Evaluation of the effect of the concentration of plasticizer di(2-ethylhexyl) phthalate on the quantity of residual monomer vinyl chloride in PVC chest drainage tubes

Y. I. KICHEVA[1]*, H. RICHTER[2], E. POPOVA[3]
[1]*Department of Physics and Biophysics, University of Medicin, Plovdiv, Bulgaria*
E-mail: icomp@plovdiv.techno-link.com
[2]*Institute of Pathology, Technical University of Aachen, Germany*
[3]*Department of Periodontology and Oral Mucosal Diseases, Faculty of Stomatology, Medical University, Plovdiv, Bulgaria*

The effect of amount of plasticizer di(2-ethylhexyl) phthalate (DEHP) amount on the amount of residual monomer vinyl chloride (VC) was determined in samples of plasticised polyvinyl chloride (PVC) with different concentration of plasticiser (22.32–33.05%), before and after sterilisation by a titrimetric method. The titrimetric method was used to determine the VC concentration in a $KMnO_4$ solution where the samples were kept immersed under the same conditions for 2 h.

The influence of PVC film extracts with different amounts of DEHP on mouse fibroblast cells L-929 in a culture medium was evaluated by using quantitative tests: the amount of cells (protein determination), viability (MTT test) and proliferation (incorporation of bromodeoxyuridine (BrDU). The amount of vinyl chloride before and after heat sterilisation at 120 °C for 30 min was found to be almost the same for all samples and without any dependence on the concentration of DEHP. The extracts of the PVC films which were tested have no toxic effect on cells in a culture medium.

Introduction

When poly(vinyl chloride) (PVC) is used for medical articles, such as drainage tubes, catheters, bags for blood and blood products, the vinyl chloride (VC) extracted from the material should be in minimal quantities and cause no toxic effects. There are several studies on the effects of the phthalate plasticizers on blood and some blood products [1–4]. The studies which deal with the interaction of drugs with di(2-ethylhexyl) phthalate (DEHP) extracted from PVC containers, used for storage medicines, suggest that the effects of DEHP are different for different drugs or nutritional solutions [5–10]. After studying the effect of VC and DEHP in plasticized PVC materials for pharmaceutical packages, Van Dooren recommends their usage [11]. There are similar studies of the toxicological effect of the DEHP in PVC used for medical devices [12, 13].

The application of polymers as biomaterials for human body implants or for medical devices is preceded by extensive testing since the material has to meet strictly determined physical and chemical requirements. On the other hand, biological testing has to be performed, not only to exclude possible deleterious effects of the biomaterials but also to assess the functionality of the device in its biological surrounding. The *in vitro* assessment of cytotoxicity forms the basis of the use of *in vitro* methods in existing national and international testing standards [14–18].

There are similar studies of the toxic effect of VC on workers involved in processing PVC [19, 20, 22], but we did not find investigations showing any relation between the amount of residual monomer VC and the amount of plasticiser DEHP in materials for chest drainage tubes. As the use of chest PVC drainage tubes is widespread [22], such investigations are necessary. Recently, the suggestion that DEHP should be considered as unlikely to be a human carcinogen has been advanced because it is claimed that the carcinogenic effects of this agent observed in rodents are due to peroxisome proliferation and that humans are not responsive to this process. An International Agency for Research working group recently graded DEHP as ''not classifiable as a

*Author to whom all correspondence should be addressed.

carcinogen to humans'' and they concluded that DEHP causes liver tumours in rats and mice by a mechanism involving peroxisome proliferation which they considered to be not relevant to humans [23]. There are studies of hepatocarcinogen in rats which were initially injected with diethylnitrosamine (20 mg/kg) and the animals were administered DEHP in their diets at a concentration of 30, 300, 3000, or 12,000 ppm. At the 30 ppm dose level, however, no morphological changes were apparent in the liver [24].

The aim of our studies is to establish the effects of the quantity of DEHP from 22.32% to 33.05% on the quantity of residual monomer vinyl chloride before and after heat sterilization and to establish the effect of DEHP from 22.32% to 33.05% in PVC films and extracts from them on the culture medium of mouse fibroblast cells (L-929) by quantitative tests.

Materials and methods

We used Bulgarian-manufactured suspension PVC (grade DEVILIT, coefficient of Fickencher $K = (70 \pm 1)$), plasticised with DEHP (grade Palatinol, BASF; acid number 0.1 mg of KOH g^{-1} and iodine number 0.8), epoxy Soya oil Edenol 82 (Henkel, Germany), Yrgavax 280, and a stabiliser Prosper 160 [C11]. The DEHP concentration varied from 32 to 48 weight parts per 100 PVC weight parts in soft and hard compositions, respectively. The rest of the components were used in identical quantities for both soft and hard compositions. The experimental samples were obtained by mixing hard and soft compositions in ratios of 1 : 0, 2 : 1, 1.5 : 1.5, 1 : 2 and 0 : 1, designated as 1, 2, 3, 4 and 5, respectively, using dry-blend extrusion on a Brabender plastograph at temperature zones of 135, 170, and 185 °C. Table I shows the percentage of DEHP and Shore A hardness in the samples.

All samples conform to the European standards [25, 26]. These materials were used in the preparation of samples for different tests.

Titrimetric method

The residual vinyl chloride in the plasticised PVC materials was determined titrimetrically [27]. We determined amount of VC before and after sterilisation in the samples at 120 °C for 30 min. The strips were 1.2 mm thick with different weights varying from 0.2 to 1 g. Twenty strips of each kind were studied. After washing the surfaces of each sample with a 50/50% solution of 90% spirit and water, we measured their weight on an electron balance in grams showing a four decimal accuracy. The samples were each placed in sterile beakers and immersed in $KMnO_4$ solution of $V_0 = 50$ ml. They were kept at room temperature entirely immersed in the solution for 24 h. At 0.001 N initial normality of solution of $KMnO_4$ we designated it as N. It remained in a dark place at room temperature for 10 days. The normality of the solution immediately prior to placing the sample in the beaker, designated as N_1 was calculated using the formula

$$N_1 = N \cdot V / V_1$$

where $V = 10$ ml is the volume of $H_2C_2O_4$, $N_0 = 0.001$ N, V_1 is the volume of $KMnO_4$ solution which titrates the mixture of 10 ml $H_2C_2O_4$ and 5 ml of H_2SO_4 to pink. After the samples were taken out of the $KMnO_4$ solution, a certain amount of this solution was pipetted on the same kind of mixture, slightly warmed, until the latter became pink coloured. The normality of $H_2C_2O_4$ designated as N_2 was 0.001 N. The volume of the $KMnO_4$ solution titrating the mixture designated as V_2 was measured. The amount of the residual monomer VC, designated as A in the solution was determined by the formula

$$A = [\{N_1 - (N_2 \cdot V / V_2)\} V_0] \cdot \text{mgE}$$

where mgE = 0.0103 is mg equivalent of vinyl chloride.

The amount of residual monomer vinyl chloride was determined with an accuracy of one hundredth of a milligram. Two titrations were made for each sample before and after heat sterilisation. The percent concentration of VC per referred to a unit of sample weight, designated by X was determined with an accuracy of five decimal places by the equation

$$X = \left(\frac{A \cdot 100}{m} \right)$$

where A is the amount of monomer in grams, and m is the sample weight in grams.

Sample biocompatibility was investigated by means of a cytotoxicity test biocompatibility: the amount of cells (protein determination), the cell viability (a MTT-test), and cell proliferation (the incorporation of bromodeoxyuridine (BrDU)) [17].

TABLE I Hardness Shore A and percentage of DEHP of the samples

Sample	Hardness shore A	DEHP%
1	96–97	22.32
2	94–95	24.50
3	92–93	25.62
4	91–92	28.91
5	90–91	33.05

Reagent and materials

Anti-BrDU antibody conjugated with FITC was supplied by Progen, Heidelberg, Germany. The BCA protein assay reagent was supplied from Pierce, Rockford, USA. Bovine serum albumin (BSA) was bought from Serva Feinbiochemica, Heidelberg, Germany. 5-bromo-2′-deoxyuridine, ethylenediamine-tetra-acetic acid (EDTA), MTT (3-[4,5-dimethyl-thiazol-2-yl]- 2,5-diphenyl tetrazolium bromide), propidium iodide, trypsin type III, Triton X-100 were supplied by Sigma Chemistry, Deisenhofen, Germany. Dulbecco's modified Eagle's medium, L-glutamine, penicillin/streptomycin solution, fetal calf serum (FCS), phosphate buffered saline solution (PBS) were supplied by Gibco BRL, Life Technologies, Eggenstein, Germany. Ethanol, methanol,

2-propanol, hydrochloric acid, Tris [hydroxymethyl]-aminomethane (Tris) were bought from Merck, Darmstadt, Germany. Filters (0.22 µm pore size; mixed cellulose-esters) were supplied by Millipore, Eschborn, Germany. Tissue culture ware and FAC Star plus flow cytometer were supplied by Becton and Dickinson, Heidelberg, Germany. Trypan blue solution was supplied by Boehringer Mannheim, Mannheim, Germany. Microplate reader, Titertek MK II, and software (EIA3) were supplied by ICN- Flow, Meckenheim, Germany.

Reference materials

The negative controls, silicone rubber tubes (poly-dimethylsiloxane; medical grade) without toxicity, was supplied by Rehau, Rehau, Germany, and the positive controls-organo-tin-stabilised PVC discs containing di-iso-octylphthalate (F formulation), with toxicity, were supplied by Huntington Research Center Ltd., Huntington, UK. The PVC strips with a different DEHP concentration were supplied by the Institute for Special Polymers, Sofia, Bulgaria.

Cells

Mouse fibroblast cells L-929 (CCL1; strain L, NCTC clone 929) were bought from the American Type Culture Collection, Rockville, USA.

Sample preparation

PVC samples, negative and positive control materials were used as received and were autoclaved at 121 °C for 30 min.

Preparation of extracts

The samples were extracted in a culture medium (Dulbecco's modified Eagle's medium; DMEM) supplemented with: L-glutamine (1.6 mM), penicillin/streptomycin (80 U/ml and 80 µg/ml respectively and 10% FCS; DMEM + 10% FCS) at 37 °C for 24 h. The ratio between the surface area of the tested materials in cm^2 and the volume of medium in ml was 1. The pH was adjusted to be between 7.2 and 7.4, the extracts were sterile filtered (pore size: 0.22 µm) and diluted 2, 4, 8, and 16 times with fresh culture medium. Positive and negative control materials were extracted in an identical way, whereas the extract of the negative control was not diluted.

Preparation of cell cultures

Mouse fibroblast cells (L-929) were cultivated in standard tissue culture ware in humidified atmosphere containing 5% CO_2. In order to prepare test wells, cells were detached from the culture flasks using a trypsin/EDTA solution (0.25%/0.25% w/v), and resuspended as a single cell suspension in a culture medium (DMEM + 10% FCS) at a density of 2×10^5 viable cells/ml (by a trypan blue exclusion test). The cells were seeded into the 60 inner-wells of microtitre plates and into the wells of a 24-well cluster, at a density of 1×10^5 cells/cm^2. After an overnight incubation, a confluent monolayer of cells was formed in these wells.

Exposure to extracts

The medium was aspirated and replaced either by fresh culture medium or medium containing a fixed dilution of the extract prepared from a biomaterial or a control material. Aliquots of 1 ml were pipetted into the wells of the 24-well clusters and 0.15 ml samples were pipetted in a fivefold repetition into the wells of the microtitre plates. After a 24-h exposure of the extracts, the cultures were used for various assays.

Protein content

The protein content of the wells, fixed with 2-propanol for 0.25 h, was determined by means of a BCA protein assay kit, using serial dilutions of a BSA solution as reference.

MTT test

Each well of the microtitre plates received 20 µl of a MTT solution (final concentration 0.44 mg/ml). The plates were shaken briefly and incubated for a further 4 h at 37 °C. After a careful aspiration of the supernatants, 100 µl of 2-propanol was added to each well. After a complete solubilization of the MTT formazan, the absorbency of each well at 590 nm was measured against blank well without cells, also incubated with MTT solution, with the aid of a microplate reader.

Labelling index

Each well, except the additional ones which served as negative control, received 10 µl of a BrDU solution (final concentration 10 mol/l). After an incubation of 120 min, the medium was aspirated, and the cells were trypsinized, resuspended, fixed in methanol/ethanol 1 : 2 v/v) and kept in this solution at 4 °C until they were analysed.

Flow cytometry

To allow the antibody to react with the incorporated BrDU, the pelletted cells were denatured in 3 ml hydrochloric acid (1 N) at 60 °C for 6 min, the solution was neutralised with 4 ml Tris buffer (1 M; pH 11) containing Triton X-100 (0.5% v/v), the cells were washed with 0.1 M Tris/Triton X-100 (0.5% v/v) and subsequently stained with fluorescent isothiocyanate (FITC)-conjugated with anti-BrDU antibody (5 µg/ml inPBS/0.5% BSA (w/v) at room temperature for 45 min. Prior to the analysis with the FAC Star plus flow cytometer, propidium iodide was added to each sample (final concentration 3 µg/ml). Only the cells, which had DNA content of 2C to 4C, as revealed by fluorescence intensity of propidium iodide bound to cellular DNA were analysed for BrDU incorporation.

All the measured values of protein content, formazan production and labelling index were expressed the values as percentage of cells incubated with fresh culture medium.

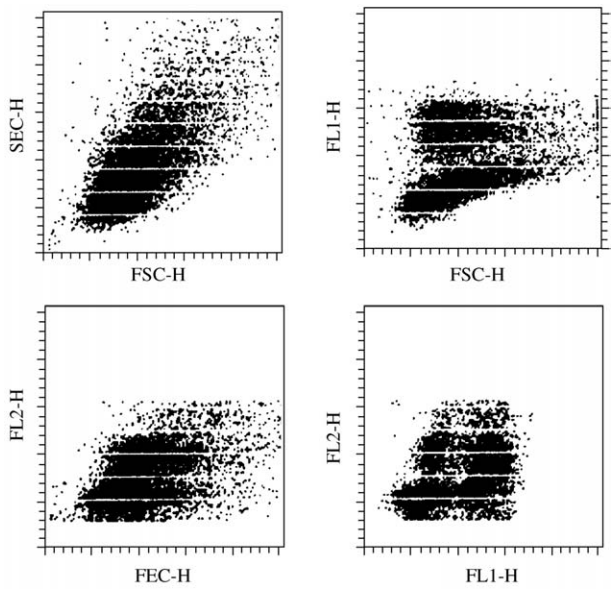

Figure 1 Dott-plot analysis.

The results can be shown on a Dott-plot diagram and on a Histogram. All samples were studied in this way. The typical Dott-plot diagram of the proliferation of mouse fibroblast cells L-929 incubated with BrDU is given in Fig. 1.

The *X*-axis of Fig. 1 represents the fluorescence 1 intensity and the *Y*-axis the fluorescence 2 intensity. The fluorescence signal 1 discriminates between non-proliferating cells and the proliferation fraction. The fluorescence signal 2 shows distinction of the phases of the DNA cycle.

Fig. 2 represents the fluorescence histogram of the cell number of the same samples. Comparing the two methods used, we established that the results were almost the same.

Results and discussion

The quantity of residual monomer VC in blood storage bags from plasticised PVC with DEHP must be up to 1 mg/kg [25, 26]. The percentage amount of VC samples referred as a unit of sample weight is designated by *X*, the

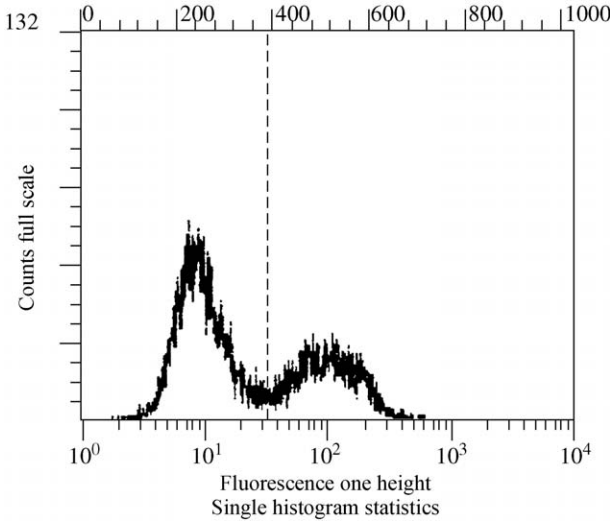

Figure 2 Single histogram.

TABLE II Percentage concentration of VC depending on the concentration of DEHP before sterilisation

Sample	Before sterilisation		
	DEHP%	X%	S_x
1	22,32	0.00267	0.00033
2	24.50	0.00320	0.00048
3	25.62	0.00324	0.00070
4	28.91	0.00342	0.00059
5	33.05	0.00392	0.00042

mean square error is designated by S_x, before and after sterilisation. The results of our study for all samples before sterilisation show that the VC amount was practically the same in all of them and did not depend on the DEHP concentration in them (Table II). The results of our study for all samples after sterilisation show that the VC amount was practically the same for all samples too and did not depend on the DEHP amount. They are given in Table III.

The results of our study show that the vinyl chloride amount was the same for all samples before and after sterilisation and did not depend on the concentration of DEHP in them, in the given concentrations. The VC amount was less than 1 mg/kg.

Flow cytometry could be a reliable test for biocompatibility [17, 28, 29]. Flow cytometry is applied to determine cell viability, cell proliferation characterization of cell populations and specific antigens. Conventional determination of the cytotoxicity of the extracts was made by a MTT-viability test. In this test insoluble formazan is built proportional to the cell activity. Formazan production is constant for every cell type. To compare the MTT test with cell proliferation measurements, we used flow cytometry and investigated five PVC samples with different DEHP concentration. The tests were carried out using the method described in the British Standard ''Evaluation of medical devices for biological hazard, Part 10. Methods of tests for cell toxicity in extract culture from medical devices.'' The test method is designed to detect the possible toxic influence of extracts from biomaterials on a confluent layer of cell culture. The samples were extracted in a culture dilution 16 times that of the primary extract which still induces cytotoxicity, the so-called cytotoxic-titre. The samples are extracted in a culture medium and diluted 2, 4, 8 and 16 times. The samples were added to a monolayer of mouse fibroblast cell L-929. After an incubation at 37 °C for 2 h, BrDU was applied to the cultures and the cells were incubated subsequently with this substance for another 2 h. BrDU is incorporated in

TABLE III Percentage concentration of VC depending on the concentration of DEHP after sterilisation

Sample	After sterilisation		
	DEHP%	X%	S_x
1	22.32	0.00291	0.00032
2	24.50	0.00305	0.00023
3	25.62	0.00319	0.00050
4	28.91	0.00293	0.00069
5	33.05	0.00306	0.00039

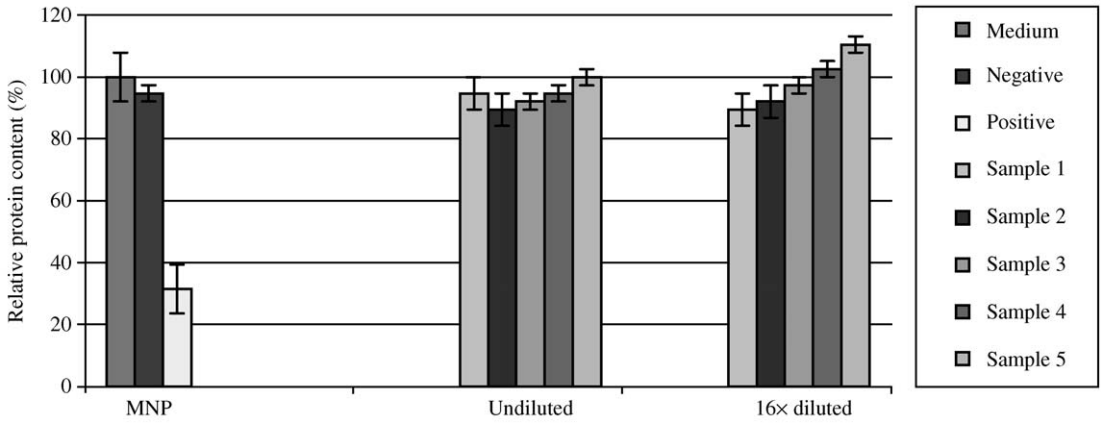

Figure 3 Protein content, extraction method.

proliferating cells as an equivalent to thymidin. Later it can be detected by a fluorescence-labelled antibody and evaluated by flow cytometry. There are some investigations which have established that the monolayer of cells after incubation of 24 h is the same as the one after 36 h [30].

The samples were designated as 1, 2, 3, 4, and 5; the culture medium without biomaterials was designated as *m*, the culture medium with non-toxic material-negative was designated as *n* and the culture medium with toxic material – positive – was designated as *p*.

The result of our study of the amount of cells (protein determination) shows that incubation of confluent layers of the cells with an extract of the negative control resulted in the amount of cellular protein, which is the same as that of the wells incubated with a fresh culture medium. The extract of the positive control material had a negative effect on the cells. The amount of cellular protein after incubation with the extracts of the PVC films or their dilutions is the same as that after incubation with a fresh culture medium or with the extract of the negative control. The relative protein content of wells with L-929 cells after incubation with a fresh culture medium, extracts of the control material, or with extracts of the PVC films and their dilutions for 24 h is expressed as a percentage of the content of wells incubated with a fresh culture medium undiluted and diluted 16 times (Fig. 3).

Cell viability

The results showed that cells incubated with a fresh culture medium as well as the extract of the negative control material produced a comparable, relatively large amount of the blue formazan product upon incubation with MTT solution. The primary extract of the positive control material, however, had a deleterious effect on the production of formazan. The relative amounts of formazan formation by L-929 cells after incubation with a fresh culture medium, extracts of the control materials or with extracts of the PVC films and their dilutions increased 16 times are expressed as a percentage of the amount produced by cells incubated with a fresh culture medium in Fig. 4. The relative amounts of formazan produced after incubation with the primary extracts of the PVC films were larger than those after incubation with a fresh culture medium or with the negative control (except film 1). The primary extract dilution decreased formazan production to the control level.

Cell proliferation

The results of our study of cell proliferation show that the labeling index for cells incubated with the primary extracts of the PVC films or their dilutions is the same as that after incubation with a fresh culture medium or with the extract of the negative control.

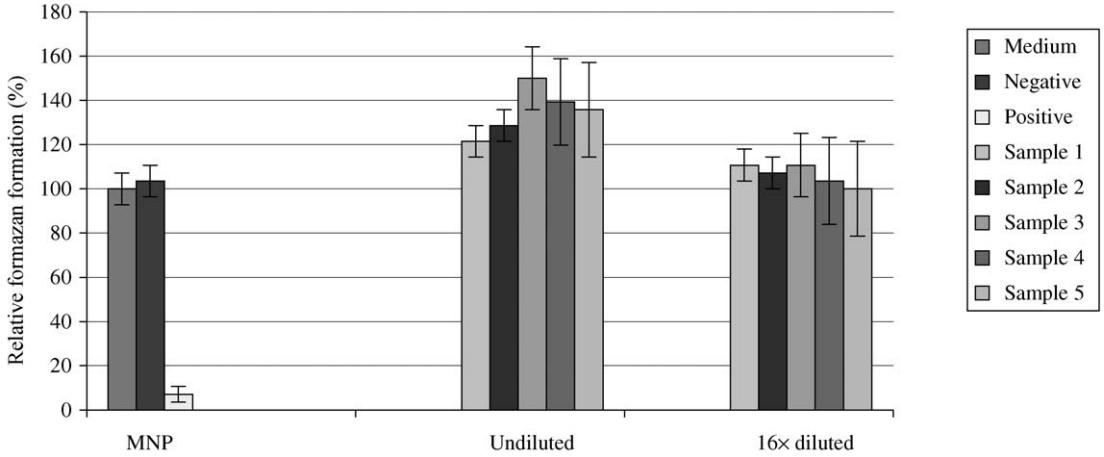

Figure 4 Formazan formation, extraction method.

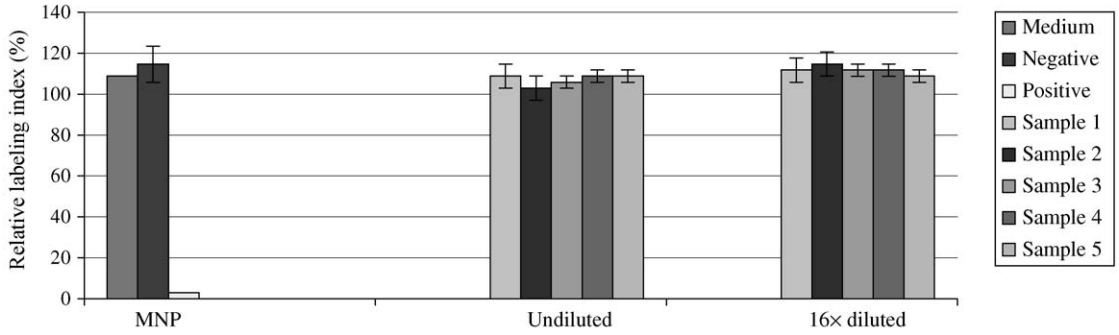

Figure 5 Labelling index, extraction method.

The relative labelling index of L-929 cells after incubation with a fresh culture medium, extracts of the control materials, or with extracts of the PVC films or their dilutions is 16-fold related to that after incubation with a fresh culture medium (Fig. 5). The extracts of the tested PVC films have no toxic effect on cells in the culture medium.

Conclusions

1. The vinyl chloride amount before and after heat sterilization in the PVC samples with different amounts of DEHP (22.32% to 33.05%) is practically the same.
2. The amount of VC monomer extracted in $KMnO_4$ solution from PVC samples does not depend on the DEHP amount extracted in the solution or the DEHP amount which migrated onto the surfaces of the samples.
3. In the cellular proliferation and viability of the PVC samples tests we found no relation between the DEHP concentration and the incorporated BrDU and formazan production on the other hand.
4. The materials made from DEHP plasticized PVC do not have a toxic effect on the cells in a culture medium. We recommend them to be used in the preparation of drainage tubes used in thoracic surgery.

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