Preservation of the liver for transplantation: Machine perfusion-based strategies for extended preservation and recovery

Bruinsma, B.G.

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ADDENDUM
CHAPTER 8

Antibiotic prophylaxis in (sub)normothermic organ preservation: *In vitro* efficacy and toxicity of cephalosporins

Bote G. Bruinsma*, Ivo C.J.H Post*, Lennart B. van Rijssen, Leonie de Boer, Michal Heger, Sebastian A.J. Zaat, Thomas M. van Gulik

*Contributed equally

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ABSTRACT

**Background.** Bacterial contamination during cold organ preservation occurs without major complications. However, with organ preservation steering toward (sub)normothermic temperatures, bacterial contamination may be detrimental with limited evidence to support the choice of antibiotic.

**Methods.** This study aimed to determine the effective antibiotic prophylaxis for (sub)normothermic preservation by investigating whether *Staphylococcus epidermidis* was capable of growing in a subnormothermia-compatible preservation solution Polysol (PS) and in solutions designed for cold preservation (University of Wisconsin solution, histidine-tryptophan-ketoglutarate solution, and Belzer-machine perfusion solution). Various *S. epidermidis* and *S. aureus* strains were exposed to ceftriaxone and cefazolin at concentrations from 0 to 1000 μg/mL under subnormothermic and normothermic conditions in PS. To mimic procedural conditions, the effect of cefazolin was determined after exposure of bacteria to 20 h incubation at 28 °C in the presence of cefazolin and subsequent incubation at 37 °C in the absence of cefazolin. The toxicity of cefazolin was assessed by cell viability and caspase activation assays in porcine kidney endothelial cells.

**Results.** Without antibiotics, PS sustained bacterial growth under sub(normothermic) conditions, whereas growth was absent in cold preservation solutions. Cefazolin exhibited greater bactericidal effect on *S. epidermidis* than ceftriaxone. However, after inoculating PS with 10⁶ colony-forming units/mL, only a cefazolin concentration of 1000 μg/mL was able to exert a complete bactericidal effect on *S. epidermidis* and *S. aureus* strains and maintain sterility after removal of cefazolin. Finally, 1000 μg/mL cefazolin showed no adverse effects on porcine kidney endothelial cells.

**Conclusions.** Based on these findings, we recommend that high-dose cefazolin be used for prophylaxis in (sub)normothermic organ preservation with PS.
INTRODUCTION

A persistent donor organ shortage has stimulated the development of novel, more advanced organ preservation techniques to increase donor organ availability. Expansion of the donor pool by including extended-criteria donor organs requires preservation techniques that minimize preservation injury. Hence, organ preservation is steering away from static cold storage, initially to hypothermic (4 °C) machine perfusion preservation followed by subnormothermic (28 °C) and even normothermic (37 °C) machine perfusion ((S)NMP). Nutrient-rich preservation solutions and extended preservation times increase the risk of bacterial graft contamination and pose a threat to the graft and the immunocompromised recipient. Graft or preservation solution contamination has a reported clinical incidence of 8–62.5% in transplantations that employ hypothermic preservation, of which up to 17.8% involves high-risk microorganisms. Although graft contamination after hypothermic preservation usually does not result in adverse outcomes, contamination may be detrimental at warmer conditions and in nutrient-rich preservation solutions, because these provide a fecund environment for bacterial growth.

A review of recent literature (1990–2012) for studies employing renal or liver (S)NMP revealed that only 14 of 33 recently published articles addressed antibiotic prophylaxis, indicating that antibiotic addition is omitted by many or grossly underreported. Tolboom et al. employed a cell culture medium for perfusion, based their choice of penicillin/streptomycin on successful in vitro work (personal communication). High-dose cephalosporin (1500 μg/mL) was used most frequently to prime the perfusion circuit or as a bolus (1000 μg/mL) per 24 h of perfusion. Cephalosporins are effective against many of the preservation-associated microorganisms in the absence of antibiotic-induced adverse effects.

The use of cephalosporins during (S)NMP has not yet been examined and no guidelines or recommendations for antibiotic prophylaxis during (S)NMP exist. The antibiotic choice must be based on optimal prevention of bacterial growth without causing additional damage to the preserved organ.

This study aimed to establish a suitable antibiotic prophylaxis regimen in renal (S)NMP. To this end, in vitro models were employed using various Staphylococcus epidermidis and Staphylococcus aureus strains to determine the bactericidal efficacy of cefazolin and ceftriaxone. Porcine kidney endothelial cells (PKEC) were used to assess toxicity. The panel of strains also included Staphylococcus small colony variants (SCV), that is, slow-growing variants that form small colonies on agar plates. These strains may have a reduced susceptibility to antibiotics and are difficult to diagnose and treat.
RESULTS

Bacterial growth in preservation solutions

Significant *S. epidermidis* RP62a growth was observed in both tryptic soy broth (TSB) and Polysol (PS). However, a 10-fold larger increase in colony-forming units (CFU)/mL was observed in TSB after 24 h (P<0.001; Fig. 1). After 20 h of incubation at 28 °C and subsequently for 48 h at 37 °C, growth of all *S. epidermidis* and *S. aureus* strains occurred in both TSB (Fig. 2A) and PS (Fig. 2B). In PS, however, the numbers of CFU of *S. aureus* 8325-4 and its SCV strain I10 decreased until 44 h but increased to numbers similar to those of the other strains at 68 h. No differences in growth in TSB and PS were observed between the wild type and the corresponding SCV strains. In general, the strains grew to higher numbers of CFU in TSB than in PS. Although there was growth of *S. epidermidis* RP62a in TSB and PS, the number of CFU decreased during the total 68-h incubation in histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), or Belzer-machine perfusion solution (Belzer-MPS) (Fig. 2C).

![Figure 1. Growth kinetics. *S. epidermidis* RP62a in PS and TSB over 24 h at 37 °C. *Significant difference from TSB. PS, Polysol; TSB, tryptic soy broth.]

Bactericidal efficacy of cefazolin and ceftriaxone

In the absence of antibiotics, *S. epidermidis* RP62a showed significant growth during the first 12 h (P<0.001). A slightly increased spectrophotometrically determined optical density (OD$_{620nm}$) was observed after 48-h incubation in TSB with 75 μg/mL ceftriaxone (NS). In all other incubations, the antibiotic inhibited the growth of the bacteria. Based on spectrophotometric measurements, cefazolin had superior bactericidal efficacy over ceftriaxone, which was confirmed by quantitative plating (Fig. 3A,B). After 2 and 12 h, 75 μg/mL cefazolin was sufficient to reduce the number of CFU/mL of *S. epidermidis* RP62a below the detection limit. In contrast, ceftriaxone required a concentration of 1000 and 300 μg/mL after 2 and 12 h, respectively, to obtain a similar bactericidal effect.
Figure 2. Growth kinetics of *S. epidermidis* and *S. aureus* strains. Bacterial growth in TSB (A) and PS (B) during incubation at 28 °C for 20 h followed by 48 h at 37 °C. (C) Growth curve of *S. epidermidis* RP62a in different preservation solutions and TSB. Belzer-MPS, Belzer-machine perfusion solution; HTK, histidine-tryptophan-ketoglutarate solution; PS, Polysol; TSB, tryptic soy broth; UW, University of Wisconsin solution. *Significant difference in bacterial concentration between PS and Belzer-MPS, UW, and HTK.*
Dose-response of cefazolin under (sub)normothermic conditions

Incubation of *S. epidermidis* and *S. aureus* strains at 37 °C in PS with cefazolin led to a complete elimination of most strains (see Fig. S1). A 1000 μg/mL concentration decreased the bacterial concentration to below the detection limit at t=44 h, whereas 300 μg/mL led to a slower elimination and was insufficient to eradicate the SCV *S. epidermidis* strain 6455-I. To mimic subnormothermic preservation and normothermic posttransplantation conditions, *S. epidermidis* was incubated in PS for 20 h at 28 °C and subsequently for 24 h at 37 °C. The bactericidal effect was less prominent at 28 °C compared with 37 °C, but subsequent warming to 37 °C greatly enhanced the effect (see Fig. S2). Again, a concentration of 1000 μg/mL was able to consistently reduce bacterial numbers to undetectable values by t=44 h, whereas the 300 μg/mL dose led to slower and incomplete eradication. Removal of the cefazolin before warming from 28 °C to 37 °C allowed *S. epidermidis* RP62a growth in the suspensions to which 300 μg/mL had initially been added (Fig. 4). However, an increase in CFU/mL was deterred up to 12 h compared with the antibiotic-free
control. Only an initial cefazolin concentration of 1000 μg/mL was capable of reducing bacterial CFU in PS to below the limit of detection after cefazolin had been removed.

Figure 4. Effect of the removal of cefazolin on continued growth of *S. epidermidis* RP62a. *S. epidermidis* RP62a were exposed to the indicated cefazolin concentrations for 20 h at 28 °C, after a temperature increase to 37 °C without any changes in the solution (solid lines) or after replacement of the cefazolin-containing solution with antibiotic-free solution.

Cytotoxicity of cefazolin to porcine kidney endothelial cells

After 8-h incubation, the change in PKEC viability relative to t=0 did not differ significantly between 0 and 1000 μg/mL cefazolin concentrations (21.1% and 26.5% increase) and all other concentrations (Fig. 5). Furthermore, caspase 3 and 7 activation remained absent regardless of the cefazolin concentration. Interestingly, incubation in 300 μg/mL cefazolin was associated with a reduced extent of apoptosis compared with control (0 μg/mL) after 8 h (−24.8% and −4.3%, respectively; P< 0.05). Accordingly, no evidence of cytotoxicity of high-dose cefazolin was observed in PKEC.

DISCUSSION

This is the first study to describe the growth of *S. epidermidis* and *S. aureus* wild type and SCV strains in different preservation media during (sub)normothermic conditions and their susceptibility to antibiotic prophylaxis. The main findings were (a) bacterial growth in (S)NMP preservation solutions is a risk at (sub)normothermic conditions; (b) in the nutrient-rich PS, the bactericidal efficacy of cefazolin is greater than that of ceftriaxone; (c) 1000 μg/mL cefazolin is bactericidal during subnormothermic and at normothermic incubation, even after cefazolin removal; and (d) 1000 μg/mL cefazolin is nontoxic to PKEC in vitro.
High rates of bacterial contamination in organ preservation solutions have always been a reason for concern. Although hypothermia appears to prevent bacterial growth and limit infectious complications\textsuperscript{6}, bacteria remain viable after hypothermic preservation even in the presence of penicillin and trimethoprim/sulfamethoxazole\textsuperscript{19}. With the current preservation techniques moving from static hypothermic storage to dynamic (sub)normothermic techniques, more aggressive antimicrobial prophylaxis is required to ensure sterility of nutrient-rich preservation solutions and the preserved organ\textsuperscript{20}. We found that HTK and UW did not support bacterial growth under (sub)normothermic conditions, which is likely due to the absence of nutritional constituents for bacteria in these solutions\textsuperscript{21}. PS, developed primarily for warm preservation, did facilitate significant bacterial growth, implying that a potential risk for other novel, (S)NMP preservation solutions exists as well\textsuperscript{22, 23}. New insights into the nature of the microorganisms responsible for significant infections should be taken into account when determining appropriate prophylaxis. In this respect, SCV are associated with a slow growth rate, atypical colony morphology, and aberrant biochemical properties that are thought to make them less susceptible to many antibiotics and therefore responsible for persistent and latent infections\textsuperscript{18}. \textit{S. aureus} 8325-4 and its SCV have been shown to be equally infective and comparably susceptible to cephapirin\textsuperscript{24}. Accordingly, our findings show similar efficacy of cefazolin across different strains and their respective SCV, supporting the broad antimicrobial coverage of cefazolin. Furthermore, the continuing bactericidal effect after removal of the antibiotic appears to be instrumental in preventing latent infections.

The in vitro model presented here allowed sensitive and controlled determination of bacterial growth and antibiotic susceptibility. However, this model is not a perfect representation of the
complex interaction between preservation solution and the various compartments of the organ. In previously performed, contaminated, isolated ex vivo renal perfusion experiments using PS at subnormothermic conditions, peritubular capillary obstruction occurred due to bacterial overgrowth, compromising perfusion (data not shown). By showing effective prophylaxis of cefazolin against high concentrations of frequent bacterial contaminants, a solid basis is provided for the use of this antibiotic in (S)NMP systems.

Prophylactic addition of antimicrobial agents to preservation solutions has been common practice for decades. However, current experimental and clinical preservation protocols frequently neglect antibiotic prophylaxis and, where reported, do not address sterility and antibiotic toxicity, despite high contamination rates and nephrotoxicity, respectively. It is likely that this stems from the absence of an evidence-based recommendation. Our results provide compelling in vitro rationalization for the use of high-dose cefazolin, which is efficacious against the highly contaminating staphylococcal species and even against their SCV. When combined with bacterial culturing according to standard operating procedures, high-dose cefazolin could help minimize infectious complication following (S)NMP.

In conclusion, a 1000 μg/mL cefazolin dose is effective against multiple S. epidermidis and S. aureus strains and their SCV at 28 °C and 37 °C in PS and was nontoxic for PKEC. It is therefore recommended to use high-dose cefazolin as antibiotic prophylaxis during warm kidney preservation with PS.

MATERIALS AND METHODS

Microorganisms and inoculum preparation

S. epidermidis strains RP62a (ATCC 35984), O-47, and the patient-derived strain 6455-I were used. hemB mutants of O-47 and 6455-I, O-47mx and 6455-II (patient strain), respectively, were included as SCVs. Additionally, S. aureus strains UAMS-1 (ATCC 49230) and 8325-4 were studied, including the SCV I10 and the hemB mutant of 8325-4.

Inocula were prepared using 50 mL of logarithmic growth phase cultures of the S. epidermidis and S. aureus strains in TSB (BD Difco, Detroit, MI). The cultures were washed thrice (15,000g, 10 min) in 30 mL of the corresponding incubation solution, resuspended, and diluted to 10⁷ CFU/mL. Concentrations were determined spectrophotometrically in 1 mL cuvettes at 620 nm (OD₆₂₀ nm, Ultraspec 2000; Pharmacia Biotech, Uppsala, Sweden), whereby an OD₆₂₀ nm of 0.3 corresponds to 10⁸ CFU/mL.

Culture conditions

Bacterial growth and dose-response experiments were performed in quadruplicate by adding 20 μL of inoculum to 180 μL of the corresponding preservation solution in polypropylene
microtiter plates (Greiner Bio-One, Kremsmuenster, Austria). The plates were then incubated while shaking at 80 rpm (Minitron Incubator Shaker; Infors HT, Bottmingen-Basel, Switzerland).

Quantification of bacteria

Bacterial concentration was quantified at OD$_{620}$ nm using a filter photometer (AR 2001; Anthos Labtec Instruments, Wals, Austria) and by quantitative culture, that is, by plating four replicate 10 μL aliquots of 10-fold serial dilutions on blood agar plates (Columbia agar and 5% sheep blood; bioMerieux, Marcy l’Etoile, France). The blood agar plates were incubated at 37 °C for 24 to 48 h, after which CFU were counted (quantitative plating).

Bacterial growth in preservation solutions

*S. epidermidis* RP62a growth was determined at 37 °C in the experimental, nutrient-rich preservation solution PS (Organoflush, Amsterdam, The Netherlands) and compared with growth in TSB over a 24-h period by quantitative plating of samples taken at predefined time points. The growth of the *S. epidermidis* and *S. aureus* strains was monitored by quantitative plating of samples taken at relevant time points during 20 h of shaking incubation at 28 °C in PS and TSB. Subsequently, the temperature was raised to 37 °C and the bacterial concentration was quantified for an additional 48 h. The growth of *S. epidermidis* RP62a was determined in HTK (Dr. F Kohller-Chemie, Bensheim, Germany), UW (Bristol-Myers Squib, New York, NY), Belzer-MPS (Bridge to Life, Columbia, SC), and PS during the same subnormothermic regimen, that is, 20 h at 28 °C followed by 48 h at 37 °C.

Efficacy of ceftriaxone and cefazolin

The bacteriostatic effect of ceftriaxone and cefazolin on *S. epidermidis* RP62a in PS was assessed at ceftriaxone (Fresenius Kabi, Bad Homburg, Germany) or cefazolin (Sandoz, Kundl, Austria) concentrations of 0, 75, 150, 300, 600, or 1000 μg/mL by measuring the absorption at OD$_{620}$ nm after 0, 2, 4, 6, 8, 12, 24, and 48 h of incubation at 37 °C. In addition, bactericidal activity was determined by quantitative culture.

Dose-response of cefazolin under (sub)normothermic conditions

Cefazolin’s bactericidal capacity against the *S. epidermidis* and *S. aureus* strains was assessed during in vitro (sub)normothermic incubation in PS. To this end, 0, 300, or 1000 μg/mL cefazolin was added to microtiter plates, prepared as described above. Plates were incubated for 68 h at 37 °C or 20 h at 28 °C followed by 48 h at 37 °C. CFU/mL were determined by quantitative culture.

Washout of cefazolin during organ reperfusion was simulated in vitro to assess its effect on residual bacterial growth. Cefazolin was added at a concentration of 0, 300, and 1000 μg/mL to 5 mL of *S. epidermidis* RP62a-inoculated PS. After 20 h of incubation at 28 °C (shaking), the cefazolin was removed by washing the bacteria three times in 5 mL PS (15,000g, 10 min, 20 °C) and resuspending the pelleted bacteria in an equal volume of PS as used for the first
incubation period. These suspensions were subsequently incubated for another 24 h at 37 °C. Bacterial growth was determined by quantitative culture and compared with suspensions in which cefazolin had not been washed out.

Isolation of porcine kidney endothelial cells

Mature PKEC were isolated from kidneys of 30 kg male Landrace pigs (van Beek Topigs, Lelystad, The Netherlands). After 5 min of warm ischemia following a cardiac resection, the endothelial cells were procured from the kidneys. Kidneys were flushed using HTK and phosphate-buffered saline (PBS; Fresenius Kabi) followed by infusion of 10 mL of 0.04% Liberase (Roche, Basel, Switzerland) in PBS through the renal artery. The kidney was incubated for 26 min at 37 °C and flushed with 20% fetal calf serum (FCS; Lonza, Verviers, Belgium)-enriched endothelial cell growth medium (ECGM; Promocel, Heidelberg, Germany) to obtain detached endothelial cells. The PKEC were subsequently cultured to confluence at 37 °C in culture flasks (T25 Primaria; BD Biosciences, Franklin Lakes, NJ) in a humidified 5% CO₂ atmosphere. After reaching confluence, cells were collected after brief treatment with 1 mL Accutase (Innovative Cell Technologies, San Diego, CA), washed in PBS (400 g, 10 min, 4 °C), and resuspended in Williams medium E (WE; Lonza) containing 20% FCS. Next, an equal volume of WE containing 20% fresh dimethyl sulfoxide was slowly added to the cell suspensions up to a final volume of 1.8 mL, after which the cell suspensions were stored in liquid nitrogen. Before use, cells were thawed and washed twice with 18 mL of 20% FCS-enriched WE (400 g, 10 min, 4 °C). The cell pellet was resuspended in 5 mL ECGM and cultured as described. Cells were used after the third passage had become confluent.

Cell viability and caspase induction

PKEC were seeded in fibronectin (Sigma-Aldrich, St. Louis, MO)-coated 24-well culture plates (Corning, Corning, NY) and cultured in ECGM. Upon reaching confluence, cell viability and caspase 3 and 7 levels were determined after 0 and 8 h. Cell viability was determined with a water-soluble tetrazolium salt assay (WST; Roche). The medium was aspirated, and the wells were incubated in WSTreagent (300 μL/well) and incubated for 30 min at 37 °C. The absorbance was measured using a Synergy HT microplate reader (λₘₚ=440 nm; BioTek, Winooski, VT).

Caspase 3 and 7 levels were determined using a fluorochrome inhibitor of caspase kit (reference#A20173; AbD Serotec, Oxford, UK) according to the manufacturer’s instructions (λₑₓ=575 nm, λₑₓ=620 nm). WST and caspase data were normalized to protein content (Bradford protein assay; Bio-Rad, Hercules, CA) and expressed as a percentage of the mean of the 0-h values.

Statistical analysis

Statistical analysis of differences in bacterial concentrations was performed using a two-way analysis of variance with a Bonferroni post hoc test between groups and unpaired-sample
Student’s t-test between time points within one group. Toxicity data were analyzed using a Kruskal-Wallis test with Dunn’s multiple comparison test (>=0.05). P<0.05 was considered statistically significant.

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REFERENCES


Figure S1. Efficacy of cefazolin on *S. epidermidis* and *S. aureus* strains under normothermic conditions. Each graph shows the concentration of the respective bacterial strains, and the three lines indicate the three different concentrations of cefazolin.
Figure S2. Efficacy of cefazolin against \textit{S. epidermidis} and \textit{S. aureus} strains. Cultured at 28 °C for 20 h, followed by 48 h at 37 °C. Each graph shows the concentration of the respective bacterial strains and the three lines indicate different concentrations of cefazolin.