The Impact of Voriconazole and liposomal Amphotericin B loaded PMMA Cement Chips in Different Concentrations on the Osteogenic Response of BM-MSCs In-Vitro Accessed by ^{99m}Tc-HDP Labeling

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INTRODUCTION: Treatment of infected non-unions and severe bone infections is already a huge challenge in modern trauma and orthopedics which is getting often catastrophic when the infection is caused by a fungus. Their treatment routinely contains the local and systemic administration of anti-fungal drugs and radical surgical debridement of the infected bone. Recent data showed that local anti-fungal drug therapy delivered by drug loaded Polymethylmethacrylate (PMMA) bone cement provides high drug concentrations at the infection site without leading to systemic side-effects. Although frequently used, little is known about the impact of these drugs on the osteogenic regenerative capabilities of the surrounding bone tissue especially upon the osteogenesis of human bone-marrow mesenchymal stem cells (BM-hMSCs). This study evaluates the effects of the two most common and approved anti-fungal drugs used in clinic, Voriconazole and liposomal Amphotericin B, upon the osteogenic response of BM-hMSCs in vitro. Within this study, we compared the ability of BM-hMSC to differentiate into osteoblast-like cells and synthesize hydroxyapatite in a monolayer cell culture under the presence of various PMMA cement chips containing these drugs in low-dose fixation concentration, high-dose spacer concentration and an extended-dose experimental spacer concentration, assessed by radioactive ^{99m}Tecnetium-Hydroxydiphosphonate (^{99m}Tc-HDP) Labeling, immunohistochemistry, and analyses of supernatants.

METHODS: Fungicides were solved in PMMA by mixing the drug powder to the PMMA powder. A 0.5 g PMMA chip was placed at the center of every cell culture dish. Then, BM-hMSCs (n=6 donors with informed consent) were seeded into 35mm-petri dishes (96 dishes in total) at a density of 10,000 cells/cm². NC: Negative control group with Dulbecco's Modified Eagle Medium Low Glucose (DMEM LG) + Fetal Bovine Serum (FCS) + 0.5 g PMMA chip, OC: Osteogenic Differentiation control group with DMEM LG + FCS + 100 nM dexamethasone, 50 µM L-ascorbic acid and 10 nM β-glycerol phosphate + 0.5 g PMMA chip, VOR1: medium like OC + 0.5 g (PMMA + 5 mg/g Voriconazole (VFEND, Pfizer)) chip, VOR2: medium like OC + 0.5 g (PMMA + 10 mg/g Voriconazole) chip, VOR3: medium like OC + 0.5g (PMMA + 15 mg/g Voriconazole) chip, AMB1: medium like OC + 0.5g (PMMA + 5 mg/g liposomal Amphotericin B (AmBisome liposomal, Gilead Sciences)) chip, AMB2: medium like OC + 0.5 g (PMMA + 10 mg/g liposomal Amphotericin B) chip, AMB3: medium like OC + 0.5 g (PMMA + 15 mg/g liposomal Amphotericin B) chip. The cells were cultured for 3 weeks, and then half of the samples were labelled with radioactive ^{99m}Tc-HDP to evaluate the amount of hydroxyapatite synthesized, as a marker of the osteogenic potential [1]. For this purpose, each dish was incubated with 5 MBq of ^{99m}Tc-HDP for 15 min. Remaining bound activity after washing the dishes was measured using a gamma camera. The other half of the samples were analyzed with DAPI Immunofluorescence staining. Calcium and phosphate concentration, as well as alkaline phosphatase activity were measured in supernatants at day 21 of cell culture. For statistical analysis, the data were examined for normal distribution using the Kolmogorov-Smirnov-Test. Then, an ANOVA analysis with Bonferroni post-hoc testing was performed. Statistical significance was set to $p \leq 0.05$. The study was approved by an Ethics Committee.

RESULTS: The mean values of the tracer uptake reflected a solid and statistically significant ($p \leq 0.005$) osteogenic differentiation in all groups except in NC (0.496 MBq). The highest uptake was measured in VOR1 (2.983 MBq), followed by AMB1 (2.527 MBq) and OC (2.496 MBq). With increasing fungicide concentration, ^{99m}Tc-HDP uptake decreased in all groups. VOR2 (1.687 MBq), VOR3 (1.436 MBq), AMB2 (2.017 MBq), AMB3 (1.337 MBq). Compared to OC ^{99m}Tc-HDP uptake was significantly lower in VOR2 ($p=0.0069$), VOR3 ($p=0.0001$) and AMB3 ($p<0.0001$). DAPI Immunoflourescence staining revealed significantly lower cell counts in every fungicide sample in comparison to OC ($p<0.0001$) (in 10^4 cells per dish): OC (90.145), VOR1 (29.186), VOR2 (9.520), VOR3 (8.905), AMB1 (38.070), AMB2 (15.311), AMB3 (6.531). Results of supernatant analyses showed significantly lower calcium concentrations in every osteogenic group compared to negative control ($p<0.005$) and significantly higher calcium concentrations in VOR2 and VOR3 compared to VOR1 ($p=0.0087$) and AMB3 compared to AMB1 ($p=0.0240$). Reporting about alkaline phosphatase activity, it was slightly but not significantly lower in fungicide probes in comparison to OC with a decreasing activity when fungicide concentration was increased. In general, phosphate concentration decreased with higher fungicide concentration both in Voriconazole and liposomal Amphotericin B groups. While VOR1 and AMB1 did not showed significantly lower phosphate levels than OC, phosphate concentrations in supernatants of VOR2, VOR3, AMB2 and AMB3 were significantly lower than in OC (VOR2,VOR3, AMB3: $p<0.0001$, AMB2: $p=0.0176$).

DISCUSSION: Our data revealed that low-dose local anti-fungal drug therapy with Voriconazole or liposomal Amphotericin B in PMMA has no negative impact on hydroxyapatite deposition and consequently on the osteogenic potential of BM-hMSC in vitro. High- and extended-dose Voriconazole have both a significant negative impact on the osteogenic potential, while only extended-dose liposomal Amphotericin B has a negative impact on the osteogenic potential of BM-hMSC in vitro. Even though we detected this negative impact, hydroxyapatite deposition was still significantly higher than in NC. Although not significant, these results are consistently with the measured calcium and phosphate concentrations in supernatants, because calcium levels act directly inverse and phosphate levels similarly to osteogenesis. According to cell amount, we showed that every fungicide in every dosage has a significantly negative impact, which means that all fungicides are cytotoxic to BM-hMSC in vitro. Although cell counts are significantly lower in VOR1, AMB1 and AMB 2 than in OC, there is no difference in hydroxyapatite deposition detectable. This shows that remaining cells in these groups are capable to compensate lower cell counts in dimension of osteogenesis. With higher fungicide concentrations (VOR2, VOR3 and AMB3) this capacity is extinguished and consequently ^{99m}Tc-HDP uptake is lowered. As a conclusion we see that negative effects on osteogenic potential due to cytotoxicity of fungicides can be compensated by BM-hMSC in low-dose Voriconazole and liposomal Amphotericin B, as they are frequently used in PMMA fixation cement (VOR1 and AMB1). We found that high-dose liposomal Amphotericin B (AMB2) does not impair osteogenic potential, while there is an impairment of osteogenic potential detectable in high-dose Voriconazole (VOR2), as both are used in PMMA-spacers. PMMA cement with extended fungicide doses (VOR3 and AMB3) both affect osteogenic potential of BM-hMSC negatively, which is most probably due to cytotoxic effects.

SIGNIFICANCE/CLINICAL RELEVANCE: According to this study correct dosage of local anti-fungal drug therapy delivered by PMMA is very important to not impair osteogenic potential of bone marrow mesenchymal stem cells. Although this study was performed in vitro, clinicians should use low-dose Voriconazole or low- or high-dose liposomal Amphotericin B in PMMA for local anti-fungal drug therapy whenever possible to avoid cytotoxicity and reduced osteogenic potential.

REFERENCES: [1] Hofmann J, Borcherding K, Thiel K, Lingner T, Sommer U, Haberkorn U, Bewersdorf T, Schmidmaier G, Grossner T: 99mTc-HDP Labeling-A Non-Destructive Method for Real-Time Surveillance of the Osteogenic Differentiation Potential of hMSC during Ongoing Cell Cultures. International journal of molecular sciences 23 (2022).