A Simple In Vito Cytotoxicity Test Using the MTT (3-(4,5)-
Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide)
Colorimetric Assay: Analysis of Eugenol Toxicity
on Dental Pulp Cells (RPC-C2A)

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Abstract—A simple colorimetric assay using MTT has been developed to monitor
mammalian cell survival and proliferation in vitro. In this study we used a clonal
fibroblastic cell line (RPC-C2A) from rat incisal dental pulp to examine the effec-
tiveness of the colorimetric assay to test for the toxicity of eugenol, which is fre-
quently used to treat inflamed dental pulp. A technical problem encountered
was the insolubility of MTT formazan, produced by the activity of mitochondria
dehydrogenases. Dimethyl sulfoxide (DMSO) seemed to be the best solvent.
Doses of eugenol causing a 50% inhibition in the colorimetric assay were calculated
as 0.6 mM and 1 mM for cells in the growing phase and for cells at confluence,
respectively. These values exist in the concentration range reported in the previous
studies. Although the correlation between spectrophotometric absorbance and
cell number was not completely linear, this method could be used effectively as a
simple preliminary assay to test for the toxicity of dental drugs and materials.

There is a need for a simple initial screening
method for the toxicity of drugs and materials
used in dentistry. The MTT colorimetric assay
has been suggested to be useful for analyzing
mammalian cell survival and proliferation of
cells in vitro (1). The advantages of this
method are simplicity, rapidity and precision.
In addition, no radioisotopes are required.
Recently, we established a clonal cell line
(RPC-C2A) from rat incisal dental pulp (2).
This cell line is the first reported cell line
established from dental pulp tissue, and the
alkaline phosphatase of RPC-C2A cells is
biochemically the same as the alkaline phos-
phatase of the dental pulp. Since these cells
have a high proliferation ratio, it is easy to
maintain this cell line in the laboratory. For
this reason RPC-C2A cells are useful for
routine investigation of the cytotoxicity of
various dental drugs and materials.
Consequently, the combination of RPC-
C2A cells and the MTT colorimetric assay ap-
peared to be useful for testing the cytotoxicity
of various dental drugs and materials. In this
study, we examined the MTT colorimetric
assay using RPC-C2A cells, and then using
this methods, we investigated the toxicity of
eugenol which is frequently used to treat in-
flamed dental pulp tissue (3).

Materials and Methods

Eagle’s minimum essential medium (MEM)
containing 60 µg/ml of kanamycin and Ca-
Mg-free phosphate-buffered saline (PBS(−))
were purchased from Nissui Pharmaceutical
Co., Ltd., Tokyo, Japan; fetal bovine serum
(FBS) from Filteron PTY., Victoria, Australia;
MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-di-
phenyl tetrazolium bromide), dimethyl
sulfoxide (DMSO), ethanol, isopropanol and
eugenol from Wako Pure Chemical In-
dustries Ltd., Osaka, Japan; mineral oil from
Sigma Chemical Co., St. Louis, U.S.A.;
trypsin (1:250) from Difco Laboratories,
Detroit, U.S.A.; 100 mm culture dishes and
96-well plates from Corning, New York,
Cell culture: The culture medium was MEM supplemented with 10% FBS, and it was controlled at pH 7.4 with NaHCO₃ under an atmosphere of 95% air, 5% CO₂ and maintained at 37°C. About 2.5×10⁵ RPC-C2A cells were inoculated into a 100-mm culture dish and cultured. After 3 or 4 days, they were washed with 5 ml of PBS(−) three times and were treated with 4 ml of PBS(±) containing 0.025% trypsin. After the cells were detached from the culture dish, 4 ml of the culture medium was added. They were collected by centrifugation (400 g for 10 min at 4°C). Then, they were resuspended in the culture medium and used for the following study.

MTT solution: MTT solution was prepared as 5 mg/ml in PBS(−) just before use and filtered through a 0.22-μm filter.

Solvents for formazan: One hundred microliters of the cell suspension containing 1.6×10⁴ cells was inoculated to each well of five 96-well plates. After 24 hr of culture, 10 μl of MTT solution was added to each well. After 4 hr incubation in CO₂ incubator, the medium containing MTT was removed by inverting and tapping the plates. The solvents examined were: ethanol, isopropanol, acid-isopropanol (0.04 N HCl in isopropanol), mineral oil and DMSO.

The MTT formazan spectrum: The spectrum of MTT formazan in DMSO was determined by a Hitachi 624 Spectrophotometer using DMSO as a blank.

Correlation between the spectrophotometric absorbance and the cell number: Cell suspensions of RPC-C2A cells at different cell concentrations were inoculated into the individual wells of a 96-well plate. After 24 hr, the cells in 4 wells of each concentration were detached by 0.025% trypsin solution and were counted with a hemocytometer. The MTT assay was performed using the cells in the remaining wells, 4 wells of each cell concentration.

The MTT assay and the cytotoxicity test of eugenol: One hundred microliters of the cell suspension of RPC-C2A cells was inoculated into 96-well plates. Usually, the initial inoculation cell density of RPC-C2A cells was 1×10⁴/cm² and their confluent cell density, 5–7×10⁴/cm². The area of each well was 0.32 cm². Thus, the inoculating cell number was 3200 cells per well for the assay of the cells in the growing phase and 16000 cells per well for the assay of cells at confluence. One hundred microliters of the culture medium was added to the bottom row of the plate to test for the effect of the drug on the production of MTT formazan in the absence of cells. After 24 hr of culture, the medium was removed by aspiration, and 100 μl of the experimental medium was added to each well. The experimental medium was also added to the bottom row of the plate wells. In this study, the experimental medium contained various concentrations of eugenol (0.125–4 mM). Forty microliters of 1.6 M eugenol in 70% ethanol was added to 4 ml of the culture medium and diluted successively with the culture medium. Eugenol, which did completely dissolve, was suspended in the culture medium. After 24 hr, cells were observed using a phase contrast microscope, and then 10 μl of MTT solution was added to each well. The plates were incubated in a CO₂ incubator for 4 hr. Next, all the medium was removed by inverting and tapping the plates, and 100 μl of DMSO was added to each well. The spectrophotometric absorbance was then measured at 540 nm using an ELISA reader (Titertek Multiskan MC).

Results

After incubation with MTT solution, dark blue formazan was observed in the cells under phase contrast microscopy. After removing the medium, a trace of formazan remained at the surface of the culture dish. This MTT formazan was soluble in ethanol, isopropanol, and acid-isopropanol after shaking for several minutes. Overnight incubation at 37°C was necessary to dissolve MTT formazan in mineral oil. However, MTT formazan dissolved in DMSO immediately.

The absorbance spectrum of MTT formazan in DMSO displayed an absorbance peak at 550 nm (Fig. 1). Since the only filter available for the ELISA reader that read close to the absorbance maximum was a 540 nm filter, this was used in all of the following MTT colorimetric assays.

The absorbance was found to increase with
the increase in cell number, although the absorbance was not directly proportional to the cell number (Fig. 2). Thus, cells at a low density tended to show a higher relative absorbance than cells at a higher density, as shown in Fig. 3.

There was no difference in absorbance among the wells on the bottom row of the plate. Since these wells contained different concentrations of eugenol only, it is clear that eugenol alone had no effect on the MTT assay.

Toxicity of eugenol to RPC-C2A cells in the growing phase and at confluence are shown in Figs. 4 and 5, respectively. Eugenol inhibited the production of MTT formazan in a dose-dependent manner in both cases. When RPC-C2A cells were in the growing phase (Fig. 4), 2 mM eugenol completely inhibited the production of MTT formazan. At this concentration, cells became rounded and almost every cell showed evidence of cell lysis, whereas 0.125 mM eugenol did not affect the MTT formazan production or cell shape. From
Fig. 5. Eugenol toxicity to cells in confluence. RPC-C2A cells were inoculated to a 96-well plate at the density 16000 cells per well. After 24 hr of culture, the culture medium was changed to the experimental medium containing various concentrations of eugenol. After 24 hr incubation, the MTT assay was performed. The mean of the absorbance of the control well, which contained no eugenol, was 0.735. Each point and bar represent a mean and one standard deviation of 7 samples.

The results, it was calculated that 0.6 mM eugenol produced 50% inhibition of MTT formazan formation. In confluent cells (Fig. 5), 2 mM eugenol completely blocked the MTT formazan production and killed cells, while 0.125 mM eugenol did not. In this case, 1 mM eugenol produced 50% inhibition.

Discussion

The principle behind the MTT colorimetric assay is that the tetrazolium ring in MTT is cleaved by dehydrogenases present in active mitochondria producing an insoluble MTT formazan product (4). Since the original method described an assay for cells in a suspension culture, there are some problems in applying this method to adherent cells in culture. The first problem is the insolubility of the MTT formazan, although several technical modifications have already been reported (5, 6).

In our preliminary experiments, we used acid-isopropanol as a solvent and added it to the remaining medium containing MTT formazan according to the original method (1). However, the insolubility of MTT formazan was a problem. In the case of adherent cells in culture, it is easy to remove the medium by inverting and tapping the plate after the production of MTT formazan. Phenol red which is usually present in culture media is known to affect the spectrophotometric absorbance of MTT formazan (5). Thus, removal of the medium before addition of the solvent for MTT formazan can prevent this effect and at the same time improves the MTT formazan insolubility.

Carmichael et al. (6) has recommended mineral oil as a solvent for the MTT formazan in the case of adherent cells in culture and DMSO for cells in suspension culture. However, the addition of mineral oil to each well is difficult because it is viscous; furthermore, overnight incubation was required to dissolve the MTT formazan. In contrast, we have found that DMSO is a more useful solvent for analyzing cells in adherent culture.

The absorbance spectrum of MTT formazan in DMSO displayed an absorbance peak at 550 nm. However, Carmichael et al. (6) reported that MTT formazan in DMSO has an absorbance peak at 503 nm. The reason of the difference is not clear. We were able to measure the absorbance around at 550 nm, using the 540 nm filter that was available for the ELISA reader.

Good correlation between cell number and absorbance has been reported in the case of cells in suspension culture (2, 5). For cells in adherent culture, the absorbance depended on cell density. Cells in adherent culture proliferate rapidly when they are sparse, and their proliferation activity decreases when they contact each other. This phenomenon is well-known as "contact inhibition". Mitochondria also grow and divide during the cell cycle (7). It is conceivable, therefore, that growing cells have more dehydrogenase activity in mitochondria than cells in the resting phase (G1 phase). This seems to be the reason why cells at a low density tended to show a higher absorbance than cells at a higher density. Although the correlation was not completely linear, a good correlation between the cell number and absorbance was observed when the cell density was between 2500 to 20000 per well. Thus, under these conditions we think this method can be applied as a preliminary toxicity test.

Eugenol is frequently used in clinical dentistry, commonly as zinc oxide-eugenol
cements (3). Zinc-oxide eugenol cement is antiseptic and application of zinc-oxide eugenol cement causes sedation of dental pulp tissue. However, both zinc-oxide eugenol cement and eugenol are toxic when they are applied to dental pulp (8) or connective tissue (9–11). In this study, we investigated the cytotoxicity of eugenol using the MTT colorimetric assay and RPC-C2A cells. Doses of eugenol producing 50% inhibition in cells in the growing phase and the cells at confluence were 0.6 mM and 1 mM, respectively.

When the MTT assay is applied for toxicological study, the results obtained in cells at low density are different from the results obtained in cells at high density. We think that the reason for this is as follows: At low density, inhibition of proliferation occurs and as such resembles the inhibition of \(^{3}\)H-thymidine uptake, reflecting the inhibition of cell number increase. At high cell density the result is due to a lethal effect on the resting cells, and it is, therefore, comparable to measurement of \(^{51}\)Cr release from prelabeled cells.

Eugenol toxicity in vitro has already been reported by several investigators. Hume (12) studied \(^{3}\)H-thymidine uptake by mouse fibroblasts following a 24 hr exposure to eugenol and reported that 0.2 mM eugenol depresses \(^{3}\)H-thymidine uptake by 50% compared to controls. He also reported that eugenol inhibits respiration of mammalian cells in the concentration range of 0.1 mM to 1 mM. Lindqvist and Otteskog (13) used \(^{3}\)H-uridine prelabeled cells and reported that 1 hr exposure to 4 mM eugenol resulted in the loss of approximately 100% of this cytoplasmic label. Kojima (14) investigated eugenol toxicity more precisely using V79 cells and reported that 24 hr exposure to 4 mM eugenol resulted in the loss of approximately 100% of this cytoplasmic label.

The difference between the toxic concentration of eugenol in this study compared to previous studies might be due to the differences in the assay method, cell susceptibility and exposure time to eugenol. However, previously published data indicated that the cytotoxicity of eugenol is in the concentration range of 0.1 mM to 4 mM, and the values obtained in this study were within this range. Consequently, we feel that the combination of RPC-C2A cells and the MTT colorimetric assay can be useful for preliminary screening for toxicity of various dental drugs and materials.

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