2023 • Vol. 1, No. 1 • pp. 37-42
*Future Dental Research (FDR)*

Print ISSN: 3006-0516
Online ISSN: 3006-0354
NABEA AL-AJYAL FOUNDATION PRESS
Future Dental Research (FDR)

# Enhancing Osteoblast Function Through Resveratrol-Loaded Methoxy Poly (Ethylene Glycol)-Polylactic Acid Nanoparticles: A Novel Approach for Osteogenic Therapy

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Access this article online

**ORIGINAL ARTICLE**
**Received:** 06.07.2023 **Revised:** 07.08.2023
**Accepted:** 13.08.2023 **DOI:** 10.57238/fdr.2023.145193.1002

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## ABSTRACT

The primary objective of this investigation was to assess the influence of resveratrol-loaded polymer nanoparticles (RSV-NPs) on osteoblast proliferation and differentiation, aiming to enhance the osteogenic properties of RSV and establish a practical drug delivery system for clinical bone and dental regeneration. The findings revealed that osteoblast proliferation was hindered in the RSV-NPs group, exhibiting more potent inhibition compared to the free RSV group. Both groups displayed increased ALP activity, with a notably greater rise in the RSV-NPs group than in the RSV group. Additionally, the expression of ALP, OCN, and BMP-2 mRNA was upregulated in both groups, demonstrating a more marked increase in the RSV-NPs group in comparison to the regular RSV group. In summary, the developed nanoparticles effectively improved the solubility of RSV and heightened its impact on osteoblast proliferation and differentiation. This innovative approach holds potential for targeted bone and dental tissue treatment, opening up new avenues for research in osteoporosis therapies.

***Keywords:*** Resveratrol, Nanoparticles, Osteoblast proliferation, Osteoblast differentiation

## 1 Introduction

Recently, the field of regenerative medicine has witnessed noteworthy advancements, particularly in the context of dental applications [1,2]. The quest for innovative strategies to promote tissue regeneration, especially within the intricate milieu of oral structures, has spurred a wave of research aimed at harnessing the therapeutic potential of bioactive compounds [3,4]. Resveratrol (RSV), a natural polyphenol found in foods like grapes and red wine, has demonstrated potential in influencing bone regeneration by affecting osteoblast proliferation and differentiation [5–7]. Studies suggest that RSV can promote osteoblast differentiation, encouraging mesenchymal stem cells to develop into mature bone-forming cells [8]. RSV's antioxidant and anti-inflammatory properties also play a role in protecting osteoblasts from oxidative stress and inflammation, factors that can hinder bone formation. Additionally, this compound can modulate various signaling pathways involved in bone metabolism, potentially leading to enhanced bone regeneration [9]. However, the use of RSV in clinical applications faces several limitations, including poor oral bioavailability, uncertain dosing and safety profiles, variability in individual responses, and a need for more robust clinical evidence [10–12]. To address these challenges, researchers have explored the encapsulation of RSV within nanoparticles (NPs). This approach offers the potential to enhance the compound's bioavailability and stability. When RSV is loaded into NPs, it can be protected from degradation in the digestive system, enabling controlled release and potentially targeted delivery to specific tissues or cells.

The aim of this study is to investigate the potential of methoxy poly (ethylene glycol)-polylactic acid (MePEG-PLA) NPs as a delivery system for RSV and evaluate their effectiveness in inhibiting MG-63 osteoblasts while pro-

 

moting the differentiation of these cells. By encapsulating RSV within MePEG-PLA NPs, we aim to overcome the limitations of RSV's bioavailability and stability, potentially enhancing its therapeutic impact on bone health. This research seeks to contribute to our understanding of how nanotechnology can improve the clinical applicability of RSV in bone regeneration and provide valuable insights into its effects on osteoblasts, crucial for advancing strategies for bone tissue engineering and regeneration.

## 2 Materials and Methods

### 2.1 Materials

The materials used in this study were RSV, polyvinyl alcohol (PVA), Mw = 31,000, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA), dimethyl sulfoxide (DMSO) (Sigma, USA), MePEG-PLA (Shandong Dagang Biotechnology Co., Ltd., China), an ultrafiltration centrifuge tube (Millipore, USA), an alkaline phosphatase (ALP) kit (Nanjing Jiancheng Bioengineering Institute, China).

### 2.2 Preparation of RSV-NPs

The RSV-NPs were synthesized using a modified spontaneous emulsifying solvent diffusion (SESD) technique. To create the organic phase, 90 mg of MePEG-PLA and a specific amount of RES were dissolved in 3 mL of acetone-ethanol (with a 3:2 volume ratio of acetone to absolute ethanol) at room temperature. The resulting solution was slowly introduced, under magnetic stirring, into a 30 mL aqueous solution containing 0.2% Tween 80. Following rotary distillation under reduced pressure for 30 minutes, residual acetone and ethanol were eliminated. The NP solution underwent centrifugation in an ultrafiltration centrifuge tube for 30 minutes to eliminate any remaining Tween 80. The NPs were then resuspended in double-distilled water, subjected to three washes, and filtered through a 0.22-$\mu$m filter membrane to yield a transparent liquid with a faint blue opalescence.

### 2.3 Osteoblast cultue

The frozen MG-63 cell line was thawed and placed in 25 mL culture flasks with DMEM containing 10% FBS at a density of 1 × 106 cells/ml. These flasks were then kept in an environment at 37 °C with 5% CO2 and high humidity. After 24 hours, the culture medium was replaced to eliminate cells that didn't adhere to the flask surface. Subsequently, the culture medium was changed every 48-72 hours. The growth and proliferation of the non-adherent cells were monitored and recorded daily. When the cells had almost completely covered the flask surface, they were rinsed with PBS and treated with 0.25% trypsin for detachment. Once the cells showed signs of detachment under an inverted microscope, a full culture medium was added to stop the trypsin treatment. The MG-63 cells that were released through trypsin digestion were collected and then subcultured at the desired cell density.

### 2.4 The effect of RSV-NPs on cell proliferation

For the MG-63 cell culture experiment, cells in the logarithmic growth phase were utilized. Initially, trypsin was applied for cell digestion. After cell counting, the cell concentration was set at 1 × 105 cells per ml, and subsequently, 1×104 cells were seeded into each well of 96-well plates, with 100 $\mu$L of cells per well. Following a 24-hour incubation period, the culture medium in each well was replaced, and the experimental groups were treated with culture medium containing RSV-NPs at a concentration of 100 $\mu$M RSV. In addition, each of the 6 wells received 100 $\mu$M RSV, and blank NPs. Another 6 wells were assigned as a blank control group. All these wells were designated for cell collection at the 72-hour time point. To assess cell viability, a 10-$\mu$L MTT detection solution was added, and incubation continued for 4 hours at 37 °C in a 5% CO2 incubator. The optical density (OD) value was measured at a wavelength of 570 nm using a microplate reader, and the results were compared with those obtained from the blank control.

### 2.5 Alkaline phosphatase activity

In the MG-63 cell culture experiment, cells in the logarithmic growth phase were utilized. Initially, trypsin was employed for cell digestion, and subsequent cell counting led to the adjustment of the cell concentration to 1 × 105 cells per ml. These cells were then seeded into 24-well plates, with 1 mL of cell suspension per well. After a 24-hour incubation period, the culture medium in each well was aspirated. In four experimental groups, we introduced RSV-NPs containing 100 $\mu$M RSV, RSV at a concentration of 100 $\mu$M, and blank NPs, each into six wells. Additionally, six wells were designated as the blank control group. Following a 72-hour incubation, the solution from each culture well was aspirated, and cells were washed twice with PBS. Subsequently, cell digestion was performed, followed by two additional PBS washes and two rounds of centrifugation (at 800–1000 rpm/min for 5 min). Following this, 200 $\mu$L of 0.1% Triton-X-100 was added to each well and left overnight at 4 °C. Cells were disrupted using an ultrasonic cell disruptor through five cycles of 5 seconds each at 4-second intervals. After centrifugation at 6000 rpm for 10 min at 4 °C, the supernatant was collected. The OD value was measured at a wavelength of 520 nm using a microplate reader, following the ALP assay kit procedure, and the results were compared with those obtained from the blank control group.

### 2.6 ALP, OCN, and BMP-2 mRNA levels

MG-63 cells in the logarithmic growth phase were cultured, trypsinized, and subsequently transferred to 75 cm2 culture flasks. Following a 24-hour incubation, the culture medium was removed, and four experimental groups, each comprising six wells, were established. These groups consisted of RSV-NPs with 100 $\mu$M RSV, RSV at a concentration of 100 $\mu$M, and blank NPs. An additional six wells were allocated for the blank control group. At the 72-hour time point, cells were harvested, and total cellular RNA was extracted. Finally, a real-time polymerase chain reaction (RT-PCR) was conducted to assess the mRNA expression of ALP, osteocalcin (OCN), and bone morphogenetic protein-2 (BMP-2).

### 2.7 Statistical methods

In each of the experimental trials, we examined a minimum of three samples, and we conducted at least three replications of the experiments. The results were presented as means along with their respective standard deviations. Statistical significance was indicated by asterisks, with * signifying a significance level of $p < 0.05$. Data analysis in this study employed either one-way or two-way analysis of variance (ANOVA) using GraphPad Prism 8.0.

## 3 Results

This research established a technique to investigate how RSV-NPs influence the proliferation and differentiation of osteoblasts. To achieve this, we utilized an MTT assay, enzyme-linked immunosorbent assay, and RT-PCR. By comparing the results of the RSV-NP group to those of the RSV group, blank NPs group, and control group, we assessed the impact of RSV-NPs on osteoblast proliferation and differentiation.

### 3.1 Morphology and size of the RSV-NPs

The appearance of RSV-NPs was examined using transmission electron microscopy (TEM), as shown in Fig.1a. The polymer NPs that were produced had a spherical shape, displayed consistent and smooth distribution, and maintained regular morphology without any adhesion or clustering. These RSV-NPs had a particle size of 37.50 nm, as depicted in Fig.1b, along with a uniform polymer dispersion index of 0.187 and an electric potential of -13.20 mV.

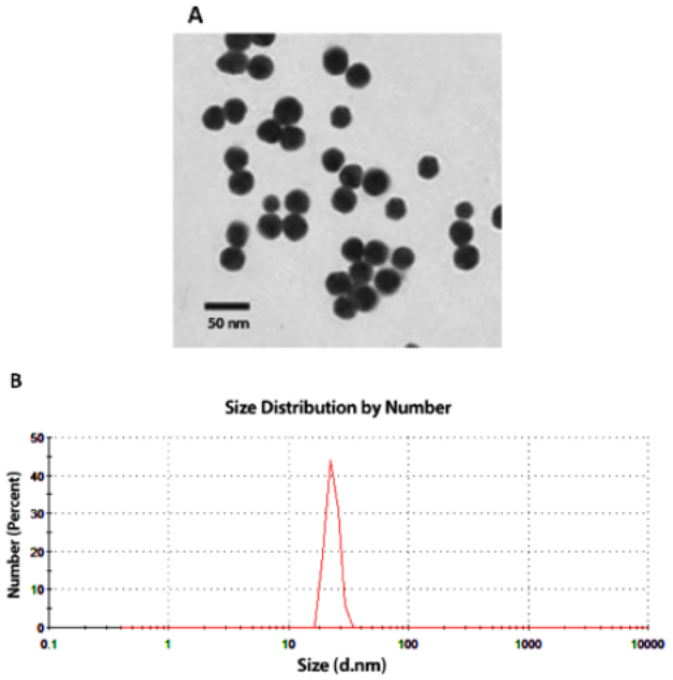


Fig. 1. (a) The transmission electron microscopy of RSV-NPs; (b) The particle size of RSV-NPs.

### 3.2 The effect of RSV-NPs on cell viability

The viability of MG-63 cells treated with NPs, RSV and RSV-NPs were evaluated after 72 hours through MTT test. According to Fig.2, it was found that RSV have no significant effect on the survival of the cells compared to the control group and the group treated with NPs, while the RSV-NPs significantly reduced the survival and proliferation of the cells compared to the other groups ($P < 0.05$).

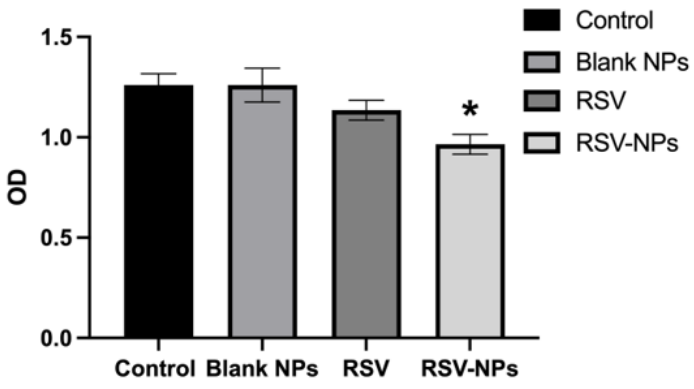


Fig. 2. MTT assay for evaluation of viability and proliferation of MG-63 cells treated with NPs, RSV and RSV-NPs. Error bars represent means $\pm$ SD ($n = 4$). *$p < 0.05$ vs control, NPs and RSV.

### 3.3 ALP activity

We assessed the influence of RSV-NPs on ALP activity through the ALP activity assay. No statistically significant differences were noted in pairwise comparisons between the control and blank NP groups.

However, a statistically significant difference ($P < 0.05$) was observed in comparisons between these two groups and the RSV and RSV-NP groups. Additionally, there was a statistically significant difference in the comparison between the RSV and RSV-NP groups ($P < 0.05$), as illustrated in detail in Fig.3.

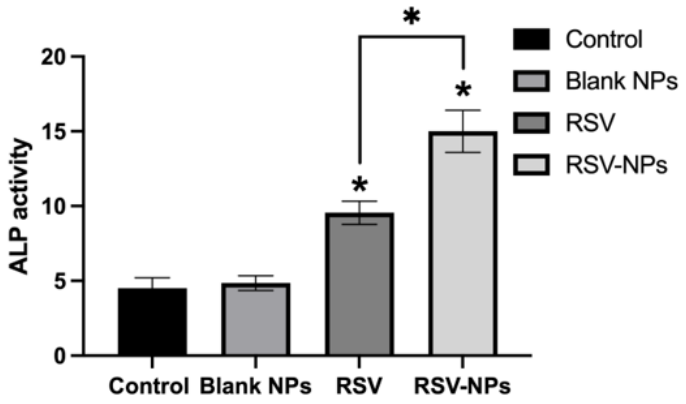


Fig. 3. Effects of each group on the ALP activity of osteoblasts. Error bars represent means ± SD ($n = 4$). *p $< 0.05$.

### 3.4 ALP, BMP-2 and OCN experssion levels

The mRNA expression of BMP ligands, such as BMP-2, plays a crucial role in osteoblast differentiation. Additionally, ALP serves as a non-specific phosphomonoesterase, which is known to effectively hydrolyze inorganic pyrophosphate, thus promoting hydroxyapatite formation.

Hence, ALP activity serves as a common early indicator for gauging osteoblast differentiation. Conversely, OCN acts as an osteoblast hormone, playing a role in binding to calcium ions and influencing bone mineralization and turnover.

OCN is employed as a biomarker to assess bone formation. As depicted in Fig.4, the mRNA levels of ALP, BMP-2, and OCN in cells treated with RSV-NPs were markedly elevated compared to cells treated with RSV alone ($P < 0.05$).

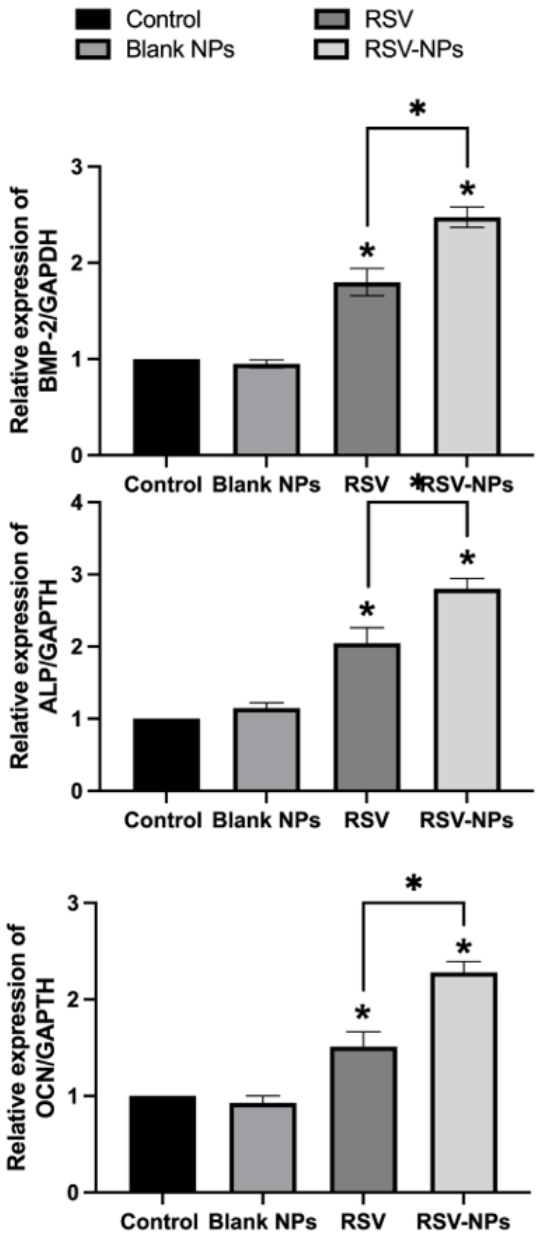


Fig. 4. Effects of each group on the mRNA expression of ALP, BMP2 and OCN in osteoblasts. Error bars represent means ± SD ($n = 4$). *p $< 0.05$.

## 4 Discussion

The utilization of nanotechnology in drug development has been an ongoing endeavor. With advancements in science and technology, the integration of nanotechnology in pharmaceutical research and development has grown progressively sophisticated [13–15]. The researchers anticipate that the future focus of nanotechnology will revolve around enhancing nanomedical delivery systems and exploring innovative drug carriers.

One significant benefit of our present investigation is the elimination of toxic dichloromethane in the process of

preparing nanoparticles. This not only mitigates the risk of toxic solvent residues but also contributes to a reduction in energy consumption. The study results demonstrate that the devised method is an effective approach for creating polymer NPs for hydrophobic drugs.

Recent investigations have delved into the potential of RSV-loaded NPs to induce osteoblast differentiation into bone cells. One study revealed that RSV-loaded NPs can boost osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) by increasing the expression of osteogenic genes [1]. Another study showed that RSV-loaded PLGA NPs can enhance the in vitro proliferation and differentiation of osteoblasts while promoting bone formation in vivo [2]. These findings indicate the promise of RSV-loaded NPs as a therapeutic strategy for bone-related diseases. In line with the results from other researchers, the current authors found that RSV-NPs enhance drug efficacy when used as drug carriers.

The present study delved into the impact of RSV-NPs on MG-63 cell proliferation and differentiation. The MTT assay revealed that RSV hindered osteoblast proliferation, with RSV-NPs exhibiting a stronger inhibitory effect on proliferation compared to RSV alone. An examination of ALP activity showed that the blank NP groups had no influence on ALP activity in the cells. In contrast, the RSV group significantly increased ALP activity compared to the blank control group, while RSV-NPs further amplified this effect, suggesting that RSV boosts osteoblast ALP activity, and RSV-NPs do so to a greater extent.

To investigate the influence of RSV on bone differentiation, the research assessed the mRNA expression levels of BMP-2, ALP, and OCN. Findings revealed that the DMSO and blank nanoparticle groups exhibited no impact on the mRNA expression levels of these genes. In contrast, the RSV group notably elevated the mRNA expression levels of BMP-2, ALP, and OCN in cells, and RSV-NPs amplified this effect, indicating that RSV enhances the mRNA expression of osteoblast BMP-2, ALP, and OCN, with RSV-NPs exhibiting a more pronounced effect. In the capacity of drug carriers, the polymer nanoparticles NPs augmented the efficacy of RSV and its osteogenic characteristics. The size of the formulated RSV-NPs was approximately $37 \pm 50$ nm, closely mirroring the dimensions of viruses, lipoproteins, and human tissue units. This size facilitated the NPs in navigating diverse physiological barriers, thereby enhancing oral bioavailability. The hydrophilic surface layer of the NPs prevented immunoglobulin adsorption and recognition by the body's immune system, allowing them to circulate in the bloodstream for prolonged periods and reducing the need for frequent drug administration.

In instances of fractures or osteomyelitis, the NPs could optimize drug efficacy and minimize toxic side effects through targeted delivery to inflamed tissues, ensuring improved penetration and retention. With a size below 100 nm, these NPs are well-suited for precise delivery to bone marrow. Consequently, RSV-NPs exhibit significant potential for applications in osteoporosis, fractures, osteomyelitis, as well as guided bone and dental regeneration [16–19].

## 5 Conclusions

In this research, we effectively utilized the modified-SESD method to prepare RSV-NPs, leading to a substantial enhancement in the water solubility of RSV. Our investigation, comparing RSV-NPs to RSV, and blank nanoparticles, revealed that RSV-NPs were capable of restraining the proliferation and differentiation of osteoblasts. Moreover, employing polymer NPs as a carrier proved to significantly improve the effectiveness of RSV.

**Conflict of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
**Financing:** The study was performed without external funding.
**Ethical consideration:** The study was approved by Islamic Azad University.
**Data Availability:** No data was used for the research described in the article.

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How to cite this article

Afshari H.; Hussain A.A.; AL-Maliki Z.K.S.; Enhancing Osteoblast Function Through Resveratrol-Loaded Methoxy Poly (Ethylene Glycol)-Polylactic Acid Nanoparticles: A Novel Approach for Osteogenic Therapy. Future Dental Research (FDR). 2023;1(1):37-42. doi: 10.57238/fdr.2023.145193.1002



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