Determination of Residual Antibiotics in Cryopreserved Heart Valve Allografts

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Keywords

Allotransplant · Cryopreservation · Heart valves · Residual antibiotics

Summary

Introduction: Cardiovascular allografts are systematically incubated in antibiotics for their decontamination, and the antibiotics are removed before allograft implantation. We studied the occurrence of antibiotic residues in allograft valves. **Methods:** 12 experimental allografts were analyzed in this study. The concentration of the residual antibiotics was determined by high-performance liquid chromatography and the results were expressed as microgram per gram of allograft tissue. **Results:** The initial analysis showed that only vancomycin HCl and lincomycin HCl were retained in the allograft, whereas no traces of polymyxin B sulfate were detected in the tissue samples. Furthermore, the values found for the antibiotic residues in the extracted solution from the allografts were similar to the initial results: Vancomycin and lincomycin were detected in very low concentrations and no polymyxin B residues were observed. According to the World Health Organization (WHO), the maximum daily doses for vancomycin, lincomycin and polymyxin B are 2.0, 1.8 and 0.4 g, respectively. The thresholds for reporting degradation products are 0.05% for vancomycin and lincomycin and 0.1% for polymyxin B. The residual values for the two detected antibiotics were largely below 0.05%. **Conclusions:** Antibiotic residues in the allograft valves do not present any risk for their recipients. Increased allograft resistance to infections is probably due to the retention of antibiotics in the tissue.

Schlüsselwörter

Allotransplantat · Kryokonservierung · Herzklappen · Restantibiotika

Zusammenfassung

Einleitung: Kardiovaskuläre Allotransplantate werden zur Dekontamination systematisch zusammen mit Antibiotika inkubiert; die Antibiotika werden vor der Allotransplantat-Implantation wieder entfernt. In der vorliegenden Studie wurden Herzklappentransplantate auf das Vorhandensein von Antibiotikaresten untersucht. **Methoden:** 12 experimentelle Allotransplantate wurden in dieser Studie untersucht. Die Konzentration von Restantibiotika wurde mittels Hochdurchsatz-Flüssigkeitschromatographie bestimmt und die Ergebnisse als Mikrogramm pro Gramm Allotransplantat-Gewebe ausgegeben. **Ergebnisse:** Die initiale Analyse ergab, dass lediglich Vancomycin HCl und Lincomycin HCl in den Allotransplantaten zurückgeblieben waren, während von Polymyxin-B-Sulfat keine Spuren in den Gewebeproben nachgewiesen werden konnten. Darüber hinaus waren die Konzentrationen der Antibiotikareste in der von den Allotransplantaten extrahierten Lösung ähnlich wie die initialen Ergebnisse: Vancomycin und Lincomycin wurden in sehr geringen Dosen gefunden, Polymyxin B ließ sich nicht mehr nachweisen. Gemäß der World Health Organization (WHO) sind die maximalen täglichen Dosen für Vancomycin, Lincomycin bzw. Polymyxin B 2,0, 1,8 bzw. 0,4 g. Die Schwellenwerte für den Nachweis von Abbauprodukten sind 0,05 % für Vancomycin und Lincomycin sowie 0,1% für Polymyxin B. Die nachgewiesenen zwei Antibiotika-Restkonzentrationen in den Herzklappen-Allotransplantaten liegen weiter unter dem Schwellenwert von 0,05%. **Schlussfolgerung:** Antibiotikareste in Herzklappen-Allotransplantaten stellen kein Risiko für die Transplantatempfänger dar. Eine erhöhte Allotransplantat-Resistenz gegen Infektionen wird möglicherweise durch die Retention von Antibiotika im Gewebe bewirkt.

Introduction

The procured human heart valves and/or arteries for allograft preparation are contaminated in approximately 25–70% of cases, depending on the donor type and the retrieval conditions [1]. Exposure to low concentrations of different antibiotics during the 20–48 h before cryopreservation reduces the contamination rate to about 5–10% [2, 3]. Different Tissue Establishments worldwide, responsible for cardiovascular allograft decontamination, have adopted different cocktails of antibiotics, under different temperature conditions and for varying duration [4–6]. Since the cell viability preservation of the heart valves signifies the valve quality preservation [7], many antibiotics such as antimycotic drugs [8] were removed from some decontamination protocols. Some long-term studies on implanted allografts have shown that allograft viability is a prerequisite for graft survival [7–9]. Although the antibiotic cocktail is referred to as low dose, the concentrations are far in excess to the doses used clinically for the treatment of systemic infections and well above the concentrations required to inhibit most microorganisms [10].

The European Homograft Bank (EHB) has been processing the heart valve and vascular allografts since 1989. The allografts are systematically incubated in the antibiotic cocktail. Before allograft implantation into the patient's body, all the processing components, such as the antibiotics, dimethyl sulfoxide (DMSO) and the medium 199, are removed by rinsing the tissue in isotonic solution (saline). Although the concentration of antibiotics is strongly reduced, for validation purposes the residues of these antibiotics in the allograft should be investigated. This study was carried out to determine the residual concentrations of the antibiotics used in the EHB cocktail for allograft decontamination. The technique used for this purpose was high-performance liquid chromatography (HPLC) carried out according to the European Pharmacopoeia 6th edition and the *Pharmeuropa* vol. 20, no. 1 (2008).

Material and Methods

The heart valves were obtained from: (a) multi-organ donors (MOD), (b) recipients of heart transplantation (RHT) or (c) cadaveric, non-heart-beating donors (NHBD), according to the EHB donor selection criteria for cardiovascular allograft preparation [10]. The heart valves that were morphologically not suitable for clinical use were discarded: 6 aortic valves (AVs) and 6 pulmonary valves (PVs) were used as the experimental allografts for this study. The cocktail of antibiotics was composed of: vancomycin HCl (vancocin, GlaxoSmithKline, Wavre, Belgium), lincomycin HCl (lincocin, Pfizer, Brussels, Belgium) and polymyxin B sulfate (polymyxin B sulfas, Fagron, Colombes, France). The antibiotics were added to 250 ml of medium 199 with Hank's salts, L-glutamine and 25 mmol/l Hepes (MEM 199; Invitrogen) to a final concentration of 50 µg/ml for vancomycin HCl, 120 µg/ml for lincomycin HCl, and 124 µg/ml for polymyxin B sulfate. The allografts were immersed in the cocktail of antibiotics at +4 °C for 48 h in sterile, hermeti-

cally closed Maco Biotech freezing-storage pots (MacoPharma, Mouvau, France). The study was carried out in 2 phases: The initial testing was conducted for each antibiotic separately, whereas during the second phase the EHB antibiotics cocktail containing all 3 antibiotics was tested.

For the preliminary testing, 3 AVs and 3 PVs were separated longitudinally into 3 equivalent fragments, each fragment containing the corresponding valve leaflet. 1 fragment was incubated in MEM 199 without antibiotic ('negative' control sample). 2 other fragments were incubated in MEM 199 containing the antibiotic to be tested. 1 of these 2 fragments was used as 'positive control' (coming directly from the antibiotic cocktail without rinsing), the other fragment (testing sample) was rinsed in the +4 °C cold saline solution, gradually added in intervals of 1 min (10 – 20 – 70 ml) following the EHB thawing and dilution protocol. Subsequently, the tissue was transferred and left for 1 min in a new receptacle containing 50 ml of cold saline solution (+4 °C) [11].

In the second phase of the study, 3 AVs and 3 PVs were decontaminated, cryopreserved and stored in the vapor phase of liquid nitrogen according to the EHB protocol, as described previously [11]. The valves were thawed and the cryoprotection solution (DMSO) was diluted from 10 to 0% following the EHB protocol [11]. One third of each valve was removed and the remaining two thirds were rinsed as mentioned above in 20 – 40 – 140 ml of the cold saline solution (+4 °C).

All tested allograft tissues were destructed and reduced to small pieces. Approximately 1 g of each sample was embedded in 10 ml HPLC water for ultrasound extraction during 30 min at room temperature. After sedimentation, 3–5 ml of this solution was filtered through a 0.45-µm filter membrane to remove particles greater than 0.45 µm, and analyzed. HPLC for each antibiotic was performed on a Pursuit reversed-phase C18 column (length: 150 mm, diameter: 4.6 mm, particles: 5 µm, at room temperature for vancomycin HCl and lincomycin HCl or at 30 °C for polymyxin B sulfate). Quantification of the residual antibiotics was accomplished using triethylamine/water/phosphoric acid/tetrahydrofuran/acetonitrile as the mobile phase at the detection wavelength of 280 nm for vancomycin HCl, phosphoric acid/water/acetonitrile/methanol at the detection wavelength of 210 nm for lincomycin HCl, and anhydrous sodium sulfate/water/acetonitrile at the detection wavelength of 215 nm for polymyxin B sulfate. The equipment used for this study was an HPLC Hitachi Lachrom Elite system and the software was EZ-Chrom – Elite. For analysis, 5 samples were taken from each solution. The results are expressed as microgram of antibiotic per milliliter of examined extraction solution.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the standard deviation of the response and the slope of the curve of each antibiotic according to the International Conference on Harmonization (ICH) guideline Q2B (Validation of Analytical Procedures, European Medicines Agency (EMA)) [12, 13], where the LOQ = 3 × LOD. The quantity of each residual antibiotic in the extraction solution was determined on the respective calibration curve.

Results

Of the tested antibiotics, 2 were retained by the allograft tissues at, on average, 3.14% for vancomycin HCl (AVs 3.56%, PVs 2.73%) and 0.61% for lincomycin HCl (AVs 0.56%, PVs 0.65%). There were no traces of residual polymyxin B sulfate in the tissue samples (table 1). There was no interference between the antibiotics and the allograft valve tissues. 2 of the antibiotics were adsorbed by the allograft tissues and leached out during the extraction procedure.

Since 2 of the 3 antibiotics were detected in the allograft tissue during the preliminary testing, additional tests were

Table 1. Initial testing for residual antibiotics after incubation in the EHB cocktail

Antibiotic	Tissue	Antibiotics residues in the tissue detected by HPLC, µg/g	Residual antibiotics based on initial concentration in MEM 199, %
Vancomycin HCl	AV	17.27	3.56
	PV	30.87	2.73
Lincomycin HCl	AV	13.4	0.56
	PV	34.76	0.65
Polymyxin B	AV	0.00	0.00
	PV	0.00	0.00

AV = Aortic valve; PV = pulmonary valve.

Table 2. Residues of antibiotics in the heart valve allograft tissue (second phase)

Antibiotic	Tissue	Antibiotics residues detected by HPLC in tested tissue, µg/g	Residual antibiotics based on initial concentration in MEM 199, %
Vancomycin HCl	AV (nr)	18.14	0.88
	AV (r)	10.74	0.65
	PV (nr)	16.67	1.01
	PV (r)	11.92	0.72
Lincomycin HCl	AV (nr)	6.72	0.14
	AV (r)	8.44	0.21
	PV (nr)	7.58	0.19
	PV (r)	8.68	0.22
Polymyxin B	AV (nr)	0.00	0.00
	AV (r)	0.00	0.00
	PV (nr)	0.00	0.00
	PV (r)	0.00	0.00

AV = Aortic valve; PV = pulmonary valve; nr = not rinsed; r = rinsed.

carried out on allografts that were treated following the EHB protocol for allograft preparation, storage and thawing. The residues of antibiotics in the EHB heart valve allografts prepared by standard method amounted to, on average, 0.81% of vancomycin HCl (AVs 0.76%, PVs 0.86%) and 0.18% of lincomycin HCl (AVs 0.17%, PVs 0.20%). Again, no residues of polymyxin B sulfate were detected. The results are expressed as percentage of the initial dose of each antibiotic for 1 g of decontaminated tissue (table 2).

The concentrations of the antibiotics in the extraction solution in the calibration curve were close to the quantity of the

Table 3. Residual antibiotics values: Percentage of antibiotics in the non-rinsed and rinsed allografts compared to the threshold of the respective antibiotic

Allograft	Vancomycin HCl, %	Lincomycin HCl, %
Aortic		
Non-rinsed	0.019	0.018
Rinsed	0.016	0.009
Pulmonary		
Non-rinsed	0.031	0.038
Rinsed	0.031	0.032

Table 4. Residue levels of the 3 antibiotics used for allograft decontamination in EHB

Allograft type	Antibiotic					
	Vancomycin HCl, %		Lincomycin HCl, %		Polymyxin HCl, %	
	AV	PV	AV	PV	AV	PV
Treated and rinsed	0.002	0.003	0.002	0.004	0.00	0.00
Treated and not rinsed	0.002	0.003	0.0009	0.003	0.00	0.00

AV = Aortic valve; PV = pulmonary valve.

Fig. 1. Chromatograms of residual antibiotics. **(A)** Polymyxin sulfate, **(B)** lincomycin HCl, **(C)** vancomycin HCl (**a**, control; **b**, aortic valve; **c**, pulmonary valve).

antibiotics in the initial testing. Further, we calculated the amount of each antibiotic starting from the calibration line. Vancomycin HCl was present at 18.14 $\mu\text{g/g}$ in the non-rinsed and at 10.74 $\mu\text{g/g}$ in the rinsed AVs and at 16.67 $\mu\text{g/g}$ in the non-rinsed and 11.92 $\mu\text{g/g}$ in the rinsed PVs. On the contrary, the concentration of lincomycin HCl in the non-rinsed allografts was 6.72 and 7.58 $\mu\text{g/g}$ for the AVs and PVs, respectively, and in the rinsed allografts it was 8.44 and 8.68 $\mu\text{g/g}$ in the AVs and PVs, respectively (table 3).

The chromatograms in figure 1 show the peak concentrations of the 3 antibiotics in the examined extraction solution and the time of their release for each of the 3 antibiotics of the EHB cocktail.

The thresholds of the residues of the 2 retained antibiotics in the allograft tissue were largely below the thresholds declared by the ICH Q3B (impurities in new drug products), which are 0.05% for vancomycin HCl and lincomycin HCl and below 0.01% for polymyxin B sulfate (table 4).

B

Discussion

The clinical use of heart valve and vascular allografts has been markedly limited by their availability [3]. Therefore, the retrieval of the allograft source material from the NHBDS was envisaged as one of the possibilities for increasing the yield of cardiovascular allografts. Consequently, some of the procured cardiovascular tissues arriving in the Tissue Establishments are primarily contaminated [8]. Earlier methods used for allograft decontamination were harmful for allograft integrity [14–19].

In 1969, Magdi Yacoub and Bryan Barratt-Boyes introduced the antibiotic decontamination of heart valve allografts [19, 20]. Different protocols (with varying combinations of antibiotics, duration of incubation, and temperature conditions) were adopted in different Tissue Establishments [3, 4, 5, 19, 20], with relatively good success in the eradication of contaminant germs. However, some antibiotics, being part of the decontamination cocktail, may have led to premature allograft deterioration, necessitating reoperation within a few years and thereby potentially endangering the patient's life. For this reason, many

Tissue Establishments have adopted the less toxic antibiotics and accepted a decrease in the yield of allografts due to insufficient decontamination. Every year, as many as 5–10% of all processed allografts have been discarded for insufficient decontamination in our Tissue Establishment [2]. However, due to the low toxicity of our antibiotics cocktail, the quality of the heart valve and vascular allografts was high and the rate of explantation of these allografts was relatively low [11, 21].

Following the European Directives 2004/23EC and 2006/86 EC, the European Association of Tissue Banking Standards

and the Belgian Standards on Tissue Banking, traceability of all the substances coming into contact with the prepared human tissues for human therapeutic application is necessary. The authors of this study analyzed the traces of residual antibiotics in the heart valve allografts after the preparation of the allograft for implantation (thawing and dilution). As at the end of incubation in the antibiotic cocktail the antibiotics are supposed to be removed from the allograft tissue, our purpose was to search for residues of these antibiotics that might have been retained in the tissue. 2 of the 3 antibiotics of the

EHB cocktail were retained in the heart valve allografts: vancomycin HCl and lincomycin HCl.

According to the World Health Organization (WHO), the minimum inhibitory concentration (MIC) is the lowest concentration of 1 antibiotic to inhibit the visible growth of 1 germ. The MIC of the 3 antibiotics of the EHB cocktail against *Staphylococcus aureus* are 4–16 µg/ml for vancomycin HCl [22], 0.125–1 µg/ml for 21 different germs for lincomycin HCl [23], and 0.01–14 µg/ml for polymyxin B sulfate [24].

The maximum daily doses of the antibiotics of our cocktail are the following: 2.0, 1.8 and 0.4 g for vancomycin HCl, lincomycin HCl and polymyxine B sulfate, respectively. Following these levels and applying the ICH Q3B standard (impurities in new drug products), the thresholds of these 3 antibiotics are 0.05% for vancomycin HCl and lincomycin HCl and 0.01% for polymyxin B sulfate. In this study, the traces of vancomycin and lincomycin found in the heart valve allografts were ≤ 0.05% (vancomycin, 0.075 for AVs and 0.031 for PVs; lincomycin, 0.0135 for AVs and 0.035 for PVs), whereas polymyxin B was not retained in any allograft tissue sample after rinsing. These concentrations do not present any risk for the patient receiving the EHB cryopreserved heart valve and/or vascular allograft.

The antibiotic cocktail used in EHB contains 3 low-concentration antibiotics that are largely below their therapeutic dosage in clinical practice. Combination of these antibiotics is supposed to cover a huge bacterial spectrum (Gram-positive, Gram-negative germs, aerobic and anaerobic germs) with low toxicity for in vitro use. Each of the 3 antibiotics has shown potentially toxic effects during in vivo use (nephrotoxicity, neurotoxicity, and hepatotoxicity), when used for the eradication of important infections and septic states of the patients. However, their use in our antibiotics cocktail is restricted to only a temporary contact with the tissue (during the allograft processing), followed by their removal from the tissue before their final preparation for implantation into the patient. Indeed, their amounts in the allograft are very low, as shown in this study. Although residues of vancomycin HCl and lincomycin HCl are detected in all heart valve allografts, their concentrations were very low and without risk for the allograft recipient.

Polymyxin B was not detected in any of the allograft samples. In vitro, after 72 h, this antibiotic is inactivated in the heart tissue because of its affinity to the phospholipids of the cell membranes [25]. This antibiotic is relatively toxic for some tissues. However, due to its ability to neutralize the endotoxin in Gram-negative infections, its use is important for the destruction of Gram-negative germs in the allograft [22]. However, polymyxin B shows important toxicity for liver

and kidneys and is known as a releaser of histamines in the body, which may provoke hypersensitivity reactions. However, due to the fact that this antibiotic is not retained in the allograft tissue at all, it is safe to use this antibiotic in vitro for allograft decontamination. Furthermore, there are no reports of any toxic effect of heart valve allografts during the EHB's long experience with allograft allocation worldwide. On the contrary, a very low recurrence of infections was reported by diverse authors [7, 14, 18–20, 26].

Heart valve and vascular allografts have shown increased resistance to infection. Therefore, they are preferably used by cardiac and/or vascular surgeons for the treatment of endocarditis or infected vascular prostheses [3]. There have been controversial opinions as to why the allografts show such an increased resistance to infection: either due to the viable cellular components or due to the remaining antibiotics in the allograft tissue [3, 5, 7, 14, 20, 26]. The reason for this increased resistance to infection might be due to residual concentrations of the antibiotics that are used for their decontamination. This is why many cardiac surgeons prefer allografts for the treatment of heart valve endocarditis rather than the prosthetic material.

Conclusions

Although residues of vancomycin HCl and lincomycin HCl were detected in the cryopreserved human heart valve allografts at the end of processing, their concentrations were largely below the daily therapeutic doses of these antibiotics and they do not present any risk for the allograft recipient. The presence of the antibiotics in the tissue may even be the reason for the increased resistance of the heart valve and vascular allografts against infection in the early period after implantation. This is particularly important when treating cases with infection problems (endocarditis, infection of prosthetic material).

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Disclosure Statement

The authors declared no conflict of interest.

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