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Measurement of Ascorbic Acid (Vitamin C) Induced Hydroxyl Radical Generation in Household Drinking Water

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Ascorbic acid (vitamin C) induced hydroxyl radical formation was measured in household drinking water samples using the hydroxyl radical sensitive probe coumarin-3-carboxylic acid. Vitamin C, a reducing agent that is commonly used as a food additive, triggered a significant hydroxyl radical generating reaction when added to the tap-water samples tested. The capacity of ascorbic acid to trigger hydroxyl radical formation in the tap-water samples was dependent on the flushing time before the samples were taken indicating that the water in the copper piping had been contaminated by copper ions. In line with this, high concentrations of copper were measured in the hydroxyl radical generating first-draw samples. Moreover, a strong correlation was found between the hydroxyl radical generation capacity seen in the coumarin-3-carboxylic acid based microplate assay and the DNA damage seen in an agarose gel assay using the pBluescript plasmid. In the water samples showing high capacity to hydroxylate coumarin-3-carboxylic acid, a rapid formation of the open circular form of the plasmid could also be seen indicating a copper assisted hydroxyl radical attack on the DNA. In conclusion, our results show that addition of vitamin C to household tap water that is contaminated with copper ions, results in Fenton type reactions that continuously generate harmful and reactive hydroxyl radicals.

Keywords: Hydroxyl radical; Water; Coumarin-3-carboxylic acid; Vitamin C; Copper

Abbreviations: $OH^{\bullet}$, hydroxyl radical; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; TRIS, tris(hydroxymethyl)aminomethane; 3-CCA, coumarin-3-carboxylic acid; H_2O_2, hydrogen peroxide; 7-OHCCA, 7-hydroxycoumarin-3-carboxylic acid; TAE, tris-acetate-EDTA

INTRODUCTION

Hydroxyl radicals ($OH^{\bullet}$) are molecules that have one unpaired electron and are therefore very reactive and easily attack any nearby molecule by taking or giving one electron. In the laboratory, hydroxyl radicals can be easily generated by UV-induced homolytic fission of hydrogen peroxide or by metal-catalyzed (copper or iron) reduction of oxygen to superoxide that reacts with hydrogen peroxide (Haber–Weiss reaction: $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{\bullet} + OH^{-}$).[1] Moreover, hydroxyl radicals can directly be generated from hydrogen peroxide by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$.[2,3] This reaction can be highly catalyzed if certain metal chelators such as EDTA, NTA and a reducing substance such as ascorbic acid (vitamin C) are present.[4] Thus, hydroxyl radicals can be generated in a system where oxygen, copper or iron and a reducing agent such as vitamin C are present.[5] The formation of hydroxyl radicals has been studied by using the spin trap technique,[6] aromatic hydroxylation of target molecules[7–9] and by studying radical attack on target molecules such as DNA[10] and deoxyribose.[11]

Tap water might be contaminated to various degrees by copper ions due to corrosion in the pipes. On the basis of this, and the fact that vitamin C is today added in high concentrations to a variety of food sources and drinks, we decided to study

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whether vitamin C can trigger hydroxyl radical generation in tap-water samples. In particular, we wanted to study hydroxyl radical reactions that might take place in tap-water samples originating from plumbing systems that had not been used for a few days "unflushed" drinking water. By using coumarin-3-carboxylic acid as hydroxyl radical indicator we demonstrate that there exists a large variability in vitamin C-induced hydroxyl radical generation in household tap waters originating from different municipal water systems.

MATERIAL AND METHODS

Chemicals

Coumarin-3-carboxylic acid and 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) were from Fluka, Switzerland. Coumarin-3-carboxylic acid was dissolved in Milli-Q water (18 MΩ cm), and pH adjusted to 8.0 with NaOH. Ascorbic acid and cuprous chloride were purchased from Riedel-deHaen, Germany. All other reagents were from Sigma, St. Louis, USA. pBluescript was purchased from Stratagene (La Jolla, CA). SeaKem, LE Agarose was from FMC Bioproducts. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of Vitamin C-induced Hydroxyl Radical Formation in Drinking Water

Hydroxyl radical formation was measured by using coumarin-3-carboxylic acid as detector molecule.[12,13] When this compound is hydroxylated to 7-hydroxycoumarin-3-carboxylic acid, a fluorescent product is formed that can easily be measured with a fluorescence spectrophotometer. The fluorescence of 7-hydroxycoumarin-3-carboxylic acid is highly pH dependent showing maximum fluorescence intensity at pH 9.0.[12]

For the assay, 200 μl of the water samples were pipetted in triplicate onto a microplate. After this, 200 μM coumarin-3-carboxylic acid was added to all wells by using a 8 channel multiwell pipette followed by 2 mM vitamin C that started the reaction. The microplate was then incubated at room temperature in dark for various time periods and the reaction was then stopped by pipetting 10 mM TRIS base (pH 9.0) to all wells. Addition of TRIS, a hydroxyl radical scavenger, stopped the reaction and adjusted the pH in the samples to 9.0. The fluorescence was measured with a spectrofluorometer capable of reading the fluorescence from microplates. (Fluoroscan II, Labsystems, Finland). The optical filter set used was excitation 380 nm and emission 460 nm. The fluorescence values were converted into 7-OHCCA formed (nM) from a standard curve where a serial dilution of 7-hydroxycoumarin-3-carboxylic acid standard in 10 mM TRIS (pH 9.0) was used. All measurements were done at room temperature.

Measurement of Hydroxyl Radical Formation in Household Drinking Water by using Plasmid DNA

The reactions were carried out in a total volume of 10 μl containing 0.3 ug pBluescript DNA in milli-Q water. After addition of 0.5 mM vitamin C the samples were incubated for 45 min at room temperature and the reaction was stopped by addition of 4 μl sample buffer (50 % glycerol, 0.15% bromphenol blue in TAE buffer). The samples were loaded on to a 1% agarose gel in TRIS–acetate–EDTA buffer (40 mM TRIS base, 1 mM EDTA, adjusted to pH 7.6 with acetate) and the supercoiled (form I) and nicked circular (form II) DNAs were separated at 70 V for 45 min. The gel was then stained with ethidium bromide (10 ug/ml) for 45 min. After washing, the gel was photographed with an AlphaDigiDog gel documentation and image analysis system, Alpha Innotech Corporation, CA.

Measurement of Copper by using Diethyldithiocarbamic Acid

The presence of trace amounts of copper in the water samples was measured using the copper specific reagent diethyldithiocarbamic acid. For the assay, 200 μl aliquots in triplicate of the water samples were pipetted onto a microwell plate followed by 300 μM of diethyldithiocarbamic acid. The yellow Cu^{2+}-diethyldithiocarbamic complex was measured at 450 nm with a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve, generated by adding known amounts of copper chloride to 300 μM diethyldithiocarbamic acid in milli-Q water.

RESULTS

Time Course of Vitamin C-induced Hydroxyl Radical Formation in Drinking Water

To measure whether hydroxyl radical formation can be generated in household drinking water we initially tested two water samples obtained from two different municipal water supplies and compared

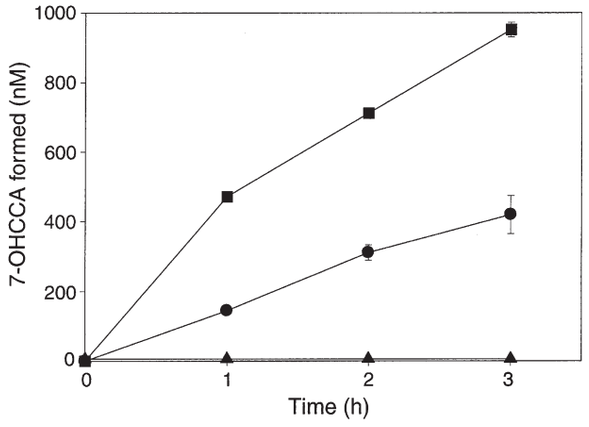

FIGURE 1 The kinetics hydroxyl radical formation in tap-water samples. At time zero, 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C were added to water sample A (●), B (■) or to milli-Q water (▲). After 1, 2 or 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. Data points are mean ± SD of triplicates from one representative experiment out of three conducted. Where absent, bars were smaller than the symbol.

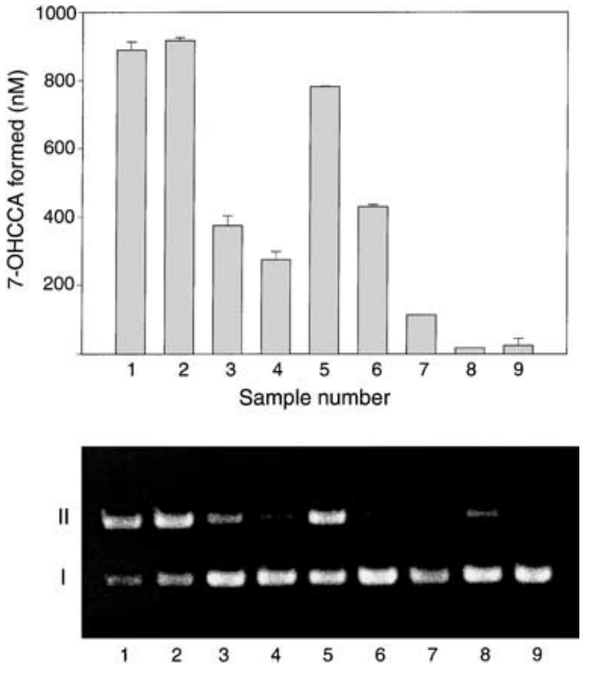

FIGURE 2 Vitamin C-induced hydroxyl radical formation in various tap-water samples. Upper panel: 200 μM coumarin-3-carboxylic acid was added to various tap-water samples followed by 0.5 mM of vitamin C. After 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. The bars show the amount of 7-hydroxycoumarin-3-carboxylic acid formed during the 3 h incubation and represent means ± SD of triplicates. Data are representative of three similar experiments. Lower panel: agarose gel electrophoresis of pBluescript DNA exposed to vitamin C in various tap-water samples. pBluescript DNA was dissolved in tap-water samples and treated for 45 min with 0.5 mM vitamin C at room temperature. The bands seen on the agarose gel are (I) supercoiled DNA and (II) open circular form DNA. The tap-water samples used in the DNA nicking assay are the same ones as used in the microplate assay. One typical gel out of four conducted is shown.

these with milli-Q water. As shown in Fig. 1, addition of 2 mM vitamin C triggered a remarkable time dependent increase in the formation of 7-hydroxycoumarin-3-carboxylic acid in one of the water samples tested (951.3 ± 20.9 nM in 3 h). In the other sample tested, the generation of 7-hydroxycoumarin-3-carboxylic acid was much weaker (419.7 ± 22.0 nM formed in 3 h). In control sample, milli-Q water, no formation of 7-hydroxycoumarin-3-carboxylic acid with time could be seen. Similarly, omission of vitamin C from the assay resulted in no formation of 7-hydroxycoumarin-3-carboxylic acid (data not shown).

Measurement of Vitamin C-induced Hydroxyl Radical Formation in Tap-water Samples Obtained from Various Municipal Water Systems

Having done the time course study, we decided to measure hydroxyl radical formation in a large number of tap-water samples and to standardize the total incubation time in our experiments to 3 h. As shown in Fig. 2 (upper panel), a large variability in vitamin C-induced formation of 7-hydroxycoumarin-3-carboxylic acid could be seen among the water samples initially tested. These samples had been taken directly from the tap without flushing the water system. In some samples, close to 900 nM 7-hydroxycoumarin-3-carboxylic acid was formed while there was hardly any hydroxylation process going on in some of the other water samples tested. The highest value obtained in our assay was 916.7 ± 8.1 nM 7-hydroxycoumarin-3-carboxylic acid formed as compared to 18.1 ± 0.4 nM found in a water sample that gave the lowest signal (Table I). In general, we found that the 7-hydroxycoumarin-3-carboxylic acid formed in tap water originating from private wells were much lower than that formed in tap water originating from public water systems (Table II). The lowest values were found in commercially sold domestic bottled water (Table II). Only one of the commercially sold bottled water samples gave a signal 142.7 ± 4.5 nM comparable with the signals obtained from tap water originating from private groundwater wells.

The hydroxyl radical formation could also be demonstrated in the water samples by using a DNA nicking assay. As shown in Fig. 2 (lower panel) addition of 0.5 mM vitamin C to pBluescript DNA dissolved in milli-Q water showed a major band corresponding to the superhelical form I DNA (lane 9). However, addition of vitamin C to the water samples containing plasmid DNA resulted in

TABLE I Vitamin C-induced hydroxyl radical generation in household tap-water samples originating from various public water systems

Sample No.	Production of 7-OHCCA (nM)
1	888.8 ± 24.8
2	916.7 ± 8.1
3	375.0 ± 28.7
4	275.0 ± 23.2
5	782.7 ± 1.9
6	430.8 ± 5.8
7	112.5 ± 0.3
8	18.1 ± 0.4
9	44.4 ± 22.6
10	786.0 ± 32.6
11	834.6 ± 24.8
12	857.5 ± 1.4
13	501.8 ± 32.6
14	911.7 ± 1.5
15	230.9 ± 24.3
16	640.8 ± 29.8
17	224.2 ± 1.0
18	289.0 ± 16.5
19	214.1 ± 2.5
20	590.0 ± 65.0
21	891.6 ± 49.4
22	85.3 ± 1.0

The reaction was started by addition of 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C. The values shown are the concentration of 7-hydroxycoumarin-3-carboxylic acid formed after 3 h incubation at room temperature and represent the means ± S.D. The results shown are triplicates from one representative experiment out of three conducted.

the appearance of an upper band corresponding to the nicked circular form II of the plasmid. The water samples used in the plasmid agarose gel assay (numbered 1–9) are identical to those used in our coumarin-3-carboxylic acid assay shown in Fig. 2, upper panel. A marked conversion of the closed circular form (form I) to the nicked circular (form II) could be detected in samples 1, 2 and 5 that also gave the highest formation of hydroxylated coumarin-3-carboxylic acid in our microplate assay.

The hydroxyl radical formation in the tap-water samples was strongly dependent on the flushing time before the samples were taken. In Fig. 3, the vitamin C-induced hydroxylation of coumarin-3-carboxylic acid in two different water samples taken either immediately or after 1, 3 or 5 min flushing is shown. As can be seen in the figure, in sample A the formation of 7-hydroxycoumarin-3-carboxylic acid within 3 h was remarkably decreased when the faucet was flushed for 5 min (from 709.5 ± 9.8 to 67.8 ± 19.2 nM). On the other hand, flushing did not decrease the formation of 7-hydroxycoumarin-3-carboxylic acid to the same extent in sample B originating from a different public water system (from 762.0 ± 38.2 to 482.7 ± 49.9 nM).

DISCUSSION

In many countries, the tap water is considered to be of good quality (taste and chemical composition) and the majority of the population use tap water instead of bottled water as their drinking water. However, the tap water reaching the homes through the pipes might be contaminated with minerals such as copper, iron, lead, chromium or arsenic. The degree of contamination is highly dependent on how corrosive the water is, the material used in the pipes and fittings and the time the water is sitting in the pipes before use.[14]

In view of the fact that drinking water can be contaminated with copper, we decided to study whether hydroxyl radical formation can be measured in tap-water samples. To initiate the hydroxyl radical generation reaction we used vitamin C, a commonly used dietary supplement, that easily can redox-cycle transition metals such as iron and copper. Vitamin C is known to be a good antioxidant but during certain circumstances, in the presence of copper or iron, vitamin C can in fact act as a strong pro-oxidant.[15,16] The concentration of vitamin C found to give the highest amount of 7-hydroxycoumarin-3-carboxylic acid in the water samples was 2 mM. Higher concentrations of vitamin C inhibited the signal (data not shown).

TABLE II Vitamin C-induced hydroxyl radical generation in drinking water

Sample No.	Production of 7-OHCCA (nM)	
	Tap water originating from various private groundwater wells	Commercially sold domestic bottled water
1	43.8 ± 22.6	4.3 ± 0.4
2	28.7 ± 0.5	142.7 ± 4.5
3	60.5 ± 22.1	0
4	119.2 ± 10.9	8.7 ± 0.5
5	80.1 ± 5.8	10.8 ± 0.4
6	105.3 ± 1.4	0
7	43.3 ± 0.8	0
8	224.2 ± 1.4	

The reaction was started by addition of 200 μM coumarin-3-carboxylic acid followed by 2 mM vitamin C. The values shown are the concentration of 7-hydroxy-coumarin-3-carboxylic acid formed after 3 h incubation at room temperature and represent the means ± S.D. The results shown are triplicates from one representative experiment out of three conducted.

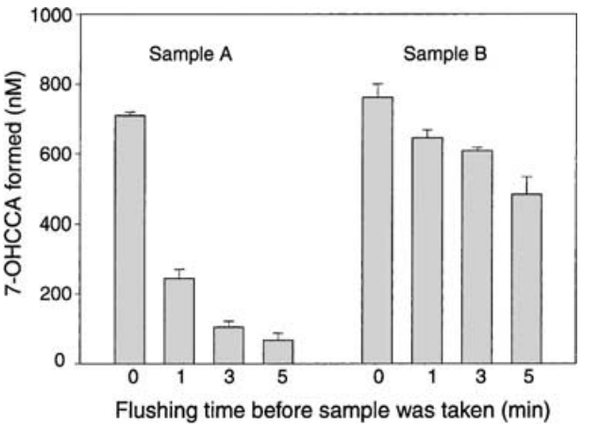

FIGURE 3 Vitamin C-induced hydroxyl radical formation in tap-water samples is dependent on flushing time. 200 μM coumarin-3-carboxylic acid and 2 mM vitamin C was added to tap-water samples taken either immediately or after 1, 3 or 5 min flushing. After 3 h incubation at room temperature, the reaction was stopped by addition of 10 mM TRIS base. The fluorescence was measured and the fluorescence values were converted into 7-OHCCA formed (nM) from the standard curve. The bars show the amount of 7-hydroxycoumarin-3-carboxylic acid formed during the 3 h incubation in two different samples originating from different municipal water systems. The bars shown represent means ± SD of triplicates. Data are representative of three similar experiments.

Our results clearly show that addition of vitamin C to the drinking water samples triggered a hydroxyl radical generating reaction that easily could be measured with coumarin-3-carboxylic acid as detector molecule. The potency of the hydroxyl radicals formed in the same tap water-samples could also be demonstrated with a DNA nicking assay. Hydroxyl radicals are known to easily damage naked DNA, both single stranded[17] and double stranded DNA[18,19] by inducing cleavage of the phosphodiester bonds. Thus, in the presence of a hydroxyl radical generating system, a plasmid DNA can easily be attacked resulting in opening and fragmentation of the DNA plasmid. A close correlation was found between our results obtained in our microplate assay using coumarin-3-carboxylic acid and the plasmid DNA assay verifying that hydroxyl radicals are formed in these water samples after vitamin C addition.

Our results indicated that there was a large variability in hydroxyl radical formation in the water samples originating from various municipal water supplies. In general, hydroxyl radical formation was most evident in household tap-water samples as compared to the hydroxyl radical formation in tap water originating from private wells or commercially sold bottled spring water. Moreover, we found that the hydroxyl radical formation in some of the tap-water samples was strongly dependent on the flushing time before the samples were taken. One explanation for this is probably that the first draw sample can be contaminated with copper from the pipes within the apartment (building).[20,21] In line with this assumption, we found measurable amounts of copper (1.04, 0.71 and 0.42 mg/l) in the samples 1, 2 and 5 used in Fig. 2. Furthermore, in the sample A, shown in Fig. 6, the copper concentration in the tap-water sample decrease from 1.85 mg/l measured in the first draw to 0.07 mg/l by flushing the faucet for 5 min. This might explain the decrease in hydroxyl radical formation seen in this sample. In sample B in the same figure, the copper concentration decreased from 1.24 to 0.18 mg/l. However, fairly high formation of 7-hydroxycoumarin-3-carboxylic acid could still be seen in the 5 min sample. This indicates that relative low concentrations of copper can assist the radical generating process or that other ions (cations or anions) than copper might be involved in the hydroxyl radical generating process in this sample.

In conclusion, we demonstrate that hydroxyl radical formation can occur in drinking water if vitamin C is added. The impact of long-term consumption of hydroxyl radical generating drinking water on human health, e.g. the incidence of ulcer or stomach cancer, remains to be studied.

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