Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants

J.C. Mayo, D.X. Tan, R.M. Sainz, M. Natarajan, S. Lopez-Burillo, R.J. Reiter

Abstract

Melatonin is a well-known hydroxyl radical (·OH) scavenger that protects DNA and lipids from free radical attack. In this paper, we studied the ability of melatonin to prevent oxidative damage to bovine serum albumin (BSA) induced by two different paradigms: the metal-catalyzed oxidation (MCO) induced by Cu²⁺/H₂O₂ and the alkoxyl and alkylperoxyl radicals formed by the azo initiator 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH, 40 mM). The protective effects of melatonin were compared with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), glutathione (GSH), ascorbate, 3,4',5-trihydroxy-trans-stilbene (resveratrol), and mannitol. Melatonin efficiently prevented protein modification induced by both models, as assayed by polyacrylamide gel electrophoresis and carbonyl content. Both trolox and ascorbate had an obvious pro-oxidant effect in the Cu²⁺/H₂O₂ model, whereas both prevented BSA damage induced by AAPH. In the MCO model, the efficacy of GSH in terms of protein protection was higher than melatonin at relatively high concentrations (250 μM–4 mM); however, at lower concentrations (50–250 μM), the efficacy of melatonin was superior to GSH. D-Mannitol (50 μM–100 mM) and resveratrol did not protect BSA from the site-specific damage induced by Cu²⁺/H₂O₂. On the other hand, the relative protective efficiency in the AAPH model was melatonin > trolox > GSH > ascorbate.

1. Introduction

Because the involvement of free radicals and oxidative damage in aging was suggested [1], the free radical theory of aging has been widely accepted as a possible means to explain age-related deterioration. Free radical damage to macromolecules is associated with a variety of pathological processes, some of which are related to aging, for example, atherosclerosis, arthritis, muscular dystrophy, cataractogenesis, pulmonary dysfunction, cancer and several neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis [2–4].

Oxygen-based reactants that escape detoxification by cellular antioxidant systems are responsible for roughly an estimated 10,000 DNA base modifications per cell per day [5]. Thus, many studies have focused on DNA damage as estimated by the levels of 8-hydroxy-deoxyguanosine after exposure to reactive oxygen species (ROS) [6]. Also lipid peroxidation as a result of free radical attack has also been widely studied; this process leads to the loss of membrane integrity and compromised cellular function. Reactive species also damage proteins but, due to rapid turnover of proteins, they are considered to contribute less prominently to total cellular damage. Recently, however, the possibility that free radical damage to proteins might contribute to aging has also received attention [7] and several groups have focused their research in this area.

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; DNPH, 2,4-dinitrophenylhydrazine; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; Resveratrol, 3,4',5-trihydroxy-trans-stilbene; ROS, reactive oxygen species; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

* Corresponding author. Tel.: +1-210-567-3859; fax: +1-210-567-6948.
E-mail address: reiter@uthscsa.edu (R.J. Reiter).
Thus, it has been shown that oxidatively modified proteins accumulate during aging and in some pathological conditions [8,9].

Exposure of proteins to $'OH$, $O_2^-$ or both leads to gross structural modifications. These oxidatively modified proteins may undergo spontaneous protein fragmentation and cross-linking or exhibit a substantial increase in proteolysis [7,10]. The principles of protein modification by ROS are well established as well as the characterized reaction products of protein interactions with $'OH$ and $O_2^-$. [10,11]. The oxidative attack of the polypeptide backbone is usually initiated by $'OH$. This has been experimentally determined by generating this radical using the radiolysis of water or from a metal-catalyzed cleavage of $H_2O_2$. This eventually leads to the formation of alkyl, alkoxyl and alkylperoxyl radical intermediates, which set the stage for cleavage of the peptide bond via several means. A wide variety of reactions between ROS and amino acid chains occur and all amino acids in proteins are susceptible to modification by $'OH$ or by $'OH$ plus $O_2^-$. However, tryptophan, histidine and cysteine are the most vulnerable [7,10,11]. In addition to fragmentation, the oxidation of lysine, arginine, proline and threonine residues may also yield carbonyl derivatives. The presence of carbonyl groups has therefore been used as a maker of ROS-mediated protein oxidation [7,11].

Oxidative modification of proteins also occurs by reactions with aldehydes produced during lipid peroxidation. Thereafter, end products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) as well as products from polyunsaturated fatty acids cause protein damage [12,13]. Moreover, peroxyl radicals initiated by thermal decomposition of 2,2$'$-azobis(2-amidinopropane) hydrochloride (AAPH) are responsible for oxidation of proteins [14].

Melatonin, or N-acetyl-5-methoxytryptamine, is a derivative of tryptophan mainly synthesized in the pineal gland of mammals [15]; however, its production is not confined exclusively to this gland and other organs and tissues including retina, Harderian glands, gut, ovary, testes, bone marrow and lens have been reported to produce it as well [15–17]. Available data regarding melatonin concentrations in tissues, cells or other body fluids are variable and range from picomolar to micromolar levels, usually estimated using radioimmunoassay, HPLC and mass spectroscopy [17–19]. Based on available data, however, it seems that the tissue melatonin concentrations are considerably higher than they are in serum, although the physiological importance of this melatonin has not yet been clarified. Melatonin is also synthesized in non-mammalian vertebrates, invertebrates [20] and in other organisms including dinoflagellates, algae and bacteria [21] and it is found in a variety of plants [22]. The presence of melatonin in such a variety of organisms suggests that this substance is phylogenetically highly conserved and plays an important role in the function and survival of organisms. In all mammals studied including man, its secretion exhibits a circadian rhythm with a peak during the dark phase [15]. Melatonin has been classically associated with circadian and circannual rhythm regulation, and with adjustments of physiology of animals to seasonal environmental changes [15].

In addition, during the last decade, a large number of articles have shown melatonin to be a potent antioxidant and to protect against a number of radical species [23–25]. Melatonin directly scavenges several oxygen-based reactants and protects against oxidative stress-related processes in experimental models of aging, of neurodegenerative disorders, etc. [25,26]. Numerous studies have reported melatonin’s protection against lipid peroxidation and DNA damage induced by ROS, both in vivo and in vitro [27–29], but fewer workers have focused on melatonin’s efficacy in reducing protein damage [30]. A few authors have noted the inefficacy of melatonin as an antioxidant in some experimental models and have thus suggested that melatonin may not be a physiological antioxidant [31,32]. The aim of the present work was to test whether melatonin counteracts protein damage caused by several pro-oxidant agents; additionally, melatonin’s actions in this regard were compared to several other known antioxidants.

Fig. 1. Time-course study of the oxidative effect of $Cu^{2+}/H_2O_2$ treatment of BSA protein. Gel electrophoresis of a representative experiment of BSA without any treatment (C) or with $Cu^{2+}/H_2O_2$ treatment at the indicated times. Lane 1 represents the known molecular weight standard (A). Graph shows the densitometric analysis of the protein bands obtained from gels shown in A $(n=4)$ (B).
2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), glutathione (GSH), ascorbate, acrylamide/bis-acrylamide solution, 3,4',5-trihydroxy-trans-stilbene (resveratrol), α-mannitol, phenol red and horseradish peroxidase were purchased from Sigma Chemicals (St. Louis, MO). 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Fluka (Neu-Ulm, Switzerland). AAPH was obtained from Wako Chemicals (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was purchased from Aldrich (Milwaukee, WI). Ultra-pure grade melatonin was a kind gift from Helsinn Chemical (Biasca, Switzerland).

2.2. Metal-catalyzed oxidation of BSA

Hydroxyl radical-mediated oxidation experiments were carried out by using a metal-catalyzed reaction. BSA (Fraction V, 98% electrophoresis grade, # A-7906) was obtained from Fluka (Neu-Ulm, Switzerland). AAPH was dissolved in air-saturated 150 mM phosphate buffer (pH 7.3) at a final concentration of 0.5 mg/ml. BSA was incubated with or without Cu²⁺ (100 μM) and H₂O₂ (2.5 mM) and 150 mM phosphate buffer (pH 7.3).

Fig. 2. PAGE profile of the BSA protein treated with Cu²⁺/H₂O₂ in the presence of the antioxidants. Gels show the protein bands obtained without treatment (C), with the Cu²⁺/H₂O₂ (T) and of the concentrations indicated for the antioxidants melatonin (MEL), glutathione (GSH), ascorbate (ASC), trolox, resveratrol (RESVT) or α-mannitol (MANN).
mM) as previously described [33], for the durations indicated in the presence or absence of antioxidants. Reactions were performed in opened tubes placed in a shaking water bath at 37 °C. Melatonin, trolox and resveratrol were dissolved in 100% ethanol and added from a 500-mM stock solution to minimize the final concentration of ethanol. Vehicle (ethanol, 0.2% v/v) was always included in the Cu²⁺/H₂O₂-treated group. GSH, ascorbate and D-mannitol were directly dissolved in 150 mM phosphate buffer.

Fig. 3. Densitometric analysis of the protein bands from gels shown in Fig. 2. Graphs summarize the analyses of at least four independent experiments. (A–F) Melatonin, glutathione, ascorbate, trolox, resveratrol and mannitol, respectively. a, P < 0.05 vs. control group (C); b, P < 0.05 vs. treatment group (T).
2.3. Peroxyl-radical-mediated protein damage

BSA was prepared as described above. AAPH was prepared as an 800-mM stock solution in water and heated for 2 min at 37 °C before adding to samples. In these experiments, the same amount of water was added to the control groups. Antioxidants were prepared as described above.

2.4. SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

To quantify protein damage, after treatment, protein samples were subjected to SDS-PAGE according to Laemli’s method [34] using the Mini-Protean® III system (Bio-Rad Laboratories, CA). Samples were mixed with loading buffer [4 × concentrated: 40% glycerol, 8% SDS, 0.25 mM Tris–HCl (pH = 6.8), 20% β-mercaptoethanol, 0.01% bromophenol blue] and heated at 100 °C for 2 min. Four micrograms of protein sample was loaded in a 12.5% polyacrylamide gel and electrophoresed. After running 1 h, gels were stained with 0.15% Coomassie brilliant blue R-250 for 30 min, washed extensively and dried in a gel dryer (Bio-Rad Laboratories) for 45 min. At least four gels of each experiment were performed.

2.5. Measurement of band intensity and graph representation

To determine the amount of protein damage, band intensity was estimated using the Scion Image Beta 4.02 for Windows™ analysis software, downloaded at the web site address http://www.scioncorp.com. The density of each band was estimated and standardized with respect to the control group. Results show the average of at least four different measurements.

2.6. Carbonyl content determination

The carbonyl content of proteins was quantified by the reaction with DNPH, using the method described by Levine et al. [35]. After stopping the reaction by adding 0.2 ml of 1 M mannitol–0.5 M EDTA solution, 2 ml of samples containing 1 mg of total protein (four samples per group) was mixed with 0.5 ml of 10 mM DNPH and reacted for 1 h at 37 °C to give a final concentration of 2 mM of DNPH. Then, 0.625 ml of 50% ice-cold trichloroacetic acid was added. After 10 min of incubation on ice, samples were centrifuged at 13,000 × g for 5 min. Pellets containing the proteins were washed three times with ethanol/ethyl acetate (1:1 v/v). Finally, the pellets were resuspended in 1 ml of 6 M guanidine in 2 N HCl (pH = 2.0) by vortexing, and incubated at 37 °C for 15 min. After this interval, samples were clarified by centrifugation at 13,000 × g for 5 min and the absorbance of supernatants was read at 375 nm in a Beckman DU 530 spectrophotometer. Protein carbonyl content, expressed in nanomoles per milligram of protein, was estimated by using the molar absorption coefficient of 22,000 M cm⁻¹ for DNPH derivatives. Guanidine–HCl solution was used as a blank. Data were standardized according to the protein content that was measured using the Bradford method and a Bio-Rad kit.

2.7. Statistical analysis

All data shown are means ± S.E. of at least three different experiments using four samples per group. One-way ANOVA was performed to compare different groups, followed by a Student–Newman–Keuls test. Statistical significance was accepted when P<0.05.
3. Results

3.1. Time-course study of the effect of Cu\(^{2+}\)/H\(_2\)O\(_2\)

A set of experiments was performed to determine the amount of protein damage induced by the metal-catalyzed reaction with Cu\(^{2+}\)/H\(_2\)O\(_2\). A time-course study was run to determine the optimal duration of treatment for the remainder of the experiments. As shown in Fig. 1A, there is rapid protein degradation when BSA is incubated in the presence of Cu\(^{2+}\)/H\(_2\)O\(_2\) and half of the protein is structurally damaged after about 50 min. Densitometric analysis of the gel electrophoretic samples is shown in Fig. 1B. Maximal effect was reached at 60–75 min after beginning the treatment. Thus, we chose 75 min for the remainder of the experiments described below. It is important to note that these samples were incubated in the presence of a final concentration of 0.2% of ethanol, as this was the concentration of the solvent used as a vehicle for antioxidants tested in this work, unless otherwise indicated. Ethanol had a slight influence in the results; it protected 10–15% of the protein from degradation (data not shown).

3.2. Inhibition of protein degradation by melatonin compared with other antioxidants

Fig. 2 is a representative gel showing the effects of the different antioxidants in preventing Cu\(^{2+}\)/H\(_2\)O\(_2\)-induced BSA degradation. The densitometric analyses are summarized in Fig. 3A–F. Both melatonin and GSH in the range of 4 mM–100 \(\mu\)M efficiently inhibited BSA degradation although the response to these antioxidants was completely different. GSH protected almost completely when it was used at the highest concentrations, while melatonin pro-

![Figure 1A](image1.png)

![Figure 1B](image2.png)

![Figure 1C](image3.png)

![Figure 1D](image4.png)

![Figure 1E](image5.png)

![Figure 1F](image6.png)

Fig. 6. Effect of melatonin and other antioxidants in the increase of carbonyl content after treatment with Cu\(^{2+}\)/H\(_2\)O\(_2\). Data represent the mean of four different experiments carried out in the absence (C) or presence of Cu\(^{2+}\)/H\(_2\)O\(_2\) (T) with the antioxidants melatonin (MEL), glutathione (GSH), ascorbate (ASC) or trolox, respectively, at the concentrations shown. a, \(P<0.05\) vs. remainder of groups; b, \(P<0.05\) vs. treatment.
protected less efficiently but at a wider range of concentrations (up to 50 μM). Two commonly used antioxidants, that is, ascorbate and trolox (hydrosoluble analogue of vitamin E) not only did not protect against protein damage but even increased the Cu2+/H2O2-induced BSA degradation, as it was expected according to the ability of these antioxidants to act as potent reducing agents in the presence of copper salts (see discussion below). This pro-oxidant effect of trolox and ascorbate was observed when used in the range of 250 μM–4 mM in the case of trolox and up to 100 μM in the case of ascorbate (Figs. 2 and 3C and D, respectively). Other ‘OH scavengers used including D-mannitol and resveratrol did not protect against the metal-catalyzed oxidation in the model described here (Figs. 2 and 3E and F, respectively).

Fig. 4 summarizes a comparative study performed with the antioxidants mentioned above in a different set of experiments. GSH completely reversed the toxic effects of Cu2+/H2O2 in terms of protein degradation. GSH in the range of 250 μM–4 mM protected BSA very efficiently and restored the protein structure almost to control levels. However, GSH showed an “all or nothing” effect; thus, highest concentrations had essentially a similar effect. At concentrations below 250 μM, however, the efficiency of GSH decreased exponentially in a concentration-dependent manner. The protective profile of melatonin was completely different; although not as effective as GSH in preventing the damage at the highest doses, the response was concentration dependent and was more effective than GSH at lower doses (50–250 μM). As noted above, both ascorbate and trolox had important pro-oxidant actions with up to a 75% increase in protein damage in the case of ascorbate.

3.3. Metal-catalyzed oxidation produced by Cu2+/H2O2 induced carbonyl group formation

Fig. 5 represents the increase in the BSA carbonyl content after treatment with Cu2+/H2O2. There is a rapid increase in the amount of carbonyl groups after 1 h of treatment, reaching a plateau when treatment is prolonged up to 6 h. Thus, a 1-h incubation duration was selected for the subsequent studies.

3.4. Melatonin and GSH protect BSA against the increase in carbonyl group formation

Results obtained with the antioxidants tested are presented in Fig. 6A–D. Melatonin partially protected BSA during the metal-catalyzed reaction (Fig. 6A), but only at

![Fig. 7. Time-course study of the effect of AAPH on BSA. BSA was incubated with AAPH for the indicated times and the gel electrophoresis band intensity (A) and densitometric analysis of the corresponding bands (B) are represented.](image)

![Fig. 8. Gel electrophoresis of BSA protein after treatment with AAPH in the presence of different antioxidants. BSA was treated without (C) or with AAPH (T) and additionally with the antioxidants melatonin (MEL), glutathione (GSH), ascorbate (ASC) or trolox added at the concentrations indicated. Arrow, protein aggregation observed in the gel after AAPH incubation.](image)
concentrations above 100 μM. Melatonin did not totally restore carbonyl levels to control values at any concentration. However, GSH as shown with the structural damage observed by PAGE, restored almost completely the carbonyl content levels to control values when used at the highest concentrations (0.5–1 mM) but rapidly lost its efficacy at lower concentrations below 0.5 mM (Fig. 6B). As predicted by previous results, both ascorbate and trolox (Fig. 6C and D, respectively) failed to protect against the increase in carbonyl accumulation and even enhanced the formation of additional carbonyl groups. Ascorbate's pro-oxidant action was even observed at concentrations as low as 10 μM. No statistically significant differences were found between negative control groups (C) and those treated only with melatonin (data not shown).

3.5. Peroxyl radical-induced BSA damage is time dependent

To establish if the protection by melatonin as well as by other antioxidants could be extended to other models of protein damage, another set of experiments using the thermal decomposition of the azo compound AAPH was performed. This substance leads to the formation of carbon-centered radicals that, under aerobic conditions, yield alkylperoxyl radicals and hydroperoxides. Fig. 7A and B shows the clear time-dependent reduction of BSA in the gel (Fig. 7A), with a

![Graphs](image)

Fig. 9. Densitometric analysis of the protein bands shown in Fig. 8. Graphs show the band intensity (black bar) or the upper band intensity (arrow, Fig. 7) corresponding to protein aggregation (white bar) from BSA proteins without (C) or with AAPH (T) in the presence of melatonin, glutathione, ascorbate or trolox (A–D, respectively). a, $P<0.05$ vs. remainder of groups; b, $P<0.05$ vs. control (C) group.
50% loss after 1.5 h of treatment (Fig. 7B). It appeared that protein aggregation (arrow) was one of the main modifications induced in BSA by AAPH.

3.6. Antioxidants inhibit protein modification caused by AAPH

Electrophoretic patterns of BSA after incubation with AAPH in the presence or absence of the different antioxidants are shown in Fig. 8 and the corresponding densitometry is presented in Fig. 9. Melatonin (Fig. 9A) at concentrations over 50 µM, reduced significantly protein damage; at concentrations over 500 µM, protein band intensity was completely restored to control levels. GSH was also effective in preventing the AAPH damage in BSA, but less efficiently than melatonin (Fig. 9B). Ascorbate only protected protein modifications when it was present at concentrations over 500 µM (Fig. 9C). Finally, trolox exhibited a protective profile similar to that of melatonin (Fig. 9D). It is noteworthy that results obtained with the main band intensity and with aggregation (upper band) were similar, so inhibition of aggregation was proportional to inhibition of protein damage in all cases.

Fig. 10 represents the comparative effects of the antioxidants described above. Although at 1–2 mM concentrations all the compounds inhibited BSA oxidative damage caused by AAPH, melatonin and trolox were more effective at lower concentrations than were the other antioxidants. Ascorbate and GSH rapidly lost their antioxidant capacity. GSH protection was not as great as that of melatonin and trolox. Table 1 shows the concentration necessary to inhibit 50% of protein band disappearance (IC50). While trolox and melatonin have a similar IC50, GSH and ascorbate required concentrations greater than 0.5 mM to confer the same protection.

4. Discussion

Herein we report the protective effects of melatonin against structural damage to proteins induced by two different free radical generating systems. In one case, the damage was mediated by the \( \text{HO}^- \) and in the other by the alkoxy/alkylperoxyl radicals. In addition, we show that melatonin is more effective and consistent in its effect than were the other antioxidants in protecting against the structural damage caused by Cu²⁺/H₂O₂ and AAPH. GSH, however, was a better protector at higher concentrations against the metal-catalyzed protein oxidative damage but this advantage disappeared when compared with melatonin at low concentrations. In the case of protein damage induced by alkoxy/alkylperoxyl radicals, melatonin showed a similar protective action as trolox (water-soluble vitamin E analogue), one of the best chain-breaking antioxidants known.

Whereas several reports have documented the protective actions of melatonin on DNA and lipids, few have focused on its effectiveness in reducing oxidative damage to proteins. An earlier report [30] demonstrated that melatonin was more effective than either trolox, vitamin C or GSH in protecting \( \beta \)-phycoerythrin after treatment with AAPH. As reported here, melatonin was also highly effective in protecting proteins from peroxyl radicals formed by the thermal decomposition of AAPH. In addition, melatonin partially protected against oxidative protein modification caused by Cu²⁺/H₂O₂ (a site-specific oxidation); in the latter situation, GSH was more effective in preserving protein structure when it was at higher concentrations. The linear concentration–response curve for melatonin, in agreement with previous results [30], shows that low doses of melatonin can be even more effective than GSH or trolox (Fig. 4). In a previous report, Kim et al. [36] demonstrated that melatonin prevented protein oxidation due to \( \text{OH}^- \) generated by ascorbate–Fe³⁺–EDTA.

When compared with other antioxidants, melatonin was shown to protect against oxidative modification to different proteins [37], although less efficiently than the flavonoid quercetin or trolox. On the other hand, melatonin was more effective in protecting red blood cell cytoskeleton protein structure after damage with ONOO⁻ [38]. Martinez-Cruz et al. [39] reported the ability of melatonin to prevent protein adduct formation of pyrrolyzed plasma proteins after H₂O₂...
treatment. In vivo melatonin has also been shown to be beneficial against protein modification \[40,41\]. Data presented herein provide additional evidence that melatonin protects against oxidative modification of proteins. In these models, melatonin was as good as trolox in protecting against alkoxyl and alkylperoxyl radicals and in contrast to ascorbate and trolox, it shows no pro-oxidant activity in the \(\text{Cu}^{2+}/\text{H}_2\text{O}_2\) model.

Ascorbate is known to be a strong reducing agent and it has been classically used to reduce transition metals such as \(\text{Fe}^{3+}\) or \(\text{Cu}^{2+}\) and to generate \('\text{OH}\) \[42\]. Also, the combination of ascorbate/\(\text{Cu}^{2+}\) causes extensive molecular damage in both proteins and DNA \[43\]. Using PAGE, we confirmed that the \(\text{Cu}^{2+}/\text{H}_2\text{O}_2\) damage promotes specific degradation of BSA and this is enhanced by ascorbate due to its pro-oxidant effects as has been reported \[44\]. Auto-oxidation of ascorbate in the presence of copper salts can also be harmful and induces protein inactivation in several proteins including lysozyme, alkaline phosphatase, angiotensin I or acetylecholine esterase \[45,46\]. Although the pro-oxidant role of ascorbate (and vitamin E) in vivo is controversial, it can occur especially after tissue damage where metal ions can be released from the proteins used by cells to sequester them \[47\].

In some studies, the \('\text{OH}\) caused site-specific damage and induction of oxidation of the imidazole ring in histidine residues has been demonstrated as the major cause of protein inactivation and/or degradation \[48\]. Although tryptophan and histidine are considered the main targets of free radicals and protein oxidative damage, the possibility exists that the \(\text{SH}\)-containing residues may also play some role in protein degradation; this could explain the protection exerted by GSH in the \(\text{Cu}^{2+}\)-dependent model.

Metal-catalyzed protein oxidation is reported to be relatively insensitive to the presence of \('\text{OH}\) scavengers \[47,48\]. Because the formation of \('\text{OH}\) is in the immediate vicinity of the protein residues, the absence of protection by some antioxidants may be attributable to their inability to be at the site of \('\text{OH}\) generation. This conclusion, however, may have exceptions because some other antioxidants such as those present in red wine bind to human lipoproteins (LDL and HDL) and to albumin and protect them from metal-catalyzed oxidation \[49\]. These findings prompted us to include one of the antioxidants obtained from red grape skin, that is, resveratrol, in the present study. Unexpectedly, however, it did not reduce the radical damage to proteins. Yet, both GSH and melatonin exhibited protection, while mannitol or the antioxidant enzyme SOD \[33\] failed as scavengers in this model. \(\alpha\)-Mannitol has the capacity to bind some metals and thus lower the in situ \('\text{OH}\) toxicity \[50\]. At the concentrations used in the current study, mannitol was ineffective in protecting proteins from oxidative damage.

The ability of \(\text{Cu}^{2+}\) to catalyze auto-oxidation of ascorbate and therefore to promote protein damage has been reported as mentioned above \[43\]. On the contrary, few studies document the pro-oxidant effect of trolox and/or vitamin E (\(\alpha\)-tocopherol) in similar situations. \(\alpha\)-Tocopherol is a classical lipophilic antioxidant well known as a scavenger of free radicals in a hydrophobic milieu. It does, however, exhibit both anti- and pro-oxidant activity in isolated low-density lipoprotein (LDL) or during lipid peroxidation \[51\]. Likewise, the hydrosoluble analogue trolox is capable of triggering similar pro-oxidant effects \[52\]. This pro-oxidant action of \(\alpha\)-tocopherol is due to the formation of \(\alpha\)-tocopheryl radical, which occurs in the presence of transition metals such as \(\text{Cu}^{2+}\); this product in turn reacts with polyunsaturated fatty acids. The rate constant for this reaction, however, is much lower than the reaction with other lipid peroxides, thus masking its pro-oxidant effects. Also, the generation of \('\text{OH}\) in the Cu–trolox complex has been shown \[52\]. These findings indicate that, in our model with BSA, the trolox promotion of \(\text{Cu}^{2+}/\text{H}_2\text{O}_2\)-dependent protein damage could be due to an “additive” effect of trolox in generating \('\text{OH}\) formation.

From the data presented here, it can be deduced that melatonin, in contrast to ascorbate or trolox, did not act as a reducing agent in the presence of \(\text{Cu}^{2+}\); this likely explains melatonin’s very low pro-oxidant potential. Melatonin, which has a redox potential of 730 mV \[53\], could theoretically have the same effect as trolox or vitamin C in the presence of \(\text{Cu}^{2+}\); however, the present findings suggest otherwise. This again demonstrates melatonin’s potential therapeutic use as an antioxidant. Additionally, the protection exerted by GSH and melatonin at site-specific oxidation, as shown herein, make them highly protective of proteins against this type of damage.

Carbonyl groups are considered a general assay of oxidative protein damage \[48\] and the presence of these products is increased in several tissues with aging \[9\]. Multiple redox reactions cause the introduction of carbonyl groups into proteins, with their number being influenced by metal cations, especially \(\text{Fe}^{2+}\) and \(\text{Cu}^{2+}\) \[48\]. Thus, the site-specific degradation caused by \(\text{Cu}^{2+}\), as it has been shown here, induced a significant increase in the level of carbonyl groups, as reported elsewhere \[33\]. Both melatonin and GSH caused a noticeable reduction in these constituents with GSH being more effective in this regard judging from an analysis of the gels. Carbonyl groups represent an early stage of protein oxidation \[11\]. Likewise, the increases in carbonyls due to trolox or ascorbate treatment were not proportional, based on the degree of protein damage seen in the gel. Thus, carbonyl levels by themselves, although a good index of protein damage, do not increase indefinitely, as protein degradation or other processes limit it. This was also apparent in the time-course study (Fig. 5) where the carbonyl content rapidly reached a plateau and the levels did not correlate with the increasing structural damage seen in the protein (Fig. 1). Furthermore, it has been also reported that enzyme inactivation mediated by oxidative damage is not necessarily correlated with an increase in carbonyl content \[54\].

Interestingly, none of the pro-oxidant effects mentioned above were observed in the AAPH-induced protein damage
model, although it has also been reported that ascorbate and trolox may have this effect in an LDL model in vitro [51]. The nature of free radicals as well as the formation at sites removed from the protein are likely some of the underlying reasons that explain the different responses. Trolox and melatonin clearly had similar effects in the current study suggesting their potential use in protecting proteins from oxidative damage induced by alkyperoxyl radicals.

AAPH-induced protein damage has been studied extensively. PAGE studies also point out that AAPH-induced amino acid modifications can also promote cross-linking between proteins, so aggregation (more than degradation) may be primarily responsible for protein modification and inactivation as shown for glutamine synthetase, LDL lipoproteins or ceruloplasmin [55,56]. Based on the PAGE results, it seems that BSA treatment with either Cu²⁺/H₂O₂ or AAPH, generates different oxidative products because no protein aggregation was observed after treatment with the metal-catalyzed reaction reagents. Kelly and Loo [57] have reported that melatonin inhibits the oxidation of LDL lipoprotein induced by AAPH although these findings have not been confirmed [58]. Our results show that BSA is oxidized by AAPH, inducing mainly protein aggregation [59], and this can be effectively prevented by peroxyl radical scavengers such as melatonin.

In summary, the results presented here, along with those summarized above, document the ability of melatonin to counteract oxidative damage to protein. Considering its lack of pro-oxidative activity, melatonin should be further tested in other models of oxidative modification of proteins.

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