

# Effects of spironolactone on cytotoxic damage in osteoblasts

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**Abstract.** Chronic hyperglycemia in diabetes elevates oxidative stress, damaging pancreatic  $\beta$ -cells and worsening complications. Oxidative stress also disrupts osteoblast function, suppresses their maturation and triggers apoptosis, thereby contributing to bone fragility. Spironolactone, an aldosterone receptor antagonist prescribed for the treatment of hypertension and heart failure, has potential protective effects on skeletal health. The present study examined whether spironolactone could protect osteoblasts from damage and restore their differentiation under oxidative conditions caused by 2-deoxy-D-ribose (dRib). MC3T3-E1 pre-osteoblastic cells were incubated with spironolactone (0-100  $\mu$ M) and 15 mM dRib. Cell toxicity (through lactate dehydrogenase release), markers of differentiation (collagen content, alkaline phosphatase activity and mineral deposition), inflammatory cytokine levels (TNF- $\alpha$  and IL-6), reactive oxygen species (ROS), mitochondrial superoxide levels, endoplasmic reticulum (ER) stress markers (activating transcription factor 6 and inositol-requiring enzyme 1), mitochondrial integrity (mitochondrial membrane potential and ATP levels), glyoxalase I activity and reduced glutathione (GSH) levels were analyzed by ELISA. Pretreatment with spironolactone reduced dRib-induced toxicity and improved differentiation markers in MC3T3-E1 cells. It also lowered the elevated cytokine levels, ROS production, mitochondrial oxidative burden and ER stress responses caused by dRib. Furthermore, spironolactone preserved mitochondrial performance and enhanced antioxidant defenses by increasing GSH levels and glyoxalase I activity, thereby promoting the detoxification of harmful byproducts. These findings suggested that spironolactone may protect osteoblasts by mitigating oxidative and inflammatory

stress, stabilizing mitochondrial function and enhancing differentiation. Overall, this highlights the possible benefits of spironolactone in the management of diabetes-related bone fragility.

## Introduction

A recent large-scale study reported that fracture risk was markedly increased in patients with type 1 diabetes, with an approximately threefold higher risk, and modestly but significantly increased in those with type 2 diabetes (20-30% higher risk), compared with non-diabetic individuals (1). In addition, recommendations for bone health management in diabetic patients have been added to the American Diabetes Association guidelines published in 2024 (2). Low bone mineral density in type 1 diabetes mellitus (3), low bone turnover, defects in microarchitecture, alterations in vitamin D regulation and diabetes-related complications, such as neuropathy and retinopathy, are associated with an increased risk of fractures in diabetic patients (4-6). Oxidative stress, advanced glycation end products (AGEs), homocysteine, and the reduction in insulin and insulin-like growth factor 1 activity can contribute to diabetes-related bone fragility (7). Research on osteoporosis treatments specifically for patients with diabetes is lacking, with the same osteoporosis treatment being recommended for both the general population and diabetic patients (8).

The environment of osteoblasts under hyperglycemic conditions can be experimentally reproduced using 2-deoxy-D-ribose (dRib), a deoxy-sugar derived from the pentose sugar ribose. dRib has a high reactivity with proteins and readily generates reactive oxygen species (ROS) through autooxidation and glycation reactions, thereby mimicking oxidative and glycative stress under diabetic conditions (9). Previous study has demonstrated that dRib induces mitochondrial dysfunction, endoplasmic reticulum (ER) stress and apoptosis in MC3T3-E1 osteoblastic cells (10), suggesting that dRib is a reliable *in vitro* model for investigating diabetes-related oxidative damage during bone metabolism.

Spironolactone, a well-known mineralocorticoid receptor (MR) antagonist used to treat hypertension, heart failure and primary aldosteronism, has attracted attention due to the diverse biological actions it exhibits, extending beyond cardiovascular effects (11). Beyond its mineralocorticoid receptor-blocking properties, spironolactone exerts a range of

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additional biological actions, such as attenuating fibrotic and inflammatory processes, reducing thrombotic activity and tissue congestion, and enhancing vascular function (11). Our previous study demonstrated that spironolactone mitigates methylglyoxal (MG)-induced oxidative injury in osteoblasts by reducing intracellular ROS levels (12). Considering that both MG and dRib are reactive carbonyl compounds that contribute to AGE formation and oxidative stress, it was hypothesized that spironolactone could also protect osteoblasts from dRib-induced cytotoxicity.

Therefore, the present study aimed to investigate the protective effects of spironolactone on bone metabolism by mitigating oxidative and glycativ stress in osteoblasts, thereby providing mechanistic insight into its potential as a therapeutic option to prevent diabetes-related bone fragility.

## Materials and methods

**Materials and reagents.** Spironolactone was obtained from MilliporeSigma. Culture media and antibiotics were supplied by Gibco (Thermo Fisher Scientific, Inc.), whereas all other reagents were sourced from MilliporeSigma, unless otherwise stated.

**Cell culture.** The osteoblastic MC3T3-E1 subclone 4 cell line was purchased from the American Type Culture Collection. Cells were grown in  $\alpha$ -modified minimal essential medium ( $\alpha$ -MEM; Gibco; Thermo Fisher Scientific, Inc) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The medium was supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and amphotericin B (25  $\mu$ g/ml). Once the cultures reached 100% confluence, they were switched to an osteogenic differentiation  $\alpha$ -MEM containing 10% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), amphotericin B (25  $\mu$ g/ml), 5 mM  $\beta$ -glycerophosphate and 50  $\mu$ g/ml ascorbic acid containing 5 mM  $\beta$ -glycerophosphate and 50  $\mu$ g/ml ascorbic acid. Spironolactone treatment was administered for 48 h at 37°C in a humidified incubator with 5% CO<sub>2</sub> after 6 days [for collagen measurement and alkaline phosphatase (ALP) activity] or 14 days (for mineralization analysis).

**Cell viability.** A preliminary study was conducted prior to the main experiments to assess the effects of a number of spironolactone and dRib concentrations on the viability of MC3T3-E1 osteoblastic cells. MC3T3-E1 cells were plated in 24-well plates at a density of 2x10<sup>4</sup> cells/well. After 48 h, the cells were exposed at 37°C for 1 h to  $\alpha$ -MEM containing 0.1% FBS and spironolactone (0, 10, 20, 50, 100, 200, 300 and 500  $\mu$ M) and then treated with dRib (0, 5, 10, 15, 20 and 30 mM) for an additional 48 h. Cell viability was determined using the water-soluble tetrazolium (WST) assay (Dojindo Molecular Technologies, Inc.). Briefly, WST reagent was added to each well and incubated at 37°C for 2 h before measurement. Absorbance was read at 570 nm with a Multiskan microplate reader (Thermo Fisher Scientific, Inc.). Untreated control cells cultured in medium alone were set as 100% viable and the percentage survival of treated cells was calculated relative to this control.

**Lactate dehydrogenase (LDH) cytotoxicity assay.** MC3T3-E1 cells were plated in 24-well dishes at a density

of 2x10<sup>4</sup> cells/well. After 48 h, the cells were pretreated at 37°C with varying spironolactone concentrations (0, 10, 20, 50, 70 and 100  $\mu$ M) for 1 h, followed by exposure to dRib 15 mM for an additional 48 h. Cell injury was assessed by measuring plasma membrane disruption. As LDH is a stable enzyme released upon membrane damage, its leakage into the culture medium was quantified using the LDH Cytotoxicity Assay Kit (BioVision, Inc.; Abcam) according to the manufacturer's protocol. Results were normalized against both positive controls (cells treated with 1% Triton X-100) and negative controls, with the Triton-treated samples defined as representing 100% cytotoxicity.

**Collagen content assay.** Osteoblast cultures were first rinsed with Dulbecco's PBS and subsequently fixed in Bouin's solution at room temperature for 1 h. After fixation, the dishes were washed under running tap water for 15 min, air-dried and then stained at room temperature with Sirius red for 1 h with gentle agitation. Excess dye was removed with 0.01N HCl and the bound stain was solubilized in 0.1N NaOH. Absorbance was recorded at 550 nm and collagen content was quantified using a calibration curve prepared from known concentrations of commercial collagen (MilliporeSigma). Absorbance was measured at 540 nm with a Zenyth 3100 multimode detector (Anthos Labtec Instruments GmbH).

**ALP activity assay.** ALP activity was determined from cell lysates using the ALP Activity Assay Kit (BioVision, Inc.; cat. no. K412-500) according to the supplier's protocol. ALP activity was normalized to total protein content. Total protein content was quantified using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). Absorbance was measured with a Zenyth 3100 multimode detector (Anthos Labtec Instruments GmbH).

**Mineralization assay.** Mineralization was evaluated by assessing calcium deposition. After fixation with 70% ethanol, cells were stained with Alizarin Red S at room temperature for 10 min and the extracted dye was quantified by measuring absorbance at 561 nm. Absorbance was measured at 540 nm with a Zenyth 3100 multimode detector (Anthos Labtec Instruments GmbH).

**Measurement of TNF- $\alpha$  and IL-6 levels.** TNF- $\alpha$  (cat. no. ELM-IL6) and IL-6 (cat. no. ELM TNF a) concentrations in the culture supernatants were determined using enzyme immunoassay kits (R&D Systems, Inc.) according to the manufacturer's guidelines. Culture supernatants were obtained from centrifugation and determined. Absorbance was measured with a Zenyth 3100 multimode detector (Anthos Labtec Instruments GmbH).

**Measurements of glyoxalase I activity and glutathione (GSH) levels.** Glyoxalase I activity was assessed according to a previously reported protocol (13). The intracellular GSH content from cell lysates was quantified using a GSH Assay Kit (BioAssay Systems) according to the manufacturer's instructions. Catalog numbers were glyoxalase I activity (DGLO-100) and GSH (EGTT-100).

**Determination of the mitochondrial membrane potential (MMP).** Cells were plated in black 96-well plates at a density of  $1 \times 10^4$  cells/well and cultured for 24 h. The cells were pretreated with numerous spironolactone concentrations (0, 50, 70 and 100  $\mu\text{M}$ ) at 37°C for 1 h, followed by exposure to dRib (15 mM) for 48 h. MMP was assessed using the JC-1 MMP Assay Kit (Cayman Chemical Company). JC-1 is a cationic, lipophilic dye that accumulates in intact mitochondria and emits red fluorescence. In depolarized mitochondria, it remains monomeric and fluoresces green. Cells were incubated with JC-1 for 20 min at 37°C, washed twice with PBS and analyzed with a fluorescence microplate reader (Molecular Devices, LLC) at 550/600 nm (red) and 485/535 nm (green). A reduction in the red/green fluorescence ratio indicated mitochondrial depolarization.

**Measurement of ATP levels.** Cells were plated in 24-well dishes at a density of  $2 \times 10^4$  cells/well. After 48 h of culture, the cells were pretreated at 37°C with numerous concentrations of spironolactone (0, 50, 70 and 100  $\mu\text{M}$ ) for 1 h and then exposed to dRib (15 mM) for 48 h. The cells were then lysed using cell lysis buffer II (cat. no. FNN0021; Thermo Fisher Scientific, Inc.) and homogenized in PBS. The homogenates were centrifuged at  $13,000 \times g$  for 15 min at 4°C and the resulting supernatants were collected for ATP and protein analyses. ATP levels were quantified by luciferase-based bioluminescence using the EnzyLight™ ATP Assay Kit (cat. no. EATP-100, BioAssay Systems) according to the manufacturer's protocol and total protein content was measured with the Bio-Rad Protein Assay Kit. Absorbance was measured with a Zenyth 3100 multimode detector (Anthos Labtec Instruments GmbH). ATP levels were normalized to total protein content.

**Measurement of intracellular ROS levels.** Cells were seeded in black 96-well plates at a density of  $1 \times 10^4$  cells per/well. After 24 h, the cells were pretreated with spironolactone (50-100  $\mu\text{M}$ ) at 37°C for 1 h, followed by the addition of dRib and incubation at 37°C for another 48 h. Subsequently, the cells were incubated with 5  $\mu\text{M}$  2',7'-dichlorofluorescein diacetate with spironolactone (50-100  $\mu\text{M}$ ) at 37°C for 1 h. Following three PBS washes, intracellular ROS levels were quantified by detecting fluorescence at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (13).

**Measurement of mitochondrial superoxide levels.** Cells were seeded in black 96-well plates at a density of  $1 \times 10^4$  cells per/well. After 24 h, the cells were pretreated with spironolactone at 37°C for 1 h, followed by the addition of dRib and incubation for another 48 h. Mitochondrial superoxide production was measured with the MitoSOX Red probe (Invitrogen; Thermo Fisher Scientific, Inc.). This fluorogenic dye specifically targets the mitochondria and exhibits fluorescence (excitation 510 nm; emission 580 nm) upon reacting with superoxide radicals (14). The cells were incubated with 2 mM MitoSOX Red at 37°C for 20 min, in accordance with the manufacturer's instructions. After washing the cells, the levels of MitoSOX Red fluorescence were measured by microplate reader.

**Measurements of inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF-6) levels.** IRE1 and ATF-6 are ER stress markers. Cytosolic concentrations were quantified using ELISA kits (MyBioSource, Inc.) following the supplier's protocols. Catalog numbers were IRE1 (MBS728814) and ATF-6 (MBS2533467).

**RNA isolation and reverse transcription-quantitative PCR (qPCR).** MC3T3-E1 osteoblastic cells were plated in 100-mm culture dishes at a density of  $2 \times 10^5$  cells/well and maintained in growth medium. After 48 h, the cells were pretreated with spironolactone (100  $\mu\text{M}$ ) for at 37°C for 1 h, followed by exposure to 100 nM dRib for 24 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmbH) and cDNA was synthesized with the PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using the SYBR® Premix Ex Taq™ Kit (Takara Bio, Inc.) on an ABI Prism 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to evaluate gene expression. The thermal cycling protocol comprised an initial denaturation at 95°C for 10 min, followed by 40 cycles of 94°C for 10 sec and 60°C for 30 sec. Reactions were performed in 20- $\mu\text{l}$  mixtures containing 0.8  $\mu\text{l}$  each primer (10  $\mu\text{M}$ ), 10  $\mu\text{l}$  SYBR Premix, 0.4  $\mu\text{l}$  ROX reference dye, 6  $\mu\text{l}$  distilled water and 2  $\mu\text{l}$  cDNA template. Table I lists the primer sequences used. All assays were conducted in quadruplicate and relative expression levels were analyzed using the  $2^{-\Delta\Delta C_q}$  method (15) with glucose-6-phosphate dehydrogenase as the reference gene, with results expressed as fold change relative to controls.

**Statistical analysis.** Experiments were carried out in at least three independent experiments. Data are presented as the mean  $\pm$  standard error of the mean. Statistical comparisons were performed using one-way ANOVA, followed by Dunnett's post-hoc test. Analyses were conducted using PASW software (version 20.0; IBM Corp.) and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cytoprotective effects of spironolactone on dRib-induced cytotoxicity in osteoblasts.** After treating MC3T3-E1 osteoblastic cells with spironolactone alone for 48 h, cell viability was measured. Cell viability decreased in response to 200-500  $\mu\text{M}$  viability, whereas concentrations  $\leq 100$   $\mu\text{M}$  did not significantly affect cell viability (Fig. S1A). Therefore, in the present study, spironolactone was used at concentrations  $\leq 100$   $\mu\text{M}$ , which did not affect cell viability. After treating MC3T3-E1 osteoblastic cells with dRib (0-30 mM) alone for 48 h, cell viability was reduced in a concentration-dependent manner (Fig. S1B). dRib was then applied to the cells at a concentration of 15 mM, which reduced the cell viability to 50%.

Osteoblasts were treated with spironolactone at concentrations ranging between 0 and 100  $\mu\text{M}$  and cultured 1 h later with 15 mM dRib for 48 h. Treatment with 15 mM dRib for 48 h significantly decreased cell viability to 19.4% of control ( $P < 0.05$ ), whereas pretreatment with spironolactone (70 and 100  $\mu\text{M}$ ) restored viability to  $32.5 \pm 5.6$  and  $79.2 \pm 12.8\%$ , respectively (Fig. S1C). LDH release increased to  $69.1 \pm 3.9\%$

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Genes	Forward primer	Reverse primer
ALP	5'-GGC CAG CTA CAC CAC AAC A-3'	5'-CTG AGC GTT GGT GTT ATA TGT CTT-3'
Collagen	5'-AGA CAT GTT CAG CTT TGT GGA C-3'	5'-CAT CCC TGA AGT CAG CTG C-3'
Osteocalcin	5'-CAC CAT GAG GAC CCT CTC TC-3'	5'-TGG ACA TGA AGG CTT TGT CA-3'
G6PD	5'-TGC AGC AGC TGT CCT CTA TG-3'	5'-ACT TCA GCT TTG CGC TCA TT-3'

ALP, alkaline phosphatase; G6PD, glucose-6-phosphate dehydrogenase.

of control after dRib exposure, but 50 and 100  $\mu\text{M}$  spironolactone reduced LDH leakage to  $19.1\pm 1.7$  and  $21.2\pm 3.9\%$ , respectively ( $P<0.05$ ; Fig. S1D). Morphological changes were photographed under an inverted microscope. Morphologically, dRib-treated cells showed marked shrinkage and detachment, whereas spironolactone treatment (100  $\mu\text{M}$ ) largely restored the normal polygonal morphology (Fig. S2).

*Effect of spironolactone on dRib-induced osteoblast differentiation.* To examine the effect of spironolactone on the differentiation of the MC3T3-E1 osteoblast cell line, collagen content, ALP activity and calcium deposition were measured. Collagen content was decreased to  $65.3\pm 2.4\%$  compared with that in the control group after dRib treatment; however, it increased again to between  $140.6\pm 1.5$  and  $142.3\pm 6.8\%$  after spironolactone treatment (50-100  $\mu\text{M}$ ;  $P<0.05$ ; Fig. 1A). ALP activity showed a similar pattern, declining to  $61.3\pm 5.3\%$  compared with the control group, then recovering to  $143.2\pm 8.5\%$  with 100  $\mu\text{M}$  spironolactone treatment ( $P<0.05$ ; Fig. 1B). Calcium deposition, measured by Alizarin Red S, fell to  $52.4\pm 4.5\%$  after dRib treatment, but was restored to between  $129.1\pm 2.6$  and  $188.1\pm 15.4\%$  of the control levels following spironolactone treatment (20-100  $\mu\text{M}$ ;  $P<0.05$ , Fig. 1C). Considering that collagen synthesis and ALP activity reflect the early stages of osteoblast differentiation, whereas mineralization represents the late stage, it is suggested that spironolactone promotes osteoblast differentiation, which is suppressed by dRib, from the early to late phase of differentiation.

*Effect of spironolactone on ER stress and inflammatory cytokine production in dRib-treated MC3T3-E1 cells.* After dRib administration, ATF-6 and IRE1 activities increased to  $268.4\pm 17.8$  and  $221.1\pm 13.3\%$  of the control levels, respectively, indicating increased ER stress ( $P<0.05$ ; Fig. 2A and B). Pretreatment with 100  $\mu\text{M}$  spironolactone significantly decreased the dRib-induced increase in ATF-6 and IRE1 activities to  $127.4\pm 7.7$  and  $142.6\pm 12.4\%$ , respectively ( $P<0.05$ ; Fig. 2A and B), suggesting that spironolactone mitigated ER stress stimulated by dRib.

Furthermore, dRib treatment (15 mM) led to a significant increase in TNF- $\alpha$  and IL-6 production to  $206.6\pm 16.3$  and  $325.5\pm 12.3\%$  of the control levels, respectively ( $P<0.05$ ; Fig. 3A and B). Increased TNF- $\alpha$  and IL-6 production was significantly reduced to  $46.3\pm 8.0$  and  $176.9\pm 16.7\%$ , respectively, by 100  $\mu\text{M}$  spironolactone pretreatment ( $P<0.05$ ;

Fig. 3A and B), suggesting that the mechanism by which spironolactone prevents osteoblast damage caused by dRib involves a reduction in inflammatory cytokine levels.

*Effect of spironolactone on glyoxalase I activity and GSH levels in dRib-treated cells.* dRib is detoxified by the glyoxalase enzyme system. Measurement of glyoxalase I activity revealed a significant decrease to  $27.7\pm 2.4\%$  of the control after dRib treatment (15 mM), whereas glyoxalase I activity was increased to  $57.5\pm 8.3\%$  when the osteoblasts were pretreated with 50  $\mu\text{M}$  spironolactone ( $P<0.05$ ; Fig. 4A). Additionally, GSH levels were increased to  $162.5\pm 4.3\%$  in dRib-treated osteoblasts after 70  $\mu\text{M}$  spironolactone treatment ( $P<0.05$ ; Fig. 4B). These results indicated that spironolactone may detoxify dRib by increasing GSH and glyoxalase I activity.

*Inhibitory effect of spironolactone on dRib-induced oxidative stress in cells.* dRib exposure markedly increased intracellular ROS generation to  $205.3\pm 14.2\%$  of the control. Spironolactone pretreatment progressively and dose-dependently reduced ROS levels to  $142.4\pm 9.6\%$  (50  $\mu\text{M}$ ),  $136.4\pm 7.8\%$  (70  $\mu\text{M}$ ) and  $130.3\pm 9.3\%$  (100  $\mu\text{M}$ ) ( $P<0.05$ ; Fig. 5A). Similarly, mitochondrial superoxide production rose to  $148.0\pm 8.2\%$  of the control after dRib treatment, but was gradually suppressed by spironolactone to  $120.5\pm 7.8$ ,  $110.4\pm 5.5$  and  $105.1\pm 5.8\%$  at 50, 70 and 100  $\mu\text{M}$ , respectively ( $P<0.05$ ; Fig. 5B). These findings indicated that spironolactone reduced dRib-induced ROS production and oxidative stress in mitochondria.

*Effect of spironolactone on the dRib-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells.* When osteoblasts were treated with dRib, MMP decreased to  $55.3\pm 7.7\%$  of the control, whereas it increased to  $66.4\pm 3.8$ ,  $85.1\pm 11.9$  and  $92.5\pm 9.8\%$  in response to 50, 70 and 100  $\mu\text{M}$  spironolactone pretreatment, respectively. ( $P<0.05$  in 70 and 100  $\mu\text{M}$  spironolactone; Fig. 6A). In addition, ATP production was reduced to  $54.3\pm 6.8\%$  of the control following dRib treatment, but was restored to  $68.4\pm 14.3$ ,  $72.4\pm 12.6$  and  $103.7\pm 6.9\%$  after 50, 70 and 100  $\mu\text{M}$  spironolactone treatment, respectively ( $P<0.05$  in 100  $\mu\text{M}$  spironolactone; Fig. 6B). These findings suggested that spironolactone may have reduced dRib toxicity by increasing mitochondrial biogenesis.

*Effect of spironolactone on the mRNA expression levels of ALP, type I collagen and osteocalcin in dRib-treated cells.* In osteoblasts treated with 15 mM dRib, the mRNA expression

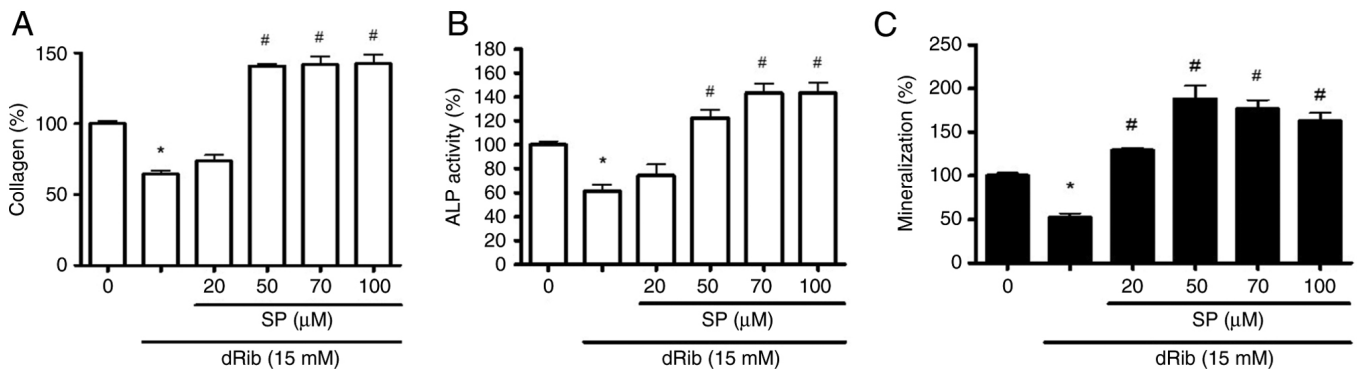


Figure 1. Effects of SP on the differentiation in dRib-treated MC3T3-E1 cells. (A) Collagen content, (B) ALP activity and (C) mineralization were assessed in cells exposed to dRib (15 mM) with or without spironolactone (20-100 μM) pretreatment. All data are presented as percentages relative to the untreated control group, which was set to 100%. Data are expressed as mean ± SEM from independent *in vitro* experiments. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells treated with dRib alone. ALP, alkaline phosphatase; SP, spironolactone; dRib, 2-deoxy-D-ribose.

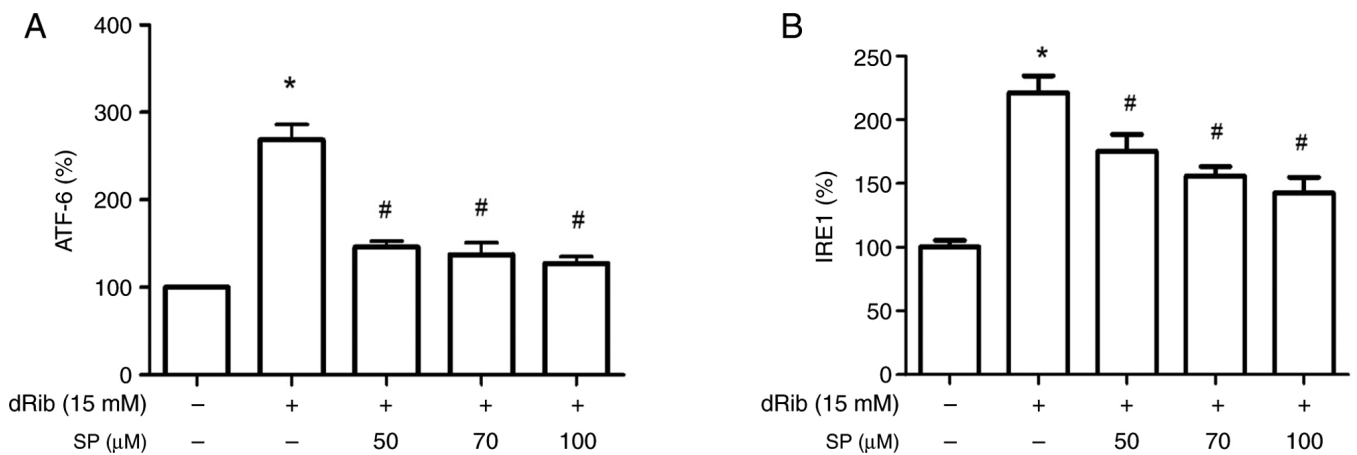


Figure 2. Effects of SP on endoplasmic reticulum stress in dRib-treated MC3T3-E1 cells. Osteoblasts were pre-incubated with spironolactone before treatment with 15 mM dRib for 24 h. The control values for (A) ATF-6 and (B) IRE1 were 49.35±0.354 and 2.845±0.154 ng/mg, respectively. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells treated with dRib alone. ATF-6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; SP, spironolactone; dRib, 2-deoxy-D-ribose.

levels of ALP, collagen and osteocalcin were significantly reduced (0.3±0.1, 0.2±0.1 and 0.42±0.1 fold of control, respectively; P<0.05), whereas treatment with spironolactone restored their expression (0.8±0.2, 0.9±0.1 and 1.1±0.1 fold of control, respectively; P<0.05) (Fig. S3).

### Discussion

Within the present study, the mechanism by which spironolactone protects osteoblastic MC3T3-E1 cells from dRib-induced oxidative and glycative stress was examined. Rather than reversing cytotoxicity, spironolactone was shown to modulate numerous interlinked stress-response pathways, including redox balance, ER stress, mitochondrial function and inflammation, ultimately preserving osteoblast differentiation capacity.

In our previous study, antioxidants such as N-acetyl-L-cysteine and α-lipoic acid were revealed to reverse dRib-induced cytotoxicity (16), indicating that oxidative stress is a key contributor to this damage. Consistent with these findings, the present study further demonstrated this mechanism by exhibiting that dRib exposure markedly increased intracellular ROS

and mitochondrial superoxide levels, implicating mitochondrial dysfunction as a key source of oxidative stress. ROS, while being important in normal cellular processes (17), can cause notable damage under oxidative stress, leading to DNA, lipid and protein damage, and enhanced apoptosis (18). The observed reduction in ROS and mitochondrial superoxide, coupled with the restoration of MMP and ATP production, implies that spironolactone stabilizes mitochondrial integrity, likely by limiting ROS-driven damage and supporting biogenesis.

The ER, which is responsible for protein synthesis and folding, also serves a role in protein quality surveillance and degradation (19). Unresolved ER stress caused by misfolded proteins activates the unfolded protein response mediated by IRE1 and ATF-6 (20-22). In the present study, both ATF-6 and IRE1 levels were elevated following dRib treatment, indicating an increase in ER stress. ER stress and mitochondrial dysfunction are associated, forming a cycle that promotes apoptosis and oxidative stress (23). Spironolactone may affect this cycle by reducing ROS accumulation, restoring MMP and ATP synthesis, and attenuating ER stress, thereby improving cell survival and osteogenic capacity. The present findings suggest that spironolactone acts as an antioxidant.

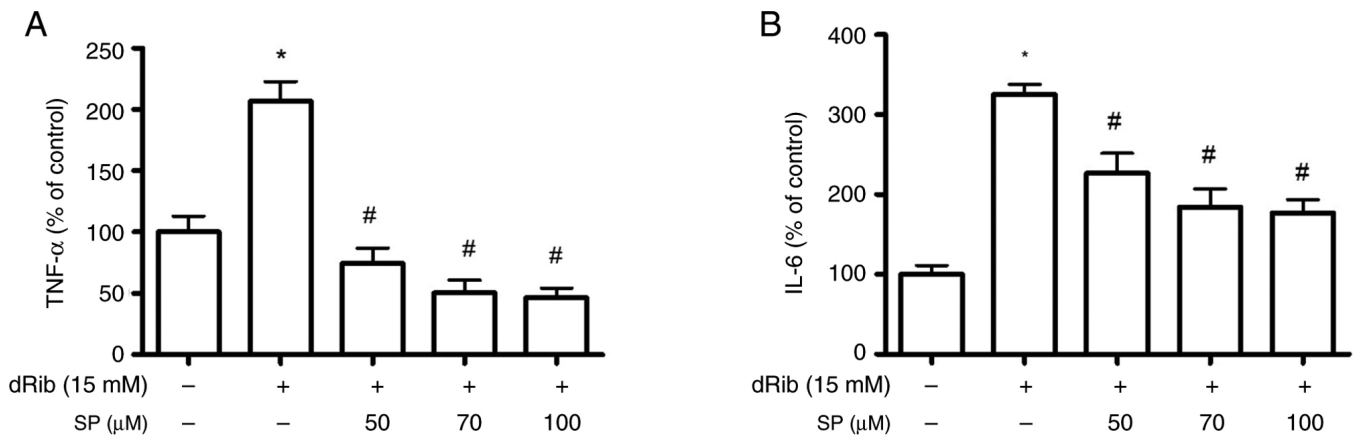


Figure 3. Effects of SP on inflammatory cytokine production in dRib-treated MC3T3-E1 cells. (A) TNF- $\alpha$  and (B) IL-6 levels were assessed in cells exposed to dRib (15 mM) with or without spironolactone (50, 70 or 100  $\mu$ M) pretreatment. Osteoblasts were pre-incubated with spironolactone before treatment with 15 mM dRib for 48 h. The control values for TNF- $\alpha$  and IL-6 were  $16.19 \pm 2.3143$  pg/mg and  $0.547 \pm 0.029$  ng/mg, respectively. \* $P < 0.05$  vs. untreated cells; # $P < 0.05$  vs. cells treated with dRib alone. SP, spironolactone; dRib, 2-deoxy-D-ribose.

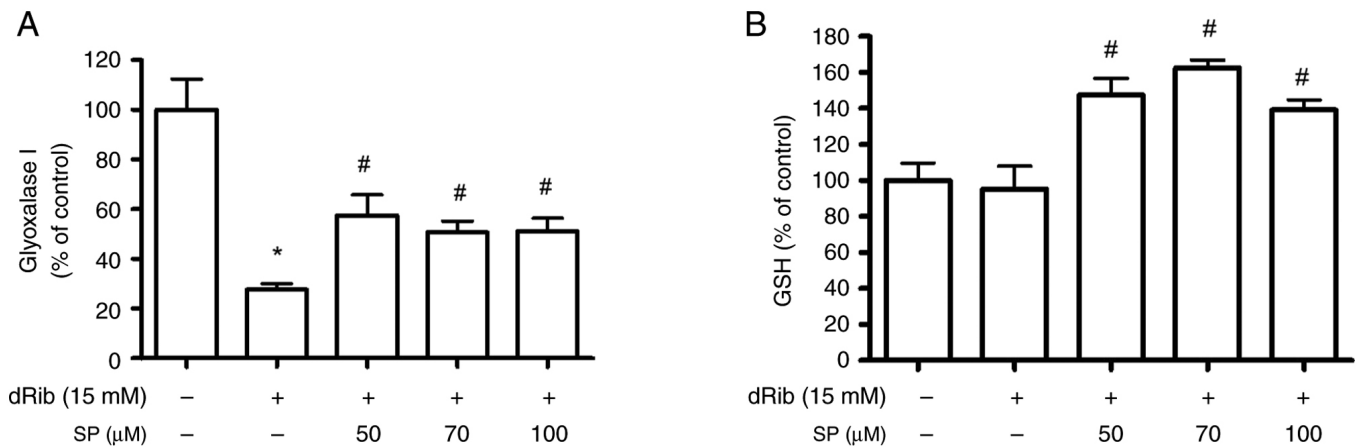


Figure 4. Effects of SP on glyoxalase I activity and reduced GSH levels in dRib-treated cells. Osteoblasts were pre-incubated with SP before treatment with 15 mM dRib for 48 h. The control values for (A) glyoxalase I activity and (B) GSH were  $0.32 \pm 0.004$   $\Delta$ OD/min/mg and  $68.05 \pm 7.47$   $\mu$ g/mg, respectively. \* $P < 0.05$  vs. untreated cells; # $P < 0.05$  vs. cells treated with dRib alone. SP, spironolactone; dRib, 2-deoxy-D-ribose; GSH, glutathione.

dRib also increased inflammatory markers TNF- $\alpha$  and IL-6, contributing to oxidative damage (24). By contrast, spironolactone suppressed the production of these inflammatory cytokines, thus reinforcing its role as an anti-inflammatory modulator. A previous *in vitro* study demonstrated that spironolactone inhibits the stimulated release of TNF- $\alpha$  and IL-6 from human peripheral blood mononuclear cells (25). Experimental studies have shown that spironolactone protects myocardial and endothelial cells by reducing oxidative stress and inflammatory responses, thereby ameliorating diabetic cardiomyopathy and endothelial dysfunction through mechanisms involving suppression of reactive oxygen species generation and inhibition of the AGE/RAGE signaling pathway (26-28).

The enhancement of GSH levels and recovery of glyoxalase I activity further reinforces the antioxidant potential of spironolactone. As glyoxalase I detoxifies MG, a precursor of AGEs (29), this mechanism may contribute to the prevention of diabetes-related bone deterioration. Thus, spironolactone not only reduces the oxidative burden, but may also improve osteoblast redox homeostasis and matrix formation through both direct and enzymatic pathways.

Patients with primary aldosteronism show increased urinary calcium excretion, leading to secondary hyperparathyroidism, which accelerates bone loss and increases fracture risk (30). Consistent with these pathophysiological mechanisms, spironolactone treatment has been shown to reduce bone loss, enhance bone strength and prevent fractures in this population (30,31). Besides the systemic effects, the present data suggest that spironolactone could exert direct osteoblastic protection through integrated antioxidant and anti-inflammatory mechanisms. This dual action underscores its therapeutic potential as an adjunct strategy for treating diabetes-related bone fragility.

The present study has some limitations. First, although bone metabolism involves osteoblasts, osteoclasts and osteocytes, only osteoblasts were examined. Nevertheless, the present study demonstrated meaningful qualitative and quantitative outcomes in terms of osteogenic activity. Further research using animal models, osteoblast-osteoclast co-culture systems, and the evaluation of bone mass and quality is needed. Second, spironolactone was tested without comparison to other antioxidants or medications, limiting

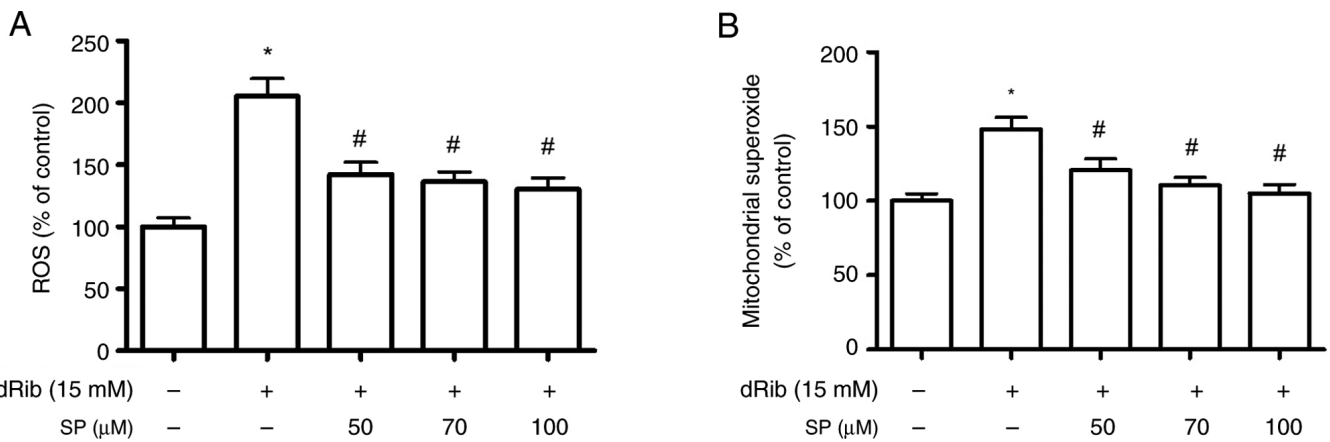


Figure 5. Inhibitory effect of SP on dRib-induced oxidative stress in cells. Osteoblasts were pre-incubated with SP before treatment with 15 mM dRib for 48 h. (A) Changes in levels of ROS, which were measured using the dichlorofluorescein fluorescence method. (B) Mitochondrial superoxide levels were detected using MitoSOX Red mitochondrial superoxide indicator. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells treated with dRib alone. SP, spironolactone; dRib, 2-deoxy-D-ribose; ROS, reactive oxygen species.

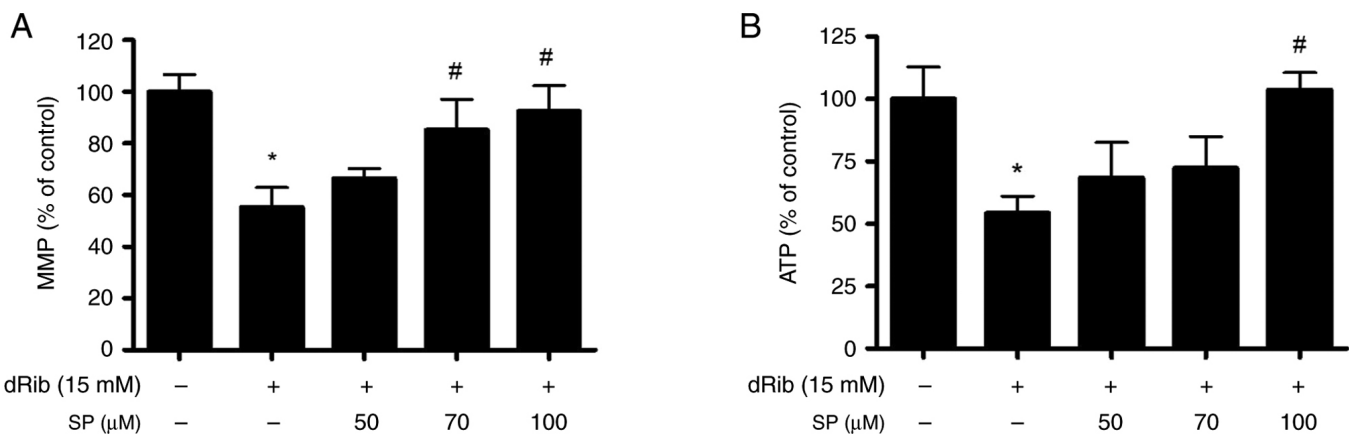


Figure 6. Effects of SP on the dRib-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. Osteoblasts were pre-incubated with SP before treatment with 15 mM dRib for 48 h. (A) Mitochondrial depolarization (loss of MMP) and (B) ATP levels were measured. \*P<0.05 vs. untreated cells; #P<0.05 vs. cells treated with dRib alone. SP, spironolactone; dRib, 2-deoxy-D-ribose; MMP, mitochondrial membrane potential.

the ability to evaluate its relative efficacy. For example, quantitative efficacy analyses through comparisons with agents such as SGLT2 inhibitors are recommended in future studies. Third, numerous factors such as hyperglycemia, insulin resistance and kidney dysfunction influence diabetes-related bone fragility and should be considered in future studies. Lastly, dRib may represent a model closer to stress-induced premature senescence rather than true aging and therefore may have limitations in fully reflecting the physiological aging process. However, considering that oxidative stress is one of the key mechanisms underlying diabetes-related bone fragility, the present findings remain meaningful.

In conclusion, the present study revealed that spironolactone protects osteoblasts from dRib-induced oxidative stress by suppressing ROS and ER stress, enhancing mitochondrial integrity and activating the endogenous antioxidant system. These findings provide mechanistic insights into the non-classical actions of spironolactone and support its potential as an adjunctive therapeutic strategy for preventing diabetes-related bone fragility.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

SYP contributed towards conducting the investigation, writing the original draft, and reviewing and editing the manuscript. SYP, SOC and KSS confirm the authenticity of all the raw data. KSS contributed towards collection of raw data, formal

analysis, conducting the investigation, experimental methodology, validation, and reviewing and editing the manuscript. HSK contributed towards collection of raw data, formal analysis, conducting the investigation, methodology, validation and reviewing and editing the manuscript. SJY contributed towards conducting the investigation, validation, and reviewing and editing the manuscript. HS contributed towards conducting the investigation, validation, and reviewing and editing the manuscript. SOC contributed towards conceptualization, funding acquisition, conducting the investigation, validation, and reviewing and editing the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

- Liao CC, Lin CS, Shih CC, Yeh CC, Chang YC, Lee YW and Chen TL: Increased risk of fracture and postfracture adverse events in patients with diabetes: Two nationwide population-based retrospective cohort studies. *Diabetes Care* 37: 2246-2252, 2014.
- American Diabetes Association Professional Practice Committee: 4. Comprehensive medical evaluation and assessment of comorbidities: Standards of care in diabetes-2024. *Diabetes Care* 47 (Suppl 1): S52-S76, 2024.
- Starup-Linde J, Hygum K, Harslof T and Langdahl B: Type 1 diabetes and bone fragility: Links and risks. *Diabetes Metab Syndr Obes* 12: 2539-2547, 2019.
- Carnevale V, Romagnoli E and D'Erasmo E: Skeletal involvement in patients with diabetes mellitus. *Diabetes Metab Res Rev* 20: 196-204, 2004.
- Hygum K, Starup-Linde J, Harslof T, Vestergaard P and Langdahl BL: MECHANISMS IN ENDOCRINOLOGY: Diabetes mellitus, a state of low bone turnover-a systematic review and meta-analysis. *Eur J Endocrinol* 176: R137-R157, 2017.
- Starup-Linde J, Lykkeboe S, Gregersen S, Hauge EM, Langdahl BL, Handberg A and Vestergaard P: Bone structure and predictors of fracture in type 1 and type 2 diabetes. *J Clin Endocrinol Metab* 101: 928-936, 2016.
- Kanazawa I and Sugimoto T: Diabetes Mellitus-induced bone fragility. *Intern Med* 57: 2773-2785, 2018.
- Sheu A, White CP and Center JR: Bone metabolism in diabetes: A Clinician's guide to understanding the bone-glucose interplay. *Diabetologia* 67: 1493-1506, 2024.
- Koh G, Suh KS, Chon S, Oh S, Woo JT, Kim SW, Kim JW and Kim YS: Elevated cAMP level attenuates 2-deoxy-d-ribose-induced oxidative damage in pancreatic beta-cells. *Arch Biochem Biophys* 438: 70-79, 2005.
- Kim HS, Suh KS, Ko A, Sul D, Choi D, Lee SK and Jung WW: The flavonoid glabridin attenuates 2-deoxy-D-ribose-induced oxidative damage and cellular dysfunction in MC3T3-E1 osteoblastic cells. *Int J Mol Med* 31: 243-251, 2013.
- Ferreira JP, Verdonschot J, Wang P, Pizard A, Collier T, Ahmed FZ, Brunner-La-Rocca HP, Clark AL, Cosmi F, Cuthbert J, *et al*: Proteomic and mechanistic analysis of spironolactone in patients at risk for HF. *JACC Heart Fail* 9: 268-277, 2021.
- Park SY, Suh KS, Jung WW and Chin SO: Spironolactone attenuates methylglyoxal-induced cellular dysfunction in MC3T3-E1 osteoblastic cells. *J Korean Med Sci* 36: e265, 2021.
- Suh KS, Chon S and Choi EM: Protective effects of piceatannol on methylglyoxal-induced cytotoxicity in MC3T3-E1 osteoblastic cells. *Free Radic Res* 52: 712-723, 2018.
- Piazena H and Kelleher D: Comments on 'Cellular response to infrared radiation involves retrograde mitochondrial signaling'. *Free Radic Biol Med* 44: 1869-1871, 2008.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Suh KS, Choi EM, Kwon M, Chon S, Oh S, Woo JT, Kim SW, Kim JW and Kim YS: Kaempferol attenuates 2-deoxy-d-ribose-induced oxidative cell damage in MC3T3-E1 osteoblastic cells. *Biol Pharm Bull* 32: 746-749, 2009.
- Rhee SG: Cell signaling. H2O2, a necessary evil for cell signaling. *Science* 312: 1882-1883, 2006.
- Jacobson MD: Reactive oxygen species and programmed cell death. *Trends Biochem Sci* 21: 83-86, 1996.
- Ellgaard L and Helenius A: Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* 4: 181-191, 2003.
- Mei Y, Thompson MD, Cohen RA and Tong X: Endoplasmic reticulum stress and related pathological processes. *J Pharmacol Biomed Anal* 1: 1000107, 2013.
- Schroder M and Kaufman RJ: The mammalian unfolded protein response. *Annu Rev Biochem* 74: 739-789, 2005.
- Ron D and Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8: 519-529, 2007.
- Bhatti JS, Bhatti GK and Reddy PH: Mitochondrial dysfunction and oxidative stress in metabolic disorders-A step towards mitochondria based therapeutic strategies. *Biochim Biophys Acta Mol Basis Dis* 1863: 1066-1077, 2017.
- Mittal M, Siddiqui MR, Tran K, Reddy SP and Malik AB: Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* 20: 1126-1167, 2014.
- Hansen PR, Rieneck K and Bendtzen K: Spironolactone inhibits production of proinflammatory cytokines by human mononuclear cells. *Immunol Lett* 91: 87-91, 2004.
- Liu W, Gong W, He M, Liu Y, Yang Y, Wang M, Wu M, Guo S, Yu Y, Wang X, *et al*: Spironolactone protects against diabetic cardiomyopathy in streptozotocin-induced diabetic rats. *J Diabetes Res* 2018: 9232065, 2018.
- Mayyas F, Alzoubi KH and Bonyan R: The role of spironolactone on myocardial oxidative stress in rat model of streptozotocin-induced diabetes. *Cardiovasc Ther* 35, e12242, 2017.
- Wang CC, Lee AS, Liu SH, Chang KC, Shen MY and Chang CT: Spironolactone ameliorates endothelial dysfunction through inhibition of the AGE/RAGE axis in a chronic renal failure rat model. *BMC Nephrol* 20: 351, 2019.
- Do MH, Hur J, Choi J, Kim M, Kim MJ, Kim Y and Ha SK: *Eucommia ulmoides* ameliorates glucotoxicity by suppressing advanced glycation end-products in diabetic mice kidney. *Nutrients* 10: 265, 2018.
- Chhokar VS, Sun Y, Bhattacharya SK, Ahokas RA, Myers LK, Xing Z, Smith RA, Gerling IC and Weber KT: Loss of bone minerals and strength in rats with aldosteronism. *Am J Physiol Heart Circ Physiol* 287: H2023-H2026, 2004.
- Carbone LD, Cross JD, Raza SH, Bush AJ, Sepanski RJ, Dhawan S, Khan BQ, Gupta M, Ahmad K, Khouzam RN, *et al*: Fracture risk in men with congestive heart failure risk reduction with spironolactone. *J Am Coll Cardiol* 52: 135-138, 2008.