약제학회지(1995), 제25권 제4호
J. Kor. Pharm. Sci., Vol. 25, No. 4, 365-369(1995)

Comparison of the Two *in Vitro* Cytotoxicity Assays in Primary Cultured Rat Hepatocytes - the Neutral Red (NR) and Lactate Dehydrogenase (LDH) Tests

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(Received October 2, 1995)

흰쥐의 배양 간세포를 이용한 세포독성시험에 있어서 뉴트랄레드 및 젖산 탈수소효소법의 비교

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(1995년 10월 2일 접수)

The acute cytotoxicities of chloroquine sulfate, propranolol, ascorbic acid, acetylsalicylic acid and acrylamide on cultured adult rat hepatocytes were evaluated by the use of LDH leakage and NR uptake test. On the basis of IC_{50} values, the rank order of cytotoxicities of these drugs in both tests was chloroquine sulfate > propranolol > acetylsalicylic acid > ascorbic acid. The IC_{50} of LDH test was very similar to that of NR uptake test. Thus, we concluded that both tests are reliable and sensitive methods in detecting toxicity in adult cultured rat hepatocytes.

Keywords—Hepatocytes, NR test, LDH test, Cytotoxicity

In vitro model systems are increasingly used to investigate mechanisms of chemical-induced toxicity and for toxicity screening of new drug families. Some of these cytotoxicity assays, such as a cell culture system would be an attractive model for elucidating the mode of interaction between a drug or chemical and a tissue at cellular, subcellular or molecular levels. The use of cultured cells enables one to examine the species- and organ-specific toxicity of a particular compound.[1)]

The values of enzyme leakage (ASAL, LDH, GOT, GPT and AP) are used as indicators for hepatotoxicity in detecting early cell injury. The percentage of LDH release correlates better than other enzymes with morphological changes observed by phase-contrast microscopy examination and cell viability assessed by trypan blue exclusion.[2,3)]

Among the different colorimetric cell viability assay, MTT assay, initially developed by Mosmann,[4)] was based on the ability of mitochondrial enzyme in viable cells to chemically reduce a yellow tetrazolium salt (MTT) to a purple formazan dye. However, many modifications[5,6)] have been performed because of the poor sensitivity and the difficulty in solublizing the final formazan product. The different colorimetric assay of NR is based on the incorporation of the supravital dye, NR, into the lysosomes of viable cells after their incubation with toxic chemicals. This weakly cationic dye penetrates cell membranes by nonionic diffusion and binds intercellularly to sites of the lysosomal matrix.[7)] Xenobiotics that injure the plasma and lysosomal membrane decrease the uptake and subsequent retention of the dye. Dead or damaged cells cannot retain the dye after the washing and fixation

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procedures. After NR is extracted from the lysosomes, it is quantitated spectrophotometrically and the amount is compared with the amount of dye extracted from control cell cultures. Quantitation of the extracted dye has been shown to be linear with cell numbers, both by direct cell counts and by protein determination of cell population.[7,8] The NR assay has been found to be more sensitive than the MTT assay.[9,10]

In this paper, we have validated the LDH leakage and NR uptake test in adult cultured rat hepatocytes *in vitro* assay using several compounds ranging from the most cytotoxic to the non- cytotoxic one.

MATERIAL AND METHODS

Chemicals and reagents

Collagenase (type I, from *Clostridium histolyticum*), bovine insulin, kanamycin and neutral red were obtained from Sigma Chemical Co (St Louis, MO, USA). Waymouth 752/1 medium, Minimal Essential medium (MEM), Medium 199, Leibovitz, streptomycin/penicillin solution and fetal calf serum (FCS) were obtained from Gibco Life Science (U.S.A). All other chemicals were from common sources and of the highest available quality.

Hepatocytes isolation and cell culture

Rat hepatocytes were obtained from male-Sprague-dawley CD rats (200~250 g) by the use of the two-step collagenase perfusion method as described by Lee.[11] Rat hepatocytes were seeded at a density of 2.2×10^6 cells/28 cm^2 petri dish in 4 m*l* culture medium containing 10% (w/v) FCS. The medium was a mixture of 75% (v/v) MEM and 25% (v/v) Medium 199 supplement with 10 μg bovine insulin per m*l*.

Incubation of rat hepatocytes with compounds

All drugs which were solubilized with medium were filtered and prepared at 5 different concentration (chloroquine: 1, 10, 50, 100 and 500 μg/m*l*, acetylsalicylic acid: 100, 500, 1000, 1500 and 2000 μg/m*l*, propranolol: 1, 5, 10, 20, 50 and 100 μg/m*l*). Each concentration was tested with 3 different dishes. The cultures were incubated for 24 hours at 37°C. After scrapping, cells were quickly frozen and stored at -70°C until LDH analysis.

LDH determination

LDH activity was measured in media and cell extract using a kit (Cat. No. 1644793) and following method from Boehringer Mannheim. Cell extracts were prepared by sonication with Ultra-Turax for 19 seconds after thawing. IC_{50} values (μg/m*l*) were calculated from the concentration-index curve. Index is expressed as the ratio of extracellular LDH to total LDH.

NR assay

NR uptake was assayed according to the procedure developed by Borenfreund and Puerner.[7] Briefly, NR-containing medium (50 μg/m*l*) was incubated overnight at 37°C and centrifuged to remove fine precipitates of dye crystals. Cultures with 0.5 m*l* of NR-containing medium were incubated for 3 hrs to allow for uptake of the vital dye into lysosomes of viable uninjured cells. After removing the medium, cells were washed rapidly with a mixture of 1% formaldehyde/1% $CaCl_2$ and 1.25 m*l* of 1% acetic acid/50% ethanol solution was added to each well to extract the dye. Culture plates were kept in dark for 15 mins before absorbance per well was read at 540 nm.

RESULTS AND DISCUSSION

The cytotoxic effects of 5 chemicals on cultured primary rat hepatocytes were determined by measuring the extent of LDH leakage in the medium and the extent of the decrease of NR uptake by viable cells when compared to untreated control cell cultures. Complete toxicity curves generated for the different drugs incubated for 24 hrs with LDH assay are shown in Fig. 1. For chloroquine, propranolol and acetylsalicylic acid, the leakage of LDH resulted in a graded response

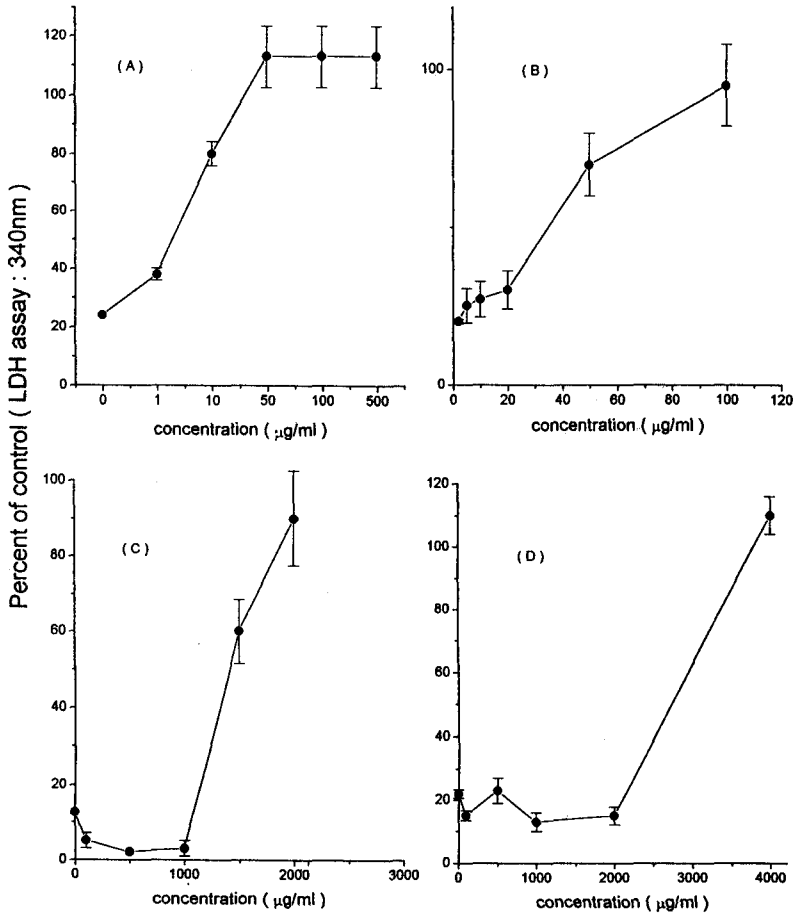

Figure 1—Concentration-response cytotoxicity curves for 1-day exposure to chemicals as dertermined with the LDH assays using cultured rat hepatocytes.
Key : (A); Chloroquine sulfate, (B); Propranolol, (C); Acetylsalicylic acid, (D); Ascorbic acid.

Table 1. *Comparison of Two In Vitro Cytotoxicity Assays-the NR and LDH Tests.*

Compounds	IC_{50}(μg/ml)	
	by NR uptake	by LDM ratio
Chloroquine sulfate	3±0.8	2.2±1.0
Propranolol	35±8	44.5±11
Acetylsalicylic acid	1,390±214	1,225±156
Ascorbic acid	⟩3,000	⟩3,000
Acrylamide	N.D	N.D

NR_{50}, LDH_{50} : midpoint cytotoxicity value after 24hr exposure to drugs. Results are the mean of three independent experiments performed with hepatocyte cultures from three rats.
N.D : no calculated. Values are mean ±S.D

with increase in drug concentration. Comparative concentrations of these drug required to obtain midpoint toxicities are shown in Table 1. IC_{50} values are calculated from the concentration-response curve.

The LDH index of chloroquine sulfate was started to increase at 1 μg/ml concentration and IC_{50} value was 3 μg/ml. Propranolol shows that

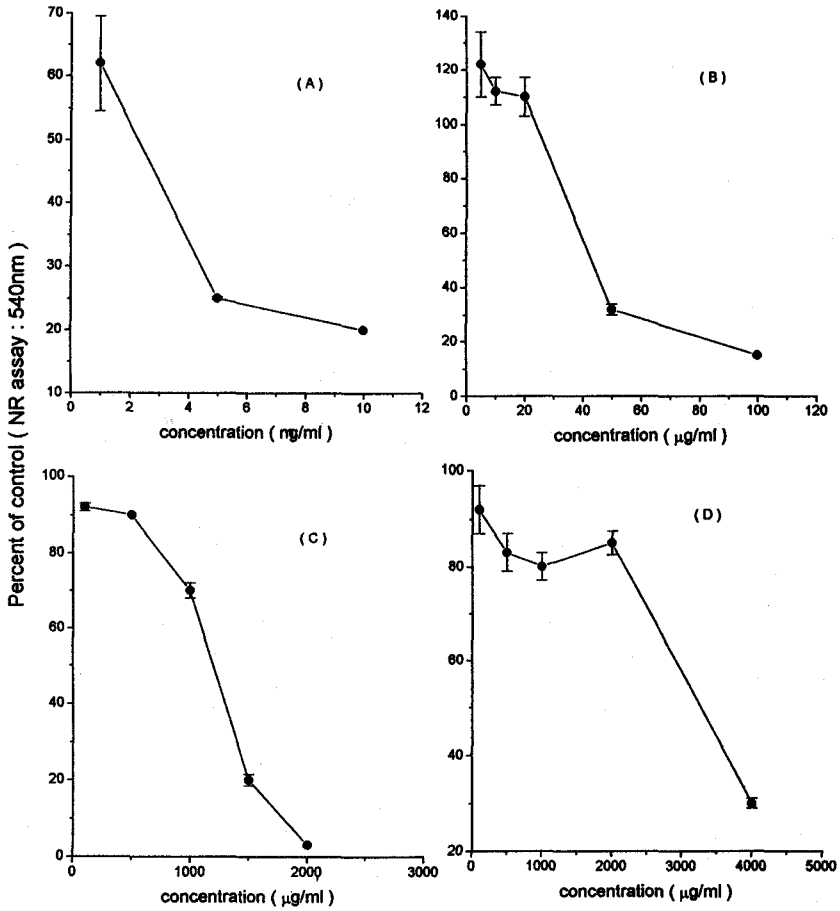

Figure 2—Concentration-response cytotoxicity curves for 1-day exposure to chemicals as dertermined with the NR assays using cultured rat hepatocytes.
Key : (A); Chloroquine sulfate, (B); Propranolol, (C); Acetylsalicylic acid, (D); Ascorbic acid .

LDH index was well correlated with the concentration of the drug and IC_{50} value is 35 μg/ml. IC_{50} value of acetylsalicylic acid is 1390 μg/ml. Sudden high toxicity at the concentration of 4 mg/ml can probably be explained by the strong acid pH of the compound itself instead of the chemical toxicologic effect. The IC_{50} value of LDH leakage by acrylamide was decreased in contrast to the increasing a concentration of compounds. This result can be expected the direct covalent binding of the compound on the LDH enzyme itself.

Treatment of cultures with chloroquine, propranolol, acetylsalicylic acid and ascorbic acid resulted in a dose-dependent decrease of NR uptake and their IC_{50} values were 3, 3.5, 1390 and 4,000 μg/ml, respectively (Fig.2). NR test showed the similar cytotoxicity compared with LDH leakage result and sudden toxicity of ascorbic acid at 4 mg/ml. Acrylamide also showed unreasonable result and it can be explained by the harmful effect on the cell structure. The values of LDH and NR in cultured hepatocytes was dependent on the compound properties.

Although the enzyme leakage test provides information on the integrity of the plasma membrane after exposure to test chemicals, we also used neutral red uptake to correlate damge in cell viability with dye uptake to lysozyme. Bas-

ed on these results, two cytotoxicity tests of LDH and NR uptake assay showed similar patterns and IC_{50} values.

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