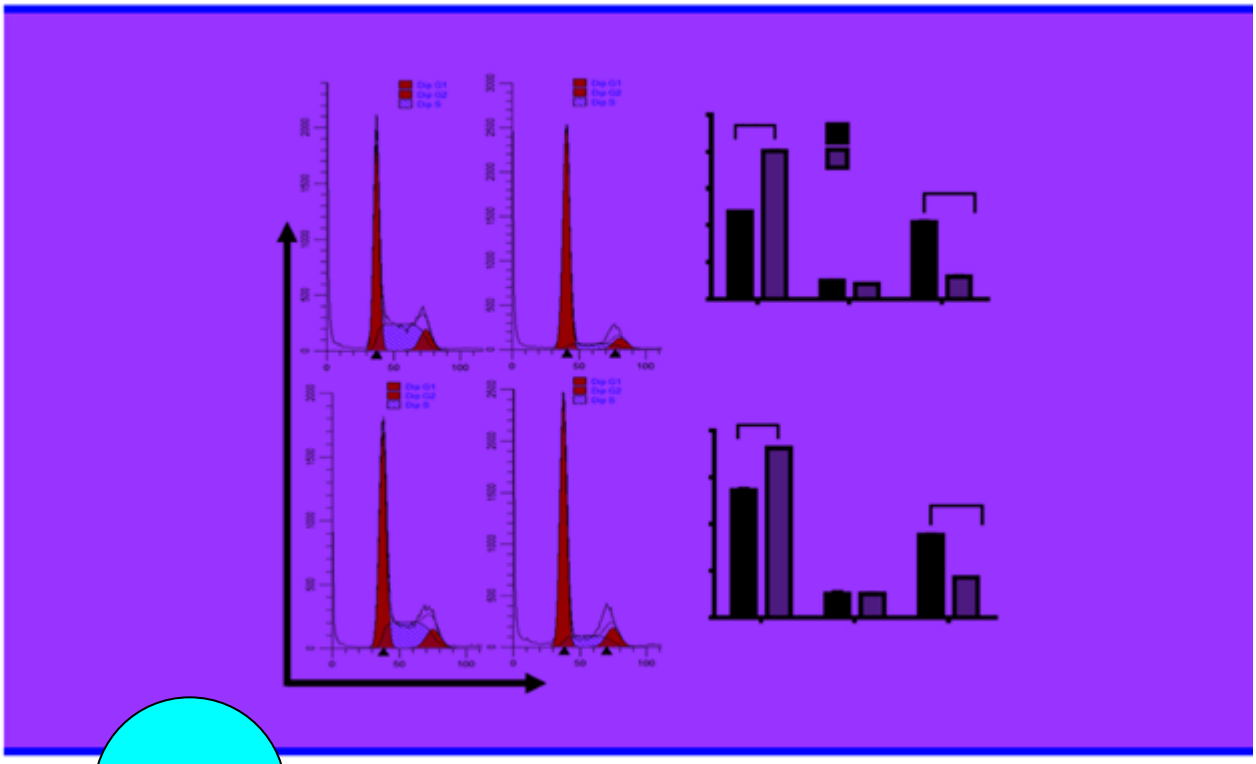

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Prednisone inhibits osteosarcoma cell by regulating the Wnt/β-catenin signaling pathway

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Abstract

Prednisone, a synthetic glucocorticoid, possesses a moderate duration of action in the human body. To investigate the effect of prednisone on osteosarcoma cells *in vitro*, CCK8, wound healing, Transwell assays, flow-cytometry, western blot, and ELISA were used to measure cell proliferation, cell migration, cell invasion, cell cycle, cell apoptosis, and the key proteins in Wnt/β-catenin pathway. The results showed prednisone could inhibit the proliferation in osteosarcoma cells and exhibit lower proliferation toxicity towards normal bone cells compared to cisplatin by 17.5% ($p<0.001$). Cell apoptosis was increased by 6.6 and 7.4%, cell migration was decreased to 62.6 and –23.9%, G0/G1 phase cells were increased by 33.0 and 17.9%, and S phase cells were reduced by 29.6 and 18.0% (all $p<0.001$) in Mg63 and Saos-2 cells respectively after prednisone treatment. The expressions of β-catenin, cMYC, cyclin D1, and MMP7 were also reduced. In conclusion, prednisone inhibits osteosarcoma cells by regulating the Wnt/β-catenin signaling pathway.

Introduction

Osteosarcoma, a highly aggressive malignancy with a pronounced incidence among children and adolescents under the age of 20, is characterized by its high metastatic potential (Xu et al., 2020), poor prognosis (Li et al., 2020), and currently limited five-year survival rates (Lei et al., 2021). Despite extensive research over the past three decades, there has been no substantial improvement in the efficacy of pharmacological treatments for osteosarcoma. Consequently, pursuing safer and more effective novel therapeutic agents against osteosarcoma holds paramount importance and urgency.

Glucocorticoids, vital small-molecule substances within the body, play pivotal roles in regulating growth (Zhao et al., 2024), development (Taves and Ashwell, 2021), metabolism (Felten and Arnaud, 2020), and immunity (Shimba and Ikuta, 2020). They exhibit extensive and significant physiological and pharmacological effects and are among the most widely used and effective anti-inflammatory and immunosuppressive agents in clinical practice. Commonly prescribed glucocorticoids include prednisone, hydrocortisone and dexamethasone, which also have demonstrated important roles in the treatment of breast cancer (Pang et al., 2020) and hematological malignancies (Ahmad, et al., 2019; Gebru et al., 2020). Prednisone, a synthetic glucocorticoid with an elimination half-life of 3-4 hours in adults and 1-2 hours in children (Puckett et al., 2024), is employed in the management of asthma (Abaya et al., 2018),

myeloma (Kaedbey et al., 2024), and prostate cancer (Duarte et al., 2019). Although the long-acting glucocorticoid dexamethasone (Kudawara et al., 2001; Yamamoto, et al., 2002) and the short-acting glucocorticoid hydrocortisone (Hindmarsh et al., 2015; Zhang et al., 2023) have been proven to have some effects on osteosarcoma inhibition, the effect of the medium-acting glucocorticoid prednisone on osteosarcoma remains unexplored. Studying the inhibitory effect of prednisone on osteosarcoma may help to fully understand the role of glucocorticoid in anti-tumor therapy and provide more comprehensive treatment options and theoretical basis for clinical practice.

Materials and Methods

Cells

The human osteosarcoma cell line Mg63, human osteosarcoma cell line Saos-2, and human normal osteocyte hFOB1.19 were all purchased from the Chinese Academy of Sciences Cell Bank in Shanghai.

Drugs and reagents

Cell counting Kit-8 (CCK8), Bradford protein quantification Kit, myelocytomatosis viral oncogene homolog (cMYC) antibody, cyclin D1 antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were purchased from Beijing Biyuntian Biotechnology Co., Ltd. Secondary antibodies were purchased from Beijing Zhongshan Jinqiao Technology Co., Ltd. High-sensitivity enhanced chemiluminescence substrate, propidium iodide cell cycle detection kit, and apoptosis detection kit were purchased from Shanghai Sizhengbai Biotechnology Co., Ltd. Prednisone was purchased from Selleck Biotechnology Co., Ltd. and dissolved in dimethyl sulfoxide (DMSO) before use. Human matrix metalloproteinase 7 (MMP7) enzyme-linked immunosorbent assay kit and crystal violet were purchased from Shanghai Biological Engineering Co., Ltd. Dulbecco's Modified Eagle Medium (DMEM) culture medium and fetal bovine serum were purchased from GIBCO Medical and Biotechnology Co., Ltd..

Cell culture

Mg63 and Saos-2 human osteosarcoma cells are cultured in a 5% CO_2-saturated incubator at 37°C, using DMEM cell culture medium supplemented with 100 IU/mL penicillin, 15% fetal bovine serum, and 100 µg/mL streptomycin. The medium is replaced every 48 hours. The human fetal osteoblastic cell line hFOB1.19 was maintained in F-12 Dulbecco's Modified Eagle Medium (GIBCO, USA) supplemented with 10% fetal bovine serum and 0.3 mg/mL of neomycin (Biyuntian Biotech, China) at 33.5°C.

CCK8 assay for cell proliferation

To assess cell proliferation, the cell suspensions were seeded into 96-well plates at 100 µL per well, containing approximately 5,000 cells per well. Different concentrations (0-1000 µM) of prednisone were added to the treatment group wells, and the plates were incubated continuously for 48 hours. CCK8 (10 µL CCK8 per 100 µL DMEM) was added to each well, and the absorbance at 450 nm was measured (Multiskan, USA). The optical density (OD) values obtained reflect the number of viable cells in each well (Jiang, et al., 2024).

Wound healing assay

MG63 and Saos-2 cells were inoculated into a 6-well plate at 5 × 10^5 cells/well at 37°C and 5% CO_2 for 24 hours to form a monolayer of cells. After 24 hours, a scratch was subsequently performed in the cell monolayer. Carefully wash three times with PBS to remove residual cell debris, and then add drug-free and prednisone-containing media to incubate without serum to avoid the effect of cell proliferation. Then the plate was incubated at 37°C and 5% CO_2 for 48 hours, and subsequently photographed (Yang et al., 2024).

Box 1: Western Blot

Principle

Western blot is based on the principle of immunochromatography where proteins are separated into polyacrylamide gel according to their molecular weight.

Requirements

BCA protein assay kit (Beyotime, China, P0010), High sensitivity enhanced chemiluminescence substrate (Beyotime, China, P0018M), Cells pellet, RIPA buffer, Protease cocktail (Beyotime, China, P1005), Blocking buffer, Primary antibodies, Secondary antibodies, Phosphate saline buffer, PVDF membrane, 1×Tris-buffered saline, 0.1%Tween, Sodium polyacrylamide gel, Transfer buffer.

Procedure

Step 1: The proteins were separated using a 1% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrotransferred onto a PVDF membrane.

Step 2: The membrane was washed, blocked, and then a primary antibody at a 1:2500 dilution was added.

Step 3: It was incubated at room temperature for 1 to 2 hours or overnight at 4°C.

Step 4: After the membrane was washed, a secondary antibody at an appropriate dilution of 1:4000 was added, and it was incubated at room temperature for 1 hour with gentle agitation.

Step 5: Following another wash, the target protein was detected, and the grayscale values were analyzed using ImageJ software.

Reference

Zheng et al., 2024

Transwell assay

The incubated upper chamber of the Transwell was placed in the 24-well plate with 5×10⁴ cells, and 600 µL culture medium containing 20% fetal bovine serum was added to the bottom of the well, which was the lower chamber. After incubation at 5% CO_2, cells were placed in 0.1% 600 µL crystal violet staining solution for 30 min at room temperature. Upon completion of staining, the crystal violet was eluted with 30% acetic acid, and the optical density values were measured at a wavelength of 570 nm for statistical analysis of the results (Yang et al., 2024).

Cell apoptosis and cell cycle detection

The cell cycle and apoptosis experiments were performed as described previously (Li et al., 2019; Le et al., 2024). Cells were harvested with trypsin without ethylenediamine-tetraacetic acid (EDTA), washed with phosphate- buffered saline (PBS), and then resuspended in binding buffer (0.1 M HEPES/NaOH, 1.4M NaCl, 25 mM $CaCl_2$) to adjust the cell concentration to between 1 × 10⁶/mL and 5 × 10⁶/mL. 100 µL of the suspension was aspirated into a 1.5 mL tube, mixed with 5 µL of annexin V/PE (Sizhengbai Technology, China), incubited at room temperature for 5 min in the dark, and then added with 10 µL of 20 µg/mL 7AAD+ (Sizhengbai Technology, China) and 400 µL of PBS before immediate flow cytometry analysis (Flowy XP, 0211, China).

Cells were digested with EDTA-free trypsin, washed with cold PBS, and fixed with precooled 75% ethanol overnight at 4°C. The cells were washed twice with PBS, centrifuged, and treated with RNase A and propidium iodide (PI) for 30 min at 37°C in the dark. Finally, the stained cells were analyzed by a flow cytometer (BD -cytometer, LSRII, USA).

Statistical analysis

All data are presented as statistical graphs generated using GraphPad Prism 5. Differences between the two groups were determined using a two-tailed t-test. Differences between three or more groups were determined using a two-way analysis of variance. Data were measured using the mean ± SEM. P values less than 0.05 were considered statistically significant.

Results

Proliferation of osteosarcoma cells and toxicity towards normal bone cells compared to cisplatin

Human normal osteocyte hFOB1.19 cells, human osteosarcoma cells Mg63, and human osteosarcoma cells Saos-2 were cultured in an incubator at 37°C and then seeded into 96-well plates. They were treated with different concentrations of prednisone (ranging from 0 to 1000 µM) or cisplatin (ranging from 0 to 40 µM), respectively, and further cultured for 48 hours. Cell viability was then measured by CCK8 assay, with the results presented in Figures 1A and 1B. At cisplatin concentrations ranging from 0 to 5 µg/mL, no significant effect on the proliferation of either normal osteocyte or osteosarcoma cells was observed. However, when cisplatin concentrations reached 10-40 µg/mL, an inhibition of proliferation was observed in three types of cells. At a cisplatin concentration of 20 µg/mL, the proliferation rate of normal osteocytes was significantly lower than that of the osteosarcoma cells (Figure 1A), suggesting a higher proliferation toxicity of cisplatin towards normal bone cells compared to osteosarcoma cells.

At lower concentrations of prednisone (0-62.5 µM), no significant inhibition of proliferation was observed in any of the three cells (Figure 1B). Nevertheless, as the concentration of prednisone increased (125-1000 µM), prednisone suppressed the proliferation of both normal osteocyte and osteosarcoma cells, leading to a significant decrease in survival rates. This finding underscores

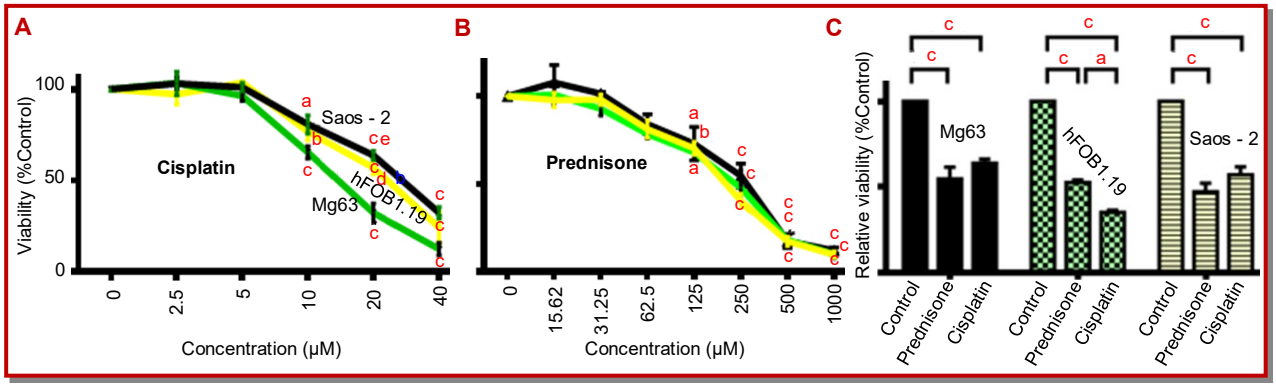

Figure 1: Prednisone inhibits osteosarcoma cells and is less toxic to the proliferation of normal osteocyte than cisplatin. Effects of cisplatin on normal bone cells hFOB1.19, osteosarcoma cells Mg63 and osteosarcoma cells Saos-2 (A). Effects of prednisone on hFOB1.19, Mg63 and Saos-2 (B). The inhibition on the proliferation of prednisone to normal bone cells was lower than that of cisplatin (C). [a]Indicates statistical results compared with the control group; [d]indicates statistical results compared with normal bone cell hFOB1.19 with $p<0.05$; [e]indicates statistical results compared with normal bone cell hFOB1.19 with $p<0.001$; [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$

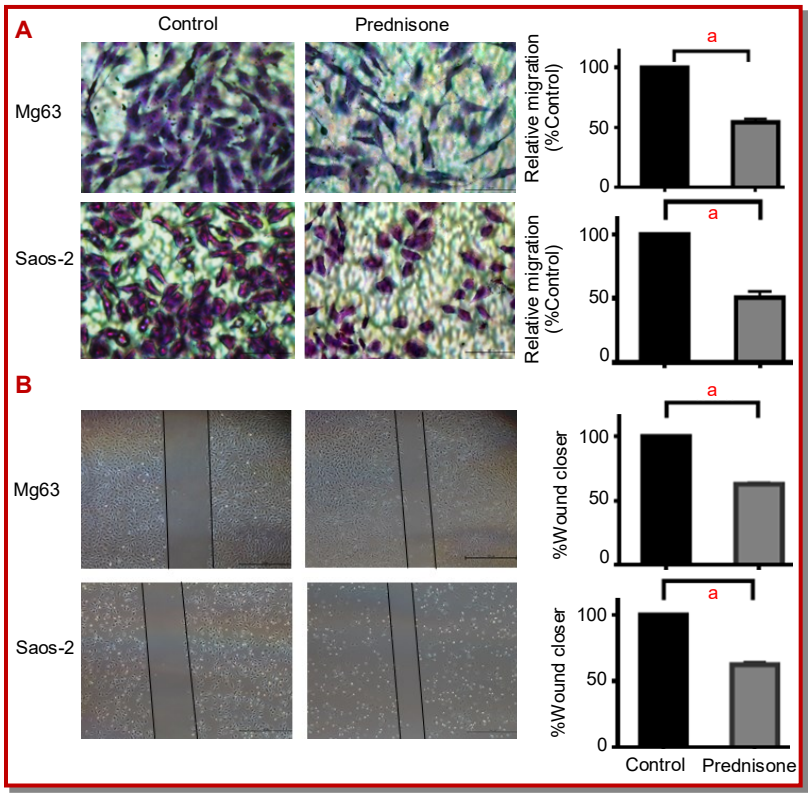

Figure 2: Effects and statistical chart of prednisone on the migration and invasion of osteosarcoma cells. The invasion number of cells was calculated by the average of 3 random horizons and analyzed in the histogram, with a scale of 5 μm (A). The effects and statistics of the migration of MG63 and Saos-2 cells. The unhealed area was measured, and the values were organized and shown in the histogram. The initial wound area was used as a 100% control (B). Scale bars: 100 μm. Data are the mean ± SEM. (n=3) and represented as the fold change. [a]p<0.001

the ability of prednisone to inhibit the proliferation of osteosarcoma cells.

To compare the differences in the inhibitory effects of prednisone and cisplatin on cell proliferation, three types of cells were treated with 20 μg/mL cisplatin or 250 μM prednisone for 48 hours (Figure 1D). The results showed that there was no significant difference in the survival rate of the two osteosarcoma cells treated with 20 μg/mL cisplatin and 250 μM prednisone (both $p>0.05$), while the survival rate of the normal osteocyte hFOB1.19 treated with prednisone was significantly higher than that of cisplatin by 17.5% ($p<0.05$). This indicates that compared to cisplatin, which has a similar inhibitory effect on osteosarcoma cell proliferation, prednisone has significantly lower toxicity in inhibiting the proliferation of normal osteocytes than cisplatin.

Migration of osteosarcoma cells

To investigate the effect of prednisone on the migration of osteosarcoma cells, human Mg63 osteosarcoma cells and human Saos-2 osteosarcoma cells were cultured in 6-well plates, scratched, and cultured in serum-free medium with prednisone treatment for 48 hours (Figure 2A). The migration of Mg63 and Saos-2 cells decreased to 62.6% ($p<0.001$) and -23.9% ($p<0.001$), respectively, compared to the control group, indicating that prednisone treatment significantly inhibited the migration of osteosarcoma cells.

Invasion of osteosarcoma cells

MG63 and Saos-2 osteosarcoma cells were grown in the chamber and treated with prednisone for 48 hours. The results showed that after 48 hours of treatment with prednisone on Mg63 and Saos-2 cells, the number of cells that had crossed the chamber membrane decreased to 54.3% ($p<0.001$) and 50.7% ($p<0.001$), respectively, compared to the control group, indicating that prednisone treatment significantly inhibited the invasion of osteosarcoma cell (Figure 2B).

Apoptosis of osteosarcoma cells

Apoptosis of osteosarcoma cells treated with prednisone for 48 hours was analyzed by flow cytometry (Figure 3A). Compared with the control group, the average apoptosis rate increased by 6.6% ($p<0.001$) and 7.4% ($p<0.001$) after treatment with prednisone in Mg63 and Saos-2 osteosarcoma cells, respectively. The results suggested that prednisone treatment significantly increased the apoptosis in osteosarcoma cells.

Osteosarcoma cell cycle

Flow cytometry was used to analyze the cell cycle in Mg63 and Saos-2 cells after treatment with 250 μM prednisone for 48 hours. As shown in Figure 3B, compared with Mg63 and Saos2 cells without any drug treatment, the number of cells in the G0/G1 phase significantly increased by 33.0% ($p<0.001$) and 17.9% ($p<0.001$), respectively, while the number of cells in the S phase decreased by 29.6% ($p<0.001$) and 18.0% ($p<0.001$), respectively. This indicated that prednisone significantly causes cell cycle arrest in osteosarcoma cells.

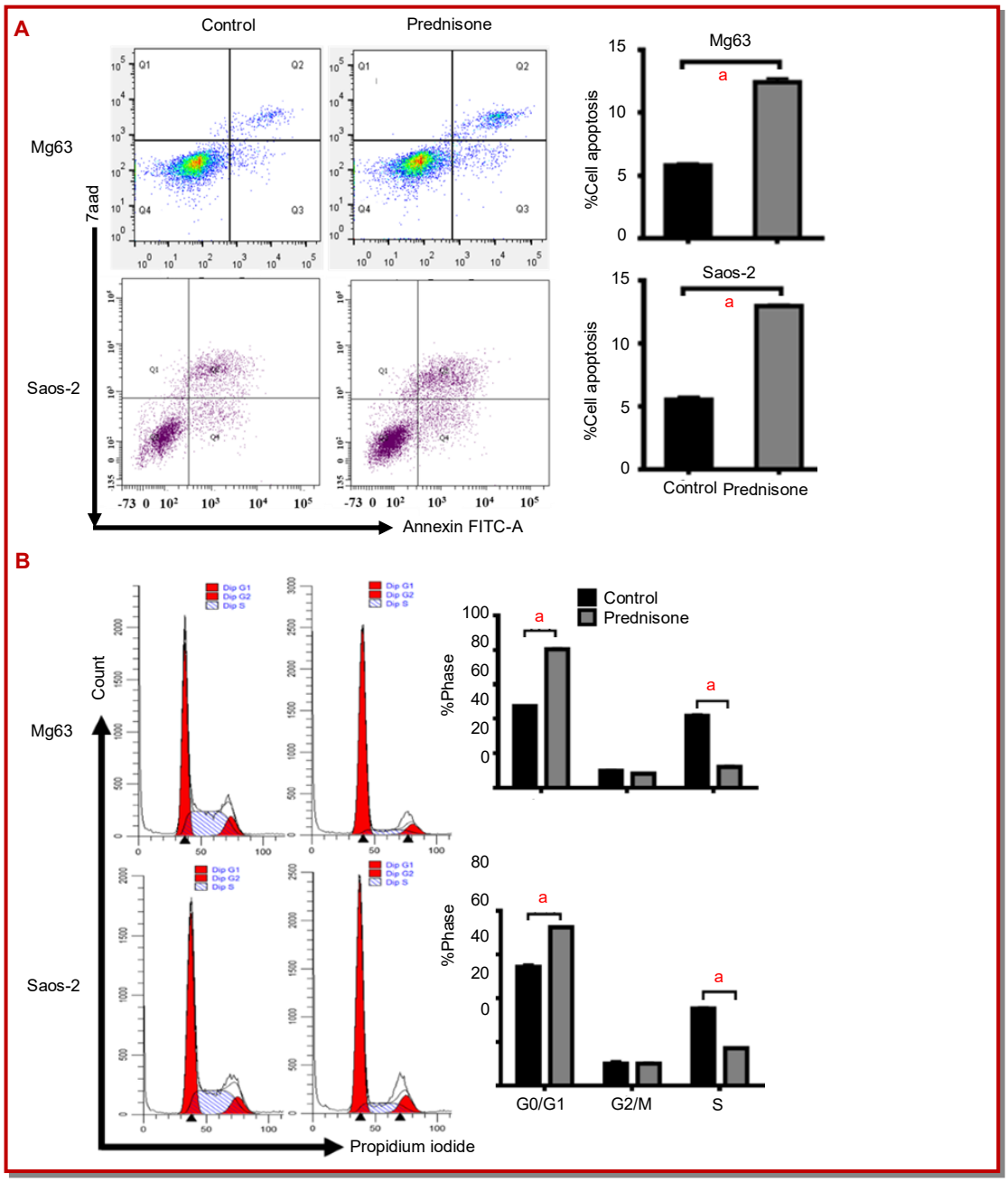

Figure 3: The treatment of prednisone increased apoptosis and cell cycle in osteosarcoma cells. Apoptosis and cell cycle of osteosarcoma cells were detected by flow cytometry (A). Cell cycle of osteosarcoma cells were detected by flow cytometry (B). Data are the mean ± SEM. n=3, [a]p<0.001

Osteosarcoma cells by regulating the Wnt/β-catenin pathway

To explore the molecular mechanism of prednisone in inhibiting osteosarcoma cells, the key proteins of the Wnt/β-catenin signaling pathway were investigated. Osteosarcoma cells Mg63 and Saos-2 were divided into control- and prednisone- (250 μM) treated groups. After treating the cells for 48 hours, total protein was collected for western blot experiment to detect the expression of key proteins β-catenin and proteins related to tumor cell proliferation, migration, and invasion in the Wnt/β-catenin pathway, and statistics were performed on protein bands. Then, the content of MMP7 in the protein lysate was detected using an ELISA kit. As shown in Figure 4, compared with cells without any drug treatment, the protein expression/concentration of cMYC, cyclin D1, β-catenin, and MMP7 significantly decreased in Mg63 and Saos-2 osteosarcoma cells after treating with 250 μM prednisone, indicating that prednisone inhibited the proliferation, migration, and invasion of osteosarcoma cells by

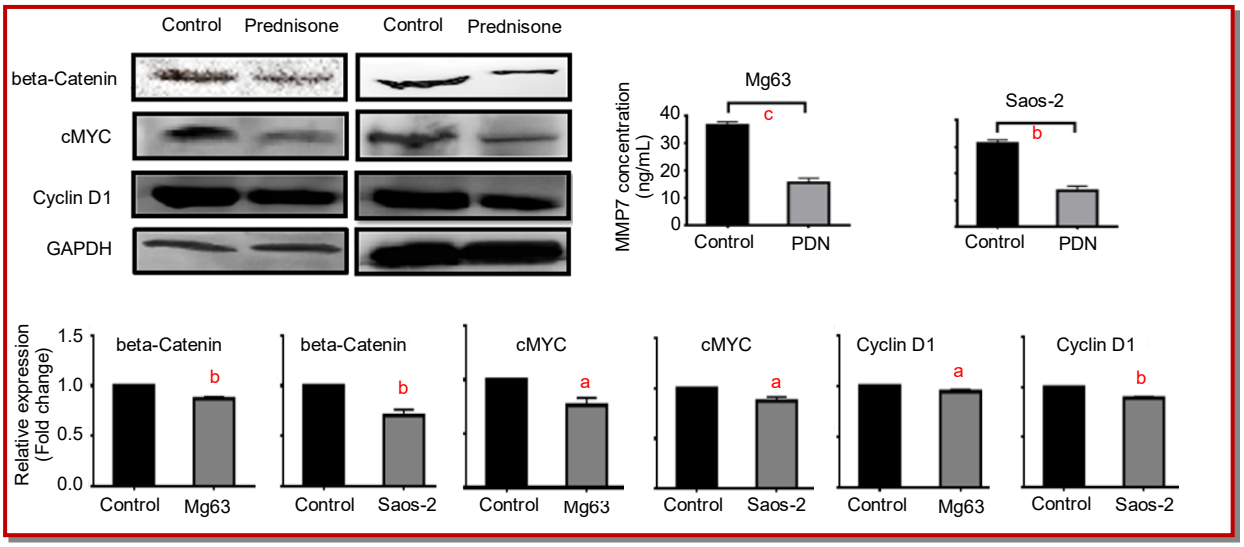

Figure 4: Effects of prednisone (PDN) on proteins of Wnt/β-catenin pathway in Saos-2 and Mg63 cells. The histograms show the quantitative analysis of the samples. Data were measured using the mean ± SEM. The statistical significance of differences between two groups was analyzed using the two-tailed unpaired t test. n=3, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$

inhibiting the Wnt/β-catenin signaling pathway.

Discussion

Literature had documented the inhibitory effects of dexamethasone and hydrocortisone on osteosarcoma cells. For instance, the proliferation of osteosarcoma cells was suppressed to varying degrees upon administering different doses of dexamethasone to Dunn cells (Yamamoto et al., 2002). A significant correlation between the inhibition of osteosarcoma proliferation and the dosage of dexamethasone in male C3H/He mice after 14 days of treatment (Kudawara et al., 2001). Hydrocortisone inhibits osteosarcoma both in vivo and *in vitro* (Zhang et al., 2023). These findings suggest that glucocorticoids may serve as a novel therapeutic agent for osteosarcoma. While hydrocortisone is prone to causing water-sodium retention and edema, dexamethasone are associated with adverse effects such as central obesity, hypertension, and diabetes. Intermediate-acting glucocorticoids (e.g., prednisone) offer a balanced anti-inflammatory and immuno-suppressive effect, making them suitable treating various autoimmune and inflammatory diseases. However, effects of prednisone on osteosarcoma remain unexplored.

To find a new drug for treating osteosarcoma with low side effects, the inhibitory effect of prednisone on osteosarcoma and its molecular mechanism was explored. First, normal bone cells hFOB1.19, osteosarcoma cells Mg63, and osteosarcoma cells Saos-2 were treated with cisplatin or prednisone. The results showed that prednisone successfully inhibited the proliferation of two types of osteosarcoma cells, with similar inhibitory effects to cisplatin but the survival rate of prednisone-treated normal bone cells hFOB1.19 was significantly higher than that of cisplatin. This indicated that prednisone has less impact on the proliferation of normal bone cells than cisplatin, which may help to reduce bone-related complications such as osteoporosis and fractures caused by drug side effects during the treatment of osteosarcoma. In addition, cell experiments also found that prednisone can inhibit the invasion and migration of osteosarcoma cells, which means that it can reduce the spread of tumor cells to tissues and distant organs and reduce the risk of tumor metastasis. Through flow cytometry experiments, it was found that prednisone can induce cell cycle arrest and apoptosis in osteosarcoma cells, which means that prednisone can affect the proliferation rate of tumor cells, thereby limiting tumor volume and disease progression. These results have not been reported before, providing a basis for the pharmacological effects of prednisone in inhibiting osteosarcoma. Moreover, the drug concentration at which prednisone exerts its inhibitory effect on osteosarcoma is lower than that of hydrocortisone (Zhang et al., 2023), potentially providing a broader therapeutic window, reducing treatment costs, and having higher application value. To date, this is the first report to show that prednisone also inhibits osteosarcoma.

The Wnt/β-catenin signaling pathway is a highly conserved pathway in biological evolution, which plays an important role in cell proliferation, differentiation, migration, and apoptosis. β-catenin is an important regulatory protein in the Wnt/β-catenin signaling pathway. Mutations at Ser33, Ser37, and Thr41 sites of β-catenin protein led to higher levels of β-catenin protein in tumor cells than in normal cells, which is observed in many tumor cell lines (Gui et al., 2021; Zhang et al., 2019; Zhang et al., 2024). β-catenin can enter the nucleus

from the cytoplasm to become a transcription factor (Nusse and Clevers, 2017), activating the transcription and expression of several downstream target proteins, such as cMYC, cyclin D1, and MMP7. cMYC is a gene that promote tumor cell proliferation, apoptosis, and division, which is associated with the occurrence and development of various tumors (McMahon, 2014). Cyclin D1 is a protein encoded by the human CCND1 gene that is involved in regulating cycle-dependent kinase (CDKs). Throughout the cell cycle, the abundance of cyclin D1 protein changes periodically (Montalto and De Amicis, 2020; Tchakarska and Sola, 2020). Matrix metalloproteinase 7 is a member of the matrix metalloproteinase (MMPs) family, which is involved in the degradation of extracellular matrix and is associated with tumor cell migration and invasion. This gene exhibits high expression levels in various human cancers (Liu et al., 2020; Nomden et al., 2020). Through western blot experiments, it was found that the expression of β-catenin, cMYC, MMP7, and cyclin D1 proteins in the Wnt/β-catenin signaling pathway in osteosarcoma cells was significantly down-regulated after prednisone treatment, indicating that prednisone can regulate the proliferation, apoptosis, cell cycle, and migration of osteosarcoma cells by regulating the Wnt/β-catenin signaling pathway, providing new ideas for novel drugs with low side effects on osteosarcoma.

Conclusion

Prednisone inhibits osteosarcoma cells by regulating β-catenin, cMYC, MMP7, and cyclin D1, proteins in the Wnt/β-catenin signaling pathway, thereby inhibiting the proliferation, apoptosis, cell cycle, migration, and invasion of osteosarcoma cells.

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Ethical Issue

The immortalized cell lines used in the experiments are not relevant materials, as they have been created outside of the human body. For research purposes, the storage and use of immortalized human cell lines do not require ethical approval.

Conflict of Interest

Authors declare no conflict of interest

Acknowledgements

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