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Anticancer effect of novel platinum nanocomposite beads on oral squamous cell carcinoma cells

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Abstract: Nanoparticles are used in industry and medicine, because of their physiochemical properties, such as size, charge, large surface area and surface reactivity. Recently, metal nanoparticles were reported to show cell toxicity on cancer cells. In this study, we focused novel platinum nanoparticles-conjugated latex beads (P2VPs), platinum nanocomposite (PtNCP) beads, and investigated the possibility to incorporate novel anti-cancer effect of these combined nanoparticles. Oral squamous cell carcinoma cell lines, HSC-3-M3 cells were injected subcutaneously into the back of nude mice to produce a xenograft model. PtNCP beads were injected locally and examined by measuring tumor volume and comparing pathological histology. PtNCP beads treatment suppressed tumor growth and identified increasing pathological necrotic areas, *in vivo*. PtNCP beads inhibited the cell viability of HSC-3-M3 cells in dose-dependent manner and induced the cytotoxicity with extracellular LDH value, in vitro. Furthermore, SEM images were morphologically observed in PtNCP beads-treated HSC-3-M3 cells. The aggregation of the PtNCP beads on the cell membrane, the destructions of the cell membrane and globular structures were observed in the SEM image. Our results indicated that a potential anti-cancer effect of the PtNCP beads, suggesting the possibility as a therapeutic tool for cancer cell-targeted therapy. © 2019 Wiley Periodicals, Inc. J Biomed Mater Res Part B: 00B: 000–000, 2019.

Key Words: Oral squamous cell carcinoma, platinum nanoparticles, anticancer effect, cell death

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INTRODUCTION

Conventional oral squamous cell carcinoma treatment includes surgery, radiotherapy, and chemotherapy. Treatment of oral cancers is generally surgery followed by radiotherapy or chemotherapy[1]. However, the 5-year survival rate of oral squamous cell carcinoma remains at 40–60%, despite rapid advances in therapeutic technologies and extensive research[2,3]. In addition, as a result of invasiveness of these treatments, significant functional side effects to surrounding normal tissues are occurred with lowering the patient's quality of life[4]. Therefore, development of therapies targeting for squamous cell carcinoma is required, and various researches on novel cancer therapeutic methods are progressing.

Nanomaterials have been developed as innovative materials in the industrial fields of electronics, cosmetics, and medicine. Some reports have suggested that various types of nanomaterials, such as carbon nanotubes, titanium dioxide and silica, exhibit harmful biological effects[5–8]. In addition, other reports have shown that the characteristics of nanoparticles, such as size and surface features, can affect their biological and pathological actions.[9,10]

Platinum nanoparticles have been utilized in cosmetics manufacturing applications. On the other hand, they were reported to be inflammatory inducer in mice and compromising the integrity of DNA in human colon carcinoma cell line.[11,12] Platinum-based agents, such as oxaliplatin, were induced for the elucidation of mechanisms of tumor resistance to platinum

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drugs, *cis*-diamminedichloroplatinum (II) (cisplatin).[13] Qi et al.[14] demonstrated that tumor hypoxia promotes oral squamous cell carcinoma resistance to the treatment of cisplatin. Currently, clinical combination studies were developed using platinum drugs with resistance modulators and new molecularly targeted drugs. Our previous study developed *in vitro* delivery and in vivo transfection systems for bleomycin and the cdtB gene into oral squamous cell carcinoma using sonoporation.[15] In addition, we also have developed anti-epidermal growth factor receptor antibody-conjugate microbubbles and evaluated their capacity to enhance anti-cancer drug cytotoxicity *in vitro* and *in vivo.*[16]

In this study, we evaluated the anticancer effect of the novel platinum nanocomposite beads (PtNCP beads) developed by New Material Development Center in Nippon Steel & Sumikin Chemical Co., Ltd. *in vivo* and *in vitro.* It was demonstrated that PtNCP beads dramatically showed cytotoxicity in HSC-3-M3 cells, and tumor growth inhibition in HSC-3-M3 xenografts, with extending histopathological necrosis lesion.

MATERIALS AND METHODS

Cell lines and culture conditions

HSC-3-M3, human oral squamous carcinoma cell lines were maintained in Eagle's Minimum Essential Medium (EMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (CORNING, NY, USA), penicillin G potassium (100 U/mL, Nacalai Tesque, Kyoto, Japan) and streptomycin sulfate (100 mg) (Nacalai Tesque) at 37°C under 5% CO_2 conditions.

Reagents

The latex beads (P2VPs) and PtNCP beads were provided by New Material Development Center in Nippon Steel & Sumikin Chemical Co., Ltd (Chiba, Japan). The PtNCP beads nuclei are composed of P2VPs and the surroundings are covered with platinum. For preparation of P2VPs decorated with platinum, the aqueous dispersion of P2VPs, 3.0 wt % 1.5 g, was mixed with H_2PtCl_6 aqueous solution, 40 m*M*, 5.0 mL at room temperature for 3 h. After removing the free metal ions from these mixtures, these mixtures were redisposed in deionized water. Dimethylamine borane (DMAB) aqueous solution (13 m*M*, 14 mL) was mixed with the aqueous dispersion of precursor particles (0.073 wt %, 48.2 g). And then, PtNCP beads were dialysis using deionized water.[17] The size distribution of PtNCP beads did not change for 8 months. PtNCP beads have long term dispersion stability. In this study, P2VPs and the PtNCP beads were used at 5 mg/mL and at 10 mg/mL in vivo experiments (the dose of PtNCP beads and P2VPs was determined according to the report by Matsumura et al.[17] and *in vitro* experiments Figure 3).

Viability and cytotoxicity tests

Cell counting kit-8 (CCK-8) (Dojindo Laboratories Co., Kumamoto, Japan) was conducted to evaluate the effect of the PtNCP beads on HSC-3-M3 cell viability. HSC-3-M3 cells were collected by trypsinization, washed twice in phosphate-buffered saline (PBS: pH 7.2) and resuspended at 2.0×10^4 cells/ml in a 96-well plate. These cells were cultured at 37°C under 5% CO_2 for 24 h. Subsequently, the supernatant was aspirated, changed to a new medium with PtNCP beads (0, 6.25, 12.5, 25, 50, and 100 μg/mL), and cultured for 48 h under the same conditions. After that, CCK-8 assay were performed according to the manufacturer's recommended protocol and absorbance was measured at 450 nm. Next, cell cytotoxicity was tested by cytotoxicity lactate dehydrogenase (LDH) assay kit (Dojindo laboratories Co.). Culture and the PtNCP beads pretreatment in the LDH assay were performed under the same conditions as in the CCK-8 assay. The assay was performed with the recovered supernatant according to the manufacture's protocol and the absorbance was measured at 490 nm.

Scanning electron microscope analysis

For preparing scanning electron microscope (SEM) specimens, HSC-3-M3 cells were seeded at 3×10^5 cells with 5% FBS (+) EMEM into 35 mm dish (Asahi Glass Co., Tokyo, Japan) containing φ 14 mm plastic sheet (Wako). Thereafter, the PtNCP beads (4.0×10^3 beads/cell) was suspended and cultured at 37°C under 5% CO_2 for 48 h. After completion of the culture, the supernatant was aspirated, washed twice with PBS and prefixed with 2% glutaraldehyde fixed solution for 1 h. Washed twice with 0.1 *M* sucrose/0.1 *M* phosphate buffer and postfixed with 1% osmium. Dehydration with 20–100% ethanol and substitution with t-butanol were carried out. After lyophilization, platinum deposition was carried out to prepare a sample of SEM. SEM observation was carried out with S-3300 N (HITACHI, Tokyo, Japan) operated with an acceleration voltage of 10 kV.

Animals and tumor xenograft model

A male of 6-week-old KSN/slc mice (body weight: 20–25 g) were obtained from SLC (Shizuoka, Japan). All mice were fed with standard feed and water, and individually reared in plastic cages in a monitored environment (temperature 22 ± 1°C, humidity 50 ± 5%, 12 h light/12 h dark cycle). HSC-3-M3 cells (5.0×10^6 cells/200 μL in serum-free EMEM) were injected into the back of mice. No significant weight loss was observed in all mice. This animal experiment was approved by the Kyushu Dental University Experimental Animal Care and Use Committee (Permit number: 17-018).

Measurement of tumor volume

In order to comprehend the influence on the tumor by administration of the PtNCP beads, beads corresponding to the experimental group were administered to the tumor, and then the volume was measured to evaluate. Tumor xenograft model mice were randomly divided into three groups, the untreated control group (n = 6), the P2VPs-treated group (5 mg/mL) (n = 6) and the PtNCP beads-treated group (10 mg/mL) (n = 6). Each bead was administered at 50 μL/day/1 tumor on day 3, 4, and 5. The long and short diameters of a tumor and the body weight were measured once every 2 or 3 days, and the tumor volume was calculated by the following formula: volume (mm^3) = a (mm) × b^2 (mm^2)/2, where a and b are long and short diameters, respectively measured with a digital caliper.[15,18]

Histological staining

After the observation period has ended, mice were sacrificed by cervical dislocation, the tumor was resected. The resected tumor was fixed in 10% formalin for 48 h, and then embedded in paraffin. The paraffin blocks were sliced in the vertical direction to the epithelium (4 μm thick sections), they were stained with hematoxylin and eosin for histopathological analysis.

Statistical analysis

All data were expressed by mean value and ± standard deviations (SDs) using Excel and analyzed by one-way ANOVA test by Tukey method.

RESULTS

Antitumor activity of PtNCP beads in subcutaneous HSC-3-M3 xenografts models

In initial experiments, we examined the effect of PtNCP beads on tumor growth in KSN/slc nude mice model, *in vivo.* Equal numbers of HSC-3-M3 cells (5.0×10^6 cells) were injected in three groups of mice. One group received the cells alone, and other with 0.75 μg P2VPs or 1.5 μg PtNCP beads. Figure 1 shows tumor images (A) and tumor growth curves (B) in the untreated group, the P2VPs group and the PtNCP beads group. HSC-3-M3 cells were injected into back of KSN/slc nude mice. After 2 days of inoculation, established subcutaneous tumor was visible (volume, 23–26 mm^3). And then, mice were treated with P2VPs or PtNCP beads for three times once every 1 day. At 14 days sacrifice, the mean tumor volumes in HSC-3-M3 cells xenografts were 91.38 ± 19.71 mm^3 (long diameter: 7.25 ± 1.13 mm, short diameter: 5.02 ± 0.79 mm) in PtNCP beads treatment group, 217.89 ± 18.84 mm^3 (long diameter: 8.27 ± 0.37 mm, short diameter: 7.25 ± 0.37 mm) in P2VPs treatment group, and 206.18 ± 37.89 mm^3 (long diameter: 9.64 ± 2.26 mm, short diameter 6.61 ± 0.61 mm) in untreated group, respectively. Tumor growth and tumor volume in PtNCP beads-treated group was significantly different in P2VPs-treated group ($^*p < 0.05$). The PtNCP beads inhibition rate on tumor volume was 44.32% compared to untreated group and 41.94% compared to P2VPs treated group. Tumors in each group were analyzed histologically by hematoxylin and eosin staining. Necrotic areas were often observed in the PtNCP beads-treated group (Fig. 2(G), (H), and (I)), compared to the untreated group (Fig. 2(A), (B), and (C)) and the P2VPs-treated group (Fig. 2(D), (E), and (F)). When the necrosis area was magnified and observed (Fig. 2(I), ×400), transplanted tumor cells existed in the surroundings, and some of them had pyknosis and disappeared cell nuclei, and they had necrosis. Mouse neutrophil infiltration was also observed around the necrotic cells (Fig. 2(I), ×400).

In vitro cell viability of HSC-3-M3 cells after PtNCP beads treatment

We examined the effects of PtNCP beads treatment on cell viability in HSC-3-M3 cells. The 50 and 100 μg/mL PtNCP beads caused a decrease in cell viability compared to untreated HSC-3-M3 cells at 48 h ($^*p < 0.005$)(Fig. 3(A)). P2VPs treatment had no effect on cell viability (Fig. 3(B)). LDH assay revealed that cytotoxicity in HSC-3-M3 cells remarkably increased at concentrations above 50 μg/mL the PtNCP beads compared to untreated HSC-3-M3 cells ($^*p < 0.005$ and $^{**}p < 0.001$). In addition, we confirmed an increase of the extracellular LDH level in HSC-3-M3 cells depending on the concentration of the PtNCP beads (Fig. 3(C)).

Cell damage of HSC-3-M3 cells induced by PtNCP beads treatment

We examined cell shape and membrane surface morphology in PtNCP beads-treated HSC-3-M3 cells utilizing SEM. The membrane surface of HSC-3-M3 cells exhibited extensions of the plasma membrane in untreated HSC-3-M3 cell (Fig. 4 (A)). As shown in Figure 4(B), the PtNCP beads aggregated around cell membrane. The cytoskeleton became unclear and the cell size expanded in PtNCP beads-treated HSC-3-M3 cells. The cell membrane was destroyed in which the PtNCP beads are aggregated, with numerous small and variable sized globular structures or blebs appearing at the cell surface.

DISCUSSION

Metal nanoparticles, such as gold, silver and platinum nanoparticles, are being used in a wide array of applications that reach far beyond therapeutics. Gold nanoparticles enhanced radiosensitization in mouse tumor model. Glucose and PEG-coated gold nanoparticles were shown to effectively enhance uptake and radiotherapy in breast adenocarcinoma cells, *in vitro* and *in vivo.*[19] Silver nanoparticles have been widely used as the therapeutic agents in anti-inflammatory or wound healing.[20] Platinum nanoparticles have been shown to possess the capacity to enter human colon carcinoma cells,[21] and compromised DNA integrity and decrease cellular glutathione,[12] leading to the hypothesis that platinum ions from nanoparticles could be the cause. The platinum-based compounds cisplatin and carboplatin belong to the group of most frequently used anti-cancer drugs in clinical practice. However, their use is limited by severe organ toxicity. In this study, we used platinum nanoparticles-coated P2VPs, PtNCP beads, and investigated the effect of PtNCP beads on anticancer activity against oral squamous cell carcinoma cells.

As shown to Figures 1 and 2, we performed in vivo experiments using subcutaneously xenografting mice with human squamous cell carcinoma cells, HSC-3-M3 cells. PtNCP beads treatment against mouse xenograft decreased the tumor volume compared to P2VPs treatment with histological necrotic lesion. Previous study reported that platinum nanoparticles (500–2000 mg/kg) have suppressive effect on tumor growth,[22,23] similar with our results. PtNCP beads were suggested that Pt nanoparticles are formed not only on the surface of P2VP particles but also inside P2VP cores.[17] Further experiments will be needed to investigate that the inhibitory effect of PtNCP beads on tumor growth by the surface and inside platinum nanoparticles.

Based on *in vivo* results, we performed in vitro experiments using HSC-3-M3 cells to investigate the tumor growth inhibitory effect of PtNCP beads. There is some evidence that nanoparticle

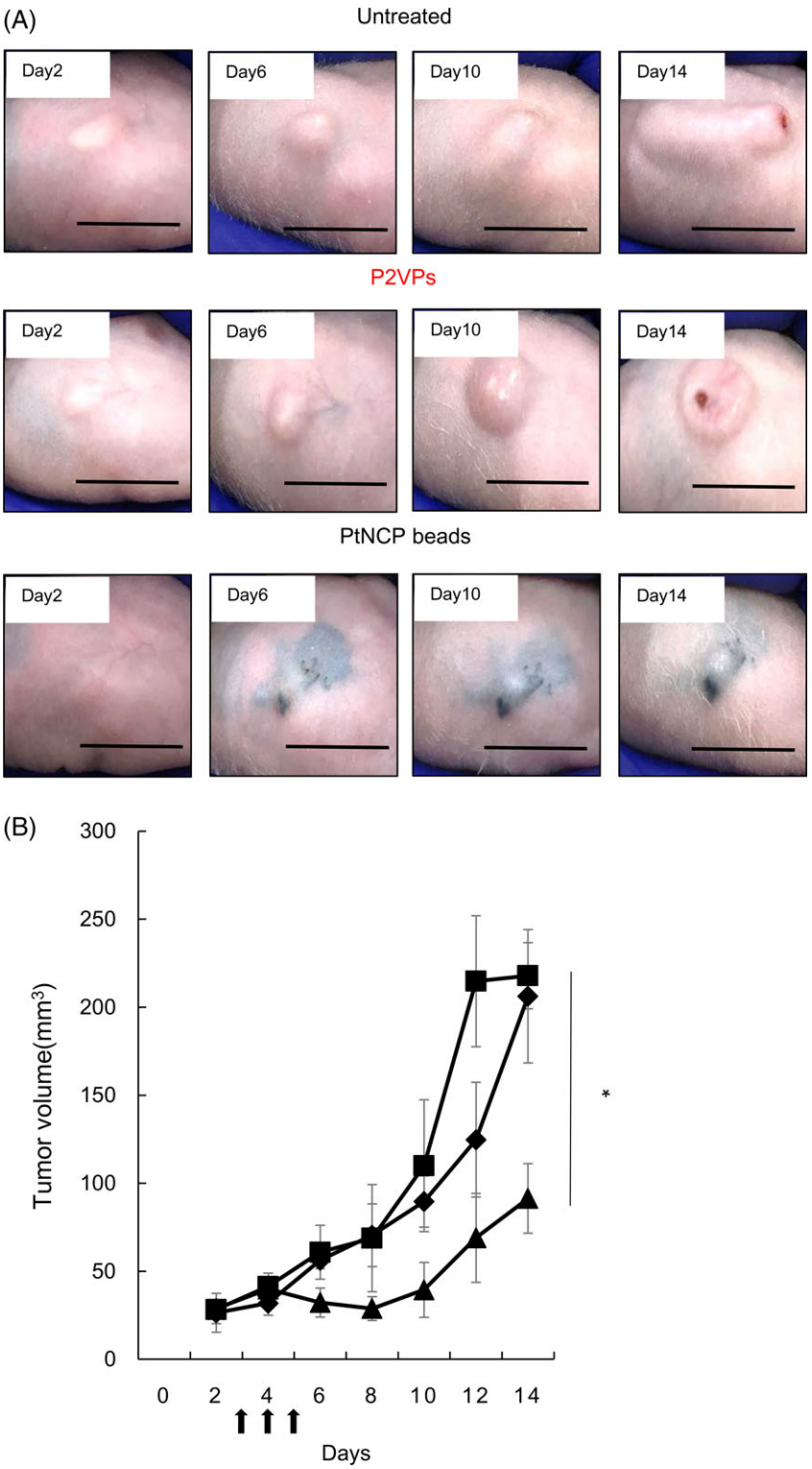

FIGURE 1. Effect of PtNCP beads on tumor growth in HSC-3-M3 cell xenografts. HSC-3-M3 cells were injected into back of KSN/slc nude mice. After 2 days of inoculation, mice were treated with 5 mg/mL P2VPs or 10 mg/mL PtNCP beads for three times once every 1 day. All mice were divided into three experimental groups: control (n = 6), P2VPs beads (n = 6), and PtNCP beads (n = 6). (A) Images of xenograft at the back of mice on three experiment groups. Bar =10 mm. (B) Growth inhibition of HSC-3-M3 cells in KSN/slc nude mice by PtNCP beads treatment. Arrows indicate the injection days (Day 3, 4, and 5) of P2VPs or PtNCP beads. Closed square: P2VPs treatment. Closed diamond: non treatment. Closed triangle: PtNCP beads treatment. Data were expressed as mean ± SDs of three independent experiments made in three replicates. Data were analyzed by one-way ANOVA test followed by Tukey's test. *$p < 0.05$.

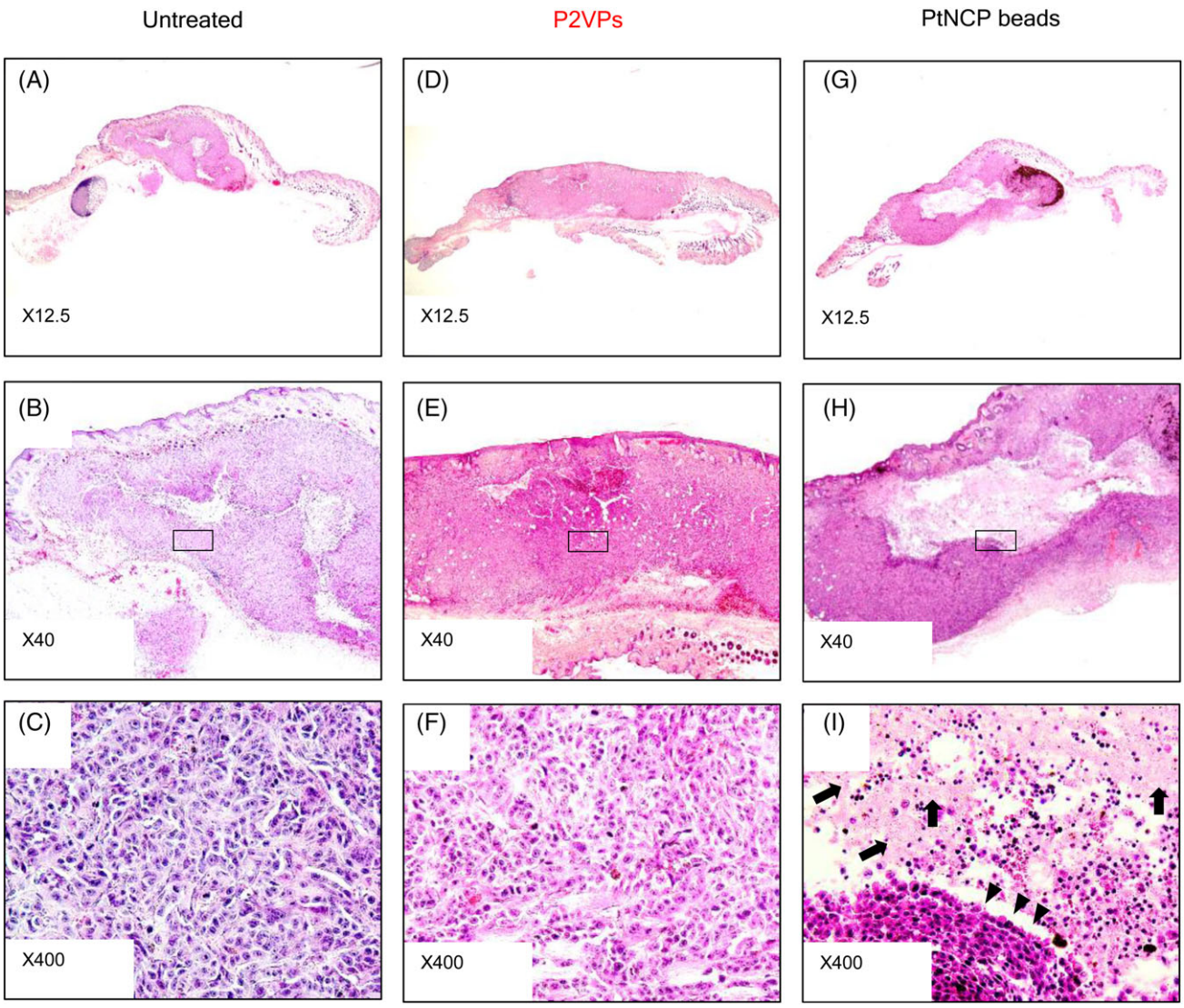

FIGURE 2. Histological analysis of xenograft tumors. Hematoxylin & Eosin staining on each group; untreated group (A), P2VPs treated group (B) and PtNCP beads treated group (C) (Magnification beginning at the top, ×12.5, ×40, and ×400). Black squares indicate enlarged areas. Arrows point to necrotic carcinoma cells. Arrowhead points to normal carcinoma cells.

pass through cell membranes and interact with cellular structures, and then have a direct impact on cell viability.[24,25] Bendale et al.[26] also have reported the cytotoxicity of platinum nanoparticles in ovarian, lung and pancreatic cancer cell lines. In contrast, no significant cytotoxic effect was observed in the normal human peripheral blood mononucleocyte.[27] In agreement with our in vivo results, the results of WST-8 assay and LDH assay revealed the suppression of cell proliferation and the extracellular leakage of intracellular LDH in PtNCP beads-treated HSC-3-M3 cells (Fig. 3). LDH is a soluble cytoplasmic enzyme that is present in cells and is released into extracellular space when the cell plasma membrane is damaged.[26] Necrotic cell death is evaluated by determining damage of the plasma membrane. In contrast to apoptosis, necrosis was once considered as a passive and accidental form of cell death. However, recent studies have clearly established that necrosis can be a programmed event, called necroptosis.[28] The SEM image (Fig. 4) in HSC-3-M3 cells suggested the possibility of cell membrane destruction by PtNCP beads. In addition, PtNCP beads treatment induced cytotoxicity with extracellular LDH release in HSC-3-M3 cells, *in vitro.* These results suggested that cell membrane destruction by PtNCP beads treatment may strongly be supported the induction of necroptosis.

Since platinum nanoparticles have been reported to induce apoptosis signaling,[28,29] we also investigated the relationship between inhibitory effect on cell proliferation and cell death induction in PtNCP beads-treated HSC-3-M3 cells. Although a necrotic rather than an apoptotic mechanism of killing is suggested, the detail by which PtNCP beads had cytotoxicity to oral squamous cell carcinoma cells is still not fully understood. To better understand the cell death mechanism, we confirmed apoptotic mechanism, such as caspase-3, −8, −9, and p53 in PtNCP beads-treated HSC-3-M3 cells. However, there was no marked difference in apoptotic

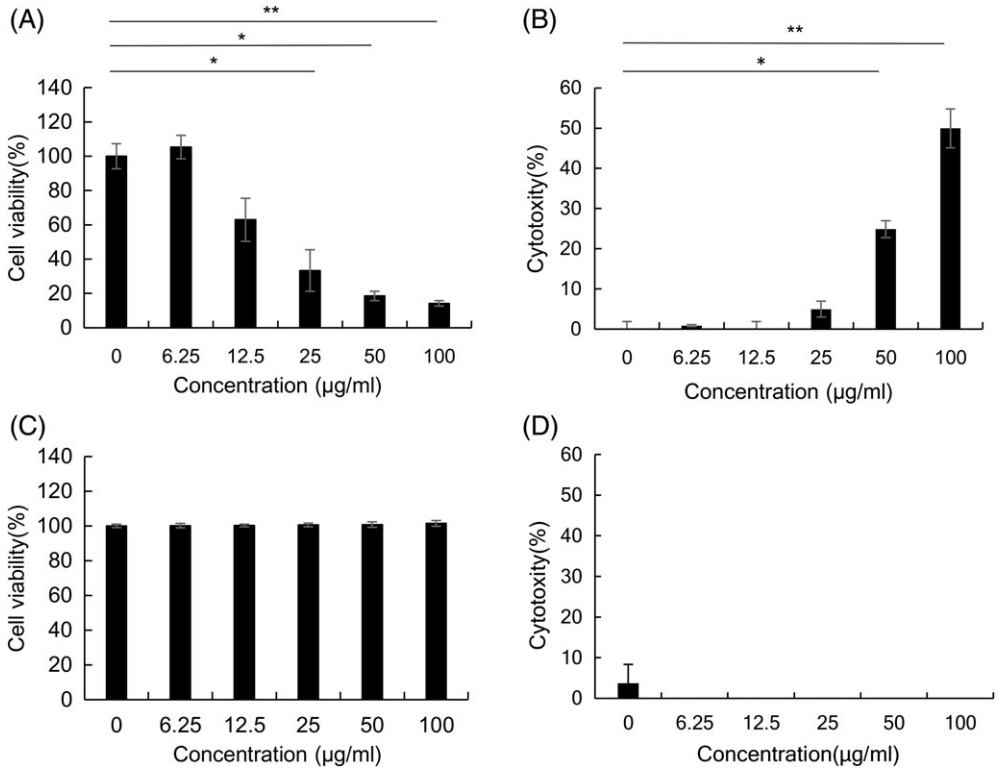

FIGURE 3. Effect of PtNCP beads on cell viability and cytotoxicity of HSC-3-M3 cells. HSC-3-M3 cells were treated with P2VPs and PtNCP beads for 48 h. (A, B) Cell viability of HSC-3-M3 cells with PtNCP beads treatment (A) ($n = 3$, $^{*}p < 0.005$ vs. untreated group) and P2VPs treatment (B) was measured using CCK-8 assay. (C, D) Cell cytotoxicity of HSC-3-M3 cells with PtNCP beads treatment (C) ($n = 3$,$^{*}p < 0.005$, and $^{**}p < 0.001$ vs. untreated group) and P2VPs treatment (D) treatment was measured using LDH assay. Data were expressed as mean ± SDs of three independent experiments made in three replicates. Data were analyzed by one-way ANOVA test followed by Tukey's test.

protein expression level under PtNCP beads treatment (data not shown). Considering expansion of cell morphology and leakage of LDH in SEM, other cell death will be suggested, such as necroptosis. In the future, it is necessary to further investigation of signal induction for several cell death types in PtNCP beads treatment.

In conclusion, PtNCP beads exhibited cytotoxic activity against oral squamous cell carcinoma cells with necrotic cell death. The antitumor efficacy of PtNCP beads for treating oral squamous cell carcinoma in xenograft mouse lends support for its use in various traditional systems of medicine. PtNCP beads can also be expected to be used as a drug transport

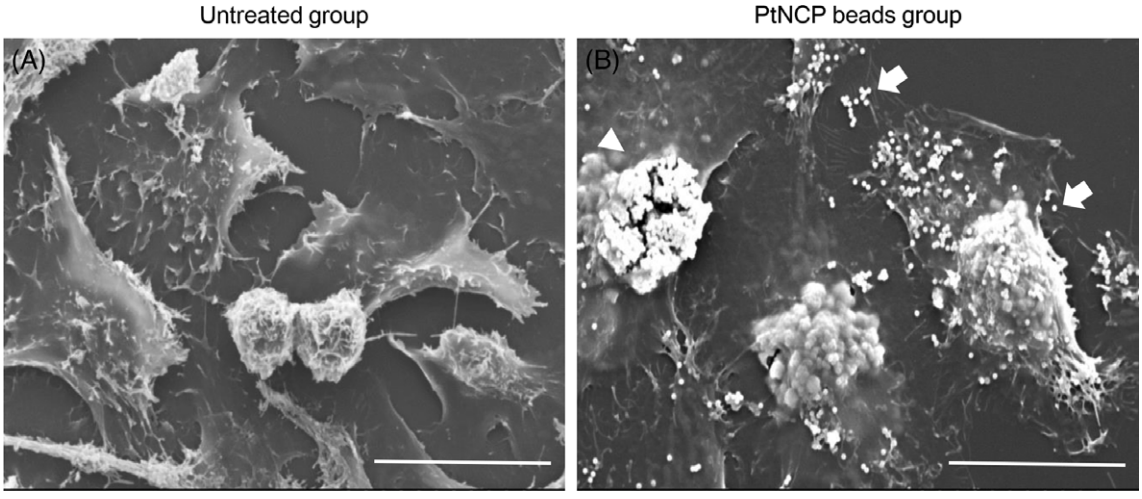

FIGURE 4. SEM images in HSC-3-M3 cells (magnification × 2000). (A) Untreated HSC-M3 cells, (B) PtNCP beads-treated HSC-3-M3 cells. Bar = 20 μm. Arrows points to a PtNCP bead and an arrowhead points to the destruction of cell membrane.

carrier and as an application for improving therapeutic effects. In addition, we have developed the intracellular drug delivery method using ultrasound and cancer specific antibodies-conjugated microbubbles.[15,16] Further studies are required to attach PtNCP specifically to cancer cells, developing cancer specific antibodies-modified PtNCP beads. The strong membranolitic effect of PtNCP beads should make it difficult for the cancer cell to develop resistance to widespread used platinum drugs. These therapeutic combinations should be explored additionally for therapeutic use.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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