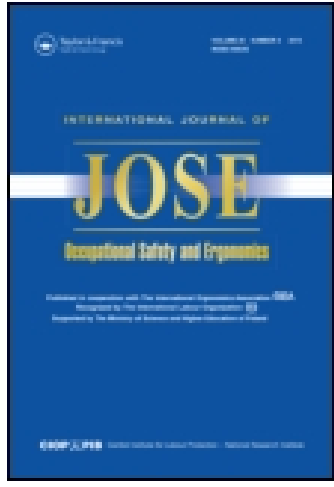


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NOTES

## The Cytotoxicity of Some Organic Solvents on Isolated Hepatocytes in Monolayer Culture

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The cytotoxic effects of volatile and water-insoluble organic solvents (ethylbenzene, tetrachloroethylene, n-hexane) were tested on isolated hepatocytes in monolayer culture by using the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay. All of the tested compounds inhibited metabolic activity of hepatocytes and this effect depended on the concentration of solvents in the incubatory medium. The presence of fetal calf serum in the medium did not change the cytotoxicity of xenobiotics.  $IC_{50}$  values calculated on the basis of the MTT assay indicated that ethylbenzene was more cytotoxic than tetrachloroethylene and n-hexane. Using hepatocyte monolayer culture and the MTT assay to assess cytotoxicity of organic solvents causes many technical problems. It seems that it cannot be used as a rapid, cheap, and credible method.

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in vitro methods    hepatocytes in monolayer culture    organic solvents  
MTT assay

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## 1. INTRODUCTION

The routine procedure for toxicity testing of chemicals requires large numbers of animals (mainly rodents). The ethical and economic problems associated with such experiments emphasize the need for the replacement of animals by *in vitro* methods in routine toxicological screening. In recent years in the framework of many international research programs (Bernson et al., 1986; Bondesson et al., 1989; Clothier, Atkinson, Garle, Ward, & Willshaw, 1995; Knox, Uphill, Fry, Benford, & Balls 1986; Walum et al., 1992) new *in vitro* methods of toxicity testing have been developed. Many of these methods concern utilisation of freshly isolated hepatocytes in suspension and in monolayer culture. These models are considered to be standardized and validated enough (Blaauboer et al., 1994; Li, 1994; Peloux, Federici, Bichet, Gouy, & Cano, 1992; Wiechetek & Karlik, 1996).

To assess the toxicity of xenobiotics many endpoints are used: cell viability, cell morphology, cell proliferation, and membrane damage. The most standardized and validated are tests that assess cell viability, that is, enzyme release, neutral red uptake, MTT reduction assay (Balls & Horner, 1985; Bernson et al., 1986; Knox et al., 1986). Tests estimating cytotoxic action of xenobiotics enable determining dose-response dependence and calculating the  $IC_{50}$  value (inhibitory concentration) similarly to dose  $LD_{50}$ , established as a result of *in vivo* experiments. Thus this method permits to establish relative acute toxicity for chemicals. The  $IC_{50}$  value also enables comparing the results of *in vitro* tests performed with the use of various examination methods and models, as well as comparing them with the results of *in vivo* experiments.

Volatile and water insoluble chemicals are the most difficult in cytotoxicity testing by *in vitro* methods (Smith, Clothier, Hillidge & Balls, 1992). Organic solvents, whose harmful influence on the human body is very strong, belong to this group of compounds. In the present study we tested toxicity action of ethylbenzene, tetrachloroethylene, and n-hexane on metabolic activities of hepatocytes in primary (monolayer) culture. To estimate the cytotoxicity of these compounds we used the MTT assay, which assesses the metabolic activities of cells by measuring the activity of succinate dehydrogenase, mostly located in mitochondria. This test is based on the principle that only viable cells (containing respiring mitochondria) can reduce significant amounts of water-soluble yellow MTT dye to an insoluble, violet MTT formazan metabolite, which can be measured colorimetrically.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Williams' E Medium (WME); fetal calf serum (FCS); Hank's buffered saline solution; trypan blue stain; dimethylsulphoxide (DMSO); 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and all chemical reagents used to isolation hepatocytes were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

The high-purity test chemicals, ethylbenzene (EB), 1,1,2,2-tetrachloroethylene (TCE), and n-hexane (n-H), were obtained from Riedel-de Haën (Germany).

### 2.2. Cells

The hepatocytes were obtained from 200–300 g Wistar male rats fed ad libitum and with free access to water. The liver was perfused by a two-stage in situ collagenase perfusion method, according to the INVITTOX Protocol No. 20 (INVITTOX, 1991). Cell number and cell viability in fresh preparations were determined in a Bürker chamber by the trypan blue exclusion method and was on average  $4 \times 10^8$  hepatocytes. For experiments cells whose viability was over 80% were used. The freshly isolated cells were suspended in WME ( $0.5 \times 10^6$  of living cells/ml). Then hepatocytes were seeded on 96-well microplates (Nunc, USA) and incubated at 37 °C for 3 hrs in an atmosphere of 5% CO<sub>2</sub> in air. After this period, non-attached cells were aspirated and test compounds were added. The tested chemicals were dissolved in WME with 6% FCS and in WME without FCS. The solutions of chemicals were prepared before each experience in 14 concentrations. These concentrations were chosen on the basis of preliminary studies. Twice-increasing concentrations were used: EB—from 0.04 to 334 mM; n-H—from 0.7 to 612 mM; TCE—from 0.025 to 195 mM. Each concentration (each sample) in 8 or 10 replication was added in plate's wells and then plates were maintained in a CO<sub>2</sub> incubator for 2 hrs. Hepatocytes not exposed to xenobiotics incubated in WME with or without FCS constituted the control group.

### 2.3. MTT Assay

The MTT assay was conducted according to the INVITTOX Protocol No. 17 (INVITTOX, 1990).

After removing the medium with tested chemicals, medium-containing MTT (5 mg/ml in Hank's buffered saline) was added to each well (100  $\mu$ l). After incubation for 3 hrs, supernatants were removed and the violet formazan product obtained (by reduction of the MTT) was dissolved in 100  $\mu$ l of pure DMSO. The plates were then shaken and absorption was measured using an ELISA microplates reader (545 and 620 nm). The results were calculated for each concentration as a percentage of medium control and the IC<sub>50</sub> value was established. IC<sub>50</sub> is defined as the concentration of a chemical that reduces cellular activities by 50% in comparison with untreated control cultures.

### 3. RESULTS

The cytotoxic action of examined solvents estimated by the MTT assay has been presented in Figures 1–3. The achieved results indicate that all of the tested compounds inhibited metabolic activity of hepatocytes and this depended on the concentration of solvents in the incubatory medium. Ethylbenzene in concentrations from 0.08 to 2.6 mM did not change the metabolic activity of mitochondria (Figure 1). Explicit cytotoxic action of EB was observed at concentration in the medium of 10.5 mM and the

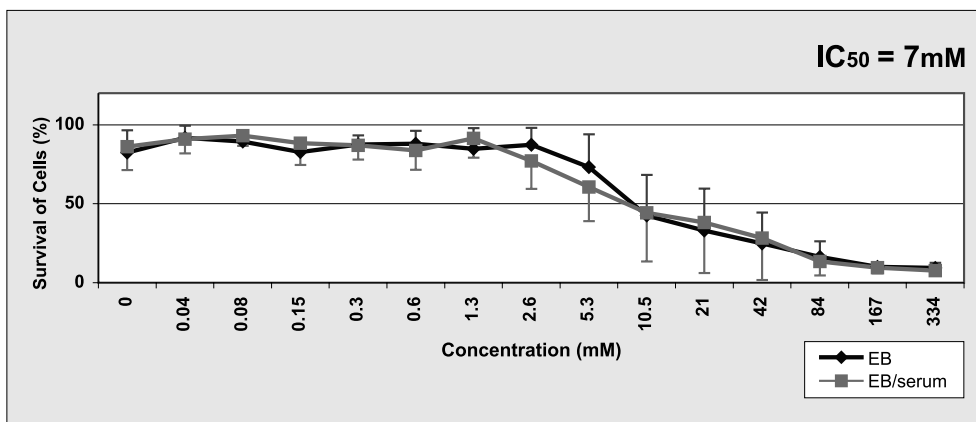


Figure 1. Toxic effect of ethylbenzene (EB) on hepatocytes, expressed as percentage survival of cells.

maximum cytotoxic effect (complete lack of MTT reduction) was observed during hepatocytes incubation in the presence of 167 mM of EB.

In the case of tetrachloroethylene, the minimum effective (leading to cytotoxic action) concentration was 3 mM and the maximum cytotoxic effect occurred during incubation of hepatocytes in the presence of 49 mM of TCE (Figure 2).

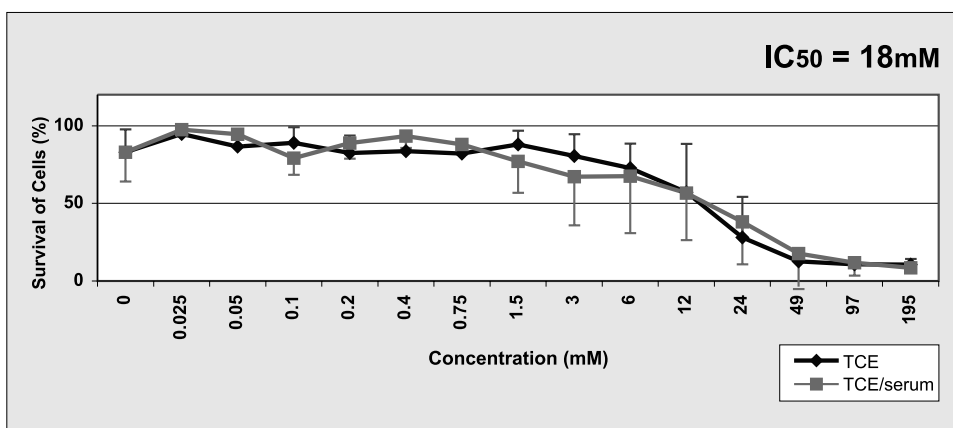


Figure 2. Toxic effect of tetrachloroethylene (TCE) on hepatocytes, expressed as percentage survival of cells.

The cytotoxic action of n-hexane was observed during incubation of hepatocytes in concentrations exceeding 76 mM (Figure 3). In the case of

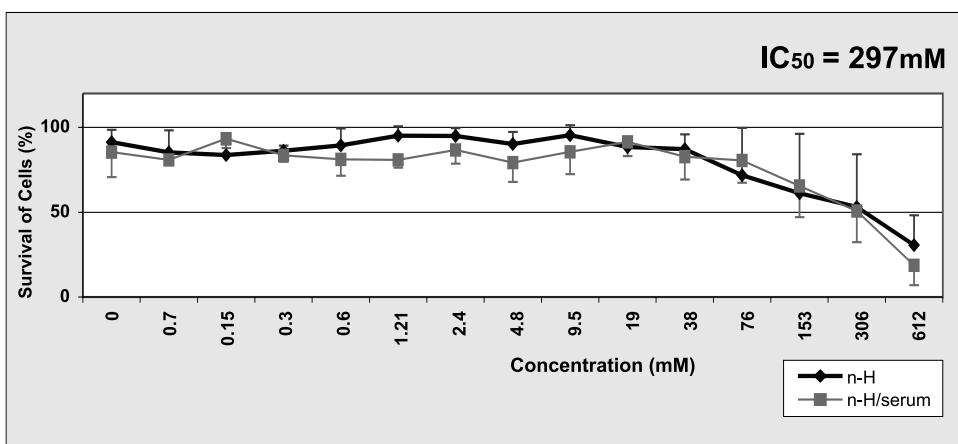


Figure 3. Toxic effect of n-hexane (n-H) on hepatocytes, expressed as percentage survival of cells.

hexane the clear dependence between the concentration of this compound in the medium and the degree of cytotoxic action was also observed, but none of the applied n-H concentrations led to the total inhibition of MTT reduction in hepatocyte mitochondria.

The presence of fetal calf serum in the incubation medium did not change the cytotoxic action of examined solvents (Figures 1–3). The calculated  $IC_{50}$  values were lowest for EB, somewhat higher for TCE, and the highest for n-H (Figures 1–3).

#### 4. DISCUSSION

The tested compounds were dissolved directly in WME culture medium or in WME with fetal calf serum (6%). It was assumed that adding serum should improve the solubility of compounds and thus facilitate their penetration into the cells. Compounds were not dissolved in DMSO or in alcohol in view of the cytotoxic action of these compounds in concentrations above 1%. Also mineral oil, the usage of which is periodically recommended (Reader et al., 1989), was not applied because hepatocytes—as cells characterized by intense metabolism—placed in a medium with oil lose their viability very quickly (these authors' observations). Adding serum to cell culture medium did not affect considerably the cytotoxic action of tested compounds. Lack of an influence of serum presence on the cytotoxic effect of the examined solvents may be considered from two points of view. Serum facilitates dissolution of lipophil substances in cell medium but at the same time it is a source of growth factors and hormones, acting as a shield for isolated cells. It is known that adding serum increases the survival rate of hepatocytes in long-term experiments and accelerates reconstruction of membrane receptors, damaged as a result of the isolating procedure. The serum also affects favourably the process of attaching hepatocytes to the bottom of microplate wells. Assuming that the cytotoxic effect of the examined solvents also leads to the detachment of damaged cells, the presence of serum might slow down this process. Therefore, adding serum may be the source of counter processes, leading to the lack of effects of its action. Obviously, the observed changes are influenced by serum concentration, which would however require additional examinations.

The achieved results indicate that all the compounds showed cytotoxic action. The dose-response dependencies had a typical sigmoidal form. It was especially clear in the case of EB and TCE. Similarly as *in vivo* (literature

data), EB was more cytotoxic than TCE and n-H. The  $IC_{50}$  values were also similar to the values obtained in cytotoxic examinations of action of the same compounds, made on hepatocytes' suspension with the usage of enzymatic tests (Zapór, Karlik, Skowroń, Gołofit-Szymczak, & Wiechetek, 2000; Zapór, Skowroń, Gołofit-Szymczak, Karlik, & Wiechetek, 2000; Zapór, Skowroń, Miranowicz-Dzierżawska, Gołofit-Szymczak, & Karlik, 1999).

The aforementioned result indicates that the applied examination model (i.e., monolayer hepatocyte culture) and the MTT reduction assay may be a screening method for examinations of the cytotoxic action of organic solvents. The problem of establishing correct time of exposure and dose of applied solvents is still of great importance at this point. The optimum time of cell incubation with tested compounds was established to be 2 hrs. Short-time tests of cytotoxicity determination were used. In preliminary experiments it was observed that longer incubation probably caused more intense evaporation of solvents so that the vapours of various concentrations of the examined compound mixed and penetrated into all the wells of microplate, causing damage of cells, even those which were not exposed to xenobiotics. In addition to this, long-lasting contact of solvent with plastic microplate material caused its damage, which made colorimetric measurements impossible. Shorter incubation time was, in turn, a reason for using high concentrations of compounds (low concentrations of xenobiotics did not manage to show cytotoxic action in time), which also led to microplate damage. The method of MTT reduction in the case of the examination of organic solvents required a very large number of preliminary experiments. That is why applying it to a cell model like freshly isolated hepatocytes in primary culture may not be reckoned as a quick and cheap method. Beside, using plastic microplates may cause an erroneous interpretation of the results.

Taking into consideration the difficulties faced in estimating the examined compounds (proper selection of examination model, methods of estimating cytotoxicity, time of exposure, vehicles of toxic substances) the more favourable method to assess cytotoxic action of volatile and non-soluble substances seems to be using other models (e.g., established cell lines) or hepatocytes in suspension. Examinations of toxicity on cell suspension are performed in glass flasks, which eliminates the aforementioned difficulties associated with performing the test.



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