Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (4): 1-7
Published online October 25, 2014 (http://www.cellmolbiol.com)
Received on June 18, 2014, Accepted on October 16, 2014.
doi : 10.14715/cmb/2014.60.4.1

Mesenchymal Stem Cell physiology can be affected by antibiotics: An *in vitro* study

I. Pountos[1, 3], T. Georgouli[1], K. Henshaw[2], B. Howard[2] and P. V. Giannoudis[1, 3]

[1] Academic Department of Trauma & Orthopaedics, School of Medicine, University of Leeds, Leeds, UK
[2] Section of musculoskeletal disease, School of Medicine, University of Leeds, Leeds, UK
[3] Leeds NIHR Biomedical Research Unit, Leeds Institute of Molecular Medicine, Beckett Street, Leeds, LS9 7TF, UK

Corresponding author: Dr Ippokratis Pountos. Academic Department of Trauma & Orthopaedics Leeds General Infirmary, Clarendon Wing Level A,Great George Street, Leeds, LS1 3EX, UK Tel: + 44-113-3922750, Fax: + 44-113-3923290, Email: pountos@doctors.org.uk

Abstract

The aim of this study was to investigate the effect of antibiotics used in clinical practice on Mesenchymal Stem Cells (MSCs) potential to proliferate and differentiate towards an osteogenic lineage. Trabecular bone was obtained from 10 patients (mean age of 36 years, range 18-72) suffering from long bone fractures. Mesenchymal Stem Cells (MSCs) were isolated and functional assays on their proliferation (CFU-F and XTT) and osteogenic differentiation (alkaline phosphatase activity and total calcium production) were performed. The effect of medium supplementation with gentamicin, vancomycin, benzyl-penicillin, flucloxacillin, cefuroxime and metronidazole was analysed. In concentrations found in peripheral circulation, none of the studies antibiotics had an effect on MSCs ability to proliferate and differentiate towards osteogenic lineage. Vancomycin and gentamicin in concentrations of 200 μg/ml and 75 μg/ml respectively, down-regulated the proliferation and osteogenic activity of MSCs. Some combination of the studied antibiotics found to inhibit both proliferation and osteogenesis. High antibiotic concentrations and the combination of different formulations can have detrimental effects on osteoprogenitor cells physiology and potentially bone healing.

Key words: Mesenchymal Stem Cells, Antibiotics, Bone healing, Non-union.

Introduction

Infection is a dreaded complication in Trauma and Orthopaedic Surgery. The administration of prophylactic antibiotics used either parenterally or together bone cement or metal implants is a common measure to reduce the risk of contamination (1,2,3). In cases of established deep seated infections treatment strategies include surgical debridement, irrigation, and reconstruction of the soft tissue envelope and the administration of a prolonged course of antibiotics (4). Prolonged courses of high doses of antibiotics however, can lead to systemic side effects including nephrotoxicity, ototoxicity and gastrointestinal complications (5). Avoidance of these systemic side effects has been achieved by the insertion of implants like polymethyl-methacrylate (PMMA) bone cement and beads which are loaded with antibiotics and release high concentrations of the antibacterial drug only locally (4,6,7,8). Moreover, injectable biodegradable antibiotic-loaded systems are currently under development as a less invasive technique to avoid the surgical stress response (9,10). It is of interest however, that such implants elute antibiotic concentration that are hundred or even thousand times higher than those expected after oral or intravenous administration (11,12,13,14).

Two commonly used antibiotics that can be mixed with cements and beads, particularly for the prevention or eradication of resistant organisms like MRSA, are preparations of gentamicin and vancomycin (6,15,16,17). Other antibacterial preparations commonly used for the treatment of post-operative trauma and orthopaedic infections in isolation or in combined formulations include benzylpenicilin, flucloxacillin, metronidazole and cefuroxime (17,18). Although all of the above preparations are very commonly used in the clinical setting, the evidence regarding their effects on bone physiology and bone repair processes is limited.

The aim of this study therefore was three-fold. Firstly, to investigate the effect of a wide range of concentrations of vancomycin and gentamicin on the proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs) aiming to model the effect of these two antibiotics when loaded to the bone cement. Secondly, to assess the effect of the expected peak plasma concentrations of benzyl-penicillin, flucloxacillin, cefuroxime and metronidazole on MSC proliferation and osteogenesis and thirdly to investigate if combinations of the above antibiotic drugs have a negative impact on the physiology of MSC's.

Materials and methods

Patients

Between January 2009 and February 2009, ten consecutive patients (6 males and 4 females) with a mean age of 36 years (range 18-72) were invited to participate in this study. Ethics committee approval was obtained (Ref number Q1206/127). Inclusion criteria included patients admitted to our institution with a lower limb long bone fracture (Tibia/Femur) requiring operative treatment. Exclusion criteria included children, pathological fractures, patients suffering from infections or other systemic inflammatory conditions and patient that were on steroids or other medications prior to the fracture.

Isolation of Mesenchymal Stem Cells

Trabecular bone (TB) was harvested intra-operatively. MSCs were isolated from trabecular bone using enzymatic digestion with collagenase 0.25% (Stem Cell Technologies, Vancouver, Canada) for 4 hours (37°C, 5% CO_2). $20x10^6$ collagenase-released TB cells were placed into 25 cm^2 flasks and grown to passage (p) 3. At p3 they were frozen in liquid nitrogen prior to further use. None of the culture media used in the study contained antibiotics except when the antibiotics were being tested. Phenotypic characterisation was performed for all donors to depict the nature of cultures grown. A standard panel of antibodies was used including CD73, CD106, CD146, CD166 (BD, Oxford, UK), CD105, CD13 (Serotec, Kidlington, UK) and CD45 (Dako, Ely, UK). Flow cytometry was performed at P0 according to standard protocols on a BD FASCan and all cells used in the study exhibited a MSCs phenotype (positive for CD13, CD73, CD105, CD106, CD146 and CD166 and negative for CD45) as shown before (19,20,21).

Antibiotics

Six antibiotics were tested in this study: Vancomycin (Hospira, Illinois, USA), gentamicin (Aventis, Surrey, UK), benzyl-penicillin sodium (Britannia, Berkshire, UK), flucloxacillin (Wockhardt, Wrexham, UK), cefuroxime (Fresenius Kabi, Cheshire, UK) and metronidazole (Braun, Melsungen, Germany). All six antibiotics were dissolved according to the manufacturer instructions and aliquoted prior to further use. Vancomycin and gentamicin were used in a range of concentrations between 25 – 800 μg/ml. As peak plasma concentrations for vancomycin and gentamicin we considered the 55 μg/ml for vancomycin and the 10 μg/ml for gentamicin. This is based on studies reporting a Cmax of vancomycin of 54.4 μg/ml following an single dose of 1gr administered intravenously and a Cmax of 10.7 of gentamicin following a dose of 4.5 mg/kg (22,23). For the rest of the antibiotics only the Cmax was used. More precisely, relevant experiments included 30 μg/ml benzyl-penicillin (based on studies reporting mean Cmax between 17 and 31 μg/ml) (23,24) 200 μg/ml for flucloxacillin (based on two studies reporting mean Cmax of 167 μg/ml and a range of 118 to 357 μg/ml after 1gr IV) (25,26) 50 μg/ml for cefuroxime (750 mg dose IV results in Cmax of 51.1-52.6 μg/ml) (27,28) and 20 μg/ml for metronidazole (500 mg dose IV results in Cmax of ~20 μg/ml) (29,30).

Analysis of proliferation

The effect of antibiotics on MSC proliferation was assessed by two different methods on cells of all 10 donors: a) according to the number of viable cells (XTT assay) and b) the number and size of the colonies formed (CFU-F assay). In both assays unsupplemented cells (no antibiotics were used in culture media) were used as control. For the XTT assay 96-well-plates were used and the cells were seeded in triplicates (3 wells per patient). The cells allowed to proliferate for 72 hours and on day 3, 50 μl of XTT dye was added according to manufacturers' instructions. After an incubation period of 4 hours (37°C, 5% CO_2) the plates were read at a plate ELISA reader at 450 nm. The optical densities were proportional to the number of viable cells.

For the CFU-F assay 6-well-plates were used. 1000 cells per well were seeded in triplicates (3 wells per patient). On the 7th day of culture, adherent cells were washed with PBS, fixed by an addition of 1 ml of 1% paraformaldehyde (Aldrich, #533998) for 15 minutes and stained with 0.5 ml Crystal Violet (1% in water, BD Lab Supplies, #C142555) for two minutes. After extensive washing with tap water, individual colonies were scored based on the size and number of their colonies blindly by two people.

Analysis of osteogenic differentiation

Osteogenic induction was performed using p3 cells from MSCs from all 10 donors using standard protocols. Briefly, the two assays used to define the osteogenic potential of MSCs were the quantitative measurement of Alkaline Phosphatase (ALP) activity in the cellular protein fraction at day 7**,** and the calcium deposition at day 21 of osteogenic differentiation. Both assays included a control group where no antibiotics were used.

For the analysis of total calcium production 10,000 cells per well in triplicates (96-well plate) were plated. The osteogenic induction medium (composed of DMEM, dexamethasone L-ascorbic acid 2-phosphate and β-glycerol phosphate) containing the respective antibiotic was added and cells stayed in culture for 21 days. For calcium extraction the cells were washed with PBS (no Ca^{2+}, no Mg^{2+}) and 50 μl of 0.1 N HCl was added for 10 minutes. The surface of each plate was

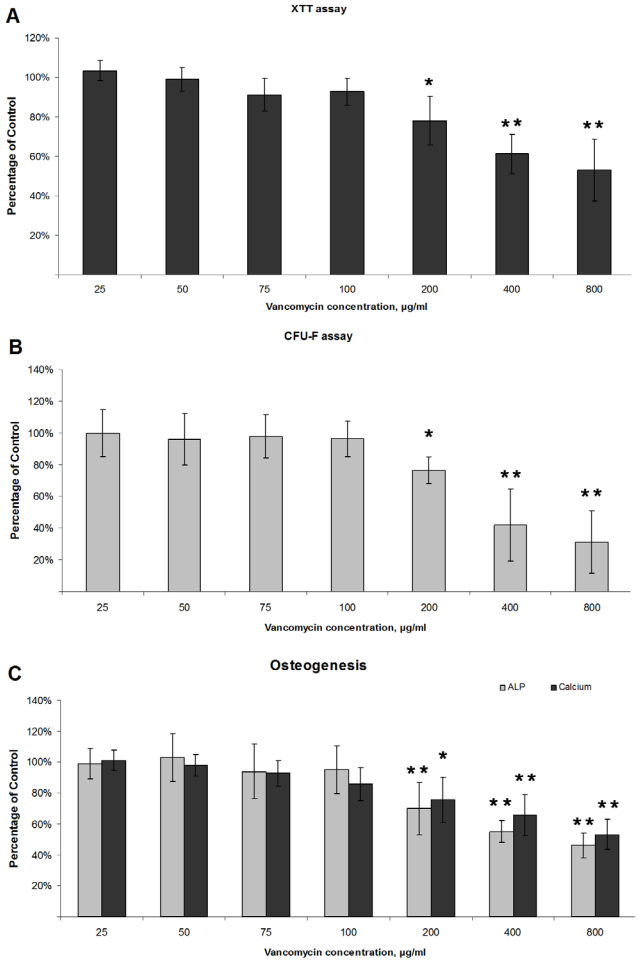

Figure 1. The effect of vancomycin on the proliferation and differentiation of MSCs. A. XTT assay, B. CFU-F assay, C. Calcium production in 21 days and ALP activity in 7 days. (n=10) * $p<0.05$, ** $p<0.01$

scraped and the content was transferred in eppendorf tubes and mixed in 4° C for 4 hours. For the quantification of calcium the Cresolphthalein Complexone method (Thermo, #TR29321) was used and values were obtained using an ELISA plate reader.

For the quantitative measurement of ALP activity cells were left to differentiate towards osteogenic lineage for 7 days under the influence of the antibiotics. At day 7, the cells were lysed and the supernatant was collected. 20 μL of cell supernatant was added to 90 μL of p-nitrophenyl phosphate (Sigma, #N-7653) and incubated for 30 minutes at 37° C. The absorption was read with the ELISA reader at 405 nm. Protein content was determined (Protein assay, BioRad, #500-0114) and normalization of ALP was performed.

Statistical Analysis

Assumption of normality was tested with a one-sample Kolmogorov-Smirnov test. Data are expressed as mean (standard deviation) or median (range) as appropriate. Parametric and nonparametric data were compared using the unpaired Student's t-test and nonparametric paired test, respectively. The result obtained from each concentration of all studied antibiotics was tested against the control (un-supplemented with antibiotics cells). The cut-off value for significance was $p = 0.05$. All calculations were done using the Statistical Package for the Social Sciences (SPSS, version 17.0, IBM, New York, USA).

Results

The effect of 25-800 μg/ml of vancomycin and gentamicin on the proliferation of MSc's

Treating proliferating MSC cultures with vancomycin, resulted in no statistical significant effect up to the concentration of 200 μg/ml **(Figure 1A,B)**. When 200 μg/ml of vancomycin was added in cuture media, a ~22 % and ~24 % (XTT and CFU-F assays respectively) downregulation of MSC proliferation was noted in comparison to untreated controls. A dose dependant effect was found and the highest inhibition was noted with the concentration of 800 μg/ml ($p<0.05$).
Similarly to the vancomycin, MSC's proliferation was found unaffected with gentamicin up to the concentration of 75 μg/ml. Higher gentamycin concentrations inhibited MSC's proliferation ($p<0.05$). **(Figure 2A,B).**

The effect of 25-800 μg/ml of vancomycin and gentamicin on the osteogenic differentiation of MSCs

Media supplementation with 200 μg/ml and higher inhibited both the ALP activity and the calcium production ($p<0.05$) while the concentration 25 – 100 μg/ml found to have no statistically significant difference to the controls **(Figure 1C)**. The inhibition noted with 200 μg/ml of vancomycin was ~30% for the ALP activity ($p<0.01$) and ~25% for the total calcium production ($p<0.05$). **(Figure 1C)**.

In terms of gentamycin, ALP activity was significantly decreased in all studied concentrations except the 25 μg/ml and a dose dependent effect was also noted (**Figure 2C**). The highest studied concentration resulted in a ~60% reduction of ALP activity ($p<0.01$). A

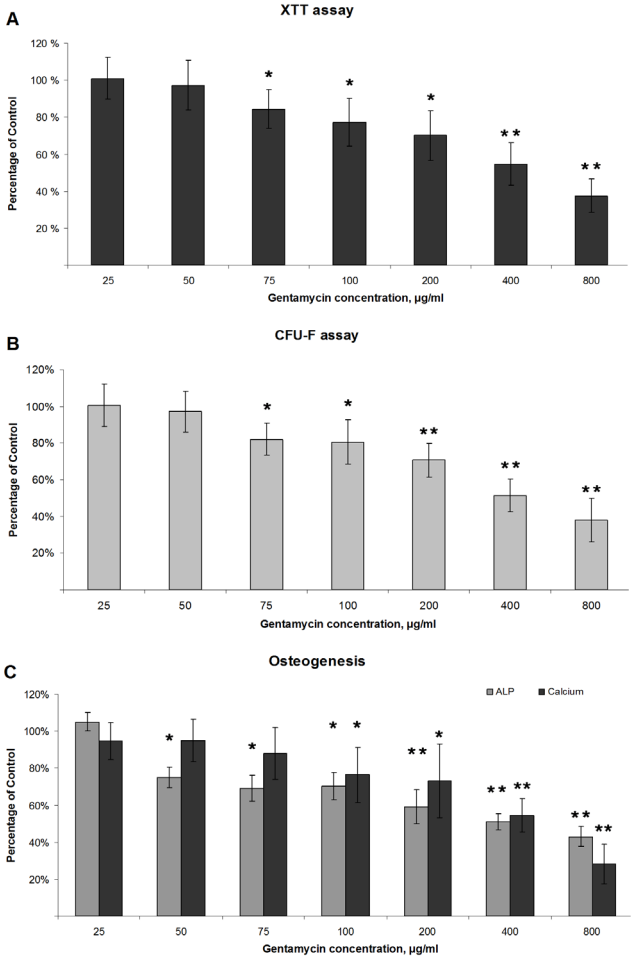

Figure 2. The effect of gentamicin on the proliferation and differentiation of MSCs. A. XTT assay, B. CFU-F assay, C. Calcium production in 21 days and ALP activity in 7 days. (n=10) * $p<0.05$, ** $p<0.01$

~25% decrease of calcium production was found with concentrations of 100 μg/ml of gentamicin and above ($p<0.05$). The lower gentamicin concentrations (25-75 μg/ml) did not result in any statistically significant difference in calcium production but a dose dependent effect was noted for the concentrations between 100 – 800 μg/ml.

Effect of the peak plasma concentrations of benzyl-penicillin, flucloxacillin, cefuroxime and metronidazole alone and in combination on MSCs ability to proliferate and differentiate towards an osteogenic lineage

None of the studied antibiotics at their peak plasma concentration affected proliferation or osteogenic differentiation. The combination of benzyl-penicillin, flucloxacillin and metronidazole however resulted in downregulation of proliferation of MSCs by ~21% and ~29% by the XTT and CFU-F assay respectively compare to the control ($p<0.05$) **(Figure 3A).**

In terms of osteogenesis, ALP activity found decreased only with cefuroxime and the combination of cefuroxime and metronidazole ($p<0.01$), **(Figure 3B).** Calcium production was statistically significantly decreased by ~31% with the combination of benzyl-penicillin, flucloxacillin and metronidazole compared to the controls whereas this combination had no effect on ALP activity ($p<0.01$), **(Figure 3B)**.

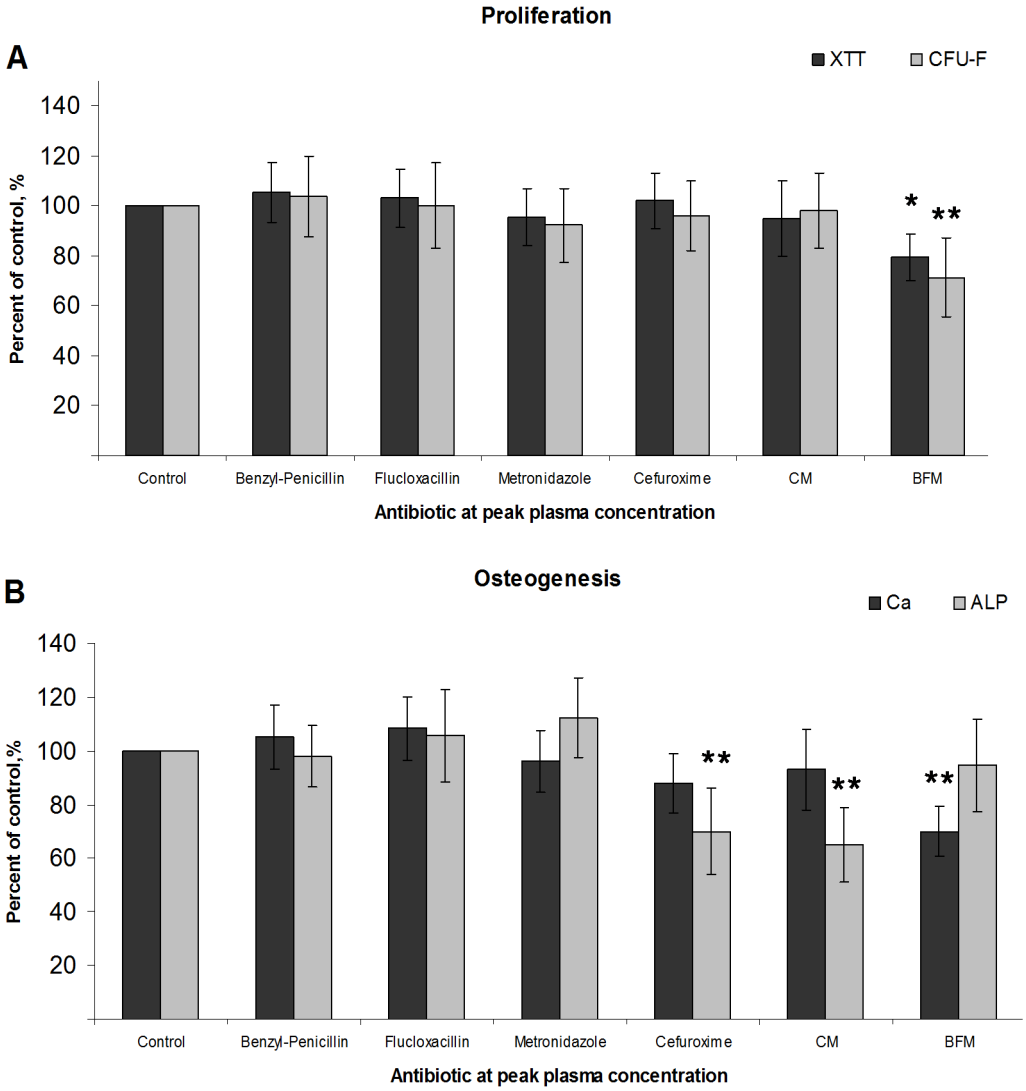

Figure 3. The effect of the expected peak plasma concentration of benzyl-penicillin, flucloxacillin, cefuroxime and metronidazol in isolation and in combination on the proliferation and differentiation of MSCs. A. XTT and CFU-F assay, B. Calcium production in 21 days and ALP activity in 7 days. (n=10) * $p<0.05$, ** $p<0.01$. CM: Cefuroxime and Metronidazole, BFM: Benzyl-penicillin, flucloxacillin and metronidazole.

Discussion

In this study, we assessed the effect a range of of vancomycin and gentamicin on MSCs potential to proliferate and differentiate towards an osteogenic lineage. In addition, four commonly prescribed antibiotics (benzyl-penicillin, flucloxacillin, cefuroxime and metronidazole) were selected to be evaluated at the peak expected plasma concentrations alone or in combination due to their wide spread usage as broad spectrum antibiotic in cases of contaminated wounds (17,18)

Vancomycin is a glycopeptide antibiotic, used in the prophylaxis and treatment of infections caused by Gram-positive bacteria and is traditionally been reserved as a drug of the last resort (22,23) Our results showed that vancomycin concentrations of 200 μg/ml and over downregulated MSC proliferation by ~22-24 % while the highest inhibition was noted with concentrations of 800 μg/ml. Similarly ALP activity and calcium production was inhibited by concentrations of 200 μg/ml and over. There are two in-vitro studies analysing the effect of vancomycin (31,32) Edin et al using osteosarcoma MG-63 osteoblasts found no effect with concentrations up to 1000 μg/ml but cell death was noted at 10,000 μg/ml (31) The second study analysed the proliferation potential of MC3T3-E1 preosteoblasts and N1511 prechondrocytes treated with vancomycin (32). Their results showed that cellular proliferation was inhibited with doses greater than 250 μg/ml, but not 4000 μg/ml for osteoblasts and all studied concentrations for chondrocytes. To the best of our knowledge, our study is the first to utilize human MSCs derived from the fracture site and the differences found could be explained by the difference of cells used in the other two studies. Our study also suggests that in a clinical scenario in which vancomycin is administered parenterally (peak plasma concentration reaches approximately 55 μg/ml after 1 gr intravenous infusion (22)), the resulted concentrations are unlikely to affect MSCs proliferation and osteogenic differentiation. In cases where vancomycin is loaded on the cement, several studies have reported concentrations prolonged high concentrations which could have detrimental effects on MSC proliferation and osteogenesis (11,14). For instance, Hsieh et al reported that cement spacers could elute vancomycin concentrations of 1538 ± 243 μg/ml in day 1 after implantation, reduced to 571.9 ± 169 μg/ml in day 7 (14). Our study showed that such concentrations could have detrimental effect on MSCs physiology.

Gentamicin is an aminoglycoside antibiotic, used

to treat many types of bacterial infections, particularly those caused by Gram-negative organisms (23) Our results showed that MSC proliferation was inhibited with concentrations of 75 μg/ml and higher. ALP activity was decreased in all concentrations except the 25 μg/ml while calcium production was decreased in concentrations of 100 μg/ml and higher. Previous studies failed to reach an agreement with some studies suggesting that osteoblast proliferation was inhibited with concentrations over 80–100 μg/ml (33,34,35) while others found either inhibitions with higher concentrations or no statistically significant difference at all (36,37). ALP activity was found down-regulated with lower concentrations, even as low as 12.5 – 25 μg/ml (36,37). sGAG and Type II & X collagen were found lower in growing chondrocytes with gentamicin levels over 100 μg/ml (33). It is of note however that a variety of cells were used including rat calvaria stromal cells, human foetal osteoblasts, murine osteoblasts (C2C12) which can contribute to the differences found. In terms of in-vivo studies Haleem et al. reported no effect with gentamicin concentrations of 1.5 mg/kg twice daily for 21 days on a rat experimental fracture healing model (38). Intravenous administration of gentamicin at a dose of 4.5 mg/kg results in a peak plasma concentration of ~11 μg/ml. Based on our results, such concentrations are unlikely to have any effect on MSC physiology. Gentamicin impregnated implants elute high concentrations for prolonged period time suggesting potential interference with the osteoprogenitor cells biology (12,39,40). Beads found to elute concentrations as high as 600 μg/ml, reduced to 120 μg/ml in day 10 and 10 μg/ml in day 80 (12). Down-regulation of MSC functions and potential should be expected with such concentrations.

Benzyl-penicillin, flucloxacillin, cefuroxime and metronidazole are all first line antibiotics used frequently in the clinical setting. Benzyl-penicillin is the gold standard type of penicillin, a beta-lactam antibiotic that is used in the treatment of bacterial infections caused by susceptible, usually Gram-positive organisms (23,24). Flucloxacillin is a narrow-spectrum β-lactam antibiotic usually used to treatment of infections caused by susceptible Gram-positive bacteria (25,26). Cefuroxime is a second generation cephalosporin, which is a subgroup of β-lactam antibiotic (27,28). Cefuroxime has increased activity against Gram-negative bacteria but also gram-positive organisms as well (27,28). Metronidazole is a nitroimidazole antibiotic used particularly for anaerobic bacteria and protozoa (28,30). The combination of either benzyl-penicillin, flucloxacillin and metronidazole or cefuroxime and metronidazole provide a wide coverage against unknown organisms and are used initially before the organism identified with cultures (17,18). Our results showed that none of the studied antibiotics at their peak plasma concentration affected MSC proliferation and osteogenic differentiation. However, the combination of benzyl-penicillin, flucloxacillin and metronidazole resulted in a statistically significant inhibition of both proliferation and calcium production of MSCs. These findings support the view that an additive effect could be exerted with different pharmacological agents. Cefuroxime and metronidazole on the other hand did not affect MSC proliferation and calcium production during osteogenesis. We failed to find any previous studies on benzyl-penicillin, flucloxacillin and metronidazole. The effect of cefuroxime was studied by Salzmann et al who suggested that TB derived osteoblast proliferation was unaltered by concentrations up to 100 μg/ml however higher concentrations resulted in an increase of proliferation (41). In addition, ALP activity was found increased with concentrations less than 100 but was inhibited in higher concentrations. In our study we failed to reproduce this effect.

The herein study suggests that all six studied antibiotics have no effect on MSCs proliferation at concentration found after parenteral administration. The combination of several agents aiming broad spectrum antibiotic cover could result in a temporary negative effect therefore focused antibiotic administration is advisable. In addition, surgical patients are exposed to a variety of pharmaceutical agents known to interfere with bone healing like NSAIDs, anticoagulants and steroids therefore it is unknown what the global risk of the combination of all these medicines is (20,42,43). Furthermore, we failed to find any variability between the patients or the site from where MSCs were harvested.

Strengths of this study include the selection of commonly used antibiotics in the clinical setting and the utilization of an *in vitro* model capable of defining the direct effect of these agents on the biological properties of MSCs. Limitations of this study include the use of an artificial culture and induction environment which is different compared to that found *in vivo* where the effect of mitogenic and osteogenic growth factors present in the fracture site is not taken into account. The requirement for cell culture expansion and the absence of other cell types could also have an impact on the final outcome. Finally, another limitation of our study is the fact that we did not investigate the molecular mechanisms that could be responsible for the results obtained. This however was not the scope of the herein study.

The mechanism by which antibiotics can inhibit proliferation and osteogenesis at a molecular level still remains obscure but several speculations can be raised. Active uptake and intracellular accumulation can occur, leading to cytotoxic concentration. In such conditions decreased mitochondria activity takes place and several studies have shown that impaired mitochondria energetics, protein synthesis and loss of mitochondrial DNA occurs (44,45). Similarly, other authors agree that toxicity seems to be the attributing pathway excluding cellular senescence as the determinant factor (32). This can also explain our finding which suggested that the highest inhibition was associated with higher doses of antibiotics or multiple agents. Another plausible pathway of inhibition of osteogenesis is that of the inhibition of matrix metalloproteinases (MMPs) and collagen synthesis (46). Studies to determine the molecular insight of the impact of high concentrations of antibiotics on the vitality of MSCs are desirable. Further analysis of the gene expression could outline the involved pathways responsible for these results. Finally, in-vivo studies as well as randomized controlled clinical trials would shed more light in this field.

As a conclusion, the expected peak plasma concentration of the studied antibiotics included in this study found to have no effect on MSCs ability to proliferate and differentiate towards osteogenic lineage. Higher

concentrations, as those eluted by some implants, can have detrimental effects on osteoprogenitor cell physiology. In addition, this study suggests that an additive effect exist when combination of antibiotics were used.

References

1. Vos, M.C., Behrendt, M.D., Melles, D.C., Mollema, F.P., de Groot, W., Parlevliet, G., Ott, A., Horst-Kreft, D., van Belkum, A. and Verbrugh, H.A., 5 years of experience implementing a methicillin-resistant Staphylococcus aureus search and destroy policy at the largest university medical center in the Netherlands. *Infect Control Hosp Epidemiol*. 2009, **30**: 977-84. doi: 10.1086/605921.

2. Tang, L., Zhao, C., Xiong, Y. and Wang, A., Preparation, antibacterial properties and biocompatibility studies on vancomycin-poly(D,L)-lactic loaded plates. *Int Orthop.* 2010, **34**: 755-9. doi: 10.1007/s00264-009-0808-z.

3. Mackain-Bremner, A.A., Owens, K., Wylde, V., Bannister, G.C. and Blom, A.W., Adherence to recommendations designed to decrease intra-operative wound contamination. *Ann R Coll Surg Engl*. 2008, **90**: 412-6. doi: 10.1308/003588408X301028.

4. Gogia, J.S., Meehan, J.P., Di Cesare PE, Jamali AA. Local antibiotic therapy in osteomyelitis. *Semin Plast Surg.* 2009, **23**: 100-7. doi: 10.1055/s-0029-1214162.

5. Popat, K.C., Eltgroth, M., Latempa, T.J., Grimes, C.A. and Desai, T.A., Decreased Staphylococcus epidermis adhesion and increased osteoblast functionality on antibiotic-loaded titania nanotubes. *Biomaterials.* 2007, **28**: 4880–4888. doi: 10.1016/j.biomaterials.2007.07.037

6. Nelson, C.L., The current status of material used for depot delivery of drugs. *Clin Orthop Relat Res.* 2004, **427**: 72-8. doi: 10.1097/01.blo.0000143741.92384.18

7. Liu, S.J., Wen-Neng Ueng, S., Lin, S.S. and Chan, E.C., In vivo release of vancomycin from biodegradable beads. *J Biomed Mater Res.* 2002, **63**: 807-13. doi: 10.1002/jbm.10406

8. Peng, K.T., Chen, C.F., Chu, I.M., Li, Y.M., Hsu, W.H., Hsu, R.W. and Chang, P.J., Treatment of osteomyelitis with teicoplanin-encapsulated biodegradable thermosensitive hydrogel nanoparticles. *Biomaterials.* 2010, **31**: 5227-36. doi: 10.1016/j.biomaterials.2010.03.027.

9. McLaren, A.C., Alternative materials to acrylic bone cement for delivery of depot antibiotics in orthopaedic infections. *Clin Orthop Relat Res.* 2004, **427**: 101-6. doi: 10.1097/01.blo.0000143554.56897.26

10. Krasko, M.Y., Golenser, J., Nyska, A., Nyska, M., Brin, Y.S. and Domb, A.J., Gentamicin extended release from an injectable polymeric implant. *J Control Release.* 2007, **117**: 90-6. doi: 10.1016/j.jconrel.2006.10.010

11. Adams, K., Couch, L., Cierny, G., Calhoun, J. and Mader J.T., In vitro and in vivo evaluation of antibiotic diffusion from antibiotic-impregnated polymethylmethacrylate beads. *Clin Orthop Relat Res.* 1992, **278**: 244-52.

12. Wahlig, H., Dingeldein, E., Bergmann, R. and Reuss, K., The release of gentamicin from polymethylmethacrylate beads. An experimental and pharmacokinetic study. *J Bone Joint Surg Br.* 1978, **60**: 270-5.

13. Nelson, C.L., McLaren, S.G., Skinner, R.A., Smeltzer, M.S., Thomas, J.R. and Olsen K.M., The treatment of experimental osteomyelitis by surgical debridement and the implantation of calcium sulfate tobramycin pellets. *J Orthop Res.* 2002, **20**: 643-7. doi: 10.1016/S0736-0266(01)00133-4

14. Hsieh, P.H., Chang, Y.H., Chen, S.H., Ueng, S.W. and Shih, C.H., High concentration and bioactivity of vancomycin and aztreonam eluted from Simplex cement spacers in two-stage revision of infected hip implants: a study of 46 patients at an average follow-up of 107 days. *J Orthop Res.* 2006, **24**: 1615-21. doi: 10.1002/jor.20214

15. Chiu, F.Y. and Lin, C.F., Antibiotic-impregnated cement in revision total knee arthroplasty. A prospective cohort study of one hundred and eighty-three knees. *J Bone Joint Surg Am.* 2009, **91**: 628-33. doi: 10.2106/JBJS.G.01570.

16. Laine, J.C., Nguyen, T.Q., Buckley, J.M. and Kim, H.T., Effects of Mixing Techniques on Vancomycin-Impregnated Polymethylmethacrylate. *J Arthroplasty.* 2011, **26**: 1562-6. doi: 10.1016/j.arth.2011.02.011.

17. Al-Arabi, Y.B., Nader, M., Hamidian-Jahromi, A.R. and Woods, D.A., The effect of the timing of antibiotics and surgical treatment on infection rates in open long-bone fractures: a 9-year prospective study from a district general hospital. *Injury.* 2007, **38**: 900-5. doi: http://dx.doi.org/10.1016/j.injury.2007.02.043

18. Roberts, P. and Walker, S., The British Military Surgery Pocket Book. 13th edition. Oxford University Press. Oxford, 2004, pp 125-52.

19. Pountos, I., Georgouli, T., Henshaw, K., Bird, H., Jones, E. and Giannoudis, P.V., The effect of bone morphogenetic protein-2, bone morphogenetic protein-7, parathyroid hormone, and platelet-derived growth factor on the proliferation and osteogenic differentiation of mesenchymal stem cells derived from osteoporotic bone. *J Orthop Trauma.* 2010, **24**: 552-6. doi: 10.1097/BOT.0b013e3181efa8fe.

20. Pountos, I., Giannoudis, P.V., Jones, E., English, A., Churchman, S., Field, S., Ponchel, F., Bird, H., Emery, P. and McGonagle, D., NSAIDS inhibit in vitro MSC chondrogenesis but not osteogenesis: implications for mechanism of bone formation inhibition in man. *J Cell Mol Med.* 2011, **15**: 525-34. doi: 10.1111/j.1582-4934.2010.01006.x.

21. Pountos, I., Georgouli, T. and Giannoudis, P.V., The effect of autologous serum obtained after fracture on the proliferation and osteogenic differentiation of mesenchymal stem cells. *Cell Mol Biol (Noisy-le-grand).* 2008, **54**: 33-9.

22. Dupon, M., Janvier, G., Vinçon, G., Winnock, S., Demotes-Mainard, F., Capeyron, O. and Saric, J., Plasma levels of piperacillin and vancomycin used as prophylaxis in liver transplant patients. *Eur J Clin Pharmacol.* 1993, **45**: 529-34. doi: 10.1007/BF00315309

23. McEvoy, G., AHFS drug Information. American Society of Health-System Pharmacists. *ASHP*: 2010. pp

24. Mavrov, G.I. and Goubenko, T.V., Treatment of pregnant women infected with syphilis with intravenous penicillin G: Pharmacokinetical and clinical study. *Gynecol Obstet Invest.* 2001, **52**: 114-8.

25. Landersdorfer, C.B., Kirkpatrick, C.M., Kinzig-Schippers, M., Bulitta, J.B., Holzgrabe, U., Drusano, G.L. and Sörgel, F., Population pharmacokinetics at two dose levels and pharmacodynamic profiling of flucloxacillin. *Antimicrob Agents Chemother.* 2007, **51**: 3290-7. doi: 10.1128/AAC.01410-06

26. Adam, D., Koeppe P. and Heilmann, H.D., Pharmacokinetics of amoxicillin and flucloxacillin following the simultaneous intravenous administration of 4 g and 1 g, respectively. *Infection.* 1983, **11**: 150-4. doi: 10.1007/BF01641294

27. Gower, P.E. and Dash, C.H. The pharmacokinetics of cefuroxime after intravenous injection. *Eur J Clin Pharmacol.* 1977, 12: 221-7. doi: 10.1007/BF00609865

28. Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S., Cefuroxime: a review of its antibacterial activity, pharmacological properties and therapeutic use. *Drugs.* 1979, **17**: 233-66. doi: 10.2165/00003495-197917040-00001

29. Houghton, G.W., Thorne, P.S., Smith, J., Templeton, R. and Collier, J., Comparison of the pharmacokinetics of metronidazole in healthy female volunteers following either a single oral or intravenous dose. *Br J Clin Pharmacol.* 1979, **8**: 337-41. doi: 10.1111/

j.1365-2125.1979.tb04715.x
30. Metronidazole datacheet http://www.medsafe.govt.nz/Profs/Datasheet/m/MetronidazoleAFTinf.pdf
31. Edin, M.L., Miclau, T., Lester, G.E., Lindsey, R.W. and Dahners, L.E., Effect of cefazolin and vancomycin on osteoblasts in vitro. *Clin Orthop Relat Res.* 1996, **333**: 245-51. doi http://dx.doi.org/10.1097/00003086-199612000-00027
32. Antoci, V., Adams, C.S., Hickok, N.J., Shapiro, I.M. and Parvizi, J., Antibiotics for local delivery systems cause skeletal cell toxicity in vitro. *Clin Orthop Relat Res.* 2007, **462**: 200-6. doi: 10.1097/BLO.0b013e31811ff866
33. Chang, Y., Goldberg, V.M. and Caplan, A.I., Toxic effects of gentamicin on marrow-derived human mesenchymal stem cells. *Clin Orthop Relat Res.* 2006, **452**: 242-9. doi: 10.1097/01.blo.0000229324.75911.c7
34. Isefuku, S., Joyner, C.J. and Simpson, A.H., Gentamicin may have an adverse effect on osteogenesis. *J Orthop Trauma.* 2003; **17**: 212-6. doi: http://dx.doi.org/10.1097/00005131-200303000-00010
35. Pedersen, J.G. and Lund, B., Effects of gentamicin and monomer on bone. An in vitro study. *J Arthroplasty.* 1988, **3**: S63-8.
36. Ince, A., Schütze, N., Hendrich, C., Jakob, F., Eulert, J. and Löhr, J.F., Effect of polyhexanide and gentamicin on human osteoblasts and endothelial cells. *Swiss Med Wkly.* 2007, **137**: 139-45.
37. Ince, A., Schütze, N., Karl, N., Löhr, J.F. and Eulert, J., Gentamicin negatively influenced osteogenic function in vitro. *Int Orthop.* 2007, **31**: 223-8. doi:10.1007/s00264-006-0144-5
38. Haleem, A.A., Rouse, M.S., Lewallen, D.G., Hanssen, A.D., Steckelberg, J.M. and Patel. R., Gentamicin and vancomycin do not impair experimental fracture healing. *Clin Orthop Relat Res.* 2004, **427**: 22-4. doi: 10.3109/17453670902884700.
39. Anagnostakos, K., Wilmes, P., Schmitt, E. and Kelm J., Elution of gentamicin and vancomycin from polymethylmethacrylate beads and hip spacers in vivo. *Acta Orthop.* 2009, **80**: 193-7. doi: 10.3109/17453670902884700.
40. Pountos, I., Georgouli, T., Bird, H., Kontakis, G. and Giannoudis, P.V., The effect of antibiotics on bone healing: current evidence. *Expert Opin Drug Saf.* 2011, **10**: 935-45. doi: 10.1517/14740338.2011.589833
41. Salzmann, G.M., Naal, F.D., von Knoch, F., Tuebel, J., Gradinger, R., Imhoff, A.B. and Schauwecker, J., Effects of cefuroxime on human osteoblasts in vitro. *J Biomed Mater Res A.* 2007, **82**: 462-8. doi: 10.1002/jbm.a.31158
42. Pountos, I., Georgouli, T., Blokhuis, T.J., Pape, H.C. and Giannoudis, P.V., Pharmacological agents and impairment of fracture healing: what is the evidence? *Injury.* 2008, **39**: 384-94. doi: 10.1016/j.injury.2007.10.035
43. Pountos, I., Georgouli, T., Calori, G.M. and Giannoudis, P.V., Do nonsteroidal anti-inflammatory drugs affect bone healing? A critical analysis. *ScientificWorldJournal.* 2012: 606404. doi: 10.1100/2012/606404.
44. Duewelhenke, N., Krut, O. and Eysel, P., Influence on mitochondria and cytotoxicity of different antibiotics administered in high concentrations on primary human osteoblasts and cell lines. *Antimicrob Agents Chemother.* 2007, **51**: 54-63. doi: 10.1128/AAC.00729-05
45. Huddleston, P.M., Steckelberg, J.M., Hanssen, A.D., Rouse, M.S., Bolander, M.E. and Patel, R., Ciprofloxacin inhibition of experimental fracture healing. *J Bone Joint Surg Am.* 2000, **82**: 161-73.
46. Lamparter, S., Slight, S.H. and Weber K.T., Doxycycline and tissue repair in rats. *J Lab Clin Med.* 2002, **139**: 295-302. doi:10.1067/mlc.2002.122624