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Limitations of the MTT Assay in Cell Viability Testing

Ograniczenia testu MTT w ocenie żywotności komórek

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Abstract

Background. The MTT assay is widely recommended for examining the cytotoxic effect of xenobiotics, assessing proliferation rates, and analyzing cell activity. The aim of this study was to evaluate the usefulness of the MTT assay in examining human lymphocyte viability in own cell culture systems which contained fluphenazine (FPh), a suspected cancer chemopreventive agent, and doxorubicin (DOX), an anticancer drug, in cell culture medium.

Material and Methods. Cell culture viability was estimated with two tests: the MTT assay and the propidium iodide (PI) exclusion assay. These results were compared using the standard ANOVA procedure.

Results. The propidium iodide exclusion-test, a microscopic assay of cell culture viability, revealed an increase in dead cell number in cultures treated with DOX and an absence of viable cell changes in cultures treated with FPh in the range of the tested concentrations. The results obtained with the MTT reduction assay in the cultures gave the opposite results; the assay did not mirror the real viable cell number in these cultures. Statistical analysis demonstrated that the results obtained with the two assays differed significantly.

Conclusions. This study shows that the MTT assay is not a feasible tool for determining cell viability in cultures in which DOX and FPh are used and possible sources of the limitations of the MTT assay are discussed (*Adv Clin Exp Med* 2008, 17, 5, 525–529).

Key words: MTT assay, PI exclusion-test, lymphocyte cultures, doxorubicin, fluphenazine.

Streszczenie

Wprowadzenie. Test MTT jest powszechnie zalecany do badania cytotoksycznego działania ksenobiotyków, znajduje także zastosowanie w ocenie proliferacji i aktywności komórek.

Cel pracy. Ocena przydatności testu MTT do analizy żywotności limfocytów w hodowlach zawierających w medium hodowlanym flufenazynę (FPh), potencjalny związek chemoprewencyjny, i doksorubicynę (DOX), przeciwnowotworowy lek cytostatyczny.

Materiał i metody. Ocenę żywotności komórek przeprowadzono w dwóch testach: redukcji MTT i eliminacji jodku propydydny (PI). Wyniki otrzymane w obu testach poddano analizie statystycznej metodą ANOVA.

Wyniki. Wyniki uzyskane w teście spektrofotometrycznej oceny redukcji MTT w hodowlach prowadzonych w obecności doksorubicyny oraz w obecności flufenazyny dały wyniki odmienne niż uzyskane w teście oceny mikroskopowej komórek po wybarwieniu hodowli jodkiem propydydny. Analiza statystyczna wykazała, że wyniki otrzymane w obu testach różniły się istotnie.

Wnioski. Przeprowadzone badania pokazały, że testu MTT nie można zastosować do oceny żywotności komórek w hodowlach prowadzonych w obecności DOX i FPh, a ograniczenia zastosowania testu MTT i ich najczęstsze przyczyny są w pracy omawiane (*Adv Clin Exp Med* 2008, 17, 5, 525–529).

Słowa kluczowe: test MTT, test eliminacji PI, hodowle limfocytów, doksorubicyna, flufenazyna.

The reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) by viable cells in culture is a method widely recommended for examining the cytotoxic effect of xenobiotics, assessing proliferation rates, and analyzing cell activity [1, 2]. MTT is reduced inside

viable cells to water-insoluble purple-colored formazan by mitochondrial dehydrogenases, although in various cell lines over 50% of the dye penetrating the cell membrane can be reduced by non-mitochondrial, cytosolic, and microsomal enzymes [3, 4]. Despite the variability of the sites of

MTT reduction within the cell, it is assumed that the MTT reduction rate is closely related to the number of actively respiring cells in the cell culture [2, 4, 5].

In the present study the validity of the MTT assay was examined by comparing its results with those obtained with the reference propidium iodide exclusion-test in estimating the toxic influence of the anticancer drug doxorubicin (DOX, 2.5–10.0 μM) on human lymphocyte cultures. The effect of fluphenazine (FPh, 0.078–5.0 μM), a suspected cancer chemopreventive agent, on the viability of lymphocyte culture was also quantified by the MTT and propidium iodide exclusion assays. The present authors previously documented that FPh exerts an antimutagenic/chemopreventive action on lymphocyte cultures damaged *in vitro* with standard genotoxic agents and established that the important mechanism of its antimutagenic action was its strong antioxidant activity [6, 7]. Recent literature data suggest that in some experimental cell culture systems containing tested compounds which exhibit antioxidant activity, the results of the MTT assay were not credible and lead to misinterpretation of cell culture viability estimation [11]. Therefore it was decided to check the validity of the MTT assay in the present authors' experimental system, which contained both fluphenazine and doxorubicin in the cell culture medium.

Material and Methods

All chemicals and cell culture media components were purchased from Sigma (St. Louis, MO, USA), except Phytohemagglutinin (PHA-M), which was obtained from Gibco (Gaithersburg, MD, USA). Cell culture viability was measured by two tests: the MTT assay (spectrophotometric examination) [1, 2] and the propidium iodide (PI) exclusion assay (microscopic examination) [8]. Their results were compared using the standard ANOVA procedure.

Lymphocytes were isolated from heparinized venous blood of healthy volunteers using the Histopaque-1077 centrifugation technique [9]. Lymphocytes were cultured for 48 hrs in the presence of a lectin, PHA-M (1% v/v), and fluphenazine (FPh, 0.078–5.0 μM). For the last 24 hrs of the culture period the cell cultures were incubated with doxorubicin (DOX, 2.5–10.0 μM). Then the MTT dye was added to the cultures (final concentration of MTT: 0.5 mg/ml) and cells were incubated for 4 hrs. The procedure of the MTT assay closely followed that given in literature [1, 2]. The cell cultures were spun out, formazan was dis-

solved with acidic isopropanol (0.04 N HCl), and the absorption of the formazan solution was estimated with a microspectrophotometer (λ_{560} – $\lambda_{630\text{nm}}$).

The microscopic estimation of cell viability was performed in parallel cultures after staining the cell pellet with propidium iodide (4.5 $\mu\text{g/ml}$ for 10 min) [8]. Afterwards, slide smears were made and the slides were examined under a fluorescence microscope (Nikon, Eclipse E-600, equipped with a G-2A filter block). Dead cells (orange fluorescence) and viable cells (unstained) were counted among 200 cells randomly found in a microscopic image. The significance of the differences between the results obtained with the MTT assay and the propidium iodide exclusion test were estimated by means of the routine ANOVA procedure.

Results

The cytotoxic effect of DOX (10 μM) added for the last 24 hrs to the lymphocyte cultures was estimated spectrophotometrically with the MTT assay and microscopically with the propidium iodide exclusion-test. The influence of FPh on the cytotoxicity of DOX-treated cells was also estimated and compared in the two tests. The results obtained in the tested cultures (E) were compared with those of control cultures (E_0), where an equivalent volume of distilled water was added instead of the water solution of DOX and FPh and, finally, the E/E_0 ratios were calculated.

As shown in Table 1, the results of the MTT reduction assay differed markedly from those obtained with the PI exclusion-test. For instance, in the cultures containing DOX an increase in MTT reduction (by 60%) was noted, whereas in the PI exclusion test there was a marked decrease (by more than 80%) of the viable cell fraction in comparison with the control cultures. In cultures containing FPh and those containing DOX and FPh together, the results obtained with the two methods were also different; however, the results of the PI exclusion-test could be considered to reflect more reliably the expected changes in cell culture viability. The statistical analysis (ANOVA) proved that the results obtained with the two methods were significantly different ($F = 112.29$, $df = 1$, $p < 10^{-4}$) and one can conclude that the two tests varied markedly in their ability to measure changes in cell culture viability.

Since MTT reduction in the presence of DOX (10 μM , 24 hrs) was unexpectedly increased, it was decided to check the effect of various concentrations of DOX (2.5–10.0 μM) on lymphocyte cultures' viability estimated with the MTT assay.

Table 1. Estimation of cell culture viability with the MTT assay and the PI exclusion test in lymphocyte cultures incubated with fluphenazine (FPh, 5 μ M, 48 hrs) and doxorubicin (DOX, 10 μ M, 24 hrs). The results obtained for the tested cultures (E) were compared with those of control cultures (E_0) and given as E/E_0 ratios

Tabela 1. Ocena żywotności hodowli komórkowych w teście MTT i teście eliminacji PI w hodowlach limfocytów inkubowanych z flufenazyną (FPh, 5 μ M, 48 godz.) i doksorubicyną (DOX, 10 μ M, 24 godz.). Wyniki uzyskane w badanych hodowlach (E) porównano z wynikami hodowli kontrolnej (E_0) i wyrażono jako E/E_0

Compounds: (Badane związki)	MTT reduction [E/E_0] <i>mean $\pm$ SD, n = 5</i> (Redukcja MTT [E/E_0]) (<i>średnia $\pm$ SD, n = 5</i>)	Number of PI-excluding (viable) cells [E/E_0] <i>mean $\pm$ SD, n = 5</i> (Liczebność komórek żywych w teście eliminacji PI [E/E_0]) (<i>średnia $\pm$ SD, n = 5</i>)
DOX _{10 μM}	1.60 $\pm$ 0.301	0.15 $\pm$ 0.029
FPh _{5 μM}	0.65 $\pm$ 0.106	0.96 $\pm$ 0.037
DOX _{10 μM} +FPh _{5 μM}	0.55 $\pm$ 0.089	0.10 $\pm$ 0.017

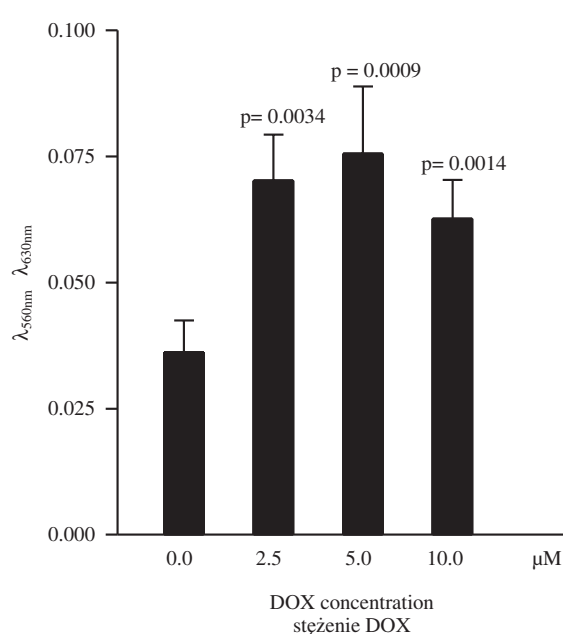


Fig. 1. Increase in MTT reduction by human lymphocytes incubated for 24 hrs with doxorubicin (DOX); mean $\pm$ SD, n = 5, p – statistical significance calculated with the paired t test

Ryc. 1. Wzrost redukcji MTT w ludzkich limfocytach w hodowlach inkubowanych przez 24 godz. z doksorubicyną (DOX); średnia $\pm$ SD; n = 5, p – istotność statystyczną obliczono w teście t

As can be seen in Figure 1, the presence of DOX (2.5–10.0 μ M) in the lymphocyte cultures led to an increase in MTT reduction (by about 60% to 100%) when compared with the control culture (without DOX). The increase in MTT reduction was highest at the DOX concentration of 5 μ M, and the results obtained in cultures containing DOX were significantly different from those of the control culture (without DOX) as estimated with the paired t test. These results would suggest, paradoxically, enhanced lymphocyte viability in the

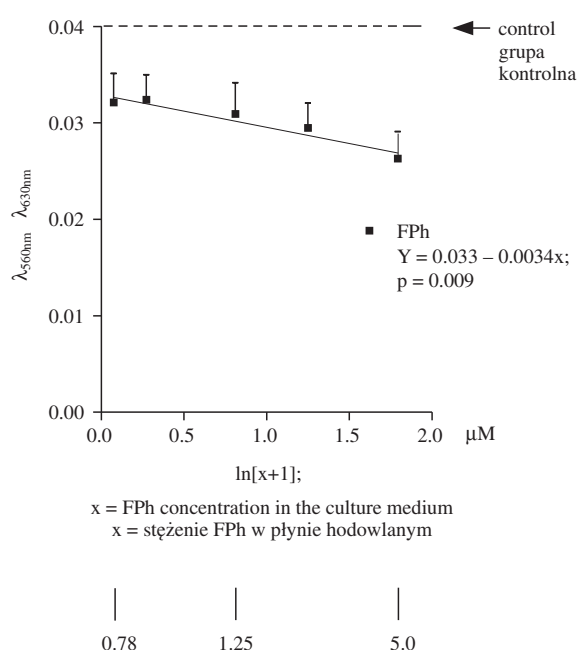


Fig. 2. Decrease in MTT reduction by human lymphocytes incubated for 48 hrs with fluphenazine (FPh); mean $\pm$ SD, n = 5, the dose-response relation was calculated and the regression equation is given

Ryc. 2. Spadek redukcji MTT w ludzkich limfocytach w hodowlach inkubowanych przez 48 godz. z flufenazyną (FPh); średnia $\pm$ SD; n = 5, zależność skutku od dawki wyliczono w równaniu regresji zamieszczonym na rycinie

presence of the cytostatic drug. This clearly shows that the MTT assay cannot be used to assess the cytotoxic action of DOX, and the alternative measure of cell viability with the PI exclusion-test showed a marked decrease in viable cell number in the presence of DOX (10 μ M).

The data given in Figure 2 show that the addition of FPh to the lymphocyte cultures led to a linear decrease in the MTT reduction level, and in the presence of FPh (5 μ M) the reduction of MTT was lower by about 35% in comparison with the control

cultures (without FPh). Therefore, one could conclude that FPh lowered the viability of the lymphocyte cultures. However, the viability of the lymphocyte cultures measured with the PI exclusion test showed an absence of cytotoxic activity of FPh. For example, the viability of the cells cultured in the presence of FPh (5 μ M) did not differ significantly from that estimated in the control cultures ($p = 0.052$). The above results demonstrate that the MTT assay is not of value in establishing the viability of lymphocyte cultures in the presence of FPh.

Discussion

The MTT assay is widely used for measuring cell viability, proliferation of living cells, and cytotoxicity of new drug candidates in the 96-well plate format [11]. MTT reduction is attributed to mitochondrial activity, although it is related either to non-mitochondrial enzymes and to lysosomes and endosomes [11]. Recently, literature data has accumulated surprising results which reveal that in some experimental systems and with many of the tested compounds, for instance: new drug candidates, MTT reduction appears to be an inadequate test of viable cell number, yielding false results which are a source of misinterpretation. The main reasons for such pitfalls and limitations of the MTT assay are:

1. Tested compounds can directly interact with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [12]. It was documented, for instance, that ascorbic acid reduced the MTT stain in a cell-free system and the reaction was markedly enhanced in the presence of retinol [13]. Also, several plant extracts, such as polyphenols (resveratrol) and flavonoids (quercetin, luteolin, kaempferol), strongly reduced MTT in the absence of living cells [14–16]. It seems necessary to rule out direct chemical interactions of the tested compounds with the MTT stain before starting routine measurements of cell viability with this assay.

2. Tested compounds could interfere with mitochondrial dehydrogenase activity and therefore lead to overestimation (activation of the MTT-reducing dehydrogenases) or underestimation (inhibition of mitochondrial dehydrogenases) of the MTT assay results. For example, such an underestimation of the MTT assay results were noted in the cases of chloroquine, β -amyloid, and interferons [12, 15] and overestimation was documented for genistein tested in cell cultures [11]. On the other hand, it was documented in literature that several compounds, such as doxorubicin, induce an intracellular generation of free radicals,

and the more radicals the greater reduction of MTT [3].

The present study confirmed this published observation: the addition of doxorubicin to lymphocyte culture markedly enhanced MTT reduction and this would lead to the paradoxical conclusion of higher cell viability in the presence of a standard cytostatic drug. It also showed that in this experimental system the activity of DOX in free-radical induction was rather strong; the MTT reduction level was as much as twice that of control cultures without DOX. Considering this, the results explain why the MTT assay could not be applied to estimate lymphocyte viability in the presence of DOX: a reduction of the dye reflects free-radical activation more than the cytotoxic action of DOX in lymphocyte cultures.

The present data also documented that the MTT test is not of value in estimating cell viability in the presence of FPh. The present authors noted previously that FPh markedly lowered the level of free radicals generated *in vitro* by human granulocyte samples [6] and in that way it probably could lower the level of MTT reduction in lymphocyte cultures. The authors suggest that the results of the MTT assay estimated a decrease in the free-radical level by FPh rather than cell culture viability. It should be kept in mind that many antimutagenic/chemopreventive as well as anti-inflammatory agents exhibit antioxidant and free-radical scavenging activity; they can lower the MTT reduction level, which can be a source of error in experiments estimating cell culture viability in the presence of antioxidants.

The authors intended to estimate the decrease in the viable cell number in lymphocyte cultures treated with FPh together with DOX in comparison with cultures containing DOX only. It is well known that FPh and other antipsychotic phenothiazines inhibit cell membrane efflux proteins (mainly P-glycoprotein) and lead to an increased accumulation of cytotoxic compounds inside the cell [10]. It was documented that compounds which influence cellular membrane efflux proteins (P-glycoprotein, MRP 1 protein) decreased MTT removal from the cell, thereby leading to elevated reduction of the stain, which could lead to erroneous conclusions in cell culture viability testing [17]. The present authors' previous data showed that FPh leads to dose-dependent inhibition of P-glycoprotein transporter function and it was considered an important mechanism of their action in late cancer chemoprevention, i.e. in an increase in the cytotoxic action of the standard cytostatic drugs [18]. In cultures exposed to DOX together with FPh, a marked decrease in cell viability was expected in comparison with cultures

exposed to DOX only, and a consecutive decrease in MTT reduction. However, the MTT assay appeared unsuitable for estimating culture cell viability in the case of the tested compounds; the results presented in Table 1 show that the MTT reduction levels in the cultures containing DOX and FPh were only slightly lower than in the cultures containing FPh only, although the results obtained with the PI exclusion test confirmed the enhanced cytotoxicity expected in those cultures.

The results given in Table 1 most probably represent the sum of the free-radical-increasing (DOX) and free-radical-decreasing (FPh) activity

of the compounds as well as their inhibition of MTT exclusion from the cells and could therefore in no way reflect culture cell viability.

The present data concur with recent literature reports documenting that the MTT assay is unreliable in estimating cell viability in the presence of compounds which influence the intracellular level of free radicals as well as in the case of compounds which lead to elevated accumulation of the stain inside a cell. The present authors strongly suggest assessment in a pilot study of the validity of the MTT assay under detailed experimental conditions before this test is designed for routine estimations of cell viability.

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