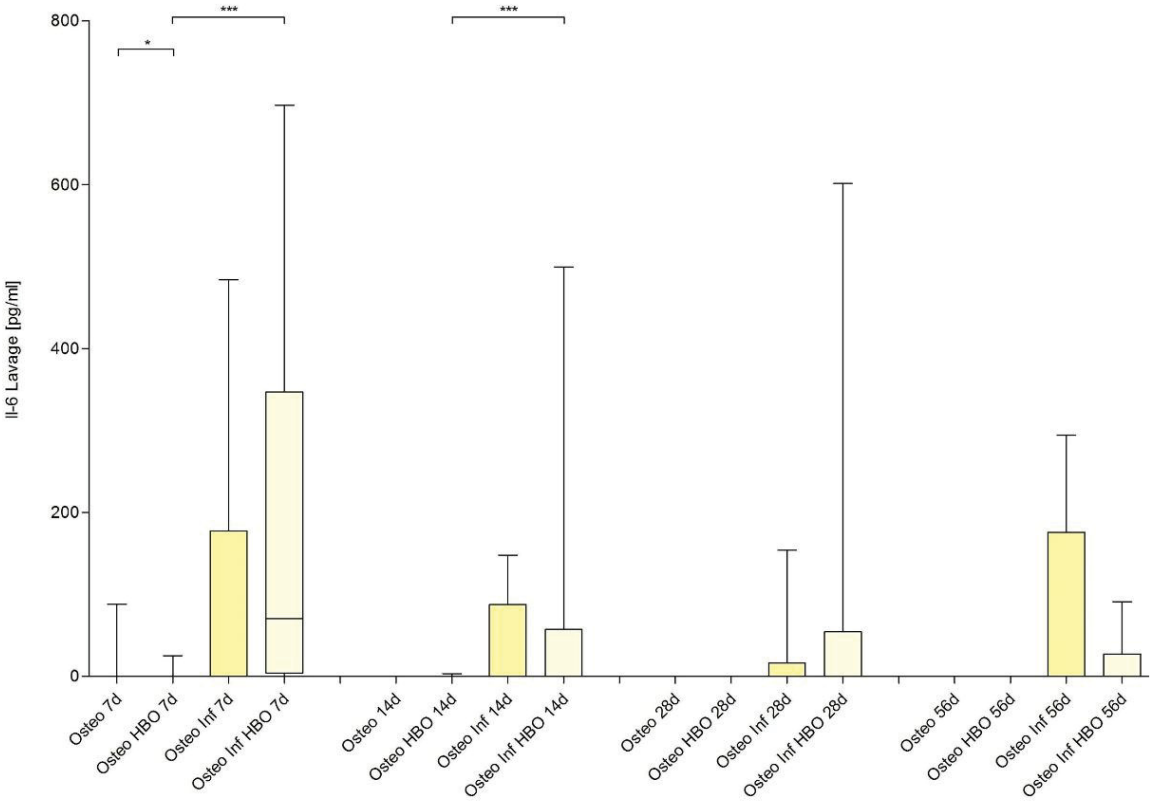

Fig 10. Quantification of Interleukin (IL)– 6 levels.

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bone cortex [2,3]. In the present study, bacterial infection was induced after osteotomy of the femur. Therefore, the present model should resemble the situation of posttraumatic / postsurgical bacterial infections. As shown in the present and the previous studies, bacterial infection involves the bone marrow, the bone cortex and the surrounding tissue. Therefore, we used the term "osteitis".

Conclusion

The present osteitis model is sufficient to study fracture healing, infection progress and immune response following implant-associated SA-mediated osteitis in mice. However, HBO did not significantly influence bone healing and local infection in the present model.

Supporting information

S1 Table. Median values and standard deviation of all outcome measures.
(XLSX)

S2 Table. Significances of all outcome measures.
(XLSX)

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Writing – original draft: Carina Büren.

Writing – review & editing: Tim Lögters, Lisa Oezel, Golnessa Rommelfanger, Armin Olaf Scholz, Joachim Windolf, Ceylan Daniela Windolf.

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PLOS ONE

RESEARCH ARTICLE

Effect of antibiotic infused calcium sulfate/ hydroxyapatite (CAS/HA) insets on implant-associated osteitis in a femur fracture model in mice

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Abstract

Cerament (Bonesupport Holding, Lund, Sweden) is a bioresorbable synthetic bone substitute consisting of calcium sulfate and hydroxyapatite which is successfully used as a bone graft in bone defects or in delayed and non-unions after fractures. Besides, calcium sulfate/ hydroxyapatite (CAS/HA) could have, attributed to its composition and osteoinductive properties, have great importance in the treatment of bone infections with critical size defects (CSD). Aim of the study was to evaluate the effects of antibiotic infused CAS/HA on inflammation and bone healing in an implant-associated osteitis mice model. In a standardized murine model, the left femur of 72 BALB/c mice were osteotomized, generating a CSD (2,5 mm) with stabilization through a 6-hole titanium locking plate. Osteitis has been induced through inoculation of *Staphylococcus aureus* (SA) into the fracture gap. To analyze the effect of CAS/HA, following groups were generated with either CAS/HA, CAS/HA with gentamycin (CAS/ HA-G) or CAS/HA with vancomycin (CAS/HA-V) insets placed into the osteotomy. Debridément and lavages were progressed on day 7 and 42 to determine the local bacterial growth and the immune reaction. Fracture healing was quantified on day 7 and 42 by x-ray and bone healing markers from blood samples. Progression of infection was assessed by estimation of colony-forming units (CFU) and immune response was analyzed by determination of Interleukin (IL)– 6 and polymorphonuclear neutrophils (PMN) in lavage samples. Osteitis induced higher IL-6 and PMN-levels in the lavage samples on day 7. Both parameters showed a reduction in all groups on day 42. CAS/HA-V revealed a significant reduction of CFU and PMNs in lavage samples on day 42. A positive effect on bone healing could only be shown in non-infected mice. Whereas, application of mere CAS/HA in infected mice did show tendencies of bone destruction and lysis, independent of impregnation with antibiotics or not. Thus, application of CAS/HA in acute implant-associated infections is not recommended. In non-infectious environments or after infect-convalescence CAS/HA could albeit serve as a suggestive tool in trauma and orthopedic surgery.

Introduction

Osteitis is described as an infection of the bone with a concomitant inflammation involving the bone marrow and the surrounding tissues [1]. These infections can originate from many different mechanisms, whereby a common cause of osteitis is a bacterial incorporation during or after surgical intervention as well as in case of open fractures caused by trauma [2]. Besides the marked progress in operating standards and amelioration of perioperative measures, especially implant-associated and perioperative infections in trauma and orthopedic surgery still represent significant complications [3]. In literature, the incidence of infection shows a wide range from 1% in primary fracture stabilization up to 55% in the treatment of open fractures [4]. Apart from that the development of an osteitis depends on individual risk factors provided by the patient such as obesity, anemia and diabetes [5]. So overall, the interaction of implants with incorporated bacteria and individual defense capacities lead to a localized infection. Regarding medical device-associated osteitis the most frequent germs are counted to be *Staphylococcus aureus* (SA) and *Staphylococcus epidermidis* [1,6]. SA has developed multiple strategies to escape from the host's immune defense. Among those, generating a protective biofilm with resistance against systemic antibiotic substances, as well as the invasion of immune host cells present fundamental mechanisms [7]. Moreover, the segregation of cytotoxic molecules, like proteases, together with onward infectious process detains fracture healing [8]. Further, these mechanisms lead to an obstructed activation of the innate immune system which consequently hamper fracture healing, e. g. the activation of polymorphonuclear neutrophils (PMN) represents an important mechanism of bacterial defense [9]. Appropriate treatment with the attempt of eradication of osteitis is necessary to prevent life threatening complications such as sepsis [10]. Osteitis is chronological distinguished into an acute and a chronic type. Time borders are set distinctly, from 4 to 8 weeks describing an acute event and all exceeding that characterizing a chronic process [11]. Both osteitis types are addressed by surgical intervention in terms of radical debridement, lavage and removal of implants as the gold standard of therapy, combined with systemic or oral antibiotic treatment [12]. Detriments of intravenous or oral antibiotic treatment are systemic side effects of antibiotics as well as potentially low effects and concentrations at the local infection site. Furthermore, impairment of local vascularity can decrease the effects of oral or parenteral antibiotic administration [13]. Application of local antibiotics can result in an increase of concentrations at the infection site. Moreover, trauma associated fractures or osteitis itself can often leave large bone defects that do not have self-healing potential and lead to even pronounced infect reactions often requiring stabilization by a bone void filler [14]. The use of autologous bone grafts is often subordinate, first in order to avoid donor site morbidity in general, secondary because most of these accrued defects are big in size leaving this option ineligible [15]. Respectively, the use of bone graft substitutes presents a reasonable option. Properties like biocompatibility, biodegradability as well a positive effect on osteoconduction and osteoinduction have high importance as they allow "one-stage" operative procedures and impel bone healing. Cerament (BoneSupport AG, Lund, Sweden) is a synthetic, bioresorbable bone substitute, composed of 60% calcium sulfate and 40% hydroxyapatite. Hereby, the calcium sulfate is intended to be quickly replaced by newly formed bone and the hydroxyapatite is supposed to act as a template to allow further bone ingrowth [16]. CAS/HA biocomposites are often used as bone graft in delayed- and non-unions after fractures [15]. Besides, several clinical studies have demonstrated the efficacy of antibiotic infused CAS/HA with gentamicin sulfate or vancomycin in the treatment of infected bone defects as well as the successful use as a coating on implants [16–19]. McNally et al. for example, present the effective treatment of chronic osteomyelitis with gentamicin loaded CAS/ HA spacer in patients [19]. Limitations of these studies seem to be that they either describe a primary infect

prevention or the treatment of a chronic osteomyelitis and results from these studies in general show a specific risk of bias [20]. Moreover, molecular analysis of bone healing, infection progress and immune response during treatment with CAS/HA has not been fully dissected in these studies. A recent animal study suggested an increased bone formation as well as a decreased rate of detectable infection using CAS/HA impregnated with gentamicin in a rat model of osteomyelitis [21]. This study analyzes the effect of CAS/HA in a plain bone defect model with no critical defect as well as no implanted device. Both criteria, namely the existence of a critical size defect (CSD) as well as the association with an implanted orthopedic device present the most challenging aspects of current septic surgery. Especially the presence of foreign surfaces like implants or prosthesis significantly increases the risk for the development of an infection [22]. Related to that, the implant type as well as the size of the bone defect might additionally influence bone healing or inflammatory processes during osteitis.

Therefore, the aim of this study was to evaluate for the first time the effect of antibiotic impregnated CAS/HA insets on fracture healing, inflammatory processes and local as well as systemic immune response in an implant-associated osteitis caused by SA in a standardized murine femur fracture model with a CSD.

Material and methods

Ethical statement

The present animal experiments were approved by the local institutional committee on animal care ("Landesamt für Naturschutz, Umwelt und Verbraucherschutz" of the federal state of North Rhine-Westphalia, Germany—file number: 84–02.04. 2014.A396) and are in line with the European Communities Council Directive (86/609/EEC). Specific effort was made to minimize the number of animals. Reporting of the results of the present study adheres to the "Animals in Research: Reporting in vivo Experiments -criteria" (ARRIVE criteria).

Animals

72 female wild-type BALB/c-mice were used for the study. The age ranged between 10 to 12 weeks with an average weight of 21 g. Mice were kept in the local animal research institution (animal facility of the Heinrich-Heine-University Düsseldorf, Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben, ZETT, Germany) in standard polycarbonate (makrolon type II) cages under a conventional 12 h light–dark cycle (7:00 a.m. / p.m.). Mice had free access to food and water. All animal procedures were carried out in accordance to local and national ethical guidelines. Special training in animal care and handling was provided for the research staff.

Before primary surgery or any lavage as well as before euthanasia, mice were anesthetized by i.p. injection of xylazine (5 mg / kg body weight) and ketamine (100 mg / kg body weight). Besides, after any invasive procedure, a single shot injection of meloxicam (5 mg / kg body weight) was administered.

Additionally, for all mice, a single shot injection of meloxicam (5 mg / kg body weight) was administered every day for the first 5 days after primary surgery to minimize suffering and distress. During the study, animal health and behavior were monitored and assessed every day.

Human endpoints to terminate the experiment were predetermined as: signs of pain for more than three days (non- weight bearing of the operated extremity, no sticking to the cage, no free running and/or moving in the cage), food refusal and weight loss > 20%, unsuccessful fracture stabilization or refracture (axial deviation of the extremity, instability), surgically not controllable wound healing disorders or defects, rectal prolapse, permanently ruffled and unkempt fur, abnormal reaction to a stimulus, permanently closed eyelids, automutilation and

immobility with inability of food consumption or fluid intake. Impartial of these endpoints, the experiment was supposed to be discontinued and the concerned animal appropriately euthanized if indicated by reasons of animal protection and welfare.

Once animals reached endpoint criteria, the experiment was immediately disrupted and affected animals were first anesthetized and subsequently euthanized.

Implant-associated osteitis model

A well-established implant-associated osteitis model in mice was used [23]. Briefly, mice were anesthetized and among sterile conditions a skin incision of 2 cm along the left lateral thigh was done and the fascia as well as the muscles were dissected to expose the femur. Subsequently, a 6-hole titanium locking plate with locking self-tapping micro-screws (MouseFix plate, RISystem, Davos, Switzerland) was applied to the femur. After plate fixation, an osteotomy using a Gigly saw (diam. 0.22 mm) was performed in midshaft of the femur to create a bone defect in terms of a CSD. Mice allocated to an osteitis group, infection was induced by inoculation of the fracture gap with 1μl of SA solution (strain ATCC 29213, averaged 1.35 x 10^5 colony forming units). After primary operation, mice of all groups were re-anaesthetized 7 and 42 days after primary surgery and a standardized lavage with phosphate buffered saline (250 μl PBS twice) and debridement of infected tissue was performed. Local debridement was implemented with a surgical spoon without involving the periosteum. The lavage fluid was recovered, and PBS added to a total volume of 1 ml. The lavage fluid was further analyzed for the number of SA colony-forming units (CFU), PMN and Interleukin-6 (IL– 6). Parallel to the surgical lavage, blood serum was obtained from the mouse cauda for further analysis of serum bone healing markers: alkaline phosphatase (AP) and amino-terminal propeptide of type I collagen (PINP). On day 42, mice were euthanized by cervical dislocation, blood was gained by cardiac punction and a final lavage was obtained. The timescale of 42 days was determined, as complete bone consolidation was assumed at that time.

Experimental groups

Mice were allocated to six experimental groups. All mice obtained a lavage and a blood extraction on day 7 and on day 42 before euthanasia. In each group an osteotomy and plate osteosynthesis of the femur was performed. Following groups were formed: Two control groups (12 mice / group), one with an isolated CSD and one with a CSD as well as a CAS/HA spacer without antibiotic augmentation. The other four groups provided the osteitis groups, one with an isolated inoculation of the fracture gap with SA, the other three groups with an inoculation of the fracture gap and additional implantation of CAS/HA, CAS/HA-G or CAS/HA-V insets.

Calcium sulfate/hydroxyapatite insets

Cerament (Bonesupport Holding, AB, Lund Sweden) is a synthetic, calcium-based bone substitute consisting of 60% calcium sulfate and 40% hydroxyapatite. CAS/HA insets were utilized to assess bone healing as well as immune reactions with regards to the CSD. The insets were generated by using a cylindric template measuring 2x2 mm creating a volume of 0,006283 ml. Either CAS/HA bone void filler itself (CAS/HA) or infused with gentamicin (CAS/HA-G, 0,110 mg gentamycin per cylinder, 17, 5 mg/ml gentamicin) or vancomycin (CAS/HA-V, 0,415 mg vancomycin per cylinder, 66 mg/ml vancomycin) was prepared according to the manufacturers guidelines to generate analogues insets.

Experimental setup and measured data

Fracture healing was examined by radiographic analysis on day 0, 7 and 42. Progress of wound infection was assessed by the counts of SA in the lavage on day 7 and 42. The local immune response was characterized by measuring the quantification of PMN and IL-6-levels in the lavage. The serum AP and PINP concentrations were evaluated on day 7 and 42.

Counts of colony-forming units (CFU)

The number of CFU was determined from the lavage on day 7 and 42. 200 μl lavage was serially diluted in PBS and four replicates of 10 μl of each dilution plated on Columbia Agar plates with 5% sheep blood. The plates were hereafter incubated at 37˚C and counted for Bacterial colonies after 24 h. Results are delineated as CFU per 1 ml lavage fluid.

Determination of polymorphonuclear neutrophils (PMN)

The local immune response was characterized by measuring the PMNs in the lavage using flow cytometry (FACSCanto II; BD Biosciences, Heidelberg, Germany). FITC rat anti-mouse Ly-6G and APC rat anti- mouse CD 11b antibodies (BD Pharmingen, Frankfurt, Germany) were used.

Quantification of Interleukin (IL)-6

IL-6 levels in the lavage samples were specified using a commercially available IL-6 ELISA kit according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The lower detection limit for IL-6 was 15, 6 pg / ml.

Radiographic adjudication

Standard anteroposterior radiographic images (MX20 Faxitron, Tucson, Arizona, USA; 40 kV, 16 mA) of the femora were taken under anesthesia on day 0, 7 and 42. The fracture gap size was measured at the plate opposing cortical bone and was classified by using a modified osteitis score [24]: 1 point was considered a healed fracture gap. Decreasing diameters of fracture gaps in terms of callus formation representing fracture healing were rated with 2 points. A constant fracture gap meaning no healing was rated with 3 points, an increasing facture gap in terms of lysis was rated with 4 points and obvious destruction of the femur with 5 points.

Blood alkaline phosphatase levels (AP)

AP activity was determined in serum. We used an AP Assay Kit measuring the AP activity directly without pretreatment (Abnova, Taipei, Taiwan). This method utilizes p-nitrophenyl phosphate that is hydrolyzed by ALP into a yellow colored product. The rate of the reaction is proportional to the enzyme activity and was measured at a wavelength of 405 nm directly at and 4 minutes after reaction (Victor3, PerkinElmer, Waltham, USA).

Amino-terminal propeptide of type I collagen (PINP)

PINP concentration in serum was measured by an ELISA assay for human N-terminal propeptide of collagen type I (Cloud-Clone Corp., Katy, USA). Manufactures instructions were followed. Intensity of color was read in a microplate reader (Victor3, PerkinElmer, Waltham, USA) and was depicted inversely proportional to the concentration of PINP in the sample. The standard range was 46, 8 pg/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software, San Diego, CA). Data was first tested for normality using D´Agostino and Pearson normality test. Further analysis was regarding the distribution of the data accomplished with either two-tailed t-test, Mann–Whitney-test or Wilcoxon test. P-values ≤ 0.05 were considered significant.

Results

Baseline data

Surgery was performed on 72 female wild-type BALB/c mice in the age range of 10–12 weeks and a weight range of 18–27 g (mean 21 g). The experimental groups were divided into respectively six groups as described before. Overall, 21 mice died during experimental procedures (mortality rate: 29,17%). 10 of these animals died peri- or postoperative (anesthesia, cardiopulmonary instability), 9 of these mice were early euthanized as they met endpoint criteria (3 mice because of immobility and non-weight bearing of the operated extremity, 6 mice because of non-controllable wound defects). 2 mice were found dead in the cage with no evident reason for death. 51 mice were considered for analysis.

Infection progress

Infection progress was verified by estimation of the numbers of CFU gained from the lavage on day 7 and 42. Overall, a significant reduction of the local infection could be detected in all groups on day 42 compared to all groups on day 7 (Fig 1). However, mice with CAS/HA-V revealed a significant reduction of detected CFU compared to the other experimental groups on day 42. (Infect solo vs CAS/HA-V: $p = 0.0004$, CAS/HA vs CAS/HA-V: $p = 0.0007$, CAS/HA-G vs CAS/HA-V: $p = 0.0256$). For clearer depiction, data is presented on an exponential scale and therefore control groups, as not being inoculated with bacteria, cannot be represented in bars.

Inflammatory response

The inflammatory response was analyzed by quantification of the PMN and IL-6 levels in lavage. The infection in corresponding groups induced a significant inflammatory response with an increase of local PMNs on day 7 compared to all control groups ($p = 0.0002$ and $p \leq 0{,}0001$) (Fig 2). Moreover, on day 7, significantly increased PMN levels could be detected in mice with CAS/HA-V compared to merely infected mice ($p = 0.0115$). On day 42, overall a reduction of PMNs in all infectious groups can be detected compared to infectious groups on day 7. Control groups also on day 42 reveal significantly lower concentrations compared to experimental groups. (Infect solo vs CSD control: $p = 0.0311$ and vs CAS/HA control: $p = 0.0021$; CAS/HA vs CSD control: $p = 0.0007$ and vs CAS/HA control: $p = 0.0006$; CAS/HA-G vs CSD control: $p = 0.0002$ and vs CAS/HA control: $p < 0.0001$; CAS/HA-V vs CAS/HA control: $p < 0.0012$).

Also, IL-6 as a marker of systemic infection, shows increased concentrations on day 7 as a response to the local infect constellation compared to day 42 (Fig 3). Additionally, all experimental groups on day 7 revealed significantly higher IL-6 concentrations compared to control groups. ($p = 0.0002$ and $p \leq 0.001$). On day 42, merely infected mice vs mice of the CAS/HA control group as well as mice with CAS/HA vs CAS/HA control showed significantly higher IL-6 values ($p = 0.0156$). Mice with CAS/HA-G vs CSD and CAS/HA control as well as mice with CAS/HA-V vs CSD and CAS/HA control showed a significant increase of IL-6 ($p = 0.0156$ and 0.0078).

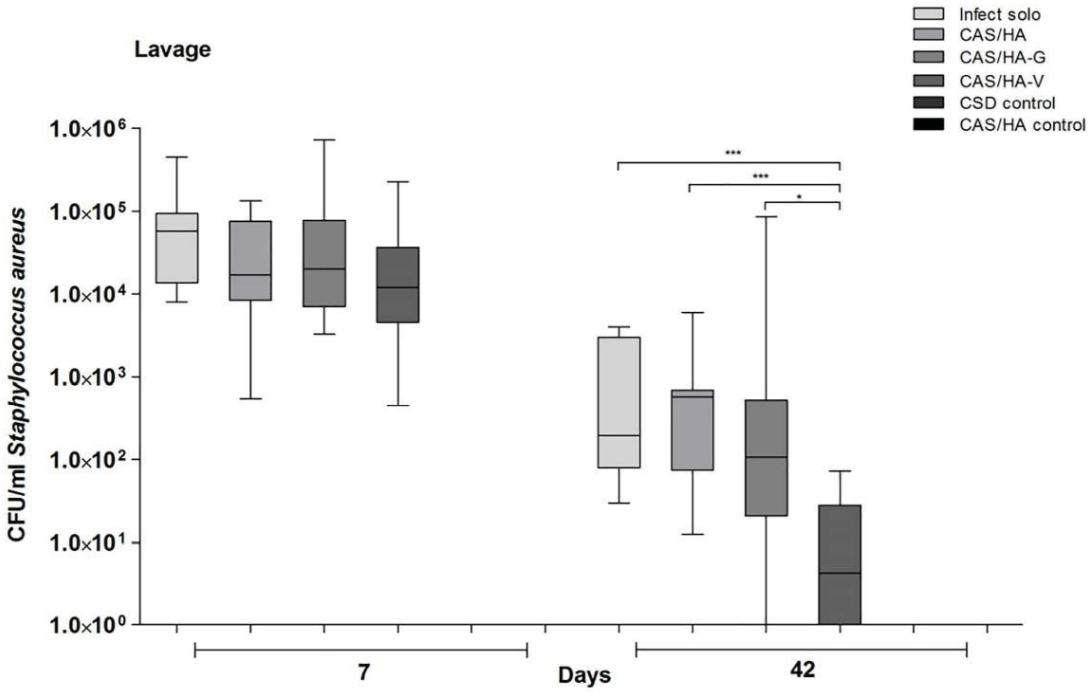

Fig 1. Detection of counts of *Staphylococcus aureus* (SA) around the facture side. Illustration of Colony-forming units (CFU) in the lavage samples obtained on day 7 and 42. On day 42, a significant reduction of CFU in mice with CAS/HA-V could be detected compared to all other experimental groups. * $p< 0.05$, ** $p<0.01$, *** $p<0.001$. Statistical analysis was performed using Mann-Whitney-test.

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Fracture healing

On days 0, 7 and 42 anteroposterior radiographic images of the femora were taken under anesthesia. All mice with infection did not show a healing fracture gap, independently of the application of CAS/HA insets or antibiotics (Fig 4A–4E). Here, significant results could be shown between infected mice as well as infected mice with CAS/HA and infected mice with CAS/HA-V, as the latter group showed a lysis of the bone whereas the other two even presented a destruction. By contrast, in experimental control groups without infection, a sufficient callus formation and fracture healing in most animals could be detected (Fig 4F).

Moreover, fracture healing was evaluated using a modified osteitis score (Fig 5). A higher frequency of nonunion, lysis and destruction could be shown in all infected mice with or without CAS/HA insets. Mice of the control groups showed a fracture healing or callus formation within the observation period of 42 days. This was reflected by mean bone healing scores of 1 (CAS/HA control) and 2 (CSD control), suggesting that CAS/HA supports bone healing in non-infected mice. Mice with infection showed different results. Mice of the groups infect solo, CAS/HA and CAS/HA-G had similar mean values of almost 5 implicating the destruction of the bone. Mice of the CAS/HA-V group suggest that vancomycin infused insets show a less distinct bone destruction, showing a mean score of 4 and therefore an early lysis of the bone.

Analysis of AP concentration in blood serum revealed that AP- synthesis in general seems to be suppressed during acute infection. On day 42, AP- values raise visibly in all groups

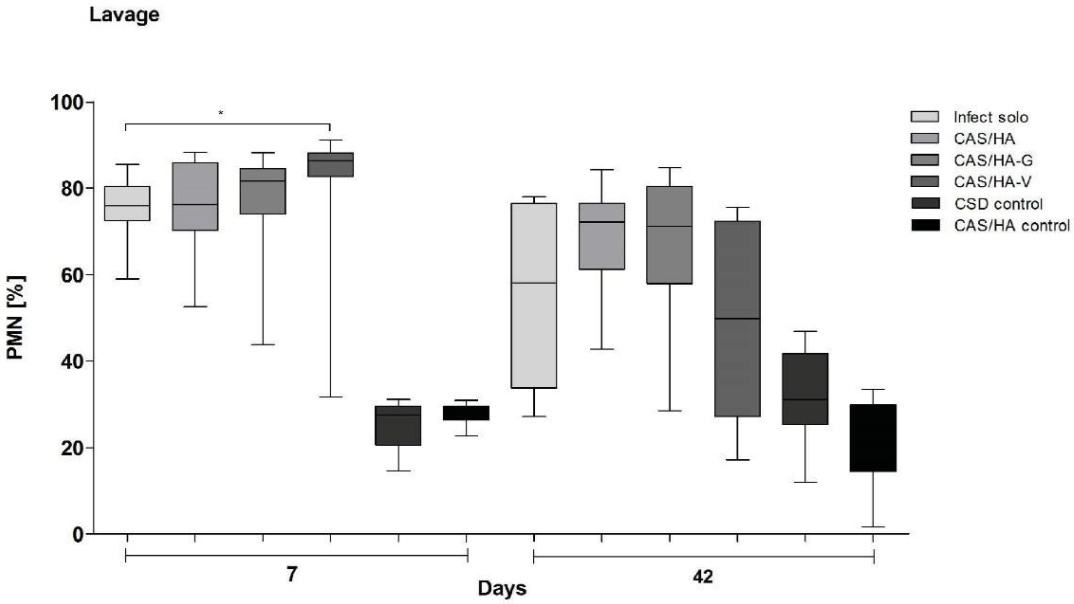

Fig 2. Quantification of polymorphonuclear neutrophils (PMNs). Illustration of PMN- levels in lavage samples on day 7 and 42. Significantly higher counts of PMNs in all experimental groups in comparison to control groups on day 7 and on day 42. On day 7, significantly higher PMNs in mice with CAS/HA-V compared to merely infected mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistical analysis was performed using two- tailed Student's t-test and Mann-Whitney-test.

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compared to AP-values in mice on day 7 (Fig 6). Moreover, on day 7 mice with CAS/HA-V showed significant higher values compared to mice with sole infection ($p = 0.0061$) and mice with CAS/HA ($p = 0.0136$). Besides, on day 42 mice with CAS/HA-V showed significant higher values compared to mice with sole infection ($p = 0.0037$), mice with CAS/HA ($p = 0.0062$) and mice with CAS/HA-G ($p = 0.0037$). Moreover, on day 7 as well as on day 42 all mice with set infection, showed significant results compared to both control groups.

PINP measured in blood serum on day 7 revealed significant higher values in all mice with caused infection compared to both experimental control groups ($p = 0.0002$ and $p \leq 0.0001$). On day 42, infected mice only showed significant higher PINP- values compared to the CSD control group ($p < 0.0001$) as well as mice with CAS/HA-V showed higher values compared to the CAS/HA control ($p = 0.0054$). In addition, on day 42 PINP- concentrations in mice of the CAS/HA group as well as with mice of the CAS/HA-G group showed significantly raised numbers of PINP- concentrations compared to mice with CAS/HA-V ($p = 0.0068$ and 0.0356). Overall, PINP- concentrations in infected mice on day 7 showed significantly raised numbers compared to infected mice on day 42 (Fig 7).

Discussion

Implant-associated infections remain one of the most feared complications in orthopedic and trauma surgery [25]. The current gold standard is radical surgical debridement along with removal of the infected osteosynthesic or prosthetic devices. Besides, initiation of systemic antibiotic therapy as well as application of local antibiotic substances complement the

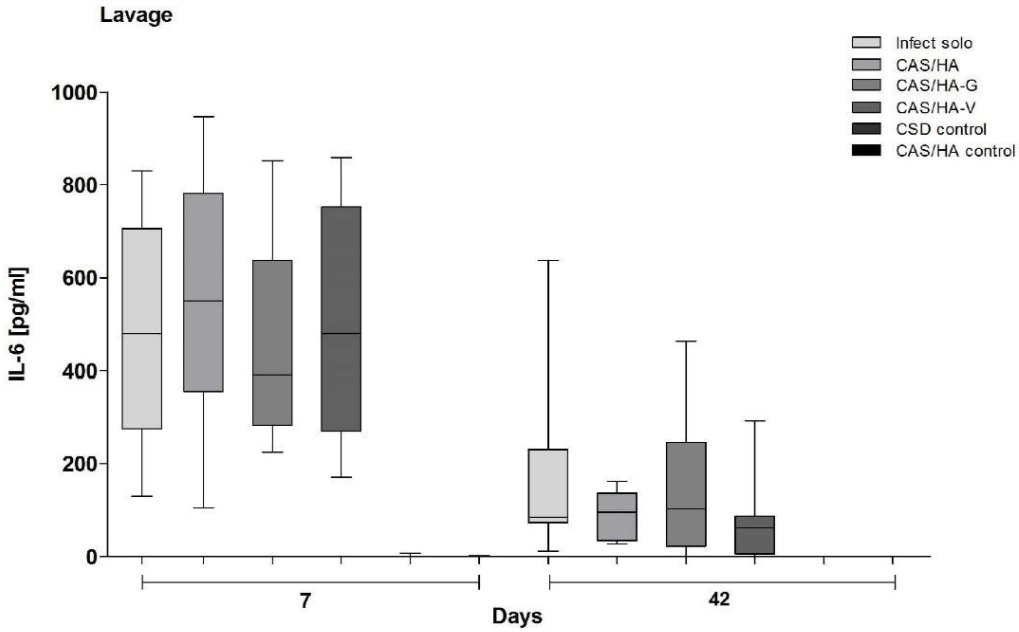

Fig 3. Quantification of Interleukin (IL)– 6 levels. Illustration of IL- 6 levels in lavage samples on day 7 and 42. A reduction of IL-6 levels in all mice groups on day 42 is detectable compared to day 7. All experimental groups on day 7 show significantly higher values of IL-6 compared to control groups ($p = 0.0002$ and $p \leq 0.001$). Also, on day 42 control groups show significantly lower IL-6 levels ($p = 0.0156$ and $p = 0.0078$). Statistical analysis was performed using two tailed Student's t test, Mann Whitney test and Wilcoxon Test.

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common therapy algorithm. In most cases, osteitis can leave a large, critical defect that requires stabilization and prevent the dispersal or recurrence of infection. Respectively, the use and development of calcium-based bone grafts acting as void fillers that feature bone-building qualities such as autografts and also serve as vehicles for antibiotic substances, is a main target in septic surgery. A systematic review by Hake *et al*. presents a wide range of various synthetic calcium-based bone substitutes either acting as bone grafts or as local antibacterial carriers [26]. Antibiotic-loaded substances serve plenty advantages, as high local concentrations of antibiotics are delivered to the infection site, devoid of all systemic detriments of a vascular therapy [27]. Moreover, bone graft substitutes can provide profitable attributes as biodegradability, osteoconduction as well as osteoinduction [20]. CAS/HA biocomposite is an injectable and moldable bone substitute, which is intended to be transformed to host bone within a few months and likewise serves as a scaffold for further bone remodeling. The use of CAS/HA was previously evaluated in studies, either in terms of a bone graft related to mere bone defects but also with respect to its potential as an antibiotic vehicle in bone infections. The aim of this present experimental study was to evaluate the use of antibiotic impregnated CAS/HA in an implant associated osteitis model to assess its effects on fracture healing, infection progress and inflammatory response on molecular level.

Several studies evaluated the use of CAS/HA in bone defects, emerged from comminuted fractures, osteotomies but also from tumorous events [28–31]. In all these cases, CAS/HA was successfully used, reporting a restored function of the patient's extremities and both clinical and radiological satisfying outcomes. However, these studies either dealt with non-infectious,

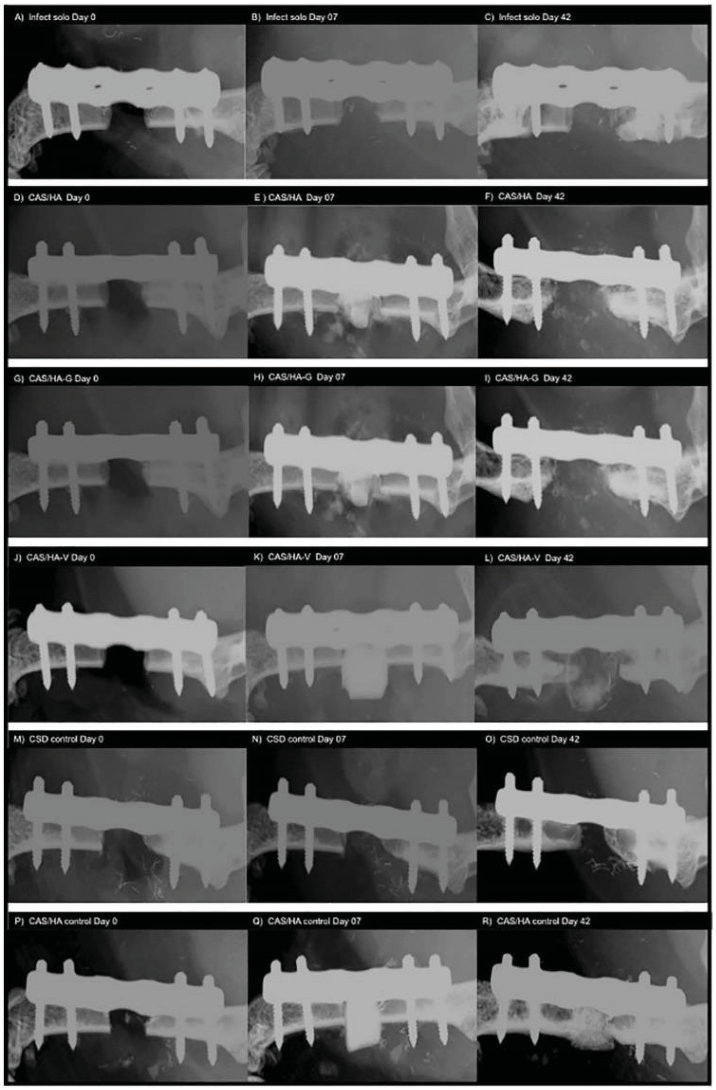

Fig 4. Radiographic analysis of fracture compartment. A-C) X-ray scans of the left femur in a solely infected mouse without implantation of a spacer, allocated to the Infect solo cohort after primary surgery, on day 7 and on day 42. The fracture gap shows evidence of destruction and lysis on day 42. **D-F)** X-ray scans of the left femur in an infected mouse allocated to CAS/HA cohort after primary surgery, after implantation of a CAS/HA inset and on day 42. The fracture gap shows a destruction and lysis on day 42 with a dissolution of the CAS/HA inset. **G-J)** X-ray scans of the left femur of a mouse allocated to the CAS-HA-G cohort after primary surgery, on day 7 and on day 42. A dissolution of the CAS/HA-G inset with destruction and lysis of the fracture gap is shown on day 42, despite the use of gentamycin. **J-L)** X-ray scans of the left femur of a mouse allocated to CAS/HA-V cohort after primary surgery, on day 7 and on day 42. Likewise, a beginning destruction and lysis of the fracture gap can be seen on day 42. In contrast to the other groups, a slighter dissolution of the CAS/HA-V inset as well as minor destruction of the fracture gap is visible. **M-O)** X-ray scans of the left femur of a mouse allocated to the CSD control group without infection of the fracture gap and without implantation of a spacer after primary surgery, on day 7 and on day 42. Tendencies of fracture healing with a decreasing fracture gap are visible on day 42. **P-R)** X-ray scans of the left femur of a mouse allocated to the CAS-HA

control group without infection of the fracture site, after primary surgery, on day 7 and on day 42. A beginning healing of the fracture gap with integration of the CAS/HA inset are seen.

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healthy bone or the presence of infection was one of the exclusion criteria for the study [31]. Moreover, an experimental work by Zampelis and coworkers conclude that CAS/HA as a coating on implants has no detrimental effects on osteointergration [17]. Another experimental thesis revealed the osteoinductive as well as osteoconductive potential of CAS/HA by presenting its induction of bone in skeletal muscle cells [32]. In this in-vitro study, expression of osteoblastic markers was proved as well as distinctly increased AP- activity as a sign for an early onset of mineralization. Compared to our study, in non-infected mice compatible results could be achieved, as in the x- ray analysis a sufficient fracture healing could be detected. Although, with regards to AP-values, an oppression was demonstrated through the set infection, visibly higher AP concentrations could be detected in experimental groups treated with CAS/HA, particularly in those groups treated with CAS/HA- V. Further, several clinical studies support the use of antibiotic-infused CAS/HA in terms of either infect prevention or addressing the treatment of chronic osteomyelitis using it as a vehicle to operate on the local infection site. Most of these works combine the use of CAS/HA with systemic antibiotic treatment and multiple lavages and debridements. Logoluso *et al.* described in a pilot study the use of CAS/HA impregnated with gentamycin and vancomycin in patients with periprosthetic joint infections as a coating of knee and hip prosthesis [16]. The patients taking part in that study, carried infections from multiple different bacteria, whereby in the majority of cases, infection was induced by *Streptococcus gallolyticus* or *Staphylococcus epidermidis*. All patients underwent a first stage procedure, including removal of all osteosynthetic material with a radical debridement of all infected tissues and the following implantation of an antibiotic loaded

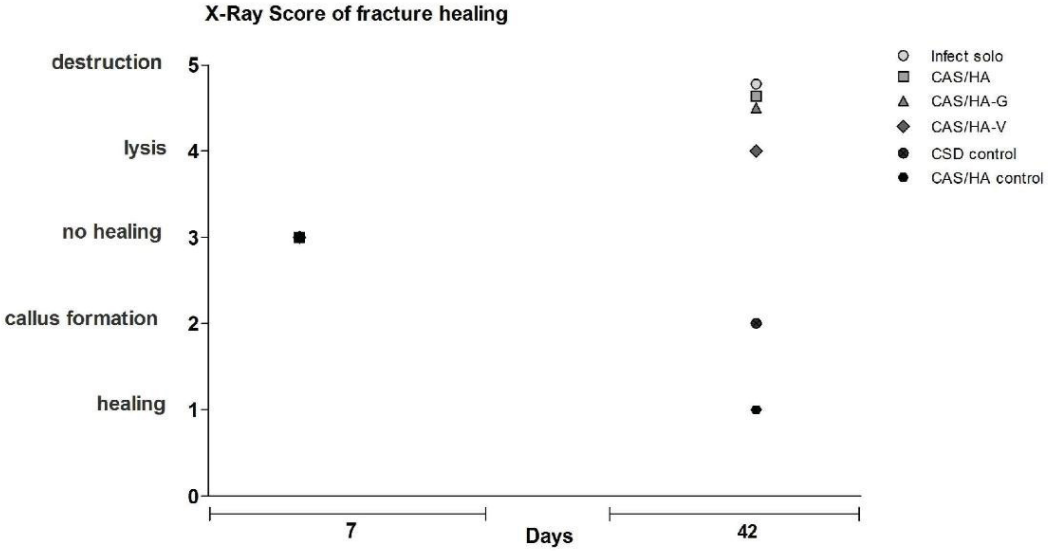

Fig 5. Mean score values of fracture healing. Mean values of the single groups are summarized. A higher frequency of nonunion, lysis and destruction could be shown in all infected mice with or without CAS/HA insets. Bone healing tendencies could only be shown in control groups.

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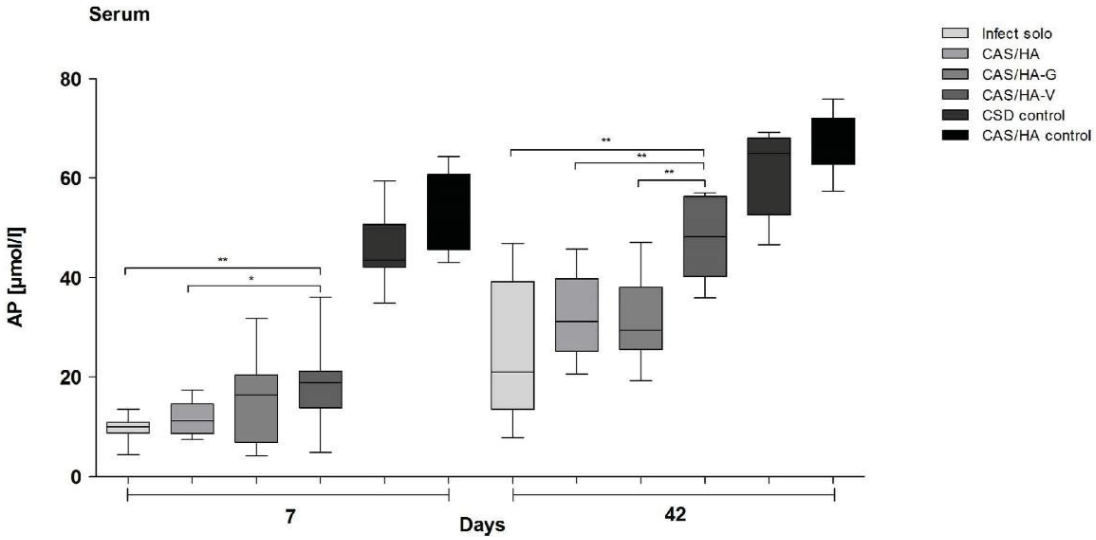

Fig 6. Blood alkaline phosphatase levels. Analysis of alkaline phosphatase (AP) in blood serum revealed significant higher concentrations in all control groups on day 7 and day 42. On day 7, infected mice as well as mice with CAS/HA showed significant lower AP concentrations compared to mice with CAS/HA-V; besides, on day 42, mice with Infect solo, CAS/HA and CAS/HA-G showed significant lower AP concentrations compared to mice with CAS/HA-V * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistical analysis was performed using two- tailed Student's t-test and Mann-Whitney-test.

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spacer. Before implantation of the CAS/HA coated prosthesis, an interval period of 8–12 weeks preceded. Logoluo and collegues described satisfying results with clinical reconvalescence of most patients, postulating that CAS/HA with gentamycin and vancomycin can be used as a coating on implants in septic bone environments. Nonetheless, accessible limitations concerning the further investigation regarding bacterial adhesion and biofilm formation were also shown. McNally and colleagues reported a prospective study about 100 patients with a chronic osteomyelitis, again induced through various germs but mainly by infection through methicillin sensitive SA [19]. All patients were treated with a single-stage protocol, including debridement, removal of implants, stabilization with gentamycin infused CAS/HA, primary skin closure and culture-specific systemic antibiotics for further 6 to 12 weeks. Infect eradication was observed in 96% of the patients at a one year follow up. Another study reports similar results, this time regarding the use of vancomycin impregnated CAS/HA Glombitza *et al.* has reported about a two-stage protocol of a chronic osteomyelitis of the lower limb. Infections were caused by methicillin-resistant SA, multi-resistant *Staphylococcus epidermidis* and polymicrobial, vancomycin-sensitive bacteria [33]. All these clinical studies do not give further insights into molecular processes, comprising different limitations without long-term results. The experimental study by Dvorzhinskiy *et al.* analyzed the effect of gentamycin impregnated CAS/HA in a rat model of osteomyelitis. Osteomyelitis was induced in rats by inoculation of SA into a drill hole. In this work, the author presumes a decreased rate of infection measured by bacterial culture and as a decrease of neutrophil reaction (histology) as well as an increased new bone growth (micro CT-analysis) with the use of CAS/HA with vancomycin compared to the use of mere CAS/HA and omission of bone void filler [21]. Important to mention is that, these results are only said to be valid in debrided and rehabilitated environments. Interestingly the same study also showed that in animals merely treated with CAS/HA, infection rates were

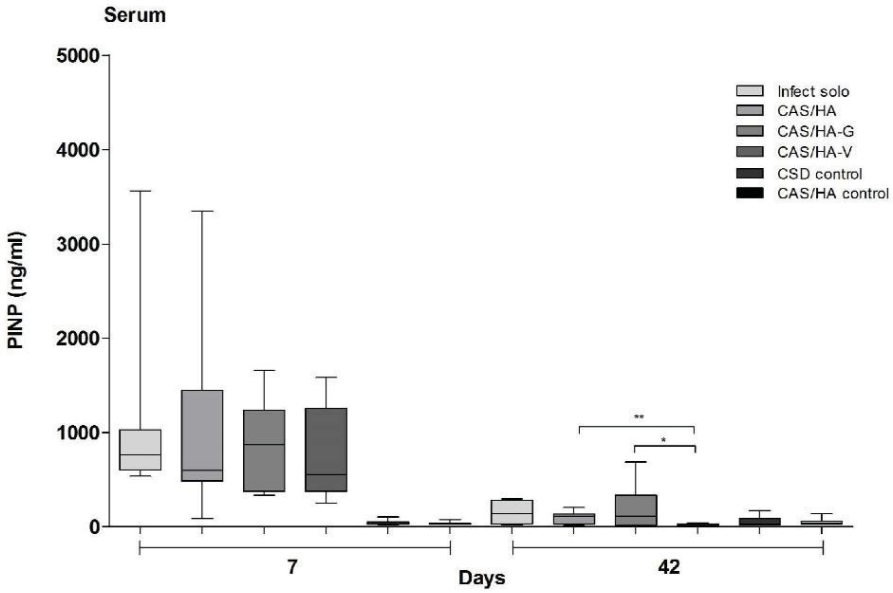

Fig 7. Blood amino-terminal propeptide of type I collagen levels. Analysis of amino-terminal propeptide of type I collagen (PINP) levels in blood samples revealed a significant upregulation of PINP in all experimental groups on day 7 compared to control groups. On day 42, mice with CAS/HA and CAS/HA-G showed significant higher levels of PINP compared to mice with CAS/HA V. * $p< 0.05$, ** $p<0.01$, *** $p<0.001$. Statistical analysis was performed using two-tailed Student's t test and Mann Whitney-test.

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higher compared to the groups with gentamycin impregnated CAS/HA and the groups without any bone void filler. These results seem to be partly compatible with the results of our study. We also declare an infect exacerbation in infected mice merely treated with CAS/HA insets, although in contrast to this, in our study also antibiotic impregnated insets did not show any signs of bone growth or regeneration, at least with regards to radiological analyses. Respectively, fracture healing markers like AP-concentrations overall seemed to be suppressed by infection comparing the values on day 7 with day 42, although in mice treated with CAS/HA higher values could be detected, strongest in the group of mice treated with CAS/HA-V. Regarding CFU, again all in all a significant reduction of the local infection could be detected in all groups on day 42 compared to all groups on day 7. In all groups on day 7 as well as in the majority of groups on day 42 stable CFU values were detected. Significant lower values could only be shown among the separate groups on day 42 compared to the group treated with CAS/HA-V, to remain unclear whether the described effect is caused by the impact of CAS/HA itself or rather is a side effect of debridement and lavage. This extends to inflammatory markers such as IL-6 and PMN- levels. Concerning IL-6, as expected lower concentrations in general could be observed on day 42, whereas among the subgroups on both days stable values were detected. Regarding PMN levels, all experimental groups on day 7 show increased PMN levels when treated with CAS/HA or CAS/HA infused with antibiotics, as well as on day 42, except the group treated with CAS/HA-V. It becomes relevant, that for the CFU, PMN and the IL-6 levels on day 42 reductions could only be detected in groups treated with CAS/HA-V, which therefore in this setting intends to show the best results among all other groups. This is partly

valid regarding AP- levels as well as radiological analyses, underlining that on the overall no healing tendencies were observed at all, but rather attenuated effects of infect exacerbation compared with the other groups.

All in all, these results can only be partly compared as not only different experimental methods were used, but also crucial differences in the experimental settings are present. On the first, Dvorzhinskiy *et al.* analyzed the effect of CAS/HA in a plain bone defect model with no critical defect, whereas our study analyses processes in an implant-associated osteitis model with a CSD. The CSD created in our study seems to provide a reasonable cause to the process of osteitis, as the effects of the set infection are presumed to be enhanced through the major bone defect. Both criteria are eminent and within the most challenging aspects of current septic surgery [22].

Limitations

We acknowledge limitations to our present study. First, our study was supposed to consist of a 12- week mice cohort next to the 6- week cohort. Mice of the 12-week cohort were supposed to be euthanized on day 84 to simulate a long-term process of bone consolidation and thus to evaluate and compare results regarding our experimental project.

For the 12 -week cohort, surgery was performed on 72 female wild-type BALB/c mice in the age range of 10–12 weeks and a weight range of 18–27 g (mean 21 g). Surgery was performed using the implant-associated osteitis model in mice as described earlier [23]. Mice allocated to an osteitis group, infection was induced by inoculation of the fracture gap with 2 μl of SA solution (strain ATCC 29213, averaged 2.55 x 10^5 colony forming units). All following procedures were implemented equivalent to the 6- week mice cohort with the difference of the mentioned bacterial load. Instead of on day 42, in the 12- week mice cohort, on day 84 after primary surgery, mice were euthanized by cervical dislocation, blood was gained by cardiac punction and a final lavage was obtained. Overall, 40 mice died during the experimental procedures (mortality rate of 55,56%). 10 of these animals died peri- or postoperative (anesthesia, cardio-pulmonary instability), 28 of these mice were early euthanized as they met endpoint criteria (3 mice because of immobility and non-weight bearing of the operated extremity, 25 mice because of non-controllable wound defects). 2 mice were found dead in the cage with no evident reason for death.

Obviously, the volume of 2 μl of SA solution was too pronounced and created an overwhelming local infection in mice of the 12-week cohort that constrained premature euthanasia. With regards to the high mortality rate and therefore the small number of animals obtained, results of the 12-week cohort were not stated as representative and could not be analyzed and merely the experimental results of the six-week cohort were considered for analysis.

Secondly, we cannot rule out that CAS/HA might have different reactions or effects regarding other bacteria or poly-microbial infections. In this study, we focused on the most popular pathogen of implant-associated osteitis [34]. Further, the presented model might not necessarily resemble the human condition in every detail, although it shows a significant bacterial load, an impairment of the natural bone healing and an inflammatory reaction, which are important features of the human condition [1]. Finally, we used the term "osteitis" for description of the SA induced bone infection in the present model. A universally accepted definition of the terms "osteomyelitis" and "osteitis" is not yet established [2,3,32,33]. Especially in Anglo-American literature, "osteomyelitis" is the preferred term to describe all kinds of bone infections [2,3]. In contrast, German literature distinguishes both terms "osteitis" and "osteomyelitis" regarding infectious origin and process [11]. Infection progress in osteomyelitis is considered to be caused by hematogenous dissemination of pathogens that firstly affect the bony marrow. By

contrast, in osteitis, pathogens intrude from the outside to the inside e.g. in open fractures or in peri-surgery. In the present study, bacterial infection is induced after osteotomy of the femur. Further, the term "osteomyelitis" refers to infection of the bone marrow itself, whereas the term "osteitis" describes an involvement of the entire bone organ, including the bone cortex [35]. Therefore, the present model should resemble the situation of posttraumatic / post-surgical bacterial infections and as bacterial infection involves the bone marrow, the bone cortex and the surrounding tissue, the term "osteitis" was preferred to be used.

Conclusion

The present osteitis model is sufficient to study fracture healing, infection progress and immune response following an acute implant-associated SA-mediated osteitis in mice. Moreover, this study supports a use of CAS/HA as void filler after infect-convalescence or in non-infectious environments. Application of CAS/HA in acute implant- associated infections, as shown in our model obviously does not lead to limitation of infection and can therefore not be recommended. Nonetheless, the use of CAS/HA in chronic septic environments combined with debridements and if necessary systemic antibiotic therapy, as described in stated studies, could serve as a suggestive tool in trauma and orthopedic surgery.

Supporting information

S1 File. NC3Rs ARRIVE guidelines checklist. A compilation of requirements in reporting in vivo experiments in animals.
(PDF)

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Visualization: Lisa Oezel.

Writing – original draft: Lisa Oezel.

Writing – review & editing: Joachim Windolf.

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ORIGINAL ARTICLE

Plate-associated localized osteitis in mini-pig by biofilm-forming Methicillin-resistant *Staphylococcus aureus* (MRSA): establishment of a novel experimental model

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Abstract

Purpose The increasing number of implant-associated infections during trauma and orthopedic surgery caused by biofilm-forming *Staphylococcus aureus* in combination with an increasing resistance of conventional antibiotics requires new therapeutic strategies. One possibility could be testing for different therapeutic strategies with differently coated plates. Therefore, a clinically realistic model is required. The pig offers the best comparability to the human situation, thus it was chosen for this model. The present study characterizes a novel model of a standardized low-grade acute osteitis with bone defect in the femur in mini-pigs, which is stabilized by a titanium locking plate to enable further studies with various coatings.

Methods A bone defect was performed on the femur of 7 Aachen mini-pigs and infected with Methicillin-resistant *S. aureus* (MRSA ATCC 33592). The defect zone was stabilized with a titanium plate. After 14 days, a plate change, wound debridement and lavage were performed. Finally, after 42 days, the animals were lavaged and debrided again, followed by euthanasia. The fracture healing was evaluated radiologically and histologically.

Results A local osteitis with radiologically visible lysis of the bone could be established. The unchanged high Colony-forming Units (CFU) in lavage, the significant differences in Interleukin (IL)-6 in blood compared to lavage and the lack of increase in Alkaline Phosphates (ALP) in serum over the entire observation period show the constant local infection.

Conclusion The study shows the successful induction of local osteitis with lysis of the bone and the lack of enzymatic activity to mineralize the bone. Therefore, this standardized mini-pig model can be used in further clinical studies, to investigate various coated implants, bone healing, biofilm formation and immune response in implant-associated osteitis.

Keywords Plate-associated osteitis · MRSA · *Staphylococcus aureus* · Mini-pig · Biofilm

Introduction

The increasing incidence of implant-associated infections induced by *Staphylococcus aureus* (SA) in combination with growing resistance to conventional antibiotics requires novel therapeutic strategies. The opportunistic SA can be found in about 30% of all humans in the nasopharynx, throat and intestinal tract [1]. In addition, SA is clinically the most important Staphylococcal species and known for a wide range of infections. Therefore, it is according to the International Consensus Conference of 2018 for Musculoskeletal Infections, not surprising that the main cause of complications in orthopedic surgery is infection with SA. In some regions, Methicillin-resistant SA (MRSA) is involved in over 50% of bone infection cases. Some studies show that the rate of recurrent or persistent infection after

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a two-stage revision is still 33% [2]. For all orthopedic subspecialties, the cost per patient with bone infection is between $61,000 and $150,000 [3]. SA's ability to form a biofilm complicates the treatment of implant-associated infections. This biofilm protects the bacteria by preventing immune cells from entering, phagocytosis and ROS killing [2]. Moreover, the biofilm increases SA's ability to acquire/disseminate plasmid-based antibiotic resistance determinants by horizontal gene transfer [4]. According to recent studies, SA migrates into a network of lacunae and canaliculi, being inaccessible to an immune response as well as an antibiotic therapy, thus surviving for several decades. This explains the high persistence of the disease. The harmful effects of SA in this network is leading to cell death of the osteocytes, which influences the entire bone metabolism and is resulting in chronic osteitis [2, 5]. Therefore, the highest priority is to prevent SA from adhering to implants and, above all, to form a biofilm.

There are numerous studies investigating implant coatings or antibiotic infused calcium sulfate/hydroxyapatite (CAS/HA) insets to prevent osteitis by SA. Silver coatings are the basis of many in vitro and in vivo studies [6–9]. However, Argawal showed that silver < 1 ppm is not able to reduce the infection rate, silver > 1.5 ppm, but can increase the cytotoxicity in the mammalian cell [10, 11]. Furthermore, gentamicin was enriched with the osteoinductive growth factor BMP2 in a rat model [12]. Also, in a rat model the implant was coated with gentamycin in a polylactide carrier matrix [13]. Li investigated vancomycin in a PEG–PLC matrix on rabbits and Diefenbeck used gentamycin coated implants on a plasma chemical oxidized titanium alloy pin in rats [14, 15]. In addition, numerous studies investigated antibiotic-enriched-hydroxyapatite coating or other surface modifications of orthopedic and dental [16–19]. Despite this large spectrum of studies, almost all were only conducted with Methicillin-sensible SA (MSSA) in small animal models. Besides the study by Stewart, who used a plate in sheep as an implant, and Li, who performed the studies with an MRSA in rabbits [20, 21].

In 2014, our working group was able to show that an implant coating with lysostaphin prevents MSSA osteomyelitis in a mouse model [22]. Unfortunately, due to the small size of the femur, it was not possible to change the plates of the mice in an existing infection. Lysostaphin destroys sessile bacteria in a biofilm and can also damage the extracellular biofilm matrix [23]. Moreover, the antibacterial potency of lysostaphin is well documented in animals and humans, even for infections with MRSA [24–26].

The immune system of mice is only 10% identical to the human immune system, whereas that of pigs is up to 80% identical. Furthermore, as pigs react similarly to infections (e.g., SA), they are very suitable for studies on infection and osteitis [27].

Therefore, the aim of this study was to establish a mini-pig model of a low-grade acute implant associated MRSA osteitis, simulating the clinical situation of a one-stage revision with plate replacement.

Materials and methods

Animals and ethics statement

For the induction of osteitis in the establishment group (control group without infection induction not relevant) seven 2-year-old Aachen mini-pigs (5 male, 2 female) with an average weight of 64 kg were used for the study (animal facility of the Heinrich-Heine University Düsseldorf; Zentrale Einrichtung für Tierforschung und wissenschaftliche Tierschutzaufgaben, ZETT, Germany). All animals were kept in separate stalls with an 12 h light/dark cycle. All animal procedures were carried out under local and national ethical guidelines and were approved by the regional ethical committee, Regional Office for Nature, Environment and Consumer Protection Nordrhein-Westfalen, Germany, with the ethical approval ID 84–02.04.2017.A181.

Bacterial inoculum

The biofilm forming MRSA strain ATCC 33592 was cultivated in BactoTryptic Soy Broth overnight and afterwards diluted 1:10. The average inoculation CFU was 10^5.

Low-grade acute osteitis model

All operations were conducted in an aseptic operating room of our local animal facility. 4 weeks after acclimatization, the operations were performed. For premedication we used ketamine 10 mg/kg i.m., azaperone 5 mg/kg i.m., diazepam 10–20 mg i.m., atropine 0.5 mg i.m.. The anesthesia was performed via thiopental 5 mg/kg i.v. and 2% isoflurane/oxygen mixture for the induction of anesthesia. The anesthesia was maintained with 1.3% isoflurane/oxygen mixture, analgesia with buprenorphin 0.3 mg i.v.. The fascia was opened after skin incision in sterile. A standardized bone defect of 2.8 × 5 mm was created in the midshaft of the femur with an LCP twist drill (DePuy Synthes). A 5-hole LCP titanium plate 3.5 (DePuy Synthes) was then modeled onto the bone and infected with 5 × 5 µl with a total average CFU of 1×10^5 (ATCC 33592 MRSA). After the bacteria dried, the plate was implanted laterally on the femur with four locking screws (Stardrive®, 3.5 mm, self-tapping, titanium, DePuy Synthes). The animals received oral analgesic treatment with meloxicam 0.4 mg/kg once a day and 3 × metamizol 20 mg/kg. buprenorphin 0.3 mg i.m. was given for the night. Systemic antibiotic therapy with enrofloxacin 2.5 mg/kg was

performed from the first to the third postoperative day. On day 14, the 5-hole LCP titan plate was removed, a debridement and a lavage were performed and an uninfected 7-hole LCP titan plate with six locking screws (Stardrive®, 3.5 mm, self-tapping, titanium, DePuy Synthes) was implanted. A blood sample was collected. On day 42, the animals were sacrificed (thiopental overdose), a blood sample was gained, and a final sample from the lavage was collected from the surgical field.

Counts of colony-forming units (CFU)

The number of CFU was elevated in the lavage on days 14 and 42. Lavage were collected during debridement as described above. 200 µl of the lavage fluid were serially diluted in Phosphate-Buffered Saline (PBS). Four replicates were made of 10 µl of each dilution planted on Columbia agar plates with 5% sheep blood. The plates were incubated for 24 h at 37 °C. Thereafter, the colonies were counted. The results are expressed in CFU/ml lavage fluid (fourfold approach).

Radiographic and histological analysis

The plate position and bone healing were controlled radiologically on days 0, 14 and 42 with a standard digital X-ray machine. After sacrificing the animals, the femora were harvested and fixed in formalin 4%. Bone fragments were generated at defined sites with an oscillating saw (Trauma Recon System, DePuy Synthes) and decalcified with a neutral EDTA solution for 8 weeks. 10 µm sections of the bone were stained with hematoxylin/eosin (HE).

Analysis of local and systemic immune response

The local and systemic immune response was measured via polymorphonuclear leukocytes (PMN) as percentages of the total number of leucocytes in the lavage and blood samples using flow cytometry (FACSCalibur™; BD Pharmingen, Heidelberg, Germany) with an antibody (FITC Mouse Anti-Pig Monocyte/Granulocyte, BD Pharmingen). The samples were tested in duplicate.

Quantification of IL-6 by ELISA

IL-6 levels in the lavage and blood were analyzed using a commercially available Swine IL-6 ELISA kit according to the manufacturer´s instructions (Thermo Scientific, Invitrogen, Waltham, USA) in a microplate reader (VICTOR X3 Plate Reader, PerkinElmer LAS, Rodgau, Germany). The samples were tested in duplicate. The lower detection limit for IL-6 was 45 pg/ml.

Alkaline phosphatase level (ALP)

ALP activity, a non-specific marker for bone healing, was determined in serum. We used an AP Assay Kit measuring the Alkaline Phosphatase (AP) activity directly without pretreatment (Abnova, Taipei, Taiwan). This method utilizes p-nitrophenyl phosphate that is hydrolyzed by ALP into a yellow-colored product. The rate of the reaction is directly proportional to the enzyme activity and was measured at wavelengths of 405 nm 0 and 4 min after reaction (Victor X3, Plate Reader, PerkinElmer LAS, Rodgau, Germany). Samples were tested in duplicates.

Statistical methods

All data are expressed as median and scatter dots. Data were tested for statistical significance with Mann–Whitney *U* test using GraphPad Prism5 (GraphPad Software, San Diego, CA): p values ≤ 0.05 were considered as significant.

Results

Clinical observations

All seven mini pigs tolerated the technique of the standardized bone defect of 2.8 × 5 mm (Fig. 1). They all returned to normal activity inside the cage and ate on their own. All

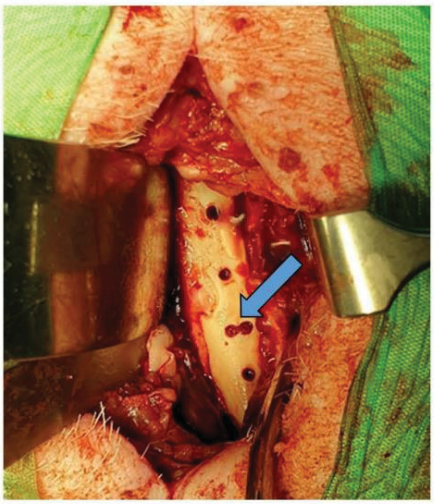

Fig. 1 Bone defect. The blue arrow shows the extended defect zone on day 14 during plate change

animals survived throughout the study period without any plate breakage, fracture or other relevant adverse events and could be euthanized according to the study protocol.

Low grade acute osteitis model

The bacterial load in the wound was determined by CFU on days 14 and 42 with an average inoculation CFU of 10^5 (Fig. 2).

Radiographic and histological analysis

Radiologically detectable lysis around the locking screws and periosteal reactions in all mini-pigs indicate osteitis on day 42. (Fig. 3a, b and c). Histologically illustrated osteitis and lysis of the bone (Fig. 4).

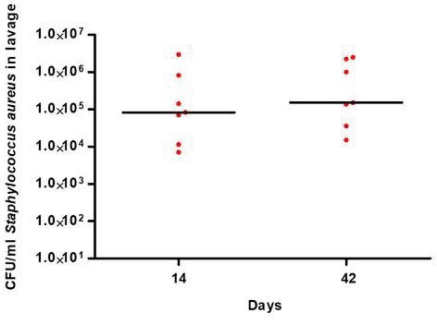

Fig. 2 Bacterial load of *Staphylococcus aureus* in lavages. CFU levels in lavage remained consistently high over the entire observation period. *CFU* colony-forming units

Immune response

Immune response was measured by detection of neutrophil granulocytes (Fig. 5) and IL-6 (Fig. 6) in lavages and serum.

The consistently low ALP values show that no notable increased mineralization of the bone had occurred (Fig. 7). This can underpin the establishment of an osteitis.

Discussion

Osteitis caused by an implant-associated infection remains one of the greatest challenges in musculoskeletal surgery. This study shows the successful induction of local osteitis with lysis of the bone and the lack of enzymatic activity to mineralize the bone. Therefore, this standardized mini-pig model can be used in further clinical studies.

As we already explain in the introduction, there are numerous studies that aim to prevent the colonization of SA in bone from the outset [28–32]. Despite this large spectrum of studies, almost all were only conducted with Methicillin-sensible SA (MSSA) in small animal models. In a standardized osteomyelitis mouse model, our research group was able to show that lysostaphin is a highly effective substance against SA [22, 33]. Lysostaphin, can penetrate biofilm and destroy the embedded bacteria even in a MRSA-caused infection [23, 34, 35].

The further established standardized osteitis mouse model has some limitations. On the one hand we could not perform a plate change in an existing infection, which

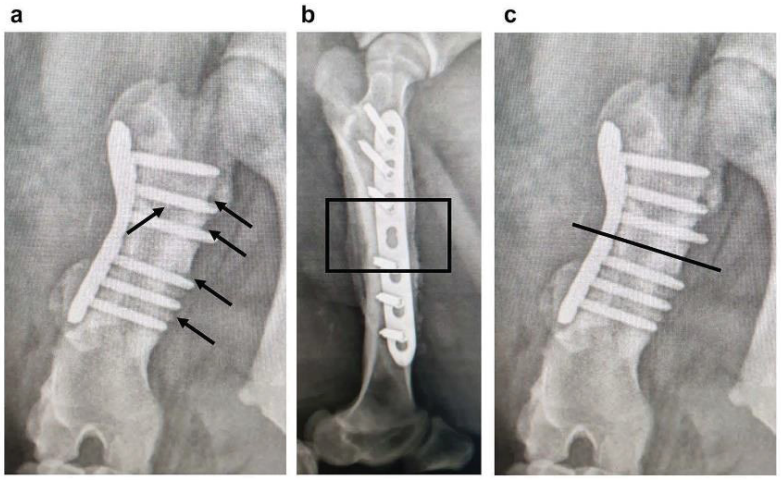

Fig. 3 Radiographic lysis around the locking screws and periosteal reactions indicate osteitis on day 42. Arrows in **a** demonstrate the bone lysis around the screws on day 42. Box **b** and line **c** show the bone section for histology

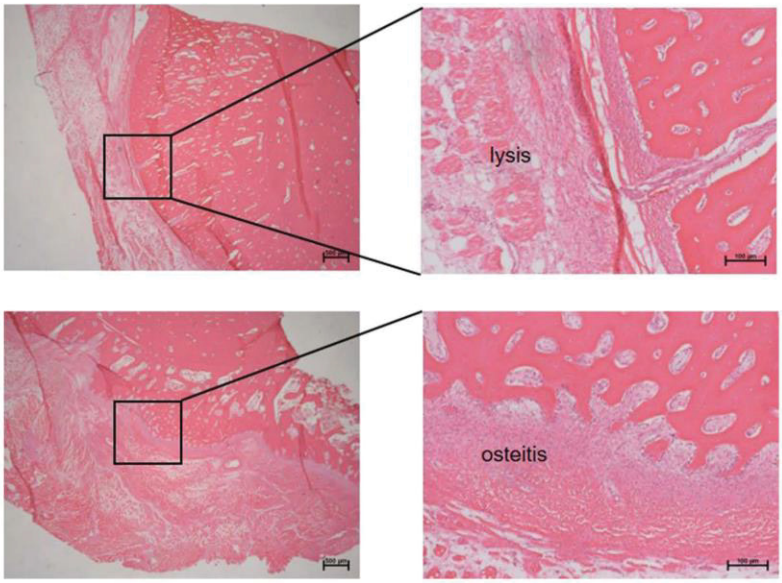

Fig. 4 Histological representation of the bony changes in the presence of osteitis. Two different areas of the same bone section in the overview of periost and cortex (box left images) with the respective magnifications (right images)

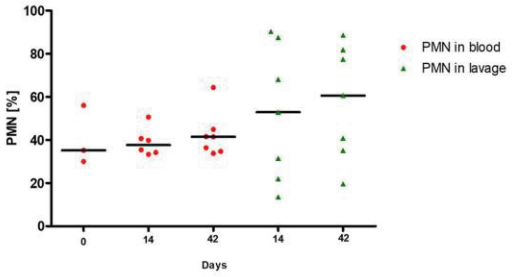

Fig. 5 Neutrophil granulocytes in blood and lavage. There were no significant differences of PMN in blood compared to lavage on days 14 and 42. *PMN*: polymorphonuclear leukocytes

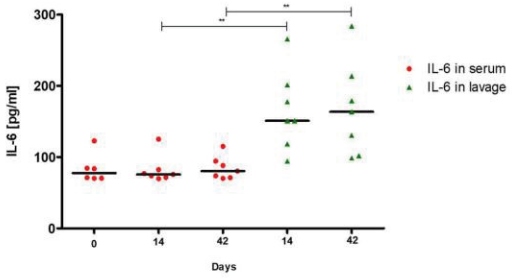

Fig. 6 IL-6 in blood and lavage. IL-6 in lavage was significantly increased both on day 14 ($p=0.0023$) and on day 42 ($p=0.0023$) in comparison to IL-6 in blood. *IL-6:* Interleukin-6

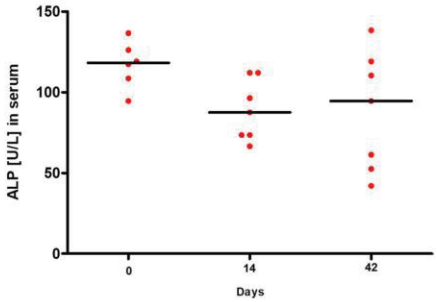

Fig. 7 ALP in blood. ALP shows an overall very low level over the entire period, which does not change at any time. *ALP*: Alkaline Phosphatase

would correspond to the clinical routine, due to the small size of the mice. On the other hand, pigs are 80% immunologically and constitutionally similar to humans and are, therefore, much more suitable for clinical models [27, 36]. Therefore, we have now established this osteitis model in mini-pigs to demonstrate the effectiveness of lysostaphin-coated plates after plate change in an established MRSA infection.

During the experiment, the mini-pigs were treated with systemic antibiotic therapy to avoid a systemic inflammation. Deliberately late we started an antibiotic therapy on the 1st post-op day, to give the bacteria time to develop a biofilm on the implant, which protects them there for

systemic antibiotic therapy. The overall low local and systemic immune response with radiological and histological evidence of osteitis confirms the success of this standardized model so that we can conduct a study with lysostaphin-coated plates in the next step.

This study has some limitations. Due to model establishment no control (negative) group was performed. This approach was chosen for various reasons. The sole aim of this model establishment was to test, whether osteitis can be induced at all in such sensitive animals as pigs. Based on the model that has now been developed, further studies are already being planned, which compare osteitis and bone regeneration in infected as well as in non-infected mini-pigs with and without lysostaphin-coated plates.

Another limitation is the previous evidence of a local and systemic infection. Immunohistochemistral detection of biofilm formation on the orthopedic implants, the detection of bacteria by means of Giemsa staining in the histological sections as well as the CFU in biofilm on plates and the bony material additional to the CFU in lavage would be great approach. Furthermore, a more detailed analyzes of the bone healing by determination of bone-specific ALP (bs-ALP), procollagen peptide (PINP) and computertomography next to the X-ray would be helpful. We want to include these in future studies.

This study shows the successful induction of osteitis with lysis of the bone and the lack of enzymatic activity to mineralize the bone in mini-pigs. Therefore, this standardized mini-pig model can be used in further clinical studies, to investigate various coated implants, bone healing, biofilm formation and immune response in implant-associated osteitis.

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Declarations

Conflict of interest All authors declare that a conflict of interest does not exist.

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Lebenslauf

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2008 bis 2013	Tutoren-Tätigkeit in der AG Medizindidaktik der Heinrich Heine Universität als Tutorin für Sonografie, Farbkodierte-Duplex-Sonografie und Röntgen-Thorax-Diagnostik in studentischen und ärztlichen Kursen

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Dissertation

Die Rolle des 20S Proteasoms in Bezug auf den Abbau pulmonaler Proteine und in der Pathogense des *Acute Respiratory Distress Syndrome (ARDS)*

bei Frau Prof. Dr. Inge Bauer, Klinik für Anästhesiologie – Experimentelle Anästhesiologie

Abschluss: magna cum laude

Sprachen

Englisch	fließend in Wort und Schrift
Latein	großes Latinum

Düsseldorf, 20.04.2022 Dr. med. Carina Jaekel