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Virucidal activity and cytotoxicity of the liposomal formulation of povidone-iodine

P. Wutzler [a,*], A. Sauerbrei [a], R. Klöcking [a], B. Brögmann [b], K. Reimer [b]

[a] *Institute for Antiviral Chemotherapy, Friedrich-Schiller University, Winzerlaer Strasse 10, 07745 Jena, Germany*
[b] *Mundipharma GmbH, Limburg, Germany*

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Abstract

Two drug formulations of povidone-iodine (PVP-I)—an aqueous PVP-I solution (Betaisodona®) and a liposomal PVP-I formulation—were tested for their virucidal activity and cytotoxicity in cell culture. As to the virudical activity against influenza A virus, herpes simplex virus type 1, adenovirus type 8 and human rhinovirus type 14, the liposomal formulation of PVP-I proved to be approximately as active as the aqueous one. Half maximum cytotoxic PVP-I concentrations were 0.01–0.07% for aqueous PVP-I and 0.03–0.27% for the liposomal PVP-I formulation (XTT reduction assay EZ4U). The detection of lactate dehydrogenase (LDH) release, DNA fragmentation (ELISA) and DNA strand breakage (TUNEL assay) after 24 h exposure of human embryonic lung fibroblasts to PVP-I revealed that necrosis predominates in cells treated with aqueous 0.08–0.32% PVP-I solutions, whereas apoptosis was the predominant type of cell death in cells treated with equivalent concentrations of liposomal PVP-I formulation. The favorable virucidal efficiency together with the preferred apoptotic route of cell death makes the liposomal PVP-I formulation a promising candidate for topical use in prevention and treatment of infections of the eye and the upper respiratory tract.

Keywords: Liposomal povidone-iodine; Aqueous povidone-iodine; Virucidal activity; Cytotoxicity; Membrane toxicity; Apoptosis

1. Introduction

Povidone-iodine (PVP-I) is a broad spectrum microbicide with in vitro activity against bacteria, viruses, fungi, and protozoans (Görtz et al., 1996). As an iodophore, PVP-I consists of elementary iodine bound to the carrier poly(1-vinyl-2-pyrrolidone) which increases the solubility and provides a reservoir of iodine. Due to its excellent antiseptic properties PVP-I is used in numerous topical formulations, e.g. for disinfection, wound antisepsis, the treatment of burns, leg ulcers and decubital ulcers. PVP-I has also been successfully applied in the treatment of bacterial and viral keratoconjunctivitis (Neuhann and Sommer, 1980; Schuhman and Vidic, 1985) but is not approved for repeated application on the eye. Possibly, PVP-I could be also suitable for the topical treatment of respiratory viral infections. However, due to its non-specific mechanism of cell killing by

* Corresponding author. Tel.: +49-3641-657-300; fax: +49-3641-657-301.
E-mail address: peter.wutzler@med.uni-jena.de (P. Wutzler).

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oxidizing effects of free iodine on SH-, OH- and NH-groups of amino acids and on double bounds of unsaturated fatty acids (Gottardi, 1991), PVP-I has a certain cytotoxic potential to mammalian cells.

Recently, a novel, liposomal PVP-I formulation was developed by liposomal drug encapsulation (Reimer et al., 1997). Liposomes are known to provide an enhanced site-specific activity, sustained release of drugs and less toxicity than conventional drug formulations in vivo. It was shown in a previous paper that the novel liposomal formulation is as virucidally active as the aqueous PVP-I solution and has a better cell tolerability (Wutzler et al., 2000). The present study was mainly directed to possible reasons for the different toxicological behavior of the two PVP-I formulations, in particular to the type of cell death. In addition, virucidal activities of aqueous and liposomal PVP-I formulations against several viruses related to infections of the eye or the respiratory tract were compared.

2. Materials and methods

2.1. Test preparations

Following test preparations were provided by Mundipharma, Limburg, Germany: (1) the commercially available aqueous PVP-I solution *Betaisodona*® containing 10% (m/v) PVP-I; (2) a liposomal PVP-I formulation containing 5% resp. 4.5% PVP-I and 4% (m/v) fully hydrogenated soy bean phosphatidylcholine (Phospholipone 90 H, Rhöne-Poulenc Rorer); (3) a drug-free isotonic liposomal preparation containing 4% of the same phosphatidylcholine.

The liposomal PVP-I formulation was prepared by heating Phospholipone 90 H above the main phase transition temperature using the high pressure homogenizer LAB40-RBFI (APV Deutschland GmbH, Lübeck). After sterile filtration, the liposomes were resuspended in an isotonic buffer at pH 6 without further separation steps. Regarding the percentage of encapsulated PVP-I in the liposomes precise data were not available. Freeze fraction of electron microscopic investigations proved a multilamellar structure of the liposomes (Reimer et al., 1997). Kinetic studies on the content of encapsulated, surface-bound and free PVP-I in the liposomal formulation are subject of current investigations.

The interaction of PVP-I and phophatidylcholine was monitored by particle size and zeta-potential measurements using the Nicomp Submicron Particle Size Analyzer Model 370 (Particle Sizing Systems, Santa Barbara, CA, USA). The particle size distribution found for the liposomal dispersion by dynamic light scattering was between 100 and 130 nm (Brögmann et al., 1997)

For the experiments, the liposomal dispersion was diluted in the corresponding phosphate-buffered cell culture medium enabling to test all PVP-I preparation at pH 7.2–7.4.

2.2. Cell cultures

Low passage human embryonic lung fibroblasts (HEF) and rabbit testes primary (RTP) cells were grown in a mixed medium containing equal parts of Leibovitz's L-15 medium and lactalbumin hydrolysate supplemented with 10% fetal calf serum (FCS). African green monkey kidney cells (Vero76) were cultured in Eagle's minimum essential medium with Hanks' salts supplemented with 5% FCS. The medium for cultivation of human lung carcinoma cells (A549) consisted of Dulbecco's modified Eagle's medium (DMEM) containing 8% FCS. Human retinal pigment (RPE) cells (Scholz et al., 1998) were grown in ISCOVE medium with 20% FCS. Chinese hamster ovary (CHO-9) cells were cultured in DMEM/Ham's F-12 (1:1 mix) supplemented with 10% FCS and 50 μg/ml gentamicin. Madin Darby canine kidney (MDCK) cells were maintained in minimum essential medium containing Earle's salts, 0.1 g/ml tricine and 10% FCS. Human epithelial-like (HeLa) cells were grown in minimum essential medium supplemented with Earle's salts, 1% non-essential amino acids, 20 mM hepes buffer, 500 μg/ml gentamicine and 5% FCS. With exception of HEF, which were incubated in a humid atmosphere containing 1% CO_2, cells were cultured at 5% CO_2 and 37 °C. If not otherwise indicated, all

media were supplemented with 2 mM L-glutamine, 100 U/ml penicilline and 100 μg/ml streptomycin sulfate.

2.3. Viruses

Influenza A virus (strain Hongkong, H3N2), herpes simplex virus type 1 (HSV-1, strain Kupka), human adenovirus type 8 (strain 1127) and human rhinovirus type 14 kindly provided by Professor Rueckert (Madison, Wisconsin, USA) were used in the present study. The test strains were propagated in MDCK cells (influenza A virus), RTP cells (HSV-1), A549 cells (adenovirus type 8) and HeLa cells (HRV-14), respectively.

2.4. Virucidal testing

Titers of viral stocks were $10^{7.3}$ cell culture infectious dose 50% ($CCID_{50}$) per milliliter for influenza A virus, 10^{11} $CCID_{50}$/ml for HSV-1, 10^{8} $CCID_{50}$/ml for adenovirus type 8 and 10^{9} $CCID_{50}$/ml for HRV-14. Two hundred and fifty microliters of viral suspensions were mixed with 2250 μl of diluted preparations to be tested. After 0.5–30 min exposure, mixtures were subsequently $\log_{10}$ diluted with ice-cold cell culture medium to stop the reaction, and 200 μl of each were seeded into microplate wells of the respective cell culture. On day 10, the remaining infectious virus was titrated by the estimation of $CCID_{50}$. Viral suspension mixed with phosphate-buffered saline instead of test preparations and experiments without virus served as controls. Each experiment was performed twice. The inhibitory effect was calculated from the difference between the virus titers with and without the exposure of the test preparations and expressed as $\log_{10}$ reduction in virus titers according to the Guidelines of the German Association for the Control of Virus Diseases (1990). A ≥ 4 $\log_{10}$ reduction of virus titer was considered virucidal effect.

2.5. XTT tetrazolium reduction assay EZ4U

PVP-I-induced cytotoxicity was quantified in microtiter plates by means of the XXT-based tetrazolium reduction assay EZ4U (Biozol, Eching, Germany) described previously (Klöcking et al., 1995). Substance concentrations at half-maximum cytotoxicity (CC_{50}) were calculated from dose–response curves by regression analysis.

2.6. Lactate dehydrogenase (LDH) assay

To detect membrane-toxic effect of PVP-I, HEF were exposed to serially diluted PVP-I formulations and to a drug-free liposomal preparation, respectively, for 24 h. Thereafter, LDH was determined in the $250 \times g$ supernatant of the cells by means of a coupled enzymatic assay (CytoTox 96®, Promega, Mannheim, Germany). The maximal LDH release of cells was determined by addition of lysis solution to equally treated cells followed by incubation at 37 °C for 45 min. Aliquots of the $700 \times g$ supernatant from all wells were transferred to a fresh 96-well plate and reconstituted substrate mixture was added. After incubation at room temperature for 30 min, the plate was read at a wavelength of 492 nm. Percent LDH release was calculated according to Lappalainen et al. (1994).

2.7. DNA fragmentation assay

DNA degradation by activated endonuclease resulting in the enrichment of histone-associated mono- and oligonucleosomes (HAN) in the cytoplasm of apoptotic cells was detected by means of the Cell Death Detection ELISA^{plus} (CDDE, Roche Diagnostics, Mannheim, Germany), a peroxidase-coupled sandwich enzyme immunoassay. The specific enrichment of HAN (HAN enrichment factor) was calculated by dividing the 405 nm absorbance of the processed PVP-I-treated cell lysates by the absorbance of the corresponding controls (untreated cell lysates).

2.8. TUNEL assay

To determine DNA strand breaks in apoptotic cells by means of the TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling] assay (Gorczyca et al., 1993), the in situ Cell Death Detection Kit AP (alkaline phosphatase) from

Roche Diagnostics was used. At bright-field microscopical inspection, apoptotic cells appeared red colored. Morphological details of the surrounding unstained cells were visualized by phase contrast microscopy in Cytomorph-b microtest plates (nerbe plus, Winsen, Germany).

2.9. Statistical analysis

Data points (at least triplicates) are presented as the arithmetic means ± standard errors of the mean (SEM). Experimental data were analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for significant differences. *P* values of the effect ≤ 0.05 were considered significant. SIGMASTAT Software (Jandel Scientific, Erkrath, Germany) was used for all statistical calculations.

3. Results

3.1. Virucidal effectiveness

The virucidal activities of the PVP-I formulations are shown in Table 1. Influenza A virus and HSV-1 were inactivated by more than 4.0 $\log_{10}$ by 0.009 (influenza A virus) and 0.11% (HSV-1) liposomal or aqueous PVP-I preparations within 0.5 min. For inactivation of human adenovirus type 8 and human rhinovirus type 14 higher concentrations and longer exposure times were necessary. Following the 15 min exposure time to 0.23% liposomal PVP-I formulation, adenovirus type 8 was inactivated by more than 4.0 $\log_{10}$. The same effect was obtained when the virus was exposed for 5 min to 0.23% aqueous Betaisodona® solution. An inactivation of human rhinovirus type 14 by more than 4.0 $\log_{10}$ was achieved after 30 min treatment with 0.45% liposomal PVP-I formulation and after 15 min treatment with the equivalent concentration of the aqueous PVP-I solution, respectively.

3.2. Cytotoxicity

Long-term cytotoxicity (exposure time: 120 h) of PVP-I formulations was evaluated in HEF and the cell lines Vero76, A549, CHO-9 and RPE. PVP-I concentrations inducing half-maximum cytotoxicity (CC_{50}) were found to range from 0.032 to 0.274% PVP-I for the liposomal PVP-I formulation and from 0.009 to 0.07% PVP-I for Betaisodona® (Table 2). With the exception of CHO-9 cells, which were equally sensitive to both PVP-I formulations, all cell lines were more sensitive to the aqueous PVP-I solution than to the comparable concentrations of the liposomal PVP-I formulation. The liposomal carrier reduced PVP-I cytotoxicity twofold in Vero76 and RPE cells, fourfold in A549 cells, and tenfold in HEF.

Table 1
Virucidal activity of liposomal and aqueous PVP-I preparations

PVP-I preparations	Dilution	PVP-I concentration (%)	Virus	Reduction of virus titer by $\log_{10}$/exposure time (min)						
				0.5	1	1.5	2	5	15	30
Liposomal PVP-I 5%	1:500	0.009	IAV	>4	>4	>4	>4	>4	>4	>4
	1:40	0.11	HSV-1	>4	>4	>4	>4	>4	>4	>4
	1:20	0.23	AV-8	0.9	1.5	1.6	1.7	2.5	>4	>4
	1:10	0.45	HRV-14	0.2	n.d.	n.d.	0.6	1.3	n.d.	>4
Betaisodona® 10% PVP-I	1:1000	0.009	IAV	>4	>4	>4	>4	>4	>4	>4
	1:80	0.11	HSV-1	>4	>4	>4	>4	>4	>4	>4
	1:40	0.23	AV-8	1.4	1.4	2.3	2.8	>4	>4	>4
	1:20	0.45	HRV-14	1.1	n.d.	n.d.	1.6	2.3	>4	>4

IAV, influenza A virus; HSV-1, herpes simplex virus type 1; AV-8, human adenovirus type 8; HRV-14, human rhinovirus type 14; n.d., not done.

Table 2
Half-maximum cytotoxicity concentrations (CC_{50}) of PVP-I applied as liposomal PVP-I formulation and aqueous Betaisodona® solution, respectively, in different cell lines; $n = 3$

Cell lines	CC_{50} (confidence limits) as % PVP-I	
	Liposomal PVP-I formulation	Betaisodona®
HEF	0.095 (0.056–0.161)	0.009 (0.008–0.010)
CHO-9	0.032 (0.026–0.039)	0.032 (0.018–0.055)
Vero76	0.074 (0.065–0.083)	0.036 (0.023–0.057)
RPE	0.102 (0.096–0.129)	0.054 (0.051–0.058)
A549	0.274 (0.268–0.279)	0.070 (0.063–0.079)

Exposure time, 120 h; serum concentration, 2.5%. XTT reduction assay EZ4U.

The results obtained after short-term (24 h) exposure of HEF to serially diluted PVP-I formulations and to the PVP-I-free liposomal preparation, respectively, demonstrated that PVP-I was significantly better tolerated in liposomal than in aqueous PVP-I formulations. No cytotoxicity was seen in the PVP-I-free liposomal preparation at equivalent liposome concentration (Fig. 1).

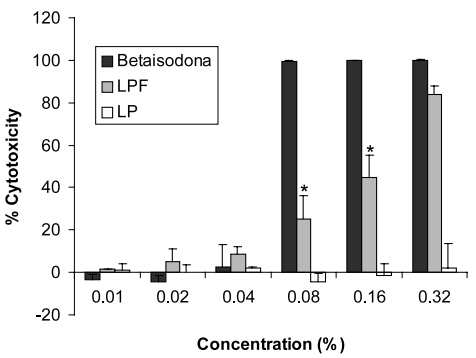

Fig. 1. Cytotoxicity of PVP-I applied to human embryonic lung fibroblasts (HEF) as aqueous solution (Betaisodona®) compared with liposomal PVP-I formulation (LPF) and PVP-I-free liposomal preparation (LP). Exposure: 24 h. *Statistically significant difference between Betaisodona® and LPF.

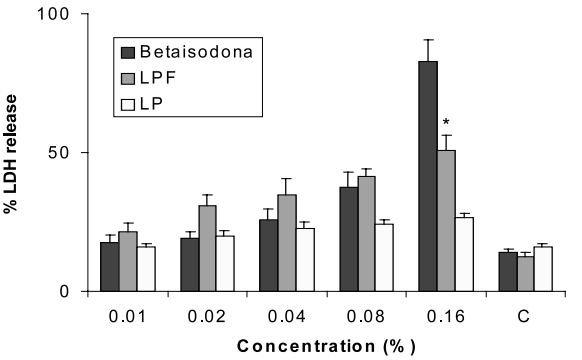

Fig. 2. Lactate dehydrogenase (LDH) release of human embryonic lung fibroblasts following a 24 h exposure to PVP-I as aqueous solution (Betaisodona®) compared with liposomal PVP-I formulation (LPF) and PVP-I-free liposomal preparation (LP). C, untreated control. *Statistically significant difference between Betaisodona® and LPF.

3.3. Membrane toxicity

To quantify the necrotic, membrane-toxic potential of test substances, both PVP-I formulations in the range of 0.01 and 0.16% as well as the PVP-I-free liposomal preparation were examined for LDH release in HEF after 24 h exposure. The results presented in Fig. 2 demonstrate that all the preparations tested showed a more or less distinct tendency to increase the LDH release in a dose-dependent fashion. However, 0.01–0.04% liposomal PVP-I formulations did not significantly exceed membrane damage caused by the drug-free liposomal control. From 0.08% onwards, both PVP-I formulations induced increasing LDH release compared with that of the corresponding controls and reached a significant difference between each other at 0.16% PVP-I, at which point the LDH release of aqueous PVP-I solution exceeded that of the liposomal PVP-I formulation 1.6-fold.

3.4. Induction of apoptosis

As shown in Fig. 3, a 24 h exposure of HEF cells to aqueous PVP-I formulations increased the concentration of cytoplasmic nucleosomes by 2- to 2.5-fold. At concentrations $\geq 0.04\%$ PVP-I, histone-associated nucleosomes in the cytoplasm declined, probably due to the increasing necrotic

effect of PVP-I, in aqueous solution. In contrast, the liposomal PVP-I formulation up to 0.08% enhanced enrichment of HAN in the cytoplasm. The difference among the HAN enrichment factors of both formulations was significant at 0.04, 0.08 and 0.16% PVP-I. The PVP-I-free liposomal preparation showed only a slight tendency to increase HAN concentration in the cytoplasm.

A later stage of PVP-I-induced apoptosis is characterized by DNA strand breaks, which were detected with a bright-field microscopy version of the TUNEL assay. Fig. 4 presents phase-contrast photographs of HEF monolayers after 48 h exposure to aqueous and liposomal PVP-I formulations, PVP-I-free liposomal preparation and drug-free cell medium, respectively. Few apoptotic cells were visible in monolayers treated with an aqueous solution of 0.4 and 0.16% PVP-I. Numerous holes in the cell monolayer, remarkable morphological alterations such as vacuolization and intensely refractive nuclei (which appear dark blue by phase contrast) were characteristic of the latter. This suggests a predominately necrotic background of PVP-I cytotoxicity. In contrast, cells treated with 0.04% PVP-I as a liposomal formulation showed numerous big, red-colored apoptotic cells, typical for apoptotic death. The apoptotic cells disintegrated at higher PVP-I concentration (0.16% PVP-I). Likewise, monolayers treated with PVP-free liposomes included clearly more apoptotic cells than the untreated cell control.

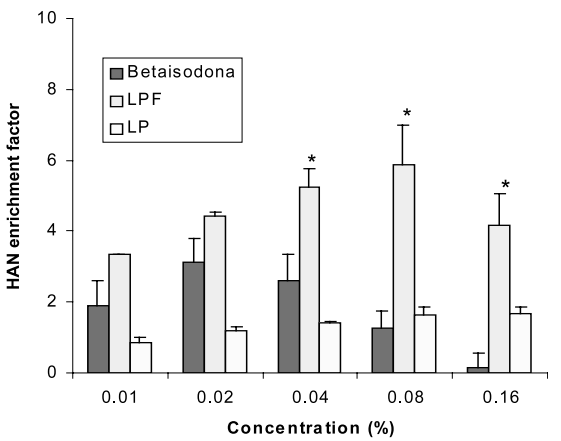

Fig. 3. Enrichment of histone-associated nucleosomes (HAN) in the cytoplasm of human embryonic lung fibroblasts following a 24 h exposure to PVP-I as aqueous solution (Betaisodona®) compared with liposomal PVP-I formulation (LPF) and PVP-I-free liposomal preparation (LP). *Statistically significant difference between Betaisodona® and LPF.

4. Discussion

In view of the intended topical treatment of viral infections of the eye and the upper respiratory tract important concerns of PVP-I formulations are their virucidal efficacy as well as cytotoxicity including membrane-toxicity and apoptosis-inducing capacity. Although the aqueous PVP-I formulation has excellent antiseptic properties, overlapping microbicidal and cytotoxic concentrations have been reported in several studies (Sanchez et al., 1988). Therefore, the development of a novel, liposomal PVP-I formulation for application to mucosal sites deserves particular attention (Reimer et al., 1997). Liposomes are not only known to decrease the drug toxicity (Allen, 1998) but they are also generally well tolerated, not immunogenic and provide a moist molecular film on skin and conjunctival surfaces. Another advantage of these liposomal preparations is that the multilamellar vesicles provide a slow release system of the microbicidal substance.

The present results of virucidal testing confirm that influenza A virus and HSV-1 belong to the most sensitive viruses against PVP-I formulations (Kawana et al., 1997). Influenza A virus was inactivated by 99.99% within 0.5 min when 0.009% liposomal or aqueous PVP-I solutions were used. For inactivation of HSV-1, 12.5-fold higher concentrations of both liposomal and aqueous PVP-I preparations were effective to the same extent. Compared with this, longer exposure times from 5 to 30 min and higher PVP-I concentrations from 0.23 to 0.45% were necessary to inactivate adenovirus type 8 and human rhinovirus type 14 which is in agreement with the findings of Kawana et al. (1997). When equivalent PVP-I concentrations were tested, the liposomal PVP-I formulation proved to be nearly as effective as the aqueous PVP-I solution Betaisodona®.

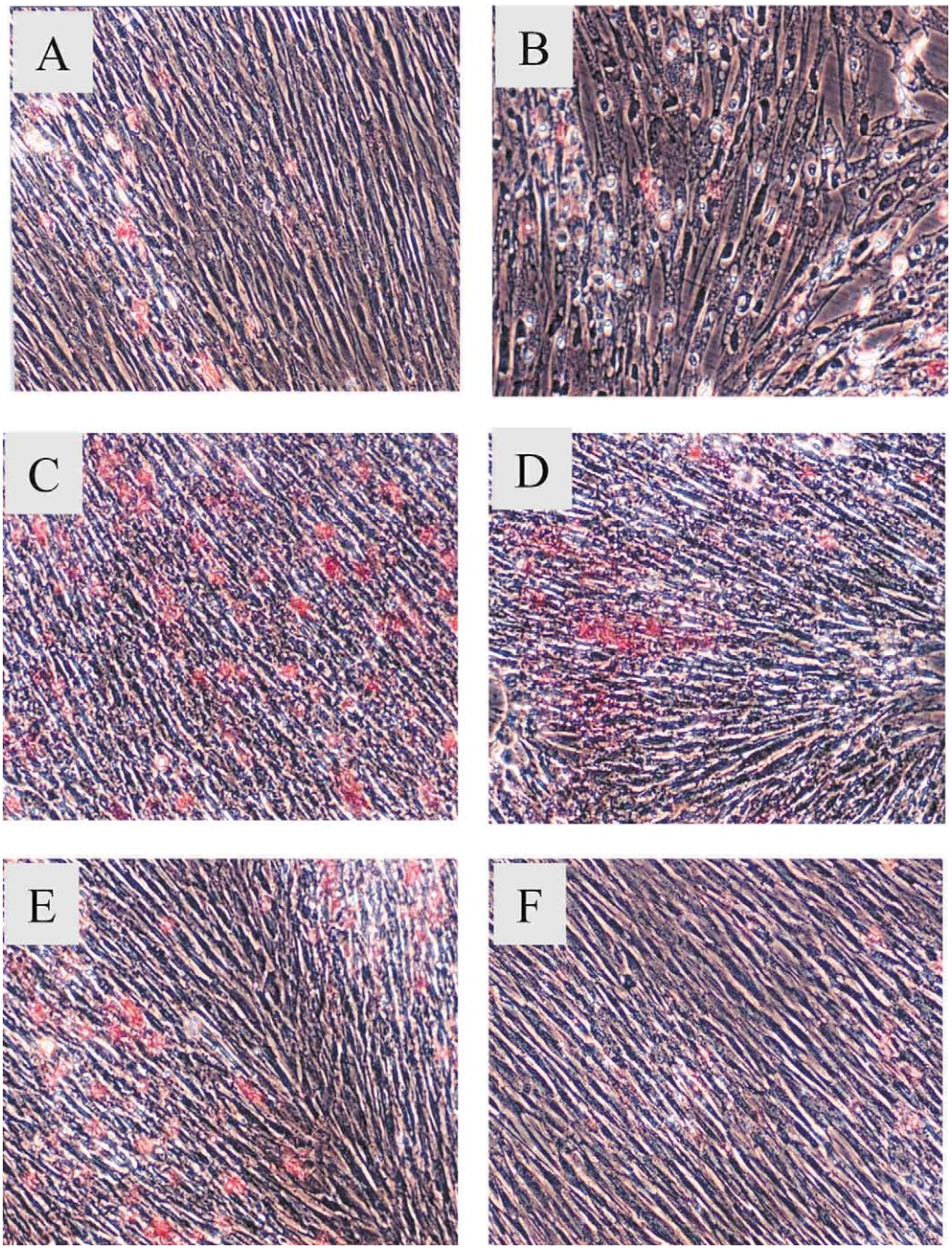

Fig. 4. Detection of DNA strand breaks in human embryonic lung fibroblasts after 48 h treatment. Few apoptotic cells are visible in monolayers treated with 0.04% (A) and 0.16% (B) PVP-I as aqueous solution (Betaisodona®). Numerous gaps, vacuolization and intensely refractive nuclei suggest a predominantly necrotic background of PVP-I cytotoxicity. Cells treated with 0.04% PVP-I as liposomal formulation (C) show numerous big, red-colored apoptotic cells that disintegrate at higher PVP-I concentrations (0.16% PVP-I, D). Monolayers treated with PVP-I-free liposomes (E) also include clearly more apoptotic cells than the untreated cell control (F). Phase contrast, 180 ×.

The differences between the enveloped influenza A virus and HSV-1, on the one hand, and the non-enveloped adenovirus and rhinovirus, on the other hand, emphasize that enveloped viruses are usually more susceptible to antiseptic compounds than non-enveloped ones (Jülich et al., 1993).

In long-term experiments (120 h exposure), the liposomal PVP-I formulation was found to be less toxic than the aqueous PVP-I solution in four of five cell lines included in the present study. Particularly, these results may be relevant to the long-term application of liposomal PVP-I hydrogel, an innovative topical drug formulation, for wound healing and infection treatment (Reimer et al., 2000). Short-time treatment of HEF cells resulted in a significantly better tolerability of liposomal PVP-I solution. The CC_{50} values found for Betaisodona® were in good agreement with published data (Sanchez et al., 1988; Damour et al., 1992; Fabreguette et al., 1994). On the basis of the 24 h cytotoxicity of test preparations shown in Fig. 1, the percentage of encapsulated PVP-I in the liposomes was assumed to be at least 70–80%. However, the presence of a not yet exactly known part of free PVP-I is likely. The present findings reflect differences of CC_{50} values well below clinically relevant concentrations and can explain the reported excellent tolerability of liposomally encapsulated PVP-I only in part. Therefore, the question was addressed, what type of cell death, apoptosis or necrosis, does occur in cells treated with different PVP-I formulations.

Using LDH release, DNA fragmentation and DNA strand breaks assays, we were able to show for the first time distinct differences in the mechanism of cell death caused by the two PVP-I formulations. The LDH release assay revealed a significantly lower membrane-toxic, i.e. necrotic effect of the liposomal PVP-I formulation compared with the aqueous PVP-I solution. The enrichment of histone-associated nucleosomes (HAN) in the cytoplasm as an early marker of apoptosis demonstrated that both PVP-I formulations showed a tendency to induce DNA fragmentation at low PVP-I concentrations (0.01–0.02%). At higher PVP-I concentrations ($\geq 0.04\%$) necrosis predominated in cells exposed to aqueous PVP-I solution whereas apoptosis was characteristic of cells treated with the liposomal PVP-I formulation. The results obtained by the TUNEL assay demonstrate that liposomal PVP-I preparations induce apoptosis in HEF cells and that drug-free liposomes obviously participate in this process. In aqueous PVP-I solution, however, the cytotoxic and membrane-toxic effect predominates and prevents the apoptotic pathway of cell death.

The good tolerability of the PVP-I liposome complex in cell culture has been confirmed by a sensitive explantation test using skin and peritoneum of the rat (Reimer et al., 2000). Growth rates of skin and peritoneum were found to be significantly higher in explants treated with a PVP-I liposomal hydrogel formulation than in those treated with PVP-I ointment. Initial results of a clinical phase II trial on the treatment of wounds with PVP-I liposome hydrogel are also in favor of the PVP-I liposome complex (Vogt et al., 2001).

On the basis of the present findings further studies on topical testing of liposomal PVP-I at mucosal sites are warranted. The high virucidal efficiency together with the preferred apoptotic route of cell death makes this formulation a promising candidate for topical use in prevention and treatment of viral infections of the eye and the upper respiratory tract.

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