Mitigating glucocorticoid - induced osteonecrosis of the femoral head in acute lymphoblastic leukemia using Metformin

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INTRODUCTION: Acute lymphoblastic leukemia is the most frequent pediatric malignancy, induced by the uncontrolled proliferation of immature lymphoid cells. Chemotherapy includes high dose Glucocorticoid [GC] administration to activate apoptosis in leukemic cells.[1] However, GC treatment has adverse effects on bone homeostasis by impairing vascular circulation and promoting apoptosis of mesenchymal stem cells [MSCs], partially due to the production of reactive oxygen species [ROS]. Osteonecrosis of the femoral head [ONFH] is an often-observed result, characterized by pain in the hip and severe functional limitations .[2] Metformin is prescripted as first line - treatment of Type 2 diabetes and additionally demonstrates antioxidative and anti - tumoral effects.[3] Studies on bone formation confirm increased osteogenic potential and anti – apoptotic effects in MSCs by Metformin.[2] Therefore, the aim of this study is the evaluation of the osteogenic, antitumoral and apoptosis - regulating effects of Metformin in GC - treated 2D cocultures of the B - cell precursor leukemia cell line RS4:11 and MSCs.

METHODS: First, mono - cultures of RS4:11 and MSCs were treated with 3; 30; 300; 1000 and 3000 ng/ml of Dexamethasone [DEX] or Prednisolone [PRED] for 1, 4 and 8 days, to simulate chemotherapy and development of ONFH. Cell Viability was assessed using the CCK – 8 Assay Kit. In addition, Annexin V/ 7AAD Assay Kits were used to identify the effective dose of GC, inducing near total – apoptosis in RS4:11 cells and MSC.
Next, 2D – transwell cocultures of MSCs and RS4:11 cells were established in a 1:4 ratio and divided into 6 groups: 1) control 2) 300 ng/ml DEX 3) 300 ng/ml PRED 4) 300 ng/ml of DEX + 10 µM Metformin 5) PRED + 10 µM Metformin 6) 10 µM Metformin. Apoptosis assays were repeated in coculture to determine differences in dose response in comparison to monoculture. Next, the effect of GC ± Metformin on oxidative stress induced ROS production was evaluated, using the DCFDA/H2DCFDA Cellular ROS Test Kit after 24 hours of treatment.

RESULTS: Cell viability assays were performed for both cell types in mono - culture after 1, 4 and 8 days of treatment with either DEX or PRED. After 1 day of treatment, a dose - dependent decrease in cell viability was observed with both types of cells and medications, first reaching significance at a concentration of 300 ng/ml. No differences were observed in MSCs after 4 and 8 days for both medications. In contrast, RS4:11 cells exposed to 3 ng/ml of DEX or PRED showed a significant increase after 4 and 8 days ($p < 0.0001$), while subsequent increasing concentrations from 60 up to 3000 ng/ ml exerted a highly significant decrease in cell – viability ($p < 0.0001$). Apoptosis - assays in monocultures of leukemia cells emphasized a time - and dose dependent increase in apoptotic cells, up to 50 % after 4 days and 75 % after 8 days using 300 ng/ml of DEX or PRED, while no alterations were observed in MSCs. In 2D - transwell cocultures, groups receiving DEX or PRED showed non – significant higher apoptosis rates in both cell types after 1 day of treatment compared to control groups. For MSCs, combining DEX + Metformin showed a decrease in apoptosis, while PRED + Metformin showed no decrease. In coculture, only a minor increase in apoptotic was detected with DEX or PRED in RS4:11 cells and in combination with Metformin no further changes were observed. Surprisingly after 8 days, apoptosis rates in MSCs showed no difference among treatment groups compared to day 1. In contrast, apoptosis rates in leukemia cells were higher than 70 % in all groups receiving DEX or PRED and even superior in groups additionally receiving Metformin ($p = 0.001$). Following, ROS – detection assays were performed to determine oxidative stress in cocultures. The production of ROS in both cell types was most intense with mono - application of Metformin and lower in groups receiving DEX or PRED ± Metformin.

DISCUSSION: Cell viability - and apoptosis assays revealed consistent results after 1 day of treatment with GC in both cell types, showing significant decreases in cell viability as well as higher apoptosis rates compared to the control group. Remarkably, after 4 and 8 days, no significant differences in cell viability were observed in MSCs, indicating superior resistance to GC - treatment in comparison with leukemia cells. Increasing apoptosis rates with addition of Metformin in coculture could possibly be explained by ROS production. Leukemic cells may be less capable to exert antioxidative properties and metabolic adaptations in comparison to MSCs. We confirmed that GC induces apoptosis directly in leukemic cells; changes in redox potential and metabolic differences and the interplay in both cell types remain to be elucidated. In summary the current experiments establish a reliable platform for evaluating the effects of Metformin on cell viability, apoptosis and the production of ROS. Next, Western blot analysis will be performed to assess changes and quantify expression of apoptosis - related proteins to corroborate the results. Future experiments will focus on alterations in bone homeostasis using histological staining for matrix maturation and calcium depositions as well as qPCR – analysis for evaluating changes in bone formation related genes by Metformin.

SIGNIFICANCE/CLINICAL RELEVANCE: Metformin is a potential therapeutic agent in preve3nting ONFH as a major adverse effect of GC – treatment in acute lymphoblastic leukemia by synergistically supporting bone homeostasis and enhancing leukemic cell death.

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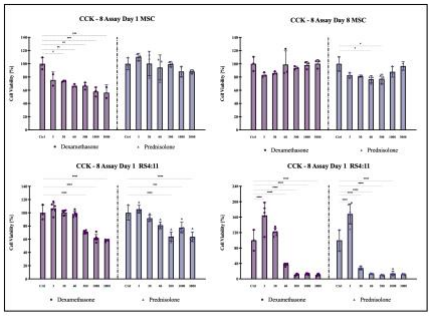

Figure 1 CCK8 cell viability assay showing a dose - dependent decrease in cell viability on day 1 for DEX . In contrast no changes were observed on day 8 in MSC. For RS4:11 cells a dose dependent decrease is visible on day 1 for DEX and PRED. On Day 8 an increase in cell viability is observed with 3 ng/ml of DEX and PRED, followed by a rapid decrease in cell viability for higher doses.

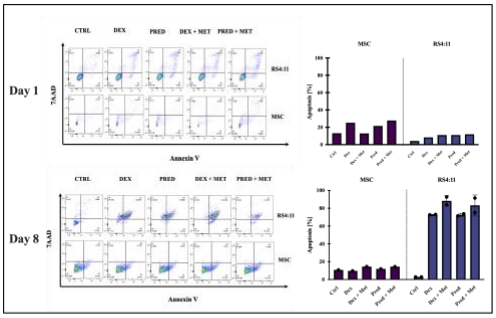

Figure 1 Annexin V/ 7AAD Apoptosis - Assay displaying major differences between MSC and RS4:11 cells on day 8. In addition, a time - dependent increase in early and late-stage apoptosis for leukemia cells up to 90 % on day 8 with treatment of GC + Metformin was observed.

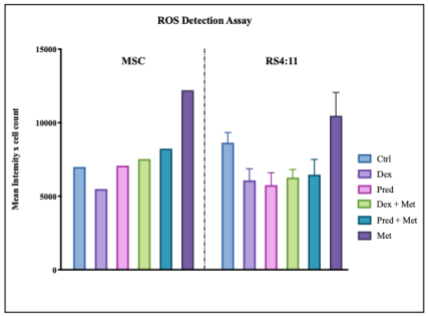

Figure 2 Detection Assay for ROS revealing an increased production in Co - cultures treated with Metformin. Application of GC and GC + Metformin decreases the production of ROS compared to control group