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Original Article

Dexamethasone-Induced Osteoblast Toxicity: Bioenergetics Dysfunction and Oxidative Stress Unmask Apoptotic Pathways

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ABSTRACT

Aim: Dexamethasone (DEX) is a glucocorticoid commonly used to treat autoimmune and non-infectious inflammatory diseases; however, its role in inducing osteoporosis is poorly understood. **Materials and Methods:** This study investigates the effects of DEX on cell toxicity, secretory functions, mitochondrial bioenergetics, oxidative stress and apoptosis in human osteoblasts. **Results:** DEX-induced cell toxicity, as evidenced by elevated Lactate Dehydrogenase (LDH) and MTT assays, with an estimated EC_{50} of approximately 50 µM. The release of "procollagen type I, osteocalcin and alkaline phosphatase" was significantly reduced. Bioenergetic assays revealed that DEX inhibited ATP synthesis, decreased "mitochondrial membrane potential" and impaired the activity of "mitochondrial complexes I and III," as well as lactate production/oxygen consumption. Additionally, DEX significantly elevated levels of "Reactive Oxygen Species (ROS), Thiobarbituric Acid Reactive Substances (TBARS) and Nuclear factor erythroid 2-related factor 2 (*Nrf2*) expression" while reducing the antioxidant levels of "catalase, reduced glutathione and superoxide dismutase." Caspase assays indicated that DEX elevated active caspases-3, -8 and -9. Furthermore, antioxidants, including "reduced glutathione, the caspase-3 inhibitor Z-VAD-FMK and Co-enzyme Q-10," significantly alleviated DEX-induced cytotoxicity. **Conclusion:** The present findings suggest that DEX induces osteoblast cytotoxicity via mitochondrial disruption, oxidative stress and apoptosis, with potential mitigation through antioxidant agents.

Keywords: Antioxidants, Bone, Dexamethasone, Mitochondria, Osteoblasts, Redox stress.

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INTRODUCTION

Dexamethasone (DEX, $C_{22}H_{29}FO_5$; Chemical Entities of Biological Interest (CHEBI:41879), a "9-fluoro-11β,17,21-trihydroxy-16 α-methylpregna-1,4-diene-3,20-dione," is a widely prescribed glucocorticoid for its anti-inflammatory action in autoimmune and other non-infectious inflammatory diseases.[1] However, long therapeutic courses and overdoses of DEX were reported to cause unfavorable side effects such as Osteoporosis (OP). DEX-induced OP is considered one of the prevalent secondary OP.[2,3] Clinical studies reported altered bone metabolism among DEX-treated patients with a higher risk for fracture and reduction of trabecular bone mass is reported during the first year of therapy.[4,5] The increased susceptibility to fractures cannot be explained solely by the reduced trabecular bone mass or Bone Mineral Density (BMD). Mineral density in DEX-treated patients is still higher than BMD of osteoporotic postmenopausal women. Hence, other mechanisms of DEX-induced bone disorders should be investigated more.[6] Thus, a comprehensive explanation of the pathology associated with DEX-induced OP is needed to help physicians efficiently prevent and treat OP.[7]

Bone is the main part of the locomotor system, which is mainly composed of mineral crystals and organic matrix. Nearly 10% of bone components are four cell types, including bone lining cells, osteocytes, osteoblasts and osteoclasts. Regular bone renewal and turnover process is ensured via a balance between the osteoclasts' resorptive activity and the osteoblasts' synthetic function.[8] This physiological continuous balance is lost in cases of osteoporosis with increased bone resorption.[9] The bone surface is lined with

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cuboidal osteoblasts that are capable of secreting osteoid proteins, including Alkaline Phosphatase (ALP), collagen (mainly type I) and osteocalcin, which are required for normal mineralization and density.[8]

This study was undertaken to address the critical gaps in understanding the mechanisms underlying dexamethasone-induced osteoblast toxicity. Given that osteoporosis induced by DEX is a significant clinical concern, the investigation of its effects on osteoblast bioenergetics, oxidative stress and apoptosis is essential for elucidating the multifactorial pathology of DEX-induced bone disorders. By employing a human osteoblast cell line, we aim to provide detailed insights into the bioenergetic dysfunction and redox imbalance that contribute to cellular apoptosis. These findings will not only enhance our understanding of DEX-related osteotoxicity but also inform the development of preventive and therapeutic strategies for managing osteoporosis in patients receiving glucocorticoid therapy.

MATERIALS AND METHODS

Chemicals

Reagents and chemicals utilized in this study were purchased from Sigma (St. Louis, MO, USA). The preparation of Modified Hank's solution was done to determine Oxygen consumption rates assay.[10]

Cell culture

Human osteoblasts (PromoCell, Heidelberg, Germany) with passages 4-5 were used as the *in vitro* model of the current study. The Human Osteoblast Growth Medium was used to culture the cells.

Cytotoxicity assessment using LDH and MTT assays

The DEX impact on the osteoblast viability and cells' membrane integrity was assessed by widely used "3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide" (MTT) and Lactate Dehydrogenase (LDH) assays. Osteoblasts were seeded in 96-well plates ($2x10^4$ cells/well) and incubated till ninety percent confluence. Then, cells were exposed to different DEX concentrations (0.1, 1, 10, 100 and 1000 µM) for 3,6,12, 24 and 48 hr. Wells containing positive control 2% Triton X-100 were used to complete cell degradation in LDH assay. LDH was performed according to the company instructions (Clontech, Mountain View, California, USA). MTT assay was conducted.[11] Plate reader 'TopCount' (Perkin Elmer, Ueberlingen, Germany) was used to estimate absorbance levels for MTT assay and LDH with reading points of 590 nm and 490 nm, respectively. For analysis, blank well average absorbance values were subtracted from samples treated and control not-treated wells values. Cell viability was determined as percentages and compared with control wells (the viability percentage in the controls is assumed to be 100%). The assessment was performed in triplicate for every time point.

Osteoblast proliferation measurements

The BrdU proliferation assays were used to evaluate osteoblast proliferation in treated DEX plates based on the company protocol (Millipore, Massachusetts, USA). Osteoblasts were seeded in 96-well plates (2×10^4 cells/well) and incubated till ninety percent confluence and occupied with DEX (5 and 50 µM) for 12, 24 hr. At the time point, the supplied "Bromodeoxyuridine (BrdU)" reagent was added to the wells within the last 2 hr of the exposure times and then the previous media was discarded. Approximately 200 µL of fixing solution was inserted into each sample and kept at room temperature. After a half hour, the solution was removed by aspiration and the well plates were cleansed. A diluted anti-BrdU monoclonal antibody was added, followed by the goat anti-mouse IgG. After washing, the plates were dried, and the peroxidase substrate was followed by the acid-stop solution prior to reading of absorbance. Plate reader 'TopCount' (Perkin Elmer, Ueberlingen, Germany) was used to read absorbance at 450 nm. The previous method was followed in triplicate in the experiment, with subtracting absorbance values of the control well from DEX-treated samples each time.

DEX effect on procollagen, osteocalcin and alkaline phosphatase secretion

Osteoblasts were cultured in 96-well plates ($4x10^3$ cells/well) incubated till 90% confluency and treated with DEX (5 and 50 µM) for 24 hr. The evaluation of osteoblast procollagen type I C-peptide secretion in DEX-treated cells was performed using an "Enzyme-Linked Immunosorbent Assay (ELISA)" kit (Abcam, Cambridge, UK) following the company's instructions. For media Procollagen type I Peptide (PIP) level, media was collected and sonicated, while cells were counted and homogenized using phosphate buffered saline composed of 1 mM "Ethylenediaminetetraacetic Acid (EDTA)," 0.5% Triton X-100 and 1 mM "Phenylmethylsulfonyl Fluoride (PMSF)." Osteoblast secretion of osteocalcin was measured in the media using an ELISA kit (Takara Shuzou, Japan) following the manufacturer's protocol. The Alkaline Phosphatase (ALP) assay commercial kit (Abcam, Cambridge, UK) was obtained to assess the Dex effect on osteoblast ALP secretion in the media. Using a plate reader 'TopCount' (Perkin Elmer, Ueberlingen, Germany), the absorbance levels were determined at 450, 405 and 450 nm for PIP, ALP and osteocalcin, respectively. Levels were estimated via plotting against a standard curve and normalized to the total cell number for each sample. The assessment was performed in triplicate for every time point.

DEX effect on intracellular adenosine triphosphate levels

Osteoblasts were occupied at $2x10^4$ cells/well in 96 well-plate and incubated till 90% confluence. Then DEX was added for final concentrations 5 and 50 µM for 24 hr. At the time point, Adenosine Triphosphate (ATP) was calculated using the supplied commercial kit with instructions (Abcam, Cambridge, UK). The interaction of ATP with added D-luciferin and luciferase caused light production. The luminescence signal was read using a 'TopCount' luminometer (Perkin Elmer, Ueberlingen, Germany). Blank readings were subtracted from all control and sample cell well values. ATP was normalized to the total tissue protein for each sample. The assessment was performed in triplicate for each concentration per experiment.

DEX effect on the osteoblasts' Mitochondrial Membrane Potential (MPP)

Mito tracker Green (MG) prob was used to assess the DEX impact on the treated osteoblast MMP. Osteoblasts were prepared and treated as in the previous section for ATP assay. At the time point, media were removed, and MG staining solution (50 nM) was added. For half an hour, tested plates were incubated at 37°C. The staining solution was removed, and the cell plates were washed. After that, freshly prepared buffered phosphate saline was added to read the fluorescence level via the "TopCount microplate reader (Perkin Elmer, Ueberlingen, Germany)" at excitation/emission filters of 490/515 nm. The uncoupler agent was selected and worked as a positive control, such as carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone. The assessment was performed in triplicate for each concentration per experiment.

DEX effect on Mitochondrial Complexes I (MCI) and complexes III (MCIII) activities

Osteoblasts were cultured in 6 well-plate ($4x10^4$ cells/well) and left to reach 90% confluence. Cells were exposed to two different DEX concentrations 5 and 50 µM for 24 hr. Mitochondrial complex I activity was evaluated,[12] and Dichloroindophenol (DCIP) was adopted as a final electron acceptor. Complex I oxidize the reduced form of "Nicotinamide Adenine Dinucleotide (NADH)", with electron reduction produced by substrate decyl-ubiquinone that later provides the electrons to DCIP. Reduction DCIP was measured at 620 nm. Mitochondrial complex III activity and cytochrome c reduction at 550 nm were assessed.[13] The activities of both complexes were evaluated by checking their activities in the presence of DEX. Bradford assay was utilized to calculate protein levels in each sample. The response of the cells MCI and MCIII to rotenone and antimycin A, respectively, was calibrated before the experiment. Experiments were repapered at least 5 samples for each treatment and controls were measured for robust data. A spectrophotometer with temperature control was used to

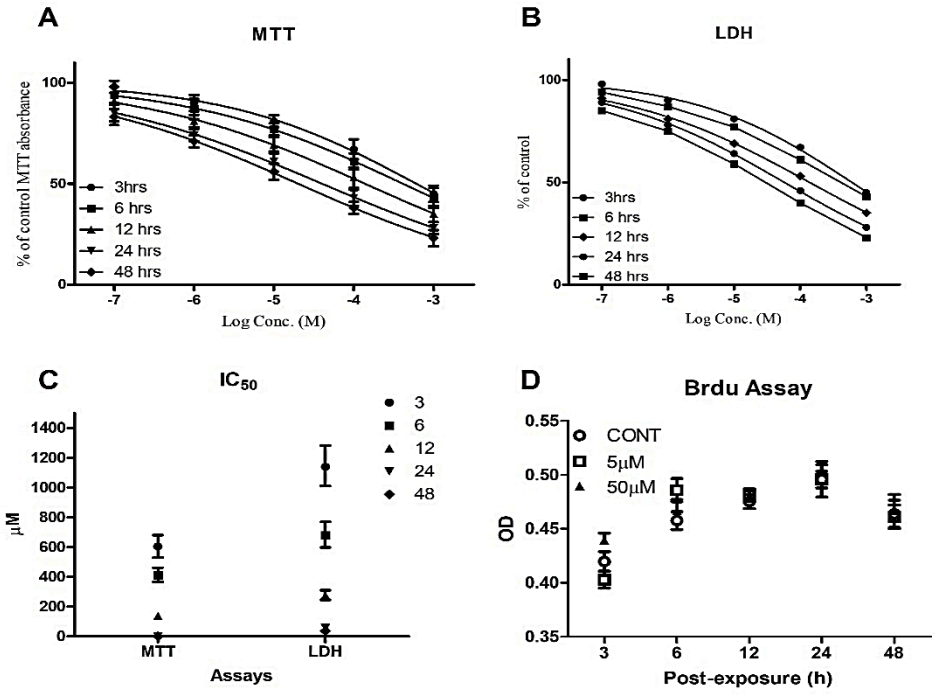

Figure 1: Dexamethasone (DEX) induced cytotoxic effect on human osteoblast cell line. DEX was shown to be cytotoxic to the cell line via MTT (1A) and LDH (1B) assays. The cells were treated with DEX at concentrations 0.1,1,10,100 and 100 µM for the exposure time range from 3-48 hr. Both assays showed that DEX was cytotoxic to the human osteoblast in concentration and exposure duration-dependent pattern. LDH assay showed higher EC_{50}s in all tested time points (1C). BrdU assay showed that DEX has no significant effect on the treated cells' proliferation at concentrations 5 and 50 µM (1D).

read absorbance (Beckman Coulter, DU 800). Both complexes' activities were expressed as nmol min^{-1} mg^{-1} of total protein.

DEX effect on osteoblast lactate production

The effect of (5 and 50 µM) DEX on the osteoblasts lactate production was assessed by "lactate assay kit (Biovision, Mountain View, California, USA)" following instructions. At the time point, media were collected, and cell protein content was measured. Media were put in a 96-well plate (25 µL/well), followed by adding 25 µL assay buffer and 50 µL reaction mix to each well. After 30 min, a Dyne MRX microplate reader (Dyne Technologies, Chantilly, VA, USA) was used to read the absorbance at 570 nm. The assay was done in triplicates.

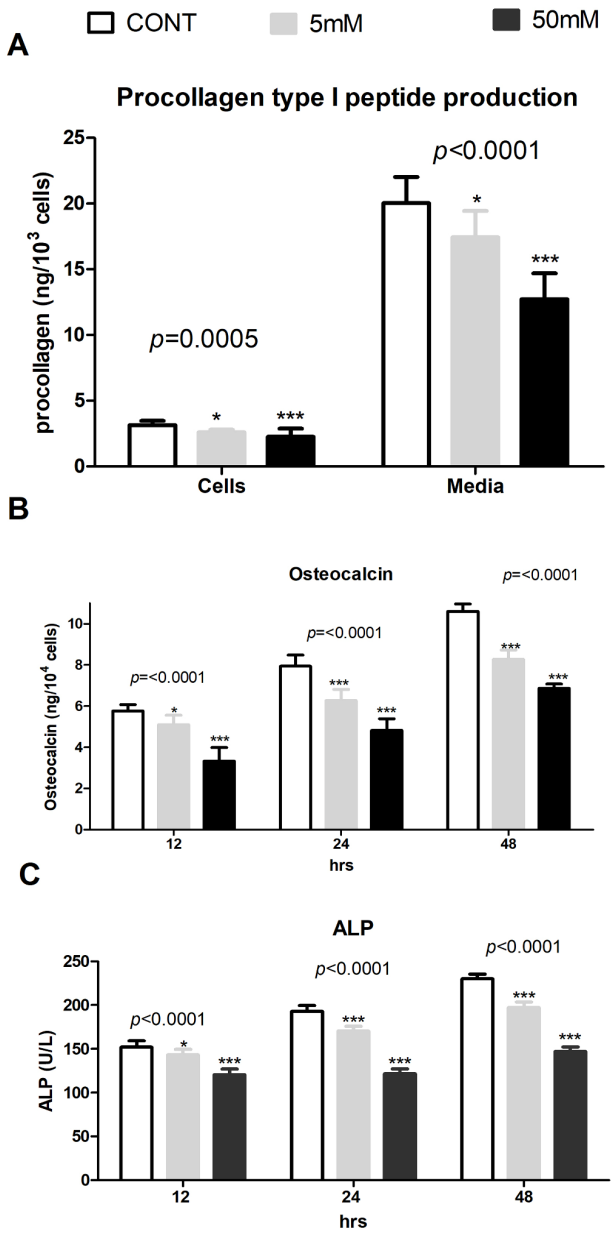

Figure 2: Effect of Dexamethasone (DEX) on the secretory functions of human osteoblast cell line. DEX was shown to decrease the osteoblast secretion of procollagen type I peptide significantly (2A), osteocalcin (2B), as well as Alkaline Phosphatase (ALP) (2C) at concentrations 5 and 50 µM for 24 hr to variable extents. Significance was evaluated by one-way ANOVA assay with "Dunn multiple comparison posthoc test." Significance was represented as * *p*-value<0.05, ** *p*-value<0.001, *** *p*-value<0.0001.

DEX effect on the osteoblasts' oxygen consumption rate

The effect of DEX on the osteoblasts' Oxygen Consumption Rate (OCR) was assessed polarographically using Clark oxygen electrodes (Rank Brothers, Bottisham, UK).[14] The electrodes were calibrated to 100% and 0 oxygen partial pressure at a voltage of 0.6 V by vigorous gassing and the addition of sodium dithionate, respectively. Osteoblasts were treated with DEX at concentrations 5 and 50 µM for 24 hr. Then, cells were trypsinized and counted and suspended in Hank's solution. The OCR was measured for 10 min and then 2 µL of 6 mM azide was added to the electrodes' chambers. The OCR was estimated via the slope of the curve representing partial oxygen tension in the electrode chamber over three hundred seconds. After 1 min of Azide addition, its effect was calculated. Experiments were repeated 5 times for each concentration for robust data.

DEX effect on reactive oxygen species in the treated osteoblasts

The production of Reactive Oxygen Species (ROS) from DEX (5 and 50 µM for 24 hr) treated osteoblasts was evaluated by using a "2,7-Dichlorodihydrofluorescein Diacetate (DCFDA)" assay.[11] Unstained well cells were utilized as negative controls, while Antimycin A (10 mM for 30 min) was used as a positive control. At the time point, media was removed, and Phosphate-Buffered Saline (PBS) was added to wash cells. Then, the DCFDA solution (25 uM in Hank's solution) was inserted, and the well plates were kept at 37 °C in a dark environment. After 45 min, cells were washed, and fluorescence was read at excitation/emission wavelengths 485/535 nm with a plate reader (Floustar). Fluorescence values of blank cells-free wells were subtracted from entirely plate reading values. The expression of ROS levels in the treated samples is a percent compared to controls (assuming the control ROS level is 100%). Experiments were done in triplicates.

DEX effect on antioxidant enzyme activity

The impact of DEX on the activities of antioxidant enzymes in osteoblasts, specifically Superoxide Dismutase (SOD) and Catalase (CAT), was assessed. Osteoblast cells were exposed to DEX at concentrations of 5 and 50 µM over duration of 24 hr. At the conclusion of this incubation period, a calorimetric assay was performed to measure CAT activity. Following treatment, cells were collected and the protein concentration for each sample was quantified. For the CAT activity assay, a reaction mixture was prepared by combining 1 mL of 0.01 M phosphate buffer (pH 7) with 0.4 mL of a 2 M hydrogen peroxide solution and 0.1 ml of the tissue homogenate. To terminate the reaction, 2 mL of

dichromate-acetic acid reagent was introduced. The absorbance of CAT was recorded at a wavelength of 620 nm and the results were expressed as micromoles of hydrogen peroxide consumed per minute and milligram of protein.

The SOD activity was determined by following a colorimetric commercial assay kit (Abcam, Cambridge, UK). Cells were harvested and lysed and Centrifuged (14,000xg for 5 min at +4ºC). After that, the resultant supernatants were collected and stored on ice till analysis. The supernatant includes all the cell content of SOD (mitochondrial and cytosolic). The SOD Absorbance rate was monitored at 440 nM via a 'TopCount' plate reader (Perkin Elmer, Ueberlingen, Germany). Units of SOD activity were measured via plotting against a standard curve and values expressed as mg of total protein. Experiments were conducted in triplicates.

DEX effect on osteoblasts reduced glutathione stores

The reduced Glutathione (GSH) in cells treated with 5 and 50 µM DEX for 24 hr was calculated according to the commercial kit (Abcam) company guide. At the designated time point, the cells were harvested, centrifuged to form a pellet and subsequently homogenized in 100 µL of a 5% sulfosalicylic acid solution, which was then kept on ice until further analysis. The absorbance was measured at 450 nm at room temperature using a plate reader (TopCount, Perkin Elmer, Ueberlingen, Germany), with readings taken dynamically over a period of 40 min. The obtained values were plotted against a standard curve to quantify the results. The levels of reduced Glutathione (GSH) were expressed as nanomoles of GSH per gram of tissue. Experiments were conducted in triplicates.

Effect of DEX on osteoblasts lipid peroxidation level

Osteoblasts were treated with DEX (5 and 50 µM) for 24 hr and Thiobarbituric Acid Reactive substances (TBARS) were quantified using a commercial kit (Abcam) based on measurement of Malondialdehyde (MDA) as a major lipid oxidation product. At the time point, cells were harvested and homogenized using the MDA lysis buffer by a Dounce homogenizer on ice. Lysate was sonicated and centrifuged at 13,000xg. Next, the supernatant was collected for analysis. Absorbance was recorded at 532 nm using a "TopCount plate reader (Perkin Elmer, Ueberlingen, Germany)," and the measurements were compared with a standard curve. All experiments were performed in triplicate.

DEX effect on osteoblast apoptotic pathways

In the treated osteoblasts with 5 and 50 µM DEX for 24 hr, the activities of caspases 3 (common pathway), 9 (intrinsic pathway) and 8 (extrinsic pathway) were assessed using "caspase fluorescent BD Apo-Alert assay kits (Clontech Laboratories, Palo Alto, CA)." Fluorescence readings were taken in a 96-well format using a "Synergy HT Fluoremeter (Bio-Tek Instruments, Inc., Winooski, VT)." The excitation/emission wavelengths were set at 400/505 nm for caspase 3, 400/505 nm for caspase 8, and 380/460 nm for caspase 9 assays.

Quantitative real-time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) assay

Total RNA extraction from the treated cells was performed using TRIzol reagent (Invitrogen) following the producer's guidelines. The "PrimeScript RT-PCR Kit (Takara, Dalian, China)" was applied for RT. Particular primers (designed by the "online primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)" were used to determine qRT-PCR (Table 1). The acquired data were adjusted to "Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)" mRNA.[15] All quality control measurements were applied for each PCR run.

Protective effect of antioxidants, anti-apoptosis and mitochondrial enhancers assay

The protective effect of GSH-R at a concentration of 10 µM, the caspase-3 inhibitor Z-VAD-FMK at 200 µM and coenzyme Q-10 at 1 µM were evaluated using MTT assays. These assays were performed in conjunction with DEX treatment at final concentrations of 5 µM and 50 µM for duration of 24 hr. The cytotoxic effects of DEX were examined both in the presence and absence of these protective agents.

Statistical analysis

Data analyses for the study were conducted using "PRISM 5 (GraphPad Software Inc., San Diego, California, USA)." Non-linear curve fitting methods were applied to determine the EC_{50} values for the non-linear MTT and LDH assays. A two-way Analysis of Variance (ANOVA) was employed to assess the influence of DEX concentrations and exposure durations on the results of the assays. For comparisons between groups, a one-way ANOVA with Dunn's multiple comparisons post-test, an unpaired Student's t-test, or a Mann-Whitney test were utilized as appropriate. Statistical significance was established at $p<0.05$.

RESULTS

Cytotoxicity effect of dexamethasone on human osteoblasts

The current study proved that exposure to DEX with different concentrations (0.1 µM to 1 mM) for variant time durations of 3, 6, 12, 24 and 48 hr is cytotoxic to human osteoblasts. DEX was cytotoxic to osteoblasts in a dose and time-dependent manner exhibited by quantified MTT and LDH parameters (Figures 1A-D). Interestingly, there was significant toxicity to treated osteoblasts at 0.1 µM at 12 hr post-DEX exposure, significantly affecting their membrane integrity in LDH assay 12 hr after exposure. A two-way ANOVA assay revealed that both concentration and exposure durations significantly affected the variation of the outcomes of both assays. The concentration

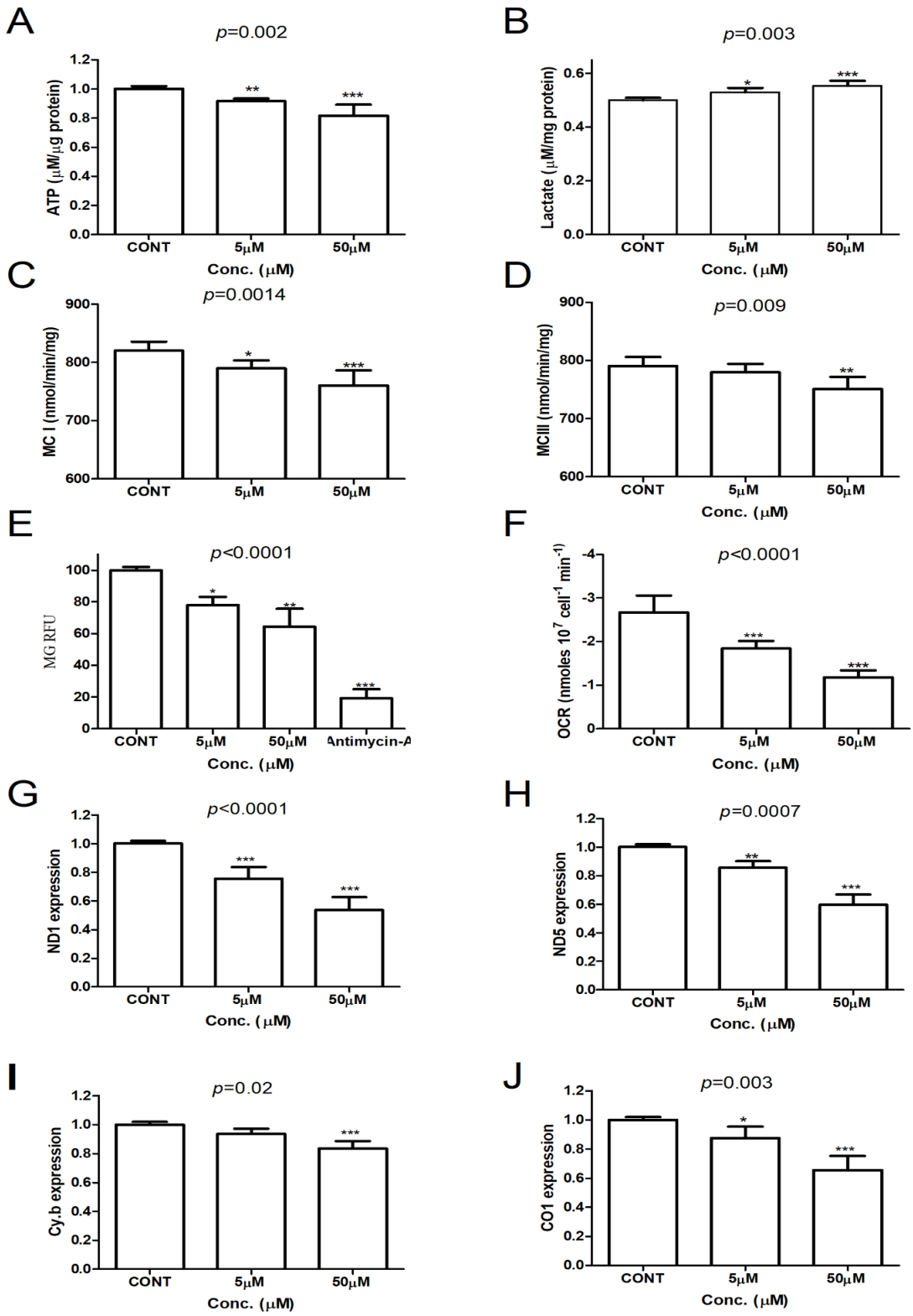

Figure 3: Effect of Dexamethasone (DEX) on the bioenergetics of human osteoblast cell line. DEX was shown to significantly decrease the osteoblast ATP production (3A), with significant increase in lactate release from the treated cells (3B) with decreased activities of electron transport chain Mitochondrial Complexes I (MC I) (3C) and III (MC III) (3D), decreased mitochondrial membrane potential measured by "Mito tracker green" (MG) as "Relative Fluorescence Unit (RFU)" (3E), decreased Oxygen Consumption Rate (OCR) (3F), "NADH dehydrogenase subunit 1/5" (ND1/5) expressions (G and H), cytochrome C oxidase subunit 1 (Cy.b) (3I) and cytochrome C Oxidase subunit 1 (CO1) (3J) at concentrations 5 and 50 μM for 24 hr to variable extents. Significance was evaluated by one-way ANOVA assay with "Dunn multiple comparison *post-hoc* test." Significance was represented as * *p*-value<0.05, ** *p*-value<0.001, *** *p*-value<0.0001.

difference was responsible for 48.3 and 50.9 *n*=% of the outcome variations for MTT and LDH assays, respectively. At the same time, exposure duration was responsible for about 39.4 and 35.8% of the outcome's variation for MTT and LDH assays, respectively. LDH showed higher half maximal Effective Concentrations (EC_{50}s) in all tested timelines. A BrdU assay was conducted to evaluate whether the effect of DEX is due to its effect on the cell's proliferation. The effects of DEX were cytotoxic without affecting osteoblast proliferation.

Effect of DEX on human osteoblast secretory functions

The secretory functions of osteoblasts treated by DEX were assessed. DEX was found to inhibit procollagen type I peptide production significantly with decreased intracellular level (p<0.0001) as well as its release in the media (p=0.0005) (Figure 2A). The intracellular level of procollagen type I peptide was decreased to 83.2±4.3 and 70.2±5.6% of the control non-treated cells levels by DEX in concentrations 5 and 50 µM, respectively. Also, there was a significant decline in osteocalcin levels based on exposure duration and concentration-dependent pattern. 48 hr post-exposure, DEX was found to decrease and osteocalcin to 79.3±5.4 and 67.2±6.2 of the control non-treated cells levels in concentrations 5 and 50 µM, respectively (Figure 2B). In comparison, ALP decreased to 86.2±5.3 and 69.2±6.4 of the control non-treated cells levels by DEX in concentrations 5 and 50 µM, respectively (Figure 2C).

Dexamethasone disrupts osteoblast bioenergetics

The osteoblast viability and cytotoxicity molecular base could be evaluated by investigation of cellular bioenergetics. Treated osteoblasts with 5 and 50 µM DEX concentrations for 24 hr

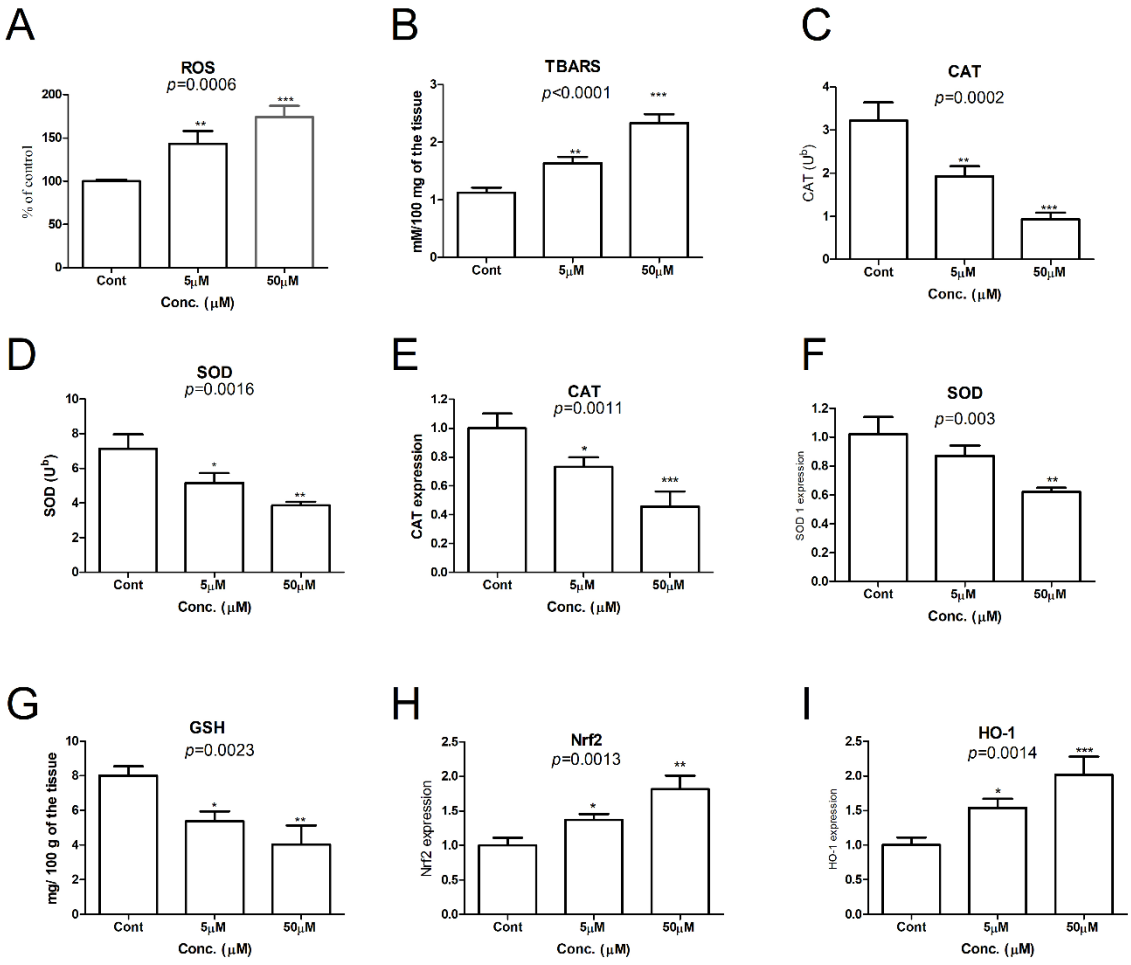

Figure 4: Effect of Dexamethasone (DEX) on the redox status of human osteoblast cell line. DEX was shown to significantly increase the osteoblast Reactive Oxygen Species (ROS) production (4A), with a significant increase in lipid peroxidation and Thiobarbituric Acid Radicle (TBARS) release (4B) and decreased activities and gene expression of antioxidant enzymes Catalase (CAT) (4C and E, respectively) and Superoxide Dismutase (SOD) (4D and F, respectively) as well as decreased the activity of Glutathione (GSH) (4G) and upregulation of the "nuclear factor erythroid 2-related factor; *Nrf2*" and "Heme Oxygenase-1; HO-1" (4H and I, respectively) at concentrations 5 and 50 µM for 24 hr to variable extents. Significance was evaluated by one-way ANOVA assay with "Dunn multiple comparison *post-hoc* test." Significance was represented as * p-value<0.05, ** p-value<0.001, *** p-value<0.0001.

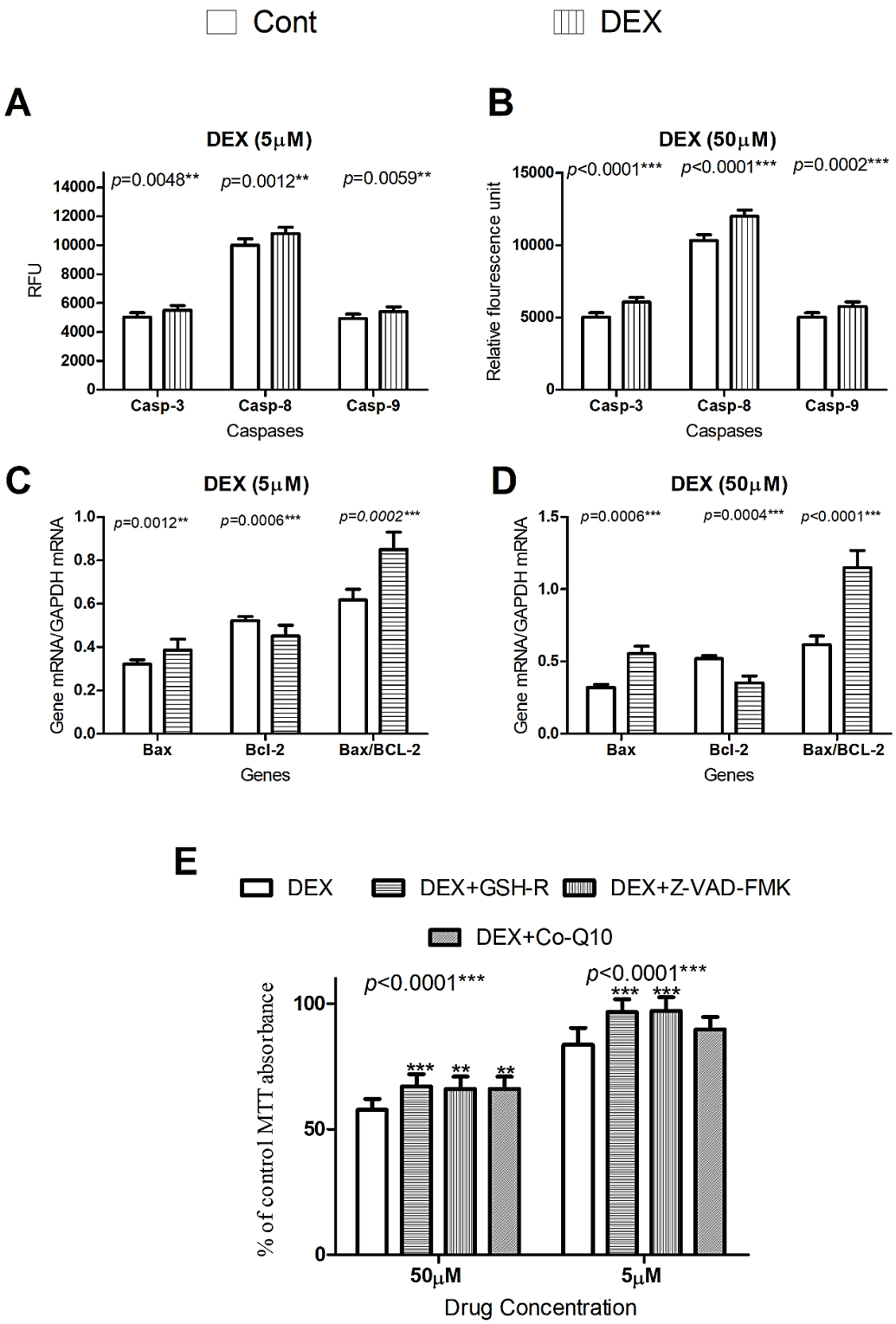

Figure 5: Figures 5A and 5B show the effect of Dexamethasone (DEX) on apoptotic pathways of the human osteoblast cell line. DEX was shown to significantly increase the activities of caspases-3, -8 and -9 at concentrations 5 and 50 μM for 24 hr, respectively, to variable extents. Figures 5C and D show a significant increase in *Bax* gene expression and decreased *Bcl2* expression, with resulting increased *Bax/Bcl2* ratio at concentrations 5 and 50 μM for 24 hr, respectively, to variable extents. Figure E showed the protective effect of antioxidant-reduced Glutathione (GSH-R) (10 μM), Caspase-3 inhibitor "Z-VAD-FMK" (200 μM) and Co-enzyme Q-10 (1 μM) on DEX induced cytotoxicity via MTT assay in presence and absence of the protective agents. Significance was evaluated by one-way ANOVA assay with Dunn multiple comparison *post-hoc* test. Significance was represented as * *p*-value<0.05, ** *p*-value<0.001, *** *p*-value<0.0001.

significantly decreased the intracellular ATP levels to 90.1±3.2 and 83.2±2.9% of controls, respectively (p=0.002) (Figure 3A). However, there is a significant elevation of lactate production (p=0.003) due to activated anaerobic pathways of glucose metabolism (Figure 3B). Given that ATP production is regulated by oxidative phosphorylation associated with the mitochondrial electron transport chain, assessments of mitochondrial membrane potential were performed along with activity assays for mitochondrial enzymes complex I and complex III. A 24 hr exposure to DEX was found to decrease MCI (p=0.001) and MCIII (p=0.009) activities significantly (Figures 3C and 3D). The mito-tracker green assay revealed that DEX significantly decreased MMP (p<0.0001) to 77.3±4.3 and 68.3±4.7 of the control MMP at concentrations 5 and 50 µM, respectively (Figure 3E). DEX significantly (p<0.0001) lessened osteoblast OCRs to about 72.3±4.3 and 46.2±3.9 at concentrations 5 and 50 µM, respectively.

The DEX effect on mitochondrial gene expression was examined. RT-PCR findings indicated that DEX notably downregulated the mitochondrial NADH Dehydrogenase 1 (*ND1*), *ND5*, cytochrome b (*cyt. b*) and Cytochrome c Oxidase subunit 1 (*CO1*) genes to 53.7±3.3, 58.6±3.7, 83.2±2.9, 68.1±3.5 of the control MMP at concentration 50 µM, respectively (Figure 3G-3J).

DEX-induced oxidative stress in the treated osteoblasts

DEX significantly raised the generation of cellular ROS (p=0.0006) by 42.3 and 75.3% over the estimated levels of the control non-treated cells at concentrations 5 and 50 µM, respectively (Figure 4A). The rise in ROS was associated with a significant surge in TBARS (p<0.0001) to about 134.5% and 203.4% of the control samples estimated values cells at concentrations 5 and 50 µM, respectively (Figure 4B). The effects of DEX on the antioxidant's enzymes CAT and SOD activities and genes expression were estimated. The present study documented that DEX significantly reduced the activities of CAT (p=0.0002) and SOD (p=0.0016) (Figures 4C, 4D) with parallel significant inhibition of expression of their coding genes (Figures 4E, 4F). On the other hand, DEX was found to significantly decrease GSH stores compared to the control cells (p=0.0023) (Figure 4G) with a concomitant significant increase in "Nuclear factor erythroid 2-related factor 2 (*Nrf2*)" gene (p=0.0021) and HO-1 gene expression (Figures 4H and 4I). All changes in oxidative stress assays were concentration dependent.

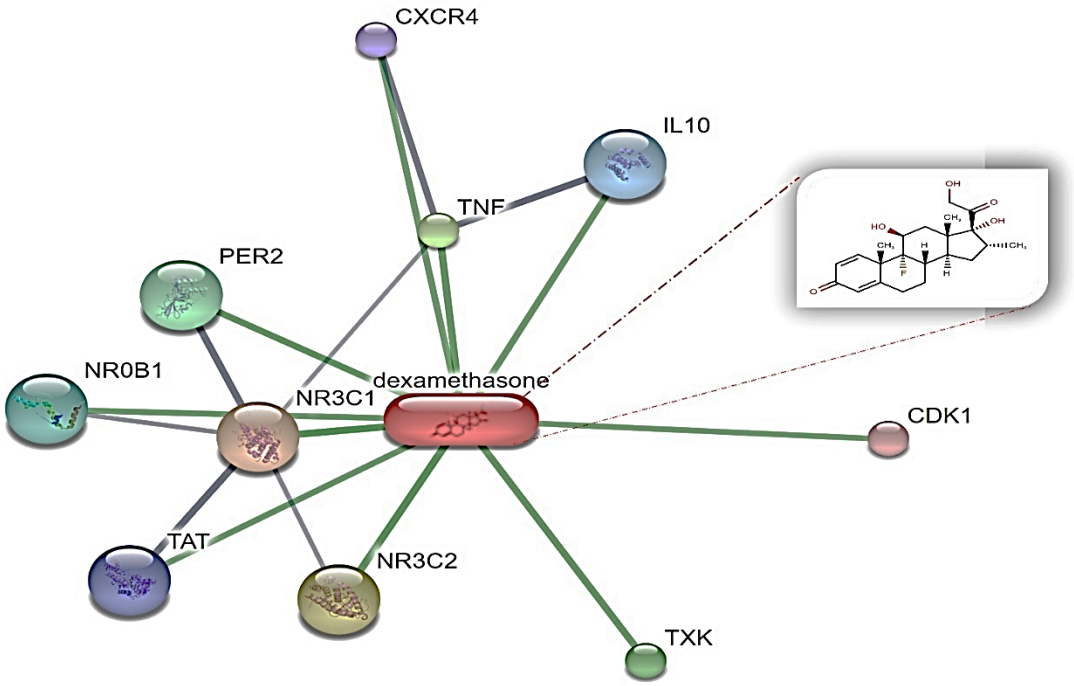

Figure 6: "Predicted Protein Interaction (PPI) Network" for dexamethasone offers a visual depiction of the possible molecular partners associated with DEX, as determined from the STITCH database (http://stitch.embl.de/) (last accessed on October 3, 2024). The identified partners encompass "NR3C1/2: nuclear receptor subfamily 3, group C, member 1/2, TNF: tumor necrosis factor, TXK: tyrosine kinase, PER2: period homolog 2; a component of the circadian clock mechanism which is essential for generating circadian rhythms. NR0B1: nuclear receptor subfamily 0, group B, member 1; Orphan nuclear receptor, IL-10: interleukin 10; inhibits the synthesis of several cytokines, TAT: tyrosine aminotransferase; involved in tyrosine breakdown, CXCR4: chemokine (C-X-C motif) receptor 4; transduces a signal by increasing intracellular calcium ion levels and enhancing MAPK1/MAPK3 activation and CDK1: cyclin-dependent kinase 1; plays a key role in the control of the eukaryotic cell cycle."

Table 1: Primers used in the study quantitative polymerase chain reaction assay.

Gene	Sense (5′-3′)	Antisense (5′-3′)
Bax	"TCCACCAAGAAGCTGAGCGAG"	"GTCCAGCCCATGATGGTTCT"
Bcl2	"CGACGACTTCTCCCGCCGCTACCGC"	"CCGCATGCTGGGGCCGTACAGTTCC"
CAT	"CTTCGACCCAAGCAACATGC"	"GCGGTGAGTGTCAGGATAGG"
SOD1	"GATGACTTGGGCAAAGGTGG"	"TACACCACAAGCCAAACGACT"
Nrf2	"CATCTACAAACGGGAATGTCTG"	"AGTGGATCTGCCAACTACTC"
Ho-1	"AGGGAATTCTCTTGGCTGGC"	"GACAGCTGCCACATTAGGGT"
ND1	"ACACTAGCAGAGACCAACCGAA"	"GGGAGAGTGCGTCATATGTTGT"
ND5	"CTATCTCGCACCTGAAACAAGC"	"GGTGGAGTAGATTAGGCGTAGG"
Cy.b	"TATTCGCCTACACAATTCTCCG"	"GCTTACTGGTTGTCCTCCGATT"
CO1	"TACGTTGTAGCCCACTTCCACT"	"GGATAGGCCGAGAAAGTGTTGT"
GAPDH	"GACAGTCAGCCGCATCTTCT"	"GCGCCCAATACGACCAAATC"

Abbreviations: "NrF2: nuclear factor erythroid 2-related factor, **CAT:** Catalase, **SOD:** superoxide dismutase, **HO-1:** heme oxygenase 1, **ND1:** NADH dehydrogenase subunit 1, **ND5:** NADH dehydrogenase subunit 5, **Cy.b:** cytochrome B, **CO1:** cytochrome C oxidase subunit 1, **ATP 6/8:** ATP synthase subunit 6/8, **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase."

Dexamethasone induces apoptosis in the treated osteoblasts

As activation of Caspase-3 has a pivot role in both extrinsic and intrinsic apoptotic pathways, its activity was evaluated in treated samples by fluorometric assay. Caspases-3, -8 and -9 activities were substantially increased in all treated samples at 5 and 50 μM concentrations (Figures 5A and 5B) with an underlying significant increase in "B-Cell Leukemia/Lymphoma 2 (BCL2)-associated X (*Bax*)" gene expression and decreased *Bcl2* expression with resulting increased *Bax*/*Bcl2* ratio Figures 5C and 5D.

Antioxidants, anti-apoptosis and mitochondrial enhancers alleviate DEX-induced cytotoxicity

To support our findings, the antioxidant GSH-R, inhibitors of caspase-3 and co-enzyme Q10 significantly improved the viability of DEX-treated cells (Figure 5E). Reduced glutathione showed the most protective effect against DEX-induced cytotoxicity in both 5 and 50 μM concentrations, followed by anti-caspase-3 Z-VAD-FMK, while Co-enzyme Q showed the least protective effect.

DISCUSSION

The present study assessed the cytotoxic effects of the widely prescribed drug DEX on human osteoblasts. Bone basic multicellular units contain mainly four types of cells with continuous dynamic interaction between them for normal bone tissue integrity and function.[8] Osteoblasts were chosen as a model in the current study due to their well-known secretory function for cartilaginous and cartilaginous proteins essential for normal bone matrix formation. Human cells were preferred to avoid the effect of interspecies variation on data robustness. An immortalized cell line was selected to get a single homogenous cell population of phenotypically similar cells for data reproducibility rather than the primary human cell line, which is difficult to obtain.[16]

Many laboratory and clinical studies suggested corticosteroid-induced osteoporosis as the most common type of secondary osteoporosis, with controversy regarding its effects on osteoblasts.[17,18] However, the DOX-induced OP is still not well established either *in vitro* or *in vivo*. The current study found that DEX inhibits human osteoblast cell line viability and markedly accelerates apoptosis in exposure time and concentration patterns. These findings agree with previous studies, which revealed that steroids mainly affect cell viability in a concentration-dependent manner.[19,20]

The current functional assays of the study revealed that DEX impaired the secretion of osteoblasts ALP, procollagen type I peptide and osteocalcin. Procollagen is essential for collagen formation, the main bone component for its structure.[21] This reduced collagen mass can explain the increased risk for osteoporosis in patients with prolonged therapeutic courses of DEX.[3,22] Additionally, osteocalcin, secreted by osteoblast, is essential for bone mineralization and density with an additional reported role in energy metabolism, brain development and cognition.[23] Hence, an induced decrease in osteocalcin secretion may also contribute to DEX-induced osteoporosis as well as DEX-reported neurological and psychological side effects with prolonged therapy.[24] Also, the effect of DEX on ALP secretion can contribute to decreased bone mineralization and osteoporosis. Secretion of ALP contributes to the release of phosphate ions in the bone matrix via phosphate compound degradation. Hence, phosphate ions play a fundamental role in enhancing the crystallization of the matrix.[25]

The current study bioenergetic assays showed that DEX is adversely affecting the osteoblasts bioenergetics treated with

decreased MMP, uncoupling of oxidative phosphorylation in mitochondrial complexes and decreased ATP generation and compensatory shifting to anaerobic glycolysis metabolism and increased lactate release. This adverse effect on mitochondria induced oxidative stress due to the leak of the reactive species from the mitochondrial electron transport chain.[26] This oxidative stress can cause damage to the different cellular structures, including the nucleus, cell membranes and mitochondria,[27,28] with continuous cycles of cellular damage and programmed cell death via induction of the apoptotic pathways.[27-30]

Interestingly, oxidative stress caused by DEX can trigger some compensatory mechanisms to maintain the cells' viability as autophagy, which was previously reported in DEX-treated osteoblasts.[7] However, the protective ability of autophagy is not absolute. With high doses of DEX and prolonged exposure, the antioxidant capacity will be significantly reduced, accompanied by activation of the apoptosis pathway.[19] Furthermore, acclamation of ROS was reported to inhibit the viability and differentiation of osteoblasts and reduce bone formation.[31,32]

Additionally, DEX was found to inhibit the antioxidant activities of CAT and SOD with reduced cellular stores of the antioxidants and reduced glutathione, which negatively affects cellular redox defense function. Accumulating the reactive radicles with the decreased ability of the antioxidant buffers will reduce the ability of enzymes to convert the superoxide radicals to water and oxygen molecules.[33]

The current study data revealed that DEX induces apoptosis in the treated osteoblasts via activation of both intrinsic and extrinsic pathways of the apoptosis cascade. These findings are consistent with prior studies supporting DEX-induced apoptosis in osteoblasts[7,34] and other different cells, such as macrophages,[35] mesenchymal stem cells,[36] and acute myeloid leukemia cells.[37]

The authors utilized the "STITCH platform (http://stitch.embl.de/)"[38] to pinpoint crucial molecular partners and interacting proteins that may mediate the cellular effects of DEX (Figure 6). Interestingly, the NR3C1, a major glucocorticoid receptor, possesses a dual mechanism of action: functioning as a transcription factor that attaches to glucocorticoid response elements in nuclear and mitochondrial DNA while also serving as a modulator of other transcription factors. It has been implicated in the "Glucocorticoid-Induced Osteoporosis (GIOP) pathway."[39] The receptor "CXCR4" for the "CXCL12" ligand was reported to be involved in skeletal homeostasis and bone development[40,41] and plays an essential role in periprosthetic osteolysis.[42] A recent report identified mechanistic insights into "how CXCR4 signaling regulates the osteogenic fate of skeletal cells and the balance between bone formation and resorption."[43] It highlighted the role of this receptor gain of function mutation in decreasing the bone mineral density in one-quarter of patients with "warts, hypogammaglobulinemia, infections and myelokathexis" syndrome and mediating osteoporosis in a mouse model of this syndrome. Cyclin-Dependent Kinase 1 (CDK1) and Period circadian Regulator 2 (PER2) were implicated in bone formation and resorption, respectively.[44-46] Also, the Tyrosine Kinase (TXK) family members were involved in bone homeostasis.[47] All these molecular players, among others, can mediate and explain, in part, the DEX cytotoxic impact on the osteoblasts and support our study's findings.

This study highlights the impact of DEX on oxidative stress and bioenergetic disruption in bone toxicity and osteoporosis. Our findings indicate that antioxidants and mitochondria-targeting drugs may play a curative and preventive role for patients undergoing long-term DEX therapy. Pre-incubation with reduced glutathione in osteoblasts significantly enhanced cell viability following DEX exposure. Additionally, promising respiratory substrate treatment agents, such as β-hydroxybutyrate, could further alleviate DEX-induced mitochondrial inhibition. While these results are promising, it is important to acknowledge the limitations of our study. Notably, our research is conducted *in vitro* using a human osteoblast cell line, which may not fully replicate the complexities of *in vivo* conditions and the systemic effects of DEX in a living organism. Furthermore, the analysis focused primarily on short-term exposures; long-term effects and the potential for chronic toxicity were not evaluated. Future studies should aim to validate these findings *in vivo* and explore the therapeutic efficacy of these interventions over extended treatment periods. Overall, the use of β-hydroxybutyrate, alongside antioxidants like glutathione and caspase-3 inhibitors, offers a multifaceted approach to mitigating DEX-induced cytotoxicity and supporting mitochondrial function, thereby improving cellular viability and metabolic health in affected tissues.

CONCLUSION

The current data support the role of mitochondrial disruption, oxidative stress and apoptosis as suggestive mechanisms of DEX-induced bone toxicity and osteoporosis with possible protective role of antioxidants and mitochondrial enhancers agents. This also highlights the importance of proper use of steroids under direct supervision, especially in cases with other predisposing factors to osteoporosis, which necessitates public awareness about the proper use of the medications, especially the widely prescribed drugs.

ABBREVIATIONS

ALP: Alkaline phosphatase; **ANOVA:** Analysis of Variance; **ATP:** Adenosine triphosphate; **BAX:** B-cell leukemia/lymphoma 2 (BCL2)-associated X; **BMD:** Bone mineral density; **BrdU:** Bromodeoxyuridine; **CAT:** Catalase; **CDK1:** Cyclin-dependent kinase 1; **CO1:** Cytochrome c oxidase subunit 1; **cyt. b:** Cytochrome b; **DCIP:** Dichloroindophenol; **DEX:**

Dexamethasone; **EC_{50}s:** Half maximal effective concentrations; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **GSH:** Reduced glutathione; **LDH:** Lactate dehydrogenase; **MCI/III:** Mitochondrial complexes I/III; **MDA:** Malondialdehyde; **MG:** Mito tracker green; **NAD:** Nicotinamide adenine dinucleotide; **ND1:** NADH dehydrogenase 1; **OCR:** Oxygen consumption rate; **OP:** Osteoporosis; **PBS:** Phosphate-buffered saline; **PER2:** Period circadian regulator 2; **qRT-PCR:** Quantitative real-time reverse-transcription polymerase chain reaction; **SOD:** Superoxide dismutase; **TBARS:** Thiobarbituric acid reactive substances; **TXK:** The tyrosine kinase.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUMMARY

This study investigates the effects of DEX on human osteoblasts, revealing its role in inducing cell toxicity and osteoporosis. DEX was shown to disrupt mitochondrial function, leading to decreased ATP synthesis and increased oxidative stress. The treatment elevated levels of reactive oxygen species and activated apoptotic pathways, indicated by increased caspase activity. Antioxidants and mitochondrial enhancers were found to mitigate DEX-induced cytotoxicity. These findings highlight the need for careful use of corticosteroids and raise awareness about their potential adverse effects on bone health.

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