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# Combining Metal Chelator and Antioxidant Improves Amelioration of Induced Lipid Peroxidation in the Human Erythrocyte Membrane

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**KEYWORDS** Ethylenediaminetetraacetic Acid. Hydrogen Peroxide. Malondialdehyde. Methylsulfonylmethane. Oxidative Stress

**ABSTRACT** The role of unliganded iron in oxidative stress (OS) suggests the therapeutic value of removing ionized iron with a chelator, such as ethylenediaminetetraacetic acid (EDTA), in combination with an antioxidant to provide more effective therapy. Hence, the present study seeks to investigate the systemic impact of EDTA and antioxidant Methylsulfonylmethane (MSM), individually and in combination, on erythrocytic lipid peroxidation marker, malondialdehyde (MDA). Blood from 60 healthy volunteers was obtained, erythrocytes separated, incubated with hydrogen peroxide to obtain OS-induced RBC ($OSI_R$) which were further incubated with EDTA, MSM or EDTA+MSM for 15/30/60 minutes. MDA showed maximum reduction after 30 min, which was 16.7, 30 and 46.8 percent respectively after incubation with EDTA, MSM and EDTA+MSM. Results indicate independent and additive roles of chelator and antioxidant in reducing lipid peroxidation. In conclusion, it is suggested that the utility of combining antioxidant with chelator is a better therapeutic strategy to ameliorate oxidative stress.

## INTRODUCTION

Reactive oxygen species (ROS) are known to increase in a variety of disease conditions, and antioxidants have been advocated as therapeutic agents in conditions where ROS are found to be high. However, the efficacy of antioxidants against disease conditions has been a subject of intense investigation in the last two decades and results have been mixed. While some studies have reported a high degree of success (Jou 2008), others have indicated no impact of antioxidants against ROS, as has been reviewed by many investigators (Oldham and Bowen 1998; Rehman et al.1998; Miller et al. 2005; Rodrigo et al. 2007; Bjelakovic et al. 2007; Valko et al. 2016).

Iron has been implicated as a major cause of this confusion (Kell 2009) because a variety of antioxidants can, in fact, act as pro-oxidants in the presence of inappropriately or inadequately liganded ferrous ions (Fe (II)), and thus actually promote the production of damaging hydroxyl radical ($OH^•$) radicals rather than mitigating them (Long et al. 2000; Hininger et al. 2005).

The mechanism of formation of the very reactive $OH^•$ and ferric ions (Fe III) involves the reaction between free or poorly liganded Fe (II) and hydrogen peroxide. Superoxide can also react with Fe (III) in the Haber-Weiss reaction (Kehrer 2000) to produce (Fe (II)) again, thereby effecting redox cycling. Thus, the focus of recent research has been the recognition of iron as a major culprit and has led to the suggestion that including iron chelators with antioxidants can be useful in scenarios where the amount of 'free' iron is high (Kell 2009), because it is important to restore and maintain iron homeostasis (Valko et al. 2016).

The benefits of combining antioxidants and chelators have been reported in some recent studies. In one study on Wistar rats, dietary iron overload led to iron toxicity in the brain, which produced several deleterious effects. These were attenuated by combining iron chelator (deferiprone) and antioxidant (N-acetyl cysteine) by reverting the impact of iron overload (Sripetchwandee et al. 2014) In another study, the same combination of chelator defiperone and antioxidant, N-acetyl cysteine were reported to decrease oxidative stress and thereby exert the greatest cardioprotective efficacy when compared with either of the monotherapies in iron-overloaded thalassemic mice (Kumfu et al. 2017).

A large body of work in this area is performed on animal models of research. However, it is important to design studies with the specific purpose of understanding the mechanisms of combining antioxidants with chelators, with a focus on human health. The erythrocyte pro-

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vides a suitable model to assess the systemic impact of combining an antioxidant and a chelator, for several reasons. The erythrocyte is particularly susceptible to oxidative stress due to its role in oxygen transport. The erythrocyte membrane is composed of an asymmetrical bilayer of phospholipids, with predominance of sphingomyelin and phosphatidylcholine on the outer side and polar phosphatidylserine and phosphatidylethanolamine on the inner cytoplasmic side. The unsaturated fatty acids on the membrane phospholipids are more susceptible to peroxidation that involve hydrogen abstraction from a carbon, with oxygen insertion in lipid peroxyl radical and hydroperoxides (Brodnitz 1968). Exposure of the membrane to hydrogen peroxide induces lipid peroxidation resulting in formation of excessive malondialdehyde which disturbs the symmetry of the membrane bilayer (Jain 1984), thereby altering its structural and physiological properties such as its fluidity, permeability to different substances and bilayer thickness (Esterbauer et al. 1991).These reactions have been recognized in the inflammatory, oxidative stress related phenomenon, and, interestingly, it has also been reported that erythrocytes when oxidized can release free iron (Ciccoli et al. 2003).

Since unliganded iron appears to have an important role in the development of oxidative stress, the next step is to choose which chelators should be used to control the damage done by it, whether alone or with antioxidants (Kell 2009). EDTA is a chelator categorized as Generally Recognized as Safe (GRAS) by Food and Drug Administration (USFDA) (http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/). It has been extensively used as a food preservative and as an anticoagulant in blood, is generally regarded as non-toxic for internal consumption in small amounts and is readily excreted in the urine after combining with metal ions in the blood (Nakhostin-Roohi et al. 2011; Zhang 2009; Anderson 2004). This chelation involves the two nitrogen atoms and two oxygen atoms in separate carboxyl (-$COO^-$) groups. Thus, EDTA can bind both Fe (II) and Fe (III); however, the stability constants are much greater for the Fe (III) chelator complexes. Therefore, it functions by binding Fe (II) and subsequently promoting oxidation of the Fe (II) to Fe (III) with a concomitant reduction of molecular oxygen to partially reduced oxygen species. EDTA can be a tetradentate or a hexadentate ligand. Since the maximal coordination number of iron is six, the hexadentate chelators can provide more consistently inert complexes due to their ability to completely saturate the coordination sphere of the iron atom and, consequently, deactivate the "free iron" completely. Chelators can function by inhibiting the production of free iron, or reducing the rate of $OH^{\bullet}$ production, or trapping the $OH^{\bullet}$ as well as by other mechanisms (Balcerczyk et al. 2007).

Although the utility of chelators in management of oxidative stress appears to be promising, their use is not widespread because their effectiveness in amelioration of oxidative stress has been found to be variable (Jayasena et al. 2007; Kalinowsky and Richardson 2005), which in the case of EDTA, is attributed to its limited ability for crossing biological membranes (Jayasena et al. 2007; Kalinowsky and Richardson 2005). Since the combination of a chelator with an antioxidant has been suggested as a treatment option for OS, it is important to choose an appropriate antioxidant to combine with EDTA.

Methylsulfonylmethane (MSM) appears to be a suitable candidate for the purpose, for several reasons. It is a small amphipathic organosulfone found in a wide range of foods including some fruits, grains and beverages (Kim et al. 2006) and is included in the USFDA's list of GRAS compounds (http://www.fda.gov/Food/IngredientsPackaging Labeling/GRAS/). It is non-toxic even at fairly high doses of 2g/kg in rats (Horvath et al. 2002), and is considered safe at recommended oral dosages for humans (Usha and Naidu 2004). Recently, it has received wide attention as an antiinflammatory, antioxidant dietary supplement in the treatment of several conditions such as allergic rhinitis, experimental colitis and autoimmune diseases (Usha and Naidu 2004; Amirshahrokhi et al. 2011). Due to its antioxidant nature, it has been found to be effective in therapy for exercise-induced muscle damage (Barmaki et al. 2012), following acute exhaustive exercise (Nakhostin-Roohi et al. 2011, 2013), and as an effective therapy against osteoarthritis (Kim et al. 2006; Gregory et al. 2008). In addition to its direct role in mitigating oxidative stress, it has also been reported to act as a permeability enhancer for EDTA, attributed to its amphipathic nature which appears to be responsible for its action on membranes. Combining EDTA with Methylsulfonylmethane (MSM) has been reported to enable the EDTA to cross the blood brain barrier and deliver ciprofloxacin into the cornea and all tested ocular tissues (Zhang et al. 2009) and ameliorated oxidation-

induced toxicity in diabetic cataract (Zhang et al. 2011). MSM is structurally related to DMSO (Shaw and Chandrasekaran 1978) which is capable of increasing the absorption rate of some compound into skin and other tissues. This was indeed found, as reported by our research group (Tripathi et al. 2011) in a study which demonstrated the efficacy of a combination of MSM and EDTA but not either alone, in reducing swelling of the lower extremities as well as systemic OS in chronic venous insufficiency.

Studies are lacking on the impact of these compounds in blood. This is important because any systemic effects require the circulation of compounds and may impact the erythrocyte which has high susceptibility to OS.

### Objectives

Based on the foregoing, the present case-control study on healthy humans was undertaken to assess whether circulating EDTA and MSM, individually and in combination, as chelator and antioxidant, impact systemic OS, especially in the erythrocyte. OS was induced *in vitro* in the erythrocytes of normal, disease-free volunteers by treatment with hydrogen peroxide, as described by Ebrahimzadeh et al. (2010), and impact was assessed on lipid peroxidation marker, malondialdehyde.

## METHODOLOGY

### Selection of Volunteers

Volunteers comprised of a cross section of adult healthy males and females, age 25-60 years, visiting physicians or pathological laboratories for routine blood tests during the period September to November 2015. Data was collected to record their age, gender, and their body weight, height was measured as per standard methodology, as recommended by the World Health Organization (WHO 1995). They were included in the study when they confirmed that they were not suffering from diseases such as diabetes, cardiac disease, hypertension, cancer etc. The study protocol was explained and written consent was obtained from all volunteers. The study was approved by the Institutional Ethics Committee of Population Resource and Research Centre, Allahabad. They were requested to volunteer for collection of about 5-6 ml of blood, and informed consent was obtained from the final sample of 60 volunteers comprising 34 men and 26 women. The mean age ± SEM of the final sample of participants was 38.63± 1.45 years, and body mass index (BMI) was 28.36± 0.80.

### Processing of Blood Samples

5 ml of venous blood was drawn into acid – citrate- dextrose (ACD) vials and kept on ice for not more than 1 hour before processing. The samples were centrifuged at 3000 rpm for 15 minutes. Plasma was collected and red blood cells (RBCs) were washed three times with normal saline. These RBC were divided into several parts and incubated in various solutions for varying durations of time, as described below.

For the non-OS induced control erythrocytes, 4 aliquots of RBC were incubated with 1:1 v/v phosphate buffer saline (PBS) (pH 7.4) for 0, 15, 30 and 60 minutes. They were then washed with normal saline. They were designated $C_R$, and hemolysate was prepared as described in an earlier paper (Tripathi et al. 2011).

### *Induction of Oxidative Stress*

Oxidative stress (OS) was induced in the remaining RBC by incubating them with1 mM $H_20_2$ (1:1 v/v)prepared in PBS buffer pH 7.4 for 30 min. $H_2O_2$ was then removed by washing the RBC with normal saline 1:1 v:v and centrifuging again to obtain OS induced RBC ($OSI_R$).

The $OSI_R$ were then incubated 1:1 v/v with either EDTA-disodium salt (2.70 g EDTA/dL of PBS) or MSM (1.35g MSM /dL of PBS) or a 1:1v/v combination of MSM and EDTA at $37^0$C for 15, 30 and 60 minutes.

All samples of incubated RBC were washed three times with normal saline to remove incubation mixtures and centrifuged to obtain treated packed RBC. These were then hemolyzed as described in Tripathi et al. (2011). The hemolysate (1: 20) was stored at -80°C, until analysis.

### *Measurement of Lipid Peroxidation*

The hemolysate was used for estimation of lipid peroxidation marker, Malondialdehyde (MDA) by the modified method of Niehaus and Samuelsson (1968), as described in Tripathi et al. (2011) and expressed as nanomoles of MDA per g of hemoglobin.

### Statistical Analysis

Mean and standard error were calculated for various groups and parameters. Statistical sig-

nificance was obtained using one-way and two way ANOVA and p- values were obtained using GraphPad Prism 5.0 version and Window Excel 10.

## RESULTS

### Effect of Hydrogen Peroxide on RBC

The effect of incubating RBC with a compound known to create oxidative stress ($H_2O_2$) on lipid peroxidation was compared with the effect of incubating them with a buffer (PBS) (Table 1, Fig. 1a).

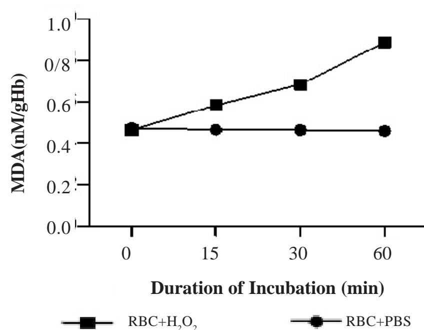


**Fig. 1(a).**

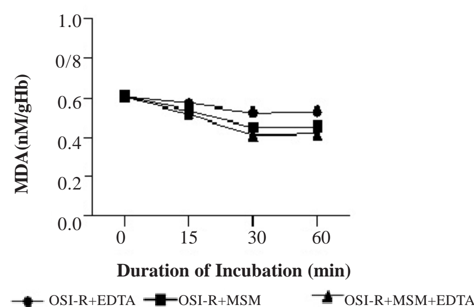


**Fig. 1(b).**

**Fig. 1. Effect of incubating (a) RBC with buffer (PBS), oxidative stress inducer, $H_2O_2$, (b) OS induced RBC ($OSI_R$) with EDTA, MSM, and a combination of these (MSM+EDTA) on malondialdehyde (MDA).**

No change in MDA was observed when the control RBC ($C_R$) were incubated in PBS for 15, 30 or 60 minutes from baseline values at 0 min. When control RBC ($C_R$) were incubated in $H_2O_2$, MDA increased from 0.465±0.021 at baseline to 0.585±0.022, 0.684±0.026 and 0.888±0.027 as duration of incubation increased from 15, 30 and 60 min respectively.

### Effect of Incubating OS Induced RBC with EDTA, MSM or EDTA+MSM

The change in MDA in $OSI_R$ on incubating them for 15, 30 or 60 minutes with metal chelator EDTA or antioxidant, MSM or their combination, MSM+EDTA is reported in Table 2 and Figure 1 b.

A decline in MDA on incubation with EDTA, MSM and EDTA+MSM respectively was indicated as baseline values of 0.611±0.040, 0.610±0.039 and 0.607±0.039 to 0.573±0.039, declined to 0.537±0.039 and 0.517±0.040 at 15 min, 0.525±0.039, 0.451±0.037 and 0.408±0.038 at 30 min and 0.530±0.039, 0.454±0.037 and 0.418±0.039 at 60 min. The decline followed the order EDTA< MSM< EDTA+MSM and the impact appeared to be maximum after 30 minutes of incubation.

The progression of change in MDA, presented graphically shows that as expected, no change in MDA was detected when normal RBC were incubated with PBS, while incubation with $H_2O_2$ led to increase throughout the 60 min of incubation (Fig.1a).

The increased MDA in $H_2O_2$ treated RBC declined when these $OSI_R$ were incubated with EDTA, MSM or EDTA+MSM, which continued till 30 minutes of incubation, but not further between 30 and 60 min (Fig.1b).

### Changes in MDA with Various Treatments on RBC of Individual Volunteers

The experiment was designed to obtain changes in MDA for each volunteer for each

**Table 1: Changes in erythrocytic MDA on incubation with hydrogen peroxide**

| *Treatment* | *MDA nM/g Hb after incubation for* | | | |
|---|---|---|---|---|
| | *0 min* | *15 min* | *30 min* | *60 min* |
| $C_R$+P | 0.474±0.017 | 0.466±0.016 | 0.465±0.015 | 0.460±0.015 |
| $C_R$+H | 0.465±0.021 | 0.585±0.022 | 0.684±0.026 | 0.888±0.027 |

All values are expressed as x ± µ

$C_R$: Control RBC; P: Phosphate buffer saline (PBS); H: Hydrogen peroxide ($H_2O_2$)

**Table 2: Changes in erythrocytic MDA in oxidative stress-induced erythrocytes ($OSI_R$) on incubation with ethylenediaminetetraacetic acid (EDTA), methylsulfonylmethane (MSM) or their combination**

| *Treatment* | *MDA nM/g Hb after incubation for* | | | |
|---|---|---|---|---|
| | *0 min* | *15 min* | *30 min* | *60 min* |
| $OSI_R$+E | 0.611±0.040 | 0.573±0.039 | 0.525±0.039 | 0.530±0.039 |
| $OSI_R$+M | 0.610±0.039 | 0.537±0.039 | 0.451±0.037 | 0.454±0.037 |
| $OSI_R$+M+E | 0.607±0.039 | 0.517±0.040 | 0.408±0.038 | 0.418±0.039 |

All values are expressed as x ± μ
$OSI_R$: Oxidative stress induced RBC; M: Methylsulfonylmethane (MSM); E: Ethylenediaminetetraacetic acid (EDTA)

time interval. For the purpose, $OSI_R$ from each volunteer were divided into 12 aliquots, four of which were incubated with each of the three test solutions, EDTA, MSM and EDTA+MSM. Four aliquots from each treatment were respectively used to obtain 0, 15, 30 and 60 min values for each volunteer, and the change in MDA was determined for the different time intervals, namely, the first 15 minutes, $\Delta_{15-0}$, the next 15 minutes, $\Delta_{30-15}$, and between 30and 60 minutes, $\Delta_{60-30}$ for each volunteer for each type of treatment. Results, expressed as mean ± SEM, are presented in Table 3.

The results of one-way ANOVA confirmed that the decrease in MDA with all three treatments were significantly different from each other after 15 minutes, (7.019, 14.47 and 18.25% respectively for EDTA, MSM and EDTA+MSM) which increased on further incubation till 30 minutes (10.65, 18.63 and 25.66% respectively for EDTA, MSM and EDTA+MSM) but not further when incubation was continued to 60 minutes. Decline in MDA after 30 minutes of incubation clearly indicated that EDTA alone produced minimal effect on reducing MDA, MSM alone produced a significant reduction in MDA which was enhanced by addition of EDTA.

The effect of MSM on reduction of MDA was more than double (106% more) over the effect of EDTA in the first 15 minutes $\Delta_{15-0}$, (-7.019±0.938 for EDTA and -14.47±1.119 for MSM), which was enhanced by another approximately sixty percent (18.25±1.369) when a combination of EDTA and MSM was used. In the next 15 minutes ($\Delta_{30-15}$), the decline in MDA on incubation with EDTA was a further twenty-five percent (from 7.019±0.938 to 10.65±1.100), which increased by another eighty percent (18.63±1.62) on incubation with MSM and a further seventy percent (25.66±1.35) with EDTA+MSM.

Since the data indicated an overall increase in MDA with $H_2O_2$ treatment in normal RBC, and a decline in MDA in $OSI_R$ with all treatments, the individual decline in MDA in $OSI_R$ of individual volunteers was assessed to check for consistency of impact of treatment with EDTA, MSM and EDTA+MSM, and is presented in Figure 2a-2d.

After 30 minutes of incubation of RBC with $H_2O_2$, MDA showed a consistent increase in the

**Table 3: Percent change in MDA in $OSI_R$ incubated in EDTA, MSM, and EDTA+MSM in the first 15 minutes, between 15 and 30 minutes and between 30 and 60 minutes**

| *Treatment* | *Percent change in MDA nM/g Hb#* | | |
|---|---|---|---|
| | Ä $_{15-0}$ | Ä $_{30-15}$ | Ä $_{60-30}$ |
| $OSI_R$ +E | -7.019±0.938 | -10.65±1.100 | 1.124±0.248 |
| $OSI_R$ + M | -14.47±1.119 | -18.63±1.62 | 1.301±0.691 |
| $OSI_R$ + M+E | -18.25±1.369 | -25.66±1.35 | 2.48±1.050 |
| F | 24.45 | 29.91 | 0.993 |
| P | <0.0001 | <0.0001 | 0.372 |

All values are expressed as x ± μ
$OSI_R$: Oxidative stress induced RBC; M: Methylsulfonylmethane (MSM); E: ethylenediaminetetraacetic acid (EDTA); Ä $_{15-0}$ = ([MDA at 15 min – MDA at 0 min)/MDA at 0 min]*100; Ä $_{30-15}$ = ([MDA at 30 min – MDA at 15 min)/MDA at 15 min]*100; Ä $_{60-30}$ = [(MDA at 60 min – MDA at 30 min)/MDA at 30min]*100.

blood of all but 2 of the 60 volunteers (96.7%) (Fig. 2a). It can be seen that MDA declined in 58 of 60 volunteers (96.7%) after incubation with EDTA (Fig 2b), in 59 of 60 volunteers (98.3%) after incubation with MSM (Fig. 2 c), and all 60 volunteers (100%) after incubation with EDTA+MSM as seen in Figure 2 (d).

This indicated a consistent effect of the three treatments on MDA in the OS induced erythrocyte of all volunteers. The effect of MSM was more than that of EDTA. The effect of the combination was maximum.

The next question that arose was whether EDTA and MSM functioned independently on MDA or there was positive or negative interaction between them, resulting in synergy or antagonism.

The percent change in MDA computed for each volunteer after incubation with EDTA (x), MSM (y) or their combination (z) are presented in Table 4. Values after 30 min of incubation were selected because, as seen from Figure 1b, maximum effect was observed at 30 min.

A positive value of change in MDA indicates reduction in ability to mitigate OS, a negative value indicates synergy and a near-zero value indicates an independent function and no impact of combining the two compounds. The difference between expected additive effect (x+y, that is 16.7 + 30.0=46.8) and actual effect (z, that is, 38.5) of the combination was found to be positive and statistically significant at p=0.016, indicating a small negative interaction of 8.2 percent between EDTA and MSM.

The results obtained indicated that the lipid peroxidation induced in erythrocyte membrane by treatment with hydrogen peroxide could be reduced by treatment with the sulphur contain-

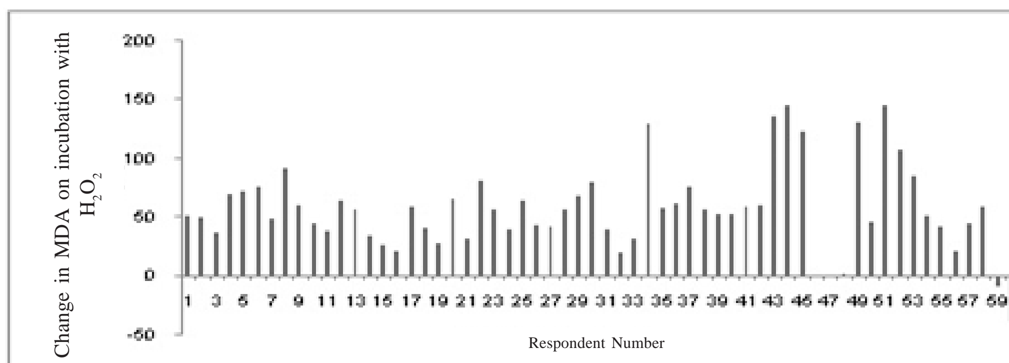


**Fig. 2(a).**

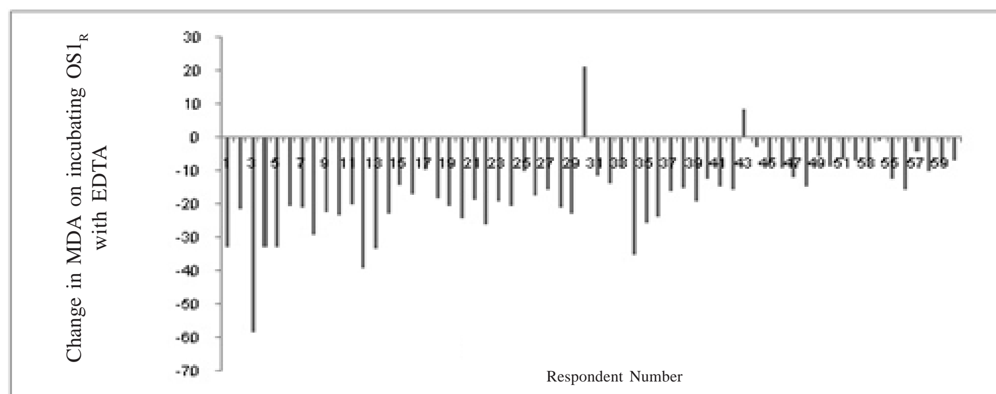


**Fig. 2(b).**

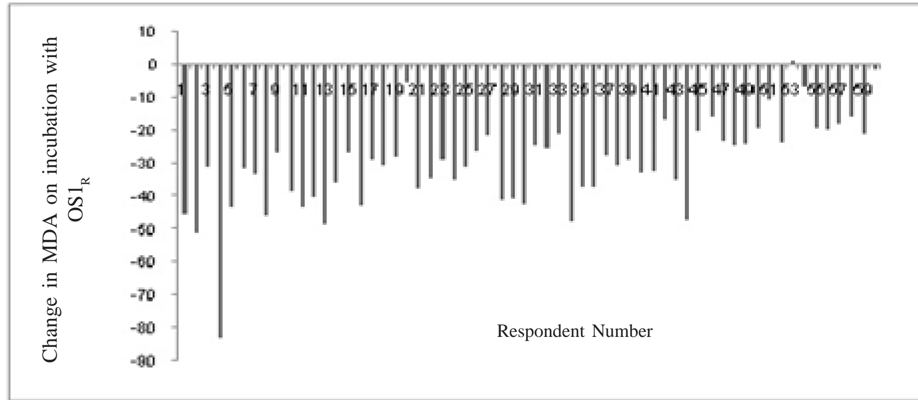


Fig. 2(c).

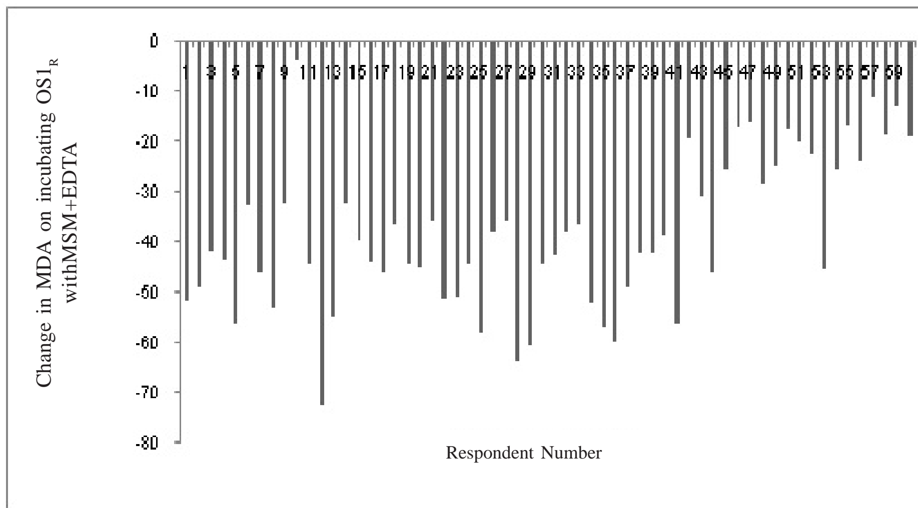


Fig. 2(d).

**Fig. 2. Change in MDA in RBCs of each respondent after 30 minutes of incubation of OS induced RBC with (a) $H_2O_2$, (b) EDTA, (c) MSM, (d) MSM+EDTA.**

**Table 4: Percent change in MDA after 30 min of incubation, to assess whether the effect of combining EDTA and MSM is additive, synergistic or antagonistic**

*Percent change in MDA after 30 min of incubation of $OSI_R$*

| *With EDTA (x)* | *With MSM (y)* | *Expected additive effect (x+y)* | *Actual effect of MSM+EDTA (z)* | *Difference between expected and actual effects z-(x+y)* |
|---|---|---|---|---|
| -16.72±1.51 | -30.0±1.8 | -46.8±2.8 | -38.5±1.9 | +8.2±2.3 |

Values are x ± μ. n = 60 volunteers

ing antioxidant, MSM as well as by the metal chelator EDTA. However, maximal antioxidant effect was obtained by combining EDTA and MSM.

## DISCUSSION

The most important function of erythrocytes (RBCs) is to carry oxygen, but they are also in-

volved in inflammatory processes. They are extremely deformable and elastic, because they are designed to withstand exposure to shear forces as they travel through the vascular system. In inflammatory conditions, and in the presence of hydroxyl radicals, they lose their discoid shape (Pretorius 2013). In the present study, inflammation was induced by incubating RBCs with hydrogen peroxide, which reacts with free or poorly liganded Fe (II) and converts it to Fe (III) and highly reactive hydroxyl radicals are produced. Lipid peroxidation is one of the major consequences of OS in the RBC, and is indexed by a number of toxic end products such as MDA (Malondialdehyde), conjugated dienes, isoprostanes, 4 hydroxynonetal (4HNE). MDA is one of the most commonly measured secondary end product of lipid peroxidation and widely used as a marker of cell membrane damage (Esterbauer et al. 1991). As expected, this was found to increase when RBC of normal, non-diseased volunteers were incubated with hydrogen peroxide. This has been reported by several investigators, and hydrogen peroxide has been used in experimental conditions to induce OS in normal RBC (Ebrahimzadeh et al. 2010). These inflamed RBC were incubated with MSM or EDTA or both to assess whether such oxidative stress can be reversed. The hypothesis was that the chelator, EDTA would remove the unliganded iron and reverse the inflamed condition in the RBC, and MSM would reduce MDA through its antioxidant function. Since their combination could result in an additive effect, or negative effect or synergistic effect, the effect of combining the two compounds on MDA was also explored.

All the three treatments, EDTA alone, MSM alone and their combination, helped in reducing MDA in the OS-induced erythrocyte membrane of all non-diseased human volunteers in the order MSM+EDTA > MSM > EDTA. The effect was maximum after 30 minutes of incubation. At this point, the MDA had been ameliorated by 16.7 percent by EDTA alone and thirty percent with MSM alone.

EDTA alone has been reported to reduce MDA in plasma while the anticoagulant citrate does not function to the same extent, indicating the efficacy of EDTA as an antioxidant (Suttnar et al. 2001). The antioxidant function of EDTA is corroborated by the present study, although its effect on the erythrocyte membrane does not seem to have been evaluated earlier.

The efficacy of using oral MSM alone in reducing oxidative stress has been demonstrated in several studies. Administration of MSM for 10 days decreased muscle damage and increased total antioxidant capacity of blood after exercise in healthy young volunteers (Barmaki et al. 2012), a single dose of MSM decreased markers of plasma antioxidant system, serum Malondialdehyde (MDA), uric acid, bilirubin, protein carbonyl (PC) and plasma vitamin E levels and increased total antioxidant capacity (TAC) (Nakhostin et al. 2013). This corroborates the present findings, although previous studies have not evaluated the impact on the RBC. The maximum reduction in oxidative stress marker MDA with MSM+EDTA confirms the advantage of using chelators along with antioxidant for ameliorating oxidative stress, as has been suggested and reviewed by (Kell 2009, 2010). Studies employing other antioxidant-chelator combinations have also reported beneficial effects on OS amelioration. The chelator defiperone is used to reduce iron toxicity in thalassemia. In a study on experimental rats (Sripetchwandee et al. 2014) iron toxicity was produced which induced brain iron accumulation, brain mitochondrial dysfunction, impaired brain synaptic plasticity and cognition, blood-brain-barrier breakdown, and brain apoptosis. Impact of oral feeding of chelator deferiprone or antioxidant N-acetyl cysteine, or both together were evaluated and it was reported that the combination worked best to attenuate the deleterious effects. In another study on thalassemic mice, experimentally induced iron overload resulted in impaired left ventricular (LV) function and heart rate variability (HRV) and increased apoptosis, and cardiac iron accumulation. Again, the same combination of chelator defiperone and antioxidant, N-acetyl cysteine were reported to decrease oxidative stress and thereby exert the greatest cardioprotective efficacy when compared with either of the monotherapies (Kumfu et al. 2017). However, no studies have been found which test the efficacy of chelator-antioxidant combinations on humans, especially employing the systemic impact, likely to be felt on erythrocytes. The present study has selected EDTA and MSM because both are GRAS compounds which have FDA approval for human ingestion and the in vitro testing of these on erythrocytes is expected to help in evaluation of their suitability for internal consumption.

An additional role for MSM as a permeability enhancer, in addition to its antioxidant func-

tion has been indicated in recent studies. Zhang et al. (2009) have reported, in an *in vitro* study, the ability of MSM to deliver EDTA into the eye, suggesting the role of MSM as an adjuvant, and also found that it could also help deliver ciprofloxacin and other chemical compounds to specific, local tissue sites. The MSM+EDTA combination has also been shown to ameliorate oxidative stress-associated neurodegeneration in rat eyes with elevated intraocular pressure (Liu et al. 2014), in reducing thermal injury in a rat model (Wang et al. 2015), in ameliorating oxidation-induced toxicity in diabetic cataract (Zhang et al. 2011) in endotoxin-induced uveitis (Ansari et al. 2007) and against dental plaque (Dadkhah et al. 2014). The researchers' have has reported the efficacy of the MSM + EDTA combination in lotion form in reducing the swelling observed in chronic venous insufficiency, which is accompanied by a reduction in some oxidative stress markers (Tripathi et al. 2011). Systemic effects of any compound depend on their presence in circulation. Therefore, it is important to assess whether the presence of MSM and EDTA, individually and in combination, as chelator, antioxidant and permeability enhancer have an impact on the erythrocyte in blood. Since the erythrocyte is highly susceptible to damages resulting from oxidative stress, this question assumes greater significance. The present study appears to be the first attempt to assess the roles of these compounds on OS in the erythrocyte membrane. Individually, both EDTA and MSM, were found to be effective in reducing OS, but MSM had a more pronounced effect. The combination worked best, but the combined effect was marginally less than the addition of the individual effects, hence, no evidence was found to suggest that MSM helps in transporting EDTA across the erythrocyte membrane. However, the combination worked better than the individual compounds, indicating independent mechanisms of their physiological function.

Further studies are required to understand the mechanisms of action of these compounds, by assessing the modulation of various antioxidant enzymes and total antioxidant capacity in the erythrocyte membrane and comparisons with the phenomena in plasma. The present study is based on a model of normal erythrocytes from non-diseased healthy volunteers and oxidative stress has been artificially induced. Since oxidative stress is known to be high in certain diseased conditions such as diabetes, obesity and other indicators of metabolic syndrome, and has been reported to modify several biochemical features of the erythrocyte membrane, further studies are also desirable to assess the impact of MSM and EDTA in diseased erythrocytes.

## CONCLUSION

In conclusion, the present study found that lipid peroxidation induced in erythrocyte membrane by treatment with hydrogen peroxide could be reduced by treatment with the sulphur containing antioxidant MSM, and to a lesser extent, by the metal chelator EDTA, and their combination produced an enhanced effect on reduction in MDA, which was marginally less than the additive effects of EDTA and MSM. Synergy was not observed. The result highlighted the independent role of chelator and antioxidant in amelioration of OS, endorsing the utility of combining antioxidant with chelator.

## RECOMMENDATIONS

It is recommended that more work is undertaken to explore the utility of various combinations of chelators with antioxidants in management of disease. Most studies dealing with OS amelioration are based on animal models which provide more homogeneous and controllable variables, and there is paucity of studies on humans. More such case-control studies are required with GRAS compounds to assess their utility on human health.

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