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Duration of extremity tourniquet application profoundly impacts soft-tissue antibiotic exposure in a rat model of ischemia-reperfusion injury

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ABSTRACT

Introduction: Extremity tourniquet (TNK) application is an effective means of achieving compressible hemorrhage control in the emergency prehospital and clinical trauma setting. Modern United States military medical doctrine recommends TNK use to prevent lethal hemorrhage from extremity injury, followed by systemic prophylactic antibiotics to prevent wound infection. Because tissue pharmacokinetics of prophylactic antimicrobials during and after TNK-induced limb ischemia are largely unknown, this study was conducted to empirically determine the relationship between TNK application time and soft tissue antibiotic exposure in order to guide medical personnel in the management of extremity trauma.

Materials and Methods: Hind limbs of anesthetized male Sprague Dawley rats were exsanguinated, and ischemia maintained by a pneumatic cuff placed at the level of the mid femur on one limb; the non-ischemic contralateral limb served as comparison tissue. Systemic prophylactic antibiotics (cefazolin, moxifloxacin, or ertapenem) were administered intravenously before or after TNK release following 2 or 4 h of ischemia with subsequent re-dosing every 12 h for 3 days. Free antibiotic in the interstitial fluid (ISF) of the tibialis anterior muscle of both hind limbs was recovered via microdialysis during ischemia and over three periods during reperfusion: immediately following TNK release, at 24 h post TNK release, and at 72 h post TNK release. Plasma and ISF free drug concentrations were determined by high-performance liquid chromatography.

Results: Tourniquet application prevented delivery of prophylactic antibiotics into distal soft tissue for the duration of ischemia, and caused a profound reduction in skeletal muscle drug exposure for up to 72 h following TNK release. A progressive decline in tissue antibiotic exposure during reperfusion was observed as TNK times increased from 2 h to 4 h. The timing and severity of reduced drug distribution in post-ischemic skeletal muscle varied substantially among the three antibiotic classes evaluated.

Conclusions: Prolonged tourniquet application can significantly reduce distribution of prophylactic antibiotics into soft tissue during and after ischemia, potentially impairing prophylaxis of extremity wound infection. Our findings support the examination of alternative approaches to wound infection prophylaxis under conditions of delayed casualty evacuation when occlusive hemorrhage control measures are utilized.

Introduction

Extremity tourniquet (TNK) application is an effective and expeditious means of achieving compressible hemorrhage control in the emergency prehospital and clinical trauma setting [1–6], as well as serving as a useful perioperative adjunct to limit blood loss in orthopedic, transplant, and tumor resection surgeries [7]. As an emergency limb isolation measure, extremity tourniquets have an additional role in the prehospital management of severe crush injury [8,9]. Regardless of indication, a persistent concern is the potential for morbidity arising from prolonged skeletal muscle ischemia. Recent evidence indicates that short-term extremity TNK application is generally safe, and significant physiological or functional deficits can be largely prevented by limiting TNK duration where possible [2,10–12]. Rapid transition from prehospital care to definitive surgical intervention is the norm in typical civilian trauma, but prolonged application of occlusive prehospital hemorrhage control devices, i.e. extremity TNK and pressure dressings,

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remains a possibility in mass casualty events, natural disaster scenarios, wilderness medicine, and military casualty care.

From the military perspective, modern United States military medical doctrine recommends tourniquet application to prevent lethal hemorrhage from extremity trauma, followed by systemic prophylactic antibiotics to prevent wound infection [13–18]. Operational conditions in future conflicts may necessitate delayed casualty evacuation requiring lifesaving hemorrhage control measures to be left in place for substantially longer durations than in prior conflicts. Acceptable time limits for TNK ischemia remain under investigation [2,4], and tissue pharmacokinetics of recommended antimicrobials [15,17,18] during and after TNK-induced limb ischemia are largely unknown. This represents a critical gap in our clinical knowledge as effective antibiotic prophylaxis is contingent on rapidly achieving drug levels sufficiently above the minimum inhibitory concentration (MIC) of a given pathogen, with higher peak drug levels or greater total drug exposure (concentration over time) within a tissue typically conferring greater benefit in preventing infection.

It is well established that early entry of antibiotics at sufficient concentration into a wound site is crucial to prevent infection. For example, a recent retrospective clinical study [19] demonstrated that administration of systemic antibiotics immediately after Type 3 open tibia fractures greatly reduces the infection rate in a civilian trauma population. With increasing time from injury to the start of antibiotic therapy, there was a progressive rise in infection occurrence from 7% in patients receiving antibiotics within an hour to 18% with antibiotics within 60 to 90 min, escalating to 28% when antibiotics are given after 90 min. These finding are further supported by animal studies of contaminated orthopedic trauma indicating a delay in antibiotics administration from to 6 to 24 h following injury has a profoundly detrimental effect on infection [20]. Unfortunately, much of what is currently known about the timing of antibiotic administration and infection in the context of extremity tourniquet application has been learned from sterile elective surgeries employing pre-TNK antibiotic dosing and relatively short ischemia durations [7,21,22]. Although these studies are informative, this patient population is likely not relevant to military or emergency civilian casualties with highly-contaminated open wounds who have tourniquets applied for long periods of time prior to antimicrobial prophylaxis.

Previous research has revealed that physiological conditions that adversely impact microvascular perfusion, such as shock or sepsis, can substantially reduce soft-tissue penetration of antibiotics [23,24]. Because a similar microvascular dysfunction has been noted in ischemia-reperfusion injury [25–29], we hypothesized that soft tissue drug levels during reperfusion following prolonged TNK application may be drastically lower than systemic levels if a perfusion deficit is present. In this scenario, delayed or inadequate penetration of antibiotics into a wound site following prolonged ischemia may have the potential to increase the risk of infection, just as delayed initial antibiotic administration favors infection in non-ischemic extremity trauma. Contaminated open wounds are common in military and civilian trauma, and the infective complication rate is extremely high [19,30–32]. In a delayed casualty evacuation scenario in which systemic prophylactic antibiotic administration may occur late in the continuum of care, or may be rendered less effective by presence of ischemia-reperfusion injury resulting from extended application of lifesaving tourniquets, the complication rates may be even higher. This study was designed to empirically determine the temporal relationship between tourniquet-induced ischemia and soft tissue pharmacokinetics of recommended prophylactic antibiotics with the goal of preventing wound infection through the improvement or synthesis of clinical practice guidelines [16] intended to guide medical personnel in management of combat-related extremity trauma.

Materials and methods

Materials

Male Sprague-Dawley rats were obtained from Envigo, Hackensack, NJ. Pneumatic tourniquet cuffs and pressure transducer-equipped inflators were obtained from D.E. Hokanson, Inc. (Bellevue, WA) and Delfi Medical Innovations (Vancouver, BC), respectively. Cefazolin for injection was purchased from Fresenius Kabi USA, Lake Zurich, IL . Moxifloxacin solution (Avelox) and Ertapenem for injection (Invanz) were obtained from Merck &Co, Kenilworth, NJ. Ceftriaxone and reference standards of cefazolin, moxifloxacin, and ertapenem for HPLC analysis were obtained from US Pharmacopeia, Rockville, MD. CMA 402 dual syringe microdialysis pumps were purchased from Harvard Apparatus, Holliston MA. Microdialysis probes (CMA 20 High MW cutoff/10 mm membrane length/100 kD cutoff), T1 perfusion fluid, and other ancillary microdialysis components were obtained from M Dialysis, Stockholm Sweden. Centrifree 30 kD MW cutoff ultrafiltration devices were purchased from Merck Millipore, Cork Ireland. Bioanalysis was performed on a Thermo Ultimate 3000 HPLC system obtained from Thermo Scientific, Waltham, MA with separation on a Phenomenex Luna 5 µm 100 Å 150 mm × 4.6 mm C18 column (Phenomenex, Torrance, CA). HPLC-grade mobile phase solvents were obtained from Fisher Scientific (Hampton, NH) and trifluoroacetic acid was purchased from Sigma-Aldrich, St. Louis, MO.

Rodent tourniquet ischemia model

An established rat (*R. norvegicus*) extremity tourniquet model [33–35] was employed for all in vivo experiments described in this study. Briefly, the hind limbs of anesthetized male Sprague Dawley rats were exsanguinated by elevation, and ischemia maintained by a commercially available pneumatic digit cuff (D.E. Hokanson, Model UDC 1.6) placed at the level of the mid femur. Tourniquets were inflated with a rapid cuff inflator equipped with a pressure transducer (Delfi Medical Innovations, P.T.S. Portable Tourniquet System) to a pressure of 300 mmHg for 2 or 4 h. For all animals, the non-ischemic contralateral hindlimb served as a comparison tissue space. Post-procedural analgesia and fluid support to prevent dehydration was accomplished by subcutaneous injection of sustained-release buprenorphine and 0.9% saline, respectively.

Antibiotic dosing and sample collection

Systemic prophylactic antibiotics (allometrically scaled on body weight from recommended human doses [15,17,18] for infection prevention) were administered via indwelling tail vein catheter, as an IV bolus in 0.9% saline according to the timelines detailed in Supplementary Fig. 1. To examine antibiotic penetration into peripheral soft tissues distal to the TNK application site, cefazolin 35 mg/kg IV was administered 10 min after TNK inflation. Free cefazolin in blood and interstitial space of the tibialis anterior muscle of both hind limbs (ischemic and non-ischemic) was recovered during 4 h TNK application. To examine the immediate and delayed effects of ischemia and reperfusion on soft tissue antibiotic distribution, cefazolin 35 mg/kg, moxifloxacin 15 mg/kg, and ertapenem 35 mg/kg IV were administered in combination 10 min after TNK release following 2 or 4 h of ischemia, with subsequent dosing every 12 h for 3 days. Circulating and local tissue free antibiotic concentrations were measured over three 120 min periods: immediately following TNK release and first drug administration, at 24 h post TNK release, and at 72 h post TNK release.

Muscle interstitial fluid (ISF) antibiotic concentrations were sampled using two microdialysis catheters (CMA 20 High MW cutoff / 10 mm membrane length / 100 kD cutoff, M Dialysis, Stockholm Sweden) implanted bilaterally into the tibialis anterior (TA) muscles prior to antibiotic administration at each sampling time point. Briefly, microdialysis allows the sampling of soluble molecules in tissue via diffusion from the extracellular space across a semipermeable membrane into the central lumen of an implanted microdialysis probe (catheter) perfused with an isotonic solution [36]. Analyte concentrations in the dialysate (outflow) recovered from the catheter are subsequently measured using standard analytical techniques. This method allows repeated samples to be taken from a precise tissue location at defined time points over the course of several hours in a minimally invasive manner. Microdialysis has an extensive history of use in mouse, rat, and human subjects for assaying both endogenous (e.g., neurotransmitters, cytokines) and exogenous (e.g., pharmaceutical) analytes in-vivo in peripheral tissues as well as the central nervous system [37–39]. By collecting microdialysate samples from both hind limbs in this model, ISF antibiotic concentrations can be assayed simultaneously in matched tissue locations representing both normal (non-ischemic) and experimental (tourniquet application) drug diffusion states. It should be noted that the high molecular weight cutoff (100 kD) microdialysis probes used for this analysis were chosen specifically to maximize free drug recovery while still effectively excluding the protein-bound drug fraction [40,41].

Microdialysis samples were taken at 10 min intervals, starting at the time of IV bolus antibiotic administration, and continuing for the following 120–240 min as described above. Probes were perfused with isotonic T1 perfusion fluid (M Dialysis, Stockholm Sweden) using CMA 402 dual syringe microdialysis pumps at a flow rate of 1.5 µL/min. Microdialysis recovery efficiency (largely influenced by tissue hemodynamic status and local capillary disruption) was determined for each individual probe (Supplementary Fig. 2) using ceftriaxone as an in vivo retrodialysis calibrator agent as previously published [42,43]. All reported ISF antibiotic concentrations were adjusted based on the measured recovery efficiency of each probe. Equivalent dialysance of ceftriaxone with cefazolin, moxifloxacin, and ertapenem at the perfusion fluid flow rate used in these experiments (1.5 µL/min) was previously verified in vitro (data not shown).

To study the relationship between systemic and local tissue antibiotic concentrations over time, serial 200 µL blood samples were collected via the tail vein catheter over a 2 h period following drug administration. Plasma was separated via centrifugation (1000 RCF for 10 min) and free (unbound) drug was recovered by ultrafiltration of plasma using Centrifree 30 kD MW cutoff UF filters (Merck Millipore, Cork Ireland) centrifuged at 1500 RCF for 30 min. Recovered samples were stored at −80 C if immediate HPLC analysis is not possible due to time or instrument availability constraints.

HPLC analysis

Free antibiotic levels were quantified in microdialysate and plasma ultrafiltrate samples using high performance liquid chromatography on a Thermo Scientific Ultimate 3000 system with separation on a Phenomenex Luna 5 µm 100 Å 150 mm × 4.6 mm C18 stationary phase. Mobile phase consisted of acetonitrile: 0.1% TFA in water at 5:95 (0–1 min), 30:70 (1–13 min), 80:20 (13–20 min), 5:95 (20–24 min) at a flow rate of 1.0 mL/min. Detection was accomplished by monitoring UV absorbance for ceftriaxone (261 nm) and cefazolin (267 nm) and fluorescence (Ex 295 nm, Em 450 nm) for moxifloxacin and ertapenem. Analyte concentrations were interpolated from external calibration curves (5 points, 0.1 µg/mL to 500 µg/mL, prepared from USP reference standards) with $1/x^2$ weighting.

Data analysis

Pharmacokinetic parameters were estimated by noncompartmental analysis from time-concentration curves of unbound antibiotic measured in plasma and ISF. Dedicated pharmacokinetic software was used (Phoenix WinNonLin v8.1, Certara USA, Inc., Princeton, NJ), and first order elimination was assumed. Differences in tissue antibiotic exposure, expressed as area under the time-concentration curve ($AUC_{0\rightarrow 120\,min}$, min*µg/mL) and peak concentration (C_{max}, µg/mL), between the experimental (TNK) and non-ischemic contralateral limb at each sampling point (0, 24, and 72 h post-TNK application) were analyzed by Wilcoxon signed rank test with Bonferroni correction at alpha = 0.05 using GraphPad Prism v7.03 (GraphPad Software, San Diego, CA). Differences in non-ischemic contralateral limb drug exposure across 0, 24, and 72 h reperfusion time sampling points were assessed for each antibiotic using the Kruskal–Wallis test at alpha = 0.05. For these comparisons, nonparametric statistical tests were utilized due to the relatively small sample sizes of each group ($N = 4$–5 animals per TNK application duration). Data are presented as mean with standard error of the mean unless otherwise noted. Tissue antibiotic concentrations are expressed as area under the concentration-time curve (AUC) and peak interstitial fluid concentration (ISF C_{max}), in the experimental limb (TNK) versus the levels at the corresponding location in the contralateral (non-ischemic) limb at each sampling time point.

Results

Experimental overview

Ischemia was induced and maintained in the hind limbs of anesthetized male Sprague Dawley rats by a pneumatic cuff placed at the level of the mid-femur. To examine antibiotic penetration into peripheral soft tissues distal to the TNK application site, cefazolin 35 mg/kg IV was administered 10 min after TNK inflation. Free cefazolin in blood and interstitial space of the tibialis anterior muscle of both hind limbs (ischemic and non-ischemic) was recovered via microdialysis for the duration of TNK application (4 h total). To examine the immediate and delayed effects of ischemia-reperfusion injury on soft tissue antibiotic distribution, cefazolin 35 mg/kg, moxifloxacin 15 mg/kg, and ertapenem 35 mg/kg IV were administered in combination 10 min after TNK release following either 2 or 4 h of ischemia. Drugs were re-dosed every 12 h for the following 72 h. Circulating and local tissue ISF free antibiotic concentrations were measured by microdialysis over three separate 120 min periods: immediately following TNK release and first drug administration, at 24 h post TNK release, and at 72 h post TNK release. In all experiments, non-ischemic contralateral limbs served as controls. Plasma and ISF pharmacokinetic parameter estimates for each antibiotic (free fraction) are detailed in Table 1.

Antibiotic soft-tissue distribution during ischemia

Tourniquet application prevented distribution of systemic prophylactic antibiotic into distal soft tissue for the duration of ischemia. Specifically, throughout 4 h TNK application, cefazolin was detectable in the contralateral limb (ISF C_{max} 48.39 ± 4.25 µg/mL at 7.5 min post drug administration), but not the ischemic limb (Fig. 1). Relative soft tissue penetration (AUC_{ISF}/AUC_{plasma}) of cefazolin in the contralateral, non-ischemic limb was 0.29 (0.33 $Cmax_{ISF}/Cmax_{plasma}$).

Table 1
Summary of free antibiotic pharmacokinetic parameter estimates.

Pharmacokinetic Parameter	Cefazolin	Moxifloxacin	Ertapenem
Plasma			
C_{max} (μg/mL) - Observed	155 ± 12	63 ± 6.5	135 ± 17
C_0 (μg/mL) - Extrapolated	304 ± 45	201 ± 43	491 ± 149
$AUC_{0\text{-}120\,min}$ (min*μg/mL)	8716 ± 231	3532 ± 295	7498 ± 1069
Terminal $t_{1/2}$ (min)	48 ± 10	33 ± 3.6	22 ± 2.4
CL (mL/min/kg)	3.4 ± 0.2	4.1 ± 0.4	5 ± 0.7
V_d (mL/kg)	220 ± 37	207 ± 43	160 ± 37
Interstitial Fluid (ISF)			
C_{max} (μg/mL) Non-ischemic Limb*	51 ± 5.9	10 ± 1.7	18 ± 3.2
$AUC_{0\text{-}120\,min}$ (min*μg/mL) Non-ischemic Limb*	2511 ± 368	589 ± 85	782 ± 121

Abbreviations: peak concentration (C_{max}), concentration at time zero (C_0), area under the concentration-time curve from 0 to 120 min ($AUC_{0\text{-}120\,min}$). half-life ($t_{1/2}$), clearance (CL), volume of distribution (V_d).
Values presented as mean ± SEM; $N = 9$ animals.
* Represents mean of interstitial fluid values in non-ischemic limbs from all collections (no significant difference in non-ischemic limb ISF values among reperfusion time points or between TNK application durations).

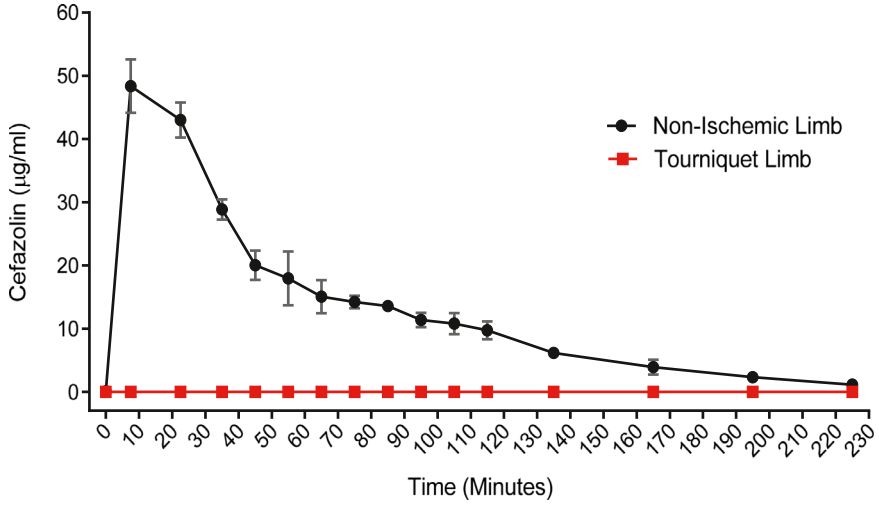

Fig. 1. Free cefazolin in interstitial space of rat tibialis anterior muscles following IV bolus. A pneumatic tourniquet placed at the level of the mid-femur on the touniquet limb was inflated 10 min prior to administration of cefazolin, 35 mg/kg IV, at 0 min. $N = 4$ animals.

Antibiotic soft-tissue distribution during reperfusion

Tourniquet application for a period of 2 h caused a significant reduction in soft tissue ISF drug penetration during the reperfusion phase immediately following restoration of arterial blood flow (Figs. 2–4A, 5A, 6A). Increasing TNK duration from 2 h to 4 h caused a pronounced decline in soft tissue antibiotic exposure during reperfusion (Fig. 2–4B), comprising both a profound decrease in peak drug levels achieved during the period immediately following TNK release as well as a substantial increase in the time required for soft tissue drug penetration in the post-ischemic limb to recover to contralateral limb levels (up to 72 h) (Figs. 5B and 6B). The timing and severity of reduced drug distribution in post-ischemic skeletal muscle varied considerably among the three antibiotic classes evaluated. In the contralateral (non-ischemic limb), mean relative soft tissue penetration (AUC_{ISF}/AUC_{plasma}) was 0.29 for cefazolin, 0.17 for moxifloxacin, and 0.10 for ertapenem ($Cmax_{ISF}/Cmax_{plasma}$ 0.33 for cefazolin, 0.16 for moxifloxacin, and 0.13 for ertapenem).

As shown in Fig. 5A, during the first 120 min of reperfusion following 2 h TNK application, cefazolin exposure in ischemic limbs was 68% ± 8% of contralateral (mean $AUC_{0->120\,min}$ TNK 1507 vs control 2327 min*μg/mL, $p = 0.012$) after IV administration. By 24 and 72 h after TNK release, cefazolin exposure in ischemic limbs was not significantly different from contralateral. Moxifloxacin exposure in ischemic limbs following 2 h TNK application was 44% ± 6% of contralateral (mean $AUC_{0->120\,min}$ TNK 287 vs control 609 min*μg/mL, $p = 0.012$) during the first 120 min of reperfusion, but was not significantly different from contralateral by 24 h post TNK application. Similarly, ertapenem exposure in ischemic limbs immediately following release of 2hr TNK reached 46% ± 7% of control (mean $AUC_{0->120\,min}$ TNK 478 vs control 852 min*μg/mL, $p = 0.016$), but was not significantly different from contralateral by 24 h post TNK release.

In contrast (Fig. 5B), during the first 120 min of reperfusion following 4 h TNK application, cefazolin exposure in ischemic limbs was 12% ± 2% of contralateral levels (mean $AUC_{0->120\,min}$ TNK 282 vs control 2355 min*μg/mL, $p = 0.004$). 24 h after TNK release, cefazolin exposure in ischemic limbs reached 47% ± 17% of contralateral (mean $AUC_{0->120\,min}$ TNK 1167 vs control 2896 min*μg/mL, $p = 0.015$) and by 72 post TNK release was not significantly different from the contralateral limb. Moxifloxacin exposure in ischemic limbs was 24% ± 7% of contralateral (mean $AUC_{0->120\,min}$ TNK 101 vs contralateral 473 min*μg/mL, $p = 0.011$) immediately following 4 h TNK application. 24 h after TNK release, moxifloxacin exposure in ischemic limbs reached 16% ± 4% of contralateral (mean $AUC_{0->120\,min}$ TNK 107 vs contralateral 705 min*μg/mL, $p = 0.002$), recovering to 45% ± 7% of contralateral levels by 72 h after reperfusion (mean $AUC_{0->120\,min}$ TNK 219 vs contralateral 491 min*μg/mL, $p = 0.005$). Ertapenem exposure in ischemic limbs

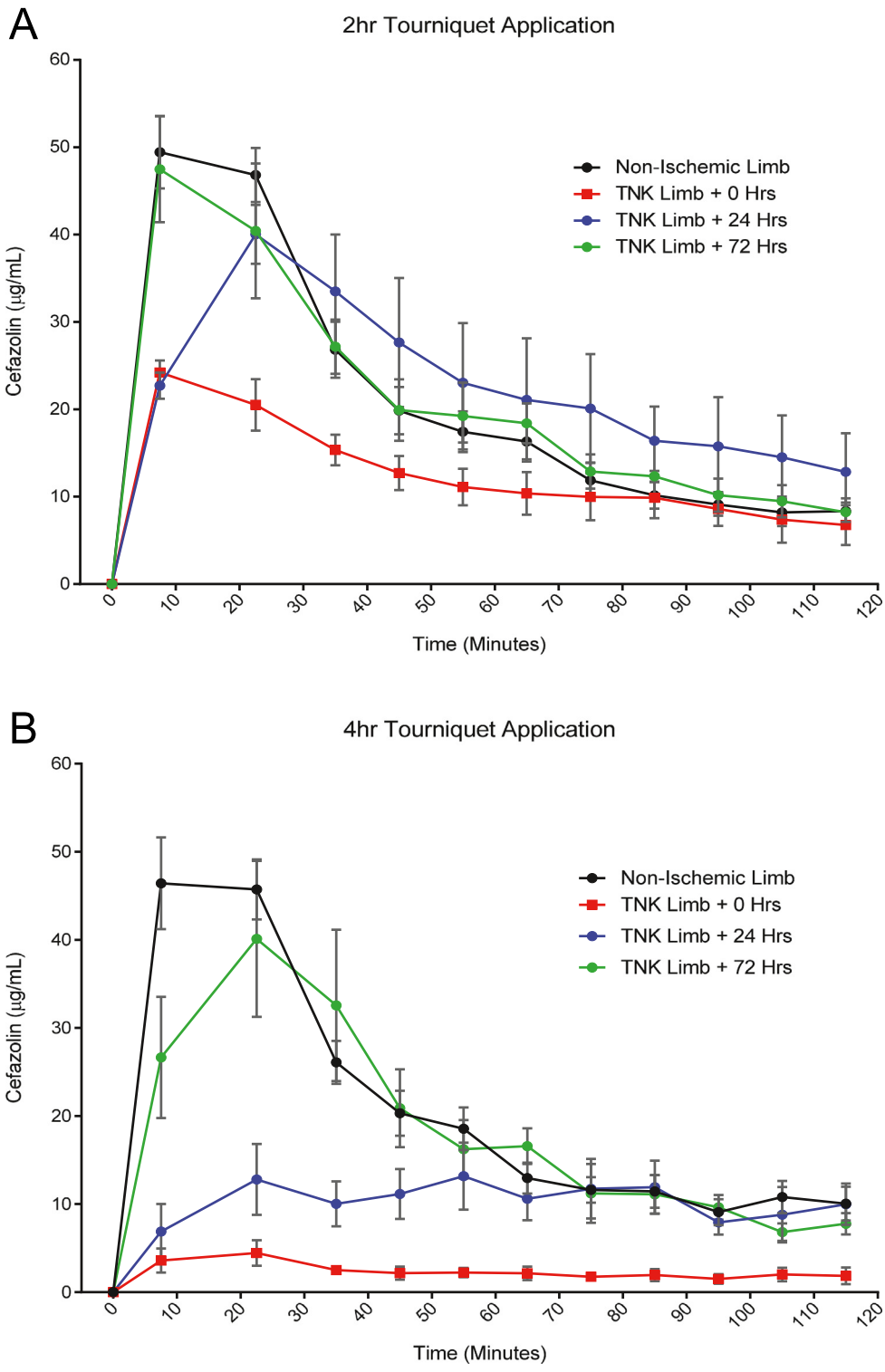

Fig. 2. Free cefazolin in the interstitial space of rat tibialis anterior muscles during 72 h reperfusion period following 2-h (A) or 4-h (B) tourniquet (TNK) application. Pneumatic tourniquet placed at the level of the mid-femur on the TNK limb was released 10 min prior to administration of first cefazolin dose, 35 mg/kg IV, at 0 min. Antibiotic was subsequently redosed every 12 h for the following 72 h. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from all relevant sampling courses for each TNK duration. $N = 4–5$ animals per TNK application duration.

immediately following 4 h TNK was 17% ± 1% of control (mean $AUC_{0->120\,min}$ TNK 132 vs contralateral 788 min*µg/mL, $p = 0.010$), falling to 15% ± 9% of control by 24 h post TNK release (mean $AUC_{0->120\,min}$ TNK 127 vs contralateral 883 min *µg/mL, $p = 0.016$). 72 h after TNK release (4 h ischemia), ertapenem exposure in ischemic limbs was not significantly different from contralateral tissue.

Discussion

This study examined the effects of prolonged tourniquet application on tissue pharmacokinetics of multiple prophylactic antibiotics in an established rat tourniquet model. We found tourniquet-induced ischemia profoundly reduces penetration of

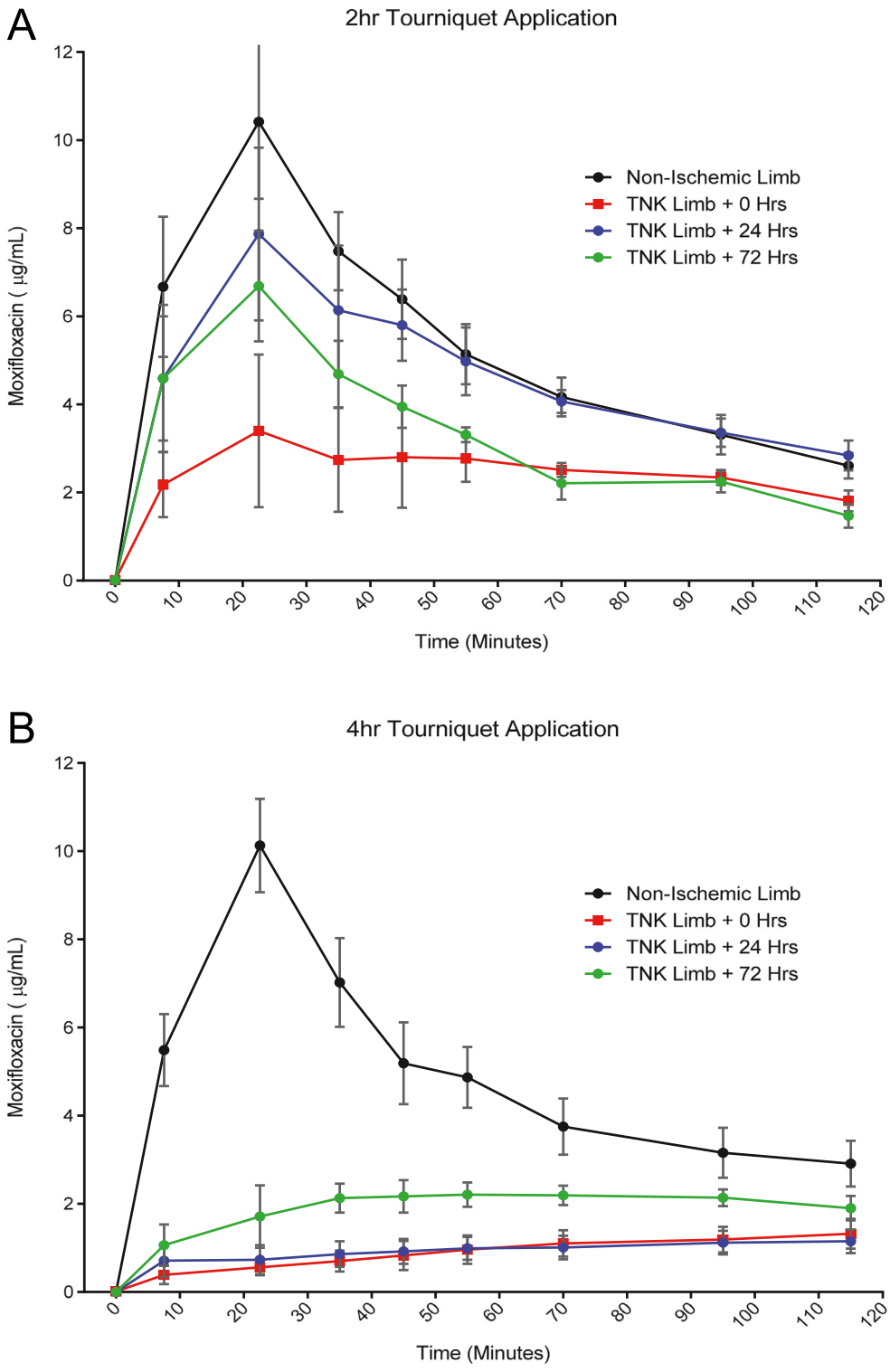

Fig. 3. Free moxifloxacin in the interstitial space of rat tibialis anterior muscles during 72 h reperfusion period following 2-h (A) or 4-h (B) tourniquet (TNK) application. Pneumatic tourniquet placed at the level of the mid-femur on the TNK limb was released 10 min prior to administration of first moxifloxacin dose, 15 mg/kg IV, at 0 min. Antibiotic was subsequently redosed every 12 h for the following 72 h. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from all relevant sampling courses for each TNK duration. $N = 4–5$ animals per TNK application duration.

systemic prophylactic antibiotics to skeletal muscle both during and after ischemia, potentially impairing prophylaxis of extremity wound infection. Our preliminary study of tissue antibiotic penetration during TNK application demonstrates that so long as a properly-applied tourniquet remains in place on an extremity, no significant amount of systemically administered antibiotic enters peripheral soft tissues distal to the impediment in blood flow. This was an expected finding, given the near-complete occlusion of the distal hindlimb achieved in this tourniquet application model. A similar scenario is very likely present in most cases of human extremity tourniquet application (emergency and otherwise) and we feel further research into the safety and efficacy of alternative wound infection prophylaxis techniques for non-perfused tissue is warranted.

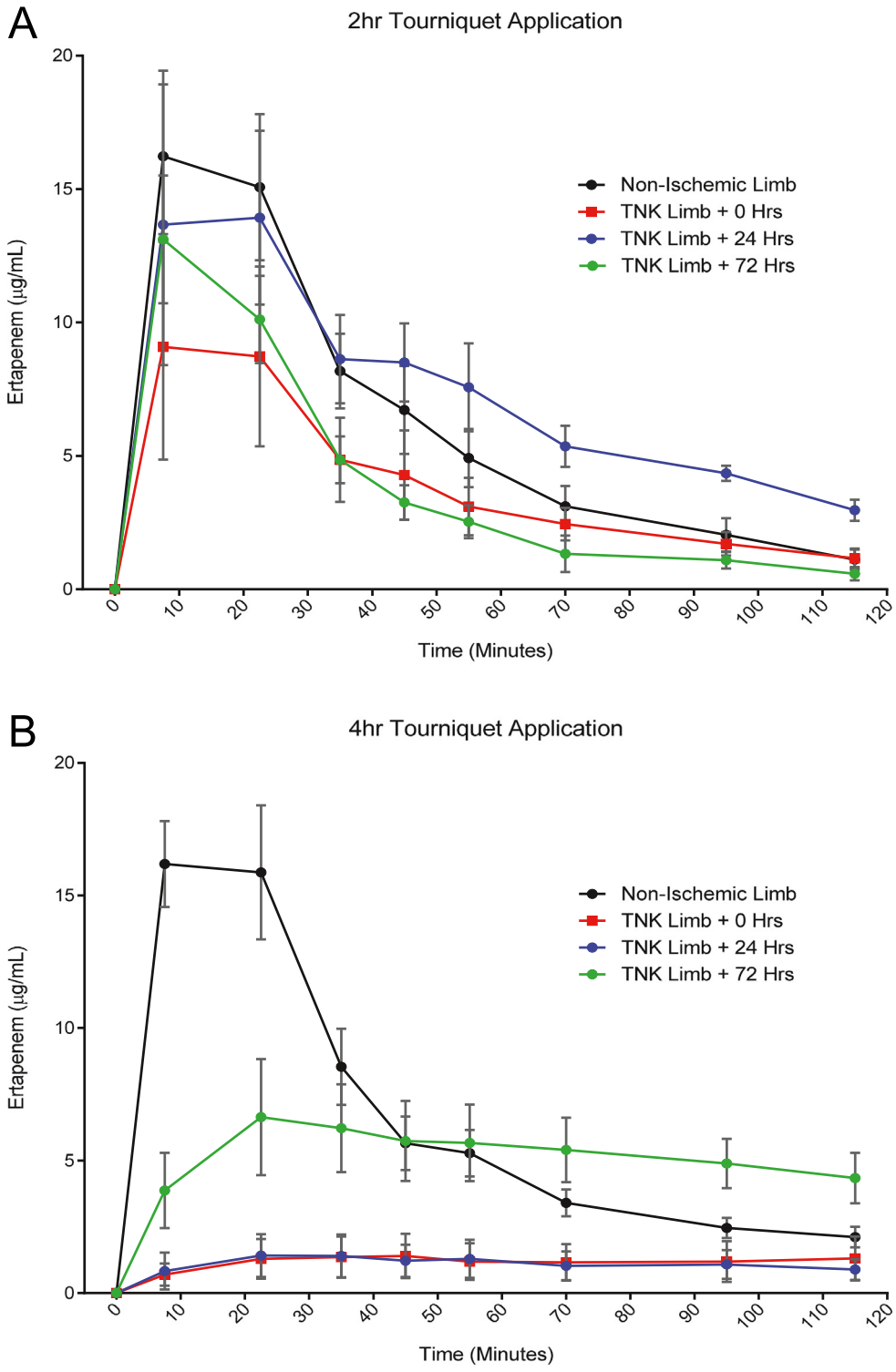

Fig. 4. Free ertapenem in the interstitial space of rat tibialis anterior muscles during 72 h reperfusion period following 2-h (A) or 4-h (B) tourniquet (TNK) application. Pneumatic tourniquet placed at the level of the mid-femur on the TNK limb was released 10 min prior to administration of first ertapenem dose, 35 mg/kg IV, at 0 min. Antibiotic was subsequently redosed every 12 h for the following 72 h. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from all relevant sampling courses for each TNK duration. $N = 4–5$ animals per TNK application duration.

Of greater relevance to the clinical management of contaminated extremity trauma, we found protracted tourniquet ischemia reduces and delays antibiotic entry into peripheral soft tissue for a significant amount of time following the restoration of arterial circulation. In our model, application of a tourniquet for a period of 2 h reduced hindlimb skeletal muscle antibiotic exposure (defined here as the area under the time-concentration curve, $AUC_{0->120\,min}$) by up to 56% in the critical period immediately following TNK release. Extending TNK ischemic time to 4 h reduced antibiotic exposure in skeletal muscle by up to 88% immediately following TNK release and by up to 85% after 24 h of reperfusion. For one tested drug (moxifloxacin), soft tissue levels recovered to only 45% of contralateral by 72 h following 4 h of ischemia. Overall, the reperfusion time required for antibiotic distribution to

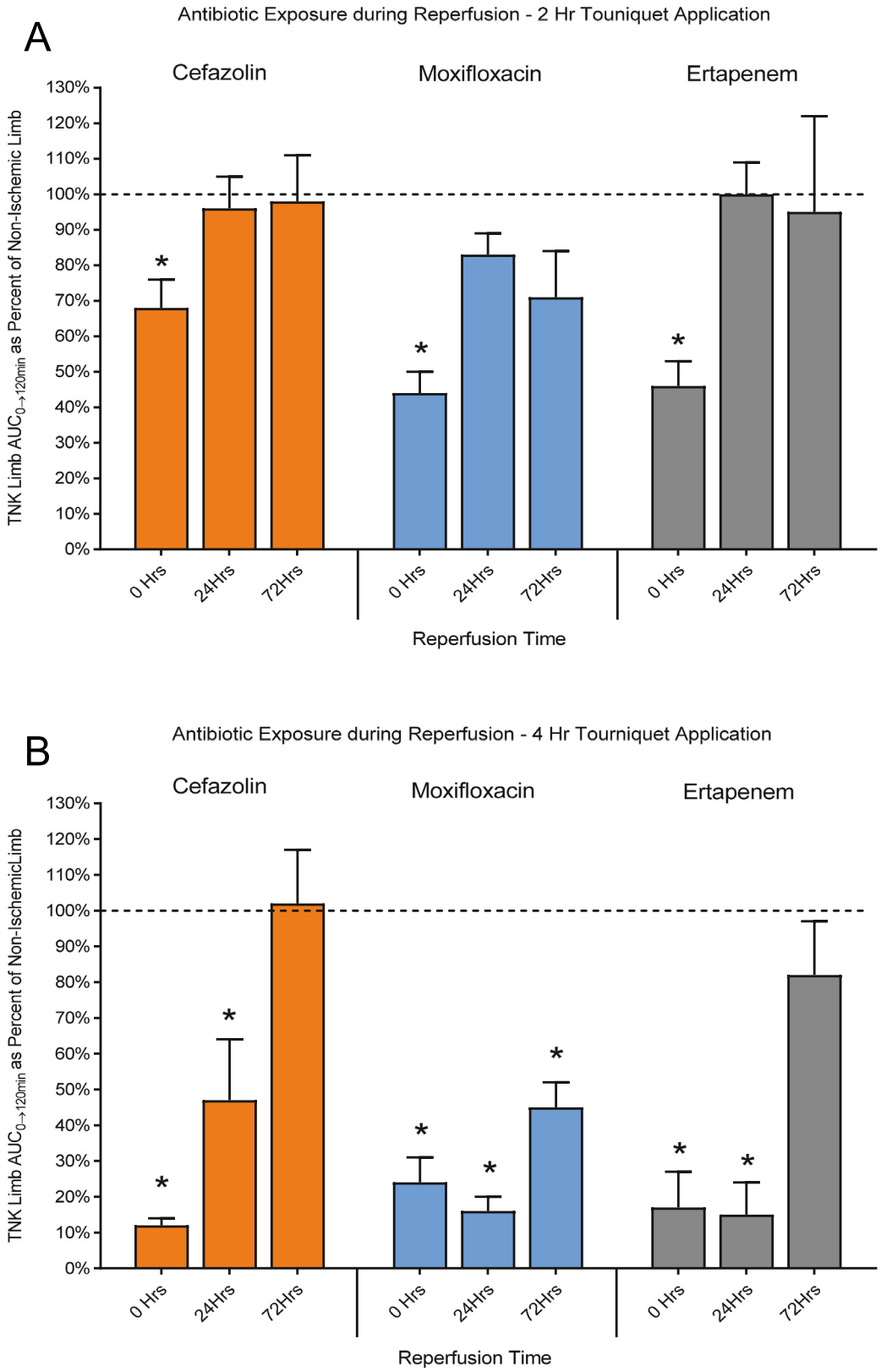

Fig. 5. Antibiotic exposure (area under the concentration-time curve, $AUC_{0\rightarrow 120\,min}$) in rat tibialis anterior muscle during 72 h reperfusion following 2-h (A) or 4-h (B) TNK application, expressed as a percentage of non-ischemic (contralateral) limb exposure. $N = 4$–5 animals per TNK application duration. *$p < 0.05$ vs non-ischemic contralateral tissue exposure – Wilcoxon signed rank test with Bonferroni correction.

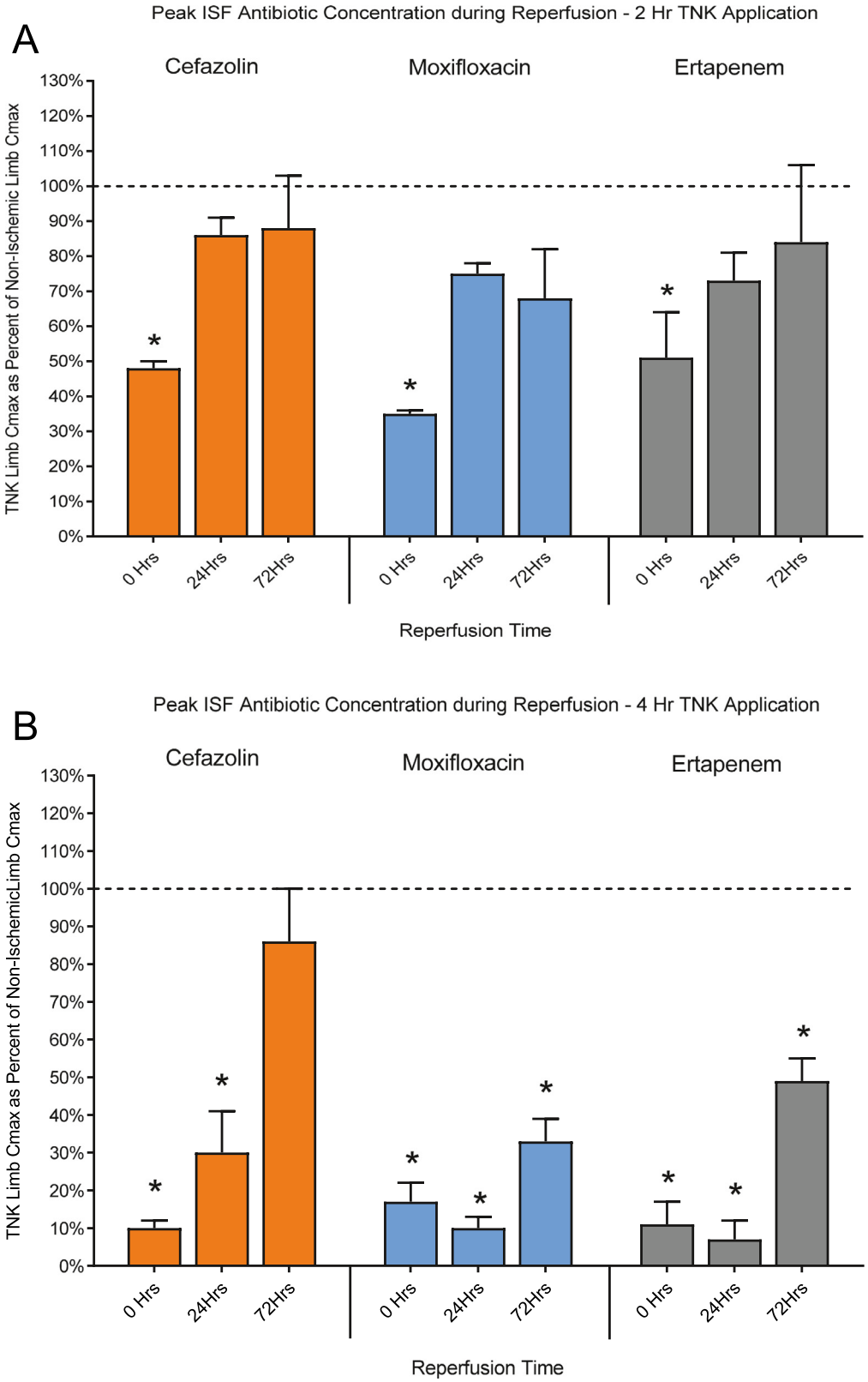

Fig. 6. Peak ISF antibiotic concentration (C_{max}) in rat tibialis anterior muscle during 72 h reperfusion following 2-h (A) or 4-h (B)TNK application, expressed as a percentage of non-ischemic (contralateral) limb C_{max}. $N = 4–5$ animals per TNK application duration. *$p < 0.05$ vs non-ischemic contralateral tissue exposure – Wilcoxon signed rank test with Bonferroni correction.

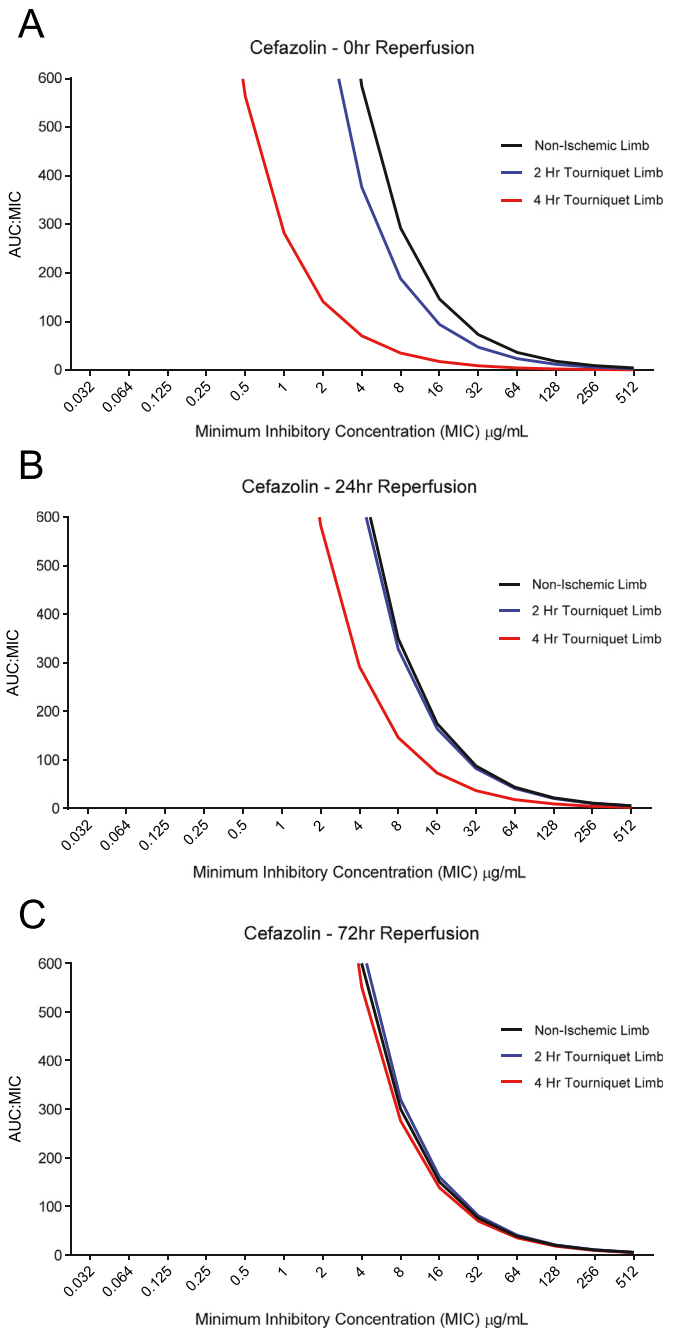

Fig. 7. Pharmacodynamic index (AUC (min*µg/mL):MIC (µg/mL)) curves of free cefazolin in the interstitial space of rat tibialis anterior muscle (A) immediately following TNK release, (B) after 24 h reperfusion, and (C) after 72 h reperfusion following 2- or 4-h TNK application. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from relevant sampling courses for both 2- and 4-h TNK durations.

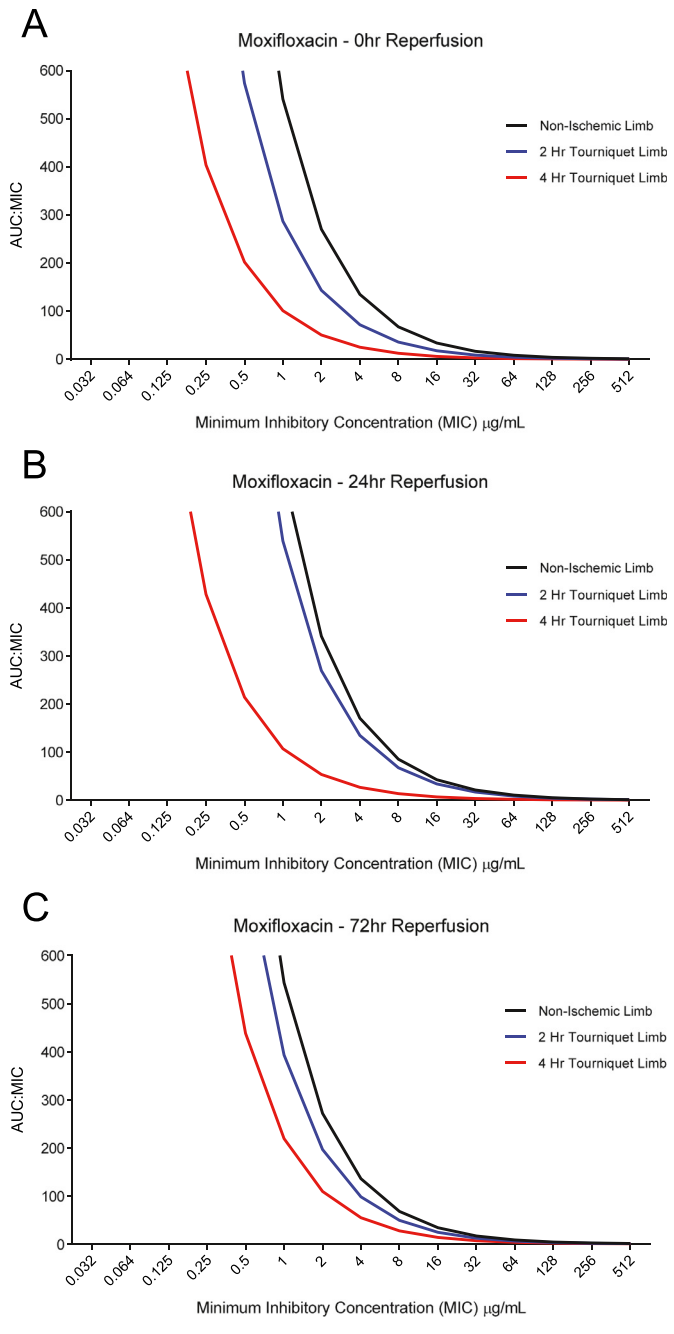

Fig. 8. Pharmacodynamic index (AUC (min*mg/mL):MIC (mg/mL)) curves of free moxifloxacin in the interstitial space of rat tibialis anterior muscle (A) immediately following TNK release, (B) after 24 h reperfusion, and C) after 72 h reperfusion following 2 or 4-h TNK application. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from relevant sampling courses for both 2- and 4-h TNK durations.

return to uninjured tissue levels following TNK application varied substantially among drug classes: lipophilic fluoroquinolones exhibited the greatest delay among tested drugs while hydrophilic cephalosporins (e.g., cefazolin) exhibited the shortest delay.

The clinical impact of the potential loss of peripheral antibiotic exposure on infective outcomes of trauma requiring TNK application remains to be elucidated, but our data clearly demonstrates that prolonged ischemia causes a progressive, persistent reduction in the antibiotic pharmacodynamic index (PDI) [44] attained in reperfused skeletal muscle after systemic drug administration. Figs. 7–9 illustrate the differential AUC:MIC PDI achieved in ischemic versus non-ischemic hindlimbs across several time points during 72 h of reperfusion following 2 or 4 h TNK application. The disparity between the TNK and non-ischemic contralateral limb curves at a given AUC:MIC target level indicates the extent to which prophylactic efficacy may be reduced by ischemia at a given time during reperfusion, i.e., nominally susceptible bacterial pathogens in post-ischemic tissue exhibiting MIC values within this gap would be exposed to antimicrobial agents at insufficient concentration to reproduce the antimicrobial effect obtained in the non-ischemic condition. It is important to note the PDI curves in Figs. 7–9 depict the ratio of free, bioactive antibiotic AUC in tissue to bacterial MIC, as

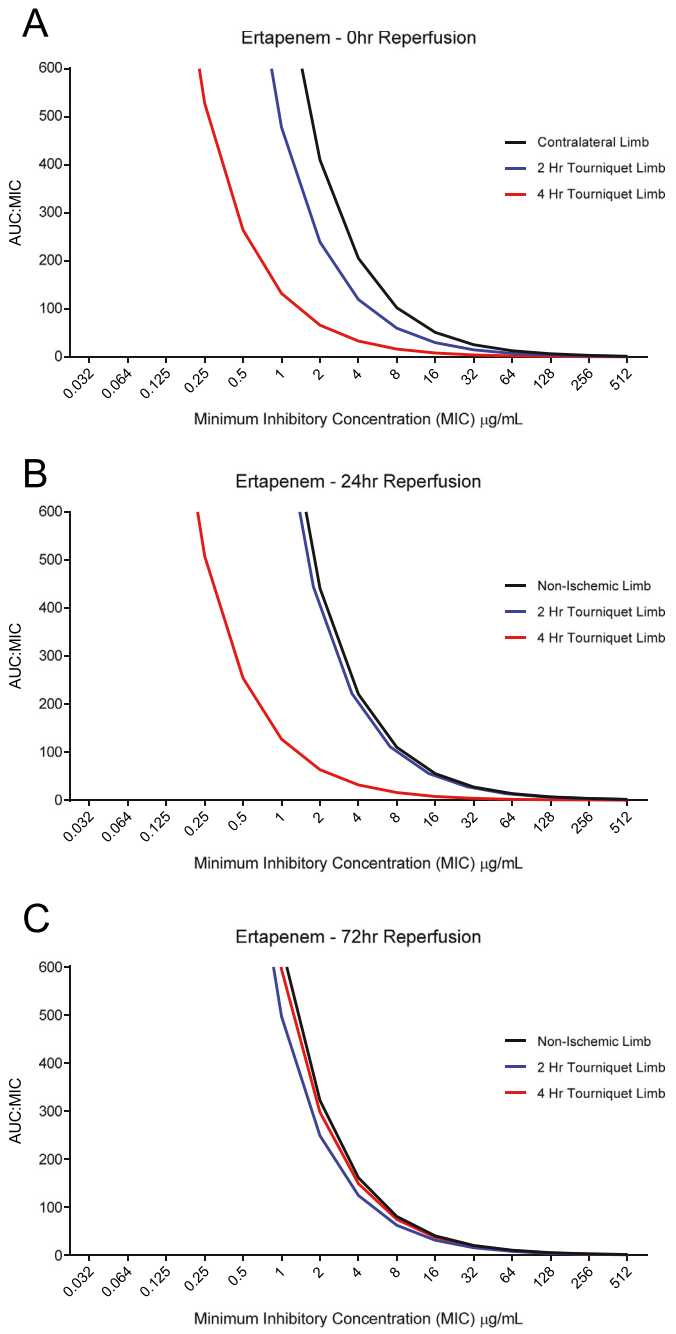

Fig. 9. Pharmacodynamic index (AUC (min*mg/mL):MIC (mg/mL)) curves of free ertapenem in the interstitial space of rat tibialis anterior muscle (A) immediately following TNK release, (B) after 24 h reperfusion, and (C) after 72 h reperfusion following 2 or 4-h TNK application. Non-ischemic (contralateral) limb curve represents interstitial fluid values in non-ischemic limb obtained from relevant sampling courses for both 2- and 4-h TNK durations.

opposed to the more conventional comparison of total drug AUC in plasma (which includes the protein bound fraction) to MIC. Unfortunately, little clinical data regarding appropriate free drug-based PDI parameters (e.g., Time>MIC, AUC:MIC, or C_{max}:MIC) or specific PDI targets for peripheral tissue prophylaxis is currently available in existing pharmacodynamic literature.

The pathophysiological mechanism underlying the observed loss of normal drug distribution in skeletal muscle following extended tourniquet-induced ischemia likely involves dysfunctional capillary reperfusion after restoration of arterial blood flow. Termed the capillary “no-reflow” phenomenon, a lack of adequate microvascular blood flow following extended ischemic injury has been described previously as arising from a loss of endothelial integrity during reperfusion that produces sufficient interstitial edema to cause capillary closure and stasis [25–28]. Previous evidence suggests increased leukocyte binding to injured post-capillary venule endothelium occurs rapidly during the initial influx of blood into post-ischemic tissue [45,46] with cytotoxic factors released by adherent leukocytes further disrupting endothelial integrity, thereby driving a progressive increase in microvascular permeability and fluid loss to the interstitial space. The resulting hemoconcentration and interstitial edema is thought to be the primary direct mechanism of capillary flow reduction: increased local pressure causes mechanical compression and narrowing of capillaries that reduces or halts the passage of blood through simple hydraulic resistance according to the Hagen–Poiseuille equation [25,46]. The scale of capillary “no-reflow” observed following tourniquet injury depends heavily on the duration of ischemia, as this directly influences the extent of initial endothelial injury and vascular leukocyte recruitment, in addition to the activation state of circulating leukocytes due to the presence or absence of other concurrent inflammatory processes [27,29].

An important limitation of this study is the utilization of a rodent model which necessarily differs from human physiology in regard to both drug metabolism and clearance, as well as skeletal muscle compliance and response to ischemia. To address this limitation, studies are underway in our laboratory to assess soft tissue antibiotic pharmacokinetics in a porcine model of extremity TNK-induced ischemia-reperfusion injury. This work will allow us to more closely replicate the human physiological response to prolonged ischemia and will provide a more clinically relevant comparison of drug pharmacokinetics during and after ischemia to established human PDI targets for infection prevention. The physiochemical factors underlying the apparent differences in soft tissue penetration of antibiotics of various classes is an additional important line of investigation we are currently pursuing. Information in this area may prove critical to the development of alternative local or regional drug delivery strategies tailored to the management of contaminated extremity wounds compounded by ischemia-reperfusion injury.

Conclusions

Extremity tourniquet application causes a profound, ischemia duration-dependent reduction in the distribution of systemic antibiotics from the circulation into peripheral soft tissue during reperfusion. These findings are agreement with previous research regarding microvascular dysfunction in this setting, and highlight the need for not only the establishment of defined “safe” time limits for tourniquet application, but also a general improvement in our understanding of the temporal relationship between vascular occlusion and capillary flow dysfunction in extremity trauma management. Given the strong evidence that increased time from injury to introduction of antibiotics causes a progressive rise in infection rate, our results support the development of alternative approaches for effective wound infection prophylaxis under conditions where extremity circulation cannot be restored in a timely manner.

Declaration of Competing Interest

The authors declare no financial or nonfinancial conflicts of interest that would inappropriately influence or bias the work described herein.

CRediT authorship contribution statement

Lee C. Mangum: Conceptualization, Data curation, Formal analysis. **Gerardo R. Garcia:** Investigation, Methodology, Formal analysis. **Kevin S. Akers:** Conceptualization, Writing - review & editing. **Joseph C. Wenke:** Conceptualization, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.injury.2019.09.025.

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