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Cytotoxicity of Resorcinol Under Short- and Long-Term Exposure in Vitro

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Cytotoxicity of resorcinol to 3T3 fibroblast in short- (3 hrs) and long-term (72 hrs or 6 weeks) exposure was investigated. The effects of resorcinol on cell viability (neutral red uptake, NRU assay), mitochondrial function (MTT assay) and total cell protein (Kenacid Blue assay) were estimated. As a model for long-term exposure an INTEGRA CL 6-WELL bioreactor was used. The concentrations of resorcinol producing 20, 50 and 80% inhibition of cell growth in the NRU test were lower than in the MTT test after 3 hrs of exposure. The use of an INTEGRA CL 6-WELL bioreactor allows continuous culturing and exposure to test chemical of cells for several weeks, but the strong adhesiveness of fibroblast and forming aggregates make it difficult to remove them from chambers. Resorcinol in concentration of 1 μg/cm^3 did not decrease the viability of cells to 50% of control in long-term exposure in the bioreactor.

resorcinol short-term toxicity long-term toxicity bioreactor in vitro methods

1. INTRODUCTION

Chemicals can have three main types of toxic effects at the cellular level: basal cytotoxicity, selective cytotoxicity and cell-specific function toxicity. All three types of effect can result in acute systemic toxicity in vivo [1].

Cytotoxicity can be defined as the interference of a chemical compound with structures and/or functions essential for survival and reproduction of almost any mammalian cell: integrity of membranes and cytoskeleton; metabolism, including energy metabolism and synthesis and degradation of cellular constituents; ion regulation; and cell division [1].

Many biological endpoints can be measured to detect cytotoxicity:

- in cell morphology: cell size and shape; cell-cell contacts; nuclear size, shape and inclusions; nucleolar vacuole formation; cytoplasmatic vacuole formation;
- in cell viability: vital dye uptake; trypan blue exclusion; cell number; replating efficiency;
- in cell adhesion: attachment to culture surface, detachment from culture surface; cell-cell adhesion;
- in cell proliferation: increase in cell number; increase in total DNA, increase in total RNA; increase in total protein; colony formation;
- in membrane damage: loss of enzymes (e.g., LDH); loss of ions or co-factors (e.g., K^+, NADPH); leakage from pre-loaded cells (e.g., vital dye); leakage across cellular membrane;
- in uptake or incorporation of radioactive precursors: thymidyne and DNA synthesis; uridine and RNA synthesis; amino acids and protein synthesis;
- in metabolic effects: inhibition of metabolic cooperation; co-factor depletion; impairment of mitochondrial function [2].

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The results from any cellular in vitro system are not only dependent on the properties of the cell type used, but also on experimental conditions such as study time, medium composition, etc. [3].

Short-term tests involve exposure to potential toxicants for periods from 1 min to about 4 hrs, and endpoint measurements focused on cell viability and cell membrane damage. Long-term tests are based on measuring cell survival and cell proliferation over periods from 24 hrs to several days [2].

Resorcinol (1,3-dihydroxybenzene, CAS No. 108-46-3) is employed in tanning and photography; in the manufacture of resorcinol-formaldehyde resins used in tire building, as well as in dyes, cosmetics, over-the-counter pharmaceutical skin creams, laminates and adhesives; and as a rubber tackifier and cross-linking agent for neoprene. Resorcinol is an irritant of the eyes and skin. Oral ingestion of resorcinol may cause methemoglobinemia, cyanosis and convulsion. Dermal exposure has been reported to cause dermatitis, hyperemia, and pruritus [4]. Resorcinol was tested for carcinogenicity in one experiment in mice and one experiment in rats by oral administration. It was also tested in mice by skin application. No carcinogenic effect was observed in these experiments. Resorcinol is water-soluble and readily conjugated and eliminated. It was tested in various genetic toxicology assays, including in vitro bacterial and mammalian assays and in vivo mammalian assays. It gave negative results in all studies, with the exception of a positive response in the two in vitro studies that assessed chromosomal aberrations in human lymphocytes from whole blood cultures; however it did not induce chromosomal aberrations in human fibroblasts. Resorcinol is not classifiable as to its carcinogenicity to humans (IARC, group 3) [5]. The mechanism of resorcinol action is not known [6].

The present study was undertaken to obtain data on resorcinol cytotoxicity under short- and long-term fibroblast exposure in vitro. We investigated the effects of resorcinol on cell viability (neutral red uptake, NRU method), mitochondrial function (MTT assay) and total cell protein (Kenacid Blue assay).

2. MATERIALS AND METHODS

2.1. Cell Culture and Treatment

3T3 mouse fibroblast (ACC 173; German Collection of Microorganisms and Cell Cultures) was established from disaggregated Swiss albino mouse embryos in 1962. It is a rapidly dividing (doubling time of ca. 40 hrs), undifferentiated cell line, with sensitivity to contact inhibition.

3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum and antibiotics (penicillin, streptomycin, amphotericin, neomycin in concentration of 1 cm^3/100 cm^3 medium) in a sterile tissue culture flask (Nagle Nunc International, USA), maintained in 5% CO_2: 95% air atmosphere. Once the cells were attached overnight, the medium was replaced. Before the experiment, the cells were removed from the flask by trypsinisation and cell suspension in medium was obtained. To determine total cell counts and the number of viable cells a trypan blue stain and a Bürker (Merck, Germany) haemocytometer was used [7]. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Viable cells were diluted with medium to give a final concentration of 8×10^3 cells/well plate for a 3-hr exposure period assay or 3×10^3 cells/well plate for a 72-hr of exposure period assay. The diluted cell suspension was put into 96 wells of multi-well plate and incubated overnight to allow adherence and recovery from exposure to trypsin. Eight wells on each plate contained the medium with cells only (control cultures). After overnight incubation, the medium was aspirated from the cells and resorcinol dissolved in the medium without serum was added in concentration: 0.01, 0.1, 0.5, 1, 10, 100, 1,000, 5,000 or 10,000 μg/cm^3 [8, 9] and incubated for 3 (NRU and MTT test) or

72 hrs (MTT and KB tests). Next the following tests were conducted: the NRU test according to INVITTOX protocol No. 64 [9], the MTT test according to INVITTOX protocol No. 17 [10] or the KB test—INVITTOX protocol No. 3b [11]. The number of cells in the presence of resorcinol was compared to that observed in control cultures and percentage inhibition of growth was calculated. For short-term exposure IC_{20}, IC_{50} and IC_{80} concentrations (i.e., concentrations producing 20, 50 and 80% inhibition of growth) were determined and expressed as $\mu g/cm^3$.

An INTEGRA CL 6-WELL bioreactor (INTEGRA Biosciences, Switzerland) was used for long-term exposure. The system comprises 6 parallel and fully independent chambers of 750 mm^3 supplemented by a nutrient supply chamber of 30 cm^3. Cells are cultured in a cell compartment separated by an upper semi-permeable membrane from the basal medium compartment. Nutrients and other small molecules pass across the semi-permeable membrane into and out of the cell compartment. Large molecules (>10,000 molecular weight) are retained within the cell compartment and need not be diluted by basal medium. The cells settle upon the bottom of the cell compartment atop of a gas exchange surface, across which oxygen and carbon dioxide rapidly diffuse. This approach leads to high concentration within the small cell compartment volume (Figure 1).

The bioreactor was divided into cells to which resorcinol was added with a medium without serum in concentration of 1 $\mu g/cm^3$ determined on the basis of 72 hrs of experiments (MTT and KB tests) and to cells with medium only (control cultures). In the cell compartment 750 mm^3 cell suspensions in concentration of 3×10^4 cell/cm^3 were added. In the nutrient medium compartment 20 cm^3 of warm medium were added. After 7 days of incubation from the cell compartment 375 mm^3 of medium were taken and 375 mm^3 of fresh medium consisting of 15% of serum and antibiotics were added. The nutrient medium was also replaced. Every 3 days the procedure of changing the medium was carried out. The procedure of removing the culture and transferring it to the 96 wells of multi-well plate was done at intervals of 1 week for 6 weeks. The nutrient medium was removed from the top compartment, from cultivation transport port after taking 375 mm^3 of medium, 375 mm^3 of 0.25% trypsin with EDTA were added. Trypsinization was done twice at intervals of 15 min. At the end the cell compartment was washed with a medium with 10% of serum. The suspension of cells prepared in this way was centrifuged for 5 min at the speed of 1,100/min.

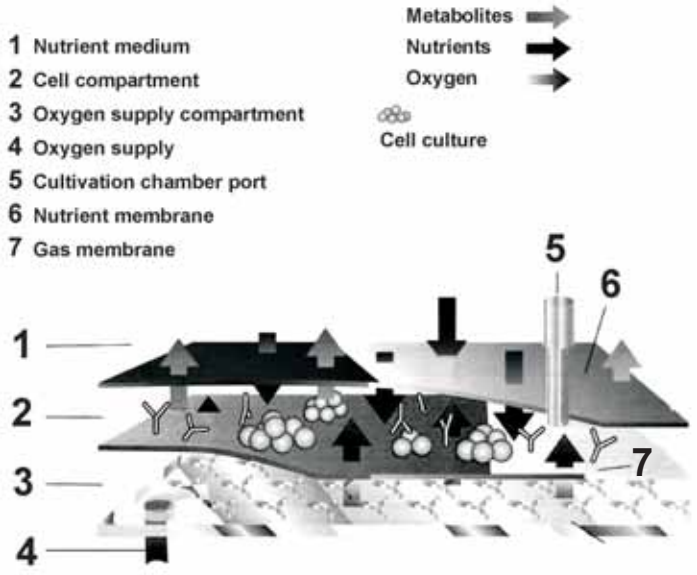

Figure 1. The structure of a membrane bioreactor.

After that the medium was removed and the cells were resuspended in 1 cm^3 of medium. To determine total cell counts and the number of viable cells the trypan blue stain was used [7]. The cells were diluted by volume according to the number of cells from the control department to obtain optimal concentrations to run tests in the 96 wells of a multi-well plate. After overnight incubation at 37 ºC in a 95% air/5% CO_2 environment the MTT test (INVITTOX protocol number 17) or the KB test (INVITTOX protocol number 3b) was done. The number of cells in the presence of resorcinol was compared to that observed in control cultures and the percentage inhibition of growth was calculated.

2.2. Chemicals

The following were used to culture the cells: culture flask Nunc, 96 wells of a multi-well plate (Nagle Nunc International, USA); bioreactor INTEGRA CL 6-WELL (INTEGRA Biosciences, Switzerland); MEM (Minimum Essential Medium) with Earle's salt, without L-glutamine; DMEM (Dulbecco's Modified Eagle's Medium); Foetal Bovine Serum; Antimycotic (an antibiotic from Gibco BRL, Life Technologies Ltd., Paisley, UK). Trypsin solution (0.25%), EDTA 0.4% and trypan blue solution (0.4%) from Sigma (Sigma Chemical Company, St. Louis, MO, USA).

The following were used in the tests: MTT [3-(4,5-dimethythiaz-2-y)-2,5-diphenyltetrazolium bromide]; dimethylsulfoxide (DMSO); Hank's Balanced Salt Solution; Brillant Blue R; Dulbeco's Phosphate Buffered Saline, potassium acetate from Sigma, acetic acid (100%) from Merck (Germany); ethanol 96% from Z.P.S. Polmos (Kutno, Poland). Tested chemical: 1,3-dihydroxybenzene from Merck.

2.3. Cell Viability Assay

The neutral red assay (NRU) is being evaluated as an alternative to the Draize test as a method for detecting potential eye irritants. The assay is based on the inability of dead and damaged mouse fibroblast 3T3 cells (lysosomes) to take up the neutral red dye. If dye-treated cells are lysed, the colour is released and it can be measured photometrically. Toxic effects of test substances are manifested as a reduction in the intensity of the extracted colour compared with untreated controls [12].

The MTT colorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt (MTT) into an insoluble formazan precipitate. The product accumulates within the cell, due to fact that it cannot pass through the plasma membrane. On solubilisation of the cells, the product is liberated and can be readily detected and quantified by a simple colorimetric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number. The assay has therefore been adapted for use with cultures of exponentially growing cells. Determination of their ability to reduce MTT to the formazan product after exposure to test compounds, compared to the control situation, enables the relative toxicity of test chemicals to be assessed [10].

The KB test is based on that of Knox et al. [13]. The measurement of total cell protein provides a quantitative indication of the number of cells present in a culture. Healthy cells, when maintained in culture continuously, divide and multiply over time. The basis of this test is that a cytotoxic chemical (regardless of site or mechanism of action) will interfere with this process and, thus, result in a reduction of growth rate as reflected by cell number. The degree of inhibition of growth, related to the concentration of test compound, provides an indication of toxicity [11].

2.4. Statistics

The obtained results were statistically evaluated by means of the Tukey (bioreactor) test for an unequal number of samples and Student's *t* tests. The values of ID_{20}, ID_{50} and ID_{80} were calculated by the nonlinear estimation method (GraphPad

Prism 3.03, San Diego, CA, USA, test version). A comparison of in vivo and in vitro acute toxicity data could be made by the Pearson correlation coefficient and linear regression analysis.

3. RESULTS

Resorcinol caused inhibition of mitochondrial activity in concentrations of 2500–4000 μg/cm^3 (MTT test) and inhibition of the intake of the neutral red dye by lysosomes in concentrations of 1500–4000 μg/cm^3 (NRU test) after 3 hrs of exposure. It did not have a cytotoxic effect on 3T3 cells in low concentrations in either test. The maximum reduction of MTT was 60–80% (Figure 2). The values of IC_{20}, IC_{50} and IC_{80} for resorcinol established in the MTT test were higher than in the NRU tests (Table 1). Good correlations were obtained when IC_{50} values from NRU and MTT assays were compared with LD_{50} values obtained in rabbits after dermal exposure. The maximum Pearson correlation coefficient was obtained for the IC_{50} value and dermal irritation coefficient [14].

Resorcinol in concentrations above 1 μg/cm^3 caused inhibition of 3T3 cell growth in the MTT and KB tests after 72 hrs of exposure (Figure 3). The concentration of resorcinol of 1 μg/cm^3 was accepted as the NOEL (no observed effect level) for long-term exposure, 6 weeks in the bioreactor.

The effects of this level of resorcinol in terms of mitochondrial activity assessed by conversion of MTT to formazon and the level of protein (KB test) in cells after 6 weeks of exposure in an INTEGRA CL 6-WELL bioreactor are shown in Figure 4. The viability of cells in the first week of exposure was higher (109.4% ± 2.2) than the control culture measurement with the the MTT test, but in the KB test viability was a little diminished in comparison with control (81% ± 5.6).

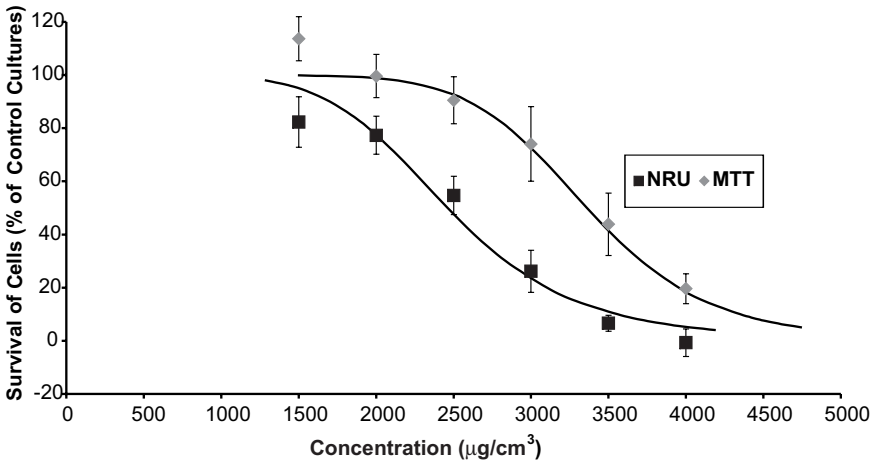

Figure 2. Cytotoxicity of resorcinol to 3T3 fibroblast assessed by NRU and MTT assays after 3 hrs of exposure. *Notes.* NRU—neutral red uptake assay, MTT—colorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt.

TABLE 1. Cytotoxicity of Resorcinol to 3T3 Fibroblasts Expressed by IC20, IC50 or IC80 Values for Neutral Red Uptake (NRU) and MTT Reduction (MTT)

Methods of Assessment	IC_{20} (μg/cm^3)				IC50 (μg/cm^3)				IC80 (μg/cm^3)			
	M	CI	*SEM*	*RSD* (%)	*M*	CI	*SEM*	*RSD* (%)	*M*	CI	*SEM*	*RSD* (%)
NRU	1934.8	234	119.6	13.8	2466.2	150	76.7	7.0	3156.3	66	33.5	2.4
MTT	2851.1	214	108.9	8.5	3366.9	177	90.3	6.0	3979.5	151	77.1	4.3

Notes. IC_{20}, IC_{50} and IC_{80}—concentrations producing 20, 50 and 80% inhibition of cell growth in comparison with control culture. CI—confidence interval, *SEM*—standard error of mean, *RSD*—relative standard deviation.

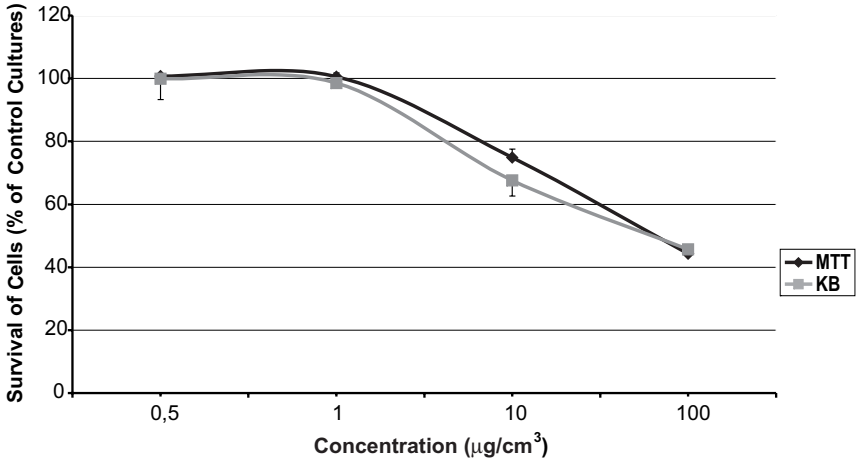

Figure 3. Cytotoxicity of resorcinol to fibroblast 3T3 assessed by MTT and KB assays after 72 hrs of exposure. *Notes.* MTT—colorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt, KB—Kenacid Blue assay determines total cell protein.

In the next week of fibroblast exposure to resorcinol the viability of cells was higher than control in both tests. In the third week of exposure there were significant differences in the viability of exposed cells compared with control. Cell proliferation measured with MTT and KB assay was elevated after exposure at 157.3% ± 3.4 and 164% ± 8.4, respectively. In the next weeks of exposure the viability of cells diminished a little in both tests and achieved about 80% viability of control cultures in 6 weeks. Resorcinol in concentration of 1 μg/cm^3 did not cause a reduction in the viability of cells to the level of 50% of control in long-term exposure in the bioreactor.

4. DISCUSSION

Cytotoxicity of resorcinol to 3T3 fibroblast in short- (3 hrs) and long-term (72 hrs, 6 weeks) exposure was investigated. For acute toxicity measurement, 3 hrs of exposure is sufficient to start the toxicity effect in the cell [15, 16, 17, 18, 19]. In the case of measuring cell proliferation, the time of exposure should be 72 hrs, because that time necessary to obtain about three cell division [2, 20, 21].

The 3T3 fibroblast chosen for this investigation are mouse undifferentiated lines, rapidly dividing and sensitive to contact inhibition. It is commonly used for testing toxicity. The ability of

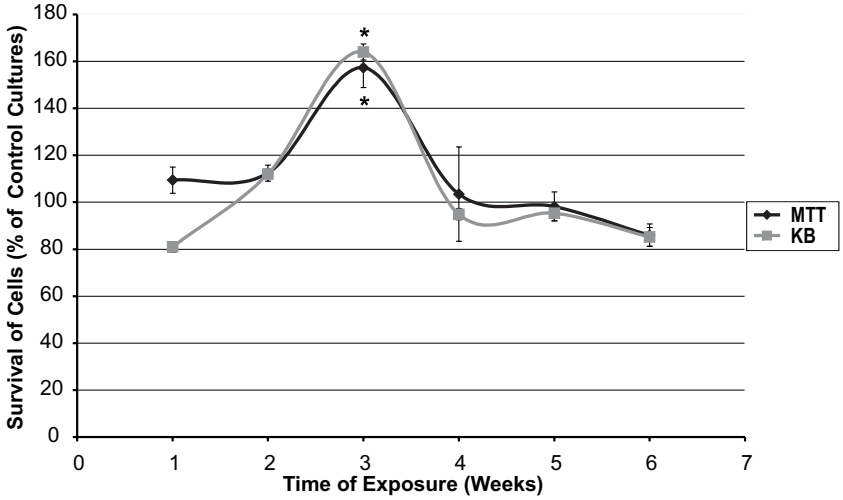

Figure 4. Cytotoxicity of resorcinol in concentration of 1 μg/cm^3 to 3T3 fibroblast after 6 weeks of exposure in an INTEGRA CL 6-WELL bioreactor (* < 0.05). *Notes.* MTT—colorimetric assay determines the ability of viable cells to convert a soluble tetrazolium salt, KB—Kenacid Blue assay determines total cell protein.

this cell to proliferate makes it possible to conduct several experiments, and also to provide long-term culture.

In accordance with European Centre for Validation of Alternative Methods (ECVAM), three tests measuring different parameters of toxicity were chosen to assess cytotoxicity of resorcinol: neutral red assay, which estimates the integrity of cell membrane (NRU test), MTT assay, which estimates the activity of mitochondrial (MTT test) and KB assay, which estimates the proliferation of cells [2, 22, 23, 24]. These methods have a very high level of standardization and validation [21, 25, 26]. They are frequently used to assess toxicity of chemicals [16, 27, 28, 29].

In our studies cytotoxicity of resorcinol to fibroblast was observed in both MTT and NRU tests after 3-hr exposure, but the sensitivity of the NRU test was greater than that of MTT. The concentrations of resorcinol producing 20, 50 and 80% inhibition of cell growth in the NRU test were lower than in the MTT test (Table 1). The dose-response curve had a typical sigmoidal form in both tests (Figure 2).

The results of NRU and MTT assays are often comparable. Some studies have revealed that differential sensitivities between the NRU and MTT tests are obtained for specific test chemicals such as antineoplastic chemicals, non-steroidal anti-inflammatory drugs or polyols. These findings demonstrate that certain chemicals may give different results in NRU and MTT assays depending on their specific mechanisms of action [27].

Good correlations of IC_{50} values from NRU and MTT assays with LD_{50} values obtained in rabbits after dermal exposure and dermal irritation coefficient may explain the local action of resorcinol in vivo. Irritation of mucosa [6] is the principal consequence of acute exposure to resorcinol. High correlation between IC_{50} and irritation explains the similarity between the in vitro and in vivo experimental systems [30]. In both cases there is direct contact between the chemicals and the cells.

Resorcinol did not have a cytotoxic effect on 3T3 cells in low concentrations, below 1000 $\mu g/cm^3$, in NRU and MTT assays after 3-hr exposure. The use of longer test periods had an influence on the viability of cells. In the 72-hr exposure experiments, there was a greater loss of viability at an exposure concentration above 1 $\mu g/cm^3$ in MTT and KB assays (Figure 3). Riddell et al. [21] concluded that a test period of 72 hrs is more suitable for the assessment of potential cytotoxicity by the KB and NRU method than a 24-hr test period, which is less likely to allow sufficient time for certain chemicals to exert their toxic effects, particularly those that inhibit cell division or affect cell viability through other long-term effects.

Many chemicals exert toxic effects following chronic low-dose treatment, a situation which occurs in the case of several various types of human exposure, such as dietary intake of foodstuffs. Most interest has been focused on developing in vitro tests for acute toxicity. Little, if any, account is taken of long-term effects. Thus, it would seem logical to develop in vitro chronic exposure assays that would make monitoring changing responses during exposure possible [31]. One alternative approach is to use a flow cell, membrane bioreactor or a hollow fibre bioreactor to allow continuous culturing and exposure to test chemicals for several weeks [32]. A system like that has a number of important variables, including initial seeding density of cells, stability of cells, target cell type, dose levels, and the use of appropriate markers of toxicity, which have not been standardized yet [31].

Hanley et al. [32] used a bioreactor system to demonstrate quantitative differences in the toxicity of 3-nitropropanoic acid, a fungal toxin, to two different continuously cultured human cell lines, depending on whether chronic or acute exposure was used. Cell populations established in the bioreactor were continuously exposed to levels of toxin below that of the NOEL for periods of up to 4 weeks and the viability of the population was determined using MTT, trypan blue and ATP assays. The effects observed were

predictive of the action of the toxin in vivo in that they suggested that high-level exposure would lead to increased mitochondrial activity and size, although they did not provide information as to the target organ in vivo.

The INTEGRA CL 6-WELL bioreactor consists of 6 independent chambers. The cells in the bioreactor are cultured in a cell compartment separated with two semi-permeable membranes: one permeable for a nutrient and metabolites and the other one for oxygen and carbon dioxide. This makes it possible to conduct a long experiment.

Resorcinol in concentration of 1 μg/cm^3 did not decrease the viability of cells to 50% of control in long-term exposure in the bioreactor. With the prolonged time of exposure the viability of cells was elevated, achieving about 150% viability of control cultures after 3 weeks in MTT and KB tests. An increase in cell viability such as that measured with MTT and KB assays was also observed when 3T3 fibroblast was exposed to benzalkonium chloride in concentration of 0.05 μg/cm^3 under long-term exposure [33].

The same effect was observed by Hanley et al. [32] when a secondary human intestinal cell line Int 401 was exposed to 3-nitropropanoic acid (3-NPA) for periods of up to 4 weeks in a flow cell bioreactor. At a higher exposure level of 8.4×10^{-10} M the mitochondrial activity as measured by the MTT assay increased compared with unexposed cells. As exposure to 3-NPA causes inhibition of succinate dehydrogenase, an alternative route for glucose catabolism—the glycolitic pathway—would be preferred. This could account for the observed increase in cell viability as determined with the MTT assay.

The mechanism of how resorcinol affects a cell is not known [6]. The observed effect of resorcinol could be explained partially by hormesis as an adaptive response to low levels of stress or damage resulting in cytotoxicity of the chemical [34]. But the adaptive mechanism of fibroblast after 4 weeks of exposure to resorcinol was broken and cell viability diminished to about 80% of unexposed cells after 6 weeks as measured with the MTT and KB assays (Figure 4).

5. CONCLUSIONS

1. Resorcinol caused inhibition of mitochondrial activity in concentrations of 2500–4000 μg/cm^3 (MTT test) and inhibition of the intake of neutral red dye by lysosomes in concentrations of 1500–4000 μg/cm^3 (NRU test) after 3 hrs of exposure.
2. The concentrations of resorcinol producing 20, 50 and 80% inhibition of cell growth in the NRU test were lower than in the MTT test.
3. Resorcinol in concentrations above 1 μg/cm^3 caused inhibition of 3T3 cell growth in the MTT and KB tests after 72 hrs of exposure. This concentration of resorcinol of 1 μg/cm^3 was accepted as the NOEL (no observed effect level) for long-term exposure, 6 weeks in the bioreactor.
4. The use of an INTEGRA CL 6-WELL bioreactor makes continuous culturing and exposure to test chemicals of cells for several weeks possible, but the strong adhesiveness of fibroblast and forming aggregates make it difficult to remove them from chambers.
5. Resorcinol in concentration 1 of μg/cm^3 did not affect the viability of cells to 50% of control in long-term exposure in the bioreactor.

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