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Yasir Hasan Siddique  
*Aligarh Muslim University, India*

Gulshan Ara  
*Aligarh Muslim University, India*

Mohammas Afzal  
*Aligarh Muslim University, India*

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## ESTIMATION OF LIPID PEROXIDATION INDUCED BY HYDROGEN PEROXIDE IN CULTURED HUMAN LYMPHOCYTES

**Yasir Hasan Siddique, Gulshan Ara, Mohammad Afzal** □ Aligarh Muslim University, India

□ Malondialdehyde (MDA) is used for the estimation of damage by reactive oxygen species. MDA is a major reactive aldehyde resulting from the peroxidation of biological membranes. The most common method used to assess MDA production is the thiobarbituric acid (TBARS) assay. However, the value of this method is curbed by low specificity and has been criticized for its use in human studies. In the present study we have used an alternative method for the estimation of MDA production i.e. reaction of MDA with a chromogenic agent 1-methyl-2-phenylindole at 45°C. The paper describes the method of preparing standards for the estimation of MDA (lipid peroxidation) after the treatment with an oxidative stress inducing agent hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In the present study, the treatments of 1, 5, 10, 20, 50, 100, 150 and 200 μM of H<sub>2</sub>O<sub>2</sub> induced significant increase in lipid peroxidation as compared to the untreated ones. The results suggest that the present method can be used to measure the lipid peroxidation in cultured human peripheral blood lymphocytes and is specific for MDA estimation.

*Keywords:* lipid peroxidation, malonaldehyde, human lymphocytes, hydrogen-peroxide, oxidative stress.

### INTRODUCTION

The major reactive aldehyde resulting from the peroxidation of biological membranes is malondialdehyde (MDA) (Vaca et al., 1988). It is used as an indicator of tissue damage by a series of chain reactions (Ohkawa et al., 1979). Figure 1 demonstrates the steps of lipid peroxidation (Alessio, 2000). MDA reacts with thiobarbituric acid to produce a red colored product. The direct assessment and measurement of free radicals is hampered by their brief half-lives, the higher reactivity and short migration distance (Bergendi et al., 1999). Indirect methods, such as the measurement of products resulting from the peroxidative decomposition of PUFA e.g. 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) are used for the estimation of the damage by reactive oxygen species (Esterbauer and Cheeseman, 1990). Lipid oxidation products, such as 4HNE, is cytotoxic, hepatotoxic, mutagenic and genotoxic. MDA reacts with DNA and forms adducts with deoxyguanosine and deoxyadenosine (Marnett 1999). It can also modify RNA, proteins and other biomolecules

Address correspondence to Yasir Hasan Siddique, Human Genetics and Toxicology Laboratory, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh (UP) – 202 002, India; Tel: 0571-2700920-3442; Email: yasir\_hasan-siddique@rediffmail.com

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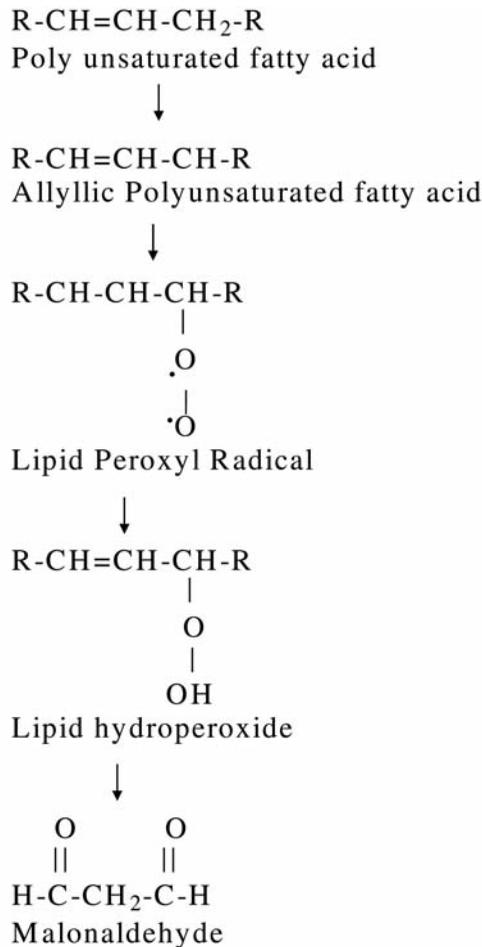


FIG. 1. Steps of lipid peroxidation (Alessio, 2000).

(Esterbauer and Cheeseman, 1990). The production of MDA is estimated by the thiobarbituric acid reactive substances assay (TBARS) (Esterbauer and Cheeseman, 1990; Marnett, 1999). However, the value of this method is reduced by low specificity and the possible reaction of malondialdehyde with other cellular constituents (Drapper and Hadley, 1990; Esterbauer and Cheeseman, 1990; Livingstone et al., 1990). Thus developing an assay MDA with an alternative method of higher specificity may prove useful in assessing lipid peroxidation. One such method of MDA determination involves the reaction of a chromogenic agent 1-methyl-2-phenylindole, with MDA at 45°C. Two molecules of 1-methyl-2-phenylindole react with one molecule of MDA to form a stable chromophore having maximal absorbance at 586 nm (Gerard-Monnier et al., 1998). In the present study, we have estimated the lipid peroxidation which is induced by hydrogen peroxide in cultured human lymphocytes.

*Estimation of Lipid Peroxidation***MATERIALS AND METHODS****Chemicals**

1-methyl-2-phenylindole and 1,1,3,3, tetra methoxypropane were purchased from Sigma. Acetonitrile and TRIS were purchased from SRL, India. Methanol and HCl were purchased from Qualigens, India. RPMI 1640, phytohaemagglutinin-M, antibiotic (Penicillin G sodium & Streptomycin) – antimycotic (amphotericin B) mixture (CAT No. 15240-096) and fetal calf serum were purchased from Gibco. All chemicals were of analytical grade.

**Human lymphocyte culture and lipid peroxidation assay**

Triplicate peripheral blood cultures were performed according to Carballo *et al.* (1993). Peripheral blood samples were aseptically collected in heparinized sterile glass tubes from three healthy individuals (22 – 25 years old). A haemocytometer was used to count the number of viable cells. Typically, each culture consisted of an initial density of  $0.5 \times 10^6$  cells in 1 mL of culture medium. 0.5 mL of blood was supplemented with 5 mL of culture medium (RPMI – 1640), 1.5 mL of fetal calf serum, 0.1 mL of antibiotic (1000 units/ml Penicillin G sodium and 10,000 µg/ml Streptomycin sulphate) – antimycotic ( 25 µg/ml amphotericin B) mixture and 0.1 mL of phytohaemagglutinin in glass culture tubes. Cells were incubated at 37°C for 24 h.

**Treatment of cells**

After 24 h, the cells were treated with 1, 5, 10, 20, 50, 100, 150 and 200 µM of hydrogen peroxide and were kept in the incubator at 37°C for another 24 and 48 h. Untreated and negative controls (200µL distilled water) were also run simultaneously.

**Preparation of buffers**

Reagent 1 (R1) was prepared by dissolving 0.064g of 1-methyl-2-phenylindole into 30 mL of acetonitrile to which 10 mL of methanol was added to bring the volume to 40 ml. The preparation of 37% HCl served as the reagent R2.

**Preparation of Standard**

The standard (S2) was prepared by dissolving 16.5µl of 1,1, 3,3-tetramethoxypropane in 10 ml of 20mM Tris HCl (0.242g of Tris HCl in 100ml H<sub>2</sub>O DW). The solution S2 was diluted to 1:100 in H<sub>2</sub>O (DW) (i.e. 20 µL of S2 was added to 2 mL of H<sub>2</sub>O). The final volumes were measured into 2 mL microcentrifuge tubes, as shown in Table 1. The tubes

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**TABLE 1.** Amounts of reagent and standard use for the preparation of the standard graph. S2 ( $\mu\text{M}$ ) is the final concentration.

R1 ( $\mu\text{L}$ )	1300	1300	1300	1300	1300
S2 ( $\mu\text{L}$ )	0	50	100	200	400
H <sub>2</sub> O ( $\mu\text{L}$ )	400	350	300	200	0
<b>S2 (<math>\mu\text{M}</math>)</b>	<b>0</b>	<b>2.5</b>	<b>5</b>	<b>10</b>	<b>20</b>

were vortexed and 300  $\mu\text{L}$  of R2 was added to each tube. The vortexed tubes were further incubated at 45°C for 40 min. After incubation, tubes were cooled in ice and centrifuged at 15000 g for 10 min. All samples were read on a double beam spectrophotometer (Analyticajena specord 5.0) at 586 nm.

### Procedure for treated cells

After incubation for 24 h, the treated blood cultures were centrifuged at 3000 g for 20 min, and subsequently the supernatants were collected. In a microcentrifuge tube 1300  $\mu\text{L}$  of R1 was taken. 1 mL of supernatant was diluted 10 times with Tris HCl and 200  $\mu\text{L}$  of this diluted supernatant from each culture was added to 200  $\mu\text{L}$  of distilled water and vortexed. 300  $\mu\text{L}$  of R2 to each tube, which were then vortexed and incubated at 45°C for 40 min. After incubation, the tubes were cooled in ice and centrifuged at 15000 g for 10 min at 4°C. All samples were read on a double beam spectrophotometer (Analyticajena specord 5.0) at 586 nm. The same procedure was adopted for the treated cells for 48 h duration.

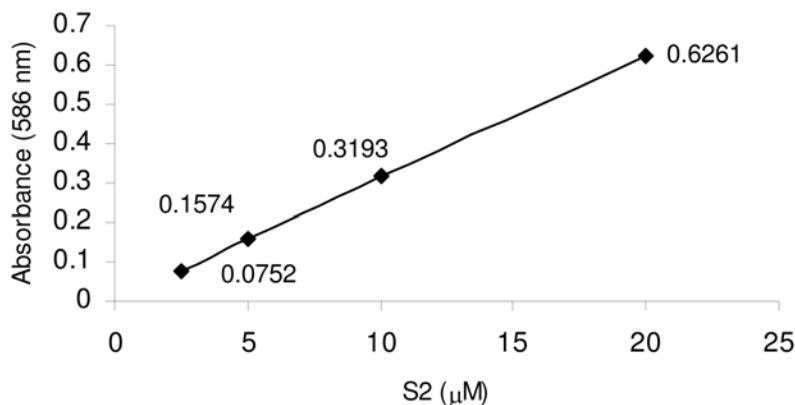
### Statistical analysis

The statistical analyses were done using Statistica Soft Inc. The student's t-test was applied to observe the significant difference between treatments and controls. Regression analysis was performed using Statistica Soft Inc.

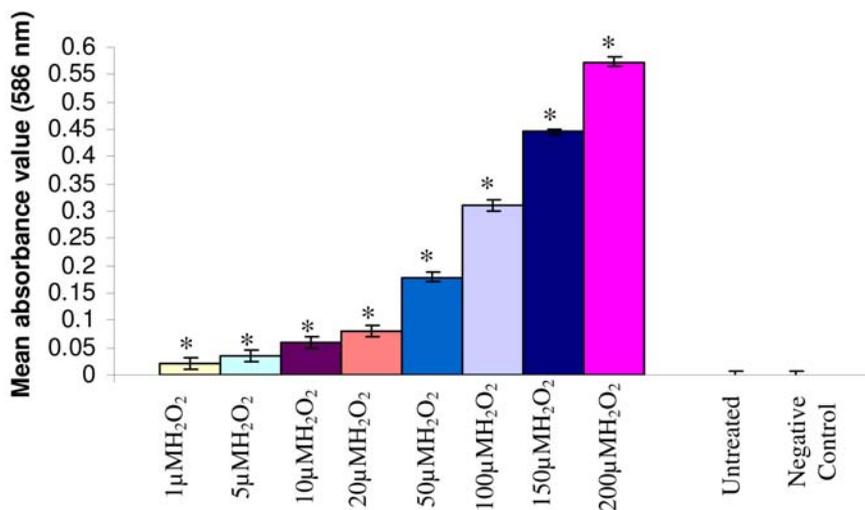
## RESULTS

Figure 2 shows the standard curve for MDA estimation. Figure 3 shows the mean absorbance value 24h after the treatments of 1, 5, 10, 20, 50, 100, 150 and 200  $\mu\text{M}$  of H<sub>2</sub>O<sub>2</sub> in cultured human lymphocytes. The significant values are denoted herewith (\*) as compared to the untreated (Fig.3). The treatments with 1, 5, 10, 20, 50, 100, 150 and 200  $\mu\text{M}$  of H<sub>2</sub>O<sub>2</sub> correspond with the mean absorbance values of  $0.0198 \pm 0.005$ ,  $0.0346 \pm 0.008$ ,  $0.0588 \pm 0.010$ ,  $0.0798 \pm 0.012$ ,  $0.1792 \pm 0.015$ ,  $0.3094 \pm 0.032$ ,  $0.4462 \pm 0.039$  and  $0.5734 \pm 0.048$ , respectively. The untreated and negative controls correspond with the mean absorbance values of  $0.0011 \pm 0.0001$  and  $0.0013 \pm 0.0002$  respectively. Similarly, the treatments of 1, 5, 10, 20, 50, 100, 150 and 200  $\mu\text{M}$  of H<sub>2</sub>O<sub>2</sub> in cultured lymphocytes after 48

## Estimation of Lipid Peroxidation



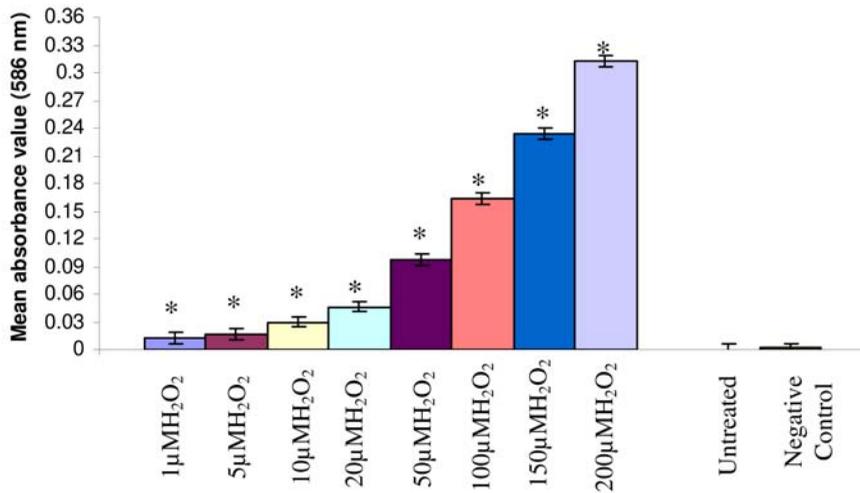
**FIG. 2.** Standard graph for the estimation of lipid peroxidation in cultured human peripheral blood lymphocytes.



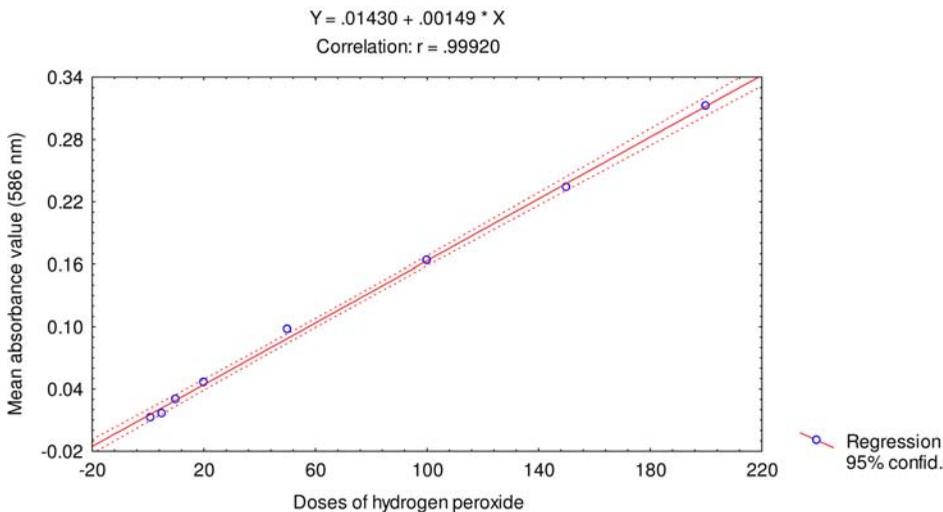
**FIG. 3.** Lipid peroxidation after 24 h treatment of the  $\text{H}_2\text{O}_2$  on cultured human lymphocytes (\* $p < 0.05$ ).

hr correspond with the mean absorbance values of  $0.0123 \pm 0.001$ ,  $0.0163 \pm 0.003$ ,  $0.0300 \pm 0.006$ ,  $0.0464 \pm 0.009$ ,  $0.0976 \pm 0.013$ ,  $0.1636 \pm 0.017$ ,  $0.2338 \pm 0.028$  and  $0.3123 \pm 0.030$ , respectively (Fig 4). The untreated and negative controls correspond with the mean absorbance values of  $0.0010 \pm 0.0001$  and  $0.0011 \pm 0.0001$ , respectively. Regression analysis was used to study the dose-time relationship of the effects of  $\text{H}_2\text{O}_2$  following 24 and 48 h of treatments based on the mean absorbance values in cultured human peripheral blood lymphocytes. An increase in the slope of linear regression lines ( $P < 0.005$ ;  $r = 0.99$ ) was observed due to the increase in the doses of the  $\text{H}_2\text{O}_2$  (Fig 5 and 6).

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**FIG. 4.** Lipid peroxidation after 48h treatment of the H<sub>2</sub>O<sub>2</sub> on cultured human lymphocytes (\*p<0.05).

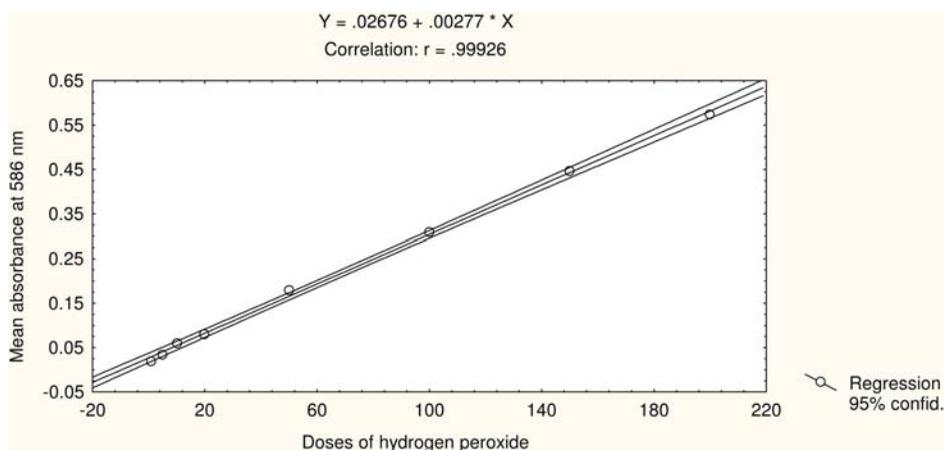


**FIG. 5.** Effect of hydrogen peroxide treatment on the mean absorbance at 586 nm in cultured human peripheral blood lymphocytes after 24 h.

## DISCUSSION

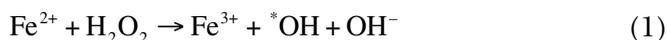
The results of the present study reveal a clear dose dependent increase in the production of MDA upon treatment with hydrogen peroxide. Ion radicals generated during oxidative stress leads to a chain reaction called lipid peroxidation. Aldehydes generated from lipid peroxidation forms the DNA adducts and lipid hydroperoxides (LOOH) that result in extensive single and double strand breaks in DNA (Devipriya et

## Estimation of Lipid Peroxidation

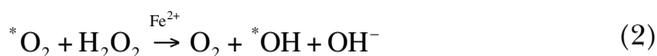


**FIG. 6.** Effect of hydrogen peroxide treatment on the mean absorbance at 586 nm in cultured human peripheral blood lymphocytes after 48 h.

al., 2008). Other compounds, such as Arochlor 1254, menadione, carbon tetrachloride, cadmium, copper, paraquat and certain nitroaromatic compounds can also increase lipid peroxidation (Digiulio et al., 1989; Halliwell and Gutteridge, 1984). Among aldehydes, malondialdehyde is used as the marker of oxidative stress (Alessio, 2000). The common method used to assess the changes in MDA production is thiobarbituric acid (TBARS) assay. This method is useful in defined membrane systems, such as microsome *in vitro* (Halliwell and Chirico, 1993), but the method has been criticized for its use in human studies of oxidative stress owing to its low specificity. This assay also shows reaction with saturated and unsaturated non-functional aldehydes, carbohydrates and prostaglandins (Alessio, 2000). Hydrogen peroxide ( $H_2O_2$ ) is the most effective species for cellular injury (Rao et al., 1996). Reactive oxygen species i.e.  $H_2O_2$  generated during oxidative stress is known to cause damage to proteins, nucleic acids, and cell membranes and has also been associated with cancer, aging and several chronic neurodegenerative diseases (Kellogg and Fridovich, 1975; Daroui et al., 2004).  $H_2O_2$  gives a Fenton reaction in the presence of  $Fe^{2+}$ . Similar reaction is involved in the presence of other transition elements such as Cu, Co and Ni.



The product can also react with  $O_2$  to initiate Haber-Weiss reaction producing  $\cdot OH$  in the presence of  $Fe^{+2}$ .



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It has been observed that certain estrogen metabolites are involved in carcinogenesis wherein  $H_2O_2$  plays an important role.  $H_2O_2$  has been associated with the induction of cancer and hence has been reported as mutagenic (Shamberger, 1972; Pryor, 1986). The compound  $H_2O_2$  is reported to induce DNA damage (Thibodeau and Paquette, 1999), chromosomal aberration (Sofni and Ishidate, 1984) and sister chromatid exchanges (MacRae and Stich, 1979). In the present study, we have estimated the lipid peroxidation in terms of quantifying malondialdehyde (MDA) level. The lipid peroxidation products of PUFAs are considered to be important for genotoxic effects (Pryor and Porter, 1990; Comporti 1989; Esterbauer et al., 1990; Eder et al., 2008). Various reactive oxygen species can induce lipid peroxidation, whereas peroxidative fatty-acid fragments and their radicals can lead to the formation of reactive oxygen species (Eder et al., 2008). Thus Malondialdehyde (MDA) and 4-hydroxy-2-noneal (HNE) are the two most prominent lipid peroxidative products (Esterbauer et al., 1990. Esterbauer et al., 1982; 1991). The present method, that we have established as a lipid peroxidation assay, gives negligible reaction with 4-hydroxynonenal. In the presence of hydrochloric acid, Malondialdehyde reacts with two molecules of 1-methyl-2-phenylindole to yield a stable chromophore having intense maximal absorbance at 586 nm (Gerard-Monnier et al., 1998). This assay performed in hydrochloric acid based medium enables one to have specific measurement of MDA in the presence of 4-hydroxyalkenals, as in the presence of hydrochloric acid, 4-hydroxyalkenals undergo an irreversible cyclization reaction (Gerard Monnier et al., 1998). In the present study, a clear dose dependent and significant increase in the concentration of MDA was obtained following  $H_2O_2$  treatment after 24h, though after 48h of treatment, the mean absorbance values were decreased. This may be due to the reduced short half life of  $H_2O_2$  or due to the internal protection mechanism of the cell. Hence, it is concluded that  $H_2O_2$  is potent enough to induce lipid peroxidation in cultured human lymphocytes and the above method, therefore, is recommended for the estimation of lipid peroxidation in blood cells in culture.

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