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Research paper

Evaluation of collagen/hydroxyapatite electrospun layers loaded with vancomycin, gentamicin and their combination: Comparison of release kinetics, antimicrobial activity and cytocompatibility

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ABSTRACT

The aim of this study was to develop a biodegradable nanostructured electrospun layer based on collagen (COL), hydroxyapatite nanoparticles (HA), vancomycin hydrochloride (V), gentamicin sulphate (G) and their combination (VG) for the treatment of prosthetic joint infections and the prevention of infection during the joint replacement procedure. COL/HA layers containing different amounts of HA (0, 5 and 15 wt%) were tested for the *in vitro* release kinetics of antibiotics, antimicrobial activity against MRSA, gentamicin-resistant *Staphylococcus epidermidis* and *Enterococcus faecalis* isolates and cytocompatibility using SAOS-2 bone-like cells. The results revealed that the COL/HA layers released high concentrations of vancomycin and gentamicin for 21 days and performed effectively against the tested clinically-relevant bacterial isolates. The presence of HA in the collagen layers was found not to affect the release kinetics of the vancomycin from the layers loaded only with vancomycin or its combination with gentamicin. Conversely, the presence of HA slowed down the release of gentamicin from the COL/HA layers loaded with gentamicin and its combination with vancomycin. The combination of both antibiotics exerted a positive effect on the prolongation of the conversion of vancomycin into its degradation products. All the layers tested with different antibiotics exhibited potential antibacterial activity with respect to both the tested staphylococci isolates and enterococci. The complemental effect of vancomycin was determined against both gentamicin-resistant *Staphylococcus epidermidis* and *Enterococcus faecalis* in contrast to the application of gentamicin as a single agent. This combination was also found to be more effective against MRSA than is vancomycin as a single agent. Importantly, this combination of vancomycin and gentamicin in the COL/HA layers exhibited sufficient cytocompatibility to SAOS-2, which was independent of the HA content. Conversely, only gentamicin caused the death of SAOS-2 independently of HA content and only vancomycin stimulated SAOS-2 behaviour with an increased concentration of HA in the COL/HA layers. In conclusion, COL/HA layers with 15 wt% of HA impregnated with vancomycin or with a combination of vancomycin and gentamicin offer a promising treatment approach and the potential to prevent infection during the joint replacement procedures.

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1. Introduction

The increasing number of primary joint replacement procedures has led to a corresponding increase in the need for endoprosthesis revision surgery due to the incidence of bacterial infections resulting either from contamination caused by microorganisms intrinsic to the patient or from external contamination initiated at the time of surgery. Thus, the need has arisen to develop novel strategies that are safe and easily implementable, and which reduce the risk of the reoccurrence of infection. The usual strategy is based on surgical revision involving the removal of the foreign material and supplementary antimicrobial therapy, in which case antibiotics must be administered at high dosages for an extended period of time [1].

In recent years *Staphylococcus aureus* resistance to vancomycin has emerged as a serious clinical problem as opposed to the more frequently encountered low-level vancomycin resistance in the form of vancomycin-intermediate *S. aureus* (VISA) and heterogenous vancomycin-intermediate *S. aureus* (hVISA). Vancomycin-resistant *Staphylococcus aureus* (VRSA) is most common in elderly patients, especially those with a history of vancomycin-resistant enterococci [2]. One possible explanation as to why infections caused by VRSA are becoming more commonly reported is that vancomycin breaks down over time to form antimicrobially ineffective crystalline degradation products [3]. A further reason for concern is the increased occurrence of a genetically different strain known as methicillin-resistant *Staphylococcus aureus* (MRSA) or multi-resistant *S. aureus*. No ideal antibiotic for the treatment of MRSA has yet been developed; therefore, the usual option in this case is the combination of antibiotics aimed at "filling the gaps" caused by the failure of individual agents [4]. Kotlus et al. [5] discovered that MRSA ocular isolates are highly sensitive to a combination of vancomycin and gentamicin and Kang et al. [6] proved the synergistic effects of vancomycin combined with gentamicin against VISA, hVISA and vancomycin-susceptible *S. aureus* (VSSA).

Currently, the gold standard for the development of bone infection therapy consists of antibiotic-loaded carriers based on inert materials such as mineral cements or polymethylmethacrylate (PMMA) beads [7–10]. The synergistic inhibitory effect of PMMA cements loaded with gentamicin and vancomycin against all the tested strains without the presence of bacterial growth has been proved *in vitro* [11–13], *in vivo* [14–16] and in clinical studies [17,18]. However, since PMMA cements are only marginally porous, the diffusion of loaded antibiotics into the surrounding bone tissue is limited. In contrast, mineral cements based on calcium phosphate are microporous and exhibit good compatibility and the sufficient release of many different types of drugs [19]. Vorndran et al. [20] reported novel calcium phosphate cement paste formulations intended for use as a vancomycin and gentamicin controlled-release system. Morley et al. [21] demonstrated the successful use of a synthetic, biodegradable and biocompatible form of calcium sulphate paste as a drug delivery system for the treatment of a deep diabetic foot infection complicated by osteomyelitis. Further, biodegradable antibiotic delivery systems such as collagen, polylactide (PLA), polylactide–polyglycolide (PLGA), polycaprolactone (PCL) and bone graft-based vehicles may be more effective than antibiotic delivery via inert carriers since it is possible to achieve complete and effective drug release without the necessity for additional surgery. Chen et al. [22] investigated the *in vitro* release of vancomycin, gentamicin and lidocaine from novel electrospun sandwich-structured PLA/PLGA/collagen nanofibrous membranes and determined that biodegradable nanofibrous membranes released concentrations of vancomycin and gentamicin well above the minimum inhibition concentration for 4 and 3 weeks respectively. Bennett-Guerrero et al. [23] demonstrated the application of the novel biodegradable modified-release gel DFA-02 containing sesame oil, soy lecithin and dehydrated alcohol as excipients and consisting of gentamicin and vancomycin. Concentrations of each antimicrobial agent were found to be at least 4 times higher than the minimum inhibitory concentration for sensitive pathogens and demonstrated high local concentrations *in vivo* over multiple days.

Due to the paucity of well-defined comparative studies [24–26], the question remains as to whether monotherapy or a combination therapy of vancomycin and gentamicin is more effective in the context of adequate biocompatibility, effective antibiotic release and the prevention of infection. This study, which provides a follow-up to work already published by the research team [27,28], addresses the development of a biodegradable nanostructured electrospun composite layer based on collagen, hydroxyapatite nanoparticles, vancomycin hydrochloride, gentamicin sulphate and on the combination thereof. The study [27] considers the sustainable release of vancomycin only, with an emphasis on the monitoring of its antibiotically active and inactive degradation products, a subject that has generally been ignored in the literature to date. Moreover, the paper [28] also provides an assessment of the influence of three different preparation techniques (lyophilization, electrospinning and impregnation) on the vancomycin release kinetics.

In addition, the study evaluates the efficiency of gentamicin and its combination with vancomycin aimed at determining the potential synergistic effect of this combination compared with that of vancomycin alone. The *in vitro* release kinetics of the antibiotic components is assessed by means of the high performance liquid chromatography method and ultra-high performance liquid chromatography with tandem mass spectrometry. The antimicrobial effects of the layers containing the various antibiotics are studied employing the agar diffusion testing technique against four different clinical bacterial isolates, an examination is conducted of the role of hydroxyapatite nanoparticles in antibiotic release, and details are provided of an *in vitro* biological evaluation which was conducted using SAOS-2 cells in direct contact with the layers, and a cytotoxicity test of 24-hour extracts thereof. It is anticipated that the layer will be used particularly in the case of known prosthetic joint infections or as a preventative procedure for primary joint replacement at a potentially contaminated site involving the controlled elution of both antibiotics so as to provide a powerful antibacterial device against a range of clinically relevant bacteria.

2. Materials and methods

2.1. Preparation of the materials

The collagen/hydroxyapatite layers (COL/HA layers) were prepared by means of the electrospinning of 8 wt% collagen (COL; type I, VUP Medical, Czech Republic), a PBS/ethanol solution modified with 8 wt% (to the COL) polyethylene oxide (PEO; Mr. 900,000, Sigma Aldrich, Germany) with dispersed 0, 5 or 15 wt% of hydroxyapatite particles (HA; avg. 150 nm, Sigma Aldrich, Germany). The detailed preparation of the electrospun mats via electrostatic spinning (4SPIN, Contipro, Czech Republic; voltage 45 kV, feeding rate130 $\mu l \cdot min^{-1}$, temperature 24 °C, relative humidity 20–25%) in combination with electroblowing (preheated air at 25 °C, 30 $l \cdot min^{-1}$) is described in detail elsewhere [27]. The stability of all the collagen layers was enhanced by means of cross-linking (24 h, 37 °C) with a 95% ethanol solution with N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) at a weight ratio of 4:1 (both Sigma Aldrich, Germany) and washed in 0.1 M Na_2HPO_4 (2 × 45 min) and deionised water (30 min), frozen at −15 °C for 5 h and lyophilised (BenchTop 4KZL, VirTis, U.S.A.). Following cross-linking, the PEO and NaCl were fully leached out. Finally, the cross-linked COL/HA mats were impregnated with an ethanol solution of vancomycin (V; vancomycin hydrochloride, Mylan S.A.S, France) or gentamicin (G; gentamicin sulphate, Sigma Aldrich, Germany) or with a V/G combination (1/1, w/w) in a final amount of 10 wt% of antibiotics per COL/HA layer and dried at room temperature in a laminar box until a constant weight was achieved (up to 2 h). The morphology of the electrospun COL/HA layers was assessed by means of electron scanning microscopy (SEM). The samples intended for SEM analysis were sputter coated with gold and analysed using a Quanta 450 microscope (FEI, USA).

2.2. Release kinetics

The *in vitro* release of vancomycin and gentamicin from the COL/HA layers prepared with V, G and V/G antibiotics was carried out by means of the high performance liquid chromatography method (HPLC) and via ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). Six samples of each type of layer were placed on a sterile gauze pad and firmly caulked prior to being transferred to separate test tubes containing a weight/volume ratio of 200 mg/20 ml of PBS (pH 7.4) which were then placed in an incubator at a temperature of 37 °C. Samples (200 µl) were taken at defined intervals (3, 6, 12, 24, 48, 96, 144, 192, 240, 288, 336, and 504 h from the start of the experiment), and stored at −70 °C. The volume was replenished with fresh phosphate buffer (95 ml of 0.2 M $NaH_2PO_4 \cdot H_2O$, 405 ml of 0.2 M Na_2HPO_4. 7 H_2O and 500 ml of water corrected to pH 7.4).

Solid phase extraction was employed for the pre-treatment of the samples containing vancomycin. The extraction cartridges, connected to a Visiprep Solid Phase Extraction Vacuum Manifold (Sigma-Aldrich), were washed firstly with 1.6 ml of methanol, and then with 0.8 ml of water. The samples were loaded onto the cartridges, and the cartridges were washed again with 1.6 ml of water. The analytes were eluted with the mixture of phosphate buffer - acetonitrile (3:7), and acetonitrile - water (1:1). The samples were then evaporated under a stream of nitrogen to the final volume 1 ml of the sample. HPLC analysis was then employed in order to characterise the *in vitro* release rates of the vancomycin and its crystalline degradation antibiotically-inactive products over a 21-day period. HPLC was performed using an Agilent 1260 system equipped with a diode array detector, quaternary pump system, column thermostat, and an auto sampler (Agilent Technologies, USA). Separation was accomplished by column Zorbax Sb-Ag (4.6 × 250 mm, particle size 5 µm) (Agilent Technologies, USA). The column was maintained at a temperature 22 °C. A gradient of mobile phases A and B was used. Mobile phase A consisted of 910 ml of phosphate buffer (2.14 g K_2HPO_4 and 11.94 g KH_2PO_4 dissolved in 2 L of distilled water, pH 7.4), 50 ml of acetonitrile and 40 ml of methanol. Mobile phase B consisted of 840 ml of the same phosphate buffer, 80 ml of acetonitrile and 80 ml of methanol. The flow rate was set at 1.5 ml/min. The total run time per sample was 28 min. The detector wavelength was set at 210 nm and the injected volume of the sample was 50 µl. Cefazolin was selected as the internal standard at a concentration of 100 mg/l. The chromatograms were processed via OpenLab (Agilent Technologies).

The UHPLC-tandem mass spectrometry method was used for the determination of gentamicin C components. Mass spectrometric detection was performed on a triple-quadrupole in the positive electrospray ionisation mode by means of multiple reaction monitoring (Agilent 1290 with Triple Quad 6470, Agilent Technologies, USA). Tobramycin (1 mg/l in 10% trichoroacetic acid) was taken as the internal standard. The developed method for the determination of gentamicin provides for the separation of the C1, C1a and C2 components. The selected reaction monitoring of the precursor-product ion transitions *m/z* of 478.5 → 322.1 (C1), 450.4 → 322.4 (C1a), 464.5 → 322.4 (C2 + C2a + C2b) for gentamicin, and 468.3 → 163.2 for tobramycin were employed for quantification purposes. 25 µl of the sample was mixed with 50 µl of the internal standard for the precipitation of the proteins. The mixture was then centrifuged. Chromatographic separation was conducted using an InfinityLab Poroshell 120 PFP column (2.1 × 100 mm, 2.7 µm, Agilent Technologies) employing the binary gradient of the mobile phases (A – water with 1% heptafluorobutyric acid, B – 90% acetonitrile with 1% heptafluorobutyric acid) at a flow rate of 0.4 ml/min; separation was accomplished at 35 °C and the analysis time was 4 min. The LC-MS/MS method was successfully validated. The intra- and inter-day accuracy and precision were evaluated on two QC samples by means of multiple analysis, and the coefficients of variation were determined at 2.2–8.5% for the intra-assay, 3.2–9.3% for the inter-assay, and 6.6–9.8% for the precision.

Table 1
Susceptibility of the microorganisms used in this study. Susceptibility to vancomycin and gentamicin was estimated using standard antibiotic discs and the diffusion test.

Microbial species	Strain ID	Vancomycin susceptibility	Gentamicin susceptibility
S. aureus	MRSA	susceptible	susceptible
S. epidermidis	SE	susceptible	resistant
E. faecalis 1	EF1	susceptible	resistant
E. faecalis 2	EF2	susceptible	susceptible

2.3. Antimicrobial activity

The antimicrobial activity of the COL/HA layers with different antibiotics was tested employing four different bacteria isolated from patients suffering from severe infections, i.e. *Staphylococcus aureus, S. epidermidis* and two *Enterococcus faecalis* isolates (Table 1), and with differing susceptibility to the antibiotics used in this study. The disc diffusion test was performed using a Mueller-Hinton agar medium (Oxoid Ltd., Hampshire, UK) with the test bacteria according to the EUCAST recommendation [40]. Discs of 6 mm in diameter ($n = 7$) prepared from the tested COL/HA layers loaded with the appropriate antibiotics were firmly applied to the dried surface of the inoculum agar plates using a sterile needle and further incubated at 37 °C for 24 h. The diameters of the inhibition zones were subsequently measured. Standard 6 mm antibiotic discs were used as standard controls, i.e. vancomycin 30 µg and/or gentamicin 10 µg for *S. aureus* and *S. epidermis*, and vancomycin 5 µg and/or gentamicin 30 µg for both *Enterococci*. COL/HA layers prepared without antibiotics were used as negative controls.

2.4. Biological evaluation of the COL/HA layers

Three different approaches were applied in order to address the cytotoxicity of the COL/HA layers. COL/HA layer extracts as well as the layers themselves were tested for the metabolic activity of SAOS-2 bone-like cells and the morphology of the SAOS-2 cultured on the COL/HA layers was analysed ($n = 4$). In addition to the effect of antibiotics, the influence of the differing HA content in the layers (0, 5 or 15 wt%) on SAOS-2 metabolic activity and morphology was also evaluated ($n = 4$).

2.4.1. SOAS-2 culture

Human osteoblast cells (SAOS-2 cell line derived from osteosarcoma, DSMZ, Germany) were cultured in McCoy's 5A medium without phenol red (PromoCell, Germany) supplemented with 15% heat inactivated foetal bovine serum (FBS) (PAA, Austria), penicillin (20 U/ml, Sigma-Aldrich, USA) and streptomycin (20 µg/ml Sigma-Aldrich, USA) at 37 °C in 5% CO_2.

2.4.2. COL/HA layer extracts test

The COL/HA layers were fixed by means of CellCrown™ inserts (Sigma-Aldrich, USA) in 48-well plate (Thermo Scientific, USA), covered by 800 µl of fully supplemented McCoy's 5A medium and incubated at 37 °C and 5% CO_2 for 24 h. Subsequently, 400 µl of the COL/HA layer extracts were transferred onto SAOS-2 pre-seeded on 48-well plate (20,000 cells/cm^2) 20 h prior to the treatment of the extracts. The metabolic activity of the SAOS-2 was measured 24 h following cultivation. SAOS-2 cultivated in a standard medium without layer extracts in 48-well plate were used as the control.

2.4.3. Direct COL/HA layer test

The layers were fixed by means of CellCrown™ inserts (Sigma-Aldrich, USA) in 48-well plate (Thermo Scientific, USA) for the direct cultivation ofSAOS-2 on the COL/HA layers. 15 000 cells/cm^2 were seeded on the COL/HA layers and the metabolic activity was measured

after 2 and 8 days of culturing. The medium was changed after 4 days of culturing. For control purposes, SAOS-2 were also cultivated on a 48-well plate standard tissue culture polystyrene surface (PS).

2.4.4. Metabolic activity measurement

The metabolic activity was measured (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, MTS, Promega, USA) (i) 24 h following SAOS-2 cultivation in COL/HA layer extracts, and (ii) 2 and 8 days following SAOS-2 cultivation directly on the COL/HA layers. The measurement of metabolic activity was performed according to the manufacturer's instructions. Absorbance (490 nm and 655 nm as the reference) was determined using a multi-detection micro-plate reader (SynergyTM 2, BioTek, USA). The results were normalised (in percentage) with respect to the controls.

2.4.5. Fluorescent microscopy of the SAOS-2 cultured on the COL/HA layers

Following the measurement of metabolic activity, the SAOS-2 cultured directly on the layers for 2 and 8 days were fixed (4% paraformaldehyde in PBS, at room temperature (RT) for 15 min; Sigma-Aldrich, USA) and permeabilised (0.1% Triton X-100 in PBS at RT for 20 min; Sigma-Aldrich, USA). The cell nuclei were then stained with DAPI (dilution 1:1000, RT for 45 min; Sigma-Aldrich, USA) and the actin filaments with Phalloidin-Alexa Fluor 488 (dilution 1:500, RT for 45 min; Life Technologies, USA). Wide field images (10x and 40x lens) of the cells on the layers were acquired using an Eclipse Ti-S microscope and a DS-U2 digital camera (Nikon, Japan) and adjusted via the use of ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2015) and Cell Profiler (Broad Institute, USA) software.

2.5. Statistical evaluation

The statistical analysis was performed using statistical software (STATGRAPHICS Centurion XVII, StatPoint, USA). The normality of the data was verified primarily by means of the Shapiro-Wilk's and Chi-Squared tests; outliers were identified via either the Grubbs' or Dixon's tests. The mean values and variability of the normally distributed numerical data were expressed as the arithmetical mean and the standard deviation (SD), while the non-normally distributed numerical data was expressed as the median and interquartile range (IQR). Homoscedasticity was verified by means of the Levene's and Bartlett's tests. Non-parametric analysis was employed since either the assumption of normality or homoscedasticity were violated and, subsequently, the Kruskal-Wallis test for multiple comparison with the subsequent post-hoc test based on the Bonferroni procedure. The Mann-Whitney W test was performed in the case of two-sample comparisons. The Games-Howell test was performed for parametric multiple comparison purposes. Statistical significance was accepted at $p \leq 0.05$.

3. Results and discussion

3.1. Preparation of the materials

The prepared COL/HA layers are illustrated in Fig. 1. The structural and mechanical properties as well as the swelling and degradation of the prepared samples were evaluated in a previous study and were described by Suchy et al. [28].

3.2. Release kinetics

The release kinetics profiles of vancomycin and gentamicin from the layers prepared in different ways were characterised by means of HPLC (vancomycin) and UHPLC-tandem mass spectrometry (gentamicin) (Fig. 2).

Initial measurements after 3 h revealed average concentrations of vancomycin of 540–700 mg/l (V15–V0) and 110 – 150 mg/l (VG0–VG15), and gentamicin concentrations of 320–630 mg/l (G5–G0) and 130–340 mg/l (VG15–VG0). The highest concentration of vancomycin was recorded with respect to the V samples immediately after 3 h (V0; approx. 700 mg/l) and the VG samples after 96 h (VG15; approx. 240 mg/l). The highest concentration of gentamicin was recorded with respect to the G samples after 96 h (G5; approx. 1020 mg/l) and the VG samples after 3 h (VG0; approx. 340 mg/l). The addition of hydroxyapatite exerted only a minor effect on vancomycin release in the case of both the V and VG layers. Conversely, the release of gentamicin was significantly higher in the case of the layers containing no hydroxyapatite (G0 and VG0), while the addition of HA decreased the rate of gentamicin elution from the 5 and 15 wt% layers within the first 6 h (G5, G15) and during the whole 21-day period (VG5 and VG15).

Vancomycin contains two basic and four acidic groups (three phenolic and one carboxylic) which participate in acid-base equilibria. Data on the protonation macroconstants of vancomycin, its isoelectric point and the distribution of its variously protonated species as a function of pH have been studied in detail by Takács-Novák et al. [29] who determined that the isoelectric point is attained at a pH of 8.3 with the neutral form H_4V. Otherwise, vancomycin exists in six perprotonated or deprotonated forms through the pH scale (from 0 to 14); all these macrospecies are composites of protonation isomers. The perprotonated H_6V^{2+} and H_5V^{+} forms (pH = 0–7.75) are able to interact with phosphate ions whereas the deprotonated H_3V^{-}, H_2V^{2-}, HV^{3-} and V^{4-} forms (pH = 8.89–14) provide groups with negative charges that are potentially able to interact with calcium ions. Vancomycin provides charged groups for the binding of both calcium and phosphate ions; however, their mutual interactions are influenced by the protonation-deprotonation equilibria of vancomycin at various pH values. At pH 7.4 penta- and tetraprotonated species are predominant which are close to the neutral form and with respect to which the average charge of vancomycin is only +0.67; therefore, it is possible to assume that bonding to phosphates is the preferred option.

In contrast, basic antibiotics such as gentamicin contain only amino groups (they do not contain carboxylic groups); though it is possible that they co-precipitate with phosphates, this mechanism is less likely than the reaction between carboxylic and calcium ions. However, Stigter et al. [24] determined that the real incorporation efficiency of antibiotics correlated not only with theoretical premises such as the presence and number of carboxylic and amino groups, but also with chemical structure and molecular weight. They also proved that vancomycin with one carboxylic group exhibited a lower incorporation rate than gentamicin. Since vancomycin has a larger molecule than gentamicin, chemical steric hindrance may be a limiting factor in terms of binding to calcium ions.

The average concentration of CDP-1 M (a major vancomycin degradation product) was found to be similar to that of the active form of vancomycin with respect to the V samples after 10 days of incubation, and the VG samples after 21 days (Fig. 3). The conversion of vancomycin to the two conformal CDP-1 s isomers was practically twice as slow with respect to all the samples with a combination of vancomycin and gentamicin (approx. 21 days) than for the vancomycin samples (approx. 10 days). The rate of the conversion of vancomycin into its degradation products was much slower than the 6 days reported by Melicherčík et al. [3] with respect to all the samples containing vancomycin and its combination with gentamicin. Moreover, despite the variable tendency of vancomycin degradation towards crystalline thermal degradation products, levels of the released form of vancomycin remained above the MIC for VRSA (16 mg/l) and *Enterococcus* spp. (32 mg/l) throughout the full 21 days of experimentation [28]. Similarly, levels of released gentamicin remained above the MIC for VRSA (16 mg/l) and *Enterococcus faecalis* (16 mg/l) [30].

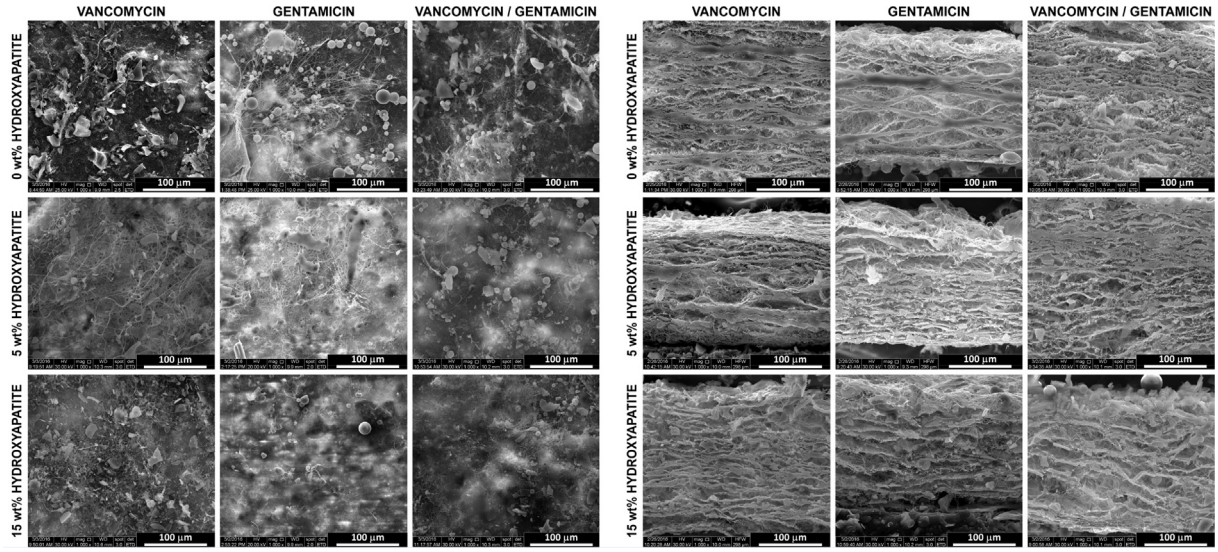

Fig. 1. Illustrative SEM images of the COL/HA layers with differing amounts of hydroxyapatite impregnated with vancomycin, gentamicin or vancomycin/gentamicin. Left panel - images of the surfaces of the layers; right panel - images of cross-sections of the layers.

3.3. Antimicrobial activity

The antimicrobial activity of all the COL/HA samples against the four bacterial strains were assessed by means of the presence or absence of inhibition zones (Fig. 4). The COL/HAlayers prepared without antibiotics and used as negative controls did not present any inhibition to the growth of the bacteria (data not shown).

The sizes of the inhibition zones surrounding the samples investigated were comparable or higher than those of standard antibiotic discs with vancomycin (CV) or gentamicin (CG), with the exception of the inhibition zone surrounding the G5 samples in the case of *E. faecalis* 2, with respect to which the inhibition zone diameter was slightly (but significantly) smaller. While no statistically significant differences between the CV and COL/HA vancomycin layers were determined with respect to *S. aureus* and *S. epidermidis*, significantly higher inhibition zones were discovered in the case of both the enterococci, as was reported in our previous vancomycin-related paper [28]. The complementary effect of the antibacterial activity was found to be present; this was visible particularly in the case of the gentamicin-resistant isolates, with respect to which vancomycin constituted the effective

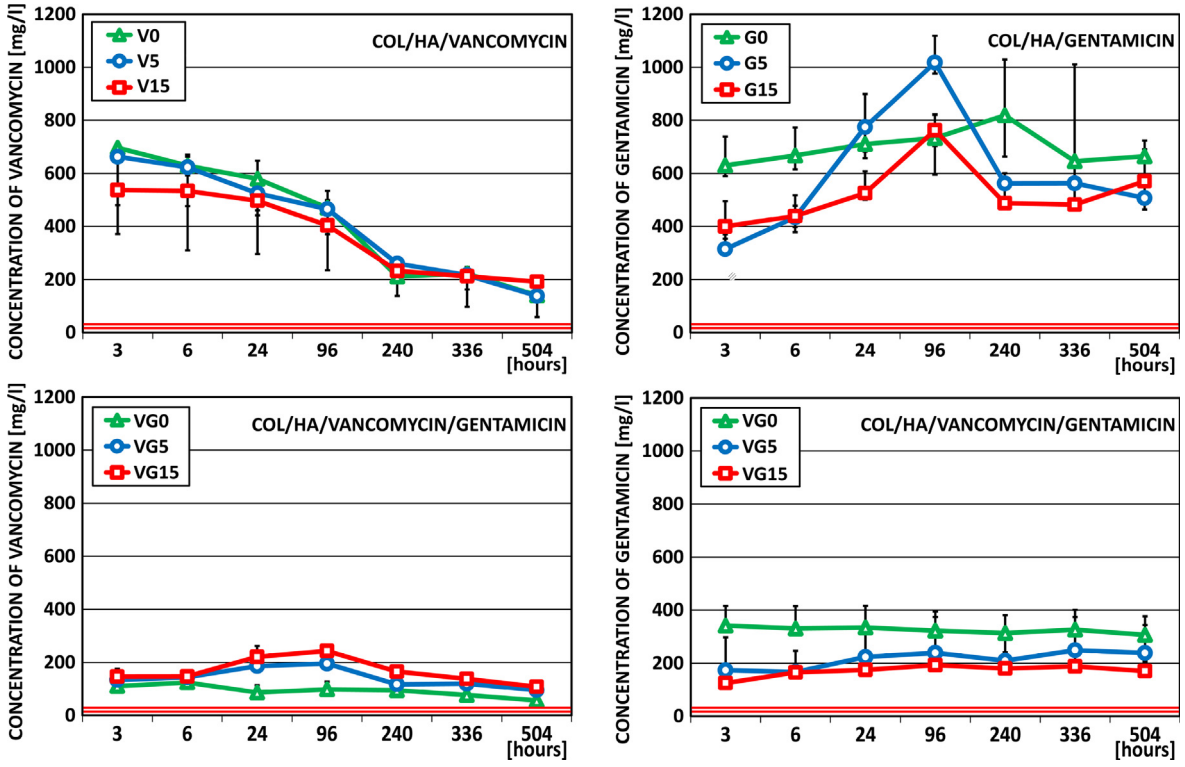

Fig. 2. Concentration of the released active forms of vancomycin and gentamicin (median, IQR, $n = 6$) from the COL/HA layers with differing amounts of hydroxyapatite and impregnated with vancomycin, gentamicin or vancomycin/gentamicin. V = vancomycin, G = gentamicin, VG = 1/2 vancomycin + 1/2 gentamicin; 0, 5, 15 = wt% of the HA in the COL/HA layers. The solid red lines denote the MIC for VRSA (16 mg/l) and *Enterococcus* spp. (32 mg/l). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

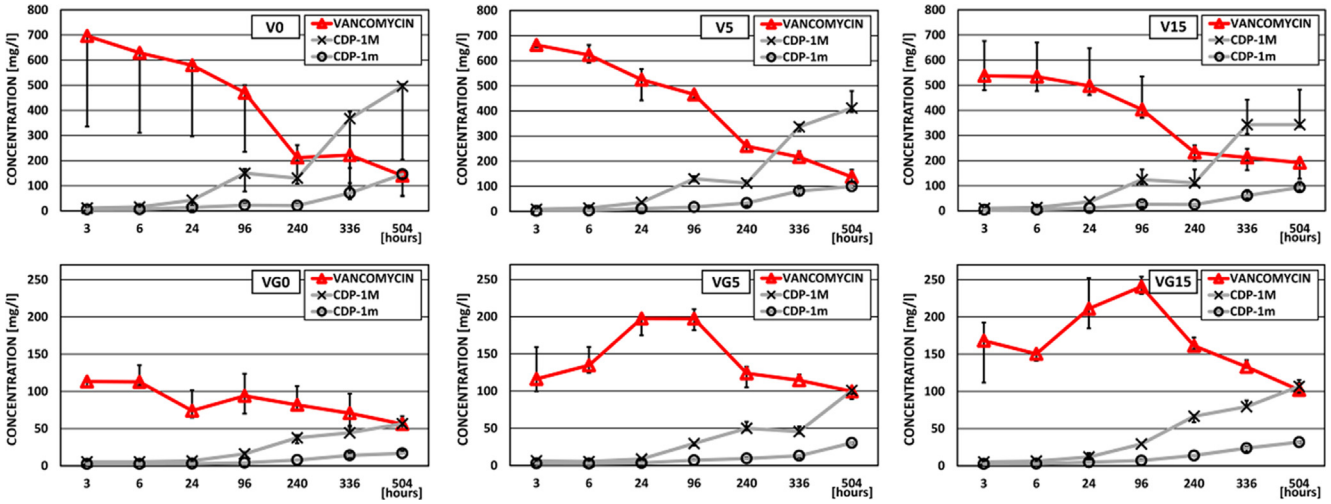

Fig. 3. Concentration of the released active form of vancomycin and its degradation products CDP-1 M and CDP-1 m (median, IQR, $n = 6$). V = vancomycin, VG = 1/2 vancomycin + 1/2 gentamicin; 0, 5, 15 = wt% of the HA in the COL/HA layers.

antimicrobial agent. Since no major antagonistic effects between vancomycin and gentamicin were determined, it is assumed that the complementary or possibly synergistic effects of the antibiotics applied will broaden the antimicrobial spectrum of the proposed material against various multiple resistant strains in a similar way to that described previously [31,32]. From this point of view, the combination of vancomycin with gentamicin, moreover at only half concentration, appears to be more effective than monotherapy with vancomycin or gentamicin. The size of the inhibition zones around the discs of the samples containing differing amounts of hydroxyapatite as a potential vancomycin and gentamicin binder appeared to exert no major influence on antibacterial activity.

3.4. Biological evaluation of the COL/HA layers

The cytotoxicity of the COL/HA layers with different antibiotics was characterised by metabolic activity and changes in the cell morphology of the SAOS-2 bone-like cells with respect both to vancomycin and gentamicin release and HA content.

Firstly, the cytotoxicity of the COL/HA layers was analysed using 1-day extracts of the layers containing released vancomycin and gentamicin. As shown in Fig. 5, the metabolic activity of SAOS-2 cultured in

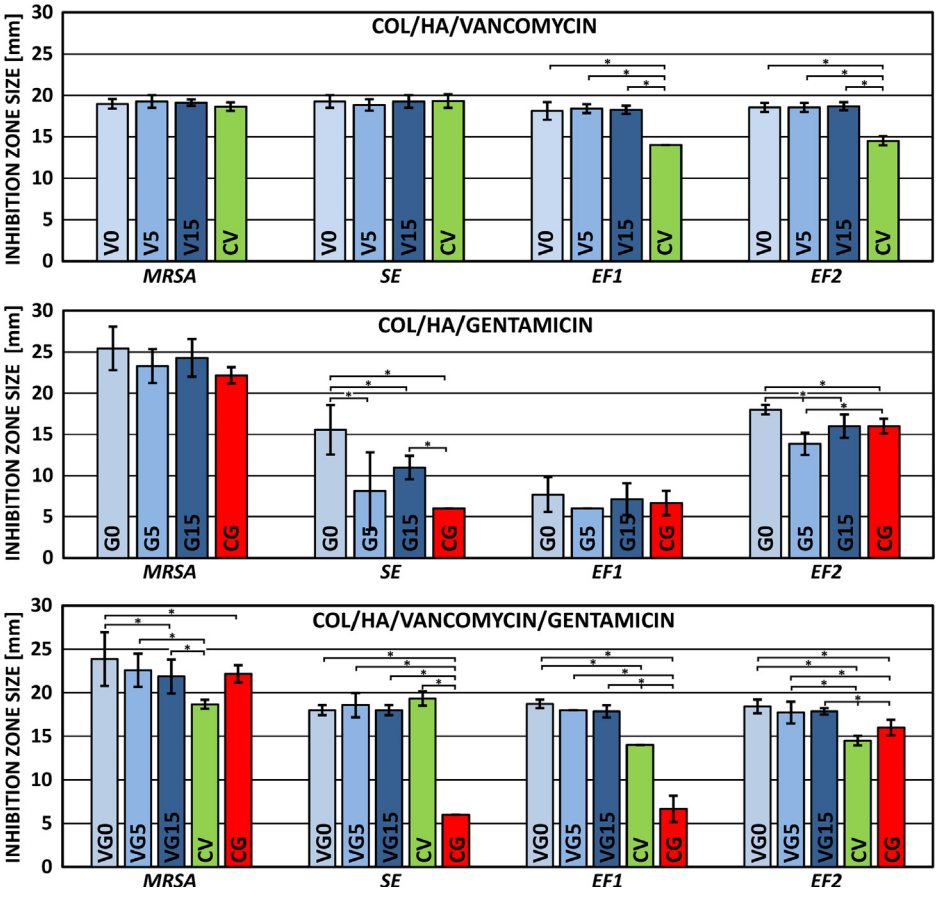

Fig. 4. Inhibition zone diameters (mean, SD) of the vancomycin, gentamicin and vancomycin/gentamicin loaded COL/HA layers with differing amounts of hydroxyapatite (0, 5, and 15 wt%) tested on clinically relevant bacterial isolates of methicillin-resistant *S. aureus* (MRSA), gentamicin-resistant *S. epidermidis* (SE) and two *E. faecalis* (EF1, EF2). The sign "*" denotes statistically significant differences (Games-Howell, *0.05, n = 7*). V = vancomycin, G = gentamicin, VG = 1/2 vancomycin + 1/2 gentamicin; 0, 5, 15 = wt% of the HA in the COL/HA layers. CV = control disc with vancomycin, CG = control disc with gentamicin.

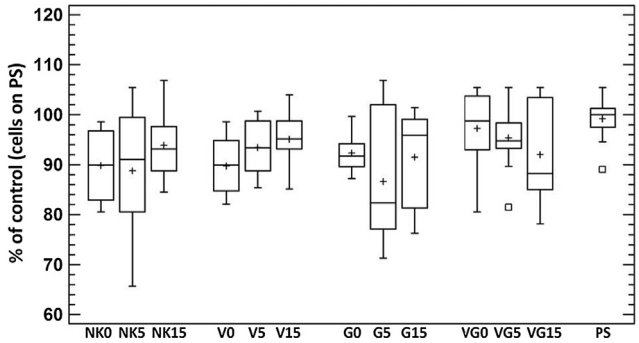

Fig. 5. Metabolic activity of SAOS-2 cultured for 24 h in 1-day COL/HA layer extracts expressed as a percentage of the metabolic activity of SAOS-2 cultured on polystyrene surfaces in culture media (PS). No statistically significant differences between the antibiotic groups (V, G, VG) and the control group (NK) were observed nor were any statistically significant differences determined between any of the COL/HA layer groups and the PS group (Kruskal-Wallis, Bonferroni procedure, *0.05*, *n* = *4*). V = vancomycin, G = gentamicin, VG = 1/2 vancomycin + 1/2 gentamicin; 0, 5, 15 = wt% of the HA in the COL/HA layers.

1-day extracts of the layers containing antibiotics was comparable to the metabolic activity of SAOS-2 cultivated on the same layers with no antibiotics (= the controls, NK0, NK5 and NK15). Similarly, the metabolic activity of SAOS-2 cultured in 1-day layer extracts of all the tested layers was found to be comparable to the metabolic activity of SAOS-2 cultivated on the standard polystyrene surfaces (PS). The only decrease in metabolic activity was indicated by the SAOS-2 cultivated in the G5 extract; however, this decrease was not only insignificant but also failed to reach the cytotoxicity level (75% of the control metabolic activity [33]). Similar results revealing the zero negative effect of vancomycin (1 g/l and less) on other bone-like cells (MG-63) have been observed previously [34,35]. In contrast to vancomycin, gentamicin doses of around 100 mg/l have been determined to be cytotoxic [34]. Thus, both the afore-mentioned studies support our observation of the general cytocompatibility of the layer extracts with the distinct cytotoxicity of gentamicin; this was further confirmed in this study by means of the investigation of the metabolic activity of SAOS-2 cultured directly on the surfaces of the layers.

The cytotoxicity of the COL/HA layers with antibiotics (V, G, VG) and without antibiotics (NKs) was estimated by means of the metabolic activity of SAOS-2 cultivated directly on the COL/HA layers (Fig. 6). The metabolic activity values of the SAOS-2 cultured on the control layers without antibiotics (NK0, NK5 and NK15) were mutually comparable (i.e. with no statistically significant differences) (data not shown). However, a decrease in the metabolic activity of SAOS-2 cultured on the COL/HA layers with antibiotics compared to the relevant controls NK0, NK5 or NK15 was apparent with respect to almost all the samples, suggesting that at least a certain proportion of the cytotoxicity was induced by the antibiotics. The results further revealed the effect of the HA in the layers on the metabolic activity of SAOS-2 which, surprisingly, is vancomycin specific. On day 2 particularly, the VO and V5 COL/HA layers caused a significant decrease in the metabolic activity of SAOS-2 of in excess of 50% that of NK0 and NK5 respectively. On day 8, a decrease, albeit statistically insignificant, was also observed. On the other hand, the SAOS-2 exhibited a slight but statistically insignificant decrease in metabolic activity with respect to the V15 COL/HA layer on day 2 (around 20% compared to that of NK15) followed by a statistically insignificant increase to 160% of NK15 on day 8. A comparison of SAOS-2 behaviour on days 2 and 8 revealed that all the COL/HA layers (V0, V5 and V15) statistically significantly stimulated the metabolic activity of SAOS-2, indicating that COL/HA layers do not directly lead to cell death and detachment. Further, the significant positive effect of increasing the amount of HA in the COL/HA layers with vancomycin on the metabolic activity of SAOS-2 was detected after both 2 and 8 days of culturing (Fig. 6). This observation was confirmed by means of the fluorescent imaging of SAOS-2 cultured on the COL/HA layers for 2 and 8 days which revealed a greater number of cells on the COL/HA layers following 8 days of cultivation than on day 2, especially with concern to the COL/HA V5 and V15 layers. Moreover, the morphology of the SAOS-2 cultured on the COL/HA V5 and V15 layers was physiological with a cuboidal-like shape (Fig. 7). For more detailed images, see the Supplementary data. The observed positive influence of HA content on cells was not surprising since the positive effect of HA on cell adhesion, spreading morphology and migration have been described previously elsewhere [36,37]. In addition, the lower cytotoxic effect of the V15 layer can be alternatively explained by the presence of bovine serum albumin (BSA; the adhesion-inhibiting protein [38,39]) in the cultivation medium. It has been demonstrated previously that vancomycin is able to interact with BSA and thus alter its conformation [40]. Hence, the observed increase in cell viability may have been caused by the BSA, which is able to reduce the cytotoxic effect of vancomycin and, simultaneously, by the vancomycin–induced alteration of BSA which may lead to improved cell adhesion (via the elevated activity of adhesion-promoting proteins from FBS).

Completely different results were obtained concerning the COL/HA G0, G5 and G15 layers with gentamicin, with respect to which the metabolic activity of SAOS-2 decreased with increasing amounts of HA, especially after 8 days of culturing. Further, all the COL/HA layers with gentamicin were found to be significantly cytotoxic when the metabolic activity of SAOS-2 was around 10–20% of the SAOS-2 cultured on the corresponding controls (NKs) (Fig. 6). The fluorescent microscopy of

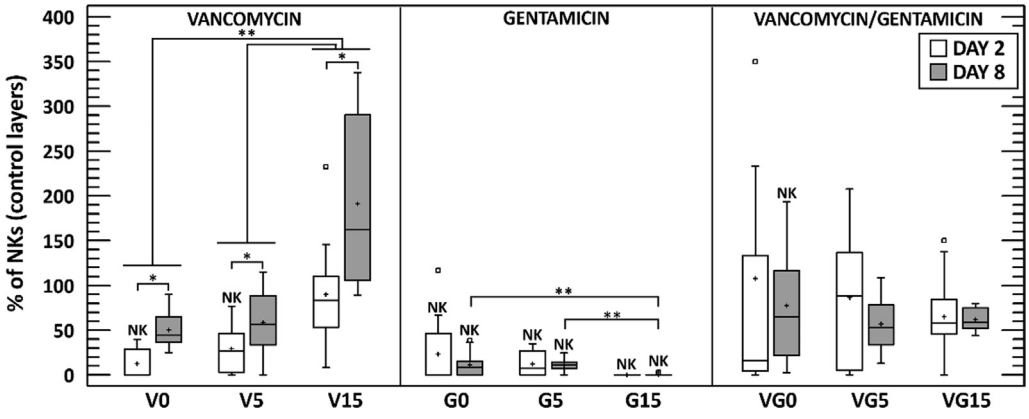

Fig. 6. Metabolic activity of SAOS-2 cultured on COL/HA layers with (V, G, VG) or without (NK) antibiotics for 2 days (white boxes) and 8 days (grey boxes). "NK" denotes statistically significant differences to the corresponding NK0, NK5 and NK15 controls (100% for each group) on the corresponding day (Kruskal-Wallis, Bonferroni procedure, *0.05*). The sign "*" denotes statistically significant differences between the time points of each of the tested layers (Mann-Whitney, *0.05, n = 4*). The sign "**" denotes statistically significant differences within the groups with the same antibiotics separately on the second and eighth days (Kruskal-Wallis, Bonferroni procedure, *0.05, n = 4*). V = vancomycin, G = gentamicin, VG = 1/2vancomycin + 1/2gentamicin; 0, 5, 15 = wt% of hydroxyapatite in the COL/HA layers.

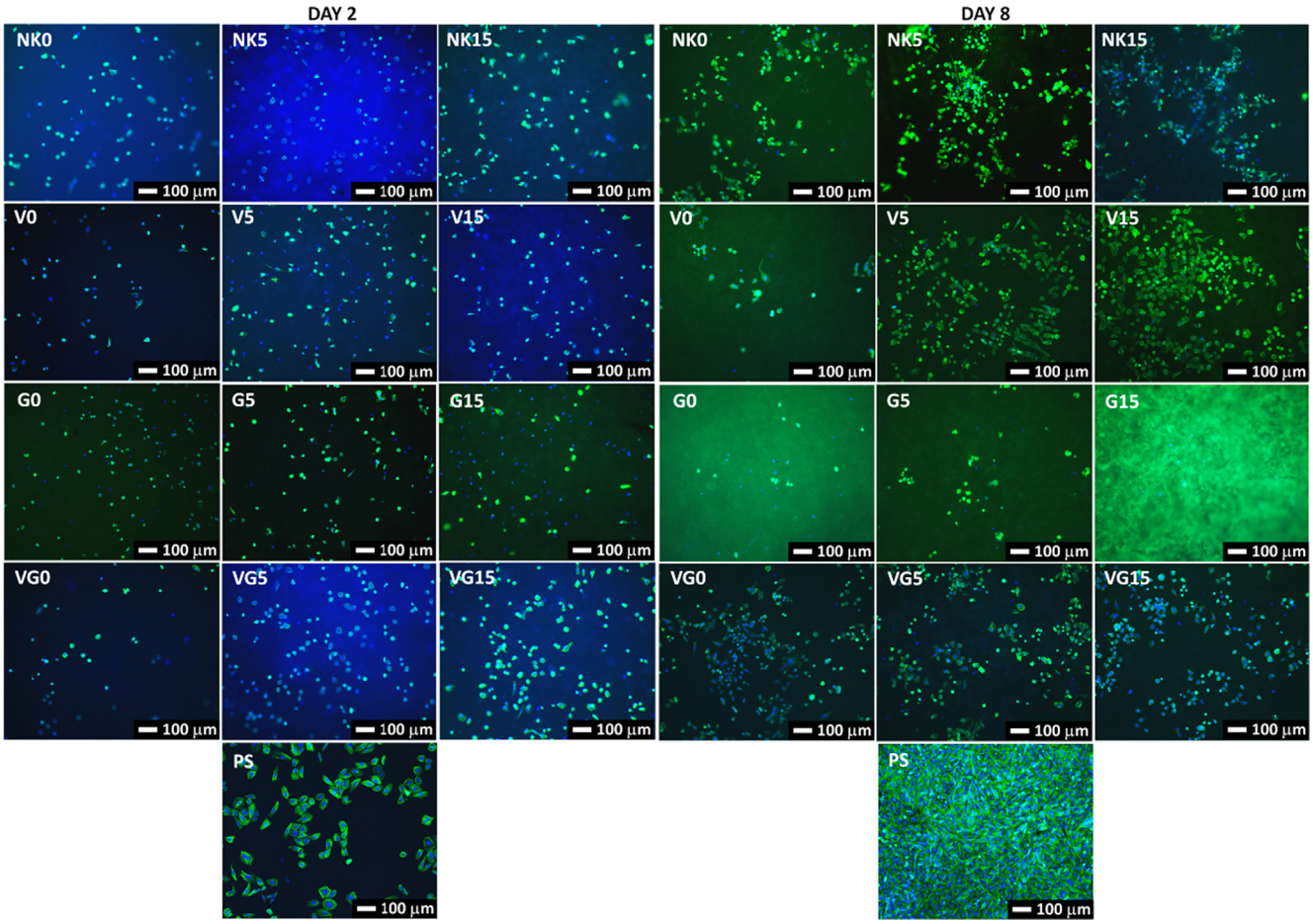

Fig. 7. Fluorescence images of SAOS-2 cultured for 2 and 8 days on COL/HA layers with (V, G, VG) and without (NK) antibiotics (mag. 10x). The actin filaments representing cell morphology are stained in green and the cell nuclei are stained in blue. V = vancomycin, G = gentamicin, VG = 1/2vancomycin + 1/2gentamicin; 0, 5, 15 = wt% of the hydroxyapatite in the COL/HA layers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the SAOS-2 cultured on these COL/HA layers proved this observation, i.e. the cells had not attached properly after 2 days of culture and had not proliferated after 8 days of culture and, moreover, their morphology exhibited a round-like shape indicating the cytotoxic effect of the gentamicin hindering the SAOS-2 attachment and proliferation processes (Fig. 7).

Finally, with respect to the COL/HA VG0, VG5 and VG15 layers, no significant effect of HA on the cytotoxicity of the antibiotics was determined; while the VG mixture exerted a moderate cytotoxic effect on SAOS-2 with a metabolic activity of around 50–80% of that of the corresponding NKs, it was not deemed statistically significant except with concern to the VG0 layer on day 8 (Fig. 6). Similar findings were obtained by means of fluorescent microscopy, according to which the cell count on the COL/HA VG layers did not increase between the 2nd and 8th days of cultivation, and the SAOS-2 exhibited both round-like and cuboidal-like morphologies indicating a non-toxic and non-stimulating cell culture environment (Fig. 7).

The limits of the positive effect of HA on SAOS-2 behaviour in relation to the type of antibiotic applied were both evident and surprising. It was supposed that this trend was closely connected to the differing release kinetics of vancomycin, gentamicin and their combination. The presence and amount of HA in the COL/HA layers did not affect therelease of antibiotics from the layers (Fig. 2) and, moreover, the antibiotics released from the layers after 1 day of extraction were not found to exhibit cytotoxicity towards the SAOS-2 (Fig. 5). However, the release of antibiotics between 2 and 8 days decreased, increased and remained constant for vancomycin, gentamicin and their combination respectively (Fig. 2). This corresponds with the SAOS-2 metabolic activity trends which increased, decreased and remained constant for vancomycin, gentamicin and their combination respectively between 2 and 8 days of culturing (Fig. 6). In addition, the detected low cytotoxicity of the COL/HA VG layers can be explained by the presence of half the amount of each type of antibiotic in the samples compared to the single antibiotic samples. The results obtained of the study of the cytotoxicity of vancomycin and gentamicin are in agreement with various previous studies on this and related topics [34,35,41,42]. As Isefuku et al. revealed with respect to osteoblasts, higher concentrations of gentamicin (≥700 mg/l) inhibit cell proliferation [41]. While the exact mechanism surrounding gentamicin toxicity is still unclear, a number of studies have related this phenomenon (gentamicin-induced nephrotoxicity in particular) to mitochondrial dysfunction and apoptosis [42,43].

4. Conclusions

The study introduces biodegradable nanostructured electrospun layers based on collagen, hydroxyapatite nanoparticles and vancomycin hydrochloride, gentamicin sulphate and their combination with respect to providing a bone/implant interface application both for cases of known prosthetic joint infection and as a preventative procedure with concern to primary joint replacement at a potentially infected site. The experimental results revealed that COL/HA layers released high concentrations of vancomycin and gentamicin well above MIC for 21 days. The most rapid and highest rates of antibiotic release were determined

for the COL/HA layers containing vancomycin, in which case a burst release was followed by a gradual decrease in vancomycin concentration. Gentamicin, used as a single agent, was released at high concentration levels throughout the full 21 days of testing without the occurrence of a burst release. The presence of HA in the collagen layers was found not to affect the release kinetics of the vancomycin from the layers loaded only with vancomycin or its combination with gentamicin. Conversely, the presence of HA slowed down the release of gentamicin from the COL/HA layers loaded with gentamicin and its combination with vancomycin. Generally, the modification of COL by means of HA nanoparticles analogically to native bone tissue may have a positive effect on bioactivity, i.e. an increase in the rate of osseointegration. Such a modification altered the release of gentamicin without decreasing the antimicrobial activity. The combination of both antibiotics exerted a positive effect on the prolongation of the conversion of vancomycin to its major degradation product (CDP-1M). The antimicrobial effects of the layers with different antibiotics were compared via the application of the agar diffusion testing technique against four different clinical isolates. All the COL/HA layers irrespective of the antibiotics employed exhibited potential antibacterial activity with respect to both the *Staphylococci* isolates and the gentamicin-resistant isolates. Complementarity was determined between vancomycin and gentamicin against gentamicin-resistant *Staphylococcus epidermidis* and both the *Enterococcus faecalis* isolates. This combination was also found to be more effective against methicillin-resistant *Staphylococcus aureus* than vancomycin as a single agent, despite the application of only half the concentrations of the two antibiotics. Finally, an *in vitro* biological evaluation was conducted employing SAOS-2 osteoblast-like cells either in direct contact with the layers or incubated extracts thereof. The results revealed the antibiotic type-specific effect of the HA content in the layers on SAOS-2. With respect to the COL/HA layers with vancomycin, the presence of HA was found to positively affect the metabolic activity of SAOS-2 while, conversely, the metabolic activity of SAOS-2 decreased with increasing amounts of HA in the COL/HA layers with concern to gentamicin. The vancomycin and vancomycin-gentamicin layers were less cytotoxic (and, moreover, their cytotoxicity was found to decrease over time) than the gentamicin layers that caused a drastic reduction in cell growth. The changes in the metabolic activity of SAOS-2 were related to the release of vancomycin, gentamicin and their combination from the layers. However, the connection between this release of antibiotics from the COL/HA layers and the effect on SAOS-2 behaviour was found to depend on the type of antibiotic rather than on HA content.

In summary, the study demonstrated the benefits and limitations of the single application and combination of vancomycin and gentamicin in collagen electrospun layers with differing amounts of hydroxyapatite. The application of vancomycin and its combination with gentamicin, unlike gentamicin as a single agent, were found to provide suitable candidates for the preparation of a powerful carrier for antibiotics. While the cytotoxicity of gentamicin at the applied concentration was determined to constitute an essential limiting factor, it was found that the cytotoxic effect can be reduced considerably via the lowering of the concentration of gentamicin and its combination with vancomycin, thus preserving the antimicrobial effect. In addition, the study revealed that collagen electrospun layers that exhibit sufficient antimicrobial activity are not directly toxic for human cells. With regard to both efficiency and safety, a combination of collagen with 15 wt % of hydroxyapatite and vancomycin or vancomycin plus gentamicin was found to present the most suitable material composition for the prevention of bone infection.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.04.021.

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