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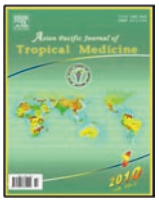

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Transitional metal ions induced damage to biomolecules: role of ferryl and perferryl radicals

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ABSTRACT

Objective: To investigate the effect of oxidized transitional metal (ferric and cupric) ions on the amino acids. **Methods**: 25 mmol/L hydroxyproline and 25 mmol/L histidine were incubated with 50 μL Fe^{3+} and Cu^{2+} ions at pH 7.4 and 37 ℃ for 30 mins in separate test tubes. Then 500 μL of 1% thiobarbituricacid (TBA) was added to the incubated amino acids followed by addition of 500 μL of glacial acetic acid. The resultant mixture was vortexed and heated at 100 ℃ for 30 min. Absorbance readings were noted after cooling to room temperature. The experiment was repeated in the presence of various reagents, like hydroxyl radical scavengers, antioxidant enzymes, and reducing agents and metal ion chelators. **Results**: The pink chromogen formed with the absorbance maxima at 524 nm, AND shifted to 560 nm in alkaline pH. The absorbance was expressed as TBA-adduct in MDA units. The TBA-adduct decreased in the presence of reducing agents and metal ion chelators. Antioxidant enzymes and hydroxyl radical scavengers did not show any effect. **Conclusion**: Transitional metal ions in their oxidized state showed significant damage to amino acids, hydroxyproline and histidine. The results indicate the possible role played by high-valent oxo-iron species, ferryl and perferry radicals in damaging biomolecules.

1. Introduction

Transitional metal ions are well known agents causing oxidative damage to biomolecules[1]. There are several reports on iron and copper mediated damage to various biomolecules [1,2]. Irradiation of aqueous solutions produces highly reactive radicals like $^{\cdot}OH$, $HO_2^{\cdot}$, $O_2^{-\cdot}$, $H^{\cdot}$, that can attack all biological molecules [3]. Several transitional metal ions react with H_2O_2 to form $^{\cdot}OH$, ferrous ions react with H_2O_2 to form $^{\cdot}OH$ by well known Fenton reaction [1].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (1)$$

(Fenton reaction)

$$Fe^{3+} + O_2^{-\cdot} \rightarrow Fe^{2+} + O_2 \quad (2)$$

(O_2 reducing the iron salts)

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe catalyst}} O_2 + OH + OH^- \quad (3)$$

(Haber-Weiss reaction)

Cu^+ salts react with H_2O_2 to form $^{\cdot}OH$ radicals with a much greater rate constant than Fe^{2+} salts[1].

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\cdot}OH + OH^- \quad (4)$$

In fact, Fenton chemistry is far more complex, and it was proposed that the initial product of reaction 1 may be an oxo-iron complex, possibly ferryl radical, which then decomposes to form $^{\cdot}OH$[4].

$$Fe^{2+} + H_2O_2 \rightarrow FeOH^{3+}(\text{or } FeO^{2+}) \rightarrow {}^{\cdot}OH + Fe^{3+} \quad (5)$$

Ferric salts in the presence of superoxide ($O^{-\cdot}$) produce another oxo-iron radical, perferyl intermediate.

$$Fe^{3+} + O_2^{-\cdot} \leftrightarrow [Fe^{3+}-O_2^- \beta\, Fe^{2+}-O_2]\, \beta\, Fe^{2+} + O_2 \quad (6)$$

In case of lipids, ferrous iron reacts with lipid hydroperoxides to give alkoxy radicals.

$$Lipid-O_2H + Fe^{2+}-complex \rightarrow Fe^{3+}-complex + OH^- + lipid-O^{\cdot} \quad (7)$$

With ferric iron a peroxy radical will form.

$$Lipid-O_2H + Fe^{3+}-complex \rightarrow lipid-O_2^{\cdot} + Fe^{2+}-complex \quad (8)$$

Both alkoxy and peroxy radicals stimulate the chain

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reaction of lipid peroxidation[1].Although all transitional metal ions can catalyze lipid peroxidation, most attention has been paid to iron, as interest in copper is increasing [5].

In all previous works, the damage to biomolecules catalyzed by transitional metal ions were studied in their reduced state or they were tried to be kept under reduced state by addition of reducing agents [5-7]. Therefore, one can conclude that the oxidized transitional metal ions are non-toxic to biomolecules in the absence of reducing agents.

Studies proving the toxicity of oxidized transitional metal ions in free state, particularly iron and copper, and damage caused by them to biomolecules in the absence of reducing agents is little.

In the current paper, we have investigated the effect of ferric and cupric ions on the amino acids hydroxyproline and histidine under various in vitro conditions. The results obtained are surprising and rise serious questions about the mechanism of transitional metal ion catalyzed damage to biomolecules.

2. Materials and methods

Hydroxyproline, histidine, thiobarbituricacid (TBA), superoxide dismutase (SOD), catalase, reduced glutathione (GSH) were obtained from Sigma chemicals company, St Louis, MO, USA. All other reagents were of analytical grade. Double distilled deionised water was used throughout the study. All glasswares wERE cleaned with warm concentrated nitric acid and thoroughly rinsed with double distilled water before experiments.

Stock solutions of 1 mmol/L of Fe^{3+}, 1 mmol/L of Cu^{2+}, 50 mmol/L hydroxyproline, 50 mmol/L histidine, 100 mmol/L ascorbate, 100 mmol/L ethelenediaminetetraaceticacid (EDTA), 1 mol/L dimethylsulfoxide (DMSO), 50 mmol/L H_2O_2 and 1 mmol/L reduced glutathione (GSH) were prepared in NaH_2PO_4/Na_2HPO_4 buffer at pH 7.4. Catalase reagent was prepared in phosphate buffer at pH 7.0 by dissolving 1 mg of catalase powder (containing 2 200 U/mg) in 1 mL of buffer. SOD reagent was prepared by dissolving 1 mg of SOD powder (containing 3 600 U/mg) in 1 mL of phosphate buffer of pH 7.4. 1 mmol/L stock solutions of ferric ammonium sulfate and copper sulfate were prepared in distilled water and prepared fresh before use. 1% TBA was prepared in 50 mmol/L NaOH. Genesys 10 UV spectrophotometer, Kontron 1 mL quartz cuvette, Metler 120-weighing balance and Elico L1-120 pH meter were used throughout study.

500 μL of 25 mmol/L hydroxyproline was incubated with 1 mmol/L of 50 μL Fe^{3+} or 1 mmol/L of Cu^{2+} ions, after volume was made to 1 000 μL by adding double distilled deionized water, at at 37 ℃ for 30 minutes in separate test tubes. Then 500 μL of 1% TBA was added to the incubated sample followed by addition of 500 μL of glacial acetic acid. The resultant mixture was vortexed and heated at 100 ℃ for 30-minute[3]. After cooling to room temp the absorption maxima of the chromogen formed was recorded and the absorbance readings were noted in that wavelength. The TBA-adduct formed is expressed in malaondialdehyde (MDA) units using extinction co-efficient 1.56×10^{-5} mentioned elsewhere[8].

500 μL of hydroxyproline was incubated with 100 μL of citric acid or 100 μL of mannitol or 100 μL of DMSO or 200 μL of H_2O_2 or 100 μL of ascorbate or 100 μL of GSH or 20 μL of catalase or 20 μL of SOD in different test tubes. Two sets of such tubes were prepared and each was incubated with 50 μL of Fe^{3+} and 50 μL of Cu^{2+}, respectively and volume in each test tube is made to 1 000 μL by adding double distilled de-ionized water, and then tubes were incubated at 37 ℃ for 30 minutes. Then 500 μL of 1% TBA was added to the incubated sample followed by addition of 500 μL of glacial acetic acid. The resultant mixture was vortexed and heated at 100 ℃ for 30 minutes[3]. After cooling to room temp the absorbance readings were noted. The above procedure was repeated with 500 μL of 25 mmol/L histidine. Appropriate blanks were always prepared. Increasing volume (100 μL to 800 μL) of 25 mmol/L hydroxyproline were incubated with fixed volume (50 μL) of 1 mmol/L Fe^{3+} and 1 mmol/L Cu^{2+} ions, and increasing volumes (25 μL to 150 μL) of 1 mmol/L Fe^{3+} and 1 mmol/L Cu^{2+} ions were taken in different test tubes with fixed volume of 25 mmol/L hydroxyproline and experimental procedure was followed as before.

3. Results

The incubation of hydroxyproline and histidine with Fe^{3+} and Cu^{2+} ions resulted in pink chromogen formation with TBA. The absorption maxima of pink chromogen was at 524 nm and on making the pH alkaline its absorbance maxima shifted from 524 nm to 560 nm. The amino acids incubated with Fe^{3+} or Cu^{2+} ions showed significant increase in TBARS. The TBA-adduct decreased neither in the presence of hydroxyl radical scavengers like mannitol, DMSO nor in the presence of antioxidant enzymes catalase and SOD (Table 1). There was gross decrease in the TBARS in the presence of metal ion chelators like EDTA and reducing agents ascorbate and reduced glutathione. Omission of the incubation stage at 37 ℃ grossly reduced the chromogen formation. There was linear increase in TBARS on increasing Fe^{3+} and Cu^{2+} ions keeping concentration of hydroxyproline constant, and on increasing concentration of hydroxyproline keeping Fe^{3+} and Cu^{2+} ions constant did not yield linear increase in MDA units.

4. Discussion

The absorption maxima of pink chromogen was at 524 nm and on making the pH alkaline its absorbance maxima shifts from 524 nm to 560 nm, which is characteristic of the TBA-MDA adduct as reported by Gutteridge[3]. To our knowledge, it is the first study on the metal ion damage to amino acids by transitional metal ions. It is believed the pink chromogen is due to the formation of TBARS from hydroxyproline and histidine in the presence of Fe^{3+} and Cu^{2+} ions and in the absence of any of the added cell membrane fractions or reducing agents. Hence, both Fe^{3+} and Cu^{2+} are damaging the amino acid hydroxyproline and histidine as indicated by the release TBARS.

In this study, the TBA-adduct decreased neither in the presence of hydroxyl radical scavengers like mannitol, DMSO nor in the presence of antioxidant enzymes catalase and SOD. Zager et al reported similar findings that hydroxyl radical scavengers and catalase did not protect Fe-induced cytotoxicity on cortical proximal tubular segments. Although GSH completely blocked Fe-mediated cell death, this protection occurred without diminution in MDA content, strongly implying that GSH mediated protection may not be through its antioxidant or antihydrogen peroxide effect. They also speculated the role OF ferryl and perferryl radicals in

Table 1
The corrected absorbance readings (at 524 nm) of the TBA-adduct.

Reagents	TBA adduct formed in MDA unites (μmoles/L)	Reagents	TBA adduct formed in MDA unites (μmoles/L)
HyPro + Fe^{3+}	1.705	HyPro + Cu^{2+}	4.487
HyPro + Fe^{3+}+ Mannitol	1.673	HyPro + Cu^{2+} + Mannitol	4.769
(100 μL of 1 mol/L) HyPro + Fe^{3+} + DMSO	1.647	(100 μL of 1 mol/L) HyPro + Cu^{2+}+ DMSO	4.673
(100 μL of 1 mol/L) HyPro + Fe^{3+} + Catalase	1.653	(100 μL of 1 mol/L) HyPro + Cu^{2+}+ Catalase	4.480
(20 μL of 2 200 U/mg) HyPro + Fe^{3+} + EDTA	0.448*	(20 μL of 2 200 U/mg) HyPro + Cu^{2+}+ EDTA	0.237*
(100 μL of 0.1 mol/L) HyPro + Fe^{3+} + Ascorbate	0.519*	(100 μL of 0.1 mol/L) HyPro + Cu^{2+}+ Ascorbate	0.038*
(100 μL of 1 mol/L) HyPro + Fe^{3+} + GSH	0.391*	(100 μL of 1 mol/L) HyPro + Cu^{2+}+ GSH	0.185*
(100 μL of 1 mol/L) HyPro + Fe^{3+} + SOD	1.679	(100 μL of 1 mol/L) HyPro + Cu^{2+}+ SOD	4.493
(20 μL of 3 600 U/mg) HyPro + Fe^{3+} + Citrate	1.801	(20 μL of 3 600 U/mg) HyPro + Cu^{2+}+ Citrate	4.589
(100 μL of 1 mol/L) His + Fe^{3+}	0.660	(100 μL of 1 mol/L) His + Cu^{2+}	2.384
His + Fe^{3+} + Ascorbate	0.003*	His + Cu^{2+} + Ascorbate	0.102*
(100 μL of 1 mol/L) His + Fe^{3+} + Mannitol	0.134	(100 μL of 1 mol/L) His + Cu^{2+} + Mannitol	1.929
(100 μL of 1 mol/L) His + Fe^{3+} + Citrate	0.474	(100 μL of 1 mol/L) His + Cu^{2+} + Citrate	1.198
(100 μL of 1 mol/L) His + Fe^{3+} + Catalase	0.538	(100 μL of 1 mol/L) His + Cu^{2+} + Catalase	1.615
(20 μL of 2 200 U/mg) His + Fe^{3+} + EDTA	0.051*	(20 μL of 2 200 U/mg) His + Cu^{2+} + EDTA	0.410*
(100 μL of 0.1 mol/L) HyPro + Fe^{3+}(without incubation)	0.397*	(100 μL of 0.1 mol/L) HyPro + Cu^{2+}(without incubation)	1.461*
HyPro + Fe^{3+}(incubation for 10 mins)	1.685	HyPro + Cu^{2+}(incubation for 10 mins)	4.846

*: Significant decrease in TBA-adduct.

cytotoxic and lipid peroxidative effects in kidney cells[9]. It has been speculated in the past that free Cu^{2+} and copper containing amino acid complexes can scavenge superoxide ($O_2^{-\cdot}$)[3].

The experiment showed that if the superoxide($O^{-\cdot}$)WAs the culprit causing damage to amino acids, it could have been scavenged by Cu^{2+} ions. However, this did not happen. Instead, Cu^{2+} showed increased chromogen formation when compared to Fe^{3+}. The only possible mechanism in this situation is the reaction of ferric with dissolved oxygen to form high-valent oxo-iron species like ferryl and perferryl radicals[4]. Ferryl radical might have caused site-specific damage to hydroxyproline and histidine. Although it was speculated about this action of ferryl radical, there are still few studies in this regard[1]. Though perferryl radicals might have caused damage to amino acids, it has been proved that its oxidizing capacity is much lower than that of ferryl radicals[1].

Recently Karlsson *et al*[10] and Rohde *et al*[11] reported the structures of two possible high-valent (Fe^{IV} = O and Fe^{V} = O) oxo-iron intermediates that may be involved in non heme iron-promoted dioxygen activation. However, this does not exclude the damage caused by $^{\cdot}OH$ radical to biomolecules in certain defined conditions. It appears non hydroxyl/non hydrogen peroxide free radicals (eg., ferryl and perferryl radicals) play significant role in oxidative damage to biomolecules.

It have displayed gross decrease in TBARS in the presence of metal ion chelators like EDTA and reducing agents ascorbate and reduced glutathione. It has been suggested that, the reducing agents may have dual roles as both an anti-and pro-oxidants[5]. Most probably , reducing agents prevented formation of high-valent oxo-iron species by (re-) reducing the ferric ions. Since omission of the incubation stage at 37 ℃ grossly reduces the chromogen formation, metal ions and hydroxyproline are significantly interacting in the incubation stage irrespective of acid heating stage that was known to liberate TBARS from biomolecules[3]. This conclusion is further confirmed by the behavior of the amino acid and oxidized metals at fixed and variable concentration.

There was linear increase in TBARS on increasing Fe^{3+} and Cu^{2+} ions to keep concentration of hydroxyproline constant, but no linear increase in TBARS on increasing concentration of hydroxyproline to keep Fe^{3+} and Cu^{2+} ions constant. Therefore it can be concluded that Fe^{3+} and Cu^{2+} ions are damaging hydroxyproline and histidine in the

incubation stage at 37 ℃ at pH 7.4. The oxidised metal ions may be loosely bound to amino acids during incubation stage, available to EDTA and reducing agents, and the oxo-iron species may cause site specific damage as in case of albumin[12]. Regarding mechanism of Cu^{2+} induced damage to hydroxyproline and histidine, it is difficult to speculate. Overall, it appears that these oxidized metal ions in the body may be sequestered with in the storage and transport proteins in such a way that they are prevented by forming the oxo-iron species. The exact mechanistic details of biological non heme iron-promoted dioxygen activation and role of high-valent oxo-iron species need further experimental designs.

Ferric or ferrous ions by Fenton chemistry yields $^{\cdot}OH$ radicals and initiates lipid peroxidation by abstracting H atom from fatty acids. The role played by ferryl and perferryl species at present are less well understood and they are definitely proved to be less reactive compared to $^{\cdot}OH$ radical. The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts, thus a reduced iron complex can react with lipid peroxide; it causes fission of O-O bonds to form alkoxyl radicals ($RO^{\cdot}$). Currently there is considerable interest in the role of free iron or non-transferrin bound iron in the body and its role in damaging biomolecules. Hider HC reported recently that NTBI is present in the serum of patients suffering from a wide range of disease states and may be induced under certain therapeutic modalities. However, the chemical nature of this NTBI pool is not clear but it is a multicomponent pool including a considerable proportion of protein bound iron.

Serum albumin is demonstrated to bind ferric iron even when transferrin is not fully saturated. At present, the nature of NTBI is not clear and it may exist in a number of isoforms. The proportion of these isoforms may depend on the nature of the disease. It is proposed that different isoforms would be cleared at different rates. It is also speculated that some isoforms would be Fenton active and others lack such activity. The nature of NTBI in vivo is far from clear, and it may be catalytically active or inactive. Free iron is found to be toxic to haematopoietic progenitors in vitro cultures and the toxic effect could be reduced with apotransferrin.

In conclusion, it is possible that the transitional metal ions can cause oxidative damage to amino acids through high-valent oxo-iron species. We recommend repetition of this study with various biomolecules and under various experimental conditions. Further research is required in this field in order to understand the exact mechanism by which these metal ions cause damage to biomolecules.

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