Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells

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Abstract The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is widely accepted as a simple and reproducible method for determining cell proliferation or cytotoxicity in vitro. In this study, we show that the flavonoids quercetin, rutin and luteolin but not apigenin can reduce MTT in the absence of live cells in the following order: quercetin >> rutin > luteolin > apigenin. Moreover, this reduction can be influenced by medium type and serum. The final concentrations of the flavonoids used were 200, 100, 50, 25 and 12.5 µg/mL. MTT reduction in Dulbecco's Modified Eagle's Medium (DMEM) is statistically higher than those in RPMI 1640 and F12 media, which are generally similar. Particularly for luteolin, MTT reduction is considerably higher with serum than without serum. In the case of quercetin at 50 µg/mL, a serum concentration of even only 0.01% is sufficient to significantly enhance MTT reduction versus that at 0% ($P < 0.05$). Serum at concentrations ranging from 0% to 5% also dose-

dependently affects the pattern of formazan crystal formation. In the presence of 0.156–5% serum, the formazan crystals gradually change from being small, numerous and scattered to being large, few and clumpy. The authors hypothesize that flavonoid structure, nutrient concentration in the culture medium as well as serum components directly affect MTT reduction by flavonoids in the absence of cells.

Keywords MTT · Flavonoids · Culture medium · Serum · Formazan crystals

Introduction

The reduction of tetrazolium salts such as MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) by live cells has been widely accepted as a reliable means of measuring cell proliferation in vitro. In principle, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of the yellow MTT to yield purple formazan crystals which are insoluble in aqueous solutions. The crystals can then be dissolved using a suitable solvent and the resulting purple solution is measured spectrophotometrically.

Recent studies, however, have shown that MTT can be reduced in the absence of live cells. Shoemaker et al. (2004) have shown that botanical

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extracts can reduce MTT in the absence of live cells and that treatment of these extracts with iodoacetic acid to alkylate free thiol groups inhibited their ability to reduce MTT. Peng et al. (2005) have also shown that the flavonoids luteolin and quercetin can also reduce MTT in the absence of live cells. Previous studies have likewise shown that some flavonoids inhibit cell growth but enhance MTT reduction (Pagliacci et al. 1993; Bernhard et al. 2003).

Flavonoids, such as those from olives, are known to have diverse biological activities (Visioli and Galli 2000; Bouaziz et al. 2005) and may be responsible for the pharmacological actions of olive leaves (Bouaziz and Sayadi 2005). Numerous studies have shown their cardioprotective and chemopreventive activities (Kris-Etherton et al. 2002; Marchand 2002); they have also been implicated in modulating protein kinase and lipid kinase signalling pathways (Williams et al. 2004).

In this study, we determine the influence of medium type and serum on MTT reduction by the flavonoids quercetin, luteolin, rutin and apigenin (Fig. 1) in the absence of cells. Because these flavonoids are relatively similar in structure, the influence of hydroxylation pattern on MTT reduction is also examined.

The simplicity, accuracy and reproducibility of the MTT assay in measuring the activity of live cells have made its use relatively widespread. Thus, a clear understanding of its limitations and pitfalls is invaluable towards its optimum use, particularly in cytotoxicity studies.

Fig. 1 Structure of flavonoids used in this study. Luteolin ($R_1=H$, $R_2=OH$), apigenin ($R_1=H$, $R_2=H$), quercetin ($R_1=OH$, $R_2=OH$), rutin ($R_1=O$ -rutinose, $R_2=OH$)

Materials and methods

Materials

Luteolin, quercetin, rutin, Dulbecco's modified Eagle's Medium (DMEM) and fetal bovine serum (Lot no. 44K3398) were purchased from Sigma (Japan). Apigenin was obtained from Fluka (Germany). RPMI 1640 medium (RPMI) and F12 Nutrient Mixture (F12) were purchased from Gibco (Invitrogen Corp., USA). 3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Dojindo (Japan). Sodium dodecyl sulfate (SDS) and 99.5% ethanol were purchased from Wako (Japan).

Preparation of flavonoids

Luteolin, quercetin, rutin and apigenin were dissolved in 99.5% ethanol at 1 mg/mL and then filtered using a 0.45 μ m filter (Millipore, Japan). The flavonoid solutions were then stored at -80°C until use.

MTT assay

The MTT assay is based on the protocol first described by Mossman (1983). To determine the influence of medium type and serum on MTT reduction by flavonoids in the absence of cells, DMEM, RPMI and F12, with or without 10% serum, were added onto 96-well plates and the flavonoids were then added at final concentrations of 200, 100, 50, 25 and 12.5 μ g/mL. The final volume per well was 100 μ L. MTT was dissolved in ultrapure water at 5 mg/mL final concentration, filter-sterilized, and then added at 10 μ L per well (0.45 mg/mL final concentration). The plates were then covered with aluminum foil and incubated at 37°C for 1.5 h, 3 h or 6 h. SDS (10%) was then added at 100 μ L per well followed by overnight incubation (18 h) at 37°C to completely dissolve the formazan crystals. Absorbances were obtained at 570 nm using a microplate reader (Powerscan HT, Dainippon Pharmaceutical, USA). Blanks containing only medium (with or without serum), MTT and SDS were used to correct the absorbances.

To determine the effect of serum concentration on MTT reduction by quercetin in the absence of cells, DMEM, RPMI and F12 were added onto 96-well plates and serum was then serially added at final concentrations ranging from 0% to 5%. Quercetin was then added at a final concentration of 50 µg/ml. Final volume per well was 105 µL. MTT was then added as described earlier and the plates incubated for 24 h. Wells were then examined and photographed to determine the influence of serum on formazan crystal formation. SDS (10%) was then added at 100 µL per well followed by overnight incubation (18 h) at 37 °C. Absorbances were then obtained as described earlier. Blanks containing only medium, MTT and SDS were used to correct the absorbances.

Statistical analyses

Two independent experiments with three trials each were carried out for each test, except for the experiment on the effect of serum concentration on MTT reduction by quercetin, in which three independent experiments were carried out. Statistical significance ($P < 0.05$) was evaluated by one-way ANOVA and, if significant, group means were compared using Tukey's post hoc comparison or Dunnett's two-sided test.

Results

The time-dependent and dose-dependent reduction of MTT by the flavonoids in DMEM, RPMI and F12 are shown in Figs. 2–4. MTT reduction potential follows this order: quercetin >> rutin > luteolin > apigenin. Apigenin cannot reduce MTT. Results further show that in the case of quercetin and rutin, the MTT reduction is dose-dependent in all types of media, with or without serum, and at all incubation times. On the other hand, MTT reduction by luteolin is dose-dependent only until 100 µg/ml; interestingly, at 200 µg/mL, MTT reduction decreases as shown by the decrease in absorbance. This is true for all types of media, with or without serum, and at all incubation times.

Results further show that MTT reduction by flavonoids in DMEM is statistically higher

($P < 0.05$) than those in RPMI and F12, which are generally similar. In the case of quercetin (Fig. 2), MTT reduction is time-dependent in the presence of serum for all types of media, whereas in the absence of serum, the absorbances at 1.5 h and 3 h are generally similar, particularly at 100 µg/mL and 200 µg/mL.

The influence of serum on MTT reduction is clearly shown in the case of luteolin (Fig. 3). Results reveal that absorbances are considerably higher with serum than without serum for all luteolin concentrations, all types of culture media and at all incubation times. On the other hand, in the case of rutin (Fig. 4), the overall effect of serum is inconclusive and does not show a distinct trend. In a number of instances, the presence of serum does not have any effect, while in some instances, the absence of serum actually resulted in higher absorbances, as shown in DMEM with 200 µg/mL rutin. However, at 6 h incubation and particularly at concentrations of 100 µg/mL and below, MTT reduction by rutin is considerably higher in the presence of serum.

Because quercetin showed the highest MTT reduction potential, it was used at a final concentration of 50 µg/mL to determine the dose-dependent effect of serum. The incubation time of 24 h was chosen to ensure maximum MTT reduction by quercetin. A serum concentration of even only 0.01% is sufficient to significantly enhance MTT reduction versus that at 0% ($P < 0.05$) in the case of RPMI and F12 media, whereas 0.02% serum is necessary in the case of DMEM (Fig. 5). Results further show that there is no significant difference ($P > 0.05$) between the absorbances at 1.25% and 2.5% for all types of media.

The dose-dependent influence of serum on MTT reduction by quercetin is not only shown by the differences in absorbance values but also in the formazan crystal formation. Results show that serum also dose-dependently affects the pattern of formazan crystal formation (Fig. 6). In the presence of 0–0.078% serum, the number of formazan crystals increases relative to an unknown powdery substance in the background, which gradually disappears as the serum concentration is increased.

In the presence of 0.156–5% serum, the unknown powdery substance completely disappears

Fig. 2 MTT reduction in the absence of cells by quercetin in Dulbecco's Modified Eagle's Medium, RPMI 1640 Medium and F12 Nutrient Mix at different concentrations and incubation times. The mean absorbances $\pm$ SD have

been corrected using blanks. Results represent the average of two independent experiments with three trials each ($n = 16$). Means without a common letter within a panel differ significantly ($P < 0.05$)

while the formazan crystals gradually change from being small, numerous and scattered to being large, few and clumpy. These crystal formation patterns are similar for all types of media. Note also that the density of the formazan crystals in DMEM is relatively higher than those in RPMI and F12. This confirms the results showing that MTT reduction in DMEM is higher than those in RPMI and F12. The results also reveal that even in the absence of quercetin and serum, the medium alone can reduce MTT to formazan; however, its effect on absorbance is not significant (data not shown).

Discussion

In this study, the authors have shown the influence of medium type and serum on MTT reduction by some flavonoids in the absence of cells. The MTT reduction potential of the flavonoids used in this study has also been shown to be of the following order: quercetin $\gg$ rutin $>$ luteolin $>$ apigenin.

The authors hypothesize that the structure of the flavonoids is directly related to their MTT reduction potential. The foremost consideration

Fig. 3 MTT reduction in the absence of cells by luteolin in Dulbecco's Modified Eagle's Medium, RPMI 1640 Medium and F12 Nutrient Mix at different concentrations and incubation times. The mean absorbances $\pm$ SD have

been corrected using blanks. Results represent the average of two independent experiments with three trials each ($n = 16$). Means without a common letter within a panel differ significantly ($P < 0.05$)

is the extent and nature of the hydroxylation pattern of the aromatic rings. Figure 1 shows that in the case of quercetin, the C-5' (R_1) and C-3 (R_2) positions are both hydroxylated and this is responsible for both its high antioxidant and MTT reduction potentials. In the case of rutin and luteolin, only the C-5' (R_2) position is hydroxylated, and thus, their MTT reduction potential is much lower than that of quercetin. On the other hand, apigenin which lacks this hydroxyl group in both the C-5' (R_1) and C-3 (R_2) positions does not show MTT reduction, suggesting that flavo-

noid structure appears to be important in determining MTT reduction potential. In addition, MTT reduction by some flavonoids may be partly the result of their chelating properties; it is hypothesized that quercetin, rutin and luteolin form strong binding complexes with MTT.

Some antioxidants have been previously shown to reduce MTT in the absence of cells (Natarajan et al. 2000). Chakrabarti et al. (2000) found that ascorbic acid reduced MTT to formazan, which was profoundly increased by a very small amount of retinol. They also showed that oxidation of

Fig. 4 MTT reduction in the absence of cells by rutin in Dulbecco's Modified Eagle's Medium, RPMI 1640 Medium and F12 Nutrient Mix at different concentrations and incubation times. The mean absorbances $\pm$ SD have

been corrected using blanks. Results represent the average of two independent experiments with three trials each ($n = 16$). Means without a common letter within a panel differ significantly ($P < 0.05$)

ascorbic acid by hydrogen peroxide destroyed its ability to reduce MTT. Quercetin and luteolin have been shown to have antioxidant activity while apigenin was found to be a prooxidant (Skerget et al. 2005).

The differences in MTT reduction in relation to medium type may be attributable to the differences in the concentrations of nutrients found in DMEM, F12 and RPMI. Nutrient concentrations are low in F12 and high in DMEM. DMEM, for instance, has twice the amino acid concentrations of Minimum Essential

Medium (MEM), has four times the vitamin concentrations, and uses twice the HCO_3^- and CO_2 concentrations to achieve better buffering (Freshney 2005). The compositions of the various media used in this study can be found in Freshney (2005). Vistica et al. (1991) have shown that the MTT assay is significantly influenced by the D-glucose concentration in the growth medium, which is 2.5-fold higher in DMEM than in F12 and RPMI.

Serum is a complex mixture of proteins, polypeptides, growth factors, amino acids, lipids,

Fig. 5 Effect of serum concentration on MTT reduction in the absence of cells by quercetin (50 $\mu\text{g/mL}$) in Dulbecco's Modified Eagle's Medium, RPMI 1640 Medium and F12 Nutrient Mix after 24 h of incubation. The mean absorbances $\pm$ SD have been corrected using blanks. Results represent the average of three independent experiments ($n = 24$). Means without a common notation differ significantly ($P < 0.05$)

carbohydrates, polyamines, urea, inorganic compounds, hormones and vitamins. A complete list of serum constituents can be found in Freshney (2005). The influence of serum on the MTT assay was previously determined by comparing the MTT results with those of haemocytometer counting. Zhang and Cox (1996) found that when smooth muscle cells were cultured in RPMI with 5% or less fetal calf serum, no significant differences were observed in cell number counted by the two methods. However, for cells cultured in 10% serum, the result was 20% higher when counted by the MTT assay. Cells grown in 5% and 10% serum showed no difference in total mitochondrial activity per cell. Because of this, Zhang and Cox (1996) consider that the MTT assay may not be accurate under certain conditions. Various enzymes that may be found in serum, such as glutathione S-transferase, as well as other energy sources required for Na^+ pumping may also affect MTT reduction (York et al. 1998; Newman et al. 2000).

While it is widely accepted that mitochondrial dehydrogenases are responsible for MTT reduction, others consider that it may also occur outside the mitochondria in some systems (Altman 1976; Burdon et al. 1993; Liu et al. 1997). Bernas and Dobrucki (2002) have also shown that MTT reduction may be catalyzed by a number of nonmitochondrial enzymes. In their study, only 25–45% of MTT-formazan was associated with mitochondria after 25 min of incubation and most MTT-formazan deposits were not coincident with

mitochondria. Liu (1999) considers that mitochondria are unlikely to play a significant role in cellular MTT reduction.

In conclusion, this study has shown that medium type and serum have a significant effect on MTT reduction by flavonoids in the absence of cells and that reduction potential may be related to flavonoid structure. Thus MTT assay results must be interpreted with caution and should take into account all factors that may influence the results, particularly when it is used to measure cytotoxicity.

To the best of our knowledge, no study has been made on the reduction by flavonoids of other tetrazolium salts such as XTT, MTS and WST in the absence of cells. We however hypothesize that since these salts are the substrates of the same cellular enzymes that can reduce MTT, they may also be reduced by quercetin or other compounds with high antioxidant potential. Thus, the results of this study can also serve as a guidepost for the use of other tetrazolium salts.

Fig. 6 Effect of serum concentration on formazan crystal formation (MTT reduction) in the absence of cells by quercetin (50 $\mu\text{g/mL}$) in Dulbecco's Modified Eagle's Medium, RPMI 1640 Medium and F12 Nutrient Mix after 24 h of incubation. All photos were taken at the same scale under 200 $\times$ magnification. Scale bars in the topmost photos represent 200 μm

Fig. 6 continued

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