Note

A Modified Colorimetric MTT Assay Adapted for Primary Cultured Hepatocytes: Application to Proliferation and Cytotoxicity Assays

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Primary culture of hepatocytes has been widely used as an in vitro system for studying multiple liver functions. In this culture, however, correct counting of the cells is difficult. This is because once isolated cells attach to a culture base, their cell-to-cell interactions become very tight, so that they cannot be dispersed perfectly by the conventional techniques (e.g., trypsin/EDTA treatment). To resolve this problem, we tried to apply a colorimetric MTT assay to primary cultured hepatocytes. The MTT assay is a rapid colorimetric microtiter assay which was originally developed to measure the living cell number of lymphocytes. This method is based on the selective ability of mitochondrial dehydrogenases in living cells to reduce the yellow soluble salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a purple insoluble formazan precipitate. The number of viable cells is, therefore, measurable as the concentration of the MTT reaction product in a spectrophotometer. The advantages of this assay system are its precision and rapidity. In addition, the absence of a requirement for dispersion of cultured cells to measure the cell number is suitable for primary cultured hepatocytes.

In this report, we modified the MTT assay to adapt it for primary cultured hepatocytes, and showed that the modified method worked for estimating the cell number of primary cultured hepatocytes. We also showed that the modified MTT assay was usable for hepatocyte proliferation assays and in vitro cytotoxicity assays using primary cultured hepatocytes.

First, we attempted to use the MTT assay for primary cultured hepatocytes following the original protocol described by Mosmann (Fig. 1). As shown in Fig. 2, although a roughly linear relationship between optical density and cell number was obtained, this protocol seemed to work incompletely because the MTT formazan crystals formed in the hepatocytes were tightly attached to the well bottoms of the plate and they could not be dissolved perfectly even by thorough mixing. Therefore we made slight modifications in the final crystal-dissolving step (Fig. 1). Instead of direct addition of acidified isopropanol to the medium including the crystals, (1) removal of the medium, (2) washing with PBS, (3) treatment by trypsin, and (4) dissolving the crystals in acidified isopropanol, were done. By these modifications, complete solubilization of the crystals was observed. Moreover, this resulted in a significant increase in sensitivity and a decrease in standard deviation compared to the result using the original method (Fig. 2). There was also a good linear relationship between optical density and cell number from 2000 to 36,000 cells/well. From these data, we considered the modified MTT assay was practical and useful for estimating the cell number of primary cultured hepatocytes.

Next, we tried to use the modified MTT assay in the hepatocytes

Fig. 1. Original and Modified Protocols for MTT Assay.
Primary hepatocytes were isolated from adult Wistar rats by Seglen's collagenase perfusion method. The living cell number of isolated hepatocytes in suspension were counted by the trypan blue exclusion method using a hemocytometer. Then hepatocytes were seeded into a flat-bottomed 96-well tissue culture plate coated with type 1 collagen, and cultured at 37°C in a 5% CO₂ atmosphere. The medium for hepatocyte cultures was WE with 10% fetal bovine serum, 100 μM dexamethasone, and 100 μM insulin. MTT solution was prepared at 5 mg/ml in PBS (pH 7.2). MTT reactions were done by the protocols shown in the figure. Acidified isopropanol in the figure is isopropanol which contains 0.4% HCl. In either the original or modified protocol, the last formazan-dissolving step was done by repeated pipetting with a multichannel pipettor. The optical densities of the formazan solutions in wells were measured by a Bio-Rad model 3500 EIA reader. Although OD₅₇₀-₆₃₀ had been used in the original report, we found that OD₅₇₀-₆₃₀ could replace OD₅₇₀-₆₃₀ with practically no problem (data not shown).

Fig. 2. Comparison between Original and Modified MTT Methods.
Isolated hepatocytes were counted by the method shown in the legend of Fig. 1 and seeded at various cell numbers between 0 and 36,000 cells/well. Then they were cultured for 4 hr to allow the cells in suspension to attach on the culture plate. Other details about cell culture and MTT assay followed the protocols and the legend of Fig. 1. The points and bars on graph represent the means and standard deviations of 4 replicates, respectively.

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; WE, Williams’ medium E; MEM, Eagle’s minimum essential medium.
proliferation assay and \textit{in vitro} cytotoxicity assay using primary cultured hepatocytes. Figure 3 shows the result of a hepatocyte proliferation assay using epidermal growth factor (EGF)\textsuperscript{1,2} and spleen soluble matrix fraction,\textsuperscript{41} which had been reported to show hepatocyte proliferation activity. In this assay system, significant mitogenic effects were detected in the samples containing either EGF or spleen soluble matrix fraction compared to the control sample (not containing mitogens). This indicates that the modified MTT assay is usable as a sensitive assay system for hepatocytes proliferation.

Figure 4 shows the result of an \textit{in vitro} cytotoxicity assay using primary cultured hepatocytes. In this assay three hepatotoxic compounds, carbon tetrachloride, \textit{N}-nitrosodimethylamine, and \textit{D}-galactosamine,\textsuperscript{1,2} were used as test chemicals. For all three compounds, this assay system was able to detect the toxic effects on hepatocytes in response to increasing concentrations of the compounds. When using the fibroblast L929, on the other hand, little or lower toxic effects were observed. These results indicate that the modified MTT assay is also available for a hepatocyte-specific cytotoxicity assay showing fine sensitivity.

As described above, the modified MTT assay worked successfully for estimating the cell number of primary cultured hepatocytes. Although the colorimetric MTT assay is an indirect method in regard to counting the cell number, there seem to be few problems if the assay is done with proper control(s).

In the hepatocyte proliferation assay, \textit{\textsuperscript{3}H} thymidine or \textit{\textsuperscript{125}I} iododeoxyuridine incorporation have usually been used.\textsuperscript{1,2} One of the advantages of the MTT assay is that it is done without using any radioisotopes. In this respect, the modified MTT assay is a useful alternative to the conventional radioisotope incorporation assay.

The cytotoxicity assay using primary cultured hepatocytes is expected to be a promising system for evaluating the toxic effects of chemicals on the human body. This is because the liver (and the hepatocyte) is a principal target for the toxic chemicals in the animal body, and the modified MTT assay is capable of detecting a slight change of hepatocyte viability as shown above. Moreover, the ability of this system to handle large numbers of samples is particularly suitable for the evaluation of combinational effects of multiple toxic compounds or the screening of toxic matters. We are now trying to apply this system to the evaluation of the toxic effects of environmental pollution.

\textbf{References}