Metal Ions Modify in Vitro DNA Damage Yields With High-LET Radiation

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Abstract

Cu^{2+} and Co^{2+} are metals known to increase DNA damage in the presence of hydrogen peroxide through a Fenton type reaction. We hypothesized that these metals could increase DNA damage following irradiations of increasing LET values as hydrogen peroxide is a product of the radiolysis of water. The reaction mixtures contain either double- or single-stranded DNA in solution with Cu^{2+} or Co^{2+} and was irradiated either with X-ray, carbon-ion or iron-ion beams or was treated with hydrogen peroxide or bleomycin at increasing radiation dosages or chemical concentrations. DNA damage was then assessed by gel electrophoresis followed by band intensity analysis. DNA in solution with metals demonstrated the most DNA damage when treated with hydrogen peroxide followed by irradiation with low-LET (X-Ray), high-LET (carbon-ion and iron-ion), respectively, and demonstrated the least damage with treatment of bleomycin. Cu^{2+} portrayed greater DNA damage than Co^{2+} following all experimental conditions. The metals effect caused more DNA damage and was observed to be LET dependent for single-strand break formation but inversely dependent for double-strand break formation. These results suggest that Cu^{2+} is more efficient than Co^{2+} at inducing both DNA single-strand and double-strand breaks following all irradiations and chemical treatments.

Introduction

The major actor responsible for radiation induced cell death is DNA double-strand breaks (DSBs), from localized single-strand breaks (SSBs). Generally, 1 Gy of radiation produces 20-40 DSBs and a few thousands of SSB in cells [1]. Radiation-induced DNA damages are caused by direct action or indirect action through the radiolysis of water. X-rays are known as low-LET (Linear energy transfer) radiation and sparsely ionizing; [2] a large amount of DNA damage is believed to be contributed by hydroxyl radical mediated indirect action. However, radiation induced cell death is not due to individual hydroxyl radicals but by the interaction of radicals at high density near DNA causing locally multiply damaged sites (LMDS) [3]. Indeed, high-LET radiation such as neutrons and heavy charged particles, are known to be densely ionizing and produce higher biological effectiveness [2]. There is an increase in direct action contribution for high-LET induced DNA damage through stronger electromagnetic interactions with the DNA molecule [4, 5]. Furthermore, dense ionization produced by high-LET radiation creates high radical concentrations from the radiolysis of water leading to more radial-radical reactions and resulting in the formation of greater G-values of hydroxyl radicals [6].

Yields of radicals in solution can be affected by factors including pH, temperature, solvents and substrates [7, 8]. As the hydroxyl radical scavengers, such as dimethyl sulfoxide (DMSO), attenuate radiation effects [9], the amount of hydroxyl radicals can interfere with DNA damage yields. This is because metal ions and hydrogen peroxide can cause Fenton reactions thus producing greater amounts of hydroxyl radicals [10]. Therefore, the interaction among radicals can be interfered with by adding metal ions and may maximize the indirect action of radiation to enhance the radiation effects on DNA damage. Superoxide dismutase and Catalase are endogenous enzymes that reduce final hydroxyl radical

formation [11]. Interestingly, another DNA DSB-inducing agent, bleomycin, has a metal binding domain and requires metals and oxygen to cleave DNA [12-15]. Bleomycin and the metal complex create radicals and contribute to DNA damage. Various studies support adding different metals to increase radiation or drug induced hydroxyl radical formation and DNA breaks [7, 16-19].

In order to better understand the underlying mechanisms behind DNA damage induced by hydroxyl radicals in high-LET radiation and investigate potential sensitization through enhancement of indirect action, we took advantage of Fenton-type reactions utilizing the metals copper and cobalt. We hypothesized that the copper ions and cobalt ions would be useful chemicals to increase DNA strand breaks. To test our hypothesis, we used *in-vitro* single- and double-stranded DNA and three different qualities of radiation with different LET values and two major chemicals to cause DNA damage to assess the metal effect for DNA damage.

Results

3.1. DNA DSB by ionizing radiation and metals

DSB formation yield measured with *in-vitro* dsDNA from lambda phage was higher in low-LET X-ray and decreased as LET value increased in carbon-ion and iron-ion, respectively. D_{50} values (dose to achieve 50% intact DNA) were achieved with 44, 142, and 299 Gy, respectively. X-ray irradiation produced significant increase of DSB with both metals compared to control. Carbon-ion irradiation produced significant increase in DSB with adding Cu^{2+} or Co^{2+} compared to control. Adding Cu^{2+} produced a significant increase in DSB formation compare to adding Co^{2+} (**Figure 1b**). Finally, iron-ion irradiation demonstrated an increase in DSB with adding both metals, most notably with Cu^{2+}, but this observed difference was not found to be significant under all experimental dosages (**Figure 1c**).

$\mathbf{D_{50,\,DSB,}}$ demonstrated fold increases of efficiency of DSB with addition of metal ions (**Table 1).**$\mathbf{D_{50,\,DSB}}$ values with adding Cu^{2+} were smaller than adding Co^{2+} for all tested radiation. Therefore, adding Cu^{2+} was more efficient at increasing DSB yields for tested radiation than adding Co^{2+}.

3.2. DNA SSB by ionizing radiation and metals

Induction of SSB was measured with *in-vitro* ssDNA originated from M13 bacteriophage. As DSB, X-ray was the most efficient to cause SSB as $\mathbf{D_{50,\,SSB}}$ values of 57 Gy among tested radiation. $\mathbf{D_{50,\,SSB}}$ values of high-LET radiation carbon-ion and iron-ion were 130 Gy and 160 Gy, respectively. Irradiation of X-ray was observed to produce a significant increase of SSB with adding Cu^{2+} and Co^{2+}. Adding Cu^{2+} and Co^{2+} showed significant increase of SSB formation compared to control (**Figure 1d**). Carbon-ion irradiation produced a significant increase of SSB formation with adding both metals compared to control (**Figure 1e**). Then, iron-ion irradiation also produced a significant increase of SSB formation with both metals (**Figure 1f**).

$D_{50, SSB}$ values demonstrated fold increases of efficiency of SSB with addition on metal ions (**Table 1**). $D_{50, SSB}$ values with adding Cu^{2+} were smaller than adding Co^{2+} for all tested radiation. Therefore, adding Cu^{2+} was more efficient at increasing DSB yields for tested radiation than adding Co^{2+}.

3.3. DNA DSB by bleomycin and hydrogen peroxide with metals

To induce DNA damages, bleomycin or hydrogen peroxide (H_2O_2) were treated with DNA at room temperature for 30 min. Bleomycin is known to produce DSB and SSB [15]. Hydrogen peroxide produces SSBs [10]. Bleomycin presented concentration dependent DSB formation up to 2000 μg/mL with $D_{50, DSB}$ value of 66.04 μg/mL (**Figure 2a**). At 20 μg/mL, adding both metals enhanced DSB formation. Interestingly, from 100 μg/mL of bleomycin, adding Co^{2+} was observed to switch from sensitization to protection in comparison to control.

Hydrogen peroxide up to 10 mM did not produce significant amount of DSB without metals. Based on regression curves, $IC_{50, DSB}$ was calculated as 20 mM. By adding either metal ions, hydrogen peroxide treatment was observed to produce a significant increase of DSB formation (**Figure 2b**).

$IC_{50, DSB}$ values demonstrated fold increases of efficiency of DNA breaks with addition of metal ions (**Table 1**). Although $IC_{50, DSB}$ values of bleomycin were decreased slightly with metals, $D_{50, DSB}$ values of hydrogen peroxide were severely decreased from 20074 μM to 21.94 μM (Cu^{2+}) and 177 μM (Co^{2+}). Therefore, bleomycin had minimum effects from metal ions to form DSB. On the other hand, hydrogen peroxide got severely affected by adding metals and copper ions affected stronger than cobalt ions for hydrogen peroxide.

3.4. DNA SSB by bleomycin and hydrogen peroxide with metals

Bleomycin produced SSB in the concentration dependent manner. As with DSB formation, bleomycin demonstrated a shift from sensitivity to protection with adding Co^{2+} in SSB formation above 10 μg/mL. Adding Cu^{2+} was observed to increase the sensitivity of bleomycin compared to control but these decreases in SSB formation were not observed to be significant (**Figure 2c**).

Hydrogen peroxide produced dose dependent SSB formation up to tested 10 μM. A significance increase in SSB was observed with adding Cu^{2+} at the lowest tested concentration of 100 pM. Adding cobalt ions increased SSBs but the effect was much smaller than adding copper (**Figure 2d**).

$IC_{50, SSB}$ values demonstrated fold increases of efficiency of DNA breaks with addition of metal ions (**Table 1**). Although $IC_{50, SSB}$ values of bleomycin showed a few times changes by adding metal ions, $D_{50, SSB}$ values of hydrogen peroxide were dramatically decreased with addition of metals. Therefore, bleomycin had minimum effects from metal ions to form SSB. On the other hand, hydrogen peroxide got severely affected by adding metals and copper ions affect was much stronger than with cobalt ions.

3.5. Metal enhancement ratio for DNA break formation

Metal enhancement ratio's (MER) were calculated from the **D_{50}** and **IC_{50}** values from **Tables 1** and described in materials and methods section. For ionizing radiation with dsDNA, the MER demonstrated a fold reduction of 5.18x, 5.09x, and 3.74x with Cu^{2+} and 2.84x, 1.77x, and 1.6x with Co^{2+} following X-ray, carbon-ion, or iron-ion, respectively. Bleomycin showed the smallest MER values. The MER demonstrated a fold reduction of 914.94x with Cu^{2+} and 113.41x with Co^{2+} following H_2O_2 (**Figure 3a**). For ionizing radiation with single strand DNA, the MER demonstrated a fold reduction of 18.37x, 21.96x, and 22.92x with Cu^{2+} and 5.97x, 2x, and 1.57x with Co^{2+} following X-ray, carbon-ion and iron-ion, respectively. The MER values for SSB were greater values than for DSB. Bleomycin showed the smallest MER values. The MER demonstrated a fold reduction of 110493x with Cu^{2+} and 15.15x with Co^{2+} following H_2O_2. (**Figure 3b**). Therefore, metal ions affected both SSB and DSB formation induced by tested chemicals and radiation. Hydrogen peroxide affected DNA damage the most followed by low-LET radiation and high-LET radiation and bleomycin affected the least. Copper ions presented stronger effects than cobalt ions. SSB formation was more enhanced by metal ions than DSBs. Metal effects for DNA damage was LET dependent for SSB formation but inversely dependent for DSB formation.

Discussion

In the present study, we observed that the induction of DSBs and SSBs reduced with increasing LET values as low-LET X-ray irradiations were the most efficient inducers of DNA strand breaks per dose (Gy) followed by the high-LET carbon-ion and iron-ion beams, respectively (**Table 1**). These results are in agreement with prior research with cell lines which indicates that the number of SSBs produced is estimated to be lower in high-LET radiation [2]. In contrast to our results, other prior studies have demonstrated an increase in DSBs with increased LET in cells [23, 24]. It is hard to compare directly our *in-vitro* DNA analysis and cells because DNA in cells has chromatin structure and are not naked forms because chromatin structure protects DNA from radiation [25].

In presence of Cu^{2+} or Co^{2+}, increase in the amounts of DSBs and SSBs were observed for both low- and high-LET radiation (**Figure 1**). Interestingly, as the LET increased the fold reduction of MER values decreased for both metals in dsDNA, as well as, with cobalt in ssDNA but increased for copper in ssDNA (**Figure 3**). A possible explanation for these observed results may be due to copper's ability over cobalt's to more efficiently interact with the hydrogen peroxide produced from the high-LET irradiations via Fenton-type reaction and resulting in the production of more hydroxyl radical formation, thus increasing the amounts of more randomly distributed SSBs (**Figure 4**). Moreover, metal ions are essential for the formations of strand breaks with hydrogen peroxide through Fenton-type reaction to produce hydroxyl radicals (**Figure 2b, d**) [10], as well as, site-specific mechanisms in the formation of DSBs mediated by Cu^{2+} Fenton reactions [7] (**Figure 4**). Our results also agreed with prior research that the Cu^{2+} -Fenton system generated a higher total yield of DNA lesions than with the Co^{2+} -Fenton system [19].

Importantly, although coppers increased ability to induce hydroxyl radical ions through Fenton-type reaction, mammalian cells are not efficiently killed by SSBs caused by hydroxyl radicals from hydrogen peroxide. Hydroxyl radicals induced singly damaged sites are efficiently and accurately repaired by cellular repair mechanisms in contrast to hydroxyl radicals produced via the radiolysis of water causing LMDS which are much more difficult for the cell to repair [26]. This is most likely due to DSBs being the major actor responsible for radiation-induced cell death which occur from localized SSBs [27]. Although, as our hydrogen peroxide results were consistent with our ionizing radiation results in which copper in solution with DNA not only increased SSBs but DSBs as well, may suggest that the hydrogen peroxide produced from the ionizing radiation, importantly from our high-LET radiation sources, was in close enough proximity to the DNA that the Fenton-type reaction production of hydroxyl radical ions is capable of inducing these LMDS. Future experiments examining direct plasmid analysis to confirm how copper influences high-LET radiation-induced DNA damage and their complexity would be beneficial to further support this reasoning [19].

Finally, we observed how copper and cobalt influence DNA breaks with bleomycin, a radiomimetic drug that binds with a metal and uses molecular oxygen to induce DSBs [28]. Prior studies have suggested that Cu^{2+}-bleomycin complex is inactive in the degradation of DNA [31, 32] well others have demonstrated that the Cu^{2+}-bleomycin complex does indeed produce DNA strand scission [33, 34]. Our results agree with the latter, in which copper ions in solution with bleomycin and DNA was observed to more efficiently induce DSBs and SSBs than in solution with control (**Figure 2a, c**). On the other hand, we observed a concentration depended switch from sensitization to protection for Co^{2+} with bleomycin in both dsDNA and ssDNA. A possible reasoning behind this may be from the fact that it has been established that Co^{2+}-bleomycin binds efficiently only at certain sites of DNA and that cleavage does not occur at all bound sites [35]. Furthermore, our observed results may also be explained as prior studies have demonstrated that as the ratio of DNA to Co^{2+}-bleomycin complex increases, the Co^{2+}-bleomycin complex becomes resistant to oxidation [36-39]. The MER values of bleomycin were the smallest among tested agents with metals. This suggested that bleomycin induced DNA damage is not strongly associated with hydroxyl radicals.

The present work demonstrates that copper and cobalt may be useful tools to enhance the indirect action of DNA damage for high-LET irradiations. Both metals were observed to be capable of increasing DNA strand breaks following irradiation. However, copper was observed to be more efficient than cobalt at inducing these strand breaks. We propose the mechanism behind this observation is due to their interaction with hydrogen peroxide produced from the radiolysis of water via Fenton-type reaction. This was supported as we also observed this increase in DNA strand breaks with the metals in solution with hydrogen peroxide and DNA. These results suggest a possible mechanism of enhancement for the indirect action of DNA damage produced by high-LET radiation.

Materials And Methods

2.1. Irradiation conditions

Low-LET irradiations were conducted utilizing the X-ray generator TITAN 320 (200 kVp, 20 mA, 5 mm aluminum and copper filter). X-ray exposure rate was 3.1 Gy/min and LET value of 2 keV/μm **[40]**. For high-LET heavy ion irradiations, spread out Bragg-peak (SOBP) carbon-ions and monoenergetic iron-ions were accelerated to 290 and 500 MeV/n, respectively, using HIMAC. Dose rates for carbon-ions and iron-ions were set at 5 and 10 Gy/min, respectively. SOBP carbon-ions and monoenergetic iron-ions contained a LET value of 50 and 200 keV/μm, respectively [40].

2.2. DNA solution preparation and chemical treatment

A total of 10 μL of reaction solution was used containing 30 ng of double strand DNA (dsDBA) of lambda phage (New England BioLabs Inc, Ipswich, MA, USA, stock concentration of 500 ng/μL) or 83 ng of single strand DNA (ssDNA) of M13mp18 phage (New England BioLabs Inc, stock concentration of 250 ng/μL) with 10 mM Tris-HCl pH7.71 with or without 0.2 mM of $CuCl_2$ or $CoCl_2$.

For chemical treatment experiments, hydrogen peroxide or bleomycin was added to a total of 10 μL of reaction solution with or without metals. Once solution was made, they were incubated at 37°C for 30 min. Following irradiation or chemical treatment incubation 1mM EDTA was added to chelate excess metals within the solution and incubated at room temperature for 5 min [7].

2.3. Electrophoresis and DNA damage quantification

Electrophoresis was carried out as previously described [41-43]. Each sample was added with 6X loading dye (15% Ficoll (w/v), 10% glycerol (v/v), 0.25% bromophenol blue (w/v), and 0.25% xylene cyanol FF (w/v) in distilled water) and electrophoresis was carried out with an 1% (w/v) Agarose gel in 1X TAE buffer containing 0.01% (w/v) ethidium bromide and ran at 100 V and for 60 min in 1x TAE buffer. After electrophoresis and destained in distilled water, gels were imaged and band intensity measurements were processed. Band intensities at intact DNA were then normalized to control that were not irradiated or treated without chemicals and calculated fraction of intact DNA after irradiation or chemical treatment.

D_{50} and IC_{50} values, dose or chemical concentration required to produce 50% intact DNA, were determined using regression curves generated by GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). From these values, Metal enhancement ratios (MER) were calculated from D_{50} (IC_{50}) of control values divided by D_{50} (IC_{50}) of tested agents values.

2.4. Statistical analysis:

All experiments were carried out with three or more independent experiments. Data points were expressed as a mean with standard errors of the means. All experimental data were analyzed via GraphPad Prism 8. One-way analysis of variance (ANOVA) and Turkey's multiple comparison test was conducted for statistical significance. Differences with *P* values of less than 0.05 were considered statistically significant.

Declarations

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Tables

Table 1. D_{50} and IC_{50} values, radiation doses (Gy) or chemical concentration required for 50% intact DNA with or without metal ions.

	Control	Cu^{2+}	Co^{2+}
DSB			
X-ray	44 Gy	8.5 Gy	15.5 Gy
Carbon-ion	142.9 Gy	28.1 Gy	80.9 Gy
Iron-ion	299.1 Gy	80 Gy	187 Gy
Bleomycin	66 μg/mL	42.3 μg/mL	56 μg/mL
H_2O_2	20074 μM	22 μM	177 μM
SSB			
X-ray	57.5 Gy	3.1 Gy	9.6 Gy
Carbon-ion	129.8 Gy	5.9 Gy	65.0 Gy
Iron-ion	160.0 Gy	7.0 Gy	102.0 Gy
Bleomycin	12.3 μg/mL	6.5 μg/mL	37 μg/mL
H_2O_2	17237 nM	0.156 nM	1138 nM

Figures

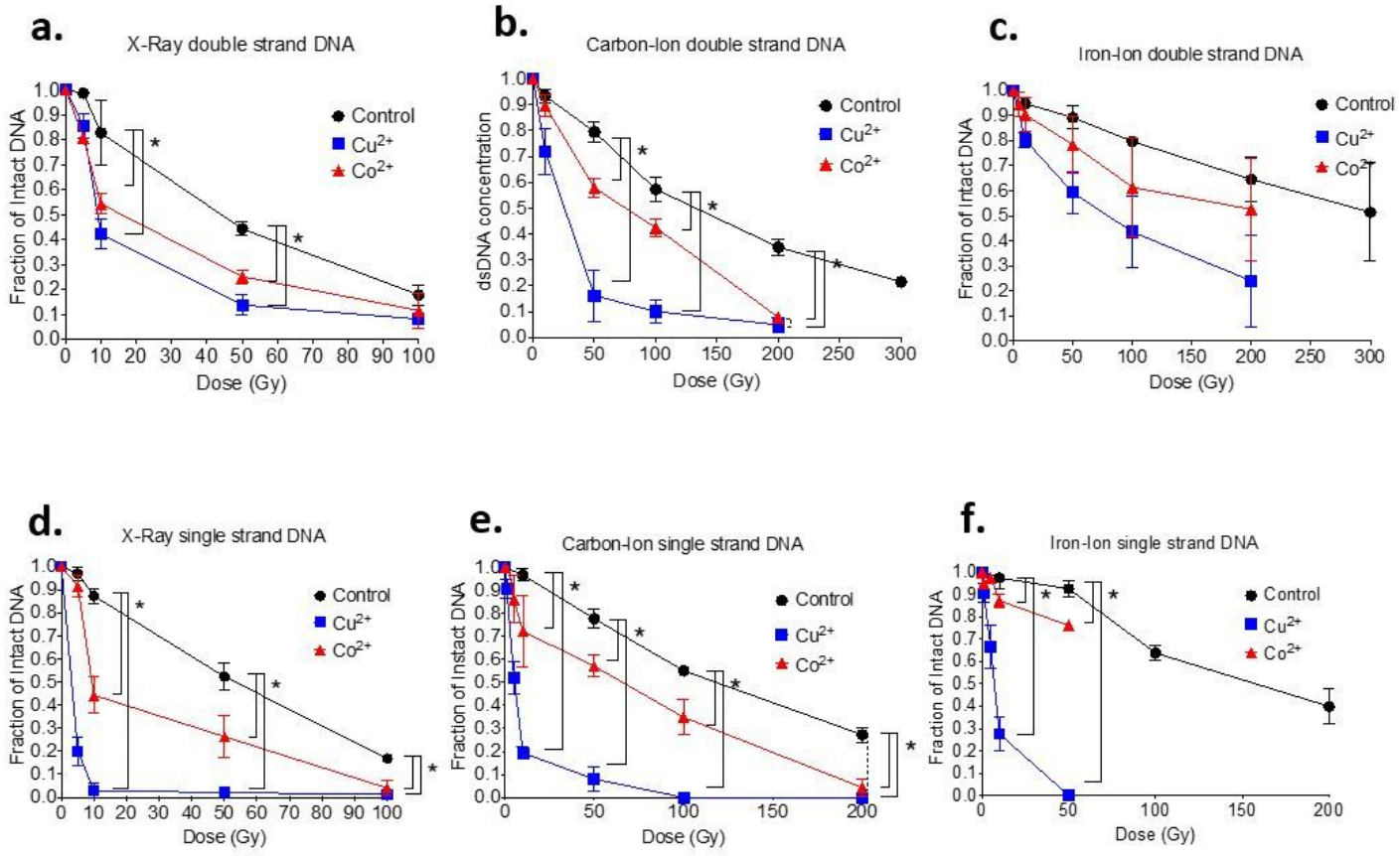

Figure 1

Cu2+ and Co2+ effect on DNA DSBs and SSBs at increasing radiation dosage (Gy) with radiation sources of increasing LET values. (a) Low-LET X-ray for DSBs; (b) High-LET carbon-ion for DSBs; (c) High-LET iron-ion for DSBs; (d) Low-LET X-ray for SSBs; (e) High-LET carbon-ion for SSBs; (f) High-LET iron-ion for SSBs. Error bars indicate standard error of the means from at least three independent experiments. * indicates statistical differences ($P < 0.05$).

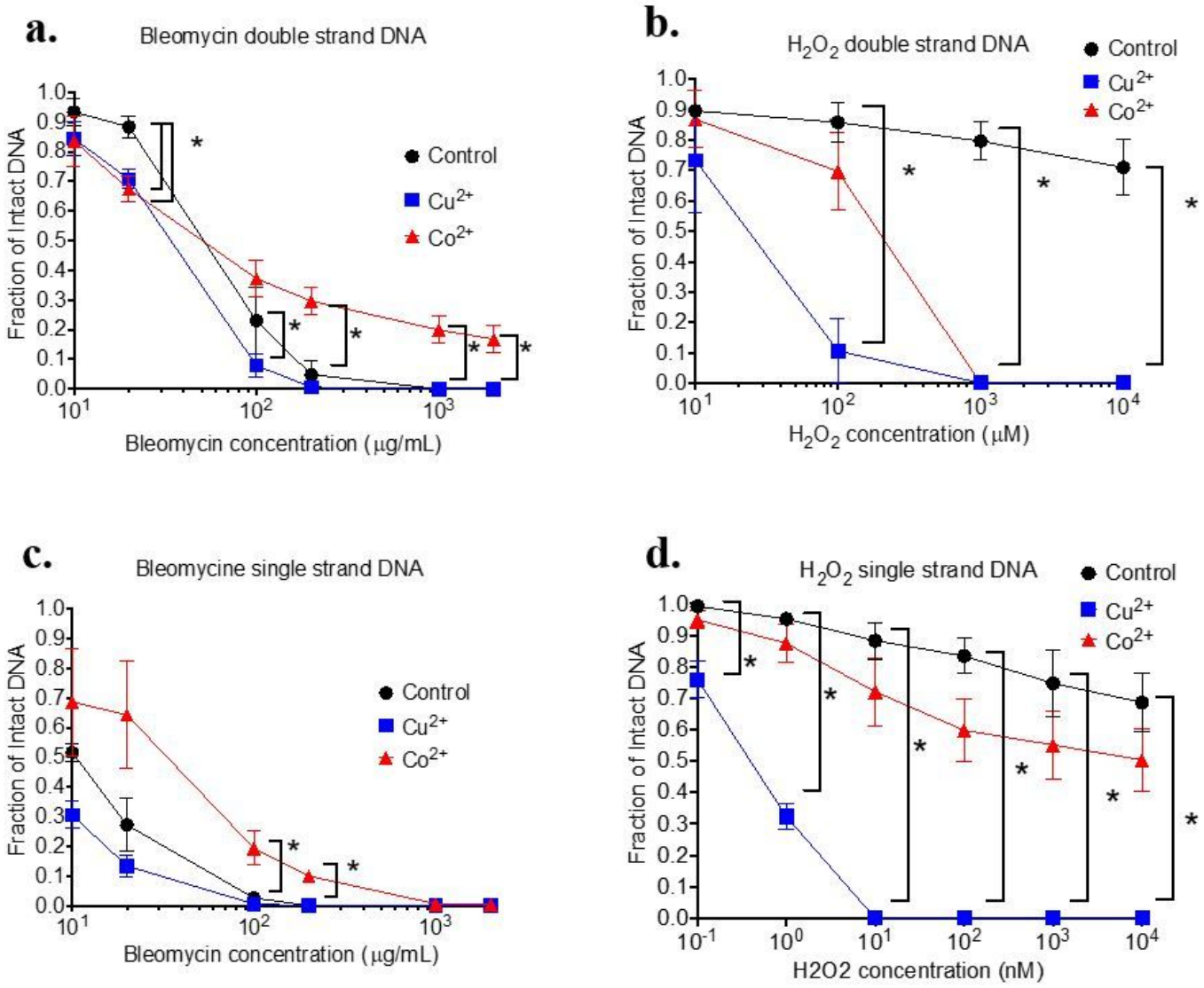

Figure 2

Cu2+ and Co2+ effect on DNA DSBs and SSBs at increasing drug concentrations of either Bleomycin or hydrogen peroxide. (a) Bleomycin for DSBs; (b) hydrogen peroxide for DSBs; (c) Bleomycin for SSBs; (d) hydrogen peroxide for SSBs Error bars indicate standard error of the means from at least three independent experiments. * indicates statistical differences ($P < 0.05$).

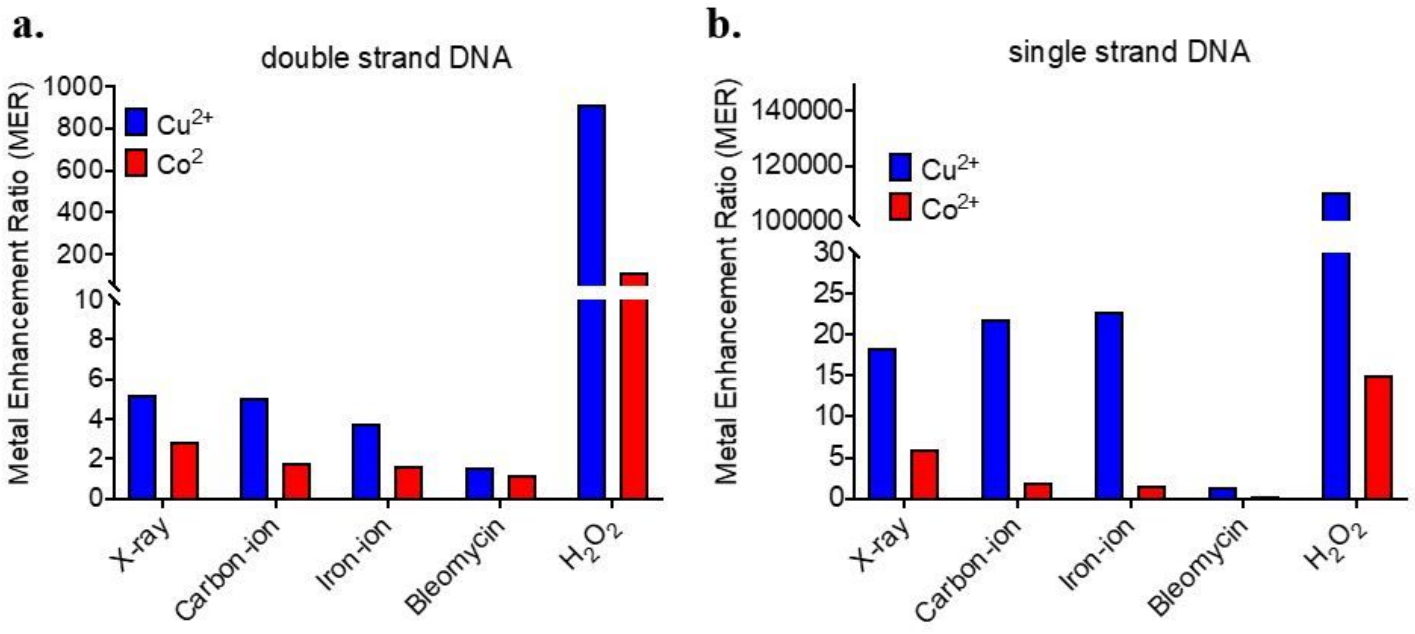

Figure 3

Metal enhancement ratio (MER) for DNA break formation comparison between ionizing radiation and chemical treatment with metals in solution with DNA (a) dsDNA; (b) ssDNA.

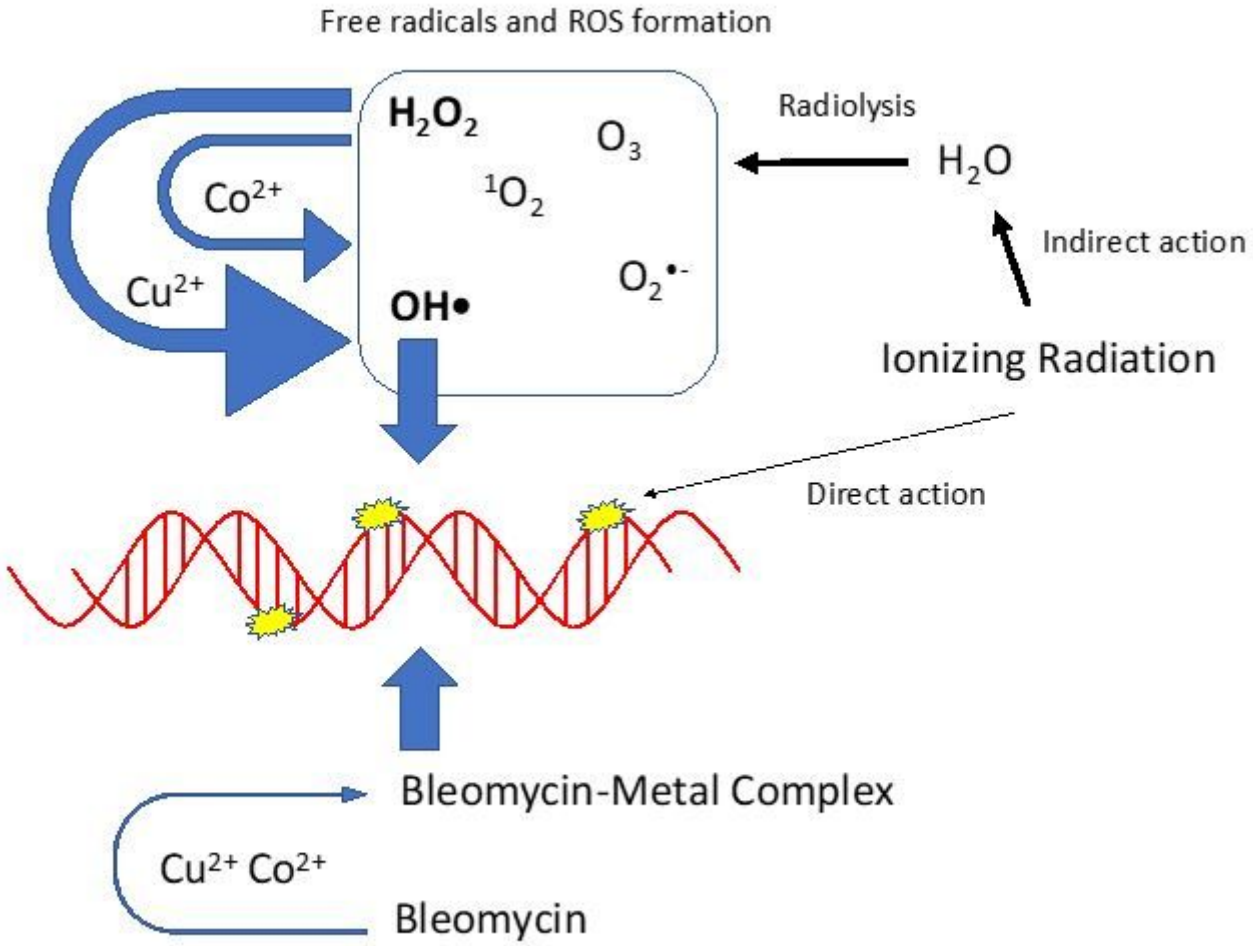

Figure 4

Proposed mechanisms of metal enhancement of ionizing radiation, bleomycin and H2O2. Thickness of arrows associated with the degree of effects.