Mitigative Effect of Erythromycin on PMMA Challenged Pre-osteoblastic MC3T3-E1 Cells

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Introduction: Aseptic loosening (AL) is mainly caused by wear particle-induced periprosthetic tissue inflammation. Polymethylmethacrylate (PMMA) particles have been previously shown to inhibit the osteogenic differentiation both in the bone marrow stromal cells and in a murine MC3T3 osteoprogenitor cell. Erythromycin (EM) has anti-inflammatory effects through targeting of NF-κB signaling. Our previous studies demonstrated that EM represents an appropriate drug candidate to inhibit wear debris-induced periprosthetic tissue inflammatory osteolysis by inhibiting osteoclast activity. However, the potential effects of EM on osteoblast growth and differentiation are still not very clear. The objective of this study was to investigate whether treatment with EM can diminish the inhibitory effects of PMMA particles on MC3T3-E1 osteoprogenitor cells in vitro.

Methods:
Cell culture Mouse MC3T3-E1 preosteoblasts were cultured in α-MEM with 10% heat-inactivated fetal bovine serum and antibiotics. For cell differentiation, cells were cultured in osteogenic media (α-MEM plus 10 mM β-glycerophosphate and 50 μg/ml L-ascorbic acid). Culture media were changed every three days.

PMMA particle PMMA particles (Polysciences) were rinsed in ethanol four times, sterilized in 70% ethanol with shaking overnight. PMMA particles were then rinsed with PBS and resuspended in serum free α-MEM.

Erythromycin (EM) EM (Sigma) stock solution (1 mg/ml) was sterilized by filtration prior to use. MC3T3 cells were pre-treated with EM at different concentrations (0, 0.2, 1, 2, 5 and 10 μg/ml) for 3 hrs before adding PMMA particles. MC3T3 cells without PMMA treatment were included as controls. Culture media and drugs were replaced every 3 days until the predetermined dates. The reason we pre-treated cells with EM prior to addition of PMMA particles was to avoid the insufficient interaction of EM with cells because of the binding of a tiny amount of EM to the PMMA particles.

Measurements Cell toxicity was determined by measuring Lactate Dehydrogenase (LDH) activity released from dead or dying cells into the culture medium. Cell proliferation was measured by cell counts. Cell differentiation was measured by cellular alkaline phosphatase (ALP) activity (BioVision) after 14 days in culture. Real Time RT-PCR was used to measure gene expression of NF-κB, Runx2, Osterix and Osteocalcin.

Statistical Analysis All treatments were repeated three times, each with duplicate samples, except RT-PCR (triplicate samples). The results were evaluated by the statistical test analysis using the ANOVA test, with the Scheffé's method of post hoc multiple comparisons.

Results:
Effects of EM on viability of PMMA-challenged MC3T3 cells Cells treated with PMMA particles (1 mg/ml) showed a significant increase of LDH release compared with untreated cells. EM treatment attenuated PMMA-induced LDH release in a dose-dependent manner. EM concentrations higher than 5 μg/ml (at 24 hours) or higher than 1 μg/ml (at 48 hours) were required to significantly reduce LDH release, indicating that EM mitigates PMMA particle-induced MC3T3 cell damage (necrotic or apoptotic change). The number of viable adherent cells was remarkably reduced after 72 hour PMMA treatment. The addition of EM at concentrations of 2, 5 and 10 μg/ml reversed this effect (p<0.05).

Effects of EM on ALP activity of PMMA-challenged MC3T3 cells PMMA treatment significantly reduced ALP activity by 49% at day 14, compared to controls. In the presence of PMMA, pretreatment with EM at the range of 2-10 μg/ml significantly elevated ALP activity in a dose-dependent manner (p<0.05).

Effects of EM on osteogenic gene expression of PMMA-challenged MC3T3 cells (Figure 1) MC3T3 cells treated with PMMA particles showed a significant reduction in gene expression of Runx2 (p<0.05) and Osterix (p<0.05). EM treatment (2-10 μg/ml) partially restored these reductions. In addition to the changes in transcription factor expression, PMMA decreased the expression of osteocalcin, a known marker for osteoblast differentiation (p<0.05). EM treatment significantly increased the osteocalcin gene expression in a dose-dependent manner (p<0.05). It should be noted that EM could not reverse the PMMA-induced gene expression changes of Runx2, osterix, and osteocalcin to the level that was similar to the controls. Gene expression of NF-κB, however, was significantly increased after PMMA particle exposure (2.8-fold, p<0.05). EM treatment inhibited PMMA-stimulated NF-κB gene expression at the concentration of 2-10 μg/ml (p<0.05).

Effects of EM on ALP activity and osteogenic gene expression under physiological condition (in the absence of PMMA stimuli) We
further invested whether EM had any effect on the differentiation and gene expression of MC3T3 cells under physiological conditions (without PMMA stimuli). We found that there was no significant difference in ALP activity between cells with and without EM treatment after 14 days in culture. There were no significant differences in MC3T3 cells in osteogenic gene expression between cells treated with and without EM.

**Discussion:** In this study, MC3T3 cells were pretreated with EM (0-10 μg/ml), then stimulated with PMMA (1 mg/ml). We found that PMMA particles reduced cellular viability and osteogenetic potential in MC3T3 cells. EM treatment mitigated the effects of PMMA particles on the proliferation, viability and differentiation of MC3T3 cells. PMMA treatment decreased the gene expression of Runx2, Osterix and osteocalcin, and EM restored their mRNA expression to levels closer to control. PMMA exposure significantly increased gene expression of NF-κB, and EM suppressed this increase. These data demonstrate that EM mitigates the effects of PMMA on MC3T3 cell viability and differentiation, in part through down regulation of NF-κB pathway. We propose that EM represents an anabolic agent on MC3T3 cells challenged with PMMA particles. The molecular mechanism of this effect warrants further investigation.

**Significance:** A mitigative effect of EM on PMMA challenged pre-osteoblastic MC3T3-E1 cells observed in this study warrants further investigation to determine whether EM has similar effects on other wear particles-induced dysfunctional changes of osteoblast and osteoblast precursors. Periprosthetic EM delivery may represent a new approach to prevent and treat AL.

**Acknowledgments:**

**References:**

![Figure 1. Effects of EM on gene expression of NF-κB, Runx2, osterix, and osteocalcin in MC3T3 cells after 7 days in culture. Values](image-url)
are means ± SEM of genes measured in triplicate, and the experiments repeated three times. *p<0.05 cells + PMMA vs. control (cell alone); **p<0.05 for PMMA with EM treatment vs. PMMA without EM treatment.

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