

Are different glucocorticoids equivalent in inducing *in vitro* osteogenesis?

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INTRODUCTION: Endogenous glucocorticoids (GC), at physiological levels, are essential for bone formation and homeostasis. However, elevated GC plasma concentrations are detrimental to skeletal health, as demonstrated by the high incidence of GC-induced osteoporosis or osteonecrosis in e.g., long-term pharmacological treatment or Cushing's syndrome.

GC are also commonly used to induce bone differentiation. Dexamethasone (DEX), a synthetic glucocorticoid available in different formulations, is mostly used to induce *in vitro* osteogenic differentiation of human mesenchymal stromal cells (MSCs), but it also has other effects including the induction of adipogenic differentiation and of a higher gene expression level of pro-inflammatory cytokines [1,2]. Betamethasone (BET) is a stereoisomer of DEX with different pharmacokinetics and pharmacodynamics profile, and it is also used clinically. To our knowledge, BET has never been tested *in vitro* for its osteogenic potential. In humans, the main endogenous GC is represented by cortisol or hydrocortisone (HYD), and its concentrations and temporal release are tightly regulated. Though HYD is still sometimes employed in protocols of osteogenic differentiation, DEX use is far more widespread. The three molecules have different EC₅₀, with HYD being ~40 times less potent than the other GC and with a shorter biological half-life.

The aim of this study is to compare the osteogenic potential of two different DEX formulations, BET, and HYD on human bone marrow derived MSCs. A particular focus is dedicated to the "side" effects of GC use, such as PPARG activation and adipocyte formation. The overall goal is to define the effect of GC on MSCs and to determine the best protocol for induction of osteogenesis *in vitro*, in order to provide better tools for basic research in the bone field. The hypothesis is that use of HYD *in vitro* might allow for a more physiological modulation of GR activation in the context of osteogenic differentiation. Its lower EC₅₀ and biological half-life compared to the synthetic GC can aid in resembling the physiological circadian oscillation of this class of hormones, preventing an excessive activation of the GC receptor.

METHODS: Human bone marrow derived MSCs (n=3 donors, samples obtained with ethical permission and written informed consent) were previously isolated using standardized methodologies [3]. Cells were induced to osteogenic differentiation in DMEM 1 g/L glucose, 10% FBS, 1% penicillin/streptomycin, 50 µg/ml ascorbic acid 2-phosphate and 5 mM β-glycerophosphate. The medium was further supplemented with 10 nM of either two different DEX formulations (one is water-soluble in a cyclodextrin complex, the other dissolved in DMSO), betamethasone (BET, solubilized in DMSO) or hydrocortisone (HYD, solubilized in DMSO). Cells maintained in growth medium, osteogenic basal medium (*i.e.*, no GC added), or DMSO-osteogenic basal medium were used as controls. Medium was refreshed every second day. Samples were collected at 7, 14, and 21 days and the effect of the different GC was assessed via Alizarin Red staining and quantification, Oil Red O staining, ALP activity assay, and gene expression analysis by RT-qPCR. Each condition was tested in triplicate for each different biological donor. To take the effect of both donor variability and *in vitro* treatments into account, two-way ANOVA with Tukey's multiple comparison test was used for statistical analysis.

RESULTS SECTION: Both DEX formulations and BET, at 10 nM concentration, have similar effects on cell mineral deposition (Fig.1). The effects of these GC on *RUNX2*, *SOX9*, and *PPARG* expression at day 7 are also comparable in *SOX9* downregulation and *PPARG* upregulation ($p < 0.0001$). The same concentration of HYD showed a milder effect on mineral deposition, but it influenced neither *SOX9* nor *PPARG* levels. *PPARG* expression at day 7 correlated with Oil Red O staining at day 21; indeed, except for one donor that did not show any adipocyte formation at all, both DEX and BET promoted adipocyte formation within the mineralizing cultures, an effect that was not observed for HYD. Other differences between HYD and the other GC can be observed in the gene expression levels of osteogenic transcription factors. The use of HYD resulted in the highest levels of *RUNX2*, *DLX5* and *SP7* expression, whilst *DLX5* and *SP7* were downregulated by both DEX formulations and BET. Synthetic GC, especially BET, induced upregulation of *IBSP* and *ALPL*, and downregulation of *SPARC* and *SPP1*. The levels of the same late osteogenic markers remained at the levels of the DMSO control for HYD.

DISCUSSION: The results suggest that different GC are not equivalent in inducing osteogenic differentiation of human bone marrow derived MSCs. Some of the differences, such as the lack of PPARG activation and of influence on late marker expression, might be explained by the lower potency of HYD compared to the other GC. However, other results suggest that different responses might also be involved, as indicated by the *DLX5* and *SP7* gene expression levels. As this study tested only one dose of GC on MSC osteogenic differentiation (above the EC₅₀ for DEX and BET, and below it for HYD), the effects of both lower and higher concentrations and the non-genomic effects of GC are next to be determined. It also remains to be investigated whether the effects on *SOX9* for higher HYD concentrations are similar to that observed for DEX, and this would have relevant implications in how *SOX9* interacts with and is regulated by GC receptor activation [4].

SIGNIFICANCE/CLINICAL RELEVANCE: The widespread use of dexamethasone for the induction of osteogenic differentiation of human MSCs might hamper the efforts of basic and preclinical bone research. Understanding the underlying mechanisms and effects of different GC on human MSC differentiation can have relevant implications in both improving *in vitro* models and in the pathophysiology of GC-related bone disorders.

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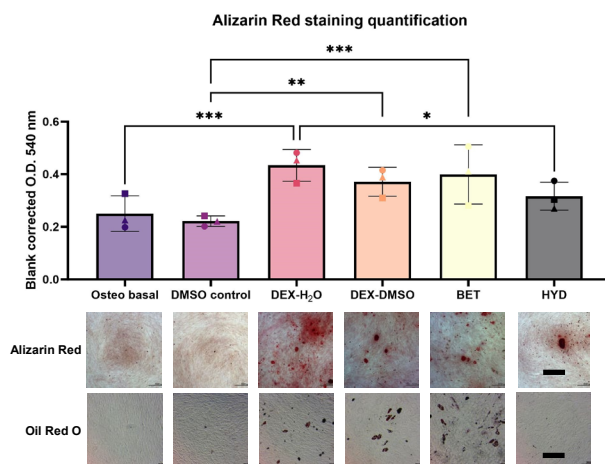


Figure 1. Alizarin Red quantification and representative pictures of Alizarin Red and Oil Red O staining for one donor. Scale bar = 500 µm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$