■ RESEARCH

The efficacy of chemical adjuvants on giant-cell tumour of bone

AN *IN VITRO* STUDY

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Various chemicals are commonly used as adjuvant treatment to surgery for giant-cell tumour (GCT) of bone. The comparative effect of these solutions on the cells of GCT is not known. In this study we evaluated the cytotoxic effect of sterile water, 95% ethanol, 5% phenol, 3% hydrogen peroxide (H_2O_2) and 50% zinc chloride ($ZnCl_2$) on GCT monolayer tumour cultures which were established from six patients. The DNA content, the metabolic activity and the viability of the cultured samples of tumour cells were assessed at various times up to 120 hours after their exposure to these solutions.

Equal cytotoxicity to the GCT monolayer culture was observed for 95% ethanol, 5% phenol, 3% H_2O_2 and 50% $ZnCl_2$. The treated samples showed significant reductions in DNA content and metabolic activity 24 hours after treatment and this was sustained for up to 120 hours. The samples treated with sterile water showed an initial decline in DNA content and viability 24 hours after treatment, but the surviving cells were viable and had proliferated. No multinucleated cell formation was seen in these cultures.

These results suggest that the use of chemical adjuvants other than water could help improve local control in the treatment of GCT of bone.

Giant-cell tumour (GCT) of bone usually affects the meta-epiphyseal region of long bones and has been found to involve almost every bone in the body in skeletally mature individuals. The mainstay of treatment of these tumours is intralesional resection, augmented with high-speed burring of the tumour cavity. Adjuvant treatment has been advocated in an attempt to reduce the rate of local recurrence, which can be as high as 50%. A wide variety of adjuvant options are commonly used, including cautery, cryotherapy, pre-operative embolisation, bone cement, bisphosphonates and argon beam coagulation.[1-6]

Chemical adjuvants such as ethanol, hydrogen peroxide (H_2O_2), phenol, zinc chloride ($ZnCI_2$) and sterile water have also been used[7-11] and are applied during surgery following curettage and burring of the involved bone. Cyclic washing of the tumour cavity is carried out with the chosen adjuvant, care being taken not to expose healthy tissue to the solution.

Most descriptions of the efficacy of these adjuvants are of clinical studies wherein one or more adjuvants have been used in conjunction with surgery and the outcome has been evaluated by determining the rate of local recurrence of the tumour. The effect of H_2O_2 and phenol have been studied on primary culture of samples of GCT and on soft-tissue sarcoma-derived cell lines (ESA 1 and ESA 2), respectively.[12,13] The tumour samples showed a decrease in metabolic activity, protein content and cell number in a dose-dependent manner when exposed to varying concentrations of H_2O_2 between 0.1 and 1000 mM. The cell content and metabolic activity were reduced by 83% and 90%, respectively, after exposure to 100 mM H_2O_2 for 48 hours. This concentration is much lower than the 3% (880 mM) commonly used in the clinical setting.[12] Treating ESA 1 and ESA 2 cells with 5% phenol resulted in the death of over 90% of the cells. A small increase in anti-tumour activity was noted with higher concentrations of 7% to 10%.[13] These studies suggest that chemical adjuvants may be appropriate in the treatment of GCT.

The purpose of this study was to determine which of the commonly used chemical adjuvants was more cytotoxic and would be the preferred choice as adjuvant treatment.

Patients and Methods

Tissue. Fresh GCT tissue was obtained from six patients at the time of surgery. All had a primary tumour, the diagnosis being confirmed histologically by an experienced

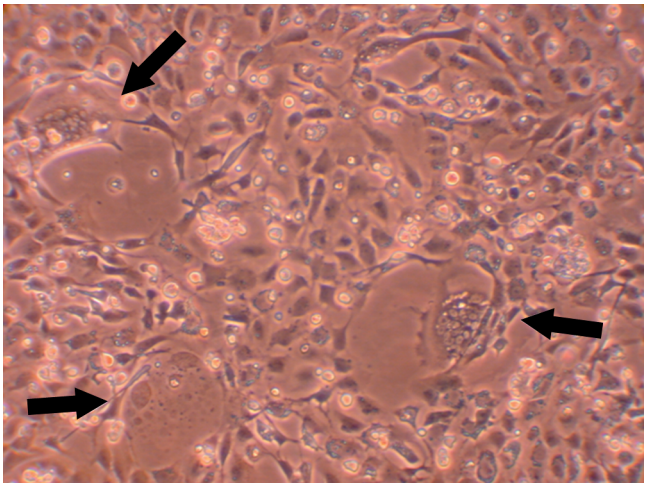

Fig. 1

Morphological appearance of cells isolated from giant-cell tumour after 24 hours in a monolayer culture. Both multinucleated giant cells (arrows) and mononuclear cells are seen (phase contrast microscopy, 40 × magnification).

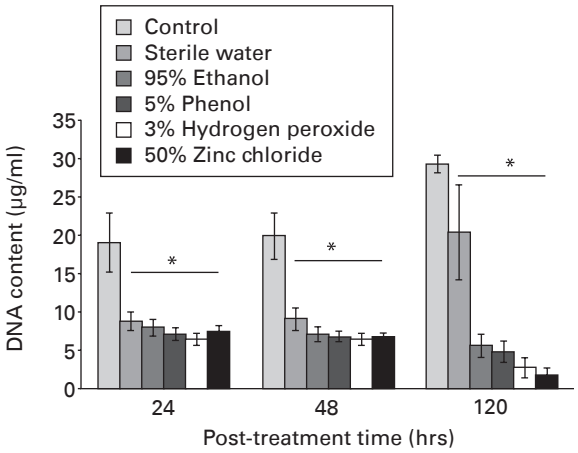

Fig. 2

Change in cellularity as measured by DNA content (ug/ml) over time of the giant-cell tumour samples treated with different adjuvant therapies. All six cases were analysed; each condition was performed in triplicate and repeated two to four times. The results were pooled and expressed as the mean ± standard error of mean. At all time points a significant reduction in cellularity was noted between the control samples and the treated samples (* $p = 0.007$, $p = 0.014$ and $p = 0.002$ at 24, 48 and 120 hours, respectively). Bar and asterisk refer to the difference between the DNA content of the treatment samples as compared to the sixth bar-control sample.

musculoskeletal pathologist (RAK). Consent was obtained from the patients prior to surgery and the use of the tissue was approved by the institutional ethics research board.

Cell culture. The fresh tissue was minced and digested in 1% collagenase and 1% dispase for two hours at 37°C. The isolated cells were placed in monolayer culture at a density of 3×10^5 cell/cm^2 in αMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After culture overnight the cells were harvested by digestion with 0.5% trypsin to obtain a mononuclear cell-enriched population. The multinucleated cells resist trypsin harvest. These cells were then plated in culture at the same density. At approximately 90% confluence the cell cultures, which contained both mono- and multinuclear components, were treated with three one-minute washes of αMEM medium, sterile water, 5% phenol, 95% ethanol, 3% H_2O_2 or 50% $ZnCI_2$ to mimic the clinical situations. The cell cultures were then returned to αMEM-containing FBS and penicillin/streptomycin and maintained for up to five days under standard conditions for tissue culture. Control specimens were prepared in an identical manner.

DNA content. In order to assess the effect of treatment on cellularity, cell samples were harvested at different times up to five days after treatment and digested in papain (Sigma; 40 µg/ml, 20 mmol/l ammonium acetate, 1 mmol/l EDTA and 2 mmol/l dithiothreitol) for 48 hours at 65°C. The DNA content was determined from aliquots of the papain digest using the Hoechst 33258 dye binding assay (Polysciences, Warrington, Pennsylvania) and fluorometry (emission wavelength 458 nm, excitation wavelength 365 nm) as described previously.[14]

MTT assay. In order to assess metabolic activity, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay was performed on cultures 24, 48 and 120 hours after treatment. This assay is based on the ability of viable cells to produce formazan from the cleavage of the tetrazolium salt by functional mitochondria. After 45 minutes incubation with MTT at 37°C, the cells were lysed with dimethyl sulphoxide (DMSO) and the formazan crystals dissolved. Absorbance was read at 550 nm using a spectrophotometric microplate reader.

Viability assay. Cell viability was measured at 24, 48 and 120 hours after treatment for the various groups using the Live/Dead kit according to the manufacturer's guidelines (Molecular Probes, Invitrogen, Carlsbad, California) under fluorescence microscopy. With this assay the nuclei of dead cells stain red, whereas the cytoplasm of live cells stains green. Dual staining can be observed in dead cells.

Statistical analysis. All the tests were carried out in triplicate and repeated two to four times for each sample, except for the Live/Dead assay, which was performed only once. The differences between the treatment groups and between various times of testing were calculated with one-way analysis of variance followed by Dunn's multiple comparison test for *post hoc* analysis. Significance was assigned at $p < 0.05$.

Results

GCT cell culture. The mononuclear cells were round and spindle shaped. There were a variable number of multinucleated cells, the nuclei of which were similar in appearance to those of the mononuclear cells. No differences in morphology between the different cases were identified by phase contrast microscopy (Fig. 1).

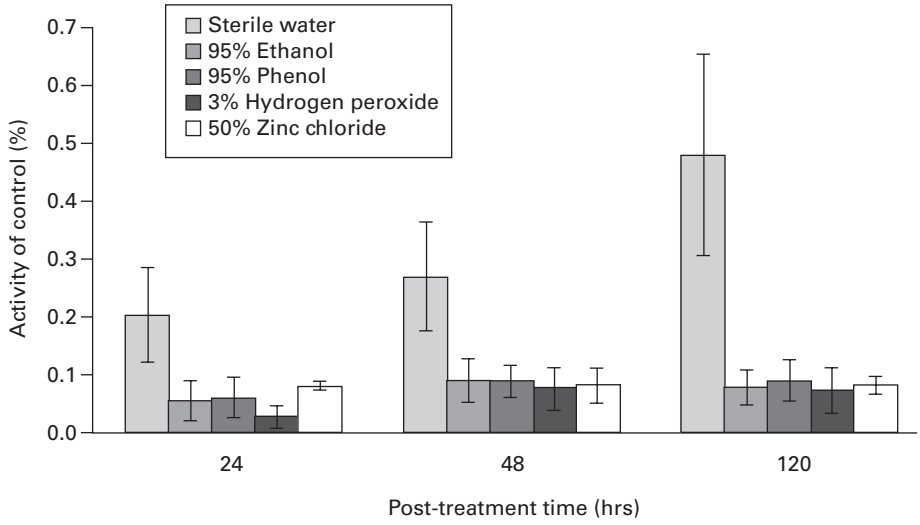

Fig. 3

Metabolic activity over time of the giant-cell tumour samples treated with specific adjuvants compared to untreated samples. All six cases were analysed; each condition was performed in triplicate and repeated four times. The results were pooled and expressed as the mean ± standard error of mean. At all time points a significant reduction in metabolic activity was noted between the control samples and the treated samples (* p = 0.001, 0.005 and 0.004, respectively, at 24, 48 and 120 hours). Bar refers to the significance in activity as measured between the control group and treatment samples as a group on ANOVA.

DNA content following treatment. The effect of the different treatments was evaluated by determining the DNA content following application of the particular adjuvant over time. By 24 hours the mean DNA content (Fig. 2) had decreased by 33.4% to 46.1%, depending on the adjuvant, in all adjuvant-treated samples (p = 0.007) compared to the untreated control samples, (19.1 (SD 3.9)) ug/ml in the control samples *versus* 6.4 (SD 0.7) to 8.8 (SD 1.1) ug/ml in the treated samples). There was a progressive decline in the mean DNA content in samples treated with ethanol, phenol, H_2O_2 and $ZnCI_2$ by 120 hours after treatment (29.4 (SD 1.1) ug/ml in control sample *versus* 2.0 (SD 1.3) to 5.7 (SD 1.4) ug/ml in treated samples, p = 0.002). The mean DNA content in samples treated with sterile water decreased to 46.1% of the controls (8.8 (SD 1.1) ug/ml) by 24 hours, but increased to 69.8% (20.5 (SD 6.2) ug/ml by 120 hours, although this increase failed to reach statistical significance (p = 0.292).

Effect of chemical adjuvants on metabolic activity. The mean metabolic activity of treated cells decreased significantly following treatment with ethanol, phenol, H_2O_2 and $ZnCI_2$ (Fig. 3). It ranged between 2.5% (SD 1.9) and 8.0% (SD 1.1) of the mean value of untreated cells (p = 0.001) at 24 hours. The cells did not recover, as the mean metabolic activity remained depressed at 120 hours, ranging from 7.1% (SD 3.9) to 8.8% (SD 2.9) of the activity of untreated cells (p = 0.001). However, cells treated with sterile water showed an initial decline in mean metabolic activity to 20.2% (SD 8.2) of that of the control cells by 24 hours, but they recovered and the mean activity increased to 47.5% (SD 17.3) of that of controls by 120 hours, although this change failed to reach statistical significance (p = 0.357).

Effect of adjuvant treatment on cell viability. The cells in the untreated cultures were mostly viable. However, all the treated samples showed many dead cells in the Live/Dead assay compared to the control samples. The samples treated with sterile water showed no viable multinucleated cells. Although there were more dead mononuclear cells than in the untreated control samples, many viable cells were seen. Solutions of 95% ethanol, 5% phenol, 3% hydrogen peroxide and 50% zinc chloride were equally cytotoxic. Both mononuclear and multinucleated cells were equally sensitive to these treatments. Only occasional viable cells could be seen in the cultures 24 and 120 hours after treatment. The sensitivity of the samples to the different treatments was variable. In some tumour samples no viable cells could be found, whereas in others clumps of viable cells remained in the culture after treatment (Fig. 4). By 24 hours after treatment 30% of the mononuclear cells were dead (Figs 5a and 5b). At 120 hours no multinucleated cells were found in the culture, but the mononuclear cells were over 95% viable.

Treatment with H_2O_2 appeared to affect both the mononuclear and the multinucleated cells. After 24 hours in culture most of the mononuclear cells that were still attached to the culture plate were not viable. The multinucleated cells that remained attached were also not viable (Figs 5c and 5d). After 120 hours no mononuclear component was seen. The multinucleated cells were still attached, but were not viable.

Discussion

The treatment of GCTs of bone remains a challenge. Despite meticulous surgery, use of a high-speed burr, chemical adjuvants and cement, and reconstruction with bone

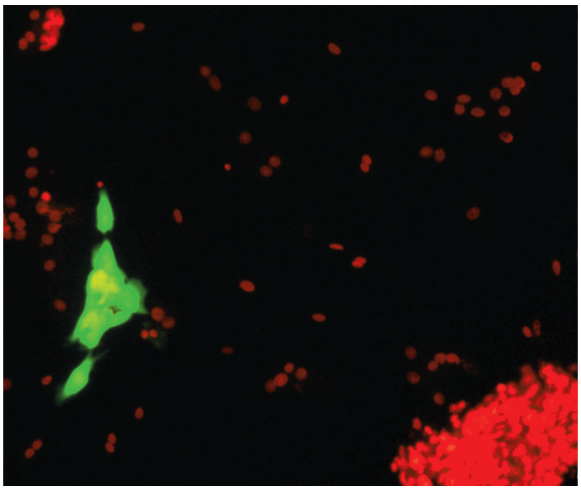

Fig. 4a

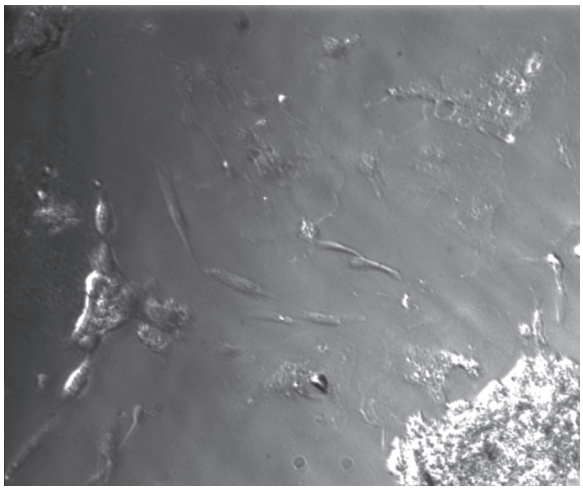

Fig. 4b

Live/Dead assay showing a fluorescent a) and a phase contrast b) sample treated with 95% ethanol 24 hours after treatment. The majority of the cells are not viable (red-staining nuclei). On the left-hand side of the image a few viable cells remain (green-staining cells, 40 ? magnification).

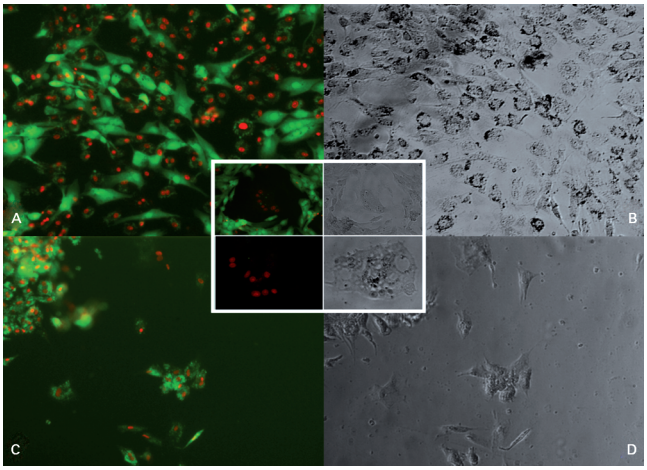

Fig. 5

Live/Dead assay showing fluorescent (A and C) and phase contrast (B and D) Images of cells 24 hours after treatment treated with sterile water (A and B) and 3% H_2O_2 (C and D). Sterile water affects both cell components in culture. Mononuclear cells that are viable stain green at 24 hours (A), whereas the multinucleated cells are not viable (red-staining nuclei, inset A). H_2O_2-treated cells are not viable at 24 hours and a large proportion of the cells are detached. The multinucleated cells are not viable (red-staining nuclei, inset C). Cells that show dual staining are considered not viable.

graft, the recurrence rate still fluctuates between 8% and 50%.[1-6] Superior results have been seen with cryosurgery, where recurrence rates as low as 2.3% have been described, but specific complications occurred in 5.9% of patients.[15]

In most clinical studies involving chemical adjuvants they are used in conjunction with another form of treatment, either chemical or mechanical, such as electrocautery, an argon laser or embolisation, which precludes evaluation of the individual cytotoxic effect of the chemical treatments.[10,11]

We compared the efficacy of different clinical adjuvants using an *in vitro* model of a monolayer culture of cells isolated from tissue of a GCT. We showed that all the chosen adjuvants had similar cytotoxic effects. The DNA content and the metabolic activity were significantly decreased in the treated samples compared to controls. The results of the Live/Dead assay revealed that the ratio of viable to dead cells was < 5%. Sterile water appeared to be less effective than the other adjuvants, as the cells that survived treatment recovered and proliferated. Cell death following this treatment is probably due to decreased osmolarity, and the cells able to withstand this change survive.

No multinucleated cells were viable at 24 hours after treatment, and multinucleated cells had not reformed by five days. This may be due to the low density of the remaining cells in culture, which was not high enough to support the formation of new multinucleated cells. Alternatively, this inability to form multinucleated cells may be because all the monocytes had been killed. This seems a more plausible explanation, as with the continuous passaging of these cells under normal culture conditions, the mononuclear cells proliferate but no multinucleated cells are formed, presumably because there is no influx of new monocytes.

H_2O_2 was toxic to both the mono- and the multinucleated cells but affected the GCT cells differentially, in that the mononuclear cells detached from the culture plates, whereas at 120 hours after treatment the non-viable multinucleated cells were still attached. In all samples viable cells could be found at all the time points, but cell proliferation was only evident in culture following treatment with sterile water. This may be due to a sustained effect of the adjuvants on the cells compared to sterile water, and may help explain their potential effectiveness *in vivo* following the typical short clinical exposure in the bone defect following

excision of the tumour. The use of H_2O_2 appears to be especially attractive in this setting, as it has a short half-life, is produced by certain cell types *in vivo*, and could be effective at lower concentrations, thereby lowering its toxicity on surrounding healthy tissue.[12] Nicholson et al[12] found that a concentration of H_2O_2 of 0.34% had the same effect as the concentration of 3% commonly used clinically.

Others have shown that H_2O_2 affects GCTs *in vitro*.[12,16] Quint et al[13] showed that 5% phenol is highly cytotoxic to soft-tissue sarcoma cell lines cultured in a monolayer. There was no significant cytotoxic advantage with higher concentrations of phenol, although solubility became a problem. The same group examined whether instillation of phenol into the tumour cavity after curettage carried a systemic risk for the patient. After measuring the rate of urinary excretion of phenol in patients treated with intralesional curettage for benign bone tumours augmented by instillation of 5% phenol, they found that an average of 2% of the phenol instilled in the bone is excreted in the urine. The effect of this is unknown.[17]

No studies have addressed the toxicity of ethanol, sterile water or Z_nCI_2 chloride on giant cell or other bone tumours *in vitro*. Alcohol is known to be toxic to bone and is associated with osteonecrosis of the femoral head.[18] Cultures of murine bone marrow cells treated with ethanol showed a decrease in the activity of alkaline phosphatase, the osteocalcin content and levels of osteocalcin mRNA expression, whereas increased numbers of empty osteocyte lacunae in the subchondral region of the femoral head were observed *in vivo*.[18] When used as an adjuvant with curettage and a high-speed burr, ethanol results in a recurrence rate of 9.5% to 20%.[7,8] The use of H_2O_2 and $ZnCI_2$, either alone or combined with other adjuvants with curettage and burring, resulted in recurrence rates of between 6.4% and 13%.[10,11]

Little information is available on the relative toxicity on the adjacent articular cartilage of the chemicals tested in this study. There is evidence that H_2O_2, ethanol and phenol cause inflammatory changes and can cause cell death in cartilage and chondrosarcoma-derived cell lines.[19,20] In one study direct injection of 10% ethanol into the joints of rats elicited minimal inflammation.[21] We would suggest caution when considering the use of chemical adjuvants in cases where there is an uncontained bone defect or when the joint has to be explored, such as when an intra-articular fracture is present.

When translating the results obtained in this study into clinical practice it must be remembered that cells cultured in a monolayer could be more susceptible to adjuvants. *In vivo*, the tissue penetration of the different adjuvants may be more limited. Also, the relative sensitivities of the different samples of tumour to the treatment regimen showed considerable variability. In order to determine definitively which chemical adjuvant is most effective in the clinical setting, a prospective study would be necessary, where the surgical method is strictly controlled and the chemical adjuvant is the only variable in the treatment. However, given the limited numbers of patients with this disease and the variation of treatments at different centres, it is unlikely that such a study with sufficient power would be feasible.

Our results suggest that 95% ethanol, 5% phenol, 3% H_2O_2 and 50% $ZnCI_2$ are equally cytotoxic to GCT *in vitro*. We suggest the use of H_2O_2, as it has a short half-life compared to the other chemicals tested in this study. Sterile water is not recommended to be used alone as an adjuvant as it was the least effective.

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