



Cisplatin loaded PMMA: mechanical properties, surface analysis and effects on Saos-2 cell culture

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Objective: Despite wide resection and systemic chemotherapy, bone tumors may present with local recurrences, metastases and pathological fractures. Application of bone cement containing antineoplastic drug to fill the defect after resection of metastatic lesions and to support implants has been suggested to prevent local tumor growth and implant failures. In this study, we aimed to demonstrate the effects of the addition of cisplatin which is a widely used antineoplastic drug for osteosarcoma, on the mechanical properties of bone cement, and to evaluate the cytotoxic effects of eluted cisplatin on Saos-2 cell culture.

Methods: Two cement samples were prepared by mixing 100 mg and 300 mg of cisplatin powder with 40 g cement powder. The bone cement of the control group did not contain cisplatin. Mechanical analyses included 4-point bending, compression and shear testing. For cytotoxicity analysis, samples were incubated in Dulbecco's Modified Eagle's medium for 15 days. Mediums were applied to Saos-2 cell culture and cell viability was measured. Surface analyses were performed by scanning electron microscope (SEM).

Results: The addition of cisplatin did not alter the mechanical properties of bone cement. It was observed that the eluted cisplatin had cytotoxic effects on Saos-2 cells. SEM analyses demonstrated cisplatin granules on the surface of cement samples.

Conclusion: Cisplatin maintains its cytotoxic property when released from bone cement without compromising the mechanical stability. Application of cisplatin loaded bone cement may help local control of tumor growth. We believe that our study will shed light on to these new practices for the treatment of bone cancers and will encourage future studies.

Key words: Bone cement; cisplatin; osteosarcoma.

The expected survival time of patients with cancer has improved in parallel with the advances in treatment modalities.^[1] As a consequence, the incidence of metastases and pathological fractures has increased. Treatment of pathological fractures addresses pain relief and survival without loss of function.^[2] To achieve this goal, internal fixation supplemented by methyl-methacrylate cement is

generally the preferred method.^[2] However, the underlying tumor may continue to grow, resulting in bone destruction, which will eventually cause implant failure.^[2] In addition, local recurrences may develop despite wide surgical resection and systemic chemotherapy.^[3]

Polymethyl methacrylate (PMMA) is an accepted deliverer for various kinds of drugs to the intended

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body parts.^[4,5] Recently, studies concerned with the use of antineoplastic pharmaceutical added PMMA have increased in number. This new method is suggested to decrease the incidence of tumor recurrences with better local tumor growth control and the number of distant metastases.^[1] In addition, implant loosening due to tumor osteolysis may be prevented and the complication free survival of patients may be prolonged.^[2,6]

To be added into PMMA, an antineoplastic drug must preserve its chemical structure against the heat generated during the polymerization process. The added drug must not interfere with the mechanical features of PMMA and must be eluted from PMMA in its active form. While mechanical features, release properties and effects of methotrexate loaded PMMA on different tumor cells have been extensively investigated previously, cisplatin added PMMA has been less studied.^[2,3,6-15]

The aim of our study was to show whether the addition of cisplatin would interfere with the mechanical properties of PMMA or not, and to evaluate the effects of eluted cisplatin on osteosarcoma cell culture. Our hypothesis was that the mechanical features of PMMA would remain unaffected and cisplatin could be eluted from PMMA exerting cytotoxic effect on osteosarcoma cells.

Materials and methods

The study conforms to the principles outlined in the Declaration of Helsinki. The study was approved by the local ethics committee.

Low viscosity PMMA (OrCem 3, Teknimed S.A.S., Vic En Bigorre, France; lot no: 043/09344) containing 40 g powder and 20 ml liquid monomer was used in the study. Cisplatin powder was obtained from Teva Pharmaceuticals Ltd. (Amsterdam, the Netherlands). For mechanical and cell culture experiments, 3 groups of PMMA samples were prepared. Powder and liquid monomer were mixed under vacuum using cement mixer (MixOR; Smith&Nephew, Memphis, TN, USA) for 100 seconds. For the groups containing cisplatin, prior to mixing, 100 mg (100-mg group) and 300 mg (300-mg group) were hand mixed with 40 g PMMA powder in a sterile pot for 100 seconds. Control group did not contain cisplatin.

To assess mechanical properties of cisplatin added PMMA; 4-point bending, compression and shear tests were performed according to ASTM-F451 and ISO-5833 standards, using a universal testing machine (MTS 858 Bionix II, Eden Prairie, MN, USA). For compression and shear testing, the samples were cylinders, 6 mm (± 0.1 mm) in diameter and 12 mm (± 0.1 mm) in height (Fig. 1a). For 4-point bending tests,

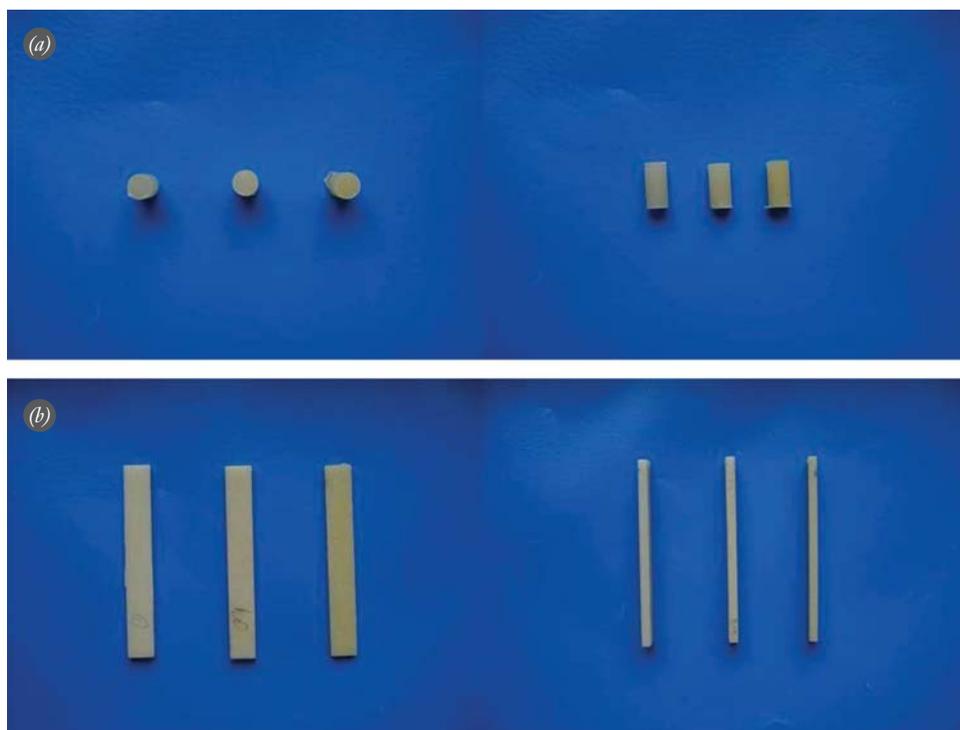


Fig. 1. Figure shows the PMMA samples prepared for (a) compression and shear tests and (b) 4-point bending tests. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]



Fig. 2. (a-c) Figures show the bending of PMMA sample in 4-point bending test.

samples were rectangles, 75 mm (± 0.1 mm) in length, 10 mm (± 0.1 mm) in width and 3 mm (± 0.1 mm) in depth (Fig. 1b). Test data were recorded to a personal computer with a data sampling of 10 Hz for further analysis. Each test group consisted of 10 samples. Samples were prepared 24 hours prior to testing.

In 4-point bending test, samples were subjected to an increasing force causing deflection with a crosshead speed of 5 mm/min (Fig. 2). Deflection values were recorded until the sample was fractured. For each sample, bending modulus (E) and bending strength (B) were calculated in megapascals (MPa).

In compression tests, the samples were subjected to a force with a crosshead speed of 20 mm/min (Fig. 3). Force, displacement and time values were recorded. Test was stopped when the sample was deformed or upper yield point was exceeded. The value of the force applied at these points was also recorded. Compressive strength (C, MPa) and stiffness of all samples were calculated.

In shear tests, the samples were subjected to a vertical shear force with a crosshead speed of 5 mm/min (Fig. 4). Force, displacement and time values were recorded. Test was stopped when the sample fractured and the applied force was also recorded. Shear strength (MPa) and stiffness were calculated for each sample.

For the cell culture experiments, Saos-2 cells (Şap Enstitüsü, Ankara, Turkey) were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, penicillin and streptomycin in an incubator (at 37°C, 5% CO₂ and 95% humidified air). At confluence, the cells were detached from flasks with trypsin. For cytotoxicity analysis, the cells were then placed in the cell culture plate with 96 wells. Each well contained approximately 5000 cells (Fig. 5). PMMA samples were cylindrical in shape, 6 mm in diameter and 12 mm in length. PMMA samples were prepared 24 hours prior to incubation with DMEM in order to avoid potential hazardous effects of heat produced during polymerization. Each group con-

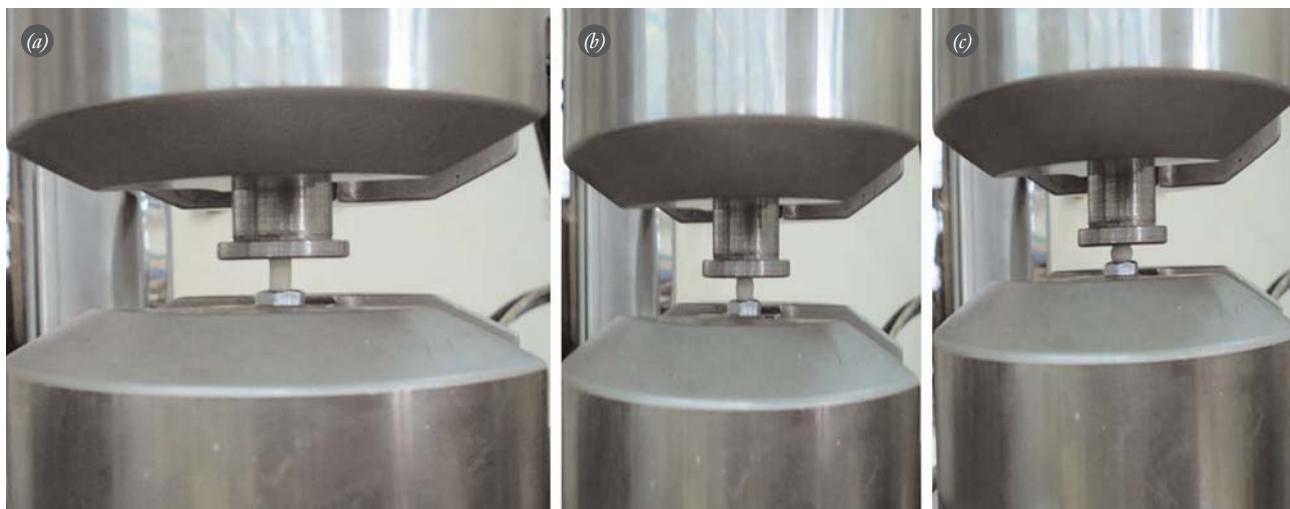


Fig. 3. (a-c) Figures show the deformation of PMMA sample in compression test. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]



Fig. 4. (a-c) Figures show the shearing of PMMA sample in shear test. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

tained 4 samples. In the first set of experiments, PMMA samples were incubated in 4 ml DMEM solution at 4°C for 24 hours. Then, the DMEM was collected, labeled as “1st day medium” and stored at -80°C. The same sample was incubated with another 4 ml of DMEM and incubated for 24 hours. After 24 hours, DMEM was col-

lected, labeled as “2nd day medium” and stored at -80°C. This process was repeated for 15 days.

In the second set of experiments, to study the effect of time on cytotoxicity, PMMA samples were prepared and stored at 4°C for 15 days. After this period, samples were incubated in 4 ml DMEM at 4°C for 24 hours.

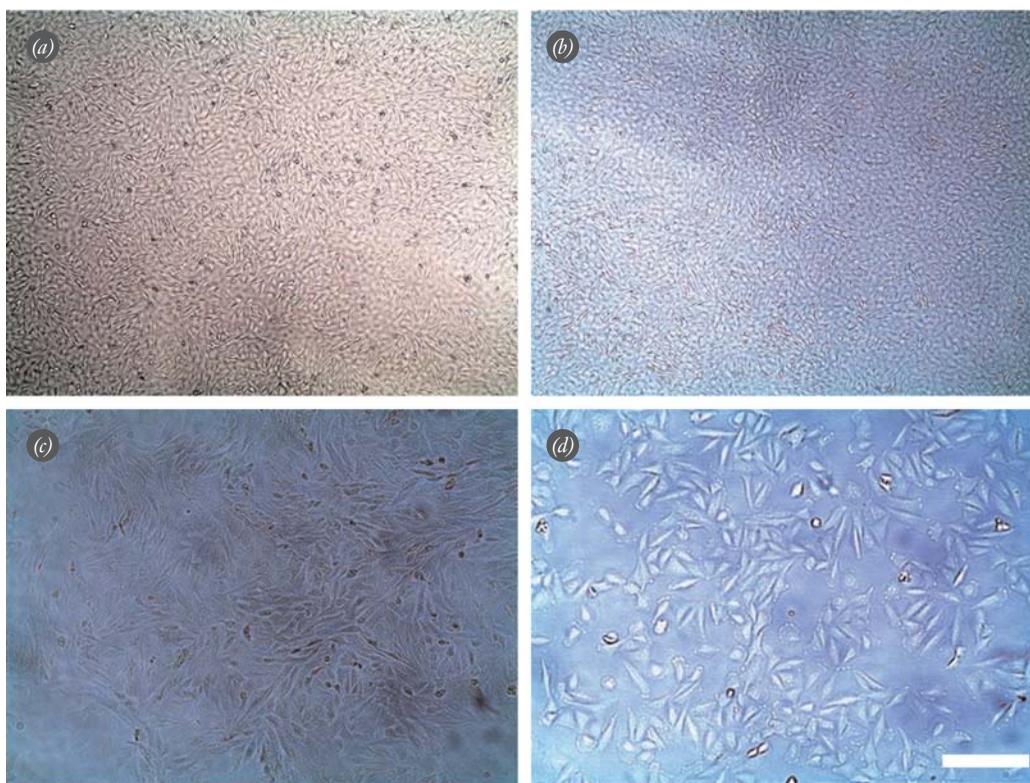


Fig. 5. (a-d) Saos-2 cells are visualized in light microscopy (scale size= 100 µm) [trypan blue x10 (a, b) and x40 (c, d)]. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 1. Results of 4-point bending test.

Group	Displacement at 15N (mm)	Displacement at 50N (mm)	Failure load (N)	Bending modulus (MPa)	Bending strength (MPa)
Control	0.49	1.74	114.62	3644.64	63.15
100 mg	0.21	1.49	113.42	3509.03	62.49
300 mg	0.07	1.32	109.81	3593.92	60.50

DMEM was collected and labeled as “delayed medium” and stored at -80°C .

Saos-2 cells were incubated with 0.1 ml of collected DMEMs for 48 hours. DMEM was removed from cell wells and wells were incubated with 0.01 ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Chemicon, Merck Millipore, Billerica, MA, USA) for 4 hours at 37° . MTT was converted to formazan by living cells. Formazan was dissolved by the addition of isopropanol and a homogenous purple color was formed. Absorbance reading was performed with 570 μm wavelength plate reader (Biotek μQuant Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Number of MTT tests performed was 15 for control group and 19 for 100 mg and 300 mg groups. Cytotoxicity effect was expressed as the ratio of viable cells.

PMMA surface analyses were performed using SEM (TEMGA-L 5400). Surface images of 2 samples of each group were taken and labeled as “before release”. These samples were then incubated in 4 ml DMEM. Mediums were replaced with new ones every 24 hours for 15 days. At the end of this period, surface images were taken and labeled as “after release”. Comparison between before and after images was done using granule number/ mm^2 .

Statistical analyses were performed with SPSS (SPSS 11.0.1 for Windows, Chicago, IL, USA). Continuous variables were expressed as mean \pm standard deviation (SD). To evaluate the statistical difference, Wilcoxon test, Mann-Whitney U test, Kruskal-Wallis test and Friedmann tests were performed. Cases where p-value was below 0.05 were accepted as statistically significance.

Results

According to the 4-point bending test results, the 3 groups were not statistically different in terms of bending modulus and bending strength ($p=0.36$ and $p=0.84$, respectively). The mean values obtained in 4-point bending tests are given in Table 1.

Mean compression strength values of the 3 groups obtained in compression tests were not statistically sig-

nificant ($p=0.26$). Stiffness values of 100-mg- and 300-mg group were statistically significantly higher than the control group ($p<0.001$ for each comparison). However, stiffness values of 100 mg and 300 mg were not statistically different ($p=0.53$). The mean values obtained in compression tests are given in Table 2.

Shear tests results revealed no significant difference between the mean shear strength and mean stiffness of the 3 groups ($p=0.13$ and $p=0.65$, respectively). The mean values obtained in shear tests are given in Table 3.

Cytotoxicity results of Saos-2 cell cultures incubated by the 1st day mediums showed significant difference in cell viability (91.65% for control group, 64.88% for 100-mg group and 37.99% for 300-mg group, $p<0.001$). Similarly, cell viability of cultures incubated with the 7th and 15th day mediums differed significantly among the 3 groups ($p<0.001$ and $p<0.05$, respectively). The results

Table 2. Results of compression test.

Group	Failure load (N)	Stiffness	Compression strength (MPa)	Modulus (MPa)
Control	2937.52	2956.79	102,86	1072,90
100-mg	3054.41	3772.91	106,43	1309,64
300-mg	3042.36	3846.43	105,30	1330,86

Table 3. Results of shear testi.

Group	Failure load (N)	Shear strength (MPa)	Stiffness
Control 1	525.97	18,57	441.24
100 mg 1	554.63	19.12	457.39
300 mg 1	528.26	18.34	442.32

Table 4. Cell viability ratios.

Viability\Group	Control	100-mg	300-mg	p value
1st day viability (%)	91.65	64.88	37.99	<0.001
7th day viability (%)	94.20	70.81	45.93	<0.001
15th day viability (%)	94.72	65.11	47.72	<0.05
Delayed medium viability (%)	91.65	65.72	37.50	<0.05

of MTT analyses are given in Table 4 and Fig. 6. Cell viability of cultures incubated with “delayed mediums” differed significantly among the groups ($p < 0.05$). However, cell viability of cultures incubated with the 1st day medium and delayed mediums did not show any statistical significance among each group ($p > 0.05$). Daily cell viability ratios did not change between control and 100 mg group ($p > 0.05$). However, in 300 mg group, the comparison of 1st and 4th days showed increased viability at the latter time point.

Cisplatin aggregates gave PMMA a granular appearance under SEM. Cisplatin granules occupied more space in 300-mg group than in 100-mg group. Number of cisplatin granules decreased after 15 days of release in both groups. Before release, on the surfaces of 100-mg and 300-mg samples, there were approximately 12,500 granules/mm² and 84,000 granules/mm², respectively. After 15 days of release, number of granules decreased to 7900 granules/mm² and 35,700 granules/mm², respectively. Since the granules differed in size, a statistical comparison could not be made according to the real cisplatin quantity. SEM images of PMMA sample were given in Figs. 7 and 8.

Discussion

Despite wide resection and high-dose systemic chemotherapy, local recurrences and distant metastases and pathological fractures may be encountered in tumors of the skeletal system.^[1-3,7,9,12] The use of antineoplastic drug loaded PMMA may be helpful in preventing local recurrence and rendering the internal fixation with a longer failure free period.^[2,3,9,11,13,14] However, the antineoplastic drug should endure against the heat generated during polymerization, should not diminish the mechanical properties of PMMA and can be released in active form from PMMA.^[1,2,5,11] Our study demonstrated that addition of cisplatin did not alter the mechanical features of PMMA and could exert cytotoxic action upon Saos-2 cells.

Our study is limited from certain aspects. We did not perform release kinetics for cisplatin from PMMA. In addition, a quantitative analysis of the cisplatin amount on the surface of PMMA samples would give a better understanding of the release kinetics. Another limitation is that the samples were not cyclically loaded over time to determine if repetitive loading affects cisplatin release from PMMA or if bone cement is more susceptible to cyclic load induced fracture. The absence of post-release mechanical analysis is another limitation.

It has been shown that, 2 grams of methotrexate can be added into PMMA without any significant effect on the compressive and bending strength of PMMA.^[1,2,13]

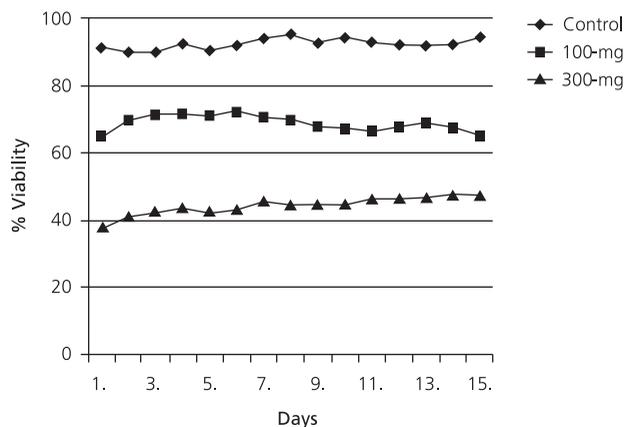


Fig. 6. The graph shows the daily changes in the viable cell ratios of different groups of Saos-2 cell cultures.

PMMA stability is not affected by addition of 50 mg doxorubicin or 50 mg cisplatin into 40 g of powder.^[1] PMMA loaded with 2 g of doxorubicin and 2 g of pamidronate was shown to preserve 87% of its compressive and tensile strength even after storage in a liquid medium for 6 months.^[15] Our results are in conjunction with the literature. Addition of 100 mg and 300 mg cisplatin into 40 g of PMMA powder did not alter bending and shear strength. Compression tests revealed an increase in stiffness values when PMMA was loaded with 100 mg and 300 mg of cisplatin. With increase in stiffness, PMMA is expected to become more brittle. However, we did not observe any difference in compression strength. Our results have showed that, up to 300 mg of cisplatin can be added into 40 g of PMMA powder without any significant compromise in mechanical strength.

Higher local concentration of antineoplastic drug may be achieved with the use of PMMA.^[2] Passage of the eluted drug into systemic circulation is via the capillary system and therefore is relatively slow. The elimination in kidney and liver will also decrease the systemic concentration and side effects.^[2] Mestri et al. studied *in vitro* and *in vivo* cisplatin release from PMMA, and found that *in vitro* cisplatin release was relative slow and dependent on the amount incorporated into PMMA in the beginning.^[14] At the end of 80 days, for samples containing 1% and 20% (cisplatin weight/PMMA powder weight), the release amount was 3% and 12% of those added in the beginning, respectively.^[14] However, they found that nearly 52% of drug added in the beginning was released during the *in vivo* tests.^[14] They concluded that increased vascularity around the cement was responsible for the increased release.^[14] In our study, we did not perform a separate kinetic analysis. However, by changing the medium every 24 hours, we tried to mimic the clearance

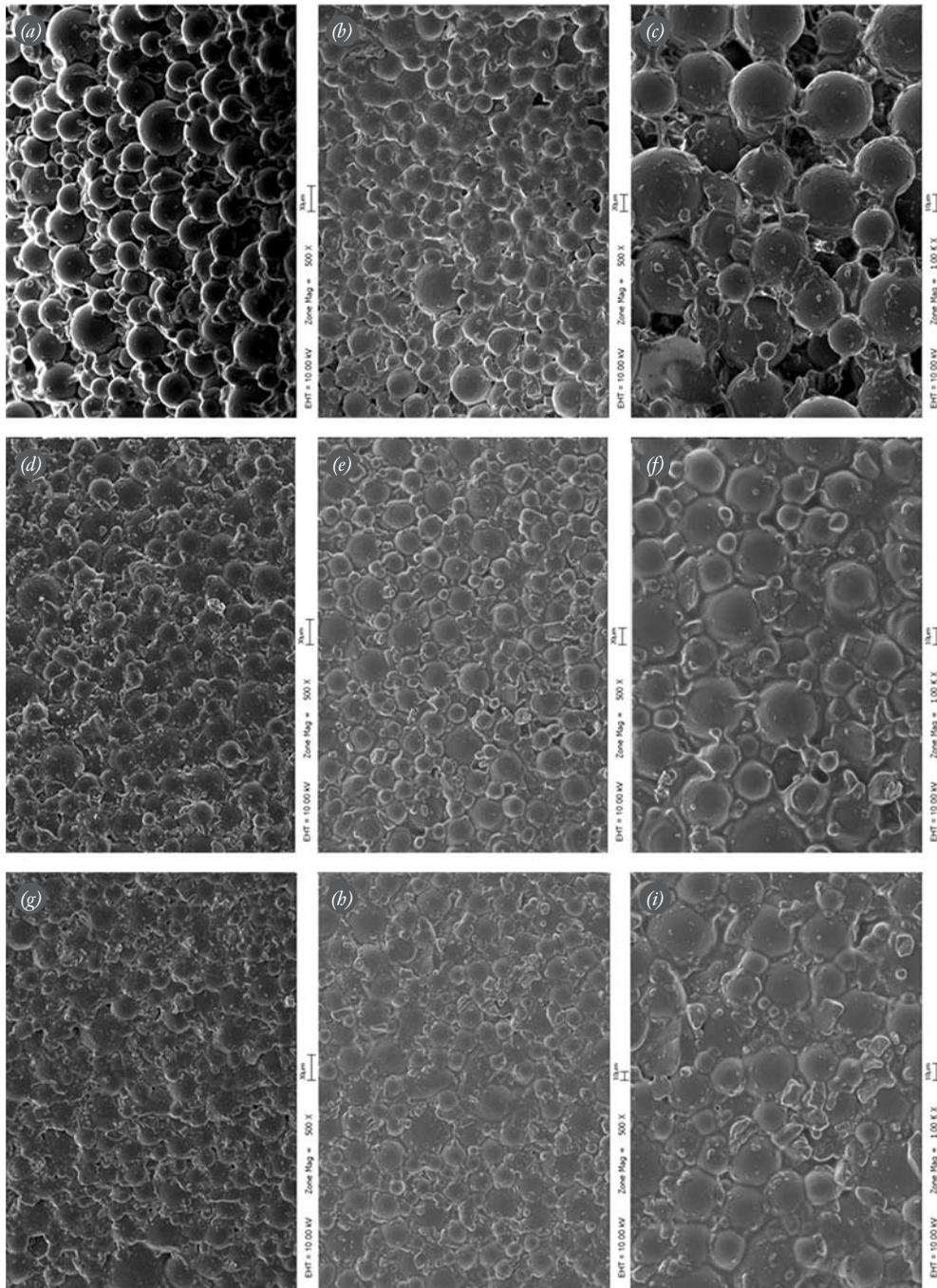


Fig. 7. Figure shows the pre-release surface electron microscopic images of **(a-c)** control, **(d-f)** 100-mg and **(g-i)** 300-mg groups.

of cisplatin from tissue with extracellular fluid. One can assume that cytotoxicity is directly proportional with the amount of drug released. Our everyday cytotoxicity values remain more or less the same indicating a constant release throughout the period of 15 days. The addition of 300 mg cisplatin resulted in 1.73 times higher cytotoxicity than 100 mg cisplatin at the end of the 1st day.

This ratio was 1.54 at the end of the 1st week and 1.36 at the end of 15 days. These results indicate that cisplatin release might depend on the amount of drug added in the initial period while that dependency decreased with time.

The cytotoxic effects of PMMA loaded with anti-neoplastic drugs have been studied *in vitro* and *in*

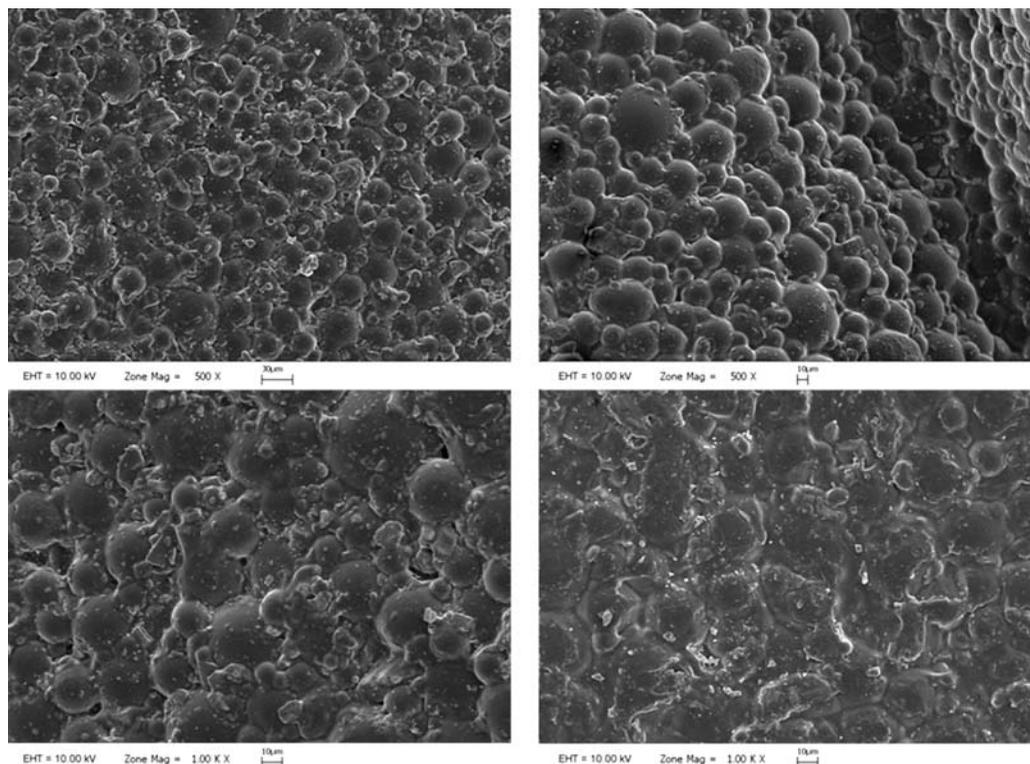


Fig. 8. Figure shows the post-release surface electron microscopic images of **(a,b)** 100-mg and **(c,d)** 300-mg groups.

in vivo.^[1,5,12] It has been reported that cisplatin,^[1,16] doxorubicin,^[1] daunorubicin^[17] and adriamycin^[16] added PMMA use can inhibit the growth of giant tumor cells, colon cancer cells, lung cancer cells and breast cancer cells *in vitro*. Our results showed that the eluted cisplatin from PMMA could successfully exert its cytotoxic action on Saos-2 cells for a fifteen-day period. This action was dose dependent, being higher in the group with higher amount of drug. Even storage for 15 days did not alter the effect of cisplatin.

The addition of cisplatin does not change the mechanical features of PMMA. The application of PMMA loaded cisplatin may supply with a high and relatively constant tissue concentration resulting in a better local control of tumor growth, while the systemic concentration and the side effects may be kept minimally. Moreover, the low amount in systemic concentration may be helpful in preventing distant metastases. Further studies investigating the release kinetic of drugs incorporated into PMMA samples of different surface areas and *in vivo* application of antineoplastic drug added PMMA with longer follow up are needed to elucidate the side effect of the maximum drug dose that can be incorporated into PMMA.

Conflicts of Interest: No conflicts declared.

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