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Metformin Modulates Cell Oxidative Stress to Mitigate Corticosteroid-Induced Suppression of Osteogenesis in a 3D Model

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Background: Corticosteroids provide well-established therapeutic benefits; however, they are also accompanied by adverse effects on bone. Metformin is a widely used medication for managing type 2 diabetes mellitus. Recent studies have highlighted additional therapeutic benefits of metformin, particularly concerning bone health and oxidative stress.

Objective: This research investigates the effects of prednisolone on cellular metabolic functions and bone formation using a 3D in vitro model. Then, we demonstrate the potential therapeutic effects of metformin on oxidative stress and the formation of calcified matrix due to corticosteroids.

Methods: Human mesenchymal stem cells (MSCs) and macrophages were cultured in a 3D GelMA scaffold and stimulated with prednisolone, with and without metformin. The adverse effects of prednisolone and metformin's therapeutic effect(s) were assessed by analyzing cell viability, osteogenesis markers, bone mineralization, and inflammatory markers. Oxidative stress was measured by evaluating reactive oxygen species (ROS) levels and ATP production.

Results: Prednisolone exhibited cytotoxic effects, reducing the viability of MSCs and macrophages. Lower osteogenesis potential was also detected in the MSC group. Metformin positively affected cell functions, including enhanced osteoblast activity and increased bone mineralization. Furthermore, metformin effectively reduced oxidative stress, as evidenced by decreased ROS levels and increased ATP production. These findings indicate that metformin protects against oxidative damage, thus supporting osteogenesis.

Conclusion: Metformin exhibits promising therapeutic potential beyond its role in diabetes management. The capacity to alleviate oxidative stress highlights the potential of metformin in supporting bone formation in inflammatory environments.

Keywords: corticosteroid, osteogenesis, inflammation, bone, metformin

Introduction

Corticosteroids are widely used in clinical practice, such as autoimmune disorders and chronic inflammatory diseases, for their potent anti-inflammatory and immunosuppressive effects.[1,2] Despite their therapeutic benefits, corticosteroids are associated with several adverse effects that can significantly impact patient health, particularly in the musculoskeletal system. Corticosteroids inhibit osteoblast proliferation, reduce bone formation, and increase bone resorption.[3,4] Additionally, corticosteroids induce oxidative stress and inflammation, which contribute to the pathology of osteonecrosis of the femoral head (ONFH); this results in progressive bone and joint damage,[5,6] which may result in hip arthroplasty. Although the development of ONFH is complex, this condition is associated with the impact of corticosteroids on bone metabolism and cellular activity.[7,8] Besides, corticosteroids can cause a dysregulation of glucose metabolism, which may lead to steroid-induced diabetes (SID). Insulin therapy is one of the most widely adopted methods for addressing diabetes.[9] However, corticosteroids increase insulin resistance and elevate levels of blood glucose. Patients with type 2 diabetes mellitus who received insulin

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therapy exhibited a more accelerated loss of bone mineral density, leading to the development of osteoporosis.[10] Understanding the underlying mechanisms is crucial in exploring potential strategies to mitigate the adverse effects of corticosteroids on the musculoskeletal system.

In the context of exploring interventions to mitigate the negative effects associated with corticosteroid use, particularly concerning ONFH and SID, the utilization of antioxidant therapies or agents that reduce oxidative damage may prove useful. Metformin is a widely used medicine in managing type 2 diabetes[11] and is known for its ability to effectively regulate blood glucose levels. Metformin may help reduce insulin resistance which might also limit the negative effects of high insulin levels.[12] While its role in glycemic control is well-established, emerging research has elucidated additional therapeutic potential uses for metformin, particularly in preventing or alleviating corticosteroid-induced complications. Osteoporosis induced by steroids is frequently aggravated by inflammatory processes. Metformin shows anti-inflammatory properties which may protect bone by reducing the inflammatory bone loss that accompanies chronic steroid treatment.[13,14] Corticosteroids also contribute to oxidative stress by increasing the production of reactive oxygen species (ROS) and impairing the body's antioxidant defenses.[15,16] For example, prednisolone has been reported to increase the level of ROS in corneal epithelial cells while simultaneously reducing the cell viability.[17] Subsequently, the elevated ROSs damage cells and affects cell viability. Recent studies have also expanded our understanding of metformin beyond its antidiabetic effects, revealing a significant impact on bone metabolism. Metformin has been shown to mitigate oxidative stress through several mechanisms. One of the major ways is by activating AMP-activated protein kinase (AMPK), a key regulator of cellular energy. AMPK activation improves mitochondrial function and reduces the overproduction of reactive oxygen species (ROS).[18,19] Besides, activation of the Akt/Nrf2 signaling pathway by metformin provides protection against oxidative stress-related damage while encouraging osteogenesis.[20] Furthermore, metformin diminishes the production of endogenous ROS and the consequent DNA damage.[21] These combined effects make metformin a potent agent for mitigating oxidative stress, which is crucial for preserving bone homeostasis and normal function.

3D cell culture models become essential tools for studying complex cellular interactions and simulating in vivo systems.[22] The 3D culture system provides a more physiologically relevant environment for exploring cellular interactions and drug effects. Mesenchymal stem cells (MSCs) have significant advantages when investigating bone formation due to their potential for differentiating into various cell types, including osteoblasts, chondrocytes, and adipocytes.[23] Macrophages contribute to the regulation of bone remodeling by secreting inflammatory cytokines that license MSCs and forming osteoclasts.[24] By incorporating both MSCs and macrophages within a 3D scaffold, our goal was to elucidate how prednisolone influences cellular interactions and how metformin might alleviate any adverse effects induced by prednisolone.

Here, we applied a 3D culture system that encapsulates MSCs and macrophages to mimic the bone microenvironment and investigated the effects of varying concentrations of prednisolone on MSCs and their interactions with macrophages. We focused on oxidative stress, osteogenic potential, and inflammatory regulation. We investigated the impact of prednisolone and metformin on MSC osteogenic potential by assessing gene expression as well as calcium deposition. Additionally, ROS and ATP production were quantified in MSCs and macrophages. Finally, we co-cultured MSCs and macrophages in one system and explored oxidative stress and osteogenesis. This comprehensive research will provide valuable insights into the mechanisms underlying corticosteroid effects on MSCs and the potential benefits of adding metformin when corticosteroids are used, contributing to the development of more effective treatment strategies in preventative and regenerative medicine.

Method

Fabrication of GelMA

GelMA scaffold was synthesized according to a previously described procedure.[25] Briefly, a solution of 15 grams of gelatin was prepared by dissolving it completely in 500 mL water. Subsequently, 15 mL of methacrylic anhydride was introduced into the solution. The mixture was then incubated in a shaker at 150 rpm and 37°C for 24 hours. The methacrylated gelatin (mGL) solution was dialyzed at room temperature for 4 days against water. The mGL was subsequently dissolved in Hank's balanced salt solution (HBSS) at a concentration of 10% (w/v), with the incorporation of 0.15% (w/v) of the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) and 1% of antibiotic-antimycotic.

Culture of MSC and Macrophage and 3D System Setup

Human bone marrow-derived MSCs used in this study were provided by Dr. Hang Lin at the University of Pittsburgh. These MSCs were isolated from the surgical waste (femoral head and trabecular bone) of de-identified patients undergoing total hip arthroplasty (THA) with Institutional Review Board (IRB) approval from both the University of Washington and the University of Pittsburgh. Human MSCs were cultured in DMEM medium supplied with 10% FBS, 1% antibiotic-antimycotic, and 1 ng/mL fibroblast growth factor (FGF). Cells were passaged upon reaching 70–80% confluency. Human buffy coats were ordered from Stanford Blood Center. Monocytes were isolated using EasySep™ Human Monocyte Isolation Kit (STEMCELL Technologies). Monocytes were cultured in a macrophage induction medium (RPMI supplemented with 10% FBS, 1% antibiotic-antimycotic, and 100 ng/mL M-CSF) for 5 days.

MSCs or macrophages were loaded onto GelMA scaffold at a concentration of 10 million/mL. 3D MSC scaffolds were cultured in osteogenic medium (DMEM, 10% FBS, 1% antibiotic-antimycotic, 50 μM L-ascorbic acid, 10 mm β-glycerophosphate, 100 mm Vitamin D_3, and 100 ng/mL bone morphogenetic proteins 7 (BMP-7)). 3D macrophage scaffolds were cultured in macrophage induction medium. In the co-culture system, scaffolds were cultured in a medium that consisted of half osteogenic medium and half macrophage induction medium.

Live/Dead Validation

Cell viabilities were measured using LIVE/DEAD™ Viability/Cytotoxicity Kit (ThermoFisher SCIENTIFIC, L3224). The working solution contains 0.1 μM calcein AM solution and 8 μM ethidium homodimer-1. Scaffolds were washed using PBS and then incubated in a working solution for 30 min in an incubator. After that, scaffolds were washed using PBS again and imaged using a Keyence microscope. Three images were taken per scaffold, and three scaffolds were measured in each group.

Cellular Oxidative Stress Assay (ROS and ATP Staining)

DCFDA - Cellular ROS Assay Kit (Abcam, ab113851) was applied to assess ROS production. Scaffolds were stained with DCFDA for 45 min in a 37°C incubator. Then, the fluorescent images were captured using a confocal microscope (Leica STELLARIS 5) with an excitation/emission at 485 nm/535 nm.

To measure the ATP production, scaffolds were stained with 5 μM BioTracker ATP-Red Live Cell Dye (Millipore Sigma, SCT045) for 15 min. After washing scaffolds with PBS buffer, the fluorescent images were captured with emission 570 nm. Three images were recorded for each scaffold, and within each group, three scaffolds were evaluated.

In the co-culture group, MSCs were pertained with LysoTracker™ Deep Red (ThermoFisher, L12492) to trace cells. Briefly, MSCs were first stained with Deep Red fluorescent dye. After staining, the cells were detached using trypsin and co-cultured with macrophages in GelMA.

Quantification of Inflammatory Cytokine by ELISA

ELISA was conducted according to the manufacturer's introduction. ELISA kits for measuring IL10 (88-7106-22), TNFα (88-7346-22), and IL6 (88-7066-86) were ordered from ThermoFisher SCIENTIFIC. ELISA kits for measuring CCL18 (DY394) and IL1b (DY201-05) were ordered from R&D Systems. We first coat the plate overnight with the corresponding antibody at 4°C. After washing, block with a blocking buffer for 1 hour at room temperature. Then, samples or standards were added to the wells and incubated for 2 hours. Subsequently, rewash and add the secondary antibody to each well. After adding substrate solution for 15 min, the reactions were stopped with a stop solution, and the absorbance was measured using a microplate reader.

Gene Expression by Real-Time PCR

mRNA was extracted by crushing scaffolds in TRIzol to release nucleic acids. After a 5-minute incubation, chloroform was added to separate the mixture. After centrifuge, the upper aqueous phase containing RNA was transferred to a new tube. Then, the RNA was precipitated by adding isopropanol and incubated for 10 minutes. After centrifugation, the RNA pellet was washed with 75% ethanol, air dry, and dissolved in RNase-free water. Finally, measure RNA concentration using nanodrop. RNA was converted to cDNA using iScript™ cDNA Synthesis Kit. Primers for qPCR were ordered from ThermoFisher

SCIENTIFIC, including CCL18 primer (Hs00268113_m1), IL6 primer (Hs00174131_m1), IL10 primer (Hs00961622_m1), IL1b primer (Hs01555410_m1), RUNX2 primer (Hs01047973_m1), ALPL primer (Hs01029144_m1), PPARG primer (Hs01115513_m1), PLIN1 primer (Hs00160173_m1), CEBPA primer (Hs00269972_s1), LPL primer (Hs00173425_m1), and GAPDH primer (Hs02786624_g1).

Osteoblast Differentiation Assay by ALP Staining and ARS Staining

For ALP quantification, scaffolds were cultured for 7 days and fixed using 4% paraformaldehyde. Then, scaffolds were stained with 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher SCIENTIFIC, 34042) for 4 hours. For Alizarin Red S staining (ARS) quantification, scaffolds were cultured for 21 days. Then, the fixed scaffolds were stained with ARS solution for 30 seconds.

The scaffolds were dehydrated by 15% sucrose and 30% sucrose solutions sequentially. Afterward, the scaffolds were embedded in optimal cutting temperature (OCT) and stored at −80°C. Microtome was applied to section frozen OCT-embedded scaffolds. A Keyence microscope was used to capture images.

Statistical Analysis

Three independent experiments were performed for all assays. Statistical analysis of data was performed with GraphPad Prism software using ANOVA. Results with a p-value of less than 0.05 were statistically significant. All results in the graphs are presented as mean ± SD.

Results

Dose Effect of Prednisolone on the Cell Viability of MSCs

We investigated the effect of prednisolone on MSC viability and whether there was a concentration dependence using a 3D GelMA system. Therefore, we tested the MSCs viability treated with 3, 30, 150, and 300 ng/mL prednisolone. Figure 1

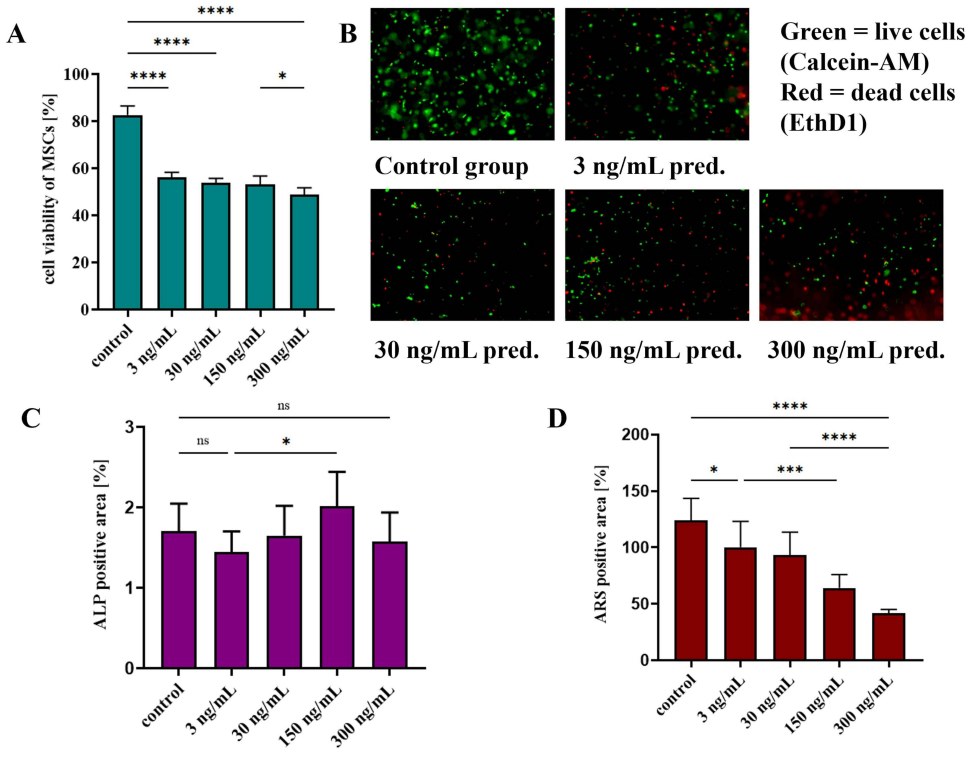

Figure 1 Impact of different concentrations of prednisolone on MSCs viability and osteogenic potential. (**A**) Comparison of MSCs' viability on day 7. (**B**) Representative fluorescent image of live/dead assay. Green dots represent live cells, while red dots represent dead cells. (**C**) Comparison of ALP level on day 7. (**D**) Comparison of ARS level on day 21. ("ns" for "no significant difference", *p < 0.05, ***p < 0.001, ****p < 0.0001).

shows reduced cell viability was observed at a concentration of 3 ng/mL on day 7 versus control with statistical significance. This suggests that even relatively low concentrations of prednisolone can have a measurable negative impact on cell viability.

We further explored the osteogenic effect of prednisolone on MSCs. MSC scaffolds were cultured in an osteogenic medium for 3 weeks. We stained our scaffolds with alkaline phosphatase (ALP) on day 7 to assess MSC osteogenic potential. We found that an increase in staining area was observed at the 150 ng/mL concentration compared to the 3 ng/mL group, at which no significant change was observed in other groups. We stained our scaffolds with Alizarin Red S (ARS) on day 21 to quantify areas of calcium deposition. A decrease in the staining area was observed in the prednisolone groups, which suggested a negative correlation with the concentration of prednisolone. Higher concentrations of prednisolone reduced the staining area. Our analysis revealed that prednisolone significantly impacts the osteogenic differentiation of MSCs.

Protective Effect of Metformin on Prednisolone-Suppressed Osteogenesis by MSCs

We assessed the impact of prednisolone and metformin on reactive oxygen species (ROS) and ATP production in MSCs on day 1, which provided valuable insights into the drug's early effects on cellular stress and energy metabolism. Cellular ROS production was quantified by cell-permeant reagent 2′,7′-dichlorofluorescin diacetate (DCFDA), which is oxidized to a fluorescent compound by ROS. 10 μM metformin was used in our experiments. The levels of ROS increased by 50% in the 3 ng/mL prednisolone group, while the levels of ROS did not change in the metformin group. The treatment of the MSC scaffold with prednisolone and metformin reduced ROS levels, which were lower than those observed in the control group. ATP level is another critical energy marker to indicate metabolic features. A significantly reduced ATP level was observed in the prednisolone group, which suggested that prednisolone impairs cellular energy metabolism. Adding 10 μM metformin resulted in a recovery of ATP levels; however, these levels remained lower than those observed in the control group. The results demonstrated that metformin contributes to the recovery of normal ROS and ATP expression when stimulated with prednisolone, although it does not achieve complete normalization.

We further investigated the protective effect of metformin on the osteogenic potential of MSCs in the context of prednisolone and metformin treatment together. MSCs were cultured with osteogenic medium with or without prednisolone for 3 weeks. Separate groups of MSCs were co-treated with metformin and prednisolone, or metformin only. Runt-related transcription factor 2 (*RUNX2*) and *ALPL* are essential markers for MSC osteogenesis. As shown in Figure 2, qPCR results indicated that the decreased expression of *RUNX2* caused by prednisolone is completely reversed by metformin. In addition, a higher expression of the *ALPL* gene was detected in the prednisolone group, and metformin enhanced *ALPL* levels. We further checked calcium matrix formation by ARS staining. Metformin increased ARS area observed in the group treated with both prednisolone and metformin. Thus, we found that metformin significantly mitigated the adverse impacts of prednisolone on osteogenesis.

The adipogenic potential of MSCs was also investigated by comparing the gene expression of peroxisome proliferator-activated receptor gamma *(PPARG)*, CCAAT/enhancer-binding protein-alpha *(CEBPA)*, lipoprotein lipase *(LPL)*, and perilipin 1 (*PLIN1)*. We found that 3 ng/mL prednisolone increased the expression of *CEBPA, LPL*, and *PLIN1* indicating an effect on adipogenesis. Metformin did not change the adipogenic potential of MSCs. However, the combination of prednisolone and metformin exhibited the most significant effect on adipogenesis.

The findings demonstrate that metformin can effectively protect against the impairment in osteogenesis caused by prednisolone-exposed MSCs. By normalizing ROS levels and supporting cellular energy metabolism, metformin helps preserve the osteogenic potential of MSCs.

Dose Effect of Prednisolone on the Macrophage Function

Macrophages serve as key regulators in both inflammatory responses and bone homeostasis. To assess the impact of prednisolone on macrophage viability at different concentrations, we also used 0 (control group), 3 ng/mL, 30 ng/mL, 150 ng/mL, and 300 ng/mL to cover a range of physiological and potentially toxic levels. Compared to the control group, significant decreases in viability were detected beginning at a dose of 3 ng/mL group, as shown in Figure 3A.

Macrophages regulate immune responses by polarization to different phenotypes and secreting various cytokines. We collected the culture supernatants on days 2 and 5 and measured the pro-inflammatory cytokines (IL1β, IL6, and TNFα)

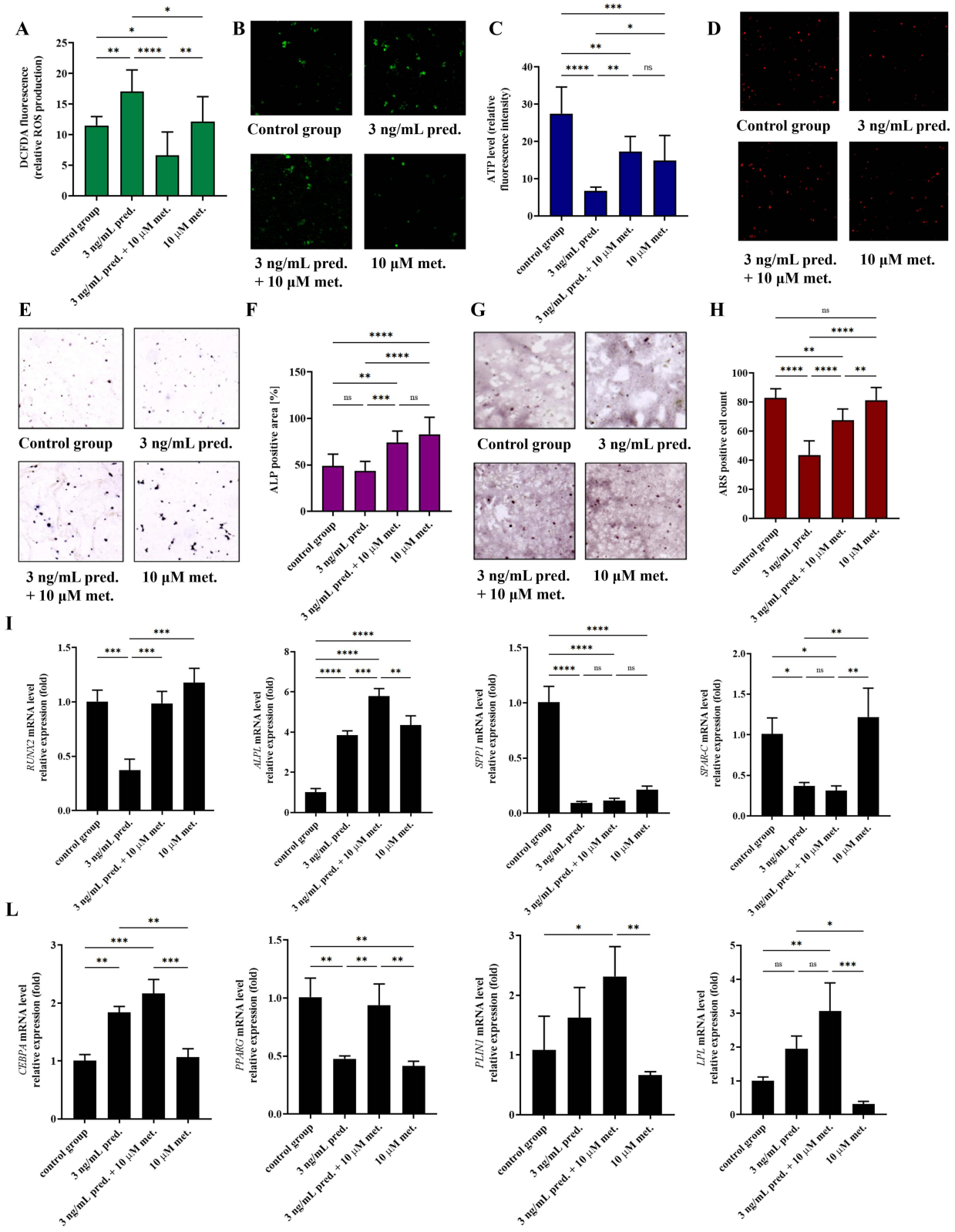

Figure 2 Metformin mitigates the osteogenic impairment by prednisolone in MSCs. (**A**) Statistical analysis of ROS level, (**B**) and the representative figures. (**C**) Statistical analysis of ATP level, (**D**) and the representative figures. (**E**) Representative figures of ALP level, and (**F**) the statistical analysis. (**G**) Representative figures of ARS expression, and (**H**) the statistical analysis. (**I**) qPCR comparison of osteogenic genes including *RUNX2, ALPL, SPP1*, and *SPARC*. (**L**) qPCR comparison of adipogenic genes including *PPARG, CEBPA, LPL*, and *PLIN1*. ("ns" for "no significant difference", **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

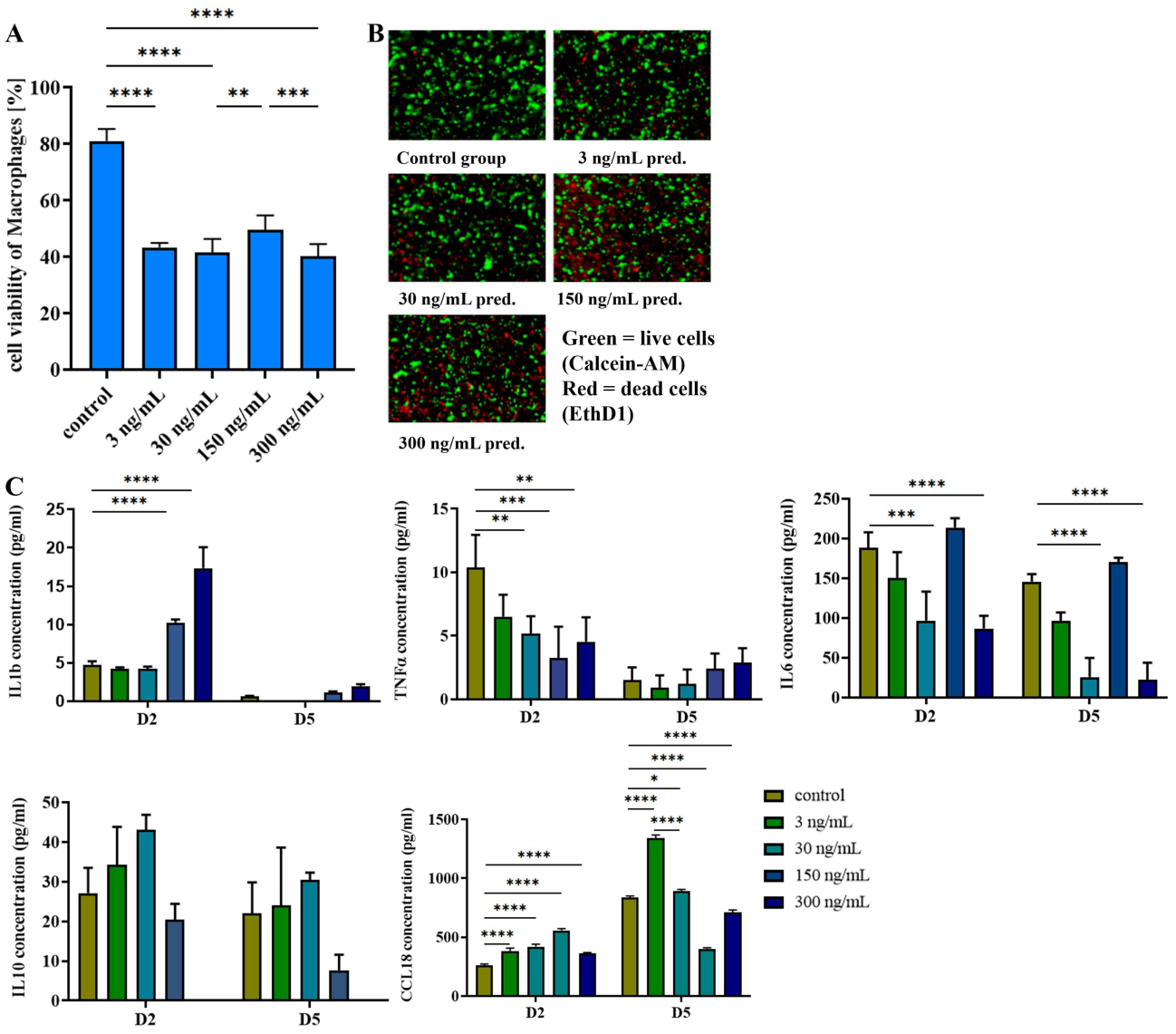

Figure 3 Impact of different concentrations of prednisolone on macrophage viability and inflammation properties. (**A**) Histogram shows macrophages' viability on day 7, and (**B**) representative fluorescent image of cells. (**C**) ELISA results of cytokine expressions of IL1b, IL6, TNFα, IL10 and CCL18. ("ns" for "no significant difference", *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

and anti-inflammatory cytokines (IL10 and CCL18) by ELISA. As shown in Figure 3, a significant increase in the levels of CCL18 was observed in the group treated with 3 ng/mL prednisolone. The expression of TNFα and IL6 was found to be reduced in the 3 ng/mL group. The level of IL10 and IL1b did not change at low concentrations of prednisolone.

Protective Effects of Metformin on Prednisolone-Treated Macrophages

Prednisolone impacts ROS production, which plays a crucial role in inflammation. We measured the ROS and ATP production in macrophages after 1 day of incubation treated with or without prednisolone. As shown in Figure 4A and B, prednisolone did not change ROS and ATP expression in macrophages. In contrast, metformin was found to enhance the expression of both ROS and ALP.

We expanded our analysis of inflammation-related cytokine levels by ELISA. As shown in Figure 4C, pro-inflammatory markers (IL-1β and IL6) and anti-inflammatory markers (CCL18 and IL-10) were measured. Within the group treated with prednisolone and metformin, the concentrations of CCL18 tend to return to their control levels. We also checked the gene expression on day 5, but most of the genes were not detectable, indicating low cytokine expression after day 5.

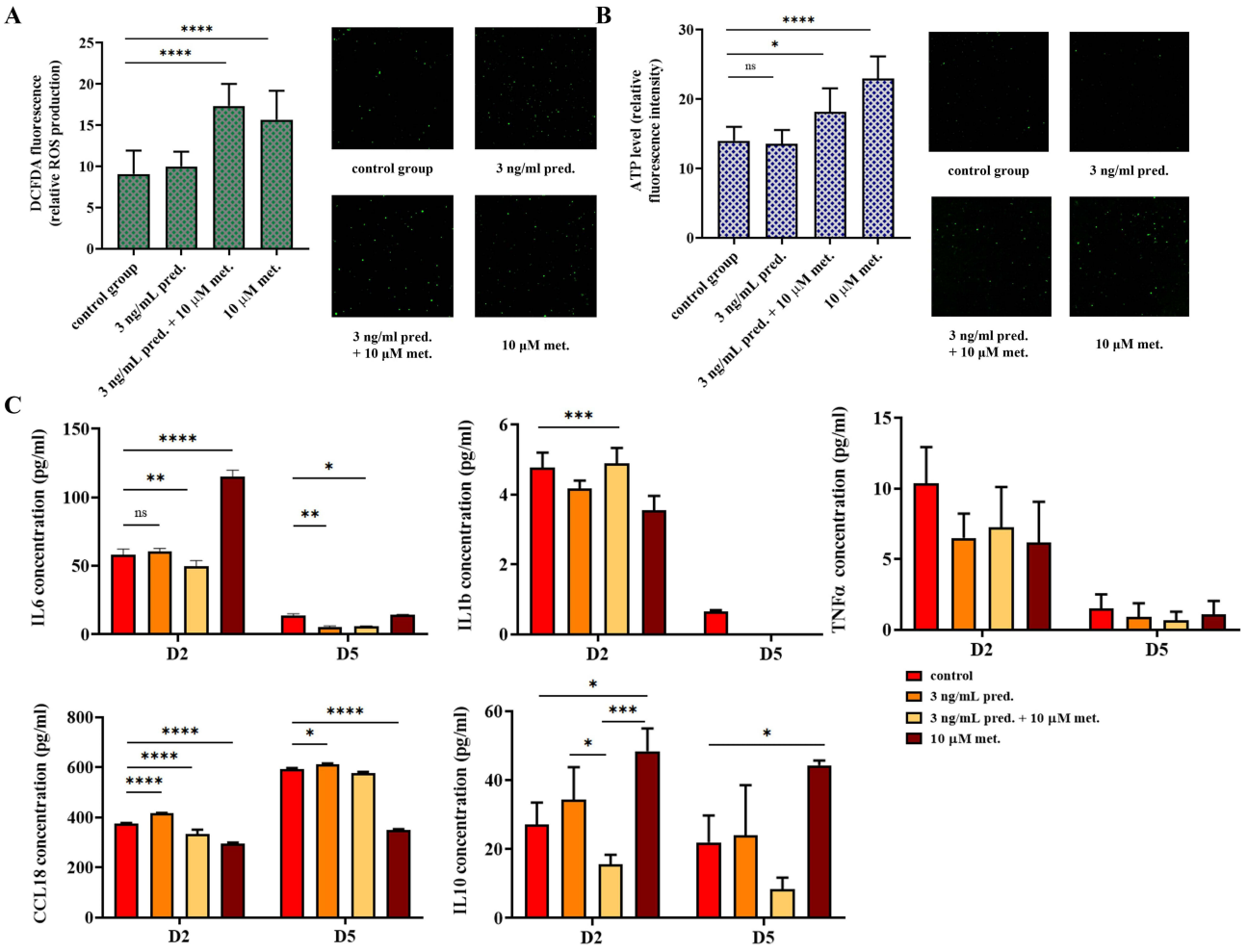

Figure 4 Metformin regulates macrophage functions. (**A** and **B**) Comparison of ROS level and ATP production in macrophages. (**C**) ELISA results of cytokine expressions of IL1b, IL6, TNFα, IL10 and CCL18. ("ns" for "no significant difference", *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Effect of Metformin on the Co-Culture of MSCs and Macrophages

We co-cultured MSCs with macrophages in a 3D scaffold to provide a more realistic system. The 3D co-culture model was applied to test the effects of prednisolone and metformin on the interactions between MSCs and macrophages. Before co-culturing, we stained the MSCs with a Deep-red dye to facilitate tracking, which allows for a clear distinction between MSCs and macrophages. Figure 5A - C shows the ROS expression level in MSCs and macrophages. The results indicated that prednisolone led to an increase in ROS levels in MSCs, but this effect was not seen in macrophages. In contrast, treatment with metformin resulted in a reduction of ROS levels, which were lower than those observed in the control group.

We evaluated the osteogenic and adipogenic markers, and the results are shown in Figure 5D and E . ALP staining on day 7 shows no significant difference among different groups. ARS staining on day 21 indicated that prednisolone reduced calcium deposition, while metformin contributed to the preservation of this effect. Gene expressions on day 21 were tested by qPCR as shown in Figure 5F. Cells subjected to prednisolone and metformin showed high expression of *SPARC*, contrasted by a decrease in *CEBPA* level. These results suggested that metformin contributes to the recovery of osteogenic capabilities in MSCs that have been adversely affected by prednisolone. We also measured cytokine levels and other secreted factors to assess the impact of co-culture conditions on inflammation. The results are shown in Figure 5G. Metformin recovered IL1b and TNFα reduced by prednisolone. However, metformin did not affect other cytokine levels.

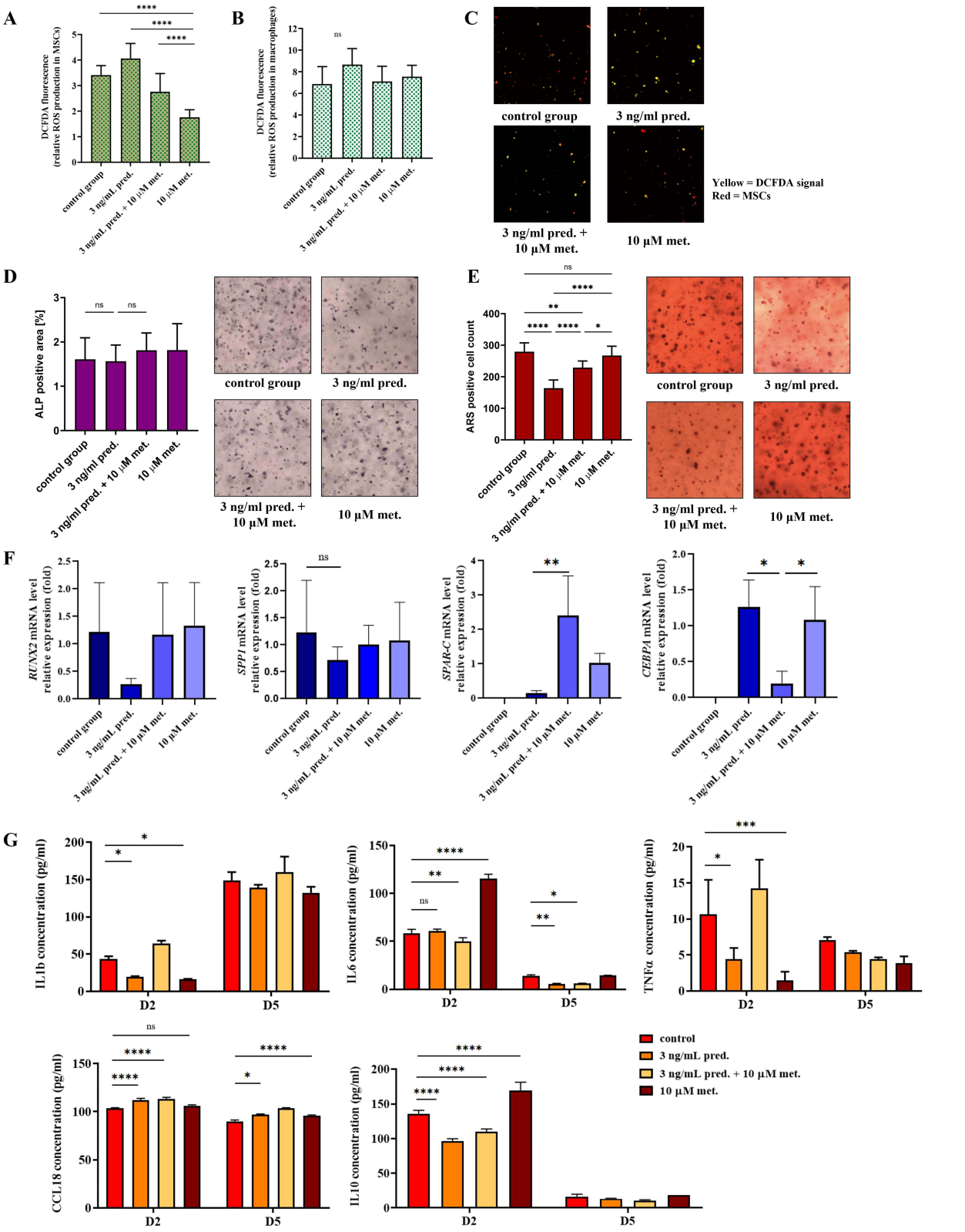

Figure 5 The therapeutic effect of metformin on MSC-macrophage co-culture system. (**A** and **B**) Comparison of ROS expression in MSCs and macrophages in the co-culture system, and (**C**) representative images. (**D** and **E**) Comparison of ALP and ARS on day 7 and day 21 representative. (**F**) qPCR comparison of osteogenic genes and adipogenic genes. (**G**) Cytokine secretion on day 2 and day 5. ("ns" for "no significant difference", *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Discussion

Prednisolone is widely used to manage inflammation and reduce pain in arthritic conditions, asthma, and many other diseases.[26,27] While prednisolone demonstrates considerable effectiveness, it is primarily utilized for short-term management due to the risk of adverse effects from long-term use. Prolonged prednisolone treatment can lead to osteoporosis due to decreased bone formation and increased bone resorption.[28] This effect has been documented in both animal models and human studies. Osteoporosis is induced through several mechanisms. For example, prednisolone promotes Notum expression and inhibits the PI3K/AKT/GSK3β/β-catenin pathway which will induce osteocyte apoptosis.[29] Prednisolone also modulates the Wnt/β-catenin signaling pathway and ERK signaling pathway.[30,31] In addition, prednisolone directly regulates the production of intracellular ROS. The increased ROS production occurs mainly through disruption of the oxidative and glycolytic balance in cells. This leads to long-term inflammation, increased cell apoptosis, and alternated cell activities. The administration of prednisolone led to a reduction in the generation of ROS at the cellular level, as assessed by the DCFH-DA assay.[32] The activation of the NOX/ROS/NF-κB signaling pathway leads to MSC apoptosis and interferes with MSC differentiation, which plays a pivotal role in the development of ONFH.[33] The effects of prednisolone on the production of intracellular ROS were also reported in human platelets, which may contribute to the anti-inflammatory actions of these agents.[34] The possible mechanisms underlying the effects of prednisolone on immune cells also include heightened oxidative stress and reduced energy production, which can account for alterations in cell functionality and phenotype. For example, prednisolone suppressed the M1 macrophage markers, including chemokine ligand 2, C-X-C chemokine motif 10, tumor necrosis factor-α and CD80. The results indicated that prednisolone inhibited the polarization of monocytes/macrophages towards the M1 phenotype.[35] ROS recruits and polarizes macrophages to the M2 phenotype.[36,37] Evaluating ROS production and ATP levels provides a comprehensive view of how prednisolone impacts MSCs and macrophages at the biochemical level. Considerable progress has been made in the development of therapeutic strategies that target ROS in stem cells for bone therapy.

Previous research has suggested that the effective concentration of prednisolone typically peaks at several hundred nanograms per milliliter.[38,39] We tested the effects of different concentrations of prednisolone (ranging from 3 ng/mL to 300 ng/mL) on cell viability and function. We found that 3 ng/mL of prednisone significantly affected the viability of MSCs and macrophages. We chose this concentration for subsequent studies. The values of plasma metformin concentrations range from 0.129 to 90 mg/L.[40] However, the therapeutic serum concentration of metformin for bone protection is poorly investigated as most research focuses on glucose modulation. Future research is needed to determine the therapeutic range of metformin in the context of corticosteroid-induced bone diseases.

One strategy to mitigate the adverse effects of prednisolone is combining corticosteroids with drugs that promote bone formation and minimize bone destruction. Metformin's potential to mitigate oxidative stress through its antioxidant properties is of significant interest.[41,42] Metformin enhances cellular antioxidant defenses and improves mitochondrial function through the activation of the AMP-activated protein kinase (AMPK) pathway, resulting in a reduction of oxidative damage.[43,44] Metformin enhances the proliferative capacity and survival of multipotent stromal stem cells, suggesting an anti-apoptotic effect that is associated with reduced ROS activity.[45] Metformin promotes cell proliferation and osteogenesis under high glucose conditions by regulating the ROS-AKT-mTOR axis.[46] Others have suggested that the mechanism by which metformin treatment decreases oxidative stress is associated with the metabolic pathway involving Sirtuin 3 (SIRT3). Activation of the SIRT3 pathway by metformin contributed to the reduction of oxidative stress in chondrocytes.[47] Besides MSCs, metformin demonstrated an inhibitory effect on the production of ROS by human M2 macrophages through the activation of AMPK.[48] One potential strategy to preserve bone tissue is by facilitating intracellular energy generation and managing the response to oxidative stress. Several clinical trials assessing the antioxidant properties of metformin have been registered.[49,50] The findings from these trials have the potential to identify additional uses for metformin.

3D culture more closely replicates in vivo tissue architecture than 2D culture and is a more authentic methodology for the assessment of biological mechanisms and drug effects on tissue regeneration. 3D culture of MSCs mimics the cellular behavior and differentiation pattern closely resembling those found in vivo.[51] This enhanced physiological relevance provides a more authentic and accurate prediction of prednisolone and metformin affecting cellular processes and interactions within a complex tissue context. Our study evaluated the effects of metformin administration following corticosteroid exposure on cellular behavior, oxidative stress mechanism, and matrix mineralization in in-vitro 3D cell cultures encapsulating MSCs ±

macrophages. Our findings reveal that prednisolone significantly impairs osteogenic differentiation, as evidenced by reduced MSC viability, mineralization, and osteogenic markers compared to controls. We also detected a high expression of anti-inflammatory cytokines in the macrophage culture group. Prior studies have demonstrated that prednisolone reduced the osteogenic potential of MSCs;[52] metformin reduces ROS, thereby enhancing the osteogenic characteristics of mesenchymal stem cells MSCs.[53] Our results agree with this previous research. We subsequently administered metformin to the prednisolone-treated MSCs, demonstrating the protective effect of metformin against the adverse effects induced by prednisolone. We further conducted a 3D co-culture of MSCs with macrophages and treated the cells with prednisolone to study the interactions between these cells and the effects of corticosteroid treatment and metformin administration. Our results suggest that metformin preserves MSC osteogenic potential in the co-culture environment. These interactions highlight the complexity of corticosteroid effects on stem cell behavior in an inflammatory context. Understanding the mechanisms may facilitate the development of more focused therapeutic approaches for mitigating corticosteroid-related adverse effects and improving regenerative treatment outcomes.

This study has a few limitations. Prednisolone modifies properties in bone diseases such as ONFH through several mechanisms. Besides decreasing MSCs osteogenesis, prednisolone also influences the vascular system, thereby impairing skeletal angiogenesis.[54] Metformin also inhibits angiogenesis.[55,56] While prednisolone and metformin are important in regulating inflammation and bone health, their effects on angiogenesis highlight the need for complementary treatments that support vascularization in bone repair. Endothelial cells were not included in our model. The interactions between endothelial cells and other cell types in the context of vascularization were not assessed. Research has also shown that prednisolone facilitates MSC differentiation into adipocytes.[57,58] We showed that prednisolone enhanced adipogenic differentiation, which is consistent with known adverse effects of corticosteroids. Future studies could focus on identifying key signaling pathways involved in the regulation of adipogenesis in the presence of corticosteroids. Exploring potential interventions that could mitigate the negative impact of corticosteroids on adipogenic potential is needed. Additionally, the long-term of safety in applying metformin for the preservation of bone health should be studied. The MSCs used in our study were derived from healthy donors. Future studies would benefit from using MSCs harvested from patients who have received prednisone treatment; this would provide data regarding the individualistic cellular response to metformin. Using MSCs derived from multiple donors would provide a more comprehensive understanding of cellular responses, as well as facilitate the development of precision medicine approaches.

Conclusion

Our findings demonstrate that prednisolone reduced cell viability even at a low dosage and negatively affected the osteogenic potential of MSCs; prednisolone was also associated with an increased ROS level. In addition, prednisolone modulated the immunomodulation properties of macrophages by increasing pro-inflammatory and anti-inflammatory cytokines. While additional research is necessary to clarify the practical use of metformin in steroid-induced diabetes, metformin helps preserve the osteogenic potential of MSCs by normalizing ROS levels and supporting cellular energy metabolism. This underscores the potential for metformin to be used as a therapeutic agent in combination with corticosteroids to mitigate adverse effects on bone formation and potentially enhance overall treatment outcomes.

Institutional Review Board Statement

Human bone marrow-derived MSCs were isolated from de-identified donors according to an exempted protocol that received approval from the Institutional Review Board (IRB) of both the University of Washington and the University of Pittsburgh (approval code PRO17070639).

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Disclosure

The authors declare no conflict of interest.

References

1. Imazio M, Brucato A, Lazaros G, et al. Anti-inflammatory therapies for pericardial diseases in the COVID-19 pandemic: safety and potentiality. *J Cardiovasc Med*. 2020;21:625–629. doi:10.2459/JCM.0000000000001059
2. Stone S, Malanga GA, Capella T. Corticosteroids: review of the history, the effectiveness, and adverse effects in the treatment of joint pain. *Pain Physician*. 2021;24:233–246.
3. Gado M, Baschant U, Hofbauer LC, Henneicke H. Bad to the bone: the effects of therapeutic glucocorticoids on osteoblasts and osteocytes. *Front Endocrinol*. 2022;13:1–14. doi:10.3389/fendo.2022.835720
4. Emkey RD, Lindsay R, Lyssy J, et al. The systemic effect of intraarticular administration of corticosteroid on markers of bone formation and bone resorption in patients with rheumatoid arthritis. *Arthritis Rheum*. 1996;39:277–282. doi:10.1002/art.1780390215
5. Chen K, Liu Y, He J, et al. Steroid-induced osteonecrosis of the femoral head reveals enhanced reactive oxygen species and hyperactive osteoclasts. *Int J Biol Sci*. 2020;16:1888–1900. doi:10.7150/ijbs.40917
6. Houdek MT, Wyles CC, Packard BD, et al. Decreased osteogenic activity of mesenchymal stem cells in patients with corticosteroid-induced osteonecrosis of the femoral head. *J Arthroplasty*. 2016;31:893–898. doi:10.1016/j.arth.2015.08.017
7. Kubo Y, Drescher W, Fragoulis A, et al. Adverse effects of oxidative stress on bone and vasculature in corticosteroid-associated osteonecrosis: potential role of nuclear factor erythroid 2-related factor 2 in cytoprotection. *Antioxid Redox Signal*. 2021;35:357–376. doi:10.1089/ars.2020.8163
8. Huang C, Qing L, Xiao Y, Tang J, Wu P. Insight into steroid-induced ONFH: the molecular mechanism and function of epigenetic modification in mesenchymal stem cells. *Biomolecules*. 2024;14:4.
9. Ostrowska-Czyżewska A, Zgliczyński W, Bednarek-Papierska L, Mrozikiewicz-Rakowska B. Is it time for a new algorithm for the pharmacotherapy of steroid-induced diabetes? *J Clin Med*. 2024;13:5801. doi:10.3390/jcm13195801
10. Ruppert K, Cauley J, Lian Y, et al. The effect of insulin on bone mineral density among women with type 2 diabetes: a SWAN pharmacoepidemiology study. *Osteoporos Int*. 2018;29:347–354. doi:10.1007/s00198-017-4276-9
11. Sanchez-Rangel E, Inzucchi SE. Metformin: clinical use in type 2 diabetes. *Diabetologia*. 2017;60:1586–1593. doi:10.1007/s00125-017-4336-x
12. Priya G, Kalra S. A review of insulin resistance in type 1 diabetes: is there a place for adjunctive metformin? *Diabetes Ther*. 2018;9:349–361. doi:10.1007/s13300-017-0333-9
13. Zhao HX, Huang YX, Tao JG. ST1926 attenuates steroid-induced osteoporosis in rats by inhibiting inflammation response. *J Cell Biochem*. 2017;118:2072–2086. doi:10.1002/jcb.25812
14. Araújo AA, Pereira ADSBF, Medeiros CACXD, et al. Effects of metformin on inflammation, oxidative stress, and bone loss in a rat model of periodontitis. *PLoS One*. 2017;12:e0183506. doi:10.1371/journal.pone.0183506
15. Kraaij MD, van der Kooij SW, Reinders MEJ, et al. Dexamethasone increases ROS production and T cell suppressive capacity by anti-inflammatory macrophages. *Mol Immunol*. 2011;49:549–557. doi:10.1016/j.molimm.2011.10.002
16. Almeida M, Han L, Ambrogini E, Weinstein RS, Manolagas SC. Glucocorticoids and tumor necrosis factor α increase oxidative stress and suppress Wnt protein signaling in osteoblasts. *J Biol Chem*. 2011;286:44326–44335. doi:10.1074/jbc.M111.283481
17. Ryu JS, Ko JH, Kim MK, Wee WR, Oh JY. Prednisolone induces apoptosis in corneal epithelial cells through the intrinsic pathway. *Sci Rep*. 2017;7:4135. doi:10.1038/s41598-017-04509-8
18. Lee S, Le NH, Kang D. Melatonin alleviates oxidative stress-inhibited osteogenesis of human bone marrow-derived mesenchymal stem cells through AMPK activation. *Int J Med Sci*. 2018;15:1083–1091. doi:10.7150/ijms.26314
19. Ha NN-Y, Huynh TKT, Phan NUP, et al. Synergistic effect of metformin and vitamin D3 on osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells under high d-glucose conditions. *Regener Ther*. 2024;25:147–156. doi:10.1016/j.reth.2023.12.003
20. Jia L, Xiong Y, Zhang W, Ma X, Xu X. Metformin promotes osteogenic differentiation and protects against oxidative stress-induced damage in periodontal ligament stem cells via activation of the Akt/Nrf2 signaling pathway. *Exp Cell Res*. 2020;386:111717. doi:10.1016/j.yexcr.2019.111717
21. Algire C, Moiseeva O, Deschênes-Simard X, et al. Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev Res*. 2012;5:536–543. doi:10.1158/1940-6207.CAPR-11-0536
22. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FDP. 3D cell culture systems: advantages and applications. *J Cell Physiol*. 2015;230:16–26. doi:10.1002/jcp.24683
23. Bartold M, Gronthos S, Haynes D, Ivanovski S. Mesenchymal stem cells and biologic factors leading to bone formation. *J Clin Periodontol*. 2019;46:12–32. doi:10.1111/jcpe.13053
24. Gao Q, Rhee C, Maruyama M, et al. The effects of macrophage phenotype on osteogenic differentiation of MSCs in the presence of polyethylene particles. *Biomedicines*. 2021;9:0–9. doi:10.3390/biomedicines9050499
25. Lin H, Cheng AW-M, Alexander PG, Beck AM, Tuan RS. Cartilage tissue engineering application of injectable gelatin hydrogel with in situ visible-light-activated gelation capability in both air and aqueous solution. *Tissue Eng Part A*. 2014;20:2402–2411. doi:10.1089/ten.tea.2013.0642
26. Mansur AH, Hassan M, Duffy J, Webster C. Development and clinical application of a prednisolone/cortisol assay to determine adherence to maintenance oral prednisolone in severe asthma. *Chest*. 2020;158:901–912. doi:10.1016/j.chest.2020.03.056
27. Krasselt M, Baerwald C. The current relevance and use of prednisone in rheumatoid arthritis. *Expert Rev Clin Immunol*. 2014;10:557–571. doi:10.1586/1744666X.2014.904746
28. Briot K, Roux C. Glucocorticoid-induced osteoporosis. *RMD Open*. 2015;1:e000014. doi:10.1136/rmdopen-2014-000014

29. Li C, Yang P, Liu B, et al. Prednisolone induces osteocytes apoptosis by promoting Notum expression and inhibiting PI3K/AKT/GSK3β/β-catenin pathway. *J Mol Histol*. 2021;52:1081–1095. doi:10.1007/s10735-021-10006-0
30. Guañabens N, Gifre L, Peris P. The role of Wnt signaling and sclerostin in the pathogenesis of glucocorticoid-induced osteoporosis. *Curr Osteoporos Rep*. 2014;12:90–97. doi:10.1007/s11914-014-0197-0
31. Aimaiti A, Wahafu T, Keremu A, Yicheng L, Li C. Strontium ameliorates glucocorticoid inhibition of osteogenesis via the ERK signaling pathway. *Biol Trace Elem Res*. 2020;197:591–598. doi:10.1007/s12011-019-02009-6
32. Jeries H, Volkova N, Grajeda-Iglesias C, et al. Prednisone and its active metabolite prednisolone attenuate lipid accumulation in macrophages. *J Cardiovasc Pharmacol Ther*. 2019;25:174–186. doi:10.1177/1074248419883591
33. Xu H, Zeng Q, Zou K, et al. Glucocorticoid-induced activation of NOX/ROS/NF-κB signaling in MSCs contributes to the development of GONFH. *Apoptosis*. 2023;28:1332–1345. doi:10.1007/s10495-023-01860-2
34. Sanner BM, Meder U, Zidek W, Tepel M. Effects of glucocorticoids on generation of reactive oxygen species in platelets. *Steroids*. 2002;67:715–719. doi:10.1016/S0039-128X(02)00024-7
35. Kim B, Son Y, Kim Su M, Kim K. Prednisolone suppresses the immunostimulatory effects of 27-hydroxycholesterol. *Exp Ther Med*. 2020;19:2335–2342. doi:10.3892/etm.2020.8458
36. Formentini L, Santacatterina F, Núñez de Arenas C, et al. Mitochondrial ROS production protects the intestine from inflammation through functional M2 macrophage polarization. *Cell Rep*. 2017;19:1202–1213. doi:10.1016/j.celrep.2017.04.036
37. Zhang B, Yang Y, Yi J, Zhao Z, Ye R. Hyperglycemia modulates M1/M2 macrophage polarization via reactive oxygen species overproduction in ligature-induced periodontitis. *J Periodontal Res*. 2021;56:991–1005. doi:10.1111/jre.12912
38. Chien K-Y, Chen -T-T, Hsu J, et al. Sub-maximal exercise altered the prednisolone absorption pattern. *J Pharm Pharm Sci*. 2010;13:58–66. doi:10.18433/J3KG6B
39. Bashar T, Apu MNH, Mostaid MS, Islam MS, Hasnat A. Pharmacokinetics and bioavailability study of a prednisolone tablet as a single oral dose in bangladeshi healthy volunteers. *Dose Response*. 2018;16:1559325818783932. doi:10.1177/1559325818783932
40. Kajbaf F, De Broe ME, Lalau J-D. Therapeutic concentrations of metformin: a systematic review. *Clin Pharmacokinet*. 2016;55:439–459. doi:10.1007/s40262-015-0323-x
41. Cahova M, Palenickova E, Dankova H, et al. Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation. *Am J Physiol Liver Physiol*. 2015;309:G100–G111.
42. Wang G, Wang Y, Yang Q, et al. Metformin prevents methylglyoxal-induced apoptosis by suppressing oxidative stress in vitro and in vivo. *Cell Death Dis*. 2022;13:29. doi:10.1038/s41419-021-04478-x
43. Ashabi G, Khalaj L, Khodagholi F, Goudarzvand M, Sarkaki A. Pre-treatment with metformin activates Nrf2 antioxidant pathways and inhibits inflammatory responses through induction of AMPK after transient global cerebral ischemia. *Metab Brain Dis*. 2015;30:747–754. doi:10.1007/s11011-014-9632-2
44. Hou X, Song J, Li X-N, et al. Metformin reduces intracellular reactive oxygen species levels by upregulating expression of the antioxidant thioredoxin via the AMPK-FOXO3 pathway. *Biochem Biophys Res Commun*. 2010;396:199–205. doi:10.1016/j.bbrc.2010.04.017
45. Smieszek A, Kornicka K, Szłapka-Kosarzewska J, et al. Metformin increases proliferative activity and viability of multipotent stromal stem cells isolated from adipose tissue derived from horses with equine metabolic syndrome. *Cells*. 2019;8:80. doi:10.3390/cells8020080
46. Zhou R, Ma Y, Qiu S, Gong Z, Zhou X. Metformin promotes cell proliferation and osteogenesis under high glucose condition by regulating the ROS-AKT-mTOR axis. *Mol Med Rep*. 2020;22:3387–3395. doi:10.3892/mmr.2020.11391
47. Wang C, Yang Y, Zhang Y, Liu J, Yao Z, Zhang C. Protective effects of metformin against osteoarthritis through upregulation of SIRT3-mediated PINK1/Parkin-dependent mitophagy in primary chondrocytes. *Biosci Trends*. 2018;12:605–612. doi:10.5582/bst.2018.01263
48. Nassif RM, Chalhoub E, Chedid P, et al. Metformin inhibits ROS production by human M2 macrophages via the activation of AMPK. *Biomedicines*. 2022;10:319. doi:10.3390/biomedicines10020319
49. Brittain EL, Niswender K, Agrawal V, et al. Mechanistic phase II clinical trial of metformin in pulmonary arterial hypertension. *J Am Heart Assoc*. 2020;9:e018349. doi:10.1161/JAHA.120.018349
50. Esteghamati A, Eskandari D, Mirmiranpour H, et al. Effects of metformin on markers of oxidative stress and antioxidant reserve in patients with newly diagnosed type 2 diabetes: a randomized clinical trial. *Clin Nutr*. 2013;32:179–185. doi:10.1016/j.clnu.2012.08.006
51. Yen BL, Hsieh -C-C, Hsu P-J, et al. Three-dimensional spheroid culture of human mesenchymal stem cells: offering therapeutic advantages and in vitro glimpses of the in vivo state. *Stem Cells Transl Med*. 2023;12:235–244. doi:10.1093/stcltm/szad011
52. Liu S, Kiyoi T, Ishida M, Mogi M. Assessment and comparison of the efficacy of methotrexate, prednisolone, adalimumab, and tocilizumab on multipotency of mesenchymal stem cells. *Front Pharmacol*. 2020;11:1–11. doi:10.3389/fphar.2020.00001
53. Marycz K, Tomaszewski KA, Kornicka K, et al. Metformin decreases reactive oxygen species, enhances osteogenic properties of adipose-derived multipotent mesenchymal stem cells in vitro, and increases bone density in vivo. *Oxid Med Cell Longev*. 2016;2016:9785890. doi:10.1155/2016/9785890
54. Peng Y, Lv S, Li Y, et al. Glucocorticoids disrupt skeletal angiogenesis through transrepression of NF-κB–mediated preosteoclast Pdgfb transcription in young mice. *J Bone Miner Res*. 2020;35:1188–1202. doi:10.1002/jbmr.3987
55. Dallaglio K, Bruno A, Cantelmo AR, et al. Paradoxic effects of metformin on endothelial cells and angiogenesis. *Carcinogenesis*. 2014;35:1055–1066. doi:10.1093/carcin/bgu001
56. Han J, Li Y, Liu X, et al. Metformin suppresses retinal angiogenesis and inflammation in vitro and in vivo. *PLoS One*. 2018;13:e0193031. doi:10.1371/journal.pone.0193031
57. Han L, Wang B, Wang R, Gong S, Chen G, Xu W. The shift in the balance between osteoblastogenesis and adipogenesis of mesenchymal stem cells mediated by glucocorticoid receptor. *Stem Cell Res Ther*. 2019;10:377.
58. Bajetto A, Pattarozzi A, Sirito R, Barbieri F, Florio T. Metformin potentiates immunosuppressant activity and adipogenic differentiation of human umbilical cord-mesenchymal stem cells. *Int Immunopharmacol*. 2023;124:111078. doi:10.1016/j.intimp.2023.111078