Bactericidal Effects of Oxidative Stress Generated by EDTA-Fe and Hydrogen Peroxide

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Hydrogen peroxide is widely used as a disinfectant by generating oxidative stress. The chelate of ethylenediaminetetraacetic acid (EDTA) and ferrous iron (EDTA-Fe) was used to increase oxidative stress and bactericidal effects. The Escherichia coli (E. coli) was added to a beef extract culture medium and treated with various doses of test reagents, including hydrogen peroxide, EDTA-Fe chelate, and antioxidants (vitamin C and vitamin E). All reagents were scanned individually or in combination to trace potential interference in optical density (OD) measurements and eliminate reagent-related interference. Medium supplemented with 13.79 mM hydrogen peroxide resulted in a large increase in the mortality of E. coli, and the highest disinfection efficiency for EDTA-Fe was observed at a neutral pH. The death of the cell of E. coli was significantly inhibited by the presence of catalase, but not vitamins C and E, suggesting that hydroxyl radicals were not generated during the EDTA-Fe-hydrogen peroxide reaction.

Key words: Antioxidants / Hydrogen peroxide / Oxidative stress / Iron chelating agents / Iron.

INTRODUCTION

Hydrogen peroxide is often used for bacterial disinfection. In response to peroxide, bacterial cells often rupture through continuous loss of electrons to peroxide in a process known as oxidation. Metal ions further enhance the oxidative power of hydrogen peroxide. For example, in the Fenton’s reaction involving ferrous or ferric ions (Walling 1975), hydrogen peroxide is catalyzed to generate hydroxyl radicals, with stronger oxidative power than that of peroxide itself (Strukul 1992). However, an acidic pH of 2.0 or below is necessary to maintain the ionic status of ferrous and ferric ions. Various iron-chelating agents have been used to capture ferrous or ferric ions to improve the reaction (Ahuja et al. 2007).

In this study, ethylenediaminetetraacetic acid (EDTA) was examined owing to its high affinity to metal ions and its extensive industrial applications, e.g., in food (Castellano et al. 2011; Wagner and Baran 2010; Whittaker et al. 2002), mining (Feng and van Deventer 2010), and manufacturing (Choppali et al. 2010; Finzgar and Lestan 2007; Udovic et al. 2007; Zhang et al. 2010; Zhu et al. 2006). Additionally, Sumaoka et al. (2006) reported the potential use of CeIV-EDTA to hydrolyze DNA at specific sites. Given these broad uses, the potential for human ingestion of EDTA is a concern. According to the US Food and Drug Administration (2017), the cumulative estimated daily intake/acceptable daily intake (CEDI/ADI) is 2.5 µg/kg-bw/day for EDTA, and 0.35 µg/kg-bw/day for EDTA-metal chelates, indicating that EDTA-metal chelates are of greater concern with respect to consumption. However, metallic chelates of EDTA are highly water soluble and can be easily rinsed with water.

Glebska et al. (2002) discovered that EDTA, but not other metal chelators, induces intensive oxidation of ascorbic acid. Fisher et al. (2004) also reported that EDTA, but not other metal chelators, activates redox-active metal ions and induces oxidative damage. In the presence of light, EDTA-Fe chelating species could generate hydrogen peroxide and serve as electron mediators, resulting in oxidative stress by mechanisms other than Fenton’s reaction (Kocot et al. 2007). EDTA has harmful effects on microbes and enzymes (Gonzalvo
et al. 1997; Mühlbachová 2011). These previous studies have established that EDTA-Fe and peroxide cause oxidative stress and have bactericidal potential, but it is not clear whether the oxidative stress is due to the production of hydroxyl radicals (Bannolker et al. 1991). Although the exact reactive mechanisms are unclear, the dispersion and suspension of ferrous and ferric irons at a neutral pH and the extension of the reaction are two obvious advantages of EDTA-Fe for bacterial disinfection.

In this study, the bactericidal effect of EDTA-Fe in the presence of hydrogen peroxide was examined. Stationary-phase *E. coli* was used for various oxidative stress tests. Additionally, the optimal pH with respect to oxidative stress was examined. Finally, reduction of oxidative stress by three antioxidants, i.e., vitamins C and E (scavengers of free radicals) and catalase (a catalyst for transforming hydrogen peroxide to oxygen and water), was investigated. In the present study, we determined the bactericidal effects of dispersed and suspended EDTA-Fe for bacterial disinfection at a neutral pH.

**MATERIAL AND METHODS**

The target bacterial species, *E. coli* (Migula Castellani and Chalmers (ATCC 23225), was purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The *E. coli* was processed by two major routes: (1) incubation to obtain a stable and fully grown bacterial culture for further oxidative tests, and (2) oxidation to examine the inhibition of cell growth in the designated experimental conditions. *E. coli* was added to culture medium including 3 and 5 grams of beef extract and peptone, respectively, in 1 L of distilled and deionized water and incubated using an incubator manufactured by FIRSTEK (Scientific Co. Ltd., Taipei, Taiwan) where special precautions were taken to prevent unnecessary contamination, all bacterial handling containers, devices, and media were autoclaved for 40 minutes using an autoclave (TM-328A; Tomin Medical Equipment Co. Ltd., Taipei, Taiwan), and all laboratory handling processes were performed under a UV-disinfected, bacterium-free working bench. For the oxidative test, within 4–7 hours, inoculated *E. coli* cells were withdrawn and treated with various doses of test reagents, including hydrogen peroxide, EDTA-Fe chelate, and antioxidants. The mixed medium was supplemented with phosphate buffer at a neutral pH.

The main test reagents were as follows: (1) EDTA and FeSO₄·7H₂O purchased from Mallinckrodt Baker, Inc. (Surrey, UK), (2) 30% hydrogen peroxide from Sigma-Aldrich Co. (St. Louis, MO, USA), (3) bovine liver catalase from Sigma-Aldrich Co. (St. Louis, MO, USA), and (4) vitamin C (1000 mg) tablets and vitamin E (400 I.U.) capsules from Member’s Mark. Stock solutions of the EDTA-Fe chelate and hydrogen peroxide were prepared at 0.216 M and 0.37 M, respectively. The initial concentrations of the antioxidants, i.e., catalase, vitamin C, and vitamin E, were 16, 17.5, and 27 µM, respectively, as the upper limit concentrations, which was approximately half of the concentration used to treat eukaryotic cells (Li-Weber et al. 2002; Wang et al. 2008). The amount of cell mass in a sample was determined by measuring the optical density (OD) using a spectrophotometer (UV-2900; HITACHI, Tokyo, Japan) at 600 nm. All reagents were scanned individually or in combination to trace potential interference in OD measurements, if any. When interference was detected, the concentration of the interference-causing reagent was reduced until the interference was eliminated. To determine *E. coli* cell survival ratio (%), the OD value of the treated sample was divided by the OD value of the blank and multiplied by 100. Each OD value was determined for triplicate samples and mean values and standard deviations are presented. The quality of all measurements was controlled by monitoring the % error (i.e., sample standard deviation divided by sample mean). Measurement quality was deemed acceptable if the % error was 5% or less.

**Interference minimization**

OD was measured at 600 nm using a spectrophotometer to determine *E. coli* cell density. However, absorbance readings are subject to interference by reagents. To minimize this interference, EDTA-Fe, EDTA only, Fe²⁺ only, phosphate buffer, and peroxide at 10.0, 10.0, 10.0, 63.4, and 44.1 mM were scanned at 200–700 nm. In this experiment without the buffer and peroxide, the reagents absorbed light at 600 nm, which would affect the OD values. To prevent reagent-related interference, the concentrations of the reagents were reduced and tested again. The test shows that EDTA-Fe, EDTA, Fe²⁺, and peroxide at 1.25, 1.25, 1.25, and 22.06 mM, respectively, were below the limit of detection, indicating a lack of interference. Additionally, no interference in the scanned spectra of catalase, vitamin C, and vitamin E at 16, 27, and 17.5 µM, respectively, was observed.

**Cell culture**

*E. coli* was incubated in a neutral culture medium in a 37°C incubator with continuous shaking at 90 rpm. The *E. coli* concentrations based on OD values were monitored over an 8-hour period. Typical growth curve for *E. coli* revealed exponential cell growth in the initial phase, followed by a stationary growth phase, and eventually a cell decay phase. The amount of *E. coli* between 4 and 7 hours of incubation was highly stable and thus bacteria at this stage were used for all oxidative stress tests.
RESULTS AND DISCUSSION

As described above, to prevent OD interference, the initial concentrations of peroxide and EDTA-Fe chelate were capped at 22.06 and 1.25 mM, respectively. A grid test using these two concentrations was performed at a neutral pH. The OD values obtained without the addition of peroxide indicated minimal inhibition of cell growth, and 8 measurements were pooled to obtain the OD value without peroxide, \((\text{OD}_0) = 0.8\) with a standard deviation of 0.02 and % error of 2.54\%. The OD, and OD values for each grid condition were used to calculate the % cell death. The cell death rate in response to peroxide alone (22.06 mM) without EDTA-Fe was approximately 15\%; meanwhile, the cell death is 34\% in combination of peroxide (22.06 mM) and EDTA-Fe (1.25 mM). On average, an increase of roughly 0.68\% in cell death per mM peroxide was observed (i.e., 15\%/22.06 mM = ~0.68\%). However, with the addition of 5.51 mM peroxide, the increase in cell death per mM EDTA-Fe addition was 28\% (i.e., 35\%/1.25 = 28\%). These results suggest that oxidative stress occurred in the presence of both peroxide and EDTA-Fe, and that the mechanism of oxidative stress was likely not Fenton’s reaction based on the neutral pH of the medium. Also, it was worth of noting that at high initial peroxide and EDTA-Fe at 22.06 and 1.25 mM, the cell death rate dropped to 34\%. This result might have two potential explanations: (1) at a higher concentration of peroxide, EDTA started to oxidize, and the oxidative stress generated by co-treatment with peroxide and EDTA-Fe diminished, and (2) cell destruction resulted in the production of certain biomolecules that exhibited absorbance at 600 nm and therefore the cell death estimates were not accurate. Additional experiments are necessary to clarify this issue.

Because cell death was fairly steady for peroxide and EDTA-Fe concentrations of 11.03-16.54 and 0.94-1.25 mM, respectively, narrower grid treatment conditions were evaluated. Overall, oxidative stress increased as the peroxide and EDTA-Fe concentrations increased and peaked at an initial peroxide concentration of 13.79 mM and an initial EDTA-Fe concentration of 1.25 mM, at which the cell death rate was 52±0.46\% (n = 3) (Table 1). For 1.25 mM EDTA-Fe, cell death did not increase as the concentration of peroxide increased to 22.06 mM. Because oxidative stress was observed at a neutral pH, the effects of variation in pH on the strength of oxidative stress were evaluated (pH 6-8). Thus, cell death was quantified at the peak concentration of peroxide and EDTA-Fe after the initial pH of the cell culture medium was adjusted to 6, 7, and 8 by the addition of sulfuric acid or sodium hydroxide. As shown in Figure 1, the maximum cell death (i.e., 52.1±1.42\%, n= 3) was detected at pH 7. At pH 6, cell death was very low, indicating low oxidative stress of peroxide and EDTA-Fe. The higher oxidative stress at neutral pH and pH = 8 and lower oxidative stress at acidic pH suggested that Fenton’s reaction (i.e., acid-preferring case) was not the mechanism underlying oxidative stress. It was speculated that at pH 7, the EDTA and iron formed a rather efficient chelate that catalyzed the oxidative reaction more, yet the hypothesis was not been proved so far. Even though, the results implied that the oxidative stress was not necessary occurred at low pH, like Fenton’s reaction. For cell disinfection using peroxide with EDTA-Fe, a neutral pH can be beneficial.

Cell death was quantified under optimal treatment conditions with or without three antioxidants, i.e., catalase, vitamin C, and vitamin E, at their maximum concentrations of 16, 27, and 17.5 \(\mu\)M, respectively. Figure 2 reveals the protective nature of the antioxidants against oxidative stress, resulting in decreased cell death. However, the extent of the anti-oxidative effects varied among the three antioxidants. Catalase completely eliminated oxidative stress at a relatively low initial concentration of 0.86 \(\mu\)M. When a higher concentration of catalase was added, the estimated cell death value was negative, indicating that the removal of oxidative

| Table 1. Cell death rates in various peroxide and EDTA-Fe doses. |
|-----------------|------------------|------------------|
| Cell death rate % and (standard deviation) | EDTA-Fe conc. (mM) |
|                 | 0.94            | 1.09            | 1.25            |
| H₂O₂ conc. (mM) |                 |                 |                 |
| 11.03           | 40              | 46              | 50              |
|                 | (0.12)          | (0.03)          | (0.11)          |
| 13.79           | 43              | 49              | 52              |
|                 | (0.19)          | (0.36)          | (0.46)          |
| 16.54           | 43              | 45              | 51              |
|                 | (0.09)          | (0.60)          | (0.03)          |

FIG. 1. Plot of cell death rates (%) at pH values of 6, 7, and 8. The results are presented as the mean with error bars showing the standard deviation (n = 3).
stress resulted in greater cell growth than that of the untreated control. Additionally, for the same initial concentrations, cell death was lower for vitamin E than vitamin C. However, only trivial antioxidant effects were observed for vitamins C and E.

The active mixture of EDTA, ferrous iron, and vitamin C enhances the effects of catechin (a weak antimicrobial tea flavanol) against *Staphylococcus aureus* and *E. coli* via H$_2$O$_2$ production (Holloway et al. 2012). Several FDA-approved pharmaceutical chelators, including dimercaprol (British Anti-Lewisite, BAL; intramuscular injection), dimercaptosuccinic acid (DMSA or succimer; oral, intravenous, suppository, or transdermal), dimercaptopropane sulfonate (DMPS; oral, intravenous, suppository, or transdermal), EDTA (intra-venous), and deferoxamine (intravenous), are used to treat metal toxicity. Additionally, N-acetyl-cysteine (NAC) and alpha lipoic acid are also used to address metal toxicity (Sears 2013). The development of safer chelators, safer administration routes, and exact dosages for increasing bactercidal effects in the human body is an important goal of future research.

In conclusion, the highest *E. coli* death rate was 52% in the presence of 1.25 mM EDTA-Fe and 13.79 mM H$_2$O$_2$. In contrast, only 6% of *E. coli* died if 13.79 mM H$_2$O$_2$ was used alone. A neutral pH yielded the greatest decrease in *E. coli*, indicating the potential for deleterious effects on bacteria in the physiological environment, particularly at a neutral pH.

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