A microtiter plate assay for superoxide using MTT reduction method

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A simple, rapid and sensitive microtiter plate assay for superoxide using the reduction of tetrazolium dye MTT to its coloured formazan has been developed. The colour formed can be measured using a microtiter plate reader and the extent of reduction of MTT indicates the amount of superoxide generation. A comparison of the sensitivities of different procedures for the quantitation of superoxide generated by X-XO system has been made. The MTT reduction due to superoxide was confirmed by inhibiting the reduction using purified superoxide dismutase. Using this method superoxide generation by mitochondria and microsomes was demonstrated and this procedure is suitable for detection of intracellularly generated superoxide. The proposed method is inexpensive and is suitable for a routine analysis of large number of samples.

Oxygen free radicals are continuously produced in small amount in normal cells and they are the side products of electron transfer reactions. Excess production of these active species result in oxidative stress which leads to altered cell function. Increased formation of free radicals occur when the cells are subjected to different stimuli such as UV light, inflammatory cytokines and low concentration of H$_2$O$_2$. Free radicals play a potential role as cytotoxic agents as in the case of stimulated neutrophils during inflammatory conditions. Various methods are available for the detection and quantitation of superoxide generated in the cells which include reduction of cytochrome c or the tetrazolium dye, NBT, by superoxide. It has been reported that some of the biological compounds interfere in these assay methods. MTT is a tetrazolium dye which can be reduced to coloured formazan and this has been used to measure cell viability. Reduction of MTT is supposed to be due to mitochondrial activity and is used to assess the mitochondrial function. Recent studies have shown that intracellular generation of superoxide is mainly due to mitochondrial electron transport chain. Nohl et al. Have reported that superoxide is generated by mitochondria when electrons are released out of mitochondrial electron transport chain sequences. In addition microsomal NADPH-cyt. c reduction system also generates superoxide. In the present study, reduction of MTT by superoxide to its formazan has been shown and using this reaction, a microtiter plate assay for superoxide has been developed and compared with other known methods.

Xanthine, xanthine oxidase (XO), cytochrome c type III, nitroblue tetrazolium (NBT), MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], MTT formazan, superoxide dismutase from bovine erythrocytes, dimethyl sulfoxide (DMSO), tertiary butyl hydroperoxide (t-buooH), cumene hydroperoxide (cuooH) and mepindone were all obtained from Sigma Chemical Co. Microtiter plates were supplied by Sterlin, England. All other chemicals used were of analytical grade.

To perform the assay, following reagents were added in microtiter wells sequentially: 15 µl of 1 mM xanthine, 6 µl of 1.25 mM MTT, 30 µl of 15 mU/ml of XO, and the volume was made up to 150 µl with 25 mM phosphate buffered saline pH 7.4 (final concentrations of the reactants were 0.1 mM xanthine, 3 mM XO and 50 µM MTT and all the reagents were prepared in PBS). The mixture was incubated for 20 min at room temperature (30°C) and the reaction was stopped with the addition of 150 µl of DMSO, which also helps to dissolve the MTT formazan crystals formed. Plates were shaken for a few minutes in an orbital shaker and were read on a microplate reader (Bio Rad Microtiter reader Model 450), using test wavelength of 570 nm and reference wavelength of 630 nm. Controls were prepared omitting either xanthine or XO in the reaction mixture. Amount of superoxide generated was calculated using the molar extinction coefficient of MTT formazan E$_{270}$ of 17,000.
Superoxide generation by X-XO system was inhibited using various concentrations of superoxide dismutase (SOD). A working solution of 1 nM SOD was prepared and different concentrations of SOD ranging from 25-300 picomolar were used. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the colour formation in the microtitre plate assay.

Superoxide dependent reduction of cytochrome C was assayed as described. Reduction of NBT by the superoxide generated by X-XO was followed at 560 nm as described.

Enterocytes were isolated from the small intestine obtained from overnight fasted rats as described. Isolated enterocytes were homogenized in a solution containing 0.25 M sucrose and 5 mM hepes buffer adjusted to pH 7.4. Mitochondria and microsomes were separated using differential centrifugation. Purity of the prepared subcellular fractions were assessed by enrichment of the marker enzymes. Protein was estimated as described.

Mitochondria or microsomes corresponding to a protein of 40-100 µg were taken in microtitre plates, added 6 µl of 1.25 mM MTT and the volume made up to 150 µl with PBS. The mixture was incubated for 20 min and 150 µl of DMSO was added. The MTT formazan formed was measured as described above. Superoxide generation by mitochondria and microsomes was also checked by inclusion of various respiratory substrates in presence and absence of SOD.

Addition of SOD to the reaction mixture causes a proportionate inhibition of the rate of MTT reduction, thus confirming the involvement of superoxide in this process and providing the basis for superoxide determination. The conversion of MTT to its formazan is shown in Fig. 1.

Fig. 2 shows the measurement of superoxide generated by X-XO system using the present MTT method as compared to the established methods using reduction of cyt. c and NBT by superoxide. There was an increase in superoxide generation with time and the MTT method was more sensitive as compared to other two methods. Fig. 3 shows the effect of varying the concentration of xanthine on superoxide generation as assayed by MTT reduction method. Increasing the xanthine concentration increased the superoxide formation. Fig. 4 shows the effect of increasing the amount of XO in superoxide generation as assayed by this method. Increased superoxide generation was detected with increasing the XO in the reaction mixture. The MTT reduction by the superoxide generated could be inhibited by SOD. Fig. 5 shows the inhibition of MTT reduction by the presence of varying concentrations of SOD. Inhibition of MTT reduction was linear at low concentration of SOD but not at higher concentrations. Using the maximum amount of SOD, only 96% inhibition could be observed. This is similar to the observations made using other methods. Heat inactivated SOD failed to inhibit MTT reduction. Various compounds of biological interest such as H2O2, NO, ascorbate-iron, i-buuoH, cuoH and menadione (1-5 mM) failed to reduce MTT.

Superoxide generation by subcellular fractions from enterocytes was tested. Mitochondria and microsomes generated superoxide as detected by this method although mitochondria generated double the amount as that of microsomes (Table 1). Presence of succinate, increased the superoxide generation by mitochondria whereas, glutamate or...
iso-citrate had no effect. These compounds had no effect on superoxide generation by microsomes. Superoxide generation by subcellular fractions was responsible for MTT reduction and this was confirmed by inclusion of SOD which prevented the reduction of MTT, both using microsomes and mitochondria (Table 1).

Any reaction inhibitable by superoxide dismutase could potentially provide the basis for an indirect assay for superoxide and SOD. According to this principle, several methods have been developed over the years. However, as pointed out by Beyer\(^{13}\), only a few procedures permit the sensitive and reliable estimation of superoxide generation and of SOD activity in tissue samples. The present MTT reduction assay is suitable for superoxide measurement since it is inhibitable by SOD and the assay is designed to use small volumes of samples. This assay is comparable to other known methods.

It was observed that 50% inhibition of MTT reduction (ie) one unit of the SOD was produced by 10 ng of pure protein whereas the activity determined by X-XO/cyt. c and X-XO/NBT system were 200 and 630 ng of pure protein respectively.\(^6\) A substantial improvement of cyt c assay has been obtained by Kirby\(^{13}\) whose procedure is less sensitive than the assay reported here. Stopping the reaction using DMSO offers convenience when a large number of samples are to be measured at a time and the colour formed is stable for many hours. In addition, use of microtiter plates for this assay considerably reduces the amount of materials to be used for the assay. The amount of superoxide generated by X-XO system measured by this assay system is higher than cyt. c and NBT assays.

This assay can also be used to measure superoxide generation by biological samples as shown.
Fig. 4—Effect of varying xanthine oxidase concentrations on superoxide production measured by MTT reduction. [Reaction was started by adding xanthine. (●), 0.1 mM xanthine, 3 mU xanthine oxidase; (▲), 0.1 mM xanthine, 6 mU xanthine oxidase, (▽), 0.1 mM xanthine, 9 mU xanthine oxidase. Each point represents mean ± S.D. of three separate determinations].

by the measurement of superoxide production by mitochondria and microsomes. These two cellular organelles are known to generate superoxide and it is known that mitochondria is a good source of superoxide in the cells\(^{22}\). Mitochondrial respiration generates superoxide and as shown here, in presence of succinate, this is increased considerably. Reduction of MTT has been used for detection of intracellularly generated superoxide in HeLa cells\(^{21}\). In conclusion this assay for superoxide is simple, rapid and sensitive and is more suitable for large number of samples.

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Table 1—Superoxide production by enterocyte subcellular fractions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT reduction (μmoles/mg protein)</th>
<th>(-SOD)</th>
<th>(+SOD)</th>
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<tbody>
<tr>
<td><strong>Mitochondria</strong></td>
<td></td>
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</tr>
<tr>
<td>None</td>
<td>52 ± 1.40</td>
<td>2 ± 1.60</td>
<td></td>
</tr>
<tr>
<td>+succinate (1 mM)</td>
<td>156 ± 6.70</td>
<td>94 ± 2.00</td>
<td></td>
</tr>
<tr>
<td>+glutamate (1 mM)</td>
<td>54 ± 2.00</td>
<td>2 ± 2.50</td>
<td></td>
</tr>
<tr>
<td>+isocitrate (1 mM)</td>
<td>51 ± 2.00</td>
<td>3 ± 1.60</td>
<td></td>
</tr>
<tr>
<td><strong>Microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>26 ± 1.60</td>
<td>1 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>+succinate (1 mM)</td>
<td>27 ± 0.10</td>
<td>1 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>+glutamate (1 mM)</td>
<td>25 ± 3.20</td>
<td>1 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>+isocitrate (1 mM)</td>
<td>26 ± 0.40</td>
<td>1 ± 0.30</td>
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References
10 Slater T F, Sawyer B & Strauli U (1963), Biochim Biophys Acta, 77, 383-393.
14 Aust S D, Roerig D L & Pederson T C (1972), Biochem Biophys Res Commun, 47, 1133-1137.
18 Pennington R J (1961), Biochem J, 80, 649.
22 Aust S D, Roerig D L & Pederson T C (1972), Biochem Biophys Res Commun, 47, 1133-1137.