Antineoplastic activity of povidone-iodine on different mesothelioma cell lines: results of in vitro study†

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Abstract

OBJECTIVE: Povidone-iodine (PVP-I) or Betadine, owing to its antineoplastic activity, is also used as an adjuvant during intra-abdominal or intrathoracic surgery. However, the protocol of PVP-I administration has not been optimized to achieve the best antitumoural efficacy. We aimed to determine the optimal concentration of PVP-I, the time of incubation and the mechanism of cell death by analysing the effect of different doses and time of administration of PVP-I on the cell viability of different mesothelioma cell lines.

METHODS: Four different cell lines (MET 5A/normal mesothelium; H2052/sarcomatoid mesothelioma; ISTMES2/epithelial mesothelioma; MSTO/biphasic mesothelioma) were incubated with increasing concentrations of diluted PVP-I (0.0001; 0.001; 0.01; 0.1; 1%) for 5, 10, 30, 60 min and 24 h, respectively. Cell viability was determined using cell direct cytotoxicity assay and cell death was determined through flow cytometry assay analysis. The superoxide dismutase activity was assessed functionally through a specific inhibitor to evaluate the mechanism of cell death.

RESULTS: The antiproliferative effect of PVP-I varied largely among different cell lines in a dose- and time-dependent manner. At 0.1% concentration for 10 min of incubation, the percentage of viable cells was 0.5 ± 0.1; 0.8 ± 0.5 and 0% (P < 0.01) for MET5A, ISTMES2 and MSTO, respectively. Conversely, the same concentration did not significantly affect the H2052 cell line which was completely suppressed at a 1% concentration of PVP-I. Double staining of Annexin V and DNA showed that PVP-I induced cell death in all four cell lines via necrosis depending on PVP-I concentration. However, H2052 was found to be more resistant than MSTO, ISTMES2 and MET 5A cells lines. The activity of superoxide dismutase was significantly inhibited in all cell lines.

CONCLUSIONS: Our results confirmed the anti-neoplastic activity of PVP-I especially on ISTMES2 and MSTO cell lines. With respect to chemotherapy pleural irrigation, washing with PVP-I is cost-effective and easy. If confirmed by larger studies, our findings suggest that the intrapleural irrigation with PVP-I (0.1% concentration for 10 min) in patients with epithelial or biphasic mesothelioma undergoing cytoreductive surgery might be applied in thoracic surgery practice to prevent neoplastic cell growth.

Keywords: Povidone-iodide • Betadine • Mesothelioma

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare and aggressive thoracic malignancy associated with poor prognosis and predicted to increase in incidence in the next decades. Patients with MPM who are not treated surgically survive at the most 12 months if they are offered the best palliative treatment [1]. A standard treatment protocol for MPM has not yet been developed, and in particular, the role of surgery including extrapleural pneumonectomy (EPP) or radical decortication, as an alternative to EPP in unfit patients, still remains controversial.

Despite attempting different multimodality therapies including surgery, MPM recurs in most patients because the mostly diffuse malignant infiltration of the surrounding tissue strictly prevents a radical resection with histologically free resection margins. Thus, other treatments such as intrathoracic perfusion chemotherapy alone or associated with hyperthermic perfusion have been applied after cytoreductive surgery with good results [2, 3]. However, such techniques are not available in all hospitals, the
been demonstrated antitumour effects on breast cancer and MPM cells have also been demonstrated in vitro [6–8]. Yet, its antitumour effects on breast cancer and MPM cells have also been demonstrated in vitro [9, 10]. The aim of the present study was to perform a comparative analysis of cell death induction by PVP-I in different cell lines of MPM focusing on dose and time of incubation dependency and the mechanism of cell death.

MATERIALS AND METHODS

Study design

To investigate the effects on proliferation and cell death of diluted PVP-I on MPM, four different cell lines (MET5A/normal mesothelium; H2052/sarcomatoid mesothelioma; ISTMES2/epithelial mesothelioma; MSTO/biphasic mesothelioma) were incubated with increasing doses of PVP-I diluted in fresh medium (0.0001; 0.001; 0.1; 1%) starting from a 10% PVP-I solution in distilled water, for different times (5, 10, 30, 60 min and 24 h). Cytotoxicity assay was used to examine the proliferation; flow cytometry was performed to detect the apoptotic rate; superoxide dismutase (SOD) activity was assessed functionally through a specific inhibitor to evaluate the mechanism of cell death.

The aim was to establish the time of incubation and the concentration of PVP-I that allowed a complete cell killing in order to evaluate its potential application in clinical practice.

Cell line and culture conditions

Four commercially available MPM cell lines were used in the present study: MET5A, ISTMES2, MSTO and H2052 cell lines (1.5 × 10^5 cells/ml) were seeded in a 6-well plate and incubated for 24 h. Cells were then treated with five different concentrations of PVP-I (0.0001; 0.001; 0.01; 0.1; 1%) and incubated for 24 h. Next, cells were harvested and processed according to the instruction of the SOD kit (Nanjing Jiancheng Biotechnology Institute SOD kit (detect total), Nanjing, China). The activity of SOD was assessed by an ultraviolet spectrophotometer (SmartSpec 3000, Bio-Rad, Philadelphia, USA).

Cell treatment with povidone-iodine and MTS assay

The cytotoxicity of PVP-I in all four cell lines was determined by MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). Compactly, MET5A/H2052/ISTMES2/MSTO (1.0 × 10^4 cells/well) were seeded in a 96-well plate with fresh medium and incubated overnight. MTS solution was added into each well at the indicated time points (5, 10, 30, 60 min, and for 24 h) following treatment with five different concentrations of PVP-I diluted in fresh medium (0.0001; 0.001; 0.01; 0.1; 1%). As a control, cells were cultured in fresh medium added with the maximum amount of water used to vehicle the higher drug concentration (1%). Cell viability was evaluated at different time points following the manufacturer’s instructions, through spectrophotometric reading at two different wavelengths (540 and 630 nm). All experiments were repeated with at least three consecutive passages.

Apoptosis analysis through flow cytometry

At the indicated incubation time, floating cells were collected together with the supernatant and adherent cells were harvested by trypsinization. Apoptosis was evaluated using Annexin V-FITC Kit (Milenyi Biotec, Inc., USA). Cells were analysed immediately using a fluorescence activated cell sorting (FACS) flow cytometer (FACS Calibur; BD Biosciences, Heidelberg, Germany) for Annexin V-FITC and Propidium iodide (PI) staining. For each measurement, 20,000 cells were counted. Dot plots and histograms were analysed by CellQuest Pro software (BD Biosciences). Annexin V-positive cells were considered in the early stage of apoptosis (lower right quadrant); Annexin V- and PI-positive cells were considered in the late stage of apoptosis or necrotic (upper right quadrant). Annexin V- and PI-negative cells correspond to the viable cell fraction (lower left quadrant) [11].

Analysis of superoxide dismutase activity

To study one of the probable mechanisms of PVP-I-induced cell death in tumour cells, the activity of SOD was detected with a colorimetric method [12]. MET5A, ISTMES2, MSTO and H2052 cell lines (1.5 × 10^5 cells/ml) were seeded in a 6-well plate and incubated for 24 h. Cells were then treated with five different concentrations of PVP-I (0.0001; 0.001; 0.01; 0.1; 1%) and incubated for 24 h. Next, cells were harvested and processed according to the instruction of the SOD kit (Nanjing Jiancheng Biotechnology Institute SOD kit (detect total), Nanjing, China). The activity of SOD was assessed by an ultraviolet spectrophotometer (SmartSpec 3000, Bio-Rad, Philadelphia, USA).

Statistical analysis

Data resulting from MTS and SOD activity experiments are expressed as means ± standard deviation (SD) of three independent experiments, each performed in triplicate. Statistically significant differences among the means of multiple groups were evaluated by one-way repeated-measures ANOVA, with Bonferroni post-test to compare pairs of data. The MedCalc statistical software (Version 12.3, Broekstraat 52; 9030 Mariakerke; Belgium) was used for the analysis. A P value of < 0.05 was considered statistically significant.

RESULTS

Povidone-iodine effect on mesothelioma cell growth

The effect of various incubations with different doses of PVP-I doses on mesothelioma cell growth is represented in Fig. 1 (A for...
MET5A, B for ISTMES2, C for MSTO and D for H2052). MET5A, ISTMES2 and MSTO cell lines presented sensitivities similar to that of PVP-I. The shortest time period necessary to observe a strong cytotoxic effect (cell viability <1%) on MET5A, ISTMES2 and MSTO cells was 10 min with a concentration of 0.1%. At a concentration of 0.1% and after 10 min of incubation, the percentage of cell viability was significantly decreased with respect to that observed after 5 min of incubation: 0.5 ± 0.1 vs 5.2 ± 0.8%, P < 0.05, respectively, for MET5A (Fig. 2A); 0.8 ± 0.5 vs 5.3 ± 0.5%, P < 0.05, respectively, for ISTMES2 (Fig. 2B); 0 vs 2.1 ± 0.1%, P < 0.05, respectively, for MSTO (Fig. 2C). Among all three different cell lines (MET5A, ISTMES2 and MSTO), no significant differences in cell viability were found after longer incubation times at 0.1% concentration (Fig. 2D for MET5A, Fig. 2E for ISTMES2 and Fig. 2F for MSTO) and/or using a higher concentration.

Conversely, 0.1% PVP-I concentration did not significantly affect the H2052 cell line which presented after 10 min of incubation a percentage of cell viability of 84 ± 0.4%. At the same concentration, the percentage of cell viability significantly decreased for longer incubation as follows: 71 ± 0.5% for 30 min (P < 0.05); 66 ± 0.2% for 60 min (P < 0.05); and 58 ± 0.9% for 24 h (P < 0.05) if compared with that observed after 5 min (88 ± 0.6%) and 10 min of incubation (84 ± 0.6%). However, these data showed that at a concentration of 0.1%, the H2052 cell line was highly resistant to PVP-I, having a survival rate above 50% also after 24 h of incubation (Fig. 3A). At 1% concentration of PVP-I, we observed a significant decrease of survival rate of 3.4 ± 0.2% (P < 0.05); of 2.2 ± 0.1% (P < 0.05) and of 0.7 ± 0.1% after 5, 10 and 30 min of incubation, respectively (Fig. 3B).

**Povidone-iodine induces cell death of mesothelioma cell lines**

FACS analysis revealed that PVP-I induced a dose response effect in all MPM cell lines (Fig. 4). In particular, the percentage of dead cells proportionally increased with PVP-I concentrations and, in each group, they were mainly in the late apoptotic stage or necrosis. In agreement with the MTS results, the sensitivity of the H2052 cell line was quite different from that of the other cell lines (MET5A, ISTMES2, MSTO). For instance, at 0.1% concentration, H2052 had a percentage of viability of 58%, whereas MET5A, ISTMES2 and MSTO had a percentage of cell viability of <1%; however, H2052 also showed dead cells, which were mainly in apoptotic late phase or necrosis (33%) whereas only 6% were in early apoptotic stage.

**Povidone-iodine inhibits superoxide dismutase activity in mesothelioma cell lines**

The SOD assay revealed that PVP-I induced cell death due to the oxidizing effects of free iodine on SH-OH-and NH-groups of...
amino acids and on the double bonds of unsaturated fatty acids. Among all four cell lines, the activity of SOD was significantly inhibited at a concentration of 0.1% with respect to lower concentrations (0.0001; 0.001; 0.01%), whereas no significant difference was found for a higher concentration (1%) for MET5A (A); for ISTMES2 (B) and for MSTO (C). Among all three different cell lines, no significant differences were found after longer incubation times (D for MET5A, E for ISTMES2 and F for MSTO).

DISCUSSION

PVP-I, a broad-spectrum microbicide against bacteria, virus, fungi and parasites in vitro, consists of elementary iodine bound to the carrier poly(1-vinyl-2-pyrrolidone) that enhances the solubility and provides a depot of iodine. Based on the oxidizing effects of free iodine on the NH-and SH-OH-groups of amino acids and on

Figure 2: After 10 min of incubation at a concentration of 0.1%, the percentage of cell viability was significantly decreased with respect to that observed using lower concentrations (0.0001; 0.001; 0.01%), whereas no significant difference was found for a higher concentration (1%) for MET5A (A); for ISTMES2 (B) and for MSTO (C). Among all three different cell lines, no significant differences were found after longer incubation times (D for MET5A, E for ISTMES2 and F for MSTO).
the double bonds of unsaturated fatty acids, PVP-I showed the ability of non-specifically induced cell death [12].

Over the last years, PVP-I has also been shown to exert antineoplastic activity in vitro as well as in vivo. PVP-I induces cell death in a variety of malignant cell lines derived from breast, colon, gastric and thymic carcinoma [6–9]. Clinically, many surgeons have been irrigating intraluminally with PVP-I just before resection attempting to prevent tumour recurrence after colorectal or breast cancer.

Figure 3: At 0.1% concentration, the H2052 cell line was highly resistant to PVP-I, having a survival rate of more than 50% also after 24 h of incubation (A). A significant decrease in survival rate in a time-dependent manner was observed using a higher PVP-I concentration (1%) (B).

Figure 4: Dot plots obtained by FACS analysis after incubation of MET5A, ISTMES2, MSTO and H2052 cell lines with different concentrations of PVP-I for 24 h. FACS analysis was performed for Annexin V-FITC (x-axis) and propidium iodide (PI) staining (y-axis). A representative experiment is shown out of three independent ones.
surgery [13, 14]. Others demonstrated the potential for this agent to prevent tumour implants in abdominal trocar wounds during laparoscopy for colon cancer [15].

In the literature, only one study from Opitz et al. [10] evaluated the antiproliferative effect of PVP-I on MPM cells with promising results. The authors found that PVP-I induced a necrotic phenotype along with the production of reactive oxygen intermediates, decreased mitochondrial membrane permeability and cell death, which was inhibited by the oxygen scavenger N-acetyl cysteine.

In our in vitro experiment, we investigated the effect of PVP-I on different MPM cell lines with the first end-point to define the concentration and the incubation time to obtain an antiproliferative effect on MPM cell in vitro. The second end-point was to evaluate the PVP-I mechanism of cell death induction.

We found that MET5A, ISTMES2 and MSTO had sensitivities similar to that of PVP-I and this effect showed dependence on the concentration and time of incubation. The shortest incubation time necessary to observe a strong cytotoxic effect (<1% of cell viability) on MET5A, ISTMES2 and MSTO cells was 10 min with a concentration of 0.1%. At these levels, the percentage of cell viability was 0.5, 0.99 and 0.1% for MET5A, ISTMES2 and MSTO, respectively. Similar results were obtained for longer incubations (30, 60 min and 24 h). No morphological differences were found at different times of incubation. Conversely, the H2052 cell lines resulted to be more resistant than MET5A, ISTMES2 and MSTO cell lines; to obtain a cell viability of 0.7%, a longer incubation (30 min or more) was required with a higher PVP-I concentration (1%). Our data are in agreement with those of Opitz et al. [10], who found that the cytotoxic effect obtained with PVP-I on MPM for different incubation times (7.5 min, 24 and 48 h) were comparable. Conversely, higher concentrations of PVP-I (2% or more) were reported to kill human colonic carcinoma cells [12].

The cell death fractions of MET5A, MSTO, ISTMES2 and H2025 treated with PVP-I were also measured by flow cytometry. Our results showed that PVP-I can induce cell death in a dose-dependent manner. In agreement with the MTS results, H2052 cells were more resistant than the other cell lines to the cytotoxic effect of PVP-I. However, in all cell lines studied, the reduction of cell viability was mirrored by the exclusive enhancement of late phase apoptosis or necrosis (upper right quadrant) without any significant effect on early apoptosis (lower right quadrant), suggesting that PVP-I inhibited MPM cell growth via necrosis. According to our results, similar findings were obtained in human embryonic fibroblasts [6] and in the study by Opitz et al. [10], who showed that the necrosis induced by PVP-I was due to a high production of reactive oxygen intermediates and low mitochondrial.

Figure 5: Inhibition of SOD activity (y-axis) induced by different concentrations of PVP-I (x-axis). Among the MET5A (A); ISTMES2 (B) and MSTO (C) cell lines, the activity of SOD in the 0.1% PVP-I-treated group was significantly inhibited compared with lower concentration groups and had a similar value compared with the 1%-treated group. The activity of SOD assay on H2052 cell line (D) was significantly inhibited at 1% concentration with respect to the other groups.
membrane permeability. Our findings seem striking in that mesothelioma cell lines in vivo and in vitro have been shown to be exquisitely resistant to apoptosis from a number of conventional stimuli, including hydrogen peroxide, asbestos fibres and calcium ionophore [16]. Yet, the necrosis of MPM cells induced by PVP-I could have important consequences in the clinical practice. Unlike apoptosis, necrosis induces an inflammatory response, which may recruit immune cells to the tumour site and therefore enhance the antitumoural response [17, 18].

The necrosis induced by PVP-I might be due to the inhibition of SOD activity. Consistently, we observed a dose-dependent inhibition of SOD activity in all cell lines, which was significant at both 0.1 and 1% PVP-I concentrations. Calcium and reactive oxygen species are key players during the propagation and execution phases of necrotic cell death, directly or indirectly provoking damage to proteins, lipids and DNA [19]. Thus, by clearing the superoxide anion, which promotes the cell generating oxidative stress, SOD activity counteracts damage to the cells and the consequent necrosis induction [20].

According to our results, PVP-I might be used in the future as local treatment of MPM after surgery. Considering the difficulty to have free margins from tumour after radical resection, continuous local infusion of intrapleural chemotherapy alone or in association with hyperthermic infusion has been utilized in the treatment of abdominal and thoracic mesothelioma in several studies. However, the technique is cumbersome and associated with significant morbidity. Perfusion (hyperthermic) chemotherapy requires inflow and outflow catheters, a pump system, temperature sensors, heat exchanger, filters and roller pump [8]. The set-up of chemotherapy is difficult and adds significant time to the duration of the procedure, and when performed as part of EPP, the mean operative time was above 6 h. Our results showed that PVP-I is potentially cytotoxic, and thus, theoretically the lavage of pleural cavity after cytoreductive surgery may help to clean the margin of resection from tumour cells reducing the recurrence of cancer. In contrast to intrapleural chemotherapy, which adds considerable complexity and time to cytoreductive surgery, PVP-I pleural lavage is simple, cost-effective, and adds little additional time to the procedure. The use of PVP-I should be considered when an epithelioid or biphasic mesothelioma is operated because such histological types resulted to be sensible to the antitumour properties of PVP-I with respect to the sarcomatoid MPM.

To have a valid effect, it is reasonable to lavage the pleural cavity with a PVP-I concentration not less than 0.1% for 10 min. Yet, our results on SOD activity suggested that the cytotoxic effect of PVP-I might be caused by the oxidizing effects of its free iodine. This is a key finding of our paper. In fact, as the free iodine level is dependent on the concentration of solution, diluted solutions of PVP-I have smaller iodine reservoirs and therefore less killing capacity. In addition, organic materials including proteins, red blood cells and free haemoglobin were found to inhibit the tumouricidal activity of PVP-I [9]; thus, the removal of organic material might be beneficial in surgical practice to achieve killing of tumour cell without increasing PVP-I concentration.

Despite safe, PVP-I presented an aspecific cytotoxic effect considering that inhibited the proliferation not only of malignant cells but also of the normal mesothelial cells (MET5A). This, however, could be of little importance because MPM usually interests all mesothelium and a radical resection of pleura is always performed with a curative intent. However, PVP-I might be toxic for other tissues. Wagenfeld et al. [21] reported three cases of bilateral severe loss of vision after thoracoscopic surgery involving resection of parts of one lung and instillation of 200–500 ml of Jodobac, a 10% PVP-I solution, into the thoracic cavity for disinfection and to cause scarring of the pleura for prophylaxis against pneumothorax. Thus, we advice to use a 0.1% PVP-I concentration for 10 min. This achieves a massive cytotoxic effect and presumably lacks significant toxicity considering that it is 110-fold lower than the concentration usually used for its antibiotic effect.

Since the risk of wound and/or trocar-site recurrence in patients undergoing cytoreductive surgery or having a biopsy done thoracoscopically for MPM is a well-known evidence, in theory the application of PVP-I at these levels could prevent recurrence through clearance of tumour cells.

**Study limitation**

Our study provided a theoretical basis on the antineoplastic activity of PVP-I on MPM cell lines at preclinical level. However, since commercially available MPM cell lines might have different responses compared with human MPMs, it remains to be assessed whether the data observed in vitro are reproducible in vivo, in terms of dose and incubation response, and can be effectively translated to the clinical practice. Thus, further studies need to be undertaken to assess the effect of PVP-I on MPM experimental animal models before its potential use in human clinical trials. In addition, in the present study, we used normal mesothelium (MET5A cell lines) as control. PVP-I reduced the proliferation of normal mesothelium as well as that of MPM cell lines (ISTMES2, and MSTO). In view of its potential clinical application, that may have little importance because MPM strictly interests all mesothelium and it is hard to differentiate normal mesothelium from cancer. In addition, the concentration of Betadine inducing an antitumoural effect was 0.1%, and was thus 110-fold significantly lower than the concentration usually used for its antiseptic effect (10%). However, as reported above, the end-point of this study was to determine the antineoplastic activity of Betadine in vitro providing the preclinical framework for the possible use of Betadine in the clinical setting. Obviously, future experiments aim to analyse in vivo the activity of Betadine and its possible side effect profiles in order to verify the possibility for an effective translation to the clinical practice and to define the final therapeutic dose.

**CONCLUSION**

Our data confirmed that low doses and short exposures to PVP-I have antiproliferative effects also on MPM cells via inducing cell death in a dose-dependent manner. H2052 cells, derived from the most aggressive sarcomatoid histotype, were more resistant than the other cell lines. Inhibition of SOD activity by PVP-I seems to underlie the mechanism of cell death. However, our in vitro data should be corroborated by in vivo studies in animal models of mesothelioma before recommending the intraoperative pleural lavage with PVP-I as adjuvant treatment to surgery for MPM.

**Conflict of interest:** none declared.
REFERENCES


APPENDIX A. CONFERENCE DISCUSSION

Dr M. Hoda (Vienna, Austria): I just wondered if you looked at the mechanistic part. Do you know how it works? How high is the toxicity? Did you look at the signalling? Did you look at Western blot maybe, or are you planning to do this to find out what the mode of action is, because it cannot only be necrotic or cytocidal. It should have some sort of influence biologically.

Dr Fiorelli: We did not perform Western blot in the laboratory. We used FACS analysis to show if the cells are in the early stage of apoptosis or in necrosis in the light of their position within quadrants.

Dr Hoda: I just wonder if you can find out if it influences any signalling pathways, like the MAP kinase pathway or PI3 kinase pathway? There is recently some evidence that at least the PI3 kinase (because we published that actually last year) has a role, for example. So maybe there is other evidence for this, maybe cell signal pathways to look at the cell signalling.

Dr Fiorelli: The only two SOD activity endpoints were cell viability and cell death.

Dr Hoda: Nice data anyway.

Dr Z. Bilgi (Istanbul, Turkey): I have two questions. First of all, we have also been using povidone-iodine in our clinic as pleural lavage after pleurectomy/decorticition. Have you ever had the chance to observe this in suspended cell cultures? Do you have any idea how penetrant povidone-iodine is, for example, how many cell-like layers?

My second question is, while you are doing your viability assays, there is a point, let’s say it is the LD 50 point, of the povidone-iodine, 50% of the cells are alive still in the culture: have you ever tried to propagate that population, like the affected but still not yet dead population, to see if there are any changes in behaviour or changes in adherence or anything like that, something observable?

Dr Fiorelli: Regarding the penetration, as it is an in vitro study, we don’t have a three-dimensional aspect; it is a planar model, and we know it is a limitation of this paper. So we were unable to evaluate the depth of penetration of Betadine in the tissue.

Dr Bilig: You didn’t use the molds. What about the propagation behaviour of the cell culture, did you ever look at it?

Dr Fiorelli: No, we didn’t.

Dr T. Grodzki (Szczecin, Poland): I can add that it will probably work in the clinical setting as well. As you may know, our centre is doing the so-called Weder’s procedure in postpneumonectomy empyema. We fill the postpneumonectomy cavity with Betadine mops for 48 hours twice, and after five years we observed, despite the healing process, the beneficial effect on the five-year survival in propensity-matched groups. I suppose it is caused just by the immunological response to chronic inflammation, but maybe povidone is additionally beneficial.

Dr Fiorelli: We think that necrosis is important; it induces an inflammatory response which may recruit immune cells to the site of the tumour and thereby enhance the anti-tumour response.