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The Role of EDTA in Malonaldehyde Formation from DNA Oxidized by Fenton Reagent Systems

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Calf thymus DNA was oxidized by various Fenton reagent systems [Fe(II)/H_2O_2] with or without ethylenediamine tetraacetic acid (EDTA) under different reaction conditions. Calf DNA was also oxidized by a modified Fenton reagent (Fe(III)/H_2O_2/ascorbic acid) with EDTA. Malonaldehyde (MA) formed from DNA was derivatized into 1-methyl hydrazine, which was subsequently analyzed by gas chromatography with a nitrogen−phosphorus detector. MA formation increased linearly with an increase of Fe(II) concentration. MA formation reached a plateau at nearly 2 mmol/L of Fe(II) with 0.5 mmol/L of H_2O_2. Addition of EDTA increased MA formation from DNA nearly 5 times. When DNA was oxidized with various amount of ethanol, MA formation decreased with an increase of ethanol concentration, either with or without EDTA. The rate of inhibition was greater without EDTA than with EDTA. When DNA was oxidized by a modified Fenton reagent, MA formation linearly increased with the increase of DNA. Ascorbic acid alone produced some MA upon oxidation.

KEYWORDS: Ascorbic acid; DNA; EDTA; Fenton reagent; malonaldehyde; oxidative damage

INTRODUCTION

In living systems, DNA is one of the target sites of reactive oxygen species, particularly the hydroxyl radical (•OH). Reactive oxygen species cause various types of damage to DNA, including breaking single- and double-strands, releasing free DNA bases, chemical changes in bases, and modification of sugar moieties (*1*−*4*). Damage to DNA promotes a series of pathological events, such as cancer and aging, (*3*, *5*, *6*).

Determination of oxidized products from DNA would provide theoretical and practical information on the mechanisms of DNA oxidation following DNA damage. Furthermore, this information could help to develop a way to prevent DNA damage caused by oxidation.

There have been many reports on investigations of DNA oxidized by a Fenton reagent or a modified Fenton reagent, which produces •OH readily (*7*). Malonaldehyde (MA), which is formed from various substrates such as deoxyribose (*8*), fatty acids (*9*), and blood plasma (*10*), has been widely and commonly used as a marker of oxidation. There is a report of the oxidation of DNA monitored by measuring MA (*3*). The most commonly used method for MA analysis is the thiobarbituric acid (TBA) assay, in which MA forms a pink complex with TBA. This complex is subsequently measured by a colorimeter. TBA reacts not only with MA but also with many other carbonyl compounds produced by oxidation. Therefore, it is not specific to MA, and the product is commonly called thiobarbituric acid reactive substance (TBARS). Moreover, this method requires high acidity (< pH 2) and elevated temperature (95 °C), which may cause alteration of the sample. Consequently, the amount of MA determined with this method is always overestimated (*11*). A highly specific and sensitive method for MA analysis was developed and applied for investigation of lipid peroxidation induced by Fenton reagent (*10*, *12*, *13*). This method involves derivatization of MA with *N*-methyl hydrazine to form stable 1-methyl pyrazole (1-MP). The reaction occurs at room temperature and neutral pH, and the resulting 1-MP is very stable and can be readily analyzed by a gas chromatograph with a nitrogen phosphorus detector (GC/NPD).

In the present study, DNA was oxidized by various Fenton reaction systems with or without EDTA, and subsequently, the MA formed was analyzed to investigate the role of EDTA in the Fenton reaction system.

MATERIALS AND METHODS

Chemicals and Materials. Calf thymus DNA, $FeCl_2$, $FeCl_3$, H_2O_2, EDTA, *N*-methylhydrazine, 2-methylpyrazole, thiourea, ethanol, and Chelex-100 were obtained from Sigma-Aldrich (St. Louis, MO). Authentic 1-MP was synthesized according to a method previously reported (*14*). All other analytical grade chemicals, reagents, and solvents were obtained from reliable sources.

A stock solution of DNA was prepared by dissolving 1 mg of DNA into 1 mL of phosphate buffer (pH 7.4). All reagents used in the oxidative reactions were prepared in Chelex-treated water. DNA, phosphate buffer, and H_2O_2 solutions were also treated with Chelex. $FeCl_2$ or $FeCl_3$ was used immediately after the preparation. Ethanol was diluted to appropriate concentrations with phosphate buffer (pH 7.4).

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Sample Preparation of Oxidized DNA. The oxidized DNA samples were prepared as follows:

I. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.5 mg/mL of DNA, 3 mmol/L of H_2O_2, and 0, 0.1, 0.5, 1.0, 2.0, or 3.0 mmol/L of $FeCl_2$ was incubated at 37 °C for 30 min.

II. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.5 mg/mL of DNA, 1 mmol/L of $FeCl_2$, and 0, 0.1, 0.25, 0.5, 0.75, 1.0, 3.0, or 5.0 mmol/L of H_2O_2 was incubated with 1.05 mmol/L of EDTA at 37 °C for 30 min. Also, a 5-mL aqueous phosphate buffer solution (0.02 mmol/mL) containing 0.5 mg/mL of DNA, 1 mmol/L of $FeCl_2$, and 0, 0.5, 1.0, 3.0, or 5.0 mmol/L of H_2O_2 was incubated without EDTA at 37 °C for 30 min. The $FeCl_2$/EDTA solution was premixed just before addition to the sample solutions.

III. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.5 mg/mL of DNA, 1 mmol/L of $FeCl_2$, and 0.5 mmol/L of H_2O_2 was incubated with or without 1.05 mmol/L of EDTA at 37 °C for 0, 15, 30, 60, 90, or 120 min.

IV. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.5 mg/mL of DNA, 1 mmol/L of $FeCl_2$, and 0.5 mmol/L of H_2O_2 was incubated with 0, 0.324, 0.648, 1.62, or 2.24 mmol/L of ethanol and with or without 1.05 mmol/L of EDTA at 37 °C for 30 min.

V. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.05 mmol/L of $FeCl_3$, 3 mmol/L of H_2O_2 and 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, or 0.6 mg/mL of DNA, was incubated with 3 mmol/L of ascorbic acid and 0.1 mmol/L of EDTA at 37 °C for 60 min.

VI. A 5-mL aqueous phosphate buffer solution (pH 7.4, 0.02 mmol/mL) containing 0.05 mmol/L of $FeCl_3$, 3 mmol/L of H_2O_2, and 0.5 mg/mL of DNA, was incubated with 0, 0.5, 1.0, 3.0, or 5.0 mmol/L of ascorbic acid and with 0.1 mmol/L of EDTA at 37 °C for 60 min.

All experiments were conducted in the dark to avoid photooxidation. Oxidation of the samples was stopped by adding a 2.5-mL aqueous solution containing 10 mmol/L of thiourea, 30 mmol/L of EDTA, and 0.6 mmol/L of sodium acetate, as previously reported (*15*).

Analysis of MA as 1-MP. MA formed in oxidized DNA was analyzed as previously reported (*10*). A 100-μL aliquot of *N*-Methylhydrazine solution (50%, v/v) was added to the above sample solutions. Immediately after the solutions were incubated at 37 °C for 30 min, they were placed into Bond Elut C18 cartridges (Varian, Inc., Harbor City, CA) connected to a vacuum manifold. After eluting the sample solutions, the cartridges were washed with 3 mL of deionized water twice, and then 1-MP was eluted with 3 mL ethyl acetate twice. The ethyl acetate solution was dried over anhydrous sodium sulfate for 30 min. After removal of sodium sulfate, the solvent was evaporated under a purified nitrogen stream. the volume of sample solution was brought up to 0.5 mL with ethyl acetate. A 20 μL aliquot of standard solution, 2-methylpyrazine (1 mg/mL), was added to the sample as an internal standard prior to GC analysis.

The resulting 1-MP was analyzed by a previously reported method (*10*, *12*, *13*). A Hewlett-Packard model 6890A GC equipped with an NPD and a 30-m × 0.25-mm i.d. ($d_f = 1$ μm) DB-Wax bonded-phased fused silica capillary column (J & W Scientific, Folsom, CA) was used. The detector and injector temperatures were 250 °C. The linear velocity of the helium carrier gas was 25 cm/sec with a split ratio of 8:1. The oven temperature was programmed from 60 to 130 °C at 3 °C/min and from 130 to 200 °C (5 min held) at 10 °C/min.

Recovery Efficiency Test on 1-MP from Bond Elut C18 Cartridge. Authentic 1-MP (8, 12, or 24 nmol/mL) was spiked into an aqueous solution (7.5 mL) containing 1 mmol/L of $FeCl_2$, 0.5 mmol/L of H_2O_2, and 0.1 mL of phosphate buffer. The solution was poured onto a SPE cartridge. 1-MP was recovered and analyzed by the same method as described above.

RESULTS AND DISCUSSION

Prior to the present study, 1-MP derived from MA was recovered using a liquid−liquid continuous extractor with dichloromethane (*9*, *10*, *13*). A liquid−liquid continuous extraction provides satisfactory recovery efficiency (82−91%) (*16*), but the process is somewhat tedious. Moreover, because a halogenated solvent—such as dichloromethane—damages the NPD, the solvent must be changed to a nonhalogenated one, such as ethyl acetate, before it is injected into GC. Therefore, SPE was used to recover 1-MP in the present study. The results of the recovery efficiency test of 1-MP were 49.8 ± 3.08% for 8 nmol/mL; 46.9 ± 2.9% for 12 nmol/mL; and 50.3 ± 2.64% for 24 nmol/mL. These values are mean ± standard deviation ($n = 6$). The recoveries were rather low. However, the values of standard deviations are significantly low, indicating that the reproducibility of recovery is satisfactory.

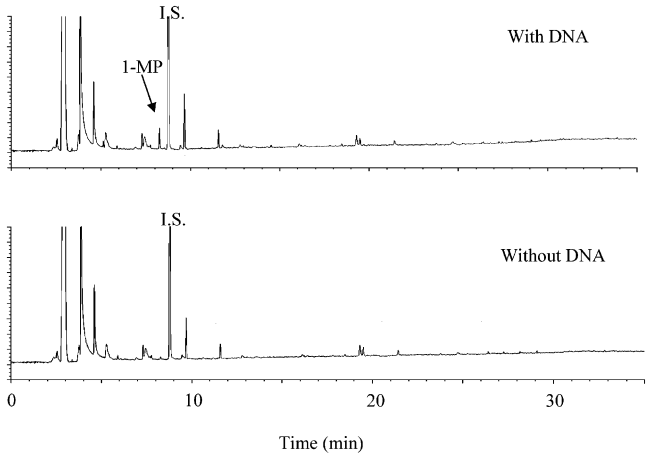

Figure 1. Typical gas chromatograms of extracts obtained from a Fenton reagent with or without DNA.

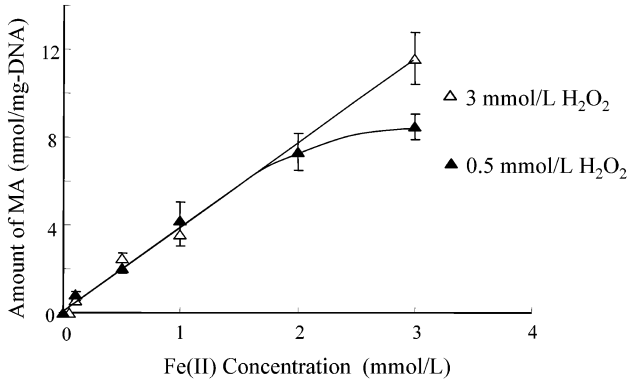

Figure 2. Effect of Fe(II) concentration on MA formation from DNA oxidized by Fenton reagent with two different concentrations of H_2O_2.

In the preliminary study, obvious formation of MA from a sample with DNA was observed but not from a sample without DNA. **Figure 1** shows typical gas chromatograms of the samples obtained with and without DNA.

Figure 2 shows the effect of Fe(II) concentration on MA formation from DNA treated with two different concentrations of H_2O_2 (refer to **I** for sample preparation). The values, which are adjusted with blank values, are mean ± standard deviation ($n = 3$). Formation of MA increased linearly with increase of Fe(II) concentration. Interestingly, the amount of MA formed reached a plateau at around 2 mmol/L of Fe(II) with 0.5 mmol/L H_2O_2. This may be due to complete consumption of 0.5 mmol/L H_2O_2 at 2 mmol/L Fe(II) concentration in the system.

Figure 3 shows the effect of H_2O_2 concentration on MA formation from DNA treated with 1 mmol/L Fe(II) and with or without EDTA (refer to II for sample preparation). MA formation reached the maximum at 0.5 mmol/L of H_2O_2 without EDTA. Addition of EDTA greatly accelerated MA formation. Fe(II) was reportedly consumed to produce several different

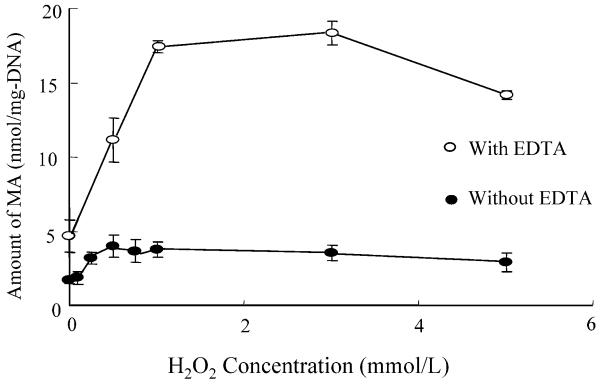

Figure 3. Effect of H_2O_2 concentration on MA formation from DNA Fenton reagent with 1 mmol/L Fe(II) and with (○) or without (●) EDTA.

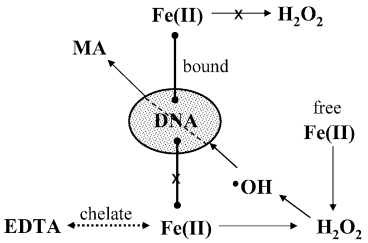

Figure 4. Hypothesized MA formation mechanisms from DNA with or without EDTA.

radicals from DNA (*1*, *17*). Therefore, one part of Fe(II) maybe reacted with DNA directly, and the remaining Fe(II) reacted with H_2O_2 to form a hydroxyl radical (•OH), which subsequently produced MA. **Figure 4** shows hypothesized MA formation mechanisms from DNA with or without EDTA. It could be hypothesized that Fe(II) binds to DNA and is involved in the production of "site-specific" radicals in the absence of EDTA, while Fe(II) trapped with EDTA does not bind to DNA but reacts with H_2O_2 stoichiometrically to produce a •OH, which contributes to the formation of MA (*18*−*21*). In the presence of EDTA, the formation of MA reached a maximum at 1 mmol/L H_2O_2 in the reaction containing 1 mmol/L Fe(II), suggesting that the reaction between Fe(II) and H_2O_2 occurred in 1:1 stoichiometry in the present study. EDTA is not found in in vivo systems. However, there are many low-molecular-weight biological complexing agents, such as ATP, ADP, and citrate as well as DNA, which form iron chelates in in vivo, and then act with the same mechanisms as EDTA (*22*). Even though these biological complexing agents are only about 10% as effective as iron-EDTA complexes, iron-EDTA complexes are normally used in in vitro studies to simulate in vivo conditions (*23*, *24*).

At least three different radical species (free •OH, bound •OH, and FeO^{2+} and/or Fe(IV)═O) were produced in the Fe(II)-EDTA/H_2O_2 system, and their formation depended on H_2O_2 concentration (*25*). Although it is suggested that these radicals were present in the Fenton reaction, there have been no reports on specific radical(s), which damage DNA. Therefore, the radical(s) which mainly contributes to biological damage is not clearly understood yet (*7*).

Figure 5 shows the effects of incubation time on the MA formation from DNA oxidized by Fenton reagent with or without EDTA (refer to III for sample preparation). The color of the sample solution changed from colorless to light yellow immediately after Fe(II) was added, suggesting that the reaction occurred immediately, and then the reaction was completed

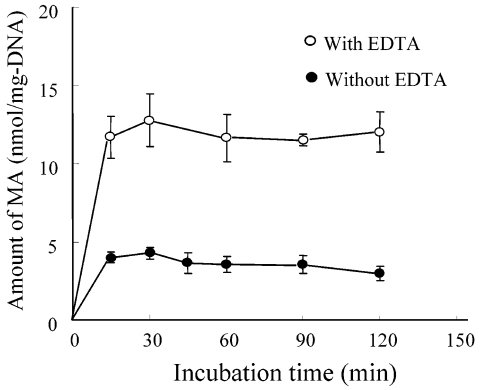

Figure 5. Effects of incubation time on the MA formation from DNA oxidized by Fenton reagent with (●) or without (○) EDTA.

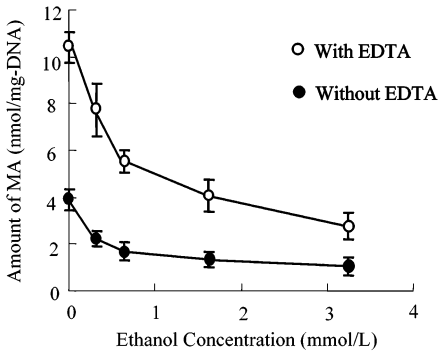

Figure 6. Effects of ethanol concentration on MA formation from DNA oxidized by Fenton reagent with (●) or without (○) EDTA.

within 15 min. The reaction responsible for the MA formation proceeds rapidly under the conditions used.

For further experiments to assess the effect of chemical(s) toward MA formation from DNA /Fenton system, the system consisting of a selected DNA (0.5 mg/mL), Fe(II) (1 mmol/L), and H_2O_2 (0.5 mmol/L) was incubated with a chemical of interest, with or without EDTA, at 37 °C for 30 min. These conditions gave the maximum MA formation in the preliminary experiments.

Figure 6 shows the effects of ethanol concentration on the MA formation from DNA under the above conditions (refer to **IV** for sample preparation). The MA formation decreased with increase of ethanol concentration either with or without EDTA. The inhibitory effect of ethanol on DNA oxidation by Fe(II)/H_2O_2 in the present study was similar to a previous report (*26*), in which inhibition of ethanol was assessed by measuring 8-oxo-dG (7,8-dihydro-8-oxo-2′-deoxyguanosine) formed from the reaction of 2′-deoxyguanosine or DNA with the Fenton reagents. Also, ethanol consumed H_2O_2 to form acetaldehyde, suggesting that ethanol inhibited DNA oxidation by reducing the amount of H_2O_2 in the Fenton reaction (*17*).

The effect of inhibition (%) and exponential ethanol concentration exhibited a linear relationship within the ethanol concentrations tested in both cases, with and without EDTA (**Figure 7**). The effect of inhibition was greater without EDTA than with EDTA, whereas the rate of inhibition (slope) was greater with EDTA than without EDTA. The results were somewhat inconsistent with one of the previous reports (*18*), in which the stimulatory effect of Fe(II) on lipid peroxidation was enhanced by EDTA when the concentration of Fe(II) in the reaction was greater than that of EDTA. It is difficult to determine the relationship of the above two results further because a nonspecific TBA method was used in the previous

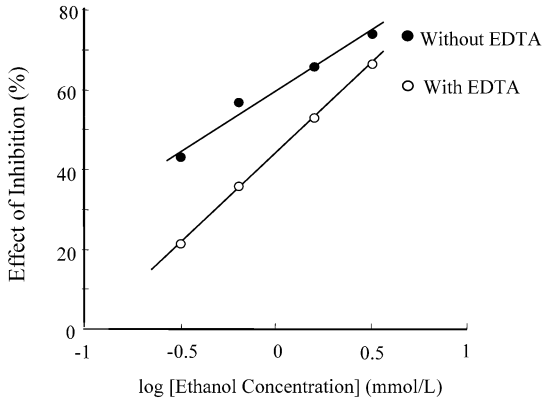

Figure 7. Rate of inhibition by ethanol plotted against log C. C = ethanol concentration.

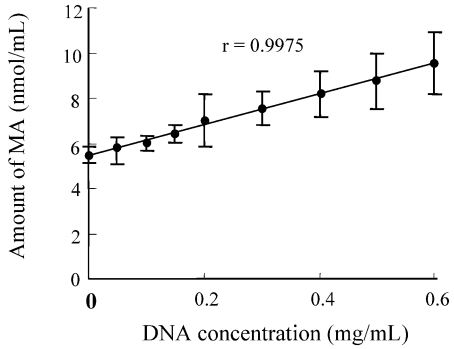

Figure 8. Amounts of MA formed from various concentrations of DNA in a modified Fenton reagent system.

report (*18*), whereas a method specific to MA was used in the present study.

The modified Fenton reagent (Fe(III)-EDTA/H_2O_2/ascorbic acid) has been preferably used to produce a hydroxyl radical for the following reasons: (1) EDTA prevents iron ions from binding to a substrate; (2) as a result, it accelerates hydroxyl radical formation at a low concentration; (3) auto-oxidation of Fe(II) to Fe(III) occurs quickly; (4) a reducing agent, such as ascorbic acid, recycles Fe(III) back to the active Fe(II) readily; and (5) ascorbic acid is present at a high level in mammalian cells (*8*, *18*, *19*, *24*−*31*). The overall reaction mechanisms of this system, which are modifications of Haber-Weiss reaction (*32*), are as follows:

$$\text{Ascorbic acid} + \text{Fe(III)-EDTA} \rightarrow \text{Oxidized ascorbic acid} + \text{Fe(II)-EDTA}$$

$$\text{Fe(II)-EDTA} + H_2O_2 \rightarrow \text{Fe(III)-EDTA} + OH^- + {}^{\bullet}OH$$

In the present study, MA formed from DNA oxidized by a (Fe(III)-EDTA/H_2O_2/ascorbic acid) system was measured to investigate a role of ascorbic acid in the modified Fenton reagent system.

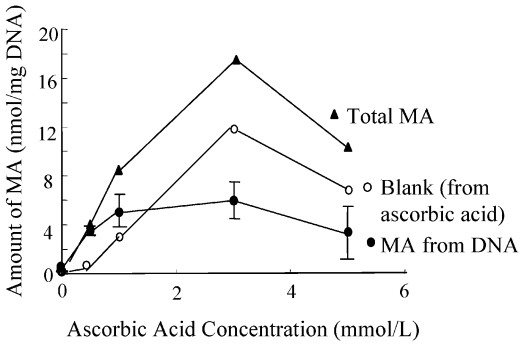

Figure 10. Amount of MA formed from DNA in a modified Fenton reagent with various amounts of ascorbic acid.

Figure 8 shows amounts of MA formed from various concentrations of DNA in a modified Fenton reagent system (refer to **V** for sample preparation). Formation of MA was linearly related to the amount of the DNA (r^2 = 0.9950). Formation of MA was observed in the sample without DNA (5.49 ± 0.36 nmol/mL), suggesting that MA was formed from ascorbic acid. Acetaldehyde was formed (122 nmol/10 μmol of ascorbic acid) from aqueous solutions of L-ascorbic acid treated with Fenton reagent. Formation of acetaldehyde and the amount of ascorbic acid used exhibited a linear relationship (r^2 = 0.978). **Figure 9** shows hypothesized mechanisms of MA formation from ascorbic acid in the Fenton reagent based on the acetaldehyde formation from ascorbic acid reported previously (*29*). It has been previously hypothesized that ascorbic acid is first oxidized to dehydroascorbic acid. Dehydroascorbic acid gives xylosone upon decarboxylation (*33*). The resulting xylosone would undergo dehydration and keto−enol reactions to form 2-hydroxy-1-keto-1,3-propanedienal, which would then break down into MA and glyoxal.

Figure 10 shows the amount of MA formed from 0.5 mmol/mL of DNA in a modified Fenton reagent with various amounts of ascorbic acid (refer to VI for sample preparation). The total amount of MA (MA from DNA + MA from ascorbic acid) found in the samples increased as the amount of ascorbic acid increased and reached a maximum value at 3 mmol/L of ascorbic acid. Formation of MA was suppressed at higher concentrations of ascorbic acid. As mentioned above, ascorbic acid itself produced MA, and amounts of MA formed in this system are estimated as blank values. Ascorbic acid promoted •OH formation as in the mechanism shown above. However, excess ascorbic acid began to behave as an antioxidant (*31*).

DNA damage caused by oxidation has been known to be associated with mutagenesis and carcinogenesis. Some chemicals, such as MA, produced from DNA upon oxidation can be an excellent marker for damage assessment. In vitro study, such as the present study, often provides important information, because direct measurement of chemicals in vivo does not always allow assessment of exposure, owing to rapid metabolism, sequestration into fatty tissues, or lack of suitable assay

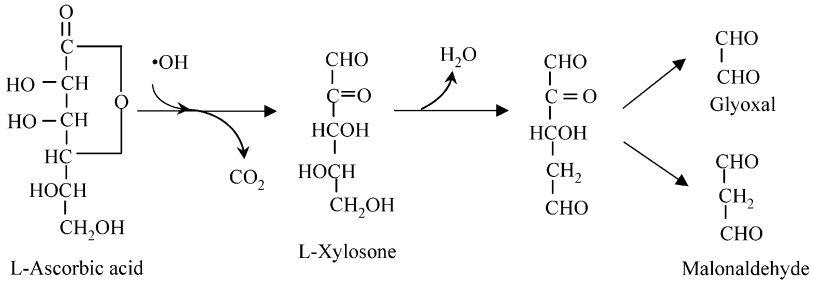

Figure 9. Hypothesized mechanisms of MA formation from ascorbic acid in the Fenton reagent.

methods. The results obtained in the present study show that EDTA plays an important role in inducing oxidation of DNA by Fenton reagents.

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