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Article

DNA damage in a solution containing copper(II) ions and ascorbic acid: Effect of the presence of sulfite

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Some antioxidant compounds have a pro-oxidant effect in the presence of transition metal ions, due to the reduction of M^{n+} to $M^{(n-1)+}$ with simultaneous formation of free radicals, which then promote DNA damage. In the present study, we evaluated the pUC19 DNA damage in a solution containing Cu(II) and ascorbic acid (AA) or S(IV) saturated with air by agarose gel electrophoresis. Our results showed that this damage decreases if AA and S(IV) are simultaneously added. This study also illustrates the importance of Cu(II) in this process, as no DNA damage was observed when AA or S(IV) were present in the absence of this metallic ion. Our data showed that DNA preservation depends on the concentration of AA and S(IV) and occurs when the [S(IV)]:[AA] ratio ranges from 1:1 to 20:1. Absorbance measurements and thermodynamic data show that no reaction occurs between AA and S(IV) when this mixture (pH 5.5) is added to pUC-19 DNA. The presence of dissolved oxygen may be the cause of AA consumption in the mixture of these two antioxidants, which subsequently decreases DNA damage.

Keywords: Gel electrophoresis. DNA damage. copper. ascorbic acid. sulfite.

INTRODUCTION

An antioxidant compound (AO) is any substance present in low concentrations when compared to an oxidizable substrate, which effectively delays or inhibits the oxidation of this substrate (Sies, Stahl, 1995). AOs have been the subject of numerous studies because it is believed that they positively contribute in reducing the risk of many diseases and in delaying cellular aging (Rohenkohl, Carniel, Colpo, 2011; Costa, Monteiro, 2009). Most AOs can be obtained through diet, by regular consumption of fruits and vegetables. Moreover, AOs can act synergistically at different levels (*Hajhashemi et al*., 2010).

On the other hand, studies have shown that in solutions that contain transition metal ions, AO compounds can act as pro-oxidants and thus contribute to the formation of free radicals, which under certain conditions can damage DNA (*Win et al*., 2002; *Yoshino et al*., 1999). In fact, it was found that melatonin (Sakano *et al*., 2004), N-acetylcysteine (Oikawa *et al*., 1999), α-tocopherol (Yamashita *et al*., 1998), resveratrol, and ascorbic acid (Sugiyama, Tsuzuki, Ogura, 1991) can act as pro-oxidants in the presence of Cu(II) and Cr(VI), thereby contributing to the formation of free radicals.

Ascorbic acid (AA) is an AO compound found in fruits and vegetables, which is also used as a food preservative (INS 300). When present in the organism, it takes part in several biochemical processes in the cell, mainly in the hydroxylation of collagen (Cerqueira, Medeiros, Augusto, 2007; Manela-Azulay *et al*., 2003). However, it is known that in a saturated air solution, AA reduces Cu(II) to Cu(I), which can lead to the formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$), which can then damage the DNA (Aruoma *et al*., 1991).

Sulfur dioxide (SO_2), another known food preservative (INS 220), is widely used in fruit juices and wines and can also be found as an atmospheric pollutant produced by industrial chemical reactions (Dong-Sheng, Xiao-Ying, Jie- Qing, 2006). It is well established that free radicals of sulfur oxide ($SO_3^{\bullet-}$, $SO_4^{\bullet-}$ e $SO_5^{\bullet-}$) are easily generated when S(IV), which may be present in

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an aqueous solution as SO_2, HSO_3^- and SO_3^{2-}, undergoes autoxidation catalyzed by transition metal ions such as Cu(II), Co(II), Mn(II), and Ni(II) (*Alipázaga et al*., 2009; Aguiar *et al*., 2007). Therefore, humans can be exposed to these sulfur derivatives through inhalation, due to the presence of sulfur oxides in the atmosphere, as well as through the consumption of processed foods (Alipázaga *et al.,* 2009; Aguiar *et al*., 2007).

Copper ions have an essential role in animal metabolism, as copper-dependent enzymes require traces of this metal to mediate chemical reactions, such as collagen and elastin biosynthesis and blood coagulation (Pedrosa, Cozzolino, 1999). A previous study has suggested that the DNA-copper association makes it possible to maintain the chromosome structure and regulate gene expression (Li, Trush, 1994). In a living system, copper ions can also be found in the cell nucleus where they interact with chromosomes, RNA, and DNA, and especially with guanine (Li, Trush, 1994). On the other hand, it was suggested that Cu(II) acts as a catalyst in redox reactions, which gives rise to free radicals that damage biomolecules and cause predisposition towards the development of various diseases (Cerqueira, Medeiros, Augusto, 2007).

In the present study, pUC-19 DNA damage was evaluated by electrophoresis, in a saturated air solution ($[O_2] \sim 250$ μM) containing Cu(II) and the two AOs compounds mentioned above, ascorbic acid and S(IV). DNA damage occurs in the presence of both AA and S(IV), which reduce Cu(II) to Cu(I). This confirms the importance of the Cu(II)/Cu(I) redox cycle. In fact, the presence of Cu(II) and Cu(I) complexing agents, such as EDTA and neocuproine, respectively, inhibits or decreases DNA damage caused by a reducing agent (Roriz, Moya, 2017).

However, when these two AO compounds (AA and S(IV)) are added simultaneously, the damage caused by AA is suppressed. Although from a biological point of view, this inhibition of DNA damage can be a positive event, this may indicate that the antioxidant capacity of a sample (e.g., processed foods) attributed to the sum of these compounds (AA and S(IV)) decreases if they are added simultaneously (mixture at pH 5.5).

Decrease in DNA damage does not happen due to a previous reaction between sodium sulfite and AA, as it is not thermodynamically favorable at pH 5.5. Moreover, the absorbance measurements using standard solutions of these compounds (sodium sulfite and AA) corroborates this conclusion. In addition, the presence of dissolved O_2, which actively participates in inducing DNA damage (Alipázaga, Moya, Coichev, 2010; Alipázaga *et al*., 2008; *Dong-Sheng, Xiao-Ying, Jie-Qing,* 2006; Moreno *et al*., 2005), might have contributed to the decrease in AA concentration in the previous mixture.

As far as we know, there are currently no studies exploring Cu(II)-mediated DNA damage (present as Cu(II) ion solution) (Dong-Sheng, Xiao-Ying, Jie-Qing, 2006) in a solution containing the two AO compounds mentioned above (ascorbic acid and sulfur dioxide), which is evaluated in the present study.

MATERIALS AND REAGENTS

The Milli-Q Plus Water System (Millipore®) was used to obtain purified water for use in the preparation of all solutions.

Absorbance measurements were performed with an HP 8453 UV (Agilent) spectrophotometer, using quartz cuvettes (1.0 cm pathlength).

pUC-19 DNA (MM 1.74×10^6 Da, 0.5 μg μL^{-1}, Fermentas) was used in all experiments. The diluted DNA solution of 18.8 ng μL^{-1} was obtained by mixing 6 μL of pUC-19 DNA (0.5 μg μL^{-1}) with 154 μL of water in an Eppendorf tube.

$Cu(NO_3)_2$ stock solution (0.20 M, pH 0.6) was prepared by dissolving 1.2719 g of copper wire (Cu, 99.99%, Sigma) in double distilled HNO_3, followed by dilution with water in a 100 mL volumetric flask as previously described (Alipázaga *et al.,* 2009).

Ascorbic acid (AA) stock solution (3.75 mM) was prepared by dissolving 0.0660 g of AA ($C_6H_8O_6$, 176.13 g mol^{-1}, Merck) in water in a 100 mL volumetric flask.

S(IV) stock solution (0.15 M, pH 4.2) was prepared by dissolving 0.3565 g of sodium metabisulfite ($Na_2S_2O_5$, 190.11 g mol^{-1}, 98.0%, Sigma) in water in a 25 mL volumetric flask.

TBE buffer (pH 8.0) stock solution (Tris 446 mM, boric acid 448 mM, and EDTA 10 mM) was prepared by dissolving 27 g of tris-(hydroxymethyl)-aminomethane ($C_4H_{11}NO_3$, 121.14 g mol^{-1}, 99.8%, Synth), 13.75 g of boric acid (H_3BO_3, 61.83 g mol^{-1}, 99.0%, Sigma Aldrich), and 1.861 g of EDTA ($C_{10}H_{16}N_2O_8$, 372.24 g mol^{-1}, 99.0%, Neon) in water in a 500 mL volumetric flask. TBE buffer working solution (Tris 89.5 mM, boric acid 89.6 mM, and EDTA 2.00 mM) was prepared by diluting 100 mL of stock solution in a 500 mL volumetric flask.

The preparation of a 0.8% (m/v) agarose gel and the Ficoll/Bromophenol blue mixture is described in Supplementary Material 1.

EXPERIMENTAL PROCEDURE

DNA with a final concentration of 5.0 ng mL^{-1} was used in all solutions (the general procedure for agarose gel electrophoresis, AGE, is described in Supplementary Material 1).

All AGE experiments (triplicates) were conducted in saturated air solutions ($[O_2]$ ~ 250 μM) under controlled temperature (21 ± 2) ºC in a PowerPac Basic Bio-Rad horizontal mini-tank (BioAgency®), with constant current (30 mA) for 80 minutes.

The gels were placed in a UV Transilluminator ZT-21 (Cristal BioGlow®) and photographed with a PowerShot G10 (Canon®) digital camera after electrophoresis. The photographs were analyzed with AlphaEase® FC software version 6.0. The DNA damage was verified by the conversion of the native form (SC = supercoiled) into an open circular (OC) form, due to single chain breaks, and a linear (L) form, due to double chain breaks.

RESULTS AND DISCUSSION

Influence of Cu(II) concentration

Under the experimental conditions used throughout this work, there was no DNA damage observed in the presence of Cu(II) concentrations ranging from 10 to 120 μM (Figure 1A). Likewise, other studies have shown that there were no lesions to the DNA in an air-saturated solution ($[O_2]$ ~ 250 μM) containing only Cu(II) *(Alipázaga et al*., 2009; *Dong-Sheng, Xiao-Ying, Jie-Qing*, 2006; *Kawanishi, Yamamoto, Inoue*, 1989). This confirms that the presence of a reducing agent is required to promote the Cu(II)/Cu(I) redox cycle and the consequent DNA damage. In fact, it is well known that Cu(I) can decompose organic peroxides (RO_2H) into alkoxyl radicals (RO•) and organic peroxyl (RO_2•) (Aguiar *et al.,* 2007) and subsequently react with H_2O_2 to produce $OH^{\bullet}$, thereby regenerating Cu(II) and resuming the Cu(II)/Cu(I) redox cycle.

However, there is no agreement regarding the Cu(II) concentration necessary (5.0, 10, 20, 50, 100, and 150 μM) to induce DNA damage (Moreno, 2005; *Frelon et al*., 2003; *Lesniak et al*., 2003; *Iwamoto et al*., 2003; *Ogawa et al*., 2003; *Husain, Hadi*, 1995).

Previous studies that evaluated the role of S(IV) showed that DNA damage was observed when Cu(II) concentration was fixed at 100 μM (Moreno, 2005). Thus, in the present study, we evaluated DNA damage in the presence of Cu(II) and AA (in the absence and presence of S(IV)) at a fixed Cu(II) concentration of 100 μM.

DNA damage in the presence of Cu(II) and AA (0.5 to 100 μM).

Addition of AA to a solution containing 100 μM Cu(II) promoted pUC-19 DNA damage, which can be observed by the appearance of the open circular (OC) configuration at AA concentrations ranging from 1.0 μM (Figure 1 B; Lane 4) to 10 μM (Figure 1 B; Lane 6). At concentrations above 50 μM AA (Figure 1 B, Lane 8), it is no longer possible to observe any of the pUC-19 DNA configurations (OC, L or SC) as shown in Table I. No lesions were observed when Cu(II) was maintained at a concentration of 5.0 μM (Figure 1 C) or 10 μM (data not shown).

Previous studies have shown that the addition of S(IV), as SO_3^{2-}, can induce DNA damage in the presence of free or complexed transition metal ions (Dong-Sheng, Xiao-Ying, Jie-Qing, 2006; Jameton, Muller, Burrows, 2002; Kawanishi, Yamamoto, Inoue, 1989).

In the present study, the presence of S(IV) in a solution containing 100 μM Cu(II) (in the absence of complexing agents) revealed that DNA damage occurs at concentrations of 100 μM S(IV) (Figure 1 D, Lane 4) and remains constant up to concentrations of 500 μM (Figure 1 D, Lane 6).

After reaching concentration of 1000 μM S(IV), an increase in the OC form is noted (Figure 1D, Lane 7), which remains constant until a concentration of 2000 μM S(IV), (Figure 1D, Lane 7 and 8). The % of (OC + L) and SC forms are shown in Figure 2.

The results presented above confirm that under these experimental conditions, S(IV) reduces free Cu(II) to Cu(I), which then effectively contributes to pUC-19 DNA damage.

Indeed, when Cu(II) 100 μM is added as a Cu(II)/EDTA complex ($\log \beta_4 = 18.8$) (Smith, Martell, 2004) in the solution containing S(IV) (5.0 to 1000 μM), pUC-19 DNA damage does not occur (data not shown). This indicates the importance of free Cu(II) in the process that promotes DNA damage. In addition, the decrease in DNA damage caused by drugs such as dipyrone (*Roriz, Moya*, 2017) and bilirubin/biliverdin (*Asad et al*., 2002) also decreases significantly when Cu(II) 100 μM is in a solution containing neocuproine (NC), in a ratio of 1Cu(II):2NC. In this case, the decrease in DNA damage is related to the formation of the Cu(I)/NC complex (log

β_2 = 19.1) (Lee *et al*., 2011), which prevents the return of Cu(I) to Cu(II). As such, this confirms that DNA damage occurs only in the presence of the Cu(II)/Cu(I) redox cycle.

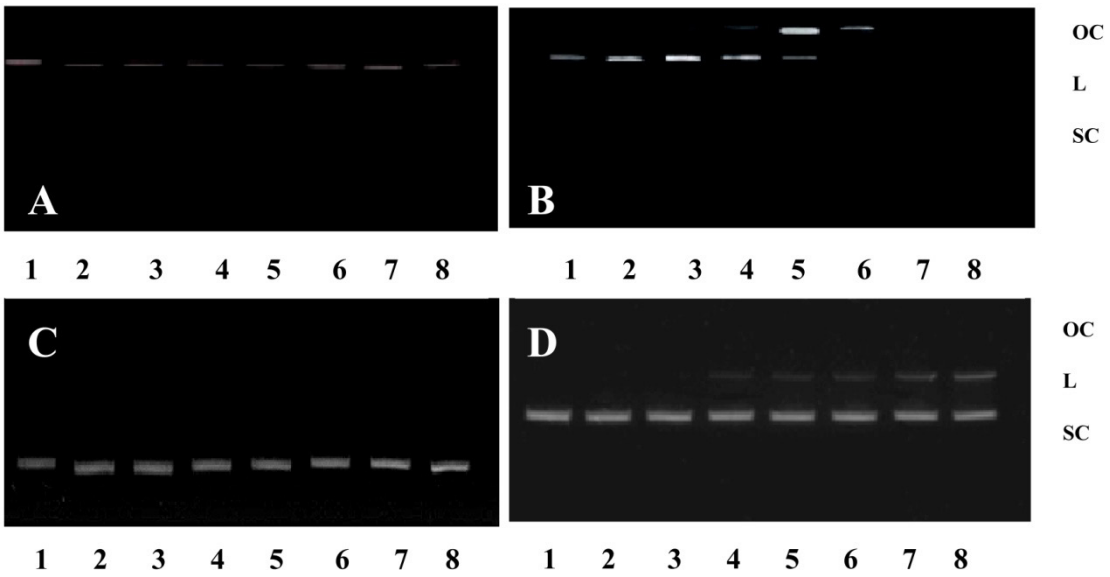

FIGURE 1 – Gel electrophoresis of pUC-19 DNA in the presence of Cu(II) and AA. Saturated air solution ($[O_2]$ ~ 250 µM). pH = 5.5; T = (21 ± 2) ºC. All lanes (1) contain only pUC-19 DNA= 5.0 ng μL^{-1}.

(A) Lane (2 to 8): Lane (1) + Cu(II) 10; 20; 40; 60; 80; 100 and 120 µM, respectively.
(B) Lane (2): Lane (1) + Cu(II) 100 µM.
Lane (3-8): Lane (2) + AA 0.5; 1.0; 5.0; 10; 50; 100 µM, respectively.
(C) Lane (2): Lane (1) + Cu(II) 5.0 µM.
Lanes (3-8): Lane (2) + AA 0.5; 1.0; 5.0; 10; 50; 100 µM, respectively.
(D) Lane (2): Lane (1) + Cu(II) 100 µM.
Lanes (3-8): Lane (2) + S(IV) 50; 100; 250; 500; 1000 e 2000 µM, respectively.

TABLE I – % of forms (OC + L) and SC of pUC-19 DNA (experimental conditions according to Figure 1 B)

Lane	[AA] µM	% (OC+L)	% SC
1	0	0	100
2	0	0	100
3	0.5	19 ± 5	81 ± 5
4	1.0	46 ± 3	54 ± 5
5	5.0	71 ± 2	29 ± 2
6	10	100	0
7	50	-	-
8	100	-	-

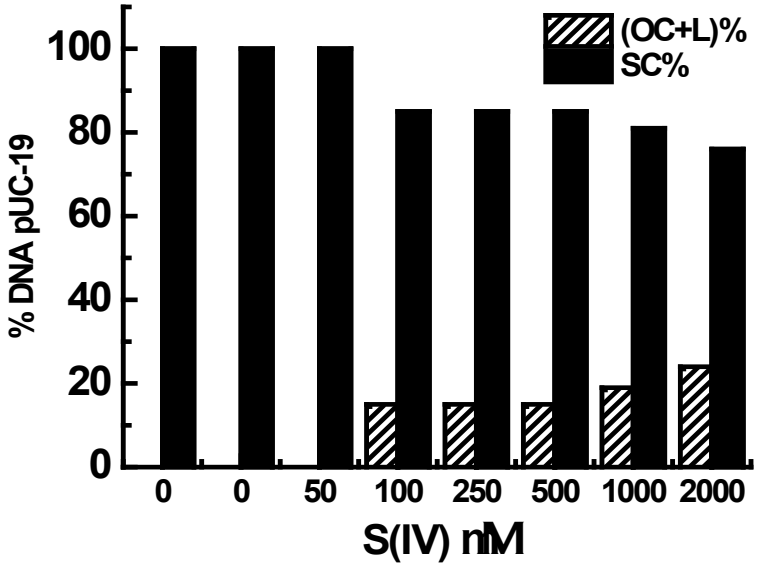

FIGURE 2 – % of (OC + L) and SC configurations of pUC-19 DNA according to the experimental conditions described in Figure 1D (Lanes 1 to 8).

DNA damage in the presence of Cu(II), AA, and S(IV).

In solutions containing either 100 μM Cu(II) or AA/S(IV) as AO agents (Figure 3, lanes 2, 3 and 4), no DNA damage was observed, lanes results which were previously described (Zheng *et al.*, 2006; Dong-Sheng, Xiao-Ying, Jie-Qing, 2006; Kawanishi, Yamamoto, Inoue, 1989). Figure 3 shows that DNA damage occurs in the presence of 100 μM Cu(II), in a solution containing either 500 μM AA or 500 μM S(IV) (Figure 3, Lane 5 and 6). Moreover, DNA damage also occurs in the mixture containing the two AO agents (500 μM AA + 500 S(IV)) (Figure 3, Lane 8).

Sodium sulfite, a preservative in the food industry in Brazil (INS 220), is used in quantities ranging from 0.0020 g to 0.30 g SO_2/100 g food (Favero, Ribeiro, Aquino, 2011), which is equivalent to concentrations ranging from 320 μM to 47 mM, respectively.

As it was previously described, DNA damage in the presence of free or complexed Cu(II) can occur at S(IV) concentrations ranging from 50 μM to 20 mM (*Alipázaga et al.*, 2009; *Dong-Sheng, Xiao-Ying, Jie-Qing,* 2006; *Kawanishi, Yamamoto, Inoue*, 1989). Therefore, to evaluate the effect of the simultaneous addition of both S(IV) and AA on DNA damage, the S(IV) concentration was fixed at 500 μM. Under these conditions, approximately 20% of the pUC-19 DNA is damaged (Figure 1 D, Lane 6 and Table II). Notably, the S(IV) concentration used corresponds to 0.0032 g SO_2/100 g food, which is close to the minimum concentration of S(IV) (350 μM), used in Brazil (Favero, Ribeiro, Aquino, 2011).

Figure 3 presents the results obtained from the addition of the mixture containing AA and S(IV) to a solution containing Cu(II) and DNA. The importance of this metallic ion is highlighted by the absence of DNA damage when these two AOs are together in the absence of Cu(II) (Figure 3, lane 7). As expected, DNA damage occurs in presence of Cu(II) at 100 μM and S(IV) at 500 μM, leading to the formation of an OC conformation (Figure 3, lane 5). On the other hand, total degradation of DNA takes place in the presence of Cu(II) at 100 μM and AA at 500 μM (Figure 3, lane 6). When AA and S(IV) (both at 500 μM) are added simultaneously, the DNA damage is also observed through the appearance of the OC and L forms (Figure 3, lane 8 and Table II). This is noteworthy as the two AO compounds do not act synergistically, whereas the native pUC-19 DNA form (SC) was preserved. This shows that the presence of S(IV) in a solution containing Cu(II) inhibited the AA-induced pUC-19 DNA damage.

By keeping Cu(II) constant at 100 μM and S(IV) at 500 μM, DNA damage (% OC) decreases as the concentration of AA decreases from 20 to 5.0 μM (Figure

4, lanes 3 to 8 and Table III). Under these experimental conditions, DNA integrity was 32% preserved, provided that the [S(IV)]:[AA] ratio was 50: 1 (Figure 4, lane 6, Table III). This can be better observed when comparing lane 4, Figure 4 (Cu(II) 100 μM + AA 500 μM) with lane 8, Figure 3 (Cu(II) 100 μM + AA 500 μM + S(IV) 500 μM). The addition of S(IV) maintains the [S(IV)]:[AA] ratio (1:1) and prevents the total degradation of the DNA originating from the OC (90%) and L (10%) forms, as shown in Table III.

By maintaining Cu(II) 100 μM and AA at 50 μM but varying the concentration of S(IV) from 10 to 1000 μM (Figure 5), we observed an inhibition of DNA damage. At an AA concentration of 50 μM, total DNA degradation occurs (Figure 1B, lane 7 and Figure 5, lane 4). However, from the first addition of the mixture containing 10 μM S(IV) (Figure 5, lane 5), inhibition of DNA damage can be observed, giving rise to (54 ± 2) % of L form. When S(IV) is added at a concentration of 50 μM in the mixture (Figure 5, lane 6), the formation of the SC conformation (23 ± 5) % is observed, which reaches a maximum of (75 ± 3) % at a concentration of 500 μM S(IV) (Figure 5, lane 7) and remains constant up to a concentration of 1000 μM S(IV) (Figure 5, lane 8), as shown in Table IV.

Agarose gel electrophoresis does not enable us to elucidate the mechanisms by which the preservation of pUC-19 DNA is achieved. However, the results presented in this study show that the [S(IV)]:[AA] ratio (ranging from (1:1) to (20:1)) is essential for this inhibition of pUC-19 DNA damage to occur.

These *in vitro* studies cannot be accurately applied to more complex mixtures (e.g. fruit juice, wines or canned food). However, it can be inferred that the presence of S(IV) and AA (in the ratio described above) would allow the association of the antioxidant capacity of these compounds with a low level of DNA damage.

Stability of the mixture containing AA and S(IV)

The inhibition of pUC-19 DNA damage in the mixture containing S(IV), AA and Cu(II) in a saturated air solution ($[O_2] \approx 250$ μM) suggested a reaction between these two AO compounds, which would result in a decrease in the concentration of AA.

Figure 6 shows the overlap of the absorption spectra of the AOs mixture (S(IV) 500 μM + AA 50 μM) and that of the standard solutions of 500 μM S(IV) and 50 μM AA. This seems to indicate that there was no reaction between AA and S(IV) in the mixture.

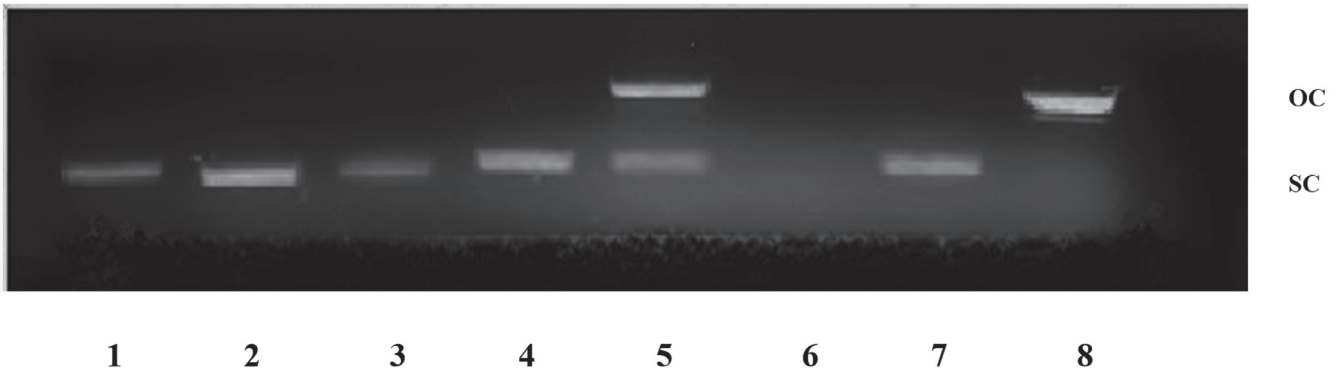

FIGURE 3 – Gel electrophoresis of pUC-19 DNA in the presence of Cu(II) and AA. Saturated air solution ($[O_2] \sim 250$ μM). pH = 5.5; T = (21 ± 2) ºC. All lanes (1) contain only pUC-19 DNA = 5.0 ng μL^{-1}.

Lane (2): Lane (1) + Cu(II) 100 μM.
Lane (3): Lane (1) + S(IV) 500 μM.
Lane (4): Lane (1) + AA 500 μM.
Lane (5): Lane (2) + S(IV) 500 μM.
Lane (6): Lane (2) + AA 500 μM.
Lane (7): Lane (1) + S(IV) 500 μM + AA 500 μM.
Lane (8): Lane (2) + S(IV) 500 μM + AA 500 μM.

TABLE II – % of OC, L and SC forms of pUC-19 DNA 5.0 ng μL^{-1} according to the experimental conditions described in Figure 3

Lane	[Cu(II)] µM	[AA] µM	[S(IV)] µM	% OC	% L	% SC
1	0	0	0	0	0	100
2	100	0	0	0	0	100
3	0	0	500	0	0	100
4	0	500	0	0	0	100
5	100	0	500	18 ± 2	0	82 ± 2
6	100	500	0	-	-	-
7	0	500	500	0	0	100
8	100	500	500	90 ± 3	10 ± 3	-

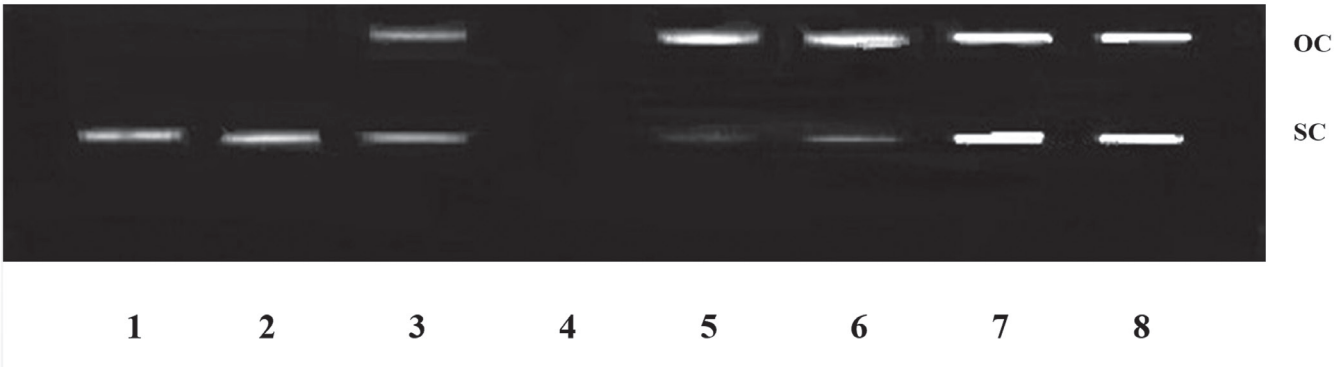

FIGURE 4 – Gel electrophoresis of pUC-19 DNA in the presence of Cu(II) and AA. Saturated air solution ($[O_2]$ ~ 250 µM). pH = 5.5; T = (21 ± 2) ºC. All lanes (1) contain only pUC 19 DNA = 5.0 ng μL^{-1}.

Lane (2): Lane (1) + Cu(II) 100 µM.
Lane (3): Lane (2) + S(IV) 500 µM.
Lane (4): Lane (2) + AA 500 µM.
Lane (5): Lane (3) + AA 20 µM.
Lane (6): Lane (3) + AA 10 µM.
Lane (7): Lane (3) + AA 6.7 µM.
Lane (8): Lane (3) + AA 5.0 µM.

TABLE III – % of (OC+L) and SC forms of pUC-19 DNA 5.0 ng μL^{-1} according to the experimental conditions described in Figure 4

Lane	[AA] µM	% (OC+L)	% SC
1	0	0	100
2	0	0	100
3	0	17 ± 10	83 ± 10
4	500	-	-
5	20	72 ± 4	28 ± 4
6	10	68 ± 9	32 ± 9
7	6.7	61 ± 10	39 ± 10
8	5.0	55 ± 7	45 ± 7

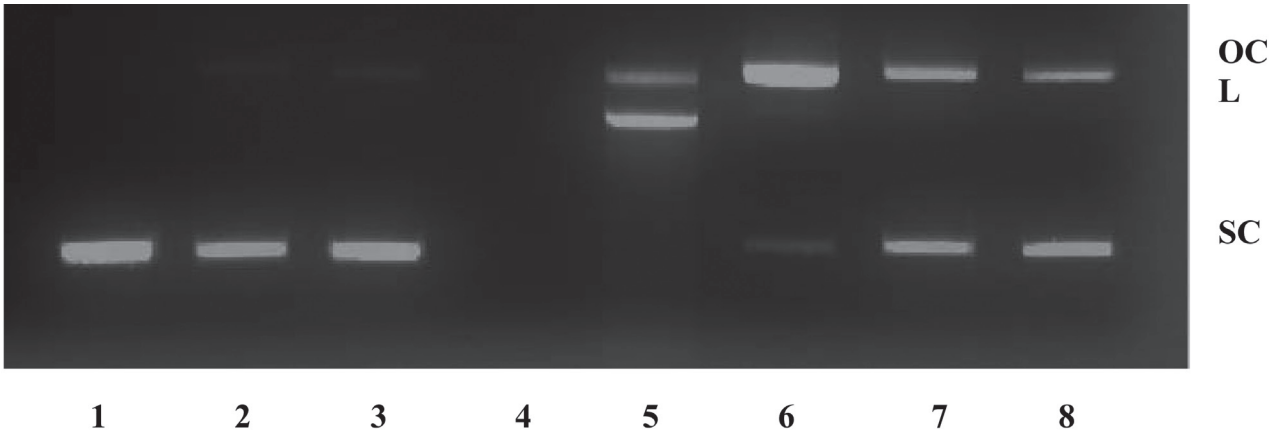

FIGURE 5 – Gel electrophoresis of pUC-19 DNA in the presence of Cu(II) and AA. Saturated air solution ($[O_2] \sim 250$ µM). pH = 5.5; T = (21 ± 2) ºC. All Lane (1) contain only pUC-19 DNA = 5.0 ng μL^{-1}.

Lane e (2): Lane (1) + Cu(II) 100 µM.
Lane (3): Lane (2) + S(IV) 500 µM.
Lane (4): Lane (2) + AA 50 µM.
Lane (5): Lane (4) + S(IV) 10 µM.
Lane (6): Lane (4) + S(IV) 50 µM.
Lane (7): Lane (4) + S(IV) 500 µM.
Lane (8): Lane (4) + S(IV) 1000 µM.

TABLE IV – % of OC, L and SC forms of pUC-19 DNA (experimental conditions according to Figure 5)

Lane	[S(IV)] µM	% OC	% L	% SC
1	0	0	0	100
2	0	0	0	100
3	500	20 ± 1	0	80 ± 1
4	0	-	-	-
5	10	46 ± 2	54 ± 2	0
6	50	77 ± 5	0	23 ± 5
7	500	25 ± 3	0	75 ± 3
8	1000	25 ± 1	0	75 ± 1

A more efficient way to verify whether AA and S(IV) react with each other in the mixture is by performing a multiwavelength linear regression analysis (Blanco *et al.,* 1989) (the mathematical deduction is described in Supplementary Material 2). Considering that $S(IV)_m$, $S(IV)_p$, AA_m, and AA_p represent sodium sulfite and ascorbic acid concentrations in the mixture (m) and in the standard solutions (p), the [S(IV)] and [AA] concentrations in the mixture (pH = 5.5) can be calculated. Using the absorbance values of these solutions ($S(IV)_m$, $S(IV)_p$, AA_m and AA_p) and the mathematical formulas presented in the Supplementary Material 3, we obtained the graph shown in Figure 6 (inserted).

The *a* value (0.95) represents the ratio of $[S(IV)_m]/[S(IV)_p]$, suggesting that sodium sulfite consumption should not occur. The *b* value (0.73), represents the ratio of $[AA_m]/[AAp]$, which indicates a 27% loss in [AA] content. However, this should not be attributed to the direct reaction with S(IV) in these experimental conditions (pH = 5.5).

According to the standard reduction potential (E^0) values of dehydroascorbic acid (DAA/AA) (Harris, 2005), E^0 = 0.390 V *vs*. NHE, and sulfate/sulfite (SO_4^{2-}/SO_3^{2-}) (Lurie, 1978), E^0 = -0.936 V *vs.* NHE, the reaction between the two AOs should not occur under these experimental conditions (pH 5.5).

As such, the mechanism by which DNA damage decreases in the presence of AA and SO_3^{2-} remains unclear. The addition of sodium sulfite to a solution of AA increases the pH value. Therefore, at the pH value of the AA + S(IV) mixture used in the present study (5.5), the AA reduction capacity seems to decrease. In addition, autoxidation of AA in a solution containing SO_3^{2-} due to the presence of dissolved oxygen ($[O_2]$ - 250 µM) may be a possible explanation for the decrease in DNA damage.

Although the gel electrophoresis analysis performed here clearly demonstrates that the presence of S(IV) in the AA solution inhibits DNA damage, studies with other techniques (e.g., HPLC or ion chromatography) should be performed to unequivocally identify the mechanism underlying this process.

CONCLUSIONS

AA (at concentrations above 0.5 µM) and S(IV) (at concentrations above 100 µM) induce damage to pUC-19 DNA in the presence of Cu(II) 100 µM in an air-saturated solution ($[O_2]$ ~ 250 µM). Under the same experimental conditions, AA at 50 µM causes total defragmentation of pUC-19 DNA. However, the simultaneous addition of AA at 50 µM and S(IV) at 500 µM minimizes pUC-19 DNA damage caused by AA. The results showed that inhibition of pUC-19 DNA damage occurs when the ratio of [S(IV)]:[AA] ranges from (1:1) to (20:1).

Absorbance measurements and thermodynamic data show that no reaction occurs between AA and S(IV) when the previously mentioned mixture (pH 5.5) is added to pUC-19 DNA. Moreover, the presence of dissolved oxygen ($[O_2] \approx 250$ µM) may be the cause of AA consumption in the mixture of these two AOs, which subsequently decreases DNA damage.

The results obtained by gel electrophoresis allowed us to observe the occurrence of pUC-19 DNA damage in the solution containing Cu(II), AA or S(IV). However, it is not possible to clarify the mechanism by which the reduction in this damage occurs after the simultaneous addition of AA and S(IV). Studies using other techniques, such as HPLC, should be performed to unequivocally identify the mechanism underlying this process.

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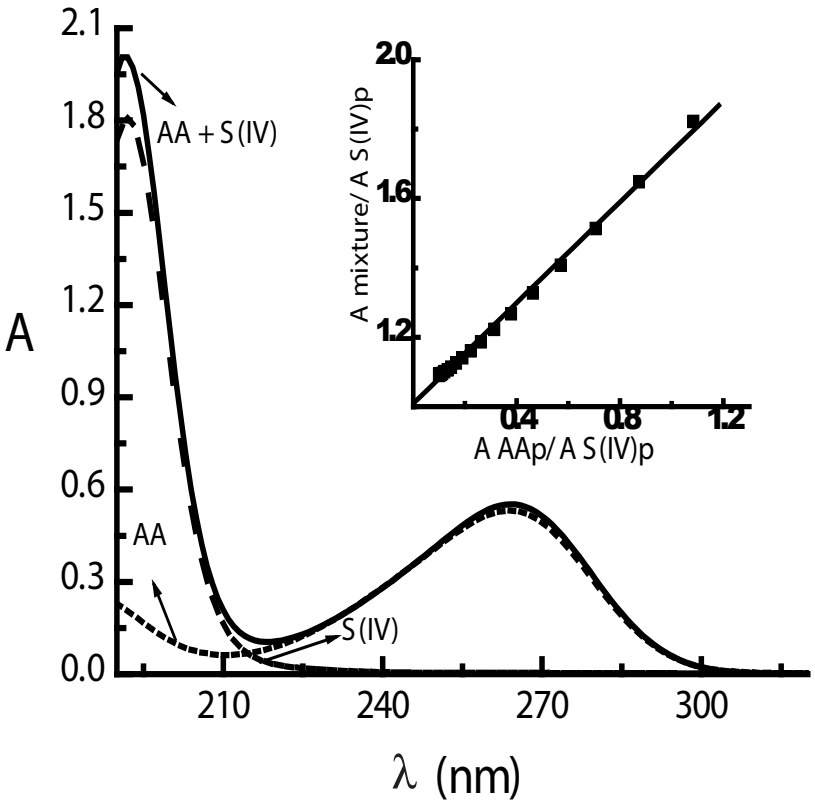

FIGURE 6 – Absorption spectra of solutions containing 50 μM AA, 500 μM S(IV) and mixture (50 μM AA + 500 μM S(IV)). pH = 5.5. Water as reference solution. 1.0 cm pathlength (quartz cuvette). Figure inserted: $A_{mixture}/A_{S(IV)p} = 1.00 + 0.727 \times A_{AAp}/A_{S(IV)p}$; $r^2 = 0.996$.

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