INTERNATIONAL JOURNAL OF OCCUPATIONAL
SAFETY AND ERGONOMICS (*JOSE*) 2002, VOL. 8, NO. 1, 121–129

NOTES

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

Lidia Zapór
Jolanta Skowroń
Małgorzata Gołofit-Szymczak

Department of Chemical and Aerosol Hazards,
Central Institute for Labour Protection, Warsaw, Poland

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.

in vitro methods hepatocytes in monolayer culture organic solvents MTT assay

This study is part of the National Strategic Programme "Occupational Safety and Health Protection in the Working Environment", supported in 1998–2001 by the State Committee for Scientific Research of Poland. The Central Institute for Labour Protection is the Programme's main co-ordinator.

The authors of the study wish to express their sincere thanks to Prof. Maria Wiechetek for enabling performing the experimental work in the Department of Toxicology of Warsaw Agricultural University and to Dr. Wojciech Karlik for his care and assistance during carrying out the work.

Correspondence and requests for offprints should be sent to Lidia Zapór, Central Institute for Labour Protection, Czerniakowska 16, 00-701 Warszawa, Poland. E-mail: <izap@ciop.pl>.

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 4×10^8 hepatocytes. For experiments cells whose viability was over 80% were used. The freshly isolated cells were suspended in WME (0.5×10^6 of living cells/ml). Then hepatocytes were seeded on 96-well microplates (Nunc, USA) and incubated at 37 ^{o}C for 3 hrs in an atmosphere of 5% CO_2 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_2 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 µl). After incubation for 3 hrs, supernatants were removed and the violet formazan product obtained (by reduction of the MTT) was dissolved in 100 µl 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_{50} value was established. IC_{50} 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

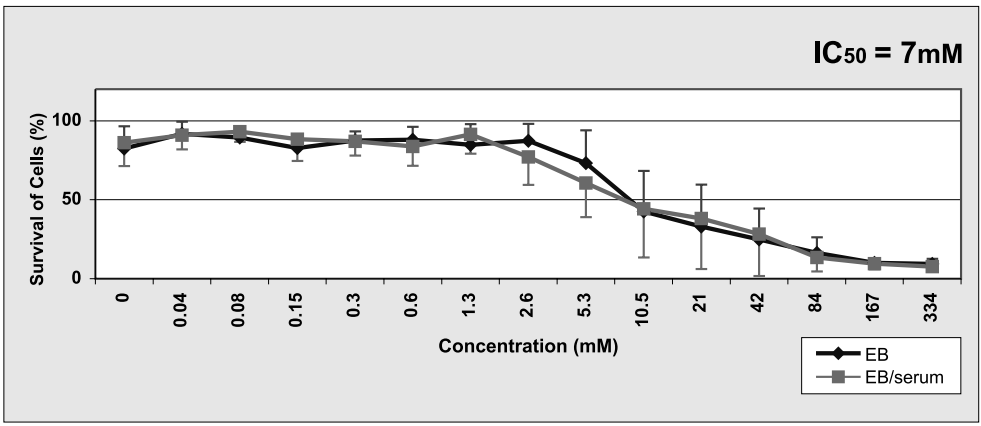

Figure 1. Toxic effect of ethylbenzene (EB) on hepatocytes, expressed as percentage survival of cells.

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).

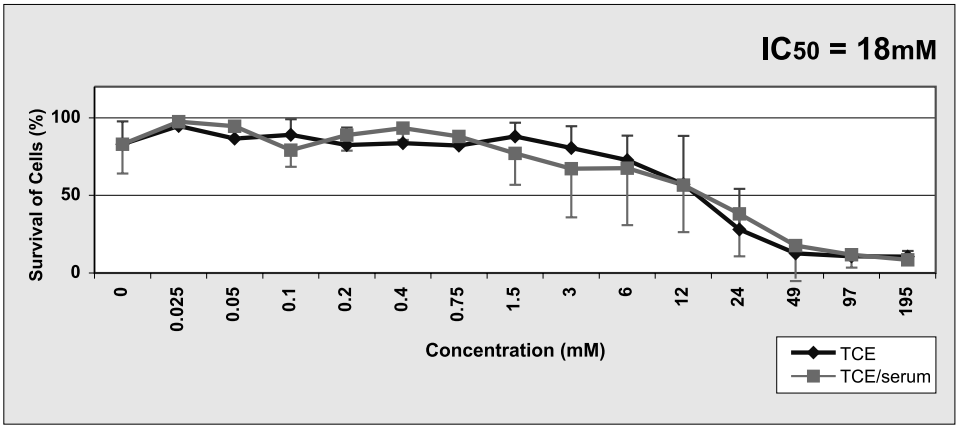

Figure 2. Toxic effect of tetrachloroethylene (TCE) on hepatocytes, expressed as percentage survival of cells.

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

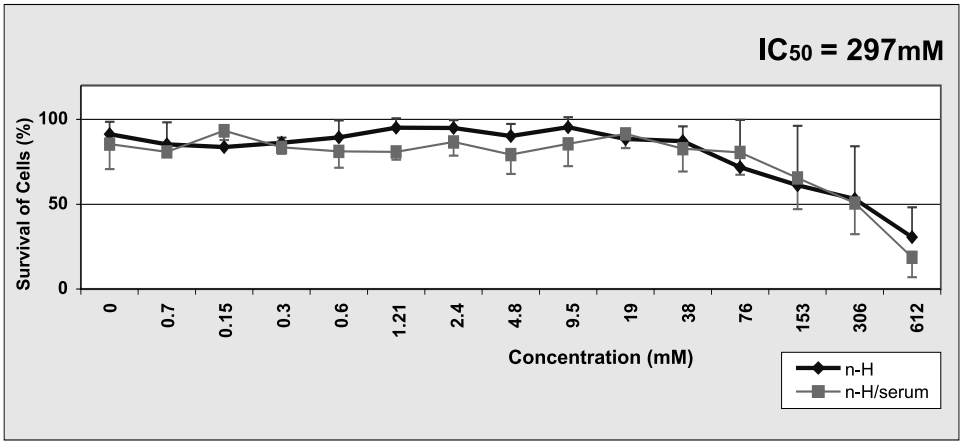

Figure 3. Toxic effect of n-hexane (n-H) on hepatocytes, expressed as percentage survival of cells.

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-tern 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.

REFERENCES

Balls, M., & Horner, S.A. (1985). The FRAME interlaboratory programme on in vitro cytotoxicology. *Food and Chemical Toxicology, 23*, 209–213.

Bernson, V., Clausen, J., Ekwall, B., Hensten-Pettersen, A., Holme, J., Hogberg, J., Niemi, M., & Walum, E. (1986). Trends in Scandinavian cell toxicology. *Alternatives to Laboratory Animals (ATLA), 13*, 162–179.

Blaauboer, B.J., Boobis, A.R., Castell, J.V., Coecke, S., Groothius, G.M.M., Guillouzo, A., Hall, T.J., Hawksworth, G. M., Lorenzon, G., Miltenburger, H.G., Rogiers, V., Skett, P., Villa, P., & Wiebel, F.J. (1994). The practical applicability of hepatocyte cultures in routine testing. The report and recommendations of ECVAM workshop 1. *Alternatives to Laboratory Animals (ATLA), 22*, 231–241.

Bondesson, I., Ekwall, B., Hellberg, S., Rombert, L., Stenberg, K., & Walum, E. (1989). MEIC—A new international multicenter project to evaluate the relevance to human toxicity of in vitro cytotoxicity tests. *Cell Biology and Toxicology, 5*, 331–347.

Clothier, R.H., Atkinson, K.A., Garle, M.J., Ward, R.K., & Willshaw, A. (1995). The development and evaluation of in vitro tests by the FRAME Alternatives Laboratory. *Alternatives to Laboratory Animals (ATLA), 23*, 75–90.

INVITTOX. (1990). *INVITTOX protocol number 17: MTT assay.* Nottingham, UK: Author.

INVITTOX. (1991). *INVITTOX protocol number 20: Isolation of rat hepatocytes.* Nottingham, UK: Author.

Knox, P., Uphill, P.F., Fry, J.R., Benford, J., & Balls M. (1986). The FRAME multicentre project on in vitro cytotoxicology. *Food and Chemical Toxicology, 24*, 457–463.

Li, A.P. (1994). Primary hepatocytes culture as an in vitro toxicological system of the liver. In S.C. Gad (Ed.), *In vitro toxicology.* New York, NY, USA: Raven Press.

Peloux, A.F., Federici, Ch., Bichet, N., Gouy, D., & Cano, J.P. (1992). Hepatocytes in primary culture; An alternative to LD_{50} testing? Validation of a predictive model by multivariate analysis. *Alternatives to Laboratory Animals (ATLA), 20*, 8–26.

Reader, S.J., Blackwell, V., O'Hara, R., Clothier, R.H., Griffin, G., & Balls, M. (1989). A vital dye release method for assessing the short-term cytotoxic effects of chemicals and formulations. *Alternatives to Laboratory Animals (ATLA), 17*(1), 28–37.

Smith, L.M., Clothier, R.H., Hillidge, S., & Balls, M. (1992). Modification of the FRAME Kenacid Blue method for cytotoxicity tests on volatile materials. *Alternatives to Laboratory Animals (ATLA), 20*, 230–234.

Walum, E., Balls, M., Bianchi, V., Blaauboer, B., Bolcsfoldi, G., Guillouzo, A., Moore, G.A., Odland, L., Reinhardt, Ch., & Spielmann, H. (1992). ECITTS: An integrated approach to the application of in vitro test systems to the hazard assessment of chemicals. *Alternatives to Laboratory Animals (ATLA), 20*, 406–428.

Wiechetek, M., & Karlik, W. (1996). Izolowane hepatocyty: Alternatywny model w badaniach toksyczności [Isolated hepatocytes: An alternative model in toxicity studies]. *Medycyna Pracy, 5*(Suppl. 6), 105–114.

Zapór, L., Karlik, W., Skowroń, J., Gołofit-Szymczak M., & Wiechetek M. (2000). Cytotoxicity of some organic solvents: In vitro studies. In *26th International Congress on Occupational Health. Singapore, 27th August–1st September 2000, Scientific Programme and Abstracts* (p. 624). Singapore: International Congress on Occupational Health (ICOH).

Zapór, L., Skowroń, J., Gołofit-Szymczak, M., Karlik, W., & Wiechetek, M. (2000). Zastosowanie izolowanych komórek wątroby do oceny toksycznego działania substancji chemicznych; cz. II pt.: Oznaczanie enzymów wewnątrzkomórkowych—jako wskaźnik cytotoksycznego działania substancji chemicznych w warunkach *in vitro* [The use of isolated liver cells for the assessment of the toxicity of chemical substances; pt. II: Leakage of cell enzymes—as an indicator of cytotoxic activity of chemical substances in in vitro conditions]. *Bromatologia i Chemia Toksykologiczna, XXXIII*(3), 269–275.

Zapór, L., Skowroń, J., Miranowicz-Dzierżawska, K., Gołofit-Szymczak, M., & Karlik, W. (1999). Zastosowanie izolowanych komórek wątroby do oceny toksycznego działania substancji chemicznych; cz. I. pt.: Aminotransferaza asparaginowa—jako wskaźnik cytotoksycznego działania substancji chemicznych w warunkach *in vitro* [The use of isolated liver cells for the assessment of the toxicity of chemical substances; pt. I: Aspartate aminotransferase—as an indicator of cytotoxic activity of chemical substances in in vitro conditions]. *Bromatologia i Chemia Toksykologiczna, XXXII*(3), 207–213.