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Cobalt-mediated oxidative DNA damage and its prevention by polyphenol antioxidants

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ABSTRACT

Although cobalt is a required nutrient, it is toxic due to its ability to generate reactive oxygen species (ROS) and damage DNA. ROS generation by Co^{2+} often has been compared to that of Fe^{2+} or Cu^{+}, disregarding the reduction potential differences among these metal ions. In plasmid DNA damage studies, a maximum of 15% DNA damage is observed with Co^{2+}/H_2O_2 treatment (up to 50 μM and 400 μM, respectively) significantly lower than the 90% damage observed for Fe^{2+}/H_2O_2 or Cu^{+}/H_2O_2 treatment. However, when ascorbate is added to the Co^{2+}/H_2O_2 system, a synergistic effect results in 90% DNA damage. DNA damage by Fe^{2+}/H_2O_2 can be prevented by polyphenol antioxidants, but polyphenols both prevent and promote DNA damage by Cu^{+}/H_2O_2. When tested for cobalt-mediated DNA damage affects, eight of ten polyphenols (epicatechin gallate, epigallocatechin gallate, propyl gallate, gallic acid, methyl-3,4,5-trihydroxybenzoate, methyl-4,5-dihydroxybenzoate, protocatechuic acid, and epicatechin) prevent cobalt-mediated DNA damage with IC_{50} values of 1.3 to 27 μM and two (epigallocatechin and vanillic acid) prevent little to no DNA damage. EPR studies demonstrate cobalt-mediated formation of $^{\bullet}OH$, $O_2^{\bullet-}$, and $^{\bullet}OOH$, but not $^1O_2$ in the presence of H_2O_2 and ascorbate. Epigallocatechin gallate and methyl-4,5-dihydroxybenzoate significantly reduce ROS generated by Co^{2+}/H_2O_2/ascorbate, consistent with their prevention of cobalt-mediated DNA damage. Thus, while cobalt, iron, and copper are all *d*-block metal ions, cobalt ROS generation and its prevention is significantly different from that of iron and copper.

1. Introduction

With the discovery of ferroptosis as a metal-controlled mechanism for cell death, the biological effects of oxidative damage in health and in disease development have been increasingly investigated. Oxidative damage by iron, copper, and chromium is extensively studied [1–7], but cobalt-mediated damage remains less understood [1,8–10]. Cobalt is an essential trace element found in vitamin B_{12}, but it can also be toxic [1,11–13]. Increased cobalt levels are found in patients with orthopedic [10,14] and orthodontic [15] appliances, and the potential for toxicity in those who consume an excess of the recommended daily allowance for vitamin B_{12} in supplements is a significant health concern [11,13].

Cobalt-mediated oxidative stress is an underlying cause of neuroinflammation [16], degeneration of neuronal cells [17,18], increased levels of β-amyloid in Alzheimer's disease [19], epilepsy [20], cancer [13], damage to liver-, kidney-, and lung- chromatin in rats [21], and reduction in kidney and liver function in mice [22]. Cobalt can cause DNA backbone cleavage [23] and base oxidation [24], and Co^{2+}, Fe^{2+}, and Cu^{+} bind to similar sites in DNA [25–28].

Among the mechanisms proposed for cobalt-mediated oxidative damage include reactive oxygen species (ROS) generation, analogous to that observed for iron and copper (Reactions 1 and 2) [1,23,29,30], despite the much lower oxidation potential for Co^{2+} oxidation compared to Fe^{2+} and Cu^{+} [31]. Since redox potentials greatly affect ROS

Abbreviations: $AscH_2$, ascorbic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTPA, diethylene triamine pentaacetic acid; EC, (−)-epicatechin; EC_{50}, 50% effective concentration; ECG, (−)-epicatechin-3-gallate; EDTA, ethylenediaminetetraacetic acid; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; EPR, electron paramagnetic resonance; GA, gallic acid; IC_{50}, 50% inhibitory concentration; MEGA, methyl-3,4,5-trihydroxybenzoate; MEPCA, methyl-3,4-diihydroxybenzoate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCA, protocatechuic acid; PREGA, *n*-propyl gallate; ROS, reactive oxygen species; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethyl-1-pyrroline-*N*-oxide; VA, vanillic acid.

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generation [32], it is unlikely that Co^{2+} generates ROS similarly to Fe^{2+} and Cu^{+}, but cobalt, iron-, and copper-mediated ROS generation and DNA damage have not been directly compared.

$$Co^{2+} + H_2O_2 \rightarrow Co^{3+} + {}^{\bullet}OH + HO^{-} \quad (1)$$

$$Fe^{2+}/Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/Cu^{+} + {}^{\bullet}OH + HO^{-} \quad (2)$$

Polyphenol antioxidants prevent Fe^{2+}/H_2O_2-mediated DNA damage *in vitro* by binding Fe^{2+} and autoxidizing it to Fe^{3+} [33,34]. In contrast, some polyphenols enhance copper-mediated DNA damage [35,36]. Because polyphenol affects on metal-mediated DNA damage differ depending on the metal ion, it is vital to test these potential antioxidants for their ability to prevent cobalt-specific DNA damage. In this work, we examine ROS generation and DNA damage caused by Co^{2+}, H_2O_2, and/or ascorbate and evaluate the affects of polyphenol compounds on cobalt-mediated DNA damage. Elucidating ROS generation and DNA damage by Co^{2+} as well as the ability of polyphenol antioxidants to prevent this damage will advance understanding of cobalt toxicity and its potential treatments.

2. Materials and methods

2.1. General details

Water was purified using a Barnstead NANOpure DIamond Life Science (UV/UF) water deionization system (Barnstead International). MES (Alfa Aesar), $CoSO_4 \bullet 7H_2O$ (Acros Organics), L-(+)-ascorbic acid (99 + %, Alfa Aesar), Chelex 100 resin (Sigma-Aldrich), and disodium dihydrogen ethylenediaminetetraacetate (EDTA; TCI America) were all used as received. Microcentrifuge tubes were rinsed in 1 M HCl, triply rinsed in deionized H_2O, and dried prior to use. Buffered solutions were treated with Chelex resin (2 g per 80 mL buffer) for 24 h prior to use. $CoSO_4$ and ascorbate solutions were prepared prior to each experiment and used immediately.

2.2. Transfection, amplification, and purification of plasmid DNA

Plasmid DNA (pBSSK) was purified from *E. coli* strain DH1 using a PerfectPrep Spin kit (Fisher). The plasmid DNA was dialyzed at 4 °C against EDTA (1 mM) and NaCl (50 mM) for 24 h and then against NaCl (130 mM) for 24 h to remove metal ions. For all experiments, the absorbance ratios for DNA solutions were $A_{250}/A_{260} \leq 0.95$ and $A_{260}/A_{280} \geq 1.8$.

2.3. Gel electrophoresis assays

In a buffered solution of MES or MOPS (10 mM, pH 6.3 or 7, respectively), NaCl (130 mM), ethanol (10 mM, as a radical scavenger to mimic organic components) [37], Co^{2+} (1–100 μM), and ascorbate (1.25–125 μM) were combined and allowed to stand. After 5 min, plasmid DNA (pBSSK in NaCl 130 mM) was added to the solution so that the final concentration of DNA was 0.1 μM. After 5 min, H_2O_2 (400 μM) was added, resulting in a total reaction volume of 10 μL. This reaction mixture was allowed to stand for 60 min before EDTA (50 μM) and loading dye (0.5% xylene cyanol, 0.25% bromophenol blue, and 40% glycerol) were added. Samples were then loaded into a 1% agarose gel. Nicked (damaged) and supercoiled (undamaged) DNA were separated by gel electrophoresis in Tris-acetate-EDTA (TAE) buffer for 60 min at 140 V and 255 mA. Gels were stained for 5 min with ethidium bromide and the bands were imaged under UV light. Intensities of the damaged and undamaged DNA gel bands were quantified using UVIproMW software (Jencons Scientific, Inc.). Ethidium stains supercoiled DNA less efficiently than nicked DNA, so supercoiled DNA band intensities were multiplied by 1.24 prior to comparison [38,39]. Intensities of the nicked and supercoiled bands were normalized for each lane so that % nicked + % supercoiled = 100%. Gel results for cobalt-mediated DNA damage are provided in the electronic supplementary information in Tables S1-S4 and Figs. S1-S4.

To evaluate polyphenol effects on Co^{2+}-mediated DNA damage, the same procedure was used, except that the indicated concentration of the polyphenol was also added with all the other components of the buffered solution 5 min prior to addition of the plasmid DNA. Gel results for cobalt-mediated DNA damage are provided in the Appendix A: Supplementary Data in Tables S5-S14 and Figs. S5-S14.

2.4. IC_{50} value calculations

IC_{50} values were calculated from fitting the average of % DNA damage inhibition of at least three trials with respect to the logarithm of polyphenol concentration with a sigmoidal dose-response curve (this gave very similar results to the mean of the IC_{50} fits from each trial and is less sensitive to data noise). IC_{50} value standard deviations were calculated from the standard deviations of the three trials' individual IC_{50} values. A p value of <0.05 was considered statistically significant. Graphs showing the relationships between the IC_{50} value for Co^{2+}/H_2O_2/ascorbic-acid-mediated DNA damage and polyphenol oxidation potential or pK_a of the most acidic hydrogen of the polyphenol are provided in Fig. S15.

2.5. Electron paramagnetic resonance spectroscopy measurements

EPR spectra were measured on a Bruker EMX spectrometer using a quartz flat cell at room temperature using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a standard ($g = 2.0036$ [40]) centered at 3500 with a sweep width of 100 G. the modulation amplitude was between 0.50 and 1.00 G, time and conversion constants were 81.92 s, and microwave power and frequency were 20.02 mW and 9.752 GHz; respectively. Samples (500 μL) were prepared and measured in <5 min at room temperature in a MES buffered solution (10 mM, pH 6.3) containing Fe^{2+} or Co^{2+} (300 μM), ascorbate (375 μM), polyphenol (300, 600, or 900 μM), and the 5,5-dimethyl-1-pyrroline-*N*-oxide or 2,2,6,6-tetramethylpiperidine (DMPO or TEMP, 30 mM) spin trap as indicated. H_2O_2 (22.5 mM) was added last to initiate the reaction. EPR spectra were processed using Bruker Xepr software, and spectra are provided in figs. S16-S23

2.6. UV-visible spectroscopy studies

Samples were measured at room temperature in an acid-washed quartz cuvette and on an Agilent 8453 spectrophotometer. Co^{2+} (2.5 μM), ascorbate (3.75 μM) where indicated, and the polyphenols at different concentrations (2.5, 5.0, 7.5, 10.0, and 12.5 μM) were combined in a buffered solution (MES, 2.5 mM, pH 6.3) in a total volume of 3.0 mL. The solutions were allowed to stand for 5 min prior to data collection. The absorbance of the component's mixture is also presented as the difference between the mixture and each individual component absorbance, prior subtraction of the blank absorbance. UV–vis data are provided in Figs. S24-S47

2.7. Mass spectrometry studies

MALDI mass spectrometry experiments were performed using a Bruker Microflex MALDI-TOF mass spectrometer with a *trans*-2[3-(4-*tert*-butylphenyl)-2-methyl-2-propenyldiene (250.3 *m/z*) matrix. Co^{2+}/polyphenol solutions (1:1) were prepared by combining aqueous solutions of $CoSO_4$ (100 μL, 100 μM), polyphenol (100 μL, 100 μM), and ascorbate (100 μL, 125 μM) as indicated. For the higher-ratio Co^{2+}/polyphenol samples, the cobalt concentration remained the same (100 μM) and polyphenol concentrations were increased (up to 500 μM). All mass spectroscopy data are provided in Table S15 and Figs. S48-S57.

3. Results and discussion

3.1. Cobalt-mediated DNA damage studies

The cobalt recommended dietary allowance (RDA) is 10–20 μg for a 70 kg adult [41], but up to 0.4–2.1 mg/day can be consumed without harmful effects [42–44]. Although typical cobalt blood concentrations are in the nanomolar range [1], blood concentrations of cobalt in the range of 1–100 μM have been reported in patients with prosthetic hip-associated cobalt toxicity [45]. Given these high cobalt concentrations and the associated toxicity, it is important to investigate cobalt-generated ROS and the DNA damage it can cause.

To evaluate cobalt-mediated DNA damage that contributes to its toxicity, the ability of Co^{2+} to cause single-strand DNA breaks under oxidative stress conditions was evaluated using a plasmid DNA damage assay. In contrast to cellular assays, these *in vitro* DNA damage assays allow a direct comparison between DNA damage and ROS generation that enables mechanistic evaluation of Co^{2+} toxicity. These DNA damage results also can be directly related to cell death [46,47]. Conditions are carefully chosen to cause only one backbone nick per plasmid, and gel electrophoresis is used to separate the undamaged (supercoiled) from damaged (nicked) plasmid DNA.

Using this DNA damage assay, we tested the ability of Co^{2+} and H_2O_2 alone as has been proposed by analogy to Fe^{2+} (Reactions 1 and 2). At a constant H_2O_2 concentration (400 μM, pH 6.3), Co^{2+} addition (1–50 μM) resulted in no significant DNA damage (Table S1). In contrast, combining Fe^{2+} (2 μM) and H_2O_2 (50 μM) results in 97% DNA damage under the same conditions (Table S1). From these results, it is clear that Fe^{2+} and Co^{2+} do not damage DNA *via* the same hydroxyl-radical-generating mechanism.

Because ascorbate is also present in blood with a typical range of 3–120 μM [48,49], and can generate ROS under certain conditions, we also examined its effect on cobalt-mediated DNA damage. Combining Co^{2+} (100 μM) and ascorbate (1.25 μM) alone does not result in significant DNA damage (lane 3, Fig. 1A). However, when Co^{2+} is combined with both H_2O_2 (400 μM) and ascorbate (1.25 equivalents) at varying concentrations, significant DNA damage is observed, with ≥90% DNA damage at high Co^{2+} concentrations (40–100 μM, lanes 10–13). This amount of damage is similar to DNA damage caused by Cu^{2+} (6 μM), ascorbate (7.5 μM), and H_2O_2 (50 μM) in the positive control (lane 4). As in the Cu^{2+} system, all three components are necessary to damage DNA damage, since DNA damage by ascorbate and H_2O_2 is significantly lower at all concentrations (Fig. 1B) than for the Co^{2+}/H_2O_2/ascorbate system. At ascorbate concentrations ≤25 μM, DNA damage is similar with or without Co^{2+}, but as the ascorbate concentration increases from 38 to 125 μM, DNA damage is approximately 40% higher when Co^{2+} is present (Fig. 2), reaching a maximum independent of ascorbate concentration. Thus, Co^{2+}, ascorbate, and H_2O_2 act synergistically to cause greater DNA damage than with ascorbate and H_2O_2 alone, or with Co^{2+} and either ascorbate or hydrogen peroxide.

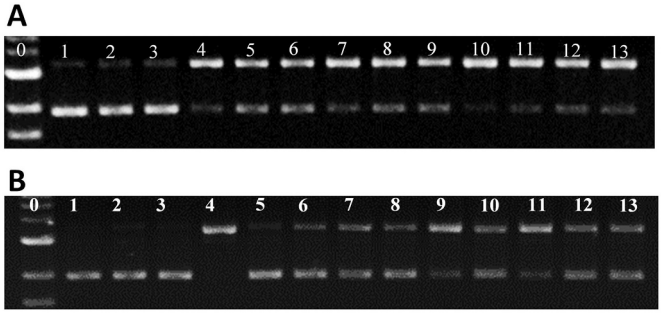

Fig. 1. A) Gel electrophoresis image of DNA damage upon treatment with Co^{2+} (1–100 μM), ascorbate (1.25–125 μM) and H_2O_2 (400 μM) at pH 6.3 (MES buffer). Lane 0: 1 kb molecular weight ladder; 1: plasmid DNA (p); 2: p + H_2O_2 (400 μM); 3: p + Co^{2+} (100 μM) + ascorbate (125 μM); 4: p + Cu^{2+} (6 μM), ascorbate (7.5 μM), and H_2O_2 (50 μM); lanes 5–13: increasing concentrations of Co^{2+} (1, 5, 10, 20, 30, 40, 50, 75, and 100 μM, respectively) with 1.25 equivalents of ascorbate per Co^{2+} (1.25–125 μM), and H_2O_2 (400 μM). B) Gel electrophoresis image upon DNA treatment with only ascorbate and H_2O_2; lanes were treated as in (A) without Co^{2+}. In both gel images, the top band is from damaged (nicked) DNA and the bottom band is undamaged (supercoiled) DNA.

Co^{2+}-mediated DNA damage is also pH-dependent, since a pH lower than 6.1 results in ≥15% DNA damage upon H_2O_2 treatment alone (data not shown). This effect has been previously observed: DNA fragmentation and apoptosis in neuroblastoma (SK-N-BE(2)) and melanoma (mel B) cells was observed upon treatment with ascorbate (1 mM) and H_2O_2 (2.5 mM) alone at pH 6 after 2–4 h [50]. H_2O_2 and ascorbate also cause DNA strand breaks from $^{\bullet}OH$, $O_2^{\bullet-}$, and $^1O_2$ [51], confirming the prooxidant potential of ascorbate.

Maximum DNA damage for this Co^{2+} system was determined to occur at pH 6.3; under similar conditions at pH 7, DNA damage by Co^{2+}/H_2O_2/ascorbate reaches a maximum of 60% damage at Co^{2+} concentrations of ≥50 μM; Fig. S4). A similar Co^{2+}/H_2O_2/ascorbate system also has been investigated for dye oxidation [52], indicating that in the presence of H_2O_2 and ascorbate, Co^{2+} generates damaging ROS.

3.2. Polyphenol prevention of cobalt-mediated DNA damage

The ability of polyphenol compounds to prevent cobalt-mediated DNA damage was evaluated using DNA damage assays with Co^{2+} (40 μM), ascorbate (50 μM), and H_2O_2 (400 μM), since these conditions result in ~90% DNA damage. By adding increasing polyphenol concentrations (0.5-800 μM, Fig. 3), their cobalt-mediated DNA damage prevention was quantified and compared. These polyphenol compounds were selected because their ability to prevent (or enhance) iron- and copper-mediated DNA damage have been reported under similar conditions [33,36,53].

As the concentration of the polyphenol EGCG increases, the amount of DNA damage decreases (Fig. 4A, lanes 5–15). The percentage of DNA damage inhibition with respect to EGCG concentration was plotted and fit with a sigmoidal dose-response curve (Fig. 4B), establishing a concentration to inhibit 50% of DNA damage (IC_{50} value) of 2.6 ± 0.4 μM for EGCG. Similar cobalt-mediated DNA damage assays were performed on the remaining nine polyphenol compounds (Fig. 3). Of the ten tested polyphenols, eight (EGCG, ECG, PREGA, GA, MEGA, MEPCA, PCA, and EC) prevent significant amounts of DNA damage, with IC_{50} values from 1.3 to 27 μM (Table 1). In contrast, EGC prevents only ~20% DNA damage at concentrations above 50 μM, and vanillic acid (VA) shows no significant ability to prevent cobalt-mediated DNA damage under these

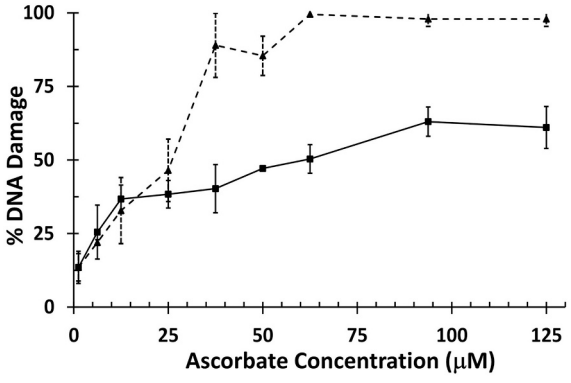

Fig. 2. Graph of percentage DNA damage with respect to ascorbate concentration after DNA treatment with A) Co^{2+} (1–100 μM), ascorbate (1.25–125 μM; 1.25 equiv. per Co^{2+}) and H_2O_2 (400 μM) for 60 min (triangles) and B) treatment with H_2O_2 and ascorbate only (squares).

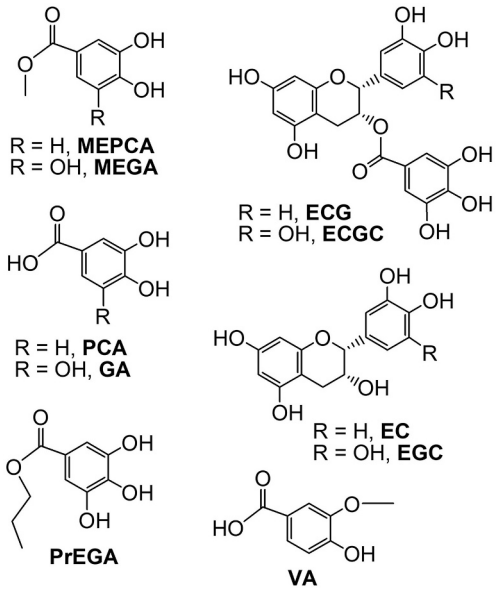

Fig. 3. Structures of polyphenol compounds examined for prevention of Co^{2+}-mediated DNA damage.

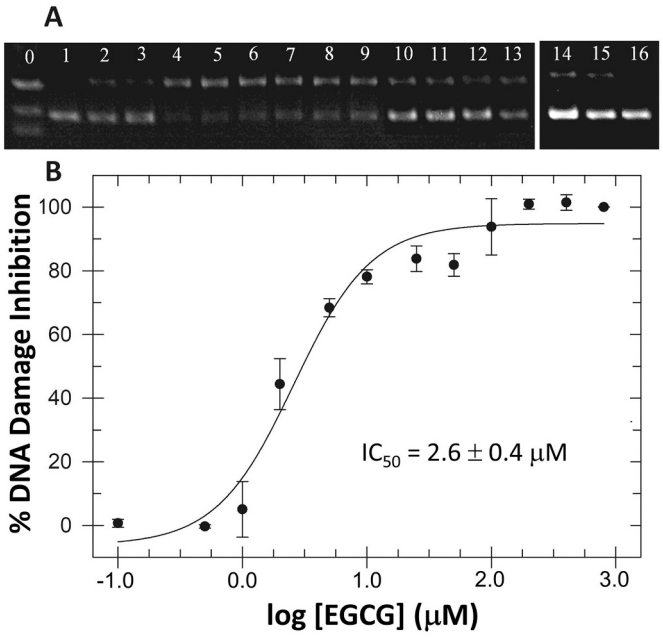

Fig. 4. A) Gel electrophoresis image of DNA treated with epigallocatechin gallate (EGCG, 0.5–800 μM) in the presence of Co^{2+}(40 μM), ascorbate (50 μM), and H_2O_2 (400 μM) at pH 6.3 (MES buffer, 10 mM) for 60 min. Lane 0: 1 kb molecular weight ladder; 1: plasmid DNA (p), 2: p + H_2O_2 (400 μM); 3: p + ECG (800 μM); 4: p + Co^{2+} (40 μM), ascorbate (50 μM), and H_2O_2 (400 μM); lanes 5–15: lane 4 with increasing concentrations of EGCG (0.5, 1, 2, 5, 10, 25, 50, 100, 200, 400, and 800 μM, respectively). The top band is from damaged (nicked) DNA and the bottom band is undamaged (supercoiled) DNA. B) Graph of the percentage of DNA damage inhibition with respect to EGCG concentration from which the IC_{50} value was determined.

conditions. Blood polyphenol levels range from 1 to 10 μM [53], so the IC_{50} values for many of the tested polyphenols are within biological polyphenol concentrations.

In every case, gallol-containing polyphenols (ECG, EGCG, PREGA, GA, and MEGA) more effectively prevent DNA damage than analogous catechol-containing polyphenols (MEPCA, PCA, EC, and EGC). No

Table 1
IC_{50} values for polyphenol prevention of Co^{2+}-, Fe^{2+}-, and Cu^{+} mediated DNA damage.

Polyphenol	IC_{50} with Co^{2+} (μM)	IC_{50} with Fe^{2+} (μM)[a]	IC_{50} with Cu^{2+} (μM)[b]
ECG	1.3 ± 0.3	2.3	53.04 ± 0.02[c]
EGCG	2.6 ± 0.4	1.1	225.9 ± 0.1
PREGA	2.6 ± 0.4	5.1	125.90 ± 0.02[c]
GA	4.1 ± 0.1	14.0	16% damage prevention at 500 μM[c]
MEGA	6 ± 1	4.0	102.3 ± 0.1[c]
MEPCA	9 ± 1	15.6	8.24 ± 0.3
PCA	15 ± 2	34.4	~482
EC	27 ± 3	59.1	Prooxidant (0.2–500 μM)
EGC	~20% inhibition at ≥50 μM	9.8	Prooxidant (0.02–1000 μM)
VA	No activity	140	No activity

[a] Fe^{2+} (2 μM) with H_2O_2 (50 μM) for 30 min; standard deviations are ±1 μM [33].
[b] Cu^{2+} (6 μM) with ascorbate (7.5 μM) and H_2O_2 (50 μM) for 30 min [36].
[c] Polyphenol exhibits prooxidant activity at low concentrations.

correlation is observed between polyphenol oxidation potential [33] and DNA damage prevention ability (R^2 = 0.15; Fig. S15A); instead, a weak correlation (R^2 = 0.67; Fig. S15B) is observed between the IC_{50} value and the first phenolic pK_a [53]. Since gallols have lower pK_a values for deprotonation of the first phenolic hydrogen atoms than catechols (~7.9 and ~ 8.5, respectively) [33], gallols are more deprotonated and capable of binding Co^{2+} at pH 6.3. This is supported by the fact that VA, which has a methylated catechol group that inhibits metal binding, prevents no cobalt-mediated DNA damage. These DNA damage results suggest that cobalt binding, rather than direct ROS scavenging, may be a primary mechanism for polyphenol prevention of cobalt-mediated DNA damage, similar to results observed for iron [33].

3.3. Reactive oxygen species identification by EPR spectroscopy

The combination of Co^{2+}, ascorbate, and H_2O_2 generates DNA-damaging ROS, likely hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and/or ascorbyl radical. Ascorbic acid ($AscH_2$) can generate $O_2^{\bullet-}$ by reduction of dioxygen, and its reaction with H_2O_2, forms $^{\bullet}OH$ and ascorbyl radical ($AscH^{\bullet}$, Reaction 3) [54]. Hydroxyl radical also can be generated from Co^{2+}-catalyzed $O_2^{\bullet-}$ decomposition in the Haber Weiss process (Reaction 4) [55].

$$AscH_2 + H_2O_2 \rightarrow AscH^{\bullet} + H_2O + HO^{\bullet} \quad (3)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + OH^{-} \quad (4)$$

To examine cobalt-generated ROS with short lifetimes, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin trap for EPR spectroscopy experiments. DMPO adducts of $^{\bullet}OH$ (a 1:2:2:1 quartet [56]) and $O_2^{\bullet-}$ (a 1:1:1:1 quartet [56]) have different EPR signals, so that these radical species can be easily differentiated. The 1:1 doublet resonance for ascorbyl radical has a long enough lifetime to be directly detected [57,58]. $^1O_2$ generation was investigated using 2,2,6,6-tetramethyl-piperidine (TEMP) as a spin trap; its $^1O_2$ adduct, 2,2,5,5-tertramethyl-1-pyrroline-*N*-oxide (TEMPO), is a 1:1:1 triplet resonance [59,60].

The EPR spectrum of Co^{2+}/H_2O_2 shows the characteristic 1:2:2:1 quartet for the DMPO-OH adduct (Fig. 5A), but it is ~25-fold lower in intensity than that resulting from Fe^{2+}/H_2O_2 (Fig. S16), consistent with the very low amount of DNA damage seen for Co^{2+}/H_2O_2 compared to Fe^{2+}/H_2O_2 conditions. Addition of ascorbate to Co^{2+}/H_2O_2 in the same ratio as in the DNA damage assays has two effects on the ROS generated: 1) the intensity of the DMPO-OH quartet is reduced two-fold, and 2) a new resonance from ascorbyl radical ($AscH^{\bullet}$) is observed (Fig. 5B). Ascorbyl radical is also generated by Co^{2+}/ascorbate alone (Fig. S17A), but no DMPO-OH resonance is observed without H_2O_2 addition.

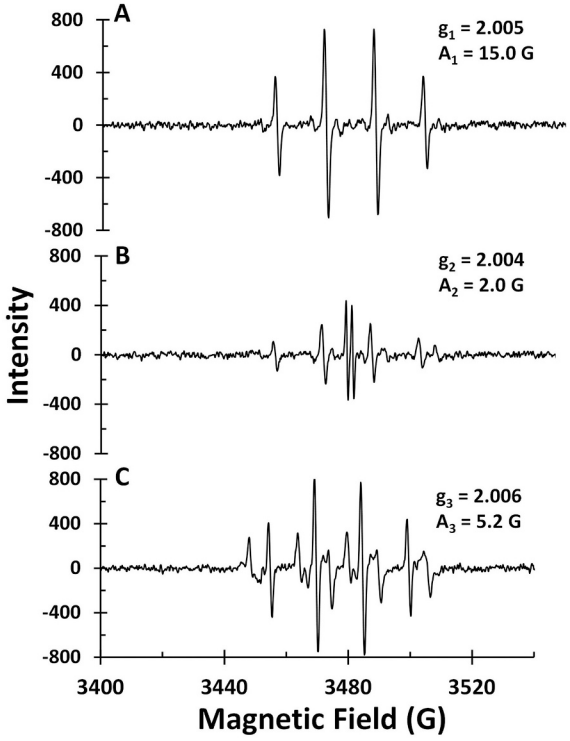

Fig. 5. EPR spectra of A) Co^{2+} (300 μM) and H_2O_2 (22.5 mM); B) Co^{2+} (300 μM), ascorbate (375 μM), and H_2O_2 (22.5 mM); and C) ascorbate (375 μM) and H_2O_2 (22.5 mM). Room temperature spectra were acquired in buffered aqueous solution at pH 6.3 (MES, 10 mM) with DMPO (30 mM) as a spin trap les than 5 min after sample preparation. Values A_1 and g_1; A_2, and g_2; and A_3 and g_3 correspond to the DMPO-OH adduct, ascorbyl radical, and DMPO-OOH adduct, respectively. Experimental conditions: time constant 81.92 ms, conversion time 81.92 ms, modulation amplitude 1.00 G, microwave power 20.02, and magnetic field 3500 ± 100 G.

The EPR spectrum of ascorbate and H_2O_2 without Co^{2+} does not show ascorbyl radical resonances, but instead shows a DMPO-OH resonance (Fig. 5C) with additional overlapping resonances similar to those reported by Finkelstein, et al. [61] for the DMPO-hydroperoxide (DMPO-OOH) adduct. This DMPO-OOH adduct forms when superoxide reacts with DMPO, and it subsequently decomposes to yield DMPO-OH. EPR studies with TEMP did not show resonances consistent with $^1O_2$ formation, but confirmed non-DNA-damaging $O_2^{\bullet-}$ generation upon observation of a TEMP-OOH resonance similar to DMPO-OOH (Fig. S17B). These EPR signals resolved into the well-defined 1:1:1:1 quartet typical of the TEMP-superoxide adduct when a higher concentration of Co^{2+} (3 mM) was added (Fig. S17C).

Hydroxyl radical generation by Co^{2+} (Reaction 1) is much less thermodynamically favorable than the analogous reaction with iron (Reaction 2), since the $Co^{2+/3+}$ oxidation potential (−1.84 V) is significantly lower than that for $Fe^{2+/3+}$ (−0.77 V) [31]. This barrier is reflected in the DNA damage results, where only 2 μM of Fe^{2+} causes >90% DNA damage in the presence of H_2O_2 (50 μM) [53], whereas even with 50 μM Co^{2+} and a higher H_2O_2 concentration (400 μM), no significant DNA damage occurs. Our EPR results comparing $^{\bullet}OH$ generation by Co^{2+}/H_2O_2 and Fe^{2+}/H_2O_2 corroborate these DNA damage results.

Several mechanisms have been proposed to explain hydroxyl radical generation by Co^{2+}/H_2O_2 despite this thermodynamic barrier. Berg, et al. [62] suggested a more complex mechanism for $^{\bullet}OH$ generation that requires three equivalents of H_2O_2 to form a Co^{2+}-peroxo complex that decomposes into $^{\bullet}OH$ [63], as well as $^1O_2$ and $^{\bullet}OH$ generation by a cobalt-dioxygen complex [64]. Under our conditions, we see no evidence of $^1O_2$ formation in the Co^{2+}/H_2O_2/ascorbate system, but the ascorbyl radical is formed, which may contribute to the increase in DNA damage observed for Co^{2+}/H_2O_2/ascorbate compared to Co^{2+}/H_2O_2 conditions.

The effect of polyphenol addition on ROS formation was also examined using EPR spectroscopy. Adding MEPCA as a representative catechol-containing polyphenol compound that prevents Co^{2+}-mediated DNA damage to a Co^{2+}/H_2O_2 solution results in a sharp drop in the intensity of the DMPO-OH adduct resonance to almost unobservable levels, even at a Co^{2+}:MEPCA ratio of 2:1 (Fig. 6). Adding EGCG as a representative gallol-containing polyphenol under the same conditions also significantly reduces the DMPO-OH resonance. At a Co^{2+}:EGCG ratio of 2:1, the intensity of the DMPO-OH adduct decreases two-fold compared to its intensity without EGCG. The DMPO-OH resonance intensity decreases as the Co^{2+}:EGCG ratio decreases, until it is almost unobservable at Co^{2+}:EGCG ratios of 1:2 and 1:3 (Fig. S18).The ability of MEPCA and EGCG to reduce hydroxyl radical generation to almost unobservable levels is consistent with their ability to prevent cobalt-mediated DNA damage at low concentrations.

When added to a solution of Co^{2+} and H_2O_2, EGC has little effect on the DMPO-OH signal intensity (Fig. S19) and adding VA results in only a slight decrease in the DMPO-OH adduct resonance intensity (Fig. S20). The inability of EGC and VA to suppress $^{\bullet}OH$ generation even at the highest polyphenol concentrations correlates with their inability to prevent significant cobalt-mediated DNA damage.

When ascorbate is combined with Co^{2+} and H_2O_2, in the same ratios used for the DNA damage assays, resonances for DMPO-OH and $AscH^{\bullet}$ are observed (Fig. 7A). Upon MEPCA addition, both the DMPO-OH and $AscH^{\bullet}$ resonance intensities significantly decrease with little change in signal intensity beyond a 2:1 Co^{2+}:MEPCA ratio (Fig. 7B-E). VA also shows EPR results similar to those observed for MEPCA (Fig. S21). In contrast, when EGC or EGCG is added to the Co^{2+}/H_2O_2/ascorbate

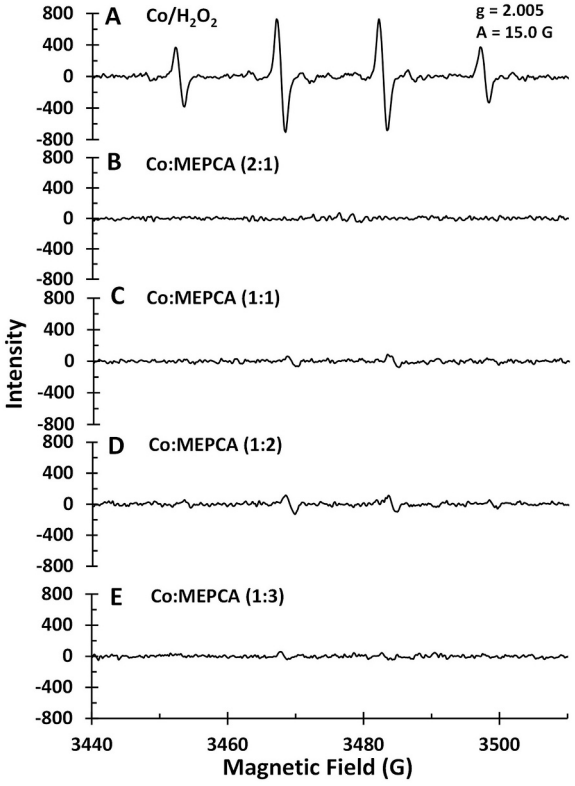

Fig. 6. A) EPR spectrum of Co^{2+}(300 μM) with H_2O_2 (22.5 mM). EPR spectra with H_2O_2 (22.5 mM) and Co^{2+}:MEPCA ratios of B) 2:1 (600 and 300 μM, respectively), C) 1:1 (both 300 μM), D) 1:2 (300 and 600 μM, respectively), and E) 1:3 (300 and 900 μM, respectively). All samples were in aqueous solution at pH 6.3 (MES, 10 mM) at room temperature.

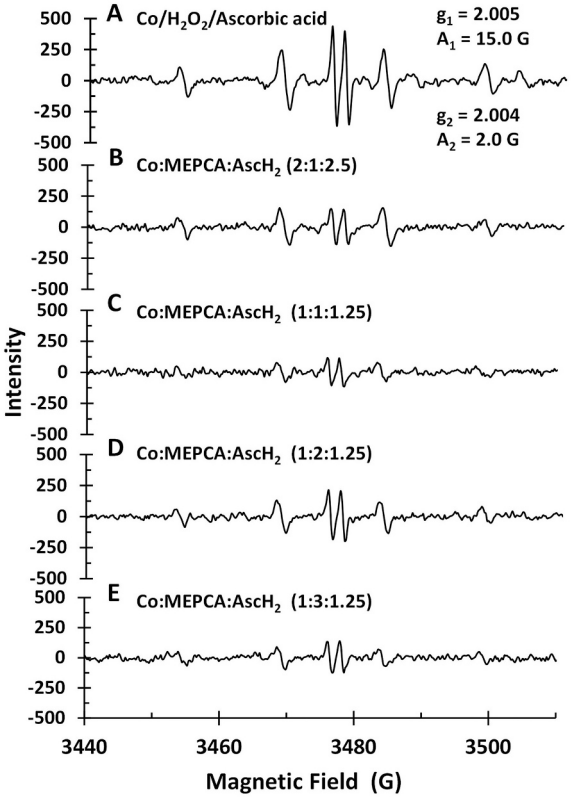

Fig. 7. A) EPR spectrum of Co^{2+}(300 μM) with H_2O_2. (22.5 mM) and ascorbate ($AscH_2$, 375 μM). EPR spectra with H_2O_2 (22.5 mM), ascorbate (375 μM), and Co^{2+}:MEPCA ratios of B) 2:1 (600 and 300 μM, respectively), C) 1:1 (both 300 μM), D) 1:2 (300 and 600 μM, respectively), and E) 1:3 (300 and 900 μM, respectively). All samples were in aqueous solution at pH 6.3 (MES, 10 mM) at room temperature. Values g_1, A_1 and g_2, A_2 correspond to DMPO-OH and ascorbyl radical signals, respectively.

system, the intensities of DMPO-OH and $AscH^{\bullet}$ resonances do not change over all Co^{2+}:polyphenol ratios (Figs. S22 and S23). Although polyphenols inhibit radical formation in the Co^{2+}/H_2O_2 system similarly to their ability to prevent cobalt-mediated DNA damage (Table 1), this same trend is not observed for the Co^{2+}/H_2O_2/ascorbate system. This unexpected effect could be due to the higher concentrations of reagents required for the EPR studies compared to the DNA damage assays that alter mechanisms of radical generation and/or Co^{2+}-polyphenol interactions in the presence of ascorbate. Formation of radical species by Co^{2+}/H_2O_2/ascorbate is complex, and further studies are necessary to determine the reactions that control ROS generation under these conditions.

3.4. Determination of Co^{2+}-polyphenol and Co^{2+}-ascorbate interactions

DNA damage prevention by polyphenols may result from Co^{2+}-polyphenol interactions rather than polyphenol ROS scavenging, and coordination of Co^{2+} to catechol and gallol compounds has been observed using UV–visible spectroscopy. Mono- and bis-catechol Co^{2+} species have characteristic UV–vis spectra [65], and Co^{2+} binding by gallic acid results in three absorption bands at 300, 389, and 675 nm [66]. Formation constants of Co^{2+}-pyrocatechol complexes were determined using spectrophotometric titrations at 276 nm with millimolar concentrations of Co^{2+} (1 mM) and pyrocatechol (1–3 mM) [67], significantly higher than those in our DNA damage assays. We used similar methods to investigate Co^{2+}-polyphenol binding in the presence of ascorbate. For these studies, only the low molecular weight polyphenols with single catechol and gallol groups were examined to avoid potentially complex stoichiometries resulting from metal binding to multiple phenolic sites on the same polyphenol. Co^{2+} (as $CoSO_4$) has no absorbance at wavelengths >230 nm, whereas ascorbate has an absorption band at 265 nm (Fig. S24). Polyphenol spectra show one absorption maximum for PREGA (273 nm) and GA (259 nm), two maxima for MEGA (266 and 294 nm), and two maxima at 250 and 290 nm for MEPCA, PCA, and VA (Figs. S25-S36), corresponding to polyphenol $\pi \rightarrow \pi^{\star}$ electronic transitions [65,68].

When PREGA, GA, MEGA, MEPCA, PCA, or VA are added to Co^{2+} alone or Co^{2+}/ascorbate solutions in Co^{2+}:polyphenol ratios of 1:1 to 1:5, no prominent bands are observed other than individual polyphenol or ascorbate absorptions. Difference spectra calculated by subtracting out the absorbances of the individual Co^{2+}, ascorbate if present, and polyphenol components at the various Co^{2+}:polyphenol ratios (1–5 equiv) showed no additional bands that could be unambiguously attributed to formation of cobalt-polyphenol complexes (Figs. S24-S47). In addition, the ascorbate absorbance obscures the most intense Co^{2+}-polyphenol complex absorption bands (270–300 nm), the most likely to be observed. Thus, we shifted to MALDI mass spectrometry to better detect polyphenol/ascorbate-Co^{2+} complexes.

Using mass spectrometry with the low-molecular weight polyphenols, aqueous solutions of Co^{2+} (33 μM) and the polyphenols (1 to 5 equiv., 33–167 μM) were combined with and without ascorbate (1.25 equiv., 42 μM). Co^{2+} binding was observed for all the tested polyphenols, in 1:2 Co:polyphenol stoichiometries for GA, MEGA, MEPCA, and PREGA and 1:3 stoichiometries for PCA, PREGA, and VA. Upon addition of ascorbate to these Co^{2+}/polyphenol solutions, molecular ion peaks for cobalt-polyphenol-ascorbate complexes are observed for MEPCA (in 1:3:1 Co:polyphenol:ascorbate stoichiometry), PCA (in 1:1:1 Co:polyphenol:ascorbate stoichiometry), and PREGA (in 1:2:2 and 1:3:1 Co:polyphenol:ascorbate stoichiometries; Table S15 and Figs. S48-S57). With ascorbate present, only Co^{2+}/polyphenol/ascorbate complexes are observed for the catechols MEPCA and PCA, whereas mass spectra with the gallol PREGA show formation of both the Co^{2+}/polyphenol and the Co^{2+}/polyphenol/ascorbate complexes.

Co^{2+}-polyphenol complexes readily form, with stability constants of $10^{7.5}$ to 10^{14} for bidentate CoL binding of catechol derivatives, $10^{5.3}$ to 10^{16} for CoL_2 complexes, and $10^{3.1}$ to $10^{4.3}$ for octahedral CoL_3 complexes [69–71]. This is consistent with our mass spectrometry results, where Co^{2+} formed 1:2 or 1:3 complexes with all the polyphenols. Although stability constants for Co^{2+}-gallol complexes are not reported, gallols have lower pK_as and therefore higher formation constants compared to analogous catechols, making gallols stronger metal-binding ligands at biological pH [53]. Stability constants for Co^{2+}-ascorbate binding range from $10^{5.6}$ to 10^{8}, depending upon ionic strength [72,73]. These similarities between Co^{2+}-ascorbate and -catechol stability constants agree with our mass spectrometry results, indicating that ascorbate competes with some polyphenols for Co^{2+} coordination under these conditions. This competition for cobalt binding is more prevalent for catechols than gallols and may be responsible for the greater efficacy of gallols compared to catechols in preventing Co^{2+}-mediated DNA damage.

3.5. Comparisons of cobalt-mediated DNA damage and polyphenol prevention

Cobalt-mediated DNA damage occurs in the presence of ascorbate and hydrogen peroxide in a synergistic manner within the range of Co^{2+} concentrations reported for in patients with prosthetic hip-associated cobalt toxicity (1–100 μM) [45]. In addition to our work, cobalt-mediated guanine base oxidation has been reported with Co^{2+} (up to 250 μM) and H_2O_2 (up to 2 mM) at pH 7.4 for 4 h [74], and DNA fragmentation occurs with Co^{2+} (50 μM) and H_2O_2 (2.5 mM) after 1 h [29]. Nackerdien, et al. [24] also observed significant DNA base oxidation upon treatment with Co^{2+} (25 μM) and H_2O_2 (2.8 mM) for 1 h that did not change upon ascorbate addition (100 μM) [24]. Other

investigations have reported DNA damage by Co^{2+} bound to chelating diethylene triamine pentaacetic acid (DTPA) [64,75] or ethylene diamine tetraacetic acid (EDTA) [24] ligands or have investigated the DNA-damaging ability of synthetic Co^{2+}-complexes [76–81]. The various conditions and endpoints for DNA damage used in these studies of cobalt-mediated DNA damage make comparing their results and potential biological relevance difficult, especially since the Co^{2+} and/or the H_2O_2 concentrations are significantly higher than the conditions described in this work (40 μM Co^{2+}, 400 μM H_2O_2, and 50 μM ascorbate). None of these prior investigations into cobalt-mediated DNA damage have closely examined a Co^{2+}/H_2O_2/ascorbate system or observed cobalt-related synergy in DNA damaging behavior.

Very few studies have examined the effects of polyphenol antioxidants on cobalt-mediated oxidative stress or DNA damage. In one, EGCG treated cells (50–200 μM for 60 min) (PC-12) challenged with $CoCl_2$ (150 μM) showed lower ROS levels and apoptosis [82]. Lower cellular ROS generation after Co^{2+} treatment was also observed upon treatment with GA (50 μM), MEGA (50 μM) and EGCG (100 μM), but only EGCG increased cell viability compared to cells treated with Co^{2+} (300 μM) and H_2O_2 (400 μM) for 24 h [83]. Similar results were observed in rat cortical neurons (E18-E19) pre-incubated with salidroside, a phenolic compound derived from glucose [84]. In addition, polyphenol-Co^{2+} binding to GA, catechin, and to a lesser degree, EGCG and tannic acid, was proposed as a mechanism for the reduction of ROS generated by Co^{2+}-H_2O_2-Se(IV) [85]. In an interesting report by Babich, et al. [86], EGCG and ECG treatment leads to higher H_2O_2 concentrations and cytotoxicity in human gingival epithelial–like S-G cells, but this toxicity is inhibited by Co^{2+} addition. Although Co^{2+}-polyphenol interactions were not directly examined, the observed reduction in cytotoxicity may be the result of Co^{2+}-polyphenol chelation that prevented polyphenol reduction of H_2O_2 to form $^{\bullet}OH$.

The antioxidant activity of polyphenols is attributed primarily to two mechanisms: metal chelation [36,53,87,88] and radical scavenging [88–90]. In our studies, polyphenol compounds prevent cobalt-mediated DNA damage, and gallol-containing polyphenols are more effective than catechol-containing polyphenols. Metal-mediated DNA damage prevention by polyphenols is highly dependent on the metal ion generating the damaging ROS (Table 1), and polyphenol-metal interactions play a significant role in this behavior. Although the trend of gallols being more effective than catechols holds true across cobalt-, iron-, and copper-mediated DNA damage prevention studies, striking individual differences in polyphenol efficacy are observed with different metal ions (Table 1). For example, EGC prevents Fe^{2+}-mediated DNA damage with an IC_{50} value of 9.8 μM [33], but prevents little Co^{2+}-mediated DNA damage, and *increases* Cu^{2+}-mediated DNA damage [36]. Generally, trends for polyphenol prevention of Co^{2+}- and Fe^{2+}-mediated DNA damage are more similar than those for Cu^{2+}-mediated DNA damage.

Since polyphenol prevention of Co^{2+}-mediated DNA damage does not correlate with oxidation potential ($R^2 = 0.15$; Fig. S15A), direct ROS scavenging is not the primary mode of antioxidant activity. In contrast, polyphenol activity is slightly correlated to the pK_a of the first phenolic hydrogen ($R^2 = 0.67$; Fig. S15B), as would be expected for a metal-binding mechanism, since polyphenol deprotonation is required for metal coordination. This correlation is not as robust for Co^{2+} as observed for polyphenol prevention of Fe^{2+}-mediated DNA damage ($R^2 = 0.91$) [53], where polyphenol-Fe^{2+} binding and subsequent autoxidation of Fe^{2+} to Fe^{3+} prevents hydroxyl radical formation (Reaction 2 [34]). Because Co^{2+} oxidation to Co^{3+} is less thermodynamically favored compared to $Fe^{2+/3+}$ oxidation and because Co^{2+} can participate in decomposition (Reaction 5 [91]) and generation of ROS (Reaction 4), it is unsurprising that its role in DNA damage and polyphenol prevention of this damage is complex.

$$H_2O_2 \rightarrow H_2O + O_2 \quad (5)$$

Ascorbate acts synergistically with Co^{2+} and H_2O_2 to generate ROS that cause DNA damage and interferes with Co^{2+}-catechol complex formation to hinder catechol prevention of cobalt-mediated DNA damage. Cobalt-generated oxidative damage and toxicity represents a human health concern, and our results suggest that the mechanisms underlying cobalt-mediated DNA damage and its prevention by polyphenols are complex. Nonetheless, many polyphenol compounds readily prevent Co^{2+}-mediated DNA damage at biological concentrations, representing a starting point to develop therapies for cobalt toxicity.

4. Conclusions

Excess Co^{2+} can result in toxicity, due to its ability to form ROS and cause oxidative damage. Although Co^{2+} toxicity has been attributed to $^{\bullet}OH$ generation by Co^{2+}, analogous to the one-electron reduction of H_2O_2 by Fe^{2+}, our results indicate that Co^{2+}-mediated DNA damage is caused by more complex mechanisms that involve $O_2^{\bullet-}$ and $^{\bullet}OH$, but not $^1O_2$, generation. Ascorbate plays an important role in this system: while a limited amount of $^{\bullet}OH$ is generated by Co^{2+} and H_2O_2 at high concentrations, this $^{\bullet}OH$ formation is not facile at lower Co^{2+} and H_2O_2 concentrations and results in insignificant DNA damage. Addition of ascorbate to the Co^{2+}/H_2O_2 system increases DNA damage in a synergistic manner.

Most polyphenol compounds reduce DNA damage by Co^{2+}/H_2O_2/ascorbate. Trends in polyphenol prevention of metal-mediated DNA damage are cobalt-dependent, suggesting that Co^{2+}-polyphenol binding plays a role in the observed antioxidant effects. Mass spectrometry studies indicated that only Co^{2+}-polyphenol complexes form without ascorbate addition, but that ascorbate competes with primarily catechol-containing polyphenols for Co^{2+} binding. Additional experiments to further explore the effect of Co^{2+}-polyphenol interactions on ROS generation and DNA damage prevention are required to fully understand this complex system, but this work establishes polyphenols as potential treatments for cobalt toxicity.

Author contribution statement

J.L.B. and C.A.-M. conceived of the presented research, conducted the DNA damage and EPR studies, and wrote and primarily revised the manuscript.

Undergraduates J.M., P.A.S., and J.H. and graduate student A.A.E.G. primarily conducted the UV–vis spectroscopy and mass spectrometry studies. They also provided assistance with some of the EPR studies and manuscript revisions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data including DNA gel data and EC_{50}/IC_{50} graphs, electron paramagnetic resonance (EPR) spectra, UV–vis spectra, and mass spectrometry data. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2022.112024.

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