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Iron prevents ascorbic acid (vitamin C) induced hydrogen peroxide accumulation in copper contaminated drinking water

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Abstract

Ascorbic acid (vitamin C) induced hydrogen peroxide (H_2O_2) formation was measured in household drinking water and metal supplemented Milli-Q water by using the FOX assay. Here we show that ascorbic acid readily induces H_2O_2 formation in Cu(II) supplemented Milli-Q water and poorly buffered household drinking water. In contrast to Cu(II), iron was not capable to support ascorbic acid induced H_2O_2 formation during acidic conditions (pH: 3.5–5). In 12 out of the 48 drinking water samples incubated with 2 mM ascorbic acid, the H_2O_2 concentration exceeded 400 μM. However, when trace amounts of Fe(III) (0.2 mg/l) was present during incubation, the ascorbic acid/Cu(II)-induced H_2O_2 accumulation was totally blocked. Of the other common divalent or trivalent metal ions tested, that are normally present in drinking water (calcium, magnesium, zinc, cobalt, manganese or aluminum), only calcium and magnesium displayed a modest inhibitory activity on the ascorbic acid/Cu(II)-induced H_2O_2 formation. Oxalic acid, one of the degradation products from ascorbic acid, was confirmed to actively participate in the iron induced degradation of H_2O_2. Ascorbic acid/Cu(II)-induced H_2O_2 formation during acidic conditions, as demonstrated here in poorly buffered drinking water, could be of importance in host defense against bacterial infections. In addition, our findings might explain the mechanism for the protective effect of iron against vitamin C induced cell toxicity.

Keywords: *Vitamin C, water, iron, copper, oxalic acid*

Introduction

Ascorbic acid (Vitamin C) is a water-soluble natural antioxidant that has been proposed to have beneficial effects on many age-related diseases such as atherosclerosis, cancer, neurodegenerative and ocular diseases [1–7]. It is believed that ascorbic acid can scavenge reactive oxygen- and nitrogen species and thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids and proteins [8–11]. On the other hand, it has also been shown that ascorbic acid can, in the presence of transition metal ions such as Cu(II) and Fe(III), function as a strong pro-oxidant [12–15].

The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions, and thereby stimulate the formation of reactive oxygen species (ROS) such as superoxide ($O\cdot_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). It is generally believed, that the cellular damage is caused by the hydroxyl radical (OH·). The hydroxyl radical can be directly formed from H_2O_2 and Fe(II) through the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$ [16,17]. This reaction can be strongly catalyzed if certain metal chelators such as EDTA and NTA and a reducing agent such as ascorbic acid are present [18–21]. Thus, in the absence of metal ions, H_2O_2 is not

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very reactive by itself, but imposes a threat due to its ability to easily diffuse through the cell membrane and then participate in metal induced free radical reactions inside the cell [22,23].

There are conflicting and confusing information regarding ascorbate and its cytotoxicity in the literature. Some reports clearly show that ascorbate is highly cytotoxic to cells [24–28], while others demonstrate that ascorbic acid can protect cells from pro-oxidative insult [1–7]. The toxic effect of ascorbic acid in cell systems has been attributed to H_2O_2 formation in the cell culture [29–31].

We have previously shown that ascorbic acid can trigger a pH dependent hydroxyl radical generating process in Cu(II) contaminated bicarbonate-buffered drinking water [32]. We found that this reaction could take place at pH levels above the pK_{al} value 4.25 of ascorbic acid. Here we have studied the chemical reactions that take place when ascorbic acid is added to either Cu(II) supplemented Milli-Q water or Cu(II) contaminated poorly buffered household drinking waters. We have addressed the question whether ascorbic acid can initiate a H_2O_2 accumulation process during acidic conditions (pH below 4.25) that do not support hydroxyl radical formation. The impact of iron on this process is studied in detail.

Materials and methods

Chemicals

Ascorbic acid, oxalic acid, ferrous chloride hexahydrate, ammonium ferrous sulfate, calcium chloride dihydrate and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Manganese chloride tetrahydrate, zinc chloride, cobalt chloride hexahydrate, xylenol orange sodium salt and 2,6-Di-tert-butyl-4-methanol-phenol were from Sigma, St. Louis, USA. Magnesium chloride hexahydrate was purchased from J.T. Baker, Denventer, Holland. 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 H_2O_2 formation in drinking water

Measurement of H_2O_2 in household drinking water and domestic bottled waters was performed by using the FOX assay as described earlier [33]. Briefly, the FOX reagent was prepared by mixing 9 volumes of FOX-1 reagent (4.4 mM 2,6-Di-tert-butyl-4-methanol-phenol in 100% HPLC grade methanol) with 1 volume of FOX-2 reagent (1 mM xylenol orange sodium salt and 2.56 mM ammonium ferrous sulfate in 250 mM sulfuric acid). In the assay, 2 mM of ascorbic acid was added to the different water samples to initiate the reaction. After various time periods, 25 μl samples were withdrawn from the tubes and pipetted into an eppendorf tube containing 750 μl FOX-reagent. The mixture was vortexed for 5 s and incubated at room temperature for 30 min. After this, 200 μl of the mixture was pipetted in triplicates onto a 96 well microtiter plate and the absorbance of the samples and standards were measured at 560 nm with a Victor plate reader, Wallac, Finland. The absorbance values were converted to concentration by comparison with a standard curve where known concentrations of H_2O_2 were used.

Results

Copper, but not iron, can support ascorbic acid induced H_2O_2 formation in Milli-Q water

We have previously shown that addition of ascorbic acid to bicarbonate buffered tap water samples contaminated with Cu(II) ions can generate hydroxyl radicals [34–35]. In this study, we have evaluated whether ascorbic acid has the ability to induce H_2O_2 formation in Cu(II) contaminated drinking water. To study this, we first used Milli-Q water as a model system. As can be seen in Table I, ascorbic acid induced a substantial increase in the H_2O_2 concentration (499.2 ± 5.5 μM) in Milli-Q water supplemented with 0.1 mg/l of Cu(II). This concentration of Cu(II) is 20 times below the amount of copper that is allowed in drinking water in Europe (Maximum Contaminant Level, MCL). The H_2O_2 formation was very rapid and 293.7 ± 5.5 μM H_2O_2 could already be detected after 1 h incubation (Figure 1). On the contrary, when Fe(III), was used in the assay, low concentrations 45.3 ± 8.9 μM of H_2O_2 could be detected after 6 h. When Ca(II), Mg(II), Zn(II), Mn(II), Co(II) or Al(III) were used in the assay, the concentration of H_2O_2 varied between 57.6 ± 3.1 and 120.9 ± 4.7 μM. (Table I). These metal ions were tested by using their highest concentration that is allowed in drinking water (MCL in Europe).

Effects of ferric iron on ascorbic acid/copper-induced H_2O_2 formation in Milli-Q water

We have recently shown that ferric iron can interfere with ascorbic acid/Cu(II)-induced hydroxyl radical formation in drinking water [35]. To examine whether ferric iron also affects ascorbic acid/Cu(II)-induced H_2O_2 formation in Milli-Q water, we next measured H_2O_2 formation in the presence of various divalent and trivalent cations (Table II). When ferric iron, 0.2 mg/l, was present in the assay, a very weak inhibition could be observed during the first hour (Table II). After 2 h incubation the concentration of

Table I. Vitamin C induced H_2O_2 formation in Milli-Q water in the presence of various metal ions.

	H_2O_2 (μM) formation							
	Metal ion added (mg/l)							
Time (h)	None	Cu(II) (0.1 mg/l)	Fe(III) (0.2 mg/l)	Mn(II) (0.5 mg/l)	Co(II) (0.2 mg/l)	Al(III) (0.2 mg/l)	Zn(II) (0.5 mg/l)	Mg(II) (50 mg/l)	Ca(II) (100 mg/l)
0.25	7.1 ± 0.1	139.4 ± 4.0	17.6 ± 0.1	6.4 ± 0.1	8.7 ± 0.1	25.0 ± 2.3	0.9 ± 3.1	6.4 ± 1.6	6.4 ± 0.1
1	18.1 ± 2.4	293.7 ± 5.5	18.4 ± 1.9	10.4 ± 1.4	21.4 ± 0.8	29.0 ± 1.7	12.0 ± 3.1	9.8 ± 1.5	14.8 ± 0.8
2	27.6 ± 1.6	402.6 ± 3.9	27.8 ± 4.9	19.2 ± 0.8	37.6 ± 0.1	32.7 ± 0.6	24.2 ± 1.6	21.4 ± 0.8	23.7 ± 0.8
3	35.9 ± 3.9	455.9 ± 0.8	34.5 ± 1.4	30.3 ± 0.8	57.0 ± 0.8	45.3 ± 1.1	40.3 ± 0.8	28.1 ± 0.8	34.8 ± 2.4
4	50.3 ± 3.9	486.4 ± 3.1	47.3 ± 4.2	40.9 ± 1.6	84.8 ± 2.4	49.4 ± 1.7	55.9 ± 2.4	40.3 ± 2.4	50.3 ± 0.8
5	47.6 ± 6.3	497.0 ± 3.9	45.6 ± 3.2	52.0 ± 1.6	96.4 ± 0.1	60.1 ± 1.3	66.4 ± 6.2	51.4 ± 2.4	49.8 ± 0.1
6	52.0 ± 9.4	499.2 ± 5.5	45.3 ± 8.9	63.1 ± 0.1	120.9 ± 4.7	71.3 ± 0.6	81.4 ± 3.9	57.6 ± 3.1	68.7 ± 3.1

Time dependent formation of H_2O_2 by ascorbic acid (2 mM) in Milli-Q water supplemented with 0.1 mg/l Cu(II). The concentrations of the other metal species used are MCL of the metals that is allowed in European drinking water. The values shown are the concentration of H_2O_2 formed during the incubation in dark at room temperature. Data are expressed as mean ± SD of triplicates of one representative experiment out of three conducted.

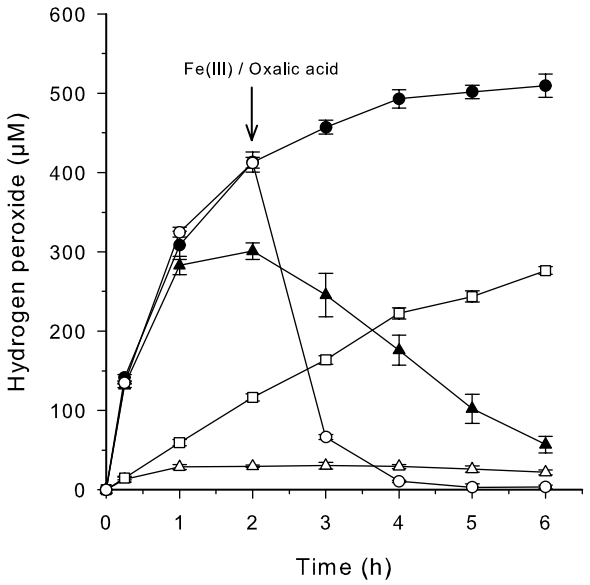

Figure 1. Effects of Iron/oxalic acid on ascorbic acid/Cu(II)-induced hydrogen peroxide accumulation in Milli-Q water. (A) 2 mM ascorbic acid was added to Milli-Q water samples containing either 0.1 mg/l Cu(II) (•); 0.1 mg/l Cu(II) and 0.2 mg/l Fe(III) (▲); 50 μM oxalic acid, 0.2 mg/l Fe(III) and 0.1 mg/l copper (△) or 50 μM oxalic acid and 0.1 mg/l copper (□). Where indicated by an arrow, 50 μM oxalic acid and 0.2 mg/l Fe(III) were added to a sample incubated for 2 h with 2 mM ascorbic acid and 0.1 mg/l Cu(II) (○). Data points are mean ± SD of three experiments. If absent, error bars are smaller than the symbols.

H_2O_2 reached 300.9 ± 10.5 μM and after this the concentration of H_2O_2 started to decrease in the water sample. After 6 h incubation, the H_2O_2 level had decreased to 57.0 ± 10.4 μM as compared to 509.5 ± 14.8 μM measured in the control sample not containing iron. Other metal ion species that might be present in drinking water, Zn(II), Co(II), Mn(II) or Al(III) did not have any impact on the ascorbic acid/(CuII)-induced H_2O_2 formation. A modest inhibition in H_2O_2 formation could be seen when Mg(II) or Ca(II) were present. However, the concentrations of Mg(II) and Ca(II) were 250- and 500-fold higher than the concentration of iron used in the assay. This is because the concentration of all the metal ions used in the assay are the amounts of these contaminants that are currently allowed in drinking water (MCL in Europe).

Iron/oxalic acid inhibits ascorbic acid/copper-catalyzed H_2O_2 formation

In our previous work, we have established that oxalic acid is one of the degradation products generated when ascorbic acid is oxidatively decomposed in copper contaminated drinking water [36]. This implies that oxalic acid might participate in the iron catalyzed H_2O_2 decomposition, since oxalic acid is known to have high affinity for ferric iron even at very low pH. To verify this, 0.1 mg/l Cu(II) and 2 mM

Table II. Effects of metal ions on ascorbic acid/copper induced H_2O_2 formation in MQ-water.

	H_2O_2 formation (μM)							
	Metal ions (mg/l) present in copper supplemented MQ-water							
Time (h)	Only Cu(II) (0.1 mg/l)	Fe(III) (0.2 mg/l)	Mn(II) (0.5 mg/l)	Co(II) (0.2 mg/l)	Al(III) (0.2 mg/l)	Zn(II) (0.5 mg/l)	Mg(II) (50 mg/l)	Ca(II) (100 mg/l)
0.25	141.4 ± 3.9	131.4 ± 2.4	144.8 ± 0.8	135.3 ± 1.6	133.9 ± 0.6	119.2 ± 3.9	132.6 ± 2.4	137.0 ± 0.8
1	308.4 ± 17.9	282.8 ± 11.6	320.9 ± 7.9	325.3 ± 6.3	310.5 ± 5.6	300.9 ± 11.0	282.0 ± 12.6	294.8 ± 5.5
2	413.1 ± 12.8	300.9 ± 10.5	399.8 ± 3.1	410.3 ± 5.5	402.0 ± 5.1	385.3 ± 6.3	358.7 ± 4.7	347.6 ± 4.7
3	457.0 ± 8.7	245.6 ± 27.4	440.3 ± 0.8	445.3 ± 3.1	459.0 ± 6.8	439.2 ± 7.1	387.6 ± 1.6	373.1 ± 4.7
4	492.8 ± 11.5	175.9 ± 18.9	471.4 ± 2.4	485.3 ± 4.7	459.8 ± 7.7	466.4 ± 3.1	407.0 ± 0.8	404.2 ± 1.6
5	501.7 ± 8.6	102.3 ± 18.4	493.7 ± 0.8	508.7 ± 3.1	486.4 ± 8.7	488.7 ± 14.1	433.1 ± 7.9	409.2 ± 2.4
6	509.5 ± 14.8	57.0 ± 10.4	502.0 ± 9.4	511.4 ± 5.5	502.0 ± 1.1	500.9 ± 6.3	434.2 ± 0.1	420.3 ± 2.4

Time dependent formation of H_2O_2 by ascorbic acid (2 mM) in Milli-Q water supplemented with 0.1 mg/l Cu(II) and various ion species (concentrations used are their MCL in Europe). The values shown are the concentration of H_2O_2 formed during the incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

ascorbic acid were added to Milli-Q water supplemented with both iron and oxalic acid. When 0.2 mg/l Fe(III) and 50 μM oxalic acid were present from the start of the reaction, extremely low concentrations of H_2O_2 could be detected in the Milli-Q water (Figure 1). Likewise, when 0.2 mg/l Fe(III) and 50 μM oxalic acid were added to the ascorbic/Cu(II) induced H_2O_2 generating reaction after 2 h incubation, the H_2O_2 concentration in the sample rapidly decreased. Furthermore, when oxalic acid alone was present in the assay from the beginning, the ascorbic acid/Cu(II)-induced H_2O_2 formation was also affected.

Ascorbic acid induced H_2O_2 accumulation in household drinking water–The relationship between copper, iron and bicarbonate

Next, we measured the amount of H_2O_2 that was formed during a 6-h incubation when 2 mM ascorbic acid was added to 40 tap water samples and 8 domestic bottled water samples (Table III). The drinking water samples tested had been sampled in the same way (directly drawn from the tap) but they originated from different municipal water suppliers. The observed levels of H_2O_2 generated varied between 0 and 488.4 μM.

When ascorbic acid was added to the low buffered drinking water samples that were contaminated with copper, H_2O_2 was generated. However, in some of the drinking water samples that were contaminated with copper but showed higher buffering capacity, much lower levels of H_2O_2 was formed in the presence of ascorbic acid. Therefore, to study the impact of bicarbonate on the H_2O_2 formation, we next performed experiments in Milli-Q water supplemented with various concentrations of bicarbonate. As shown in Figure 2, the ascorbic acid induced accumulation of H_2O_2 in the presence of 0.1 mg/l copper was significantly decreased when the concentrations of bicarbonate was increased. Consequently, in the presence of bicarbonate, lower concentrations of ferric iron was required to inhibit the hydrogen peroxide accumulation.

Discussion

We have previously shown that addition of ascorbic acid (vitamin C) to tap water samples contaminated with Cu(II) ions can generate hydroxyl radicals [32,34,35]. Here, we have studied whether ascorbic acid can initiate a H_2O_2 accumulating reaction in Cu(II) contaminated poorly buffered drinking water or Cu(II) supplemented Milli-Q water. Our results show that ascorbic acid can initiate a time dependent accumulation of H_2O_2 in copper contaminated poorly buffered drinking water. The H_2O_2 formation occurred at very low concentration of Cu(II) ions

Table III. Vitamin C induced H_2O_2 formation in drinking water.

	Household drinking water (from taps)								Bottled water	
Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	H_2O_2 (μM) formation	Sample number	Sample number	H_2O_2 (μM) formation
1	417.3 ± 17.4	9	219.5 ± 8.2	17	0	25	282.8 ± 20.3	33	106.7 ± 27.4	41	43.7 ± 12.8
2	20.1 ± 13.2	10	92.0 ± 13.1	18	396.4 ± 7.1	26	62.6 ± 3.5	34	294.5 ± 19.0	42	123.9 ± 2.5
3	252.6 ± 22.9	11	96.4 ± 20.6	19	408.1 ± 10.8	27	398.9 ± 9.2	35	175.9 ± 8.2	43	97.3 ± 13.4
4	85.6 ± 4.3	12	482.0 ± 19.0	20	426.7 ± 16.2	28	361.7 ± 3.9	36	175.9 ± 4.9	44	71.2 ± 42.7
5	481.7 ± 13.6	13	115.9 ± 7.8	21	160.3 ± 3.8	29	142.8 ± 8.8	37	356.7 ± 12.3	45	226.2 ± 11.7
6	488.4 ± 8.8	14	177.6 ± 32.2	22	476.4 ± 30.2	30	440.3 ± 8.7	38	188.9 ± 6.8	46	44.2 ± 8.5
7	488.4 ± 16.6	15	454.2 ± 9.1	23	121.4 ± 5.9	31	252.6 ± 3.9	39	375.9 ± 8.6	47	457.8 ± 12.5
8	219.2 ± 7.1	16	326.4 ± 12.8	24	67.3 ± 6.9	32	305.1 ± 7.4	40	135.6 ± 42.7	48	474.2 ± 4.4

Vitamin C (2 mM) induced H_2O_2 formation in tap water samples (numbered 1–40) and domestic bottle water samples (numbered 41–48). The values shown are the concentration of H_2O_2 formed during a 6-h incubation in dark at room temperature. Data are expressed as means ± SD of triplicates of one representative experiment out of three conducted.

Figure 2. Effect of bicarbonate and iron on ascorbic acid induced hydrogen peroxide accumulation. Ascorbic acid (2 mM) and various concentrations of Fe(III) were added to Milli-Q water supplemented with 0.1 mg/l Cu(II) and 0 mg/l (•); 25 mg/l (○); 50 mg/l (▲) and 100 mg/l (△) HCO_3. The H_2O_2 concentration was measured in the samples after a 6 h incubation at room temperature. Data points are mean ± SD of three experiments.

(20 times below the amount of copper that is allowed in drinking water (MCL in Europe)). Of the other metals tested, only Co(II) generated significant amounts of H_2O_2 (Table I).

The ascorbic acid induced H_2O_2 accumulation in copper supplemented Milli-Q water was significantly higher than the H_2O_2 formation observed earlier in cell culture and cell culture medium [37,38]. However, addition of ascorbic acid to the Milli-Q water model system used in our experiments, resulted in a much more acidic milieu (pH 3.5) than the one normally seen in buffered cell culture medium. Also the pH in our drinking water samples were close to or under the pK_{al} value of ascorbic acid (4.25). During more neutral conditions (pH 6–6.5) that was obtained when magnesium ascorbate, calcium ascorbate or sodium ascorbate were added to Cu(II) supplemented Milli-Q water, less amount of H_2O_2 was generated within 6 h (data not shown). These results are in better agreement with the amounts of H_2O_2 generated in cell cultures and cell culture medium [37,38]. This emphasizes the importance of pH in the ability of ascorbic acid to induce H_2O_2 accumulation in the presence of Cu(II) ions.

In our hands, of the metal ions tested, only Fe(III) was found to strongly affect the ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water during acidic conditions (pH 3.5) (Table II). The inhibition of the H_2O_2 accumulation could be observed after 2 h incubation, and after 6 h incubation the majority of the H_2O_2 formed had been eliminated from the water

sample (Table II, Figure 1). The shape of the ascorbic acid/Cu(II) induced H_2O_2 curve, obtained in the presence of ferric iron, strongly indicated that a metabolite from ascorbic acid might be involved in the H_2O_2 decomposition reaction. Consistent with this assumption, we found that oxalic acid, together with Fe(III) induced a prompt inhibition of ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water (Figure 1). The reason why we used oxalic acid was because we have previously demonstrated that oxalic acid is one of the degradation products when ascorbic acid is oxidatively decomposed in Cu(II) contaminated drinking water [36].

Oxalic acid also decreased the ascorbic acid/Cu(II) induced H_2O_2 formation in Milli-Q water, implying direct interaction between Cu(II) and oxalic acid. The mechanism for the oxalic acid mediated inhibitory effect on ascorbic acid/copper induced H_2O_2 formation in Milli-Q water might be due to its ability to interfere with copper redox-cycling. This is because oxalic acid is known to have affinity for both Fe(III) and Cu(II), even at very low pH. Alternatively, copper/oxalic acid could catalyze H_2O_2 to hydroxyl radicals. However, we could not detect any ascorbic acid induced hydroxyl radical formation when oxalic acid was added to copper supplemented water (data not shown), implying reduced copper redox-activity when oxalic acid was present. Interestingly, it has been suggested that oxalic acid could act as an antioxidant in some systems, because oxalic acid reduces the rate of ascorbic acid oxidation in the presence of H_2O_2 and Cu(II) [39].

The ability of Fe(III) and oxalic to regulate ascorbic acid/Cu(II)-induced H_2O_2 formation during acidic conditions as shown in our experiments could be of importance *in vivo* when acidic conditions prevail. This could emphasize the importance of vitamin C, oxalic acid and iron during certain conditions such as the inflammatory process. Thus, when Cu(II) ions and Vitamin C are present during acidic conditions, the presence or absence of free redox-active iron and oxalic acid will determine the amount of H_2O_2 that will be accumulated. In particular, during conditions where catalase is not present or not functioning properly e.g. in the presence of ascorbic acid and copper [40,41], the iron/oxalic acid complex could be of importance in regulating H_2O_2 toxicity during acidic conditions in various biological systems *in vivo*.

The vitamin C induced accumulation of H_2O_2 could also be demonstrated in 40 household tap water samples and 8 domestic bottled water samples. In 25% of the drinking water samples tested, over 400 μM of H_2O_2 was formed during the 6 h incubation and some drinking waters generated close to 500 μM of H_2O_2 within 6 h (Table III). The kinetics for the H_2O_2 formation in these drinking waters was relative fast, reaching close to 300 μM H_2O_2 in only 2 h (data not shown). The ascorbic acid induced hydrogen peroxide formation was particularly evident in the poorly buffered copper contaminated drinking water samples. When bicarbonate was added to Milli-Q water supplemented with copper, the hydrogen peroxide accumulation was much lower (Figure 2). During these conditions the hydrogen peroxide is converted to hydroxyl radicals [32].

Hydrogen peroxide has been shown to have strong antibacterial effects [42]. One might speculate, whether the ascorbic acid/Cu(II)-induced H_2O_2 formation and the ability of Fe(III) and oxalic acid to regulate H_2O_2 formation, as demonstrated here in drinking water, could be involved in controlling the survival of pathogenic bacteria in the human gastrointestinal tract. On the other hand, our results demonstrating that vitamin C induces H_2O_2 formation in drinking water could, in fact, result in increased oxidative stress *in vivo*. In particular, in the absence of Fe (III) ions, ascorbic acid/Cu(II)-induced H_2O_2 formation in drinking water, as demonstrated here, could strongly enhance the total H_2O_2 load and H_2O_2 induced oxidative stress in some individuals.

In conclusion, our results show that copper, but not iron, can support vitamin C induced H_2O_2 formation in weakly buffered drinking water. Moreover, we show that iron, together with ascorbic acid-derived oxalic acid prevents ascorbic acid/Cu(II) induced H_2O_2 accumulation. Our results strongly indicate that the iron oxalic acid complex might function as an important regulator of ascorbic acid/copper-induced H_2O_2 formation and copper mediated tissue damage. The iron/oxalic acid induced inhibition of H_2O_2 formation, as demonstrated here in drinking waters, might explain the mechanism for the protective effect of iron against ascorbic acid (vitamin C) induced cell toxicity in cell cultures.

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