Quantitative evaluation of EDTA and ethanol for their ability to afford protection against ^{o}OH and ROS generating chemical system: DNA strand breaks in pMTa4 plasmid DNA *in vitro*

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Abstract:

It is known that majority of cellular H_2O_2 is involved in reducing transition metals to generate °OH via classical Fenton or Haber-Weiss reaction inflicting strand break type of damage to DNA. In this report the effects of a metal chelator, EDTA, and a free radical scavenger, ethanol, were studied to assess the protective ability of the modulators on plasmid DNA strand breaks induced by Fenton's oxidants and Haber-Weiss oxidants. Both EDTA and ethanol reduced single strand breaks (SSB) and abolished the induction of double strand breaks (DSB), which implicates that these agents protected plasmid DNA strand breaks *in vitro*. However, ethanol afforded significantly better protection than EDTA. The report discuses implication of this in human radio-protection program.

Key words: Plasmid pMTa4 DNA, Fenton oxidant, Haber-Weiss oxidant, Hydroxyl radicals (°OH), Hydrogen peroxide (H_2O_2), Chelator, Free radical scavengers.

Introduction:

Oxidative damage to DNA by reactive oxygen species (ROS) and free radicals (FR) are considered to be the major causes of induction of metabolic degenerative processes including mutagenesis, carcinogenesis and senescence [1, 2]. The highly reactive °OH produces a number of damages on the DNA, notably oxidation of bases and sugars, single strand breaks (SSB) and double strand breaks (DSB) [2]. Therefore, containment of the cellular load of ROS and FR is likely to slow down the degenerative processes leading to healthy living. In principle, this may be achievable by the two approaches. In the first, enzyme driven metabolic reactions generating ROS and FR may be inhibited by use of metal chelators, which complex with metal co-factors and inhibit the chemical reaction [3]. Iron (Fe^{2+}) and copper (Cu^{2+}) are two transition metals of biological relevance. The free metal ions of both of these readily participate in the degenerative chemical reactions mediated by co-metabolite product, H_2O_2 and produce highly reactive °OH [4, 5]. In the second approach, free radical scavengers (FRS) or antioxidants may be used. FRS are chemicals that have the ability to neutralize FR by donating one of their own electrons. Ethanol at low concentrations is metabolized very efficiently by low K_m alcohol dehydrogenase to acetaldehyde and then by aldehyde dehydrogenase to acetate, producing NADH in both

reactions. NADH is a key component of the electron transport chain and, due to its high reducing potential functions to promote regeneration of endogenous vitamin radicals back to their reduced form. This ability of ethanol at low concentrations to create a strong reducing environment, makes it an attractive free radical scavenger [6].

Using a plasmid DNA model (pMTa4) we have earlier reported the strand breaking abilities of γ-radiation as well as two FR generating reactants, namely the Fenton and the Haber-Weiss oxidants *in vitro* [7]. We measured qualitatively different DNA strand breaking abilities of the Fenton and Haber-Weiss oxidants on one hand and γ-rays on the other, either separately or in combination. Among the three interventions, the Fe^{2+} mediated Fenton oxidant was found to be most efficient DNA damaging genotoxin. The damage inflicted upon DNA via Fenton or Haber-Weiss oxidants and that by ionizing radiation were similar but not identical. We also reported that at least under *in vitro* condition, which is devoid of any biological repair pathway, the secondary or the final damaging entity decided the quantum and quality of inflicted damage on DNA [7]. As strand breaks can potentially lead to large mutations, it is of biological and medical significance to understand how to contain the oxidant mediated ROS and FR induced damage onto DNA. This investigation was designed to evaluate the efficiency of a common metal chelator and a FRS in reducing the ROS and FR load created by oxidants *in vitro*. Extending our earlier work, we have used ethylenediaminetertaacetic acid (EDTA) as a metal chelator and ethanol as a FRS in this investigation, and report the result of EDTA and ethanol mediated protection to Fenton and Haber-Weiss oxidants induced strand breaks in pMTa4 DNA *in vitro*.

Methods and materials

Chemicals:

Analytical grade $CuSo_4$ (S.D. fine - chem. Ltd), $FeCl_2$ (Fluka), EDTA and H_2O_2 (Qualigens) were used in the investigation without further purification. Other chemicals used in the investigation were of highest purity grade. All solutions were made in sterile ultra pure (Milli Q) water.

Plasmid DNA isolation:

92 The plasmid, pMTa4 (6173 bp), used in this investigation has been described earlier [7, 8, 9, 10].
93 The pMTa4 was propagated in and routinely purified from *Escherichia coli* K12 strain AB1157
94 (wild type) growing on LB medium supplemented with 100 µg ml^{-1} Ampicillin under standard
95 conditions. As detailed earlier [7] the isolation was performed by a modified alkaline lysis
96 method in which EDTA was not used at any stage. The isolated pMTa4 was dissolved in sterile
97 ultrapure water (Milli-Q) and stored refrigerated.

98

99 *Exposure of pMTa4 to Fe^{2+} mediated Fenton oxidant in the presence of EDTA or ethanol*

100 The plasmid aqueous solution (5 µg) was mixed with Fenton oxidant (88 mM H_2O_2 and 20 µM
101 $FeCl_2$) in the presence of either 10 mM EDTA in 10 µM Tris-Cl buffer (pH 7) or 400 mM
102 ethanol in a final volume of 25 µl. The mixture was incubated at 37˚C for 10, 20, 30, 40, 50 & 60
103 min. The reaction was stopped by addition of 2 µl of 10 mM EDTA to the reactants and pMTa4
104 was analyzed by Agarose gel electrophoresis (AGE).

105

106 *Exposure of pMTa4 to Cu^{2+} mediated Haber-Weiss oxidant in the presence of EDTA or ethanol*

107 The plasmid aqueous solution (5 µg) was mixed with Haber-Weiss oxidant (2.8 mM H_2O_2 and
108 25 µM $CuSO_4$) and 10 mM EDTA in 10 µM Tris-Cl buffer (pH 7) or 400 mM ethanol in a final
109 volume of 25 µl. The mixture was incubated at 37˚C for 30, 60, 90 &120 min. The reaction was
110 stopped by addition of 2 µl of 10 mM EDTA to the reactants and pMTa4 was analyzed by AGE.

111

112 *Agarose gel electrophoresis (AGE)*

113 The pMTa4 samples were analyzed by 1% AGE using Tris-Acetate-EDTA buffer system as
114 described earlier [7, 8]. The gels were stained with 0.3 µg ml^{-1} Ethidium bromide and visualized
115 over a UV-transilluminator (BioRad).

116

117 *Analysis*
118 The gel images were digitized using a digital camera (Kodak) and the net pixel density of three
119 topological forms of the plasmid was calculated using KDS1D Image Analysis (Kodak)
120 software. Each experiment had at least three independent replicates. The mean±SD was
121 calculated from each of the three independent experiment and plots were made in the form of bar
122 diagrams.

123

Results:

Effect of EDTA or ethanol on Fenton oxidant induced strand breaks on pMTa4 DNA

The AGE of the control and pMTa4 DNA samples exposed to Fenton oxidant in the presence of 10 mM EDTA (Fig. 1A) and 400 mM ethanol (Fig. 2A) showed complete absence of L form and degradation of the plasmid DNA, which were prominent in the absence of EDTA or ethanol [7]. However, a persistent dose dependent induction of OC from of pMTa4 DNA by Fenton oxidant was observed prominently in the presence of EDTA only (Fig. 1). It was virtually absent in the presence of ethanol (Fig. 2). The quantum of induced SSB in the presence of EDTA, as shown by increase in pixel density of the OC from, recorded a time dependent increase reaching a maximum of about 85% of the control after 60 min exposure to Fenton oxidant (Fig. 1B). On the other hand, it was almost invariant or variations were non- significant in case of ethanol (Fig. 2B).

Effect of EDTA or ethanol on Haber-Weiss oxidants induced strand breaks on pMTa4 DNA

The AGE of the control and pMTa4 DNA samples exposed to Haber-Weiss oxidant in the presence of 10 mM EDTA (Fig. 3A) and 400 mM ethanol (Fig. 4A) also showed complete absence of L form and degradation of the plasmid DNA, which were otherwise prominent in the absence of EDTA or ethanol [7]. However, a persistent but weaker dose dependent induction of OC from of pMTa4 DNA by Haber-Weiss oxidant was observed prominently in the presence of EDTA only (Fig. 3A), which was virtually absent in the presence of ethanol (Fig. 4A). The quantum of induced SSB in the presence of EDTA, as shown by increase in pixel density of the OC from, recorded a time dependent increase reaching a maximum of about 40% of the control after 60 min exposure to Haber-Weiss oxidant (Fig. 3B). On the other hand, it was almost invariant in case of ethanol (Fig. 4B).

Discussion:

The pMTa4 DNA has been a convenient tool for monitoring strand break type of damage on DNA as its native CC form get converted to OC or L forms depending on induction of SSB or DSB, respectively, onto the plasmid DNA [7, 8, 9, 10]. Coincidentally, these three topological

forms of the plasmid also migrate differently on Agarose gel, thereby, making it possible to conveniently and quantitatively monitor the induction of SSB and/or DSB on DNA [3, 4, 10]. We have been using this model for gaining insight into the effects of FR generating radiomimetic chemicals [7] as well as UV [8], γ-rays [7, 9] and swift charged particles [10] on DNA. The investigation has also exploited these special characteristics of pMTa4 DNA molecule to elucidate and understand the inhibition of oxidative damage by the metal chelator, EDTA, and antioxidant or FRS, ethanol, on strand breaks induced by Fenton and Haber-Weiss oxidants *in vitro*.

EDTA is a novel molecule for complexing metal ions. It contains four carboxylic acid groups and two amine groups with lone pair electrons. The fully deprotonated form of EDTA binds to metal ions in the ratio of 1:1. It forms complexes with Fe^{2+} and Cu^{2+} and, hence, this chelating property has been employed to inhibit the production of ROS and FR via Fenton reactions in this study [3, 4, 5]. Ethanol is an oxidant with low scavenging reaction rates. Ethanol scavenging action is exerted via the production of hydroxyethyl radicals giving rise to acetaldehyde.

We have earlier shown that the Fenton and Haber-Weiss oxidants as well as γ-rays induced qualitatively and quantitatively different patterns of strand breaks on the pMTa4 DNA [7]. The efficiency of induction of strand break by the Fe^{2+} mediated Fenton oxidant was found to be the highest [7]. Our results presented in this report show that presence of EDTA or ethanol completely abolished induction of DSB by both ROS and FR generating Fenton (Figs. 1 & 2) and Haber-Weiss (Figs. 3 & 4) oxidants in sharp contrast to their reported effects in their absence [7]. Even though the induction of SSB by Fenton or Haber-Weiss oxidants continued prominently in the presence of EDTA (Figs. 1 and 3) and weakly in the presence of ethanol (Figs. 2 & 4), the overall magnitude was noticeably low. From the results it is apparent that protection of pMTa4 DNA from ROS and FR induced strand break type of damage to DNA was significantly high in case of 400 mM ethanol (Figs. 2 & 4) as compared to 10 mM EDTA (Figs. 1 & 3). We have chosen the concentration for EDTA as 10 mM and ethanol as 400 mM as in our experimental conditions these were the smallest concentrations, which inhibited DSB to a measurable level. Moreover, since EDTA tends to be toxic at a higher molarity, effort was to keep the concentration of EDTA at the lowest possible [3].

Both the interventions reduce the ROS and FR loads differently. EDTA, a metal scavenger, does it by preventing classical Fenton-type reaction and ethanol, a FRS, by scavenging generated FR from the system. In the *in vitro* setup used in this investigation, EDTA must have interfered with ROS and FR generation by complexing with Fe^{2+} or Cu^{2+} in case of Fenton and Haber-Weiss oxidants, respectively. Therefore, no free Fe^{2+} or Cu^{2+} was available for interactions with H_2O_2 to

195 generate ROS and FR. Thus, ROS and FR mediated damage to DNA was prevented. However,
196 this would also leave reactive H_2O_2 free in the system to interact with pMTa4 DNA directly
197 leading to time dependent SSB induction, which we observe in Figs. 1 & 3. In case of ethanol the
198 situation was different. Presence of ethanol did not prevent Fe^{2+} or Cu^{2+} interaction with H_2O_2.
199 Therefore, ROS and FR were generated via the classical Fenton-type reactions, which would
200 progressively consume the reactive H_2O_2. As ethanol is an FRS, it would efficiently scavenge the
201 generated FR. Consequently, neither FR nor the reactive H_2O_2 would be left to inflict damage
202 onto pMTa4 DNA. This may be the reason why 400 mM ethanol virtually prevented induction of
203 SSB by either Fenton (Fig. 2) or Haber-weiss (Fig. 4) oxidants. This makes ethanol, a FRS, more
204 superior protector of DNA damage than EDTA, a metal chelator, *in vitro*. Alcohol administration
205 has been reported to decrease chromosomal aberrations (dicentrics) in spleen cells by γ-radiation
206 [11]. This protection was thought to be mediated via °OH radical scavenging as 70% of γ-ray
207 induced damage is via the indirect effect and through the formation of °OH. Alcohol or FRS
208 mediated protection was also reported in other prokaryotic and eukaryotic cells [12, 13, 14, 15].

209

210 Thus, our results show that there were qualitative and quantitative reduction in the induction of
211 FR induced strand breaks on pMTa4 due to the presence of the metal chelator, EDTA, as well as
212 the FRS, ethanol. Results further show that ethanol afforded better protection in comparison to
213 EDTA suggesting that use of FRS (alcohol) may be a better approach for human radio-protection
214 program than metal chelation (EDTA).

215

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219 **References:**

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Figure legends:

Figure 1: Effect of EDTA on Fenton oxidant induced damage to pMTa4 DNA: Electropherogram of pMTa4 exposed to Fenton oxidant in the presence of 10 mM EDTA *in vitro* for increasing exposure periods (panel A). The bands marked CC (closed circle) and OC (open circle) represent the native and SSB forms of plasmid, respectively. Quantitative bar diagram with closed and open bars represent the CC (native) and OC (SSB) forms of pMTa4, respectively (panel B).

Figure 2: Effect of ethanol on Fenton oxidant induced damage to pMTa4 DNA: Electropherogram of pMTa4 exposed to Fenton oxidant in the presence of 400 mM ethanol *in vitro* for increasing exposure periods (panel A). The bands marked CC (closed circle) and OC (open circle) represent the native and SSB forms of plasmid, respectively. Quantitative bar diagram with closed and open bars represent the CC (native) and OC (SSB) forms of pMTa4, respectively (panel B).

Figure 3: Effect of EDTA on Haber-Weiss oxidant induced damage to pMTa4 DNA: Electropherogram of pMTa4 exposed to Heber-Weiss oxidant in the presence of 10 mM EDTA *in vitro* for increasing exposure periods (panel A). The bands marked CC (closed circle) and OC (open circle) represent the native and SSB forms of plasmid, respectively. Quantitative bar diagram with closed and open bars represent the CC (native) and OC (SSB) forms of pMTa4, respectively (panel B).

Figure 4: Effect of ethanol on Haber-Weiss oxidant induced damage to pMTa4 DNA: Electropherogram of pMTa4 exposed to Haber-Weiss oxidant in the presence of 400 mM ethanol *in vitro* for increasing exposure periods (panel A). The bands marked CC (closed circle) and OC (open circle) represent the native and SSB forms of plasmid, respectively. Quantitative bar diagram with closed and open bars represent the CC (native) and OC (SSB) forms of pMTa4, respectively (panel B).

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292 **Figure 1**

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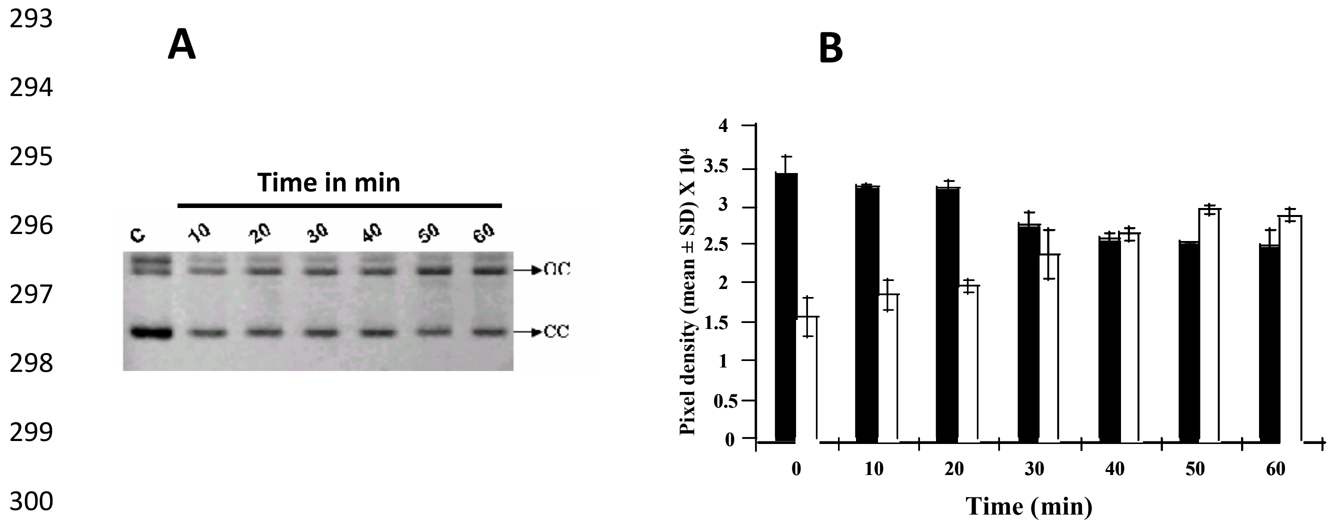

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303 **Figure 2**

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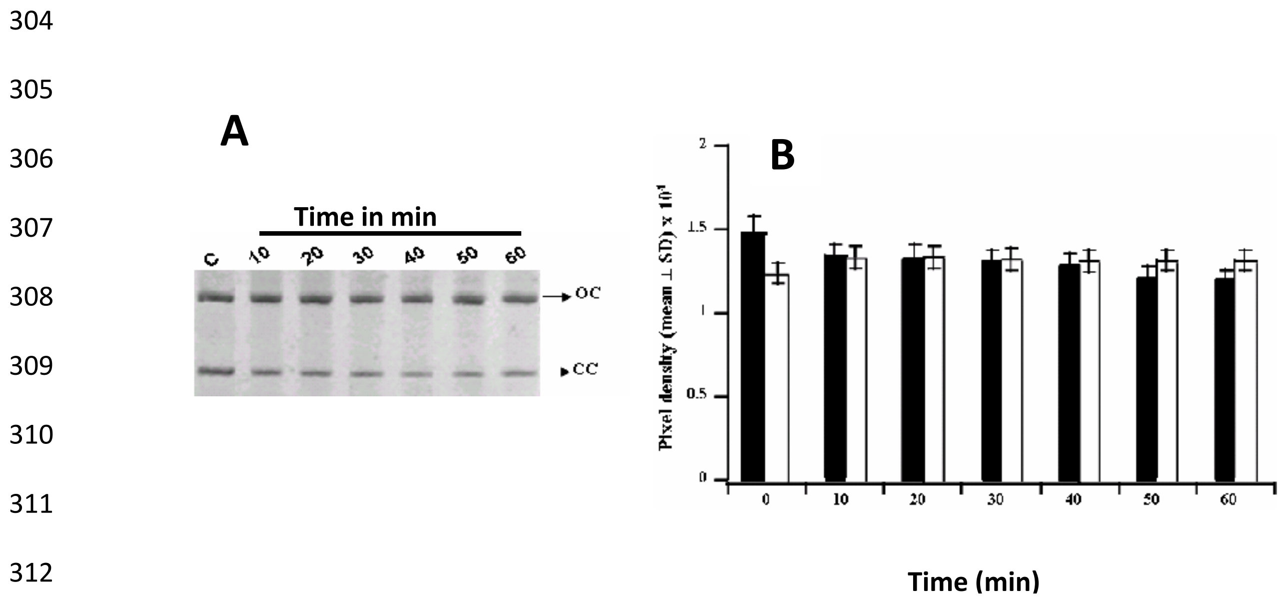

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317 **Figure 3**

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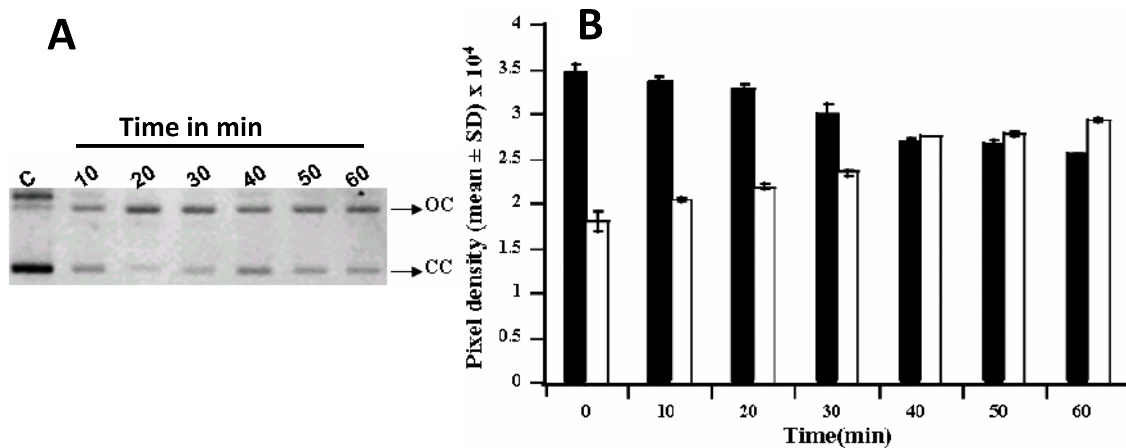

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328 **Figure 4**

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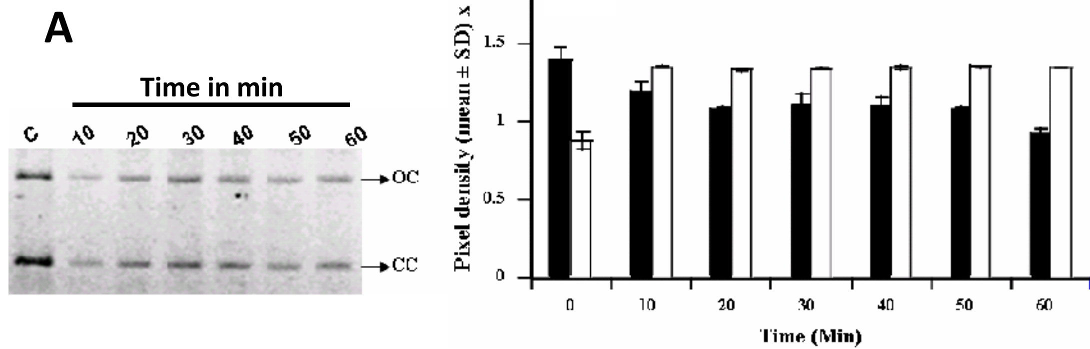

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