The handle http://hdl.handle.net/1887/43475 holds various files of this Leiden University dissertation

**Author:** Verdegaal, Suzan H.M.  
**Title:** Diagnostics and treatment options in low-grade central chondrosarcomas  
**Issue Date:** 2016-10-12
Chapter 3
The cytotoxic effect of phenol and ethanol on the chondrosarcoma-derived cell line OUMS-27, an in vitro experiment
Summary

Surgery is considered to be the most effective treatment for cartilaginous tumours. In recent years, a trend has emerged for patients with low-grade tumours to be treated less invasively using curettage followed by various forms of adjuvant therapy. We investigated the potential for phenol to be used as an adjuvant. Using a human chondrosarcoma-derived cartilage-producing cell line OUMS-27 as an in vitro model we studied the cytotoxic effect of phenol and ethanol. Since ethanol is the standard substance used to rinse phenol out of a bone cavity, we included an assessment of ethanol to see whether this was an important secondary factor with respect to cell death. The latter was assessed by flow cytometry. A cytotoxic effect was found for concentrations of phenol of 1.5% and of ethanol of 42.5%. These results may provide a clinical rationale for the use of both phenol and ethanol as adjuvant therapy after intralesional curettage in low-grade central chondrosarcoma and justify further investigation.

Introduction

Malignant cartilaginous tumours are the second largest group of primary bone tumours. Approximately 90% of chondrosarcomas are of the conventional type while the remainder in decreasing order of malignancy are dedifferentiated, extra-osseous, juxtacortical, mesenchymal and clear-cell chondrosarcomas. An enchondroma is a hyaline cartilaginous tumour which is found centrally in the medullary cavity and may contain some secondary calcification and/or ossification. The malignant counterpart is conventional chondrosarcoma, which is subdivided into a peripheral and central subtype according to the clinicoradiological presentation and the oncogenic pathway. A primary intramedullary presentation and the production of a hyaline cartilaginous matrix are the hallmarks of a central chondrosarcoma. Most arise de novo but a small subset can appear secondary to a pre-existing enchondroma (secondary chondrosarcoma). Central chondrosarcomas constitute about 75% of all chondrosarcomas, of which most are also low grade (grade I, 55%; grade II, 37%; grade III, 8%). These tumours primarily occur in adults between the ages of 30 years and 70 years with an equal gender distribution. Grade-I tumours are characterised by local destruction and a tendency to recur locally after excision without adequate margins.
They do not seem to metastasise or behave in a lethal manner except for those located at the base of the skull and in the pelvis.\textsuperscript{4} Surgery remains the most effective treatment for chondrosarcomas. Additionally, there is strong evidence that disease-free survival is closely related to the grade of the tumour and to the adequacy of the resection margins.\textsuperscript{4} With the exception of proton-beam irradiation for small lesions located at the base of the skull,\textsuperscript{6} it has not been shown that radiation therapy has any effect on chondrosarcomas.\textsuperscript{7} However, the type of surgical procedure required may vary according to the grade of malignancy and the extent of the tumour.\textsuperscript{8} Patients with grade I central chondrosarcoma may benefit from curettage given that this avoids resection and reconstruction, thus minimising functional impairment. This approach is only applicable for tumours which will not metastasise and thus only require local control.

Curettage without adjuvant therapy results in a high rate of recurrence of up to 40\% for patients with grade I tumours.\textsuperscript{4} Different forms of adjuvant therapy have been reported. Polymethylmethacrylate (PMMA) was first proposed by Persson and Wouters\textsuperscript{9} during the mid-1970s based on the hypothesis that it may kill residual tumour cells. Another advantage of using PMMA was the possibility of early weight-bearing. The maximum peripheral limit of a thermal lesion induced by PMMA varies from 2 mm to 5 mm in cancellous bone and 0 mm to 5 mm in cortical bone.\textsuperscript{10} Cryosurgery uses low temperature to induce tissue necrosis with reported cycles of freezing and thawing.\textsuperscript{11} Cryosurgery can affect tissue at least 7 mm to 12 mm beyond the surgical margin.\textsuperscript{12} The side-effects of cryosurgery are temporary nerve damage and a risk of fracture.\textsuperscript{13} By contrast, an 85\% solution of phenol (Figure 1) may be applied as adjuvant therapy with subsequent washing of the cavity with a 96\% solution of ethanol. Despite the fact that several unpublished series have demonstrated a positive effect on the local recurrence rate in the treatment of cartilaginous tumours, the direct anti-tumour effect of phenol has been debated.\textsuperscript{14,15} We have studied the cytotoxic action of phenol on different cell lines by flow cytometry in order to provide a rationale for its use as an adjuvant and to identify potentially effective concentrations \textit{in vitro}, which may be extended to clinical use. Given the standard use of ethanol to wash the phenol out of the bone cavity, we assessed whether this was an important independent factor with respect to cell necrosis. Ethanol was tested at different concentrations on chondrosarcoma cell lines to assess its effect.
Figure 1.
Diagram of the chemical formula of phenol which is a benzene ring in which one H+ is replaced by a hydroxyl group. Its chemical formula is C₆H₅OH and its structure is that of a hydroxyl group (-OH) bonded to a phenyl ring. Phenol is a carboxylic acid, a colourless crystalline solid. One of its properties is its limited solubility in water and it is known for its capacity to denature proteins.

Materials and methods

Cell lines and culture conditions

We used a chondrosarcoma-derived cell line (OUMS-27) as well as a cervical carcinoma cell line (SiHa). The characteristics of these cell lines have been reported previously. All the cells were cultured in monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies), 2 mM L-glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin (ICN Biomedicals, Aurora, Ohio) at 37°C in 5.0% CO₂. Detachment and dissociation of the cells before for flow cytometric analysis were performed when the cultures had just reached confluence. The cells were harvested using Hank’s Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, Missouri) buffered in 5 mM EDTA/0.25% trypsin (Invitrogen Life Technologies) at pH 7.2 and at 37°C, as described previously. 14 different concentrations of phenol diluted in phosphate-buffered saline (PBS) ranging from 0% to 1.5% and 14 concentrations of ethanol ranging from 0% to 47.5% were used.
Phenol and Ethanol treatment

Two sets of experiments were undertaken. In the first the SiHa cell line was used to investigate the effective range of concentration of phenol (Liquid Phenol, Ph Ned Ed VI quality; BUFA bv, Pharmaceutical Products, Uitgeest, The Netherlands) (Table 1) and ethanol (Merck, Darmstadt, Germany) (Table 2). Initially, concentrations of phenol from 0.04% to 10.63% were used, but the ranges were narrowed after cell death was observed at low concentrations. For ethanol, concentrations ranging from 0% to 96% were applied.

<table>
<thead>
<tr>
<th>phenol (%)</th>
<th>SiHa cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.6</td>
</tr>
<tr>
<td>0.65</td>
<td>56.9</td>
</tr>
<tr>
<td>1.33</td>
<td>97.1</td>
</tr>
<tr>
<td>2.66</td>
<td>98.5</td>
</tr>
<tr>
<td>5.32</td>
<td>97.7</td>
</tr>
<tr>
<td>10.63</td>
<td>88.1</td>
</tr>
</tbody>
</table>

The second set of experiments was undertaken on the OUMS-27 cell line using the concentrations defined in the first experiment. Harvested cells were subjected to 14 different concentrations of phenol diluted in phosphate-buffered saline (PBS) ranging from 0% to 1.5% and 14 concentrations of ethanol ranging from 0% to 47.5%.

Harvested cells were washed twice with HBSS and cell concentrations were determined using a Bürker counting chamber (Omnilabo, Breda, The Netherlands), 500 000 cells pelleted at 500 g (centrifugal force), for five minutes at 4°C. The supernatant was decanted and 50 μl of PBS were added to the pellet. Next, 50 μl of a phenol or ethanol dilution were added to the cells while gently stirring. The cells were subsequently incubated on ice. After five minutes, 1000 μl of PBS were added and the cells were deposited by centrifugation. They were then rewashed with PBS and finally resuspended in 500 μl of PBS containing 1.0 μM propidium iodide to stain for dead and necrotic cells. This is a cell-membrane-impermeable fluorescent dye which binds to RNA and DNA thereby allowing the presence of dead and vi-
able cells to be determined by flow cytometry. After incubation for 30 minutes on ice, the cells were ready for flow cytometric analysis (Figure 2). Each experiment was repeated four times.

| Table 2. Initial tests performed to determine the dose-effect curves of ethanol on a SiHa cell line showed that a concentration of ethanol of 42.5% killed all cells |
|----------------|----------------|
| ethanol (%) | SiHa cell death (%) |
| 0 | 2.52 |
| 0.65 | 2.20 |
| 1.33 | 1.46 |
| 2.66 | 1.38 |
| 5.31 | 1.80 |
| 10.63 | 1.86 |
| 21.25 | 27.80 |
| 42.5 | 99.54 |
| 63.75 | 99.78 |
| 85 | 99.76 |
| 96 | 99.38 |

Flow cytometry

The flow cytometric analysis of stained cells was performed as described previously. Briefly, for each measurement 10 000 events were collected using a standard FACS-calibur (BD Biosciences, San Jose, California) flow cytometer. Autofluorescence was measured using the green fluorescent parameter (FL1, 530/30 nm). Propidium iodide fluorescence was collected using the deep-red fluorescence detector (FL3, > 670 nm). Simultaneously, forward-scatter and side-scatter data were monitored and collected. Listmode files were analysed using WinList 6.0 software (Verity Software House Inc, Topsham, Maine).

Statistical analysis

For each experiment, the observed data consisted of a series of X and Y values where X was the concentration of phenol or ethanol and Y was the percentage of cells kil-
The cytotoxic effect of phenol and ethanol on the chondrosarcoma-derived cell line

Figure 2. Flow cytometric dot plots of forward scatter (abscissa) vs propidium iodide fluorescence (dead cells, ordinate) showing the death and disintegration of OUMS-27 cells after exposure to increasing concentrations of phenol and ethanol. Upper row, increasing concentrations of phenol: A=0.2%; B=0.4%; C=0.6%; D=0.9%; E=1.2%; F=1.5%. At a concentration of phenol of 1.5% the cells were killed. Lower row, increasing concentrations of ethanol: G=10%; H=20%; I=25%; J=30%; K=35%; L=45%.

A logistic regression curve was applied to these data points, usually around 15 points, using the standard formula: $$Y = Y_{\text{min}} + (Y_{\text{max}} - Y_{\text{min}}) \frac{1}{1 + \exp(-\text{slope}(X - X_{\text{half}}))}$$ where $Y_{\text{min}}$ was the estimated horizontal asymptote of the curve for very low values of $X$, $Y_{\text{max}}$ was the estimated horizontal asymptote of the curve for very high values of $X$, slope was the parameter to quantify the curvature of the logistic curve and $X_{\text{half}}$ was the concentration where the curve had maximum slope.

The values of lethal dose $xx$ ($x$) when $xx$ was 50% or 95%, were calculated using the estimated parameters by determining $X$ when $xx$ denoted the relative distance between $Y_{\text{min}}$ and $Y_{\text{max}}$.

The experiments showed that both the mean lethal dose $xx$ and its associated 95% confidence interval could be computed, thus summarising the evidence from all experiments together.
Figure 3.
Graph showing 100% cell death at concentrations of phenol higher than 1.4%. Lethal dose$_{50}$ and lethal dose$_{95}$ were corrected for the relatively high amount of cell death at T$_0$.

Results

The first series of experiments with different concentrations of phenol and ethanol on SiHa cells showed that a concentration window of 0% to 1.5% for phenol and 0% to 47.5% for ethanol could be used.

In experiment 2, this concentration range was applied to the chondrosarcoma cell line. For phenol, 100% of OUMS-27 cells were killed by a concentration of phenol of 1.5%. This experiment was repeated four times with identical results (Figure 3). In these tests the proportion of cells dying without exposure to any toxic insult ranged from 20% to 37% because of the relatively high vulnerability during the harvesting of these cells. The data in Figure 2 are presented as logistic saturation curves, which also show the dose effect. Due to the relatively high amount of death of OUMS-27 cells by T$_0$, the lethal dose$_{50}$ was corrected as explained earlier.

In the 14 different concentrations of ethanol tested on 500 000 cells per test, five repeated tests all OUMS-27 cells were killed by a concentration of ethanol of 42.5%.
Figure 4.
Graph showing 100% cell death with higher concentrations of ethanol. Lethal dose$_{50}$ and lethal dose$_{95}$ were corrected for the relatively high amount of cell death at T$_0$.

and higher. The dose-effect curves for ethanol in the tests, which were performed separately, are presented in Figure 4. For ethanol a correction was made in calculating the lethal dose$_{50}$ and lethal dose$_{95}$.

Discussion

In recent years, intralesional curettage of low-grade central chondrosarcoma followed by local adjuvant therapy has become an accepted method of treatment. In chondrosarcoma, the use of cryotherapy has been shown to have particularly good clinical results with regard to local control of the tumour but serious complications may occur. The effect of using phenol as an adjuvant in low-grade chondrosarcoma has been debated in the literature. Surprisingly, the effect of ethanol as adjuvant therapy has never been investigated.

Our study has shown that both phenol and ethanol can kill chondrosarcoma cells in vitro. For phenol, all the cells were killed by a relatively low concentration of 1.5%.
Ethanol also had the potential to kill cells when used as a neo-adjuvant in the treatment of low-grade chondrosarcoma. In our study, all OUMS-27 cells were killed by ethanol in vitro using concentrations of 42.5%.

Our study used OUMS-27, a cartilage-forming chondrosarcoma-derived cell line.\textsuperscript{16} While we acknowledge that all tests were performed in an in vitro environment, OUMS-27 may be regarded as the most appropriate cell line currently available given that it retains its cartilaginous phenotype in vitro, thereby simulating the clinical situation as closely as possible. The high number of dead cells at the start of the tests is comparable with that of other recorded experiments with this cell line, which is known to be quite fragile. Test III of experiment 2 (phenol vs OUMS-27) showed a curve, which, while starting later than the other four tests performed, displayed the same slope and also resulted in 100% cell death.

Normally, using flow cytometry, a forward scatter threshold is set in order to reduce the collection of system noise during measurement. Once a cell is prone to necrosis, it becomes permeable to propidium iodide, which can be readily measured by flow cytometry. However, when cells are exposed to a high concentration of phenol or ethanol, they disintegrate completely into small fragments, which generate a similar scatter. As a result, the final percentage of cells killed decreases by a few percent from 100\% (Figures 3 and 4).

There have been a few reports\textsuperscript{13,14} on the testing of the cytotoxic effects of phenol on cartilage-forming tumours. These studies, however, have focused on the necrotising effect of phenol, rather than impaired tumour cell viability. Lang et al.\textsuperscript{14} and Lack et al.\textsuperscript{15} concluded that while phenol might kill different benign and malignant cells it had no cytotoxic effect on cartilaginous tumours. They suggested that this was probably the result of the cartilaginous matrix.

In an investigation of the necrotising effect of phenol on vertebral bodies of fresh animal cadavers a defect was made in four vertebrae and phenol in different concentrations was applied to the defects for 30 to 120 seconds.\textsuperscript{14} Necrosis was measured by determining the thickness of the cell layer demonstrating nuclear pyknosis or necrosis. The cellular effects on the bone-marrow cells were evaluated visually and the thickness of necrosis was measured microscopically. At concentrations of phenol of 10\% to 25\%, cells started to show aberrant nuclear structures. At concentrations of 50\% to 75\% there was a uniform zone of necrosis with a width of 630 to 747 μm. At a concentration of phenol of 90\% less necrosis was identified when judged by the width of the zone of necrosis. In their discussion, the authors doubted whether a zone of necrosis of 0.7 mm to 0.8 mm was generally sufficient to prevent local recurrence.
In another study on the necrotising effect of a solution of phenol of 75% in 20% ethanol on normal tissue, which had been harvested during surgery or at post-mortem, cell death was also determined by measuring the thickness of the cell layers demonstrating nuclear pyknosis or necrosis using light microscopy, and taking ocular measurements in μm. It was concluded that phenol induced varying extents of necrosis within different tissues, but that it did not have any apparent effect on cartilaginous tissue or cartilaginous tumours in any case.

By contrast, our study did not focus on the necrotising effect of phenol but instead investigated how phenol impaired tumour-cell viability. It showed that both phenol and ethanol had profound cytotoxic potential on \textit{in vitro} chondrosarcoma cell lines. Furthermore, it also indicated that this potential had a concentration range, which could be applied in a clinical setting. On this basis we plan to perform an additional investigation into the effect of different concentrations of phenol and ethanol on chondrosarcoma pellets in which the matrix between the chondrosarcoma cells is present.
Acknowledgements

We want to thank A. Yavas for her expert technical assistance, and R. Brand for his help with the statistics. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.
References