Int J Clin Exp Med 2026;19(2):55-65
www.ijcem.com /ISSN:1940-5901/IJCEM0167884

Original Article

The inhibitory effect of zoledronic acid on prostate cancer cells is significantly less in a three-dimensional polyurethane-based culture system compared to a two-dimensional monolayer culture

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Received August 3, 2025; Accepted January 21, 2026; Epub February 15, 2026; Published February 28, 2026

Abstract: Objective: Conventional two-dimensional (2D) *in vitro* models do not adequately reproduce the structural and functional complexity of the three-dimensional (3D) tumor microenvironment. *In vivo* animal studies, although physiologically relevant, often encounter limitations such as low reproducibility and ethical constraints. In contrast, *in vitro* 3D culture systems have gained increasing attention in cancer research owing to their improved ability to mimic *in vivo* conditions, enhanced reproducibility, and cost-effectiveness. Methods: In this study, we established a 3D prostate cancer model using a polyurethane foam scaffold to evaluate the pharmacodynamic effect of the antitumor agent zoledronic acid. Cell viability and inhibition rates of PC-3 cells were quantified using the CCK-8 assay. The growth patterns and spatial distribution of cells on the scaffold were further examined by scanning electron microscopy (SEM). Results obtained from the 3D model were compared directly with those from conventional 2D cultures. Results: Increasing the concentration of zoledronic acid or prolonging the treatment period produced a dose- and time-dependent inhibition of PC-3 cell proliferation. Across all tested conditions, inhibition rates in the 2D model were significantly higher than those observed in the 3D model. Stated differently, PC-3 cells exhibit greater drug tolerance within the 3D scaffold system. The 3D polyurethane foam-based prostate cancer model demonstrated growth characteristics and drug-response patterns more closely aligned with *in vivo* tumor behavior than traditional 2D cultures. Conclusion: The 3D model provides superior predictive value for assessing anticancer drug efficacy and represents a more reliable platform for pharmacodynamic evaluation compared to conventional 2D culture systems.

Keywords: Polyurethane foam, 3D prostate cancer model, zoledronic acid, drug susceptibility test

Introduction

Prostate cancer (PCa) represents one of the most prevalent male urinary system cancers and is the sixth leading cause of male mortality worldwide [1]. The scientific community has made substantial progress in cancer research but the intricate relationships between cancer cells and their metastatic tissue environment still need further investigation [2]. The most common *in vitro* platforms for studying tumor physiology have used two-dimensional (2D) cell culture models for decades. The research community has used two-dimensional PCa cell line cultures since the beginning, but these models do not properly demonstrate how PCa progresses in patients, while they also lack the diverse genetic and molecular characteristics that most preclinical PCa research requires [3, 4]. The scientific community now understands that 2D models fail to provide accurate drug screening results for tumor drug screening because they lack sufficient complexity [5]. The evaluation of *in vivo* models reveals their usefulness but their complexity makes it difficult to study particular protein functions and cell-cell interactions. Three-dimensional (3D) culture systems have become a focus of research [6, 7]

https://doi.org/10.62347/TBPX8704

during the last few years because they represent human disease conditions more accurately than two-dimensional systems [8, 9]. The creation of 3D *in vitro* systems marks an important achievement because these models effectively duplicate the conditions that exist inside living tumors [10-12]. The development of tissue-engineered 3D models that duplicate tumor distribution patterns enables scientists to study cancer biology better while helping them create new therapeutic approaches [13, 14]. Researchers now use 3D tissue-engineered models because these models provide a better balance than traditional 2D and *in vivo* testing methods [15]. The development of 3D *in vitro* culture systems by tissue engineers and cell biologists during the last twenty years has produced multiple systems that better duplicate how cells behave in living tissues [16]. The systems that contain essential components of tumor extracellular matrix serve as effective tools to study cancer cell invasion capabilities and drug sensitivity [17, 18]. The development of tumor drug susceptibility testing requires scientists to move from 2D to 3D culture systems because this change has become essential for scientific progress [19].

The field has seen significant progress through the creation of three-dimensional scaffolds, which first emerged during tissue engineering research. The scaffolds establish a three-dimensional framework that enables cells to grow properly while maintaining their correct spatial arrangement and cell-to-cell connections. The material polyurethane porous foam demonstrates exceptional properties because it contains a porous structure that combines low weight with strong mechanical properties and extensive surface area [20, 21] that enables optimal cell attachment in three-dimensional cell culture systems [22]. Zoledronic acid serves as a standard treatment for bone metastases in cancer patients because it blocks osteoclast bone destruction and stops bone-related problems that occur with metastasis [23, 24]. Zoledronic acid is used to treat bone diseases. Still, scientific studies show that it directly inhibits tumor cell proliferation and induces apoptosis, making it a promising therapeutic agent for malignant tumors [25-27].

Our study used polyurethane foam to create a 3D prostate cancer model for testing the effectiveness of zoledronic acid. The CCK-8 assay measured PC-3 cell viability to calculate the growth inhibition rate, and scanning electron microscopy examined their scaffold growth patterns. The research team compared their results with data from conventional 2D cell cultures.

Materials and methods

Drugs and reagents

Prostate cancer PC-3 cells (iCell); RPMI-1640 medium (Gibco); Fetal bovine serum (Procell); Penicillin-streptomycin double antibiotic (Beyotime); 48-well cell culture plate (Corning). Polyurethane foam (Guangzhou Peitai Instrument Equipment Co., LTD.). Rat tail collagen Type I (Xinyou Biotechnology Co., LTD., 5 $mg{\cdot}mL^{-1}$) is diluted to 2 $mg{\cdot}mL^{-1}$ with 0.36 $g{\cdot}L^{-1}$ sterile acetic acid on ice surface when used. Zoledronic acid (MACKLIN, 98%) was dissolved in a small amount of complete medium, filtered through a 0.22 μm filter membrane, and then prepared with complete medium for working concentration. Cell Counting Kit-8 (CCK-8 Kit, Beyotime); CO_2 incubator (Thermo BB150), inverted phase contrast fluorescence microscope (IX73, Olympus, Japan), multifunctional enzyme label instrument (Shanghai Pudan Optical Instrument Co., LTD.), Mechanical testing machine (QX-W300, Shanghai Qixiang), Scanning electron microscope (SU8010, Hitachi, Japan).

Polyurethane characterization

According to the literature [28], the water absorption of the polyurethane foam material was measured using the formula: water absorption = (Mb-Ma)/Ma × 100%, where Ma is the dry weight of the sample, and Mb is the weight of the sample at saturation. Compression resistance was evaluated using a tensile testing machine [28], and porosity was determined using Archimedes' principle [29]. The pore size, pore structure, and perforation of the polyurethane foam were observed using both a scanning electron microscope and an inverted microscope.

Polyurethane treatment and surface modification

The polyurethane foam received cylindrical shape processing which produced samples

with 8 mm diameter and 2.5 mm height. The ultrasonic cleaning machine treated these samples through a series of cleaning steps, which included 0.5 mol·L^{-1} hydrochloric acid, followed by distilled water, 0.5 mol·L^{-1} NaOH, and finally distilled water. The samples underwent a 1-hour ethanol disinfection using a 75% ethanol solution, followed by double-distilled water rinsing and air drying. One sample from each group is positioned into separate wells of a 48-well culture plate. 50 μL of a 2 mg·mL^{-1} rat tail type I collagen solution was applied to each sample, then the samples were placed in a 37°C incubator for 20 minutes. The prepared samples were used to conduct cell culture experiments.

Cell suspension preparation

PC-3 prostate cancer cells were routinely cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were harvested in the logarithmic growth phase and suspended to prepare a cell suspension.

Two-dimensional drug susceptibility test

A 3.75 × 10^4 cells·mL^{-1} PC-3 cell suspension was prepared, and 400 μL (1.5 × 10^4 cells/well) was added to each well of a 48-well plate. The cells were cultured for 24 hours before the medium was removed. The experiments were then conducted as follows: (i) Dose-effect experiment: Different concentrations of zoledronic acid (0 [Control], 5, 10, 20, and 40 μg·mL^{-1}) were added to each well (400 μL per well), with triplicate parallel wells per concentration. After 48 hours of incubation, cell growth was observed, followed by CCK-8 detection. (ii) Time-effect experiment: A concentration of 10 μg·mL^{-1} of Zoledronic acid was added at 400 μL per well, and the cells were incubated for 24, 48, and 72 hours, respectively. CCK-8 detection was conducted at each time point, with triplicate parallel wells per condition. The control group was maintained without any drug intervention.

Three-dimensional drug susceptibility test

A PC-3 cell suspension was prepared at a concentration of 5 × 10^5 cells·mL^{-1}. A 30 μL aliquot of the suspension was carefully added to each type I collagen-modified polyurethane foam scaffold and incubated at 37°C for 1 hour. Subsequently, 370 μL of complete medium was added to each well, achieving a final cell density of 1.5 × 10^4 cells/well. The cells were cultured for 24 hours, after which the medium was removed. Dose-effect experiment and time-effect experiment were then conducted as described in the section "Two-dimensional drug susceptibility test". The 3D drug sensitivity assays were performed in five replicate wells per dose, with three wells designated for CCK-8 detection and the remaining two for SEM observation. The cell morphology, growth, and proliferation on the scaffold were examined using scanning electron microscopy following fixation, dehydration, and gold spray coating.

Cell inhibition rate detected by CCK-8

A 48-well plate was used for the 2D/3D drug sensitivity assay. After replacing the medium with fresh culture medium, 40 μL of CCK-8 reaction solution per well was added and incubated at 37°C for 3 hours. 100 μL of sample from each well was transferred to a 96-well plate for OD_{450} measurement by a microplate reader. Each concentration and time point was tested in triplicate. The cell inhibition rate was calculated using formula: Cell inhibition rate = [1 - (experimental group OD value/control group OD value)] × 100%.

Statistical analysis

GraphPad Prism 9 was used in statistical analysis. Data were expressed as mean ± SD, and the comparison between the two groups was conducted using the paired T-test of two independent samples. When $P<0.05$, the difference was considered significant.

Results

The polyurethane foam scaffold material possessed excellent biological properties

The polyurethane foam owns three main properties including water absorption at 715%, elastic modulus at 31.6 kPa and porosity at 82.7%. The pore size spanned between 130 μm and 210 μm while maintaining excellent internal permeability and well-connected spaces which

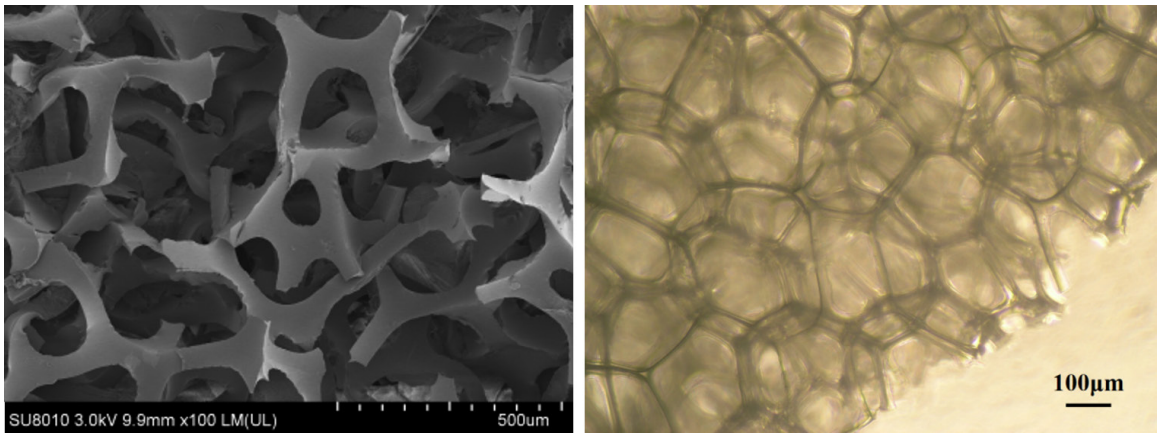

Figure 1. Polyurethane foam possessed excellent biological properties. The left image shows observation of scanning electron microscope (100×, the scale =500 μm), and the right image shows observation of inverted phase contrast microscope (100×, the scale =100 μm). The pore size of this polyurethane foam ranged from 130 to 210 μm, while maintaining excellent internal permeability and well-connected spaces for cell development and drug reaction evaluation.

offring an appropriate microenvironment for cell growth and drug evaluation. Please refer to **Figure 1**.

Zoledronic acid showed a significantly stronger inhibitory effect on PC-3 cells in two-dimensional culture compared to three-dimensional culture

Dose-effect experiment: The treatment of cells with different zoledronic acid concentrations during 48 hours under 2D culture conditions showed that higher concentrations led to decreasing living cell numbers. The PC-3 cells suffered visible transformations including cell rounding, losing their shiny appearance and developing vacuoles before detaching from the surface to float while their intercellular spaces expanded. This indicated that zoledronic acid blocked PC-3 cell growth in a dose-dependent manner. The growth inhibition rate surpassed 50% when the concentration reached ≥10 $\mu g \cdot mL^{-1}$ and cell death became extensive at 40 $\mu g \cdot mL^{-1}$. Please refer to **Figure 2**.

Time-effect experiment: The time-effect experiment showed that zoledronic acid at 10 $\mu g \cdot mL^{-1}$ concentration inhibited 2D-cultured PC-3 cells growth more strongly with each passing day, and PC-3 cells exhibited obvious morphological changes similar to those in the aforementioned 2D dose-effect experiment. This effect was time-dependent. After 48 and 72 hours, the growth inhibition rate exceeded 50%, with substantial cell death recorded at 72 hours. Please refer to **Figure 3**.

Zoledronic acid exhibited a significantly weaker inhibitory effect on PC-3 cells in 3D polyurethane-based culture system compared to 2D culture

Dose-effect experiment: The cell viability of PC-3 cells decreased in a concentration-dependent manner when treated with zoledronic acid for 48 hours under 3D culture conditions while the number of dead cells simultaneously rose. As the concentration of zoledronic acid increased, the PC-3 cells changed from their original spreading, elongated, and adherent growth state to a shortened and rounded shape, losing their intercellular connections and adhesion abilities, detaching from the growth surface, resulting in an expansion of the gap and an increasing number of dead cells. The addition of 10 $\mu g \cdot mL^{-1}$ zoledronic acid resulted in growth inhibition to some extent, and at 20 $\mu g \cdot mL^{-1}$, the inhibition increased. By 40 $\mu g \cdot mL^{-1}$, growth inhibition was significant, with a marked increase in cell death, and the inhibition rate was close to 50%. Please refer to **Figure 4**. Compared to 2D culture, the drug action speed, cell morphology change speed, and cell death speed of the 3D culture model were significantly slower, while the drug resistance was significantly stronger.

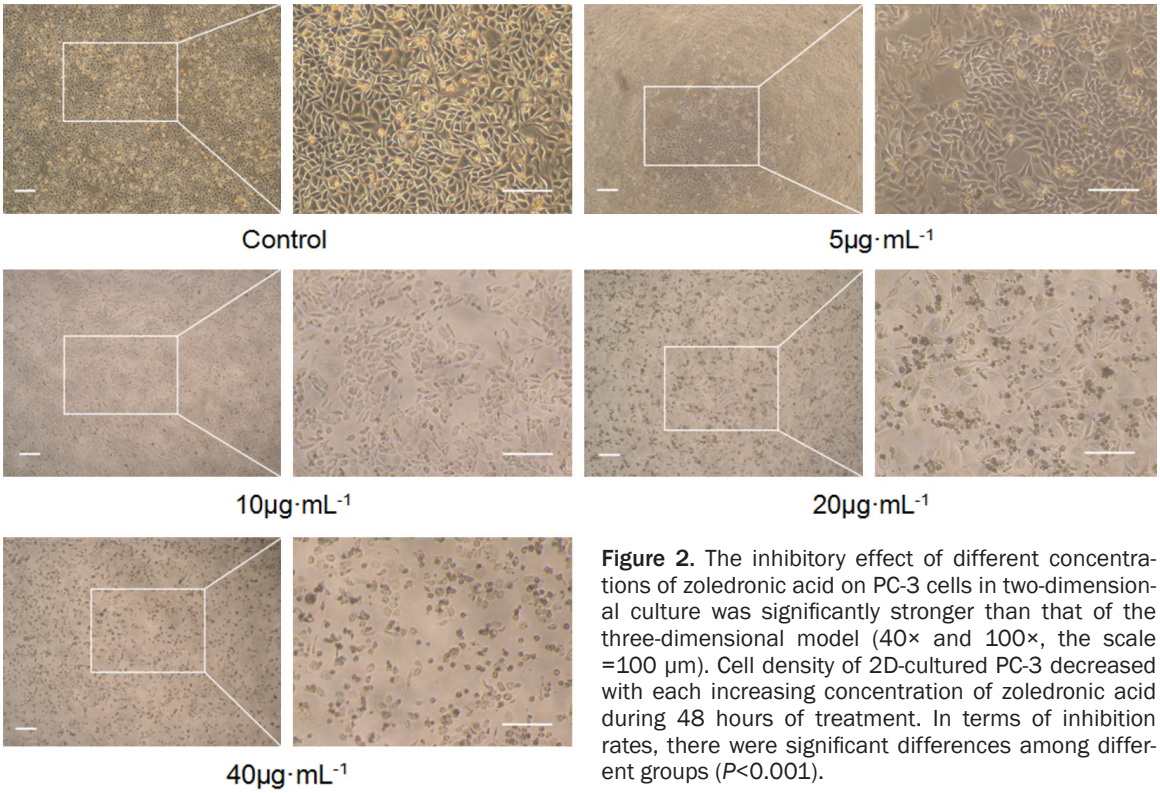

Figure 2. The inhibitory effect of different concentrations of zoledronic acid on PC-3 cells in two-dimensional culture was significantly stronger than that of the three-dimensional model (40× and 100×, the scale =100 μm). Cell density of 2D-cultured PC-3 decreased with each increasing concentration of zoledronic acid during 48 hours of treatment. In terms of inhibition rates, there were significant differences among different groups (P<0.001).

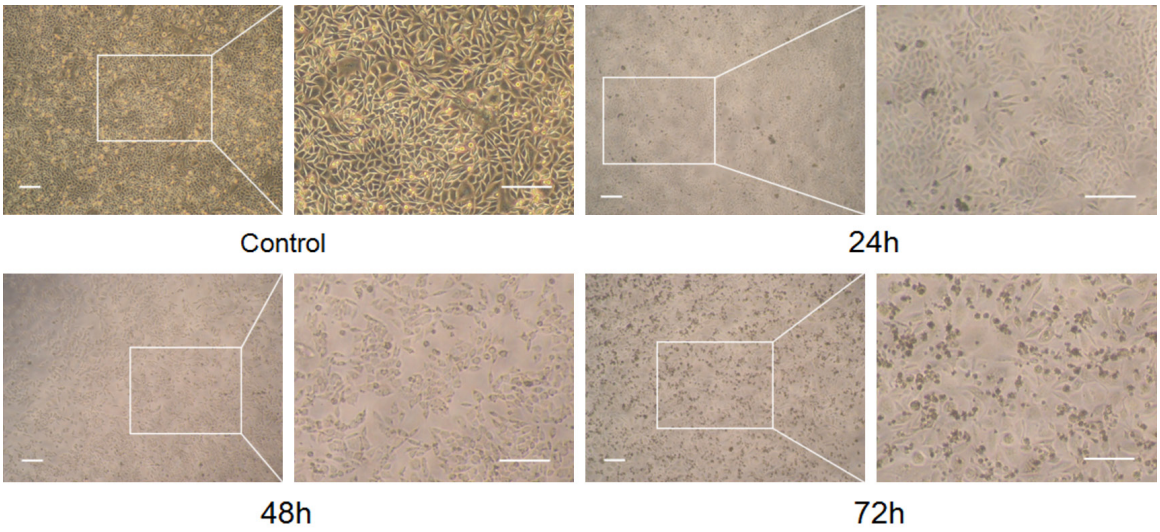

Figure 3. The inhibitory effect of zoledronic acid on PC-3 cells in two-dimensional culture was significantly stronger than that of the three-dimensional model at different times. Inverted microscope observation of the time-effect experiment of PC-3 cells treated with 10 μg·mL^{-1} zoledronic acid at different time points under 2D culture (40× and 100×, the scale =100 μm). With respect to inhibition rates, there were significant differences among different groups (P<0.001).

Time-effect experiment: When treated with 10 μg·mL^{-1} zoledronic acid, the growth of 3D-cultured PC-3 cells was progressively inhibited over time, suggesting that prolonged exposure further suppresses cell proliferation. The PC-3 cells exhibited obvious morphologic changes similar to those observed in the aforementioned 3D dose-effect experiment. However, the inhibition rates at 24, 48, and 72 hours were all below 50%. Please refer to **Figure 5**.

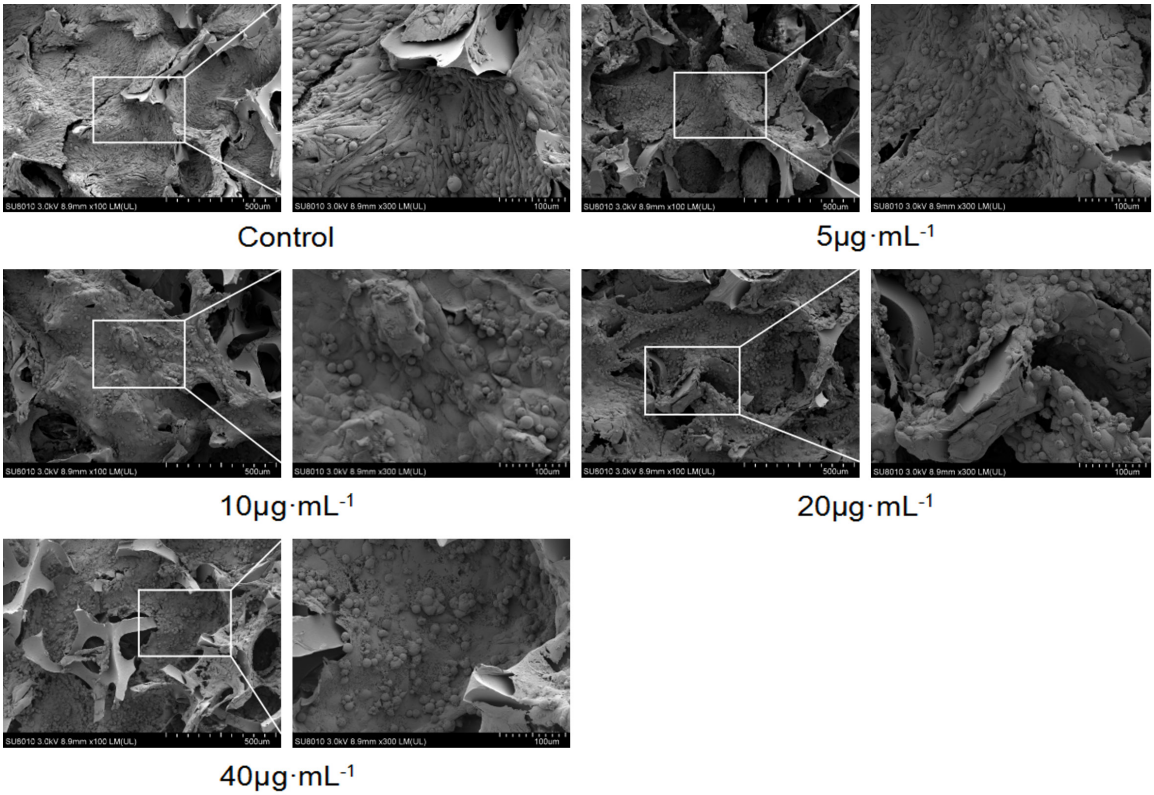

Figure 4. The inhibitory effect of different concentrations of zoledronic acid on PC-3 cells in three-dimensional culture was significantly weaker than that of two-dimensional culture. Scanning electron microscopy observation of the dose-effect experiment on PC-3 cell growth under 3D culture conditions with varying doses of zoledronic acid for 48 hours (100×, the scale =500 μm, and 300×, the scale =100 μm). Following the treatment of PC-3 cells with zoledronic acid for 48 hours, a dose-dependent decrease in cell viability was observed as the concentration of zoledronic acid increased, accompanied by a corresponding increase in the number of dead cells. In terms of inhibition rates, significant differences were observed among different groups (P<0.001).

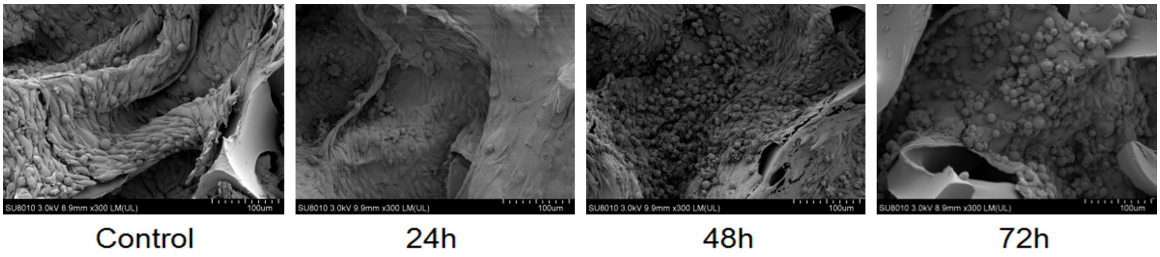

Figure 5. The inhibitory effect of zoledronic acid on PC-3 cells in three-dimensional culture model was significantly weaker than that in two-dimensional culture at different times. Scanning electron microscopy observations of the time-effect experiment of PC-3 cells treated with 10 μg·mL^{-1} zoledronic acid under 3D culture conditions at various time points (300×, the scale =100 μm). In terms of inhibition rate, there were significant differences across the various groups (P<0.001).

The inhibitory rate of zoledronic acid on PC-3 cells in 3D culture system was significantly lower than that in 2D culture

Dose-effect experiment: When PC-3 cells were exposed to increasing concentrations of zoledronic acid for 48 hours under both 2D and 3D culture conditions, growth inhibition rose progressively in a dose-dependent manner. However, the inhibition rate observed in 3D cultures was markedly lower than that in 2D cultures. As the zoledronic acid concentration

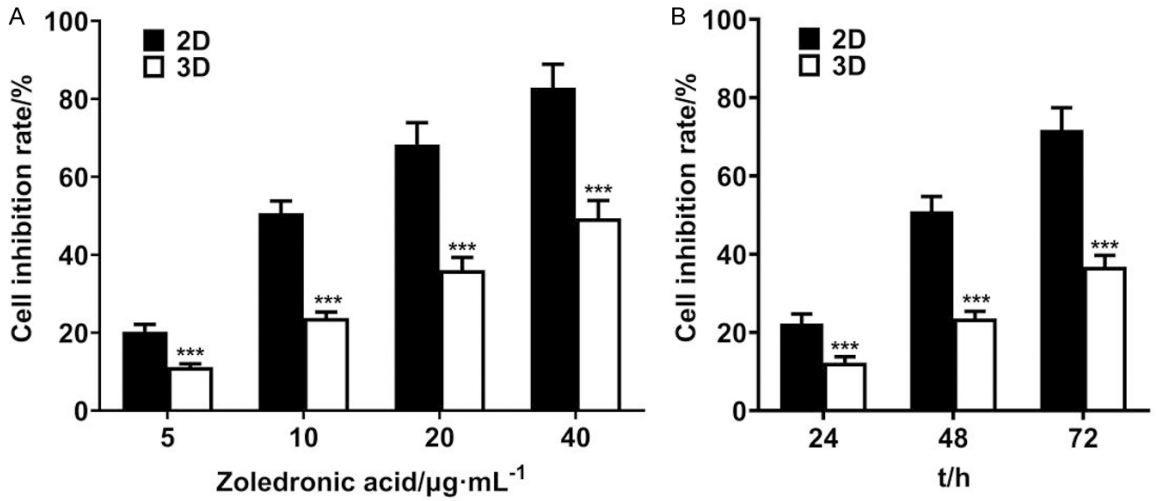

Figure 6. Regardless of the different dosages or at different times, zoledronic acid showed significantly weaker inhibitory effects on PC-3 cells in three-dimensional culture compared to two-dimensional culture (mean ± SD, n=3). A. Dose-effect experiment (t/48 h). When PC-3 cells were treated with varying concentrations of zoledronic acid for 48 hours under both 2D and 3D culture conditions, the inhibition of PC-3 cell growth increased gradually in a dose-dependent manner. However, the inhibition rate in 3D culture was significantly lower than in 2D culture. B. Time-effect experiment (zoledronic acid/10 μg·mL^{-1}). PC-3 cell growth inhibition increased over time under both 2D and 3D conditions. Same higher drug resistance observed in the 3D cell culture model. ***Compared to 2D culture, P<0.001.

increased, the number of viable PC-3 cells declined while the number of dead cells increased. Notably, the inhibition rate in 3D cultures remained significantly reduced compared to 2D cultures, indicating substantially greater drug resistance in the 3D system. Please refer to **Figure 6A**.

Time-effect experiment: When the concentration of zoledronic acid was 10 μg·mL^{-1}, PC-3 cell growth inhibition increased over time under both 2D and 3D conditions. However, the inhibition rate in 3D cultures was again significantly lower than in 2D cultures, confirming the higher drug resistance observed in the 3D cell culture model. Please refer to **Figure 6B**.

Discussion

Prostate cancer (PCa) is one of the leading cancers affecting men and there are no known treatments for its advanced stages [30]. The current standard treatments for PCa include chemotherapy, immunotherapy, radiation therapy, and endocrine therapy [31]. Although early diagnosis and treatment have shown promising success, managing advanced and metastatic PCa remains a significant challenge [32], which highlights a pressing need for innovative approaches to elucidate tumor growth and metastatic progression [33]. Conventional *in vitro* assessments of anticancer effects are typically performed using 2D monolayer cultures and subsequently validated in *in vivo* animal models. However, the complex cell-cell communication and cell-matrix interactions that play essential roles in cancer metastasis are absent in 2D systems. Their lack of spatial depth, limited cell connectivity, and inability to replicate the tumor microenvironment often lead to results that are not reproducible *in vivo*. Consequently, results from 2D studies might provide unreliable information which does not accurately forecast clinical outcomes effectively [31]. Animal models serve as valuable tools, but they come with high costs and are time-consuming, and biologically different from human systems. Thus, developing 3D *in vitro* models that mimic the complex structural and physiologic environment of tumors is essential for advancing cancer research [34]. These models connect 2D cell cultures to animal models and provide scientists with a better method to study cancer biology and develop new cancer treatments [19]. In this study, polyurethane foam was used to construct a 3D PCa model. Drug sensitivity tests with zoledronic acid demonstrated significantly lower sensitivity in

3D-cultured PC-3 cells compared to 2D cultures, reflecting more realistic *in vivo* drug responses and better predictability of therapeutic efficacy. Experiments using this 3D model yielded more reliable data, suggesting it may eventually replace extensive *in vivo* validation.

Polyurethane foam is an ideal scaffold material for 3D models due to its excellent biocompatibility and mechanical properties [35, 36]. Its open-cell structure and interconnected pores provide ample space for cell attachment and biologic activities. The uniform and continuous pore network enhances cell interactions, nutrient exchange, and waste elimination, creating an optimal environment for cell growth [37]. The foam's high water absorption further supports substance exchange and cell proliferation [38, 39]. The study demonstrated that the polyurethane porous foam used in this experiment exhibits excellent biocompatibility, mechanical stability, and favorable porosity, making it a suitable 3D cell culture carrier. This biomimetic scaffold holds significant potential for *in vitro* high-throughput cancer research, particularly for drug discovery and treatment screening [40].

Zoledronic acid is a third-generation bisphosphonate and an anti-resorptive agent. It has been approved for the treatment of bone complications associated with metastatic breast cancer and prostate cancer. Zoledronic acid not only targets tumor cells but also enhances apoptosis and inhibits proliferation, migration, and invasion [41, 42]. This effect is significantly amplified when combined with chemotherapy drugs [43, 44]. Notably, it demonstrates particular efficacy for suppressing the growth of breast tumors [45].

The findings of this study reveal that, under 2D culture conditions, in the dose-effect experiment (t/48 h), when the concentration of zoledronic acid reached 10 $\mu g \cdot mL^{-1}$, the inhibition rate of prostate cancer PC-3 cell growth exceeded 50%. However, under 3D culture conditions, when the concentration of zoledronic acid was =40 $\mu g \cdot mL^{-1}$, the inhibition rate of PC-3 cell growth was approximately 50%. In the time-effect experiment (zoledronic acid/10 $\mu g \cdot mL^{-1}$), after 48 and 72 hours under 2D culture conditions, the growth inhibition rate of PC-3 cells exceeded 50%, with substantial cell death observed at 72 hours. However, the inhibition rate under 3D culture conditions was significantly lower than that under 2D conditions, confirming the 3D culture model's higher drug resistance. Statistical analysis indicated that, compared to 2D culture conditions, the sensitivity of prostate cancer PC-3 cells to different concentrations of zoledronic acid under 3D culture conditions was significantly reduced ($P< 0.001$). This suggests that tumor cells cultured under 3D conditions exhibit markedly increased drug resistance.

This phenomenon can be attributed to the dense fibrous structure formed by most PC-3 cells cultured on the polyurethane-based 3D model, with a minority forming three-dimensional spheroids. The three-dimensional structure limits drug penetration into the interior, resulting in insufficient contact and extremely low intracellular drug concentrations, thereby hindering drug efficacy. In contrast, monolayer tumor cells cultured under 2D conditions are more likely to achieve complete contact with the drug. These findings indicate that a dense structure reduces drug diffusion, promotes cell survival, and induces drug resistance, aligning with the research results of Comito et al. [46]. Drug resistance in tumor cells poses major challenges for chemotherapy efficiency, thereby shortening patient survival. The three-dimensional *in vitro* prostate cancer culture model using polyurethane scaffolds developed in this study shows better resistance to zoledronic acid than two-dimensional cell cultures while providing a more realistic environment for drug-tumor interactions, which suggest that this 3D model could serve as a robust *in vitro* screening platform for anti-tumor drugs, offering valuable theoretical insight for drug development and clinical applications. Further investigation into the underlying mechanisms is warranted.

Conclusion

Experimental assessment of the anticancer drug zoledronic acid revealed that the 3D tumor model exhibited greater drug tolerance than traditional 2D cultures. The construction of an *in vitro* 3D PCa model became possible using polyurethane scaffolds, which allowed tumor cells to develop drug resistance and become more malignant. Given its promising

predictive accuracy, the 3D model seems more suitable for representing actual drug responses that occur in living organisms. This makes the 3D model model suitable for drug susceptibility testing as well as a promising platform for *in vitro* drug screening, providing theoretical guidance for drug development and clinical applications. However, further studies are needed to clarify the underlying process.

Acknowledgements

This research was supported by Basic Scientific Research Funds of Department of Education of Zhejiang Province (KYYB202103), and Zhejiang Provincial Traditional Chinese Medicine Science and Technology Plan Project of China (2023ZL358).

Disclosure of conflict of interest

None.

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