

## Pentosidine and Nε-carboxymethyl-Lysine (CML) Alter Gene Expression of Bone Formation and Resorption Markers in OCY454-12H Cells

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**INTRODUCTION:** Type 2 Diabetes (T2D) is a metabolic disease marked by disrupted control of glucose, which results in continual high blood sugar levels. This elevated glucose concentration triggers dysfunction at the cellular level, affecting various physiological systems, with heightened susceptibility to bone fractures and skeletal aberrations. This vulnerability may be due to Advanced Glycation End Products (AGEs), which are harmful crosslinks that form in presence of sugar. Notably, the AGEs Pentosidine (PEN) and Carboxymethyl-lysine (CML) can escalate cellular oxidative stress via the upregulation of the receptor for AGEs, known as RAGE [1]. Among the discerned AGEs, PEN has a well-defined chemical structure and has a documented association with compromised bone strength in diabetic patients [2]. Similarly, CML is implicated in contributing to deterioration of bone quality and consequently exacerbating bone fracture risk within the context of T2D [3]. However, a knowledge gap exists in understanding the precise implications of PEN and CML in the context of diabetes-related cellular alterations and how these impacts contribute to altered bone function overall. Our study focuses on evaluating changes in osteocyte behavior by subjecting osteocytes to a hyperglycemic milieu while being exposed to PEN or CML. We aim to elucidate how they impact the bone turnover process, and we hypothesize that the exposure of osteocytes to PEN or CML in conjunction with elevated glucose levels will result in lower cell proliferation, elevated cell apoptosis, and upregulation of gene expression of SOST, RANKL, and RAGE.

**METHODS:** In six independent experiments (3 studies of PEN, 3 studies of CML), Ocy454 12-H osteocytes were grown in complete  $\alpha$ -MEM with the addition of 25 mM HEPES for pH maintenance at 33°C until confluent. Cells were then moved to 37°C and cultured for 9 days to allow differentiation. Cells were cultured in 3 different media groups: 1) osmotic control (OC; complete  $\alpha$ -MEM with 22 mM mannitol), 2) high glucose (HG; complete  $\alpha$ -MEM with 22 mM glucose), and either 3) HG + PEN (complete  $\alpha$ -MEM with 22 mM glucose + 2  $\mu$ M PEN) or HG + CML (complete  $\alpha$ -MEM with 22 mM glucose + 10  $\mu$ M CML). RNA was extracted from cell lysates at day 9 of incubation in culture, and gene expression was quantified using Real Time PCR with SYBR green chemistry and  $\beta$ -actin as housekeeping gene. Cell proliferation was assessed using an MTT assay. Cell apoptosis was assayed using EnChek caspase – 3 activity kit (Invitrogen). Fold difference (FD) was calculated using the Delta-delta Ct method, relative to osmotic control (OC). One-way ANOVA testing was performed on the qPCR data with significance set at  $\alpha = 0.05$ . One-way ANOVA with Tukey post-hoc testing was performed on MTT and apoptosis data to identify differences between group.

**RESULTS:** SOST gene expression after 9 days of incubation was upregulated in the HG + PEN group compared to OC group (2-fold,  $p \leq 0.05$ ) and to the PEN group (2-fold,  $p \leq 0.05$ ). SOST gene expression was upregulated in HG + CML group compared to the CML group (2-fold,  $p \leq 0.05$ ). RANKL gene expression was upregulated in HG + PEN group compared to OC group (4-fold,  $p \leq 0.05$ ) and to the PEN group (4-fold,  $p \leq 0.05$ ). RAGE gene expression was not different between groups (neither PEN nor CML). Cellular proliferation did not differ between groups ( $p > 0.05$ ). The PEN and CML groups appeared to have a trend in proliferation at day 7 compared to the OC group (+40%,  $p = 0.06$ ; +30%,  $p = 0.09$ ; respectively). Cellular apoptosis did not differ between groups except that the PEN exposed groups had a trend for higher caspase 3 activity than their relative non-PEN group ( $p = 0.09$ ). Results are illustrated in Fig 1.

**DISCUSSION:** HG + PEN and HG + CML significantly increased SOST gene expression, compared to only PEN or only CML, respectively. This suggests that the combination of PEN or CML with hyperglycemic conditions has pronounced negative effects on bone turnover, as it upregulates the gene responsible for sclerostin protein, an inhibitor of bone formation via osteoblasts. RANKL gene exhibited higher expression with HG + PEN compared to OC and only PEN, indicating that the addition of PEN in presence of hyperglycemia amplifies the potential for differentiation of osteoblasts as triggered through RANKL upregulation. Neither of the AGEs had an impact on oxidative stress and/or inflammation as shown by RAGE gene expression, nor did they impact cellular apoptosis or proliferation results. Together the data suggest these AGEs affect bone remodeling via sclerostin and RANKL produced by osteocytes, but they do not necessarily lead to osteocyte cell death nor proliferation. Further investigation with additional experiments and employing additional doses of PEN and CML will help provide clarify our data and offer further insight.

**SIGNIFICANCE/CLINICAL RELEVANCE:** T2D leads to increased fracture risk, which may be due to altered bone remodeling and deterioration of bone quality. We show for the first time the impact of PEN and CML on mature osteocyte behavior to help elucidate these cellular changes.

**REFERENCES:** [1] Murray CE, et al. (2019) [2] Yamamoto M, et al. (2008) [3] Dhaliwal R, et al. (2022) [4] Bonewald LF. (2011)

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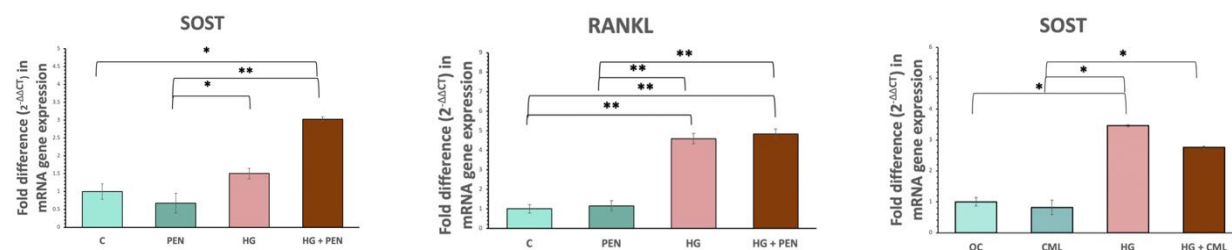


Fig 1: Fold difference in osteocyte gene expression of (A) SOST in PEN, (B) RANKL in PEN, and (C) SOST in CML. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$

