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Methotrexate-added acrylic cement: biological and physical properties

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Abstract
Background Previous reports have demonstrated the suitability of adding different chemotherapeutic drugs to acrylic cement for the treatment of bone metastases. The best results so far have been obtained with methotrexate (MTX) for which diffusion from the implanted cement has been demonstrated both in vitro and in vivo. In this study the suitability of adding MTX to acrylic cement as local adjuvant chemotherapy was investigated.
Methods Using an in vitro model of human breast cancer cells we demonstrated that the drug is eluted in an active form able to exert a cytotoxic effect over a long period of time. The use of different concentrations of drug on the kinetic of elution and on the mechanical properties of cement was also evaluated.
Results The results obtained suggest that the release of MTX is higher at the beginning and progressively decreases over time being affected by the concentration of drug used. Our results also demonstrated that the addition and the subsequent elution of MTX does not alter the compressive properties of the cement.
Conclusion These findings confirm the suitability of MTX-supplemented cement and support its use as an effective aid for the management of bone metastases requiring surgical curettage and acrylic cement implantation for structural support.

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1 Introduction

Acrylic cement (PMMA) is widely used in Orthopaedic surgery either for anchoring total hip and knee replacements and for filling bone cavities after curettage of bone tumours. It is also used in the treatment of infections [1] for the capability of antibiotics to be eluted from PMMA for a long time after implantation. More recently the use of PMMA has become progressively more relevant in bone oncology since the long-term survival of cancer patients has increased the possibility to develop bone metastases. Indeed, due to its mechanical properties, PMMA is widely used together with plates and or intramedullary nails in the treatment of bone metastases. However, it has been recently suggested the possibility to add chemotherapeutic drugs to PMMA which might improve the local control of skeletal neoplasms by exerting a direct cytotoxic effect on residual cancer cells [2]. In particular the possibility of adding chemotherapeutic agents such as Methotrexate (MTX) to bone cement has been reported and the diffusion of the drug from the implanted cement has been demonstrated both in vitro

and in vivo [3, 4]. We previously reported the possibility to add different chemotherapeutic drugs (such as Methotrexate, Doxorubicin and Cisplatinum) to PMMA and demonstrated, using an in vitro model of human breast cancer cells, that drugs were eluted in an active form able to exert a cytotoxic effect [5]. We also demonstrated that MTX is the drug showing the slowest release and a more prolonged toxic effect over time thus confirming its suitability for this type of application [5]. In this paper we extend our previous study by evaluating how addition of MTX affects the mechanical properties of PMMA. To this aim we analysed how and whether the addition of different concentrations of MTX affect the properties of PMMA. Moreover, we evaluated how the drug is released by PMMA and whether released drug can exert a cytotoxic effect on cell cultures in vitro.

2 Materials and methods

2.1 Technique of cement mixing and specimens preparation

Surgical Simplex P® radiopaque bone cement (Howmedica International Inc. Clare, Ireland) was used for the preparation of five different mixtures whose compositions are summarised in Table 1. Except for the cement dough C, used as control, the other ones were loaded with MTX (Lederle). In the mixtures MTX1, MTX2, MTX3, and MTX4, the MTX previously milled was added to 40 g of the powder in the amount (expressed in mg) reported in the Table 1.

2.2 Manufacturer of implantable cylinders

The antiblastic-containing cement mixtures were obtained mixing the MTX with acrylic cement powder and adding the liquid monomer under vacuum using dedicated instrumentation (Simplex Enhancement Mixer, Howmedica). Vacuum was removed after one hundred seconds of mixing. The mixture was then poured on the moulds [6, 7] for the manufacture of cement cylinders by compression. Test cylinders of acrylic cement without drug were obtained as controls in a similar way. All the cylinders were 10 mm in length and 4 mm in diameter. (Fig. 1)

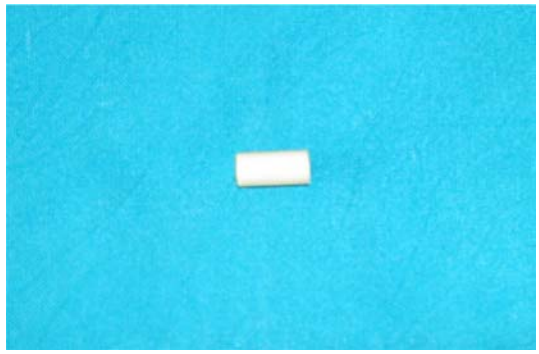

Fig. 1 Cylinder manufactured according to ASTM F451 and ISO 5833

2.3 Morphological analysis

The cylinders were coated with gold-palladium and examined by Scanning Electron Microscopy (SEM 515 Philips).

2.4 Methotrexate release tests

The cylindrical specimens-containing cement alone (C) or supplemented with MTX (MTX1, MTX 2, MTX 3 and MTX 4) were immersed in 3 ml saline solution (NaCl 0.9%) and stirred at 37 °C. At sampling intervals (5 min; 1, 24 and 48 h; 5, 16 and 30 days,

Table 1 Composition of different mixtures of MTX-loaded cements, expressed as a percentage of the total powder and liquid components, respectively[a]

	C (w/w %)	MTX1 (w/w %)	MTX2 (w/w %)	MTX3 (w/w %)	MTX4 (w/w %)
Powder					
Poly(MMA/styrene)	82.26	82.11	82.05	81.90	81.74
Poly(methylmethacrylate)	6.55	6.54	6.53	6.52	6.51
$BaSO_4$	10.00	9.98	9.98	9.96	9.94
Benzoyl peroxide	1.19	1.19	1.19	1.18	1.18
Methotrexate[b]	0.00	0.18	0.25	0.44	0.63
Liquid					
Methylmethacrylate	97.51	97.51	97.51	97.51	97.51
N,N-Dymethyl-*p*-toluidine	2.48	2.48	2.48	2.48	2.48
Hydroquinone	75 ppm	75 ppm	75ppm	75 ppm	75 ppm

[a] Compositions are in percent by weight (w/w %) of powder and liquid component, respectively [b] Hydroquinone content is expressed in particles per million

calculated from the start of the immersion), the cylinders were removed and placed in 3 ml of fresh saline solution. All the incubating solutions were collected and stored under sterile conditions at –0.80 °C

The MTX released in the solution was analysed by high performance liquid chromatography (Varian HPLC) [8, 9] using an UV–VIS detector fixed at a wavelength $\lambda = 301$ nm, column LiChroCart RP18 5 μm (250 × 4 mm I.D.) and using 50% methanol in Tris-sodium dihydrogen phosphate (both 0.1 M, pH 6.7) as eluent at flow rate 1.0 ml/min.

Five experimental runs were performed for each composition and data were examined for statistical significance using the Student *t*-test. Significance level was defined as $p < 0.05$.

2.5 Cell cultures

The MCF-7 breast cancer cell line was obtained from the American Type Culture Collection and cultured according with the instructions of the supplier in RPMI medium (Gibco) supplemented with 10% heat inactivated FBS [5].

2.6 Cell proliferation assay

Cylinders of specimen MTX2 were incubated in cell culture medium (1 cyclinder/2 ml). The medium was changed every day from 1 to 30 days. The medium was collected at each time point and immediately frozen at –80 °C.

The effects of media collected at different time points on the proliferation of MCF-7 cells were evaluated using the MTT test [5]. Briefly, MCF-7 cells were seeded at a final concentration of 2.5×10^4 cells/well in the 24 wells of a multiwell plate and left for 36 h to obtain a good adhesion on the substrate. Then medium was changed with fresh medium or with medium collected after incubation with cylinders of cement alone or supplemented with MTX. After 48 h, medium was removed and cultures were incubated with medium containing 1 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) for 2 h at 37 °C. Medium was then discarded and 500 μl acid-isopropanol (0.04 N HCL in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes which convert MTT to an insoluble purple formazan in living cells. Plates were then kept in agitation at room temperature for about 15–20 min. and the level of the coloured formazan derivative was determined on a multiscan reader at a wavelength of 540 nm (reference wavelength 630 nm). Each experiment was repeated in triplicate.

Results were expressed as the percentage of surviving cells as compared to control cells incubated with fresh medium (mean ± standard deviation).

2.7 Compression tests

The end faces of the cylindrical specimens were machined with a low-speed diamond wheel saw in order to ensure parallel end surfaces. The uniaxial static compressive tests were performed by a universal mechanical testing machine (Model Mayes ESM100) operating at a constant cross-head speed of 1.3 mm/min. For each sample the Ultimate Compressive strength (UCS) was calculate, according to ASTM F-451 and ISO 5833 [6, 7].

The tests were performed on the specimens at two different times: before and after the immersion for 30 days in the saline solution used in the MTX release test in order to assess the effects of ageing on the compressive property. The cylinders after immersion were respectively called Ci, MTX1i, MTX2i, MTX3i, and MTX4i. Each compressive test was performed on five samples.

The UCS values were expressed as the mean ± standard deviation. The Student's *t*-test was used in determining the significance of the difference between the UCS results of the control cement C and the mixture samples, before and after the immersion of 30 days. Significance was defined as $p < 0.05$.

3 Results

3.1 Morphological analysis

SEM analysis showed that the cylinders without drugs had a beaded surface while granules of powder were detected on the surface of cylinders-containing MTX (Fig. 2).

3.2 Methotrexate release test

The results of the release test are reported in Table 2 and are shown as the cumulative release of the drug from MTX-supplemented cylinders over time.

For all the concentrations of drug tested, a significant and rapid release of MTX was observed in the first five minutes followed by a progressive decay in MTX elution al later time points. However, MTX

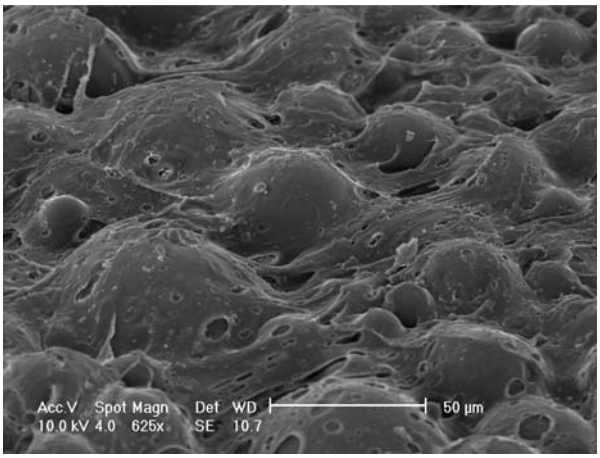

Fig. 2 SEM showing powders dispersed on the surface of cement

continued to be released although at a lower rate up to 30 days from all the mixtures tested.

The results obtained also demonstrated that the efficiency of MTX elution is influenced by the amount of the drug incorporated in the cement but the kinetic profiling of drug release is the same for all of the concentrations tested. In fact, if the release process is diffusion controlled, then the release will be governed by the equation, for a short cylinder:

$$M_t = M_\infty\{1 - 1/8\,\pi^2 \sum_1^\infty a^2\,\alpha_n a^2 \exp(D\,\alpha_n^2 t) \sum_0^\infty 1/(n+1)2\exp[-\pi^2 D(2n+1)^2 t]/L^2\}$$

where M_t is the amount released at time t M_∞ the amount finally released, a is the cylinder radius, L its length, D the Diffusion Coefficient and a α_n the roots of

$$J_0(a\,\alpha_n) = 0$$

where J_0 is the Bessel Function of order zero.

For sufficient long times, only the first term of the above equation is needed, from which it can be shown that:

$$\ln(1 - M_t/M_\infty) = \ln(32\,M_\infty/\pi^2\,\alpha_1^2 a^2) - [D(\alpha_1^2 + \pi^2/L^2)t]$$

This predicts a straight line when $\ln(1–M_t/M_\infty)$is plotted against t, of slope $= D(\alpha_1^2 + \pi/L)^2$ from which D may be calculated.

The graph in Fig. 3 shows that all points approximately fit a common line, from which a value of $D = 6.37 \times 10^{-11}$ m^2 s^{-1} is obtained for the MTX1 mixture which is not significantly different from the values obtained for the other mixtures (Fig. 3 and data not shown).

3.3 Cell cultures

The biological activity of the drug released by MTX-supplemented cylinders was evaluated by repeating the release test using cell culture medium as incubating solution, rather than a simple saline solution. Cylinders were incubated in cell culture medium (1 cylinder/2 ml) and the medium was changed every day up to 30 days. Media collected at each time point were used

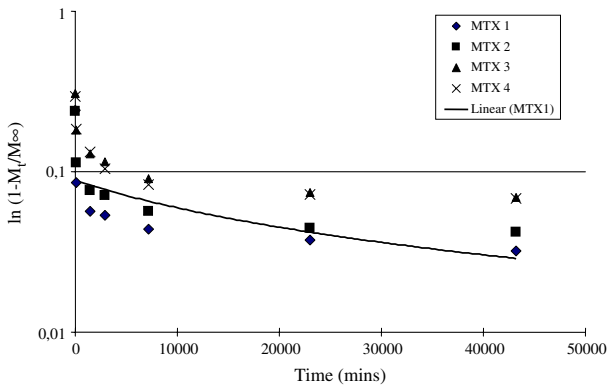

Fig. 3 Release of methotrexate at different concentration from cylinders in time

Table 2 Cumulative release of MTX, expressed in µg, from different mixtures of MTX-loaded cements[a]

[a] The amount of released MTX was assessed by HPLC and the data are expressed as total MTX released over time (µg ± S.D)

Time of immersion	MTX1	MTX2	MTX3	MTX4
5 min	2.92 ± 0.59	4.12 ± 0.82	5.61 ± 1.20	8.39 ± 0.27
60 min	7.99 ± 0.81	8.37 ± 1.40	9.41 ± 1.98	13.27 ± 0.65
24 h	11.70 ± 0.79	12.25 ± 1.50	13.08 ± 1.40	18.30 ± 1.11
48 h	12.20 ± 0.77	13.18 ± 0.96	14.60 ± 1.3	22.89 ± 0.82
5 days	14.64 ± 0.40	16.22 ± 0.80	18.35 ± 0.95	28.43 ± 2.52
16 days	16.94 ± 0.64	20.08 ± 0.75	22.10 ± 1.06	32.73 ± 2.35
30 days	19.35 ± 0.83	21.18 ± 0.70	23.56 ± 0.83	34.50 ± 2.20

Table 3 Effects of the drug released from MTX-loaded cement on the proliferation of breast cancer cells[a]

Medium	Cell survival (%)
Control	100
1 day medium[b]	51.6 ± 4.1
2 days medium	55.5 ± 5.5
3 days medium	60.9 ± 5.1
7 days medium	74.7 ± 3.5
15 days medium	83.0 ± 5.2
30 days medium	90.5 ± 4.8

[a] Experiments were repeated three times and data are expressed as percentage of control (mean ± SD)

[b] Cylinders were incubated in cell culture medium and the medium was changed every day up to 30 days. Media collected at each time point were used in the MTT test. See text for details

in the MTT test and their ability to inhibit proliferation of breast cancer cells was assessed. The results obtained for the mixture MTX2 are summarised in Table 3 and demonstrate that media incubated with MTX-supplemented cylinders were able to exert an inhibitory effect on the proliferation of breast cancer cells which was strongly evident at the beginning of the experiment (first change) and progressively decreased thereafter, being still evident after 30 days (corresponding to 30 changes). No toxic effect was exerted by the medium collected after incubation with control cylinders not containing the drug (data not shown).

3.4 Compression tests

The results of the compression test are given in Tables 4 and 5.

The data show that the Ultimate Compressive Strength of the samples, irrespective of the amount of MTX loaded, met the minimum standard of 70 Mpa as reported in the specific standards ASTM F451 and ISO 5833. This observation indicates that in any case

Table 4 Ultimate Compressive Strength (UCS) of different mixtures of MTX-loaded cements

Cement bone	Property	Statistical analysis[b]
	UCS[a](MPa)	
Control	77.0 ± 3.0	
MTX1	82.1 ± 1.4	Significant
MTX2	84.9 ± 0.7	Significant
MTX3	92.1 ± 1.5	Significant
MTX4	78.1 ± 2.2	Not significant

[a] Values are given as mean ± standard deviation of a number $n = 5$ of test specimens

[b] Means of UCS of mixtures MTX1, MTX2, MTX3 and MTX4 were evaluated against the control cement

Table 5 Ultimate Compressive Strength (UCS) of different mixtures of MTX-loaded cements analysed after 30 days immersion in the saline solution

Cement bone	Property	Statistical analysis[b]
	UCS[a](MPa)	
Ci	75.0 ± 2.8	
MTX1i	83.2 ± 1.9	Significant
MTX2i	87.2 ± 2.1	Significant
MTX3i	91.6 ± 1.8	Significant
MTX4i	80.2 ± 3.2	Not significant

[a] Values are given as mean ± standard deviation of a number $n = 5$ of test specimens

[b] Means of UCS of mixtures MTX1, MTX2, MTX3 and MTX4 were evaluated against the control cement

compressive properties are not negatively affected by the addition of the drug, at least within the range of concentrations used in this study thus suggesting that MTX is likely well incorporated in the polymer matrix.

As can be seen in Table 3, the UCS values of the MTX-loaded cylinders were even significantly higher ($p < 0.05$) for mixtures MTX1, MTX2 and MTX3 as compared to the control specimens. No statistically significant differences were observed for the specimen MTX4 compared to control cylinders. Similar results were obtained after incubating cylinders for 30 days in a saline solution (Table 4).

4 Discussion

We previously reported the suitability of adding different antineoplastic drugs to acrylic cement obtaining the release of biologically active drug [5]. The best results in term of amount of drug eluted and duration of release was obtained with MTX, thus confirming previous reports on the possibility to use MTX-supplemented cement for the treatment of skeletal metastases. In this study we extended the previous report by analyzing the effects of using different concentrations of MTX on the release of the drug and on the physical properties of cement. The results obtained confirm that MTX, a widely used drug for the treatment of breast and many other human cancers, when mixed to PMMA, is eluted in active form able to exert a cytotoxic effect on cancer cells. Compressive strength is not a highly sensitive parameter to assess the effect of drug addition. However, the results obtained suggest that drugs-added PMMA displays good mechanical properties thus supporting the need for further studies to evaluate the suitability of MTX-PMMA mixture as an effective aid for the management

of bone metastases requiring surgical curettage and acrylic cement implantation for structural support.

The cylinders used for this study were manufactured to ensure acceptable mechanical compressive strengths according to ASTM F451-95 and ISO 5833 [6, 7]. SEM showed that drug powders could be identified on the surface and presumably within the cement. In a previous study we demonstrated, using SEM coupled with EDAX, that powders identified on the surface of chemotherapeutic drug-supplemented cylinders contained drug [5].

Evaluation by HPLC of the drug released from the MTX-PMMA mixtures showed that the highest proportion of drug is released in the first five minutes (Table 2 and Fig. 3). A rapid decay in the rate of MTX elution occurred for all concentrations of drug after the first 5 min, and the release was dependent on the concentration used. However, MTX continued to be released up to 30 days for all concentrations of MTX used with a diffusion coefficient remaining constant over the time and being not significantly different amongst the various mixtures tested (Fig. 3). Furthermore, the results demonstrated that the mixture containing the lowest concentration of drug released the highest percentage of it during the time course compared with the mixtures containing higher amount of drug (Table 2). We believe that, taken together, these findings suggest that at the beginning the drug present on the surface of the cement is likely eluted and it relates to the concentration of drug used. At later time points, the efficiency of the MTX elution is influenced by the amount of the drug incorporated into the cement and our results support the hypothesis that at increasing concentrations of drug higher amount of it are entrapped into the PMMA and could not be released through the cement pore. Further experiments will be needed to confirm this hypothesis.

We also verified and confirmed that MTX is released in an active form able to exert a toxic effect on breast cancer cells. This effect, although progressively reduced over time, was still evident, in our experimental model, after 30 days incubation in an aqueous solution (cell culture medium) changed every day (Table 3), thus mimicking the in vivo situation.

Finally our results also demonstrated for the first time that the addition and the subsequent elution of MTX likely does not alter the mechanical properties of cement. In fact, compressive strength of cement was not reduced at any of the concentration of MTX used (Tables 4, 5). On the contrary, within a range of MTX concentration the compressive strength appeared to be significantly improved by the addition of drug and was not further affected by its (Tables 4, 5). Although unexpected, this finding was not surprising. In fact, it is an agreement with a previous report by Raimondi et al. demonstrating that the addition of antibiothics to cement increases the compressive strength of bone cement [10]. The latter Authors attributed this effect to the good integration of added powders in the structure of final material which might well occur also with MTX, although our results cannot definitively prove it.

In conclusions, our results support the clinical suitability of MTX-supplemented cement as a relevant therapeutic option for the treatment of bone metastases and justify the need for further studies to conclusively evaluate the distribution of MTX within the cement and its effect on the mechanical properties of the mixture, especially in term of tensile properties and fatigue which, together with the compressive properties of bone cement, play a relevant role in the clinical setting.

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