Antimutagenic Effects of Vitamin C Against Oxidative Changes Induced by Quinolones

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Summary

Quinolones are broad-spectrum antibiotics effective against both Gram-positive and Gram-negative bacteria. Reactive oxygen species (ROS) generated by quinolones may damage cell structures and could be a risk to health. The use of vitamin C to reduce such risks may have the opposite effects: vitamin C in the presence of divalent metal ions can induce the Fenton reaction, leading to hydroxyl radical (HO$^\bullet$) generation and oxidative damage. The purpose of this study is to evaluate the antioxidant and prooxidant properties of vitamin C by measuring its effects on both lipid peroxidation and mutagenesis induced by quinolones nalidixic acid (NLX) or norfloxacin (NOR) in *Salmonella typhimurium* TA102. Mutagenicity was evaluated by the Ames test and the results were expressed as (histidine$^+$ revertants/ng of quinolone), while lipoperoxidation was measured as thiobarbituric acid reactive substances (μmol malondialdehyde/(mL·h)). The effects of different concentrations of nalidixic acid (10–1000 ng) or norfloxacin (7–700 ng) on *S. typhimurium* TA102 were studied, employing the S9 mix (liver homogenate from rats pre-treated with Arochlor 1254) in the presence of 10–1000 μg of ascorbic acid (AA) with 0.1 mM FeCl$_3$ or EDTA. Minimal inhibitory concentrations of NOR and NLX against 25 uropathogenic *Escherichia coli* strains were obtained using the plate dilution method in the presence of vitamin C. Vitamin C (1 mg) together with 0.1 mM FeCl$_3$ showed a prooxidant effect in the S9 mix and enhanced the lipoperoxidation induced by either NOR or NLX. Mutagenic potency was also increased for both NOR and NLX. When metal ions were chelated with EDTA, ascorbate showed both antimutagenic and antioxidant properties. Mutagenic potency and lipoperoxidation were reduced for both NOR and NLX. The addition of vitamin C did not change the minimal *in vitro* inhibitory concentrations of NLX or NOR against the 25 uropathogenic *E. coli* strains. The antimutagenic and antioxidant effects of vitamin C were especially marked when the *Salmonella* strain was exposed to NOR or NLX in the presence of EDTA. In contrast, the vitamin C in the presence of FeCl$_3$ increased ROS generation, enhancing both the mutagenic effect of the quinolones and malondialdehyde production from lipoperoxidation induced in the bacterial membranes. Therapeutic use of quinolones together with vitamin C and divalent cations might induce the Fenton reaction involving norfloxacin and nalidixic acid. However, our results suggest that vitamin C could be a good alternative for reducing the genotoxic risk of these therapeutic drugs if it is carefully handled.

Key words: ascorbic acid, vitamin C, norfloxacin, nalidixic acid, antioxidant, antimutagenesis, prooxidant

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Introduction

Fluoroquinolones are widely-used antibiotics because they are effective against Gram-positive and Gram-negative bacteria (*1*). Their therapeutic use cannot be avoided even though it has been shown that they induce oxidative damage in *Salmonella typhimurium* TA102 (*2*). These antibiotics are photosensitive mutagens, with toxic and genotoxic effects that may be due to the singlet oxygen reactive species (ROS) generated by quinolones that absorb light energy (*3,4*). As DNA mutations by ROS are important in the induction of cancers, it is worthwhile to evaluate procedures that might reduce this genotoxic risk. The induction of ROS in *S. typhimurium* by nalidixic acid (NLX) or norfloxacin (NOR) can be inhibited by antioxidants such as β-carotene or by plants rich in antioxidants (*5,6*).

The antimutagenic effects and mechanisms of action of several vitamins against diverse mutagenic agents (*6,7*) have been studied in different short test systems; vitamin C (ascorbic acid, AA) and β-carotene are particular examples. While the antimutagenicity of β-carotene was well demonstrated, vitamin C (AA) only slightly reduced the genotoxic effects of potent mutagens such as 2-nitrofurans, according to the Ames test (*7–9*). In fact, vitamin C (AA) might have a dual activity. It readily scavenges reactive oxygen and nitrogen species and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids and proteins. On the other hand, owing to its oxidoreductive properties, AA also reduces redox-active transition of metal ions in the active sites of specific biosynthetic enzymes. This interaction with catalytically active metal ions could contribute to oxidative damage through the production of hydroxyl and alkoxyl radicals (*10*), so AA could behave as a prooxidant rather than an antioxidant and produce adverse effects. Thus, it significantly increases the frequency of spontaneous revertants in the *S. typhimurium* strains TA102 and TA104, which are sensitive to oxidative mutagens (*11*), but it can utilize H_2O_2 during *in vitro* oxidation and generate hydroxyl radicals in the Fenton type reaction, inducing non-disulphide covalent cross-links in biomolecules (*12*). This study was designed to investigate the antioxidant and prooxidant properties of vitamin C (AA) on *S. typhimurium* strain TA102, using nalidixic acid (NLX) and norfloxacin (NOR) as mutagenic and ROS-generating agents, with the Arochlor 1254-induced rat liver (S9 mix) in the presence of Fe^{3+} ions or EDTA as metal chelator (*13*). Mutagenicity was evaluated by the Ames test and the results were expressed as histidine⁺ revertants/ng of quinolone, while lipoperoxidation was determined by the production of thiobarbituric acid reactive substances (TBARS; μmol of malondialdehyde/(mL·h)).

Materials and Methods

Reagents and strains

Norfloxacin (NOR) was kindly donated by Aplicaciones Farmacéuticas, SA, México, DF, México. Nalidixic acid (NLX) was kindly provided by Winthrop Laboratories, SA, México, DF, México. The antibiotics were tested as chemically pure salts since they are used as commercial preparations. Sodium-L-ascorbate, ampicillin, tetracycline, D-biotin, D-glucose-6-phosphate dipotassium salt (G6P), β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), mitomycin C, 2-aminoanthracene, 1,1,3,3-tetramethoxypropane (malonaldehyde bis(dimethyl acetal)) and 2-thiobarbituric acid were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dimethylsulphoxide (DMSO) and L-histidine were purchased from Merck, Darmstadt, Germany. Arochlor 1254-induced rat liver homogenate (S9 mix) was prepared as described by Maron and Ames (*14*). *S. typhimurium* strain TA102 (hisG428, pQ1, pKM101) was kindly provided by Dr B.N. Ames, University of California (Berkeley, CA, USA). Uropathogenic strains of *E. coli* were obtained from patients with symptomatic urinary infections (*15*).

Mutagenicity assays

Stationary phase cultures of *S. typhimurium* TA102 were prepared in Nutrient Broth No. 2 (Oxoid) in a shaking water bath at 37 °C for 16 h. Aliquots (100 μL) of this culture were exposed to non-bactericidal doses of antibiotics in screw-top sterile tubes: 0.007–0.7 ng/Petri dish of NOR or 0.01–1.0 ng/Petri dish of NLX, or to appropriate positive controls: mitomycin C (2 ng/Petri dish) or 2-aminoanthracene (10 μg/Petri dish). Negative controls (1000 μg/Petri dish) were either the solvent used (distilled water, 10 μL of 0.1 mM EDTA or 0.1 mM $FeCl_3$), DMSO, or AA. Assays were performed with or without 500 μL of Arochlor 1254-induced rat liver (S9 mix). Tubes were incubated for 60 min at 37 °C, and 90 rpm in a Scientific-BF-23 shaking water bath, then 2.0 mL of soft agar were added, as described in the modified preincubation Ames test (*14*). The tube contents were mixed in a vortex, plated on Vogel-Bonner plates and incubated for 48 h at 37 °C. Histidine (His⁺) revertants were counted in a Fisher colony-counter.

Antimutagenicity assays

Cultures were exposed to different doses of NOR or NLX as described above, then 1000, 100 or 10 μg/Petri dish of AA were added. The AA was dissolved in (*i*) distilled water free of metal cations, (*ii*) 0.1 mM EDTA, or (*iii*) 0.1 mM $FeCl_3$, and tested with S9 mix. Negative and positive controls are described above and the reference values were the His⁺ values induced by NOR and NLX in the absence of AA.

Thiobarbituric acid assay (TBARS)

Tubes (100 μL) of overnight *S. typhimurium* TA102 cultures were exposed to either NOR or NLX and ascorbate under the three different experimental conditions described above, then 200 μL of 0.6 M H_2SO_4 and 0.3 M Na_2WO_4 were added. After centrifugation (1000×*g*, 10 min) and addition of 1 mL of 0.375 % thiobarbituric acid, the mixture was boiled for 15 min. After cooling, 0.5 mL of HCl were added and the absorbance was read at 535 nm (*16*). Malondyaldehyde bis(dimethyl acetal), 1,1,3,3-tetramethoxypropane, was used as standard. Lipoperoxidation was measured as thiobarbituric acid reactive substances (TBARS; μmol of malondialdehyde/(mL·h)). Results were calculated as described by Hicks *et al.* (*16*).

In vitro bactericidal studies

Serial dilutions of NOR or NLX of 1:2 were prepared from stock solutions containing 640 µg/mL of NOR or NLX in 0.01 M NaOH (0.5 to 320 µg/mL) (*17*). Volumes of 1 mL of these solutions, with or without 1000 µg of AA, were poured into sterile Petri dishes. They were further diluted (1:10) with 9.0 mL of Mueller-Hinton agar at 45 °C. The plates were gently shaken and allowed to dry at room temperature overnight. Uropathogenic *E. coli* strains were cultured on MacConkey agar and suspensions were adjusted to a McFarland tube no. 0.5 and diluted to 1:10. The final strain dilutions deposited on a Steers replicator were 10^4 CFU^{-1}. NOR or NLX plates with or without AA were inoculated with microcolonies. Initial and final controls of strain growth were also observed. All plates were incubated at 37 °C for 18 h and the number of resistant strains was evaluated (*17*).

Statistical methods

Mutagenicity results were considered positive when the chemical tested induced twice as many His+ revertants as were obtained by spontaneous reversion, and a dose-response curve was plotted. The gradient of the curve was estimated by a multivariate ANOVA test, using Salmonella Assay Analysis software from the US Environmental Protection Agency. The gradient indicates the number of His+ revertants induced per ng of quinolone. Antimutagenesis and antioxidant results were analyzed by Dunnett´s Multiple Comparison test, using GraphPad Prism software version 2.01.

Results and Discussion

Mutagenicity and antimutagenicity assays

Ascorbic acid (1 mg) slightly increased the spontaneous reversion of *S. typhimurium* TA102 from 263±3.84 to 424.33±47.67, without fitting any of the Maron and

Ames (*14*) criteria of mutagenicity. These results were similar to those reported by D'Agostini *et al.* (*18*), who found that AA slightly increased the spontaneous reversion of *S. typhimurium hisG428* strains. This result showed that AA is suitable for evaluation by this short test system (Table 1).

When rat liver was exposed to Arochlor 1254 (S9 mix), and the cytochrome P450 (CYP450) was induced under standard Ames test conditions (*14*), AA reduced the mutagenic effects of both NOR and NLX acid on *S. typhimurium hisG428* TA102 in a dose-dependent manner (Table 1). Chelating of the metal ions on the plate with 0.1 mM EDTA significantly reduced the mutagenic potency of the quinolones compared to the data obtained under standard Ames test conditions. This result is attributable to the sequestering of ions needed for optimum *in vitro* metabolic activation in the Ames test. This problem was partially solved by a twofold increase in the volume of $MgCl_2$-KCl solution recommended by Maron and Ames in the S9 mix for *in vitro* microsomal *Salmonella* mutagenicity assays (Table 1, Fig. 1) (*14*). When AA was added (Table 1, Figs. 1a and 1c), the mutagenic potencies of NOR and NLX were reduced from 9916 to 1605 and from 8727 to 2351 His+ revertants/ng of quinolone (p<0.05), respectively. When AA was added in the presence of $FeCl_3$ and S9 mix, the mutagenic potencies of NOR and NLX were increased from 10800 to 14587 (p<0.05) and from 9052 to 13473 His+ revertants/ng of quinolones (p<0.001) respectively (Table 1, Figs. 1b and 1d). These results were statistically significant.

Lipoperoxidation reactions

Under the assay conditions normally employed for the Ames test, both NOR and NLX induced TBARS in *S. typhimurium* membranes. Chelating metal ions with EDTA resulted in a dose-dependent antioxidant effect of AA (Fig. 2): TBARS were decreased from 48.5 to 12.8 nmol/ (mL·h) (p<0.001) for NOR and from 19.6 to 5.86 nmol/

Table 1. Induced mutagenic potency expressed as His+ revertants/ng of quinolone

	m(ascorbic acid)	*m*(norfloxacin)	S.D.	*m*(nalidixic acid)	S.D.
	µg/assay	0–700 ng/plate		0–1000 ng/plate	
(+S9 mix)	none	22402.3*	93.0	9733.0	149.3
	10.0	19540.0	529.0	9393.0	910.0
	100.0	20400.0	245.0	6257.0	1315.0
	1000.0	18850.0	1641.9	3724.0	649.0
(+EDTA)	none	9916.0	610.0	8727.0	470.2
	10.0	9300.0	871.2	6057.0	172.3
	100.0	5035.0	97.5	6011.0	589.2
	1000.0	1605.0	181.5	2351.0	504.0
(+$FeCl_3$)	none	10800.0	1183.0	9052.8	282.5
	10.0	10870.0	1037.0	10778.0	65.5
	100.0	11547.5	919.0	10472.0	120.0
	1000.0	14587.0	368.5	13472.6	1750.0

*Mutagenic potency calculated as the induced revertants by the gradient, expressed as His+ revertants induced by 1 ng of quinolone. Values were obtained by multi-variance analysis using SALANAL software (EPA, USA). S.D.: standard deviation. The mutagenic potency of ascorbic acid alone was 0.04886±0.016
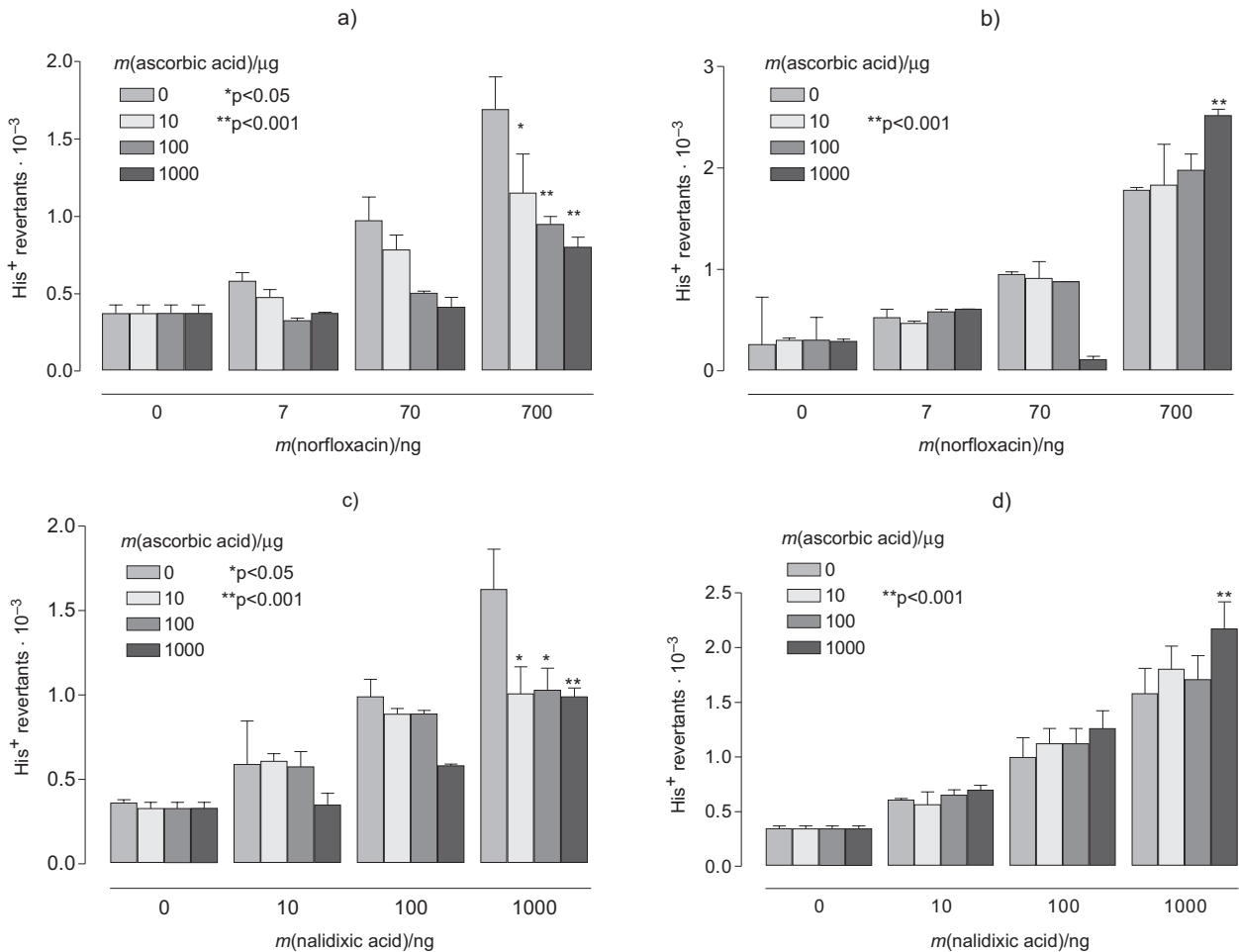
Fig. 1. Antimutagenic activity of ascorbic acid (AA) against mutations induced by: (a) norfloxacin (NOR) in a cation-free medium, (b) norfloxacin (NOR) in Fe^{3+} solution, (c) nalidixic acid (NLX) in a cation-free medium, (d) nalidixic acid (NLX) in Fe^{3+} solution. Each point is the mean of six plates from two independent assays. *Salmonella typhimurium* TA102 spontaneous reversion: 325±45.80. Positive controls: mitomycin C, 2 ng/plate, 2543±83.10; 2-aminoanthracene, 10 µg/plate, 2113.00±190.33. Each point represents the mean of nine Petri dishes from three independent assays. AA in Fe^{3+} solution, 433±71.24; in EDTA, 440±28.90

(mL·h) (p<0.001) for NLX (Figs. 2a and 2c). In contrast, AA in the presence of Fe^{3+} increased the oxidant effect of quinolones: it enhanced the measurable TBARS induced by NOR and NLX from 25.9 to 75.6 nmol/(mL·h) and 18 to 35 nmol/(mL·h), respectively (p<0.001) (Figs. 2b and 2d).

In vitro bactericidal studies

Among the uropathogenic strains evaluated, 16.6 % were resistant to NOR and 33.5 % were resistant to NLX. The addition of vitamin C dissolved in distilled water did not affect the resistance to these antibiotics with or without the S9 mix.

The C-5 fluoroquinolone, norfloxacin (NOR), is a potent antibiotic which induces mutation independently of structural changes that may occur through *in vitro* metabolism. It has been shown to induce oxidative damage in *S. typhimurium* TA102 (*2,3*). After incubation for 60 min at 37 °C and 90 rpm, as described in the Materials and Methods section, NOR shows high mutagenic potency towards *S. typhimurium* TA102. Under these assay condi-

tions, AA reduced its mutagenic potency only slightly (Table 1). Our results agree with those reported by Bala and Grover (*9*), who used distilled water under standard Ames test conditions and reported that AA was only weakly antimutagenic when it was added in a chemically pure form or as citrus juice to potent mutagens. Similar data were also reported by Gajewska *et al.* (*8*), who showed that the effects of the potent mutagens nitrofurazone and furazolidone on *S. typhimurium* TA98 and TA102 were slightly reduced by AA under the Ames test conditions. In contrast, the mutagenic potency of NLX, a less potent mutagen, was reduced in a dose-dependent way by the antioxidant effect of AA (Table 1).

When EDTA was used to sequester the divalent ions present in distilled water, the antimutagenicity of AA was enhanced against both NOR and NLX (Table 1, Fig. 1). The use of weaker, naturally-occurring chelators must be further evaluated to identify the one that can reduce the genotoxic risk of the Fenton reaction without interfering with normal iron metabolism (*13*). When the mutagenicity assays were performed in the presence of Fe^{3+}, the mutagenic potency was reduced, probably because the

cytochrome P450 oxidase system was less efficient and less able to metabolize the quinolones (Table 1). Instead of being an antimutagen under these conditions, AA increases the DNA damage induced by quinolones. These results are consistent with other reports, which indicate that vitamin C (AA) induces the Fenton reaction, producing DNA breaks and biomolecule damage and enhances the genotoxicity of drugs (*13,19*). The value of antimutagens in reducing genotoxic risk is still uncertain, despite several years of study and several assays on naturally-occurring plant derivatives (*20*). The interaction of antioxidants with other organic and inorganic compounds needs to be further evaluated. Espinosa--Aguirre *et al.* (*21*) reported that high doses of a complex mixture of chili extract, which also contains ascorbic acid and inorganic compounds, enhance the mutagenic potency of aromatic hydrocarbons present in polluted air samples, rather than reducing it, as observed with pure antioxidants (*21*). From our results, it is possible to consider the participation of ROS generated by both NOR and NLX (*4*) in the lipoxygenation process indicative of cell membrane breakdown and DNA-damaging mutagenicity in *S. typhimurium* strains (Fig. 2).

In fact, it is well known that quinolones that decompose without generating singlet oxygen may still form ROS or give rise to toxic breakdown products (*22*), producing mutagenic effects at the A/T hot-points in *S.*

typhimurium TA102 strains. Metabolic activation by the NADP-CYP450 oxidase chain (S9 mix), used under the Ames test conditions, involves direct electron transfer from the quinone group to AA, producing ascorbyl free radicals (*23*) that lead to DNA oxidation (a mutagenic effect) and ascorbyl radical monoelectronic reduction, generating semidehydroascorbic acid (*24*). A similar effect might be produced in the presence of iron. AA can be an antioxidant or prooxidant agent, depending on chemical or physiological conditions. Franke *et al.* (*25*) also reported a dual effect of higher and lower doses of AA employed to reduce genotoxic damage induced by methylmethanesulphonate using the comet assay. Ascorbate reduces ferric to ferrous iron, producing the chemical conditions for the Fenton reaction (*26*). The results presented here support the possibility that AA in the presence of free iron and CYP450 enhances the oxidative damage to DNA induced by quinolones. Our results are consistent with the evidence that a combination of AA and iron induces the Fenton reaction in the presence of certain phenolic compounds that can induce DNA breaks, biomolecule damage and increase drug genotoxicity. Indeed, Li *et al.* (*27*) reported that when DNA breaks were induced by the Fenton reagent (ascorbate plus iron), four different phenolic compounds added *in vitro* inhibited the oxidative injury because of their scavenging properties.
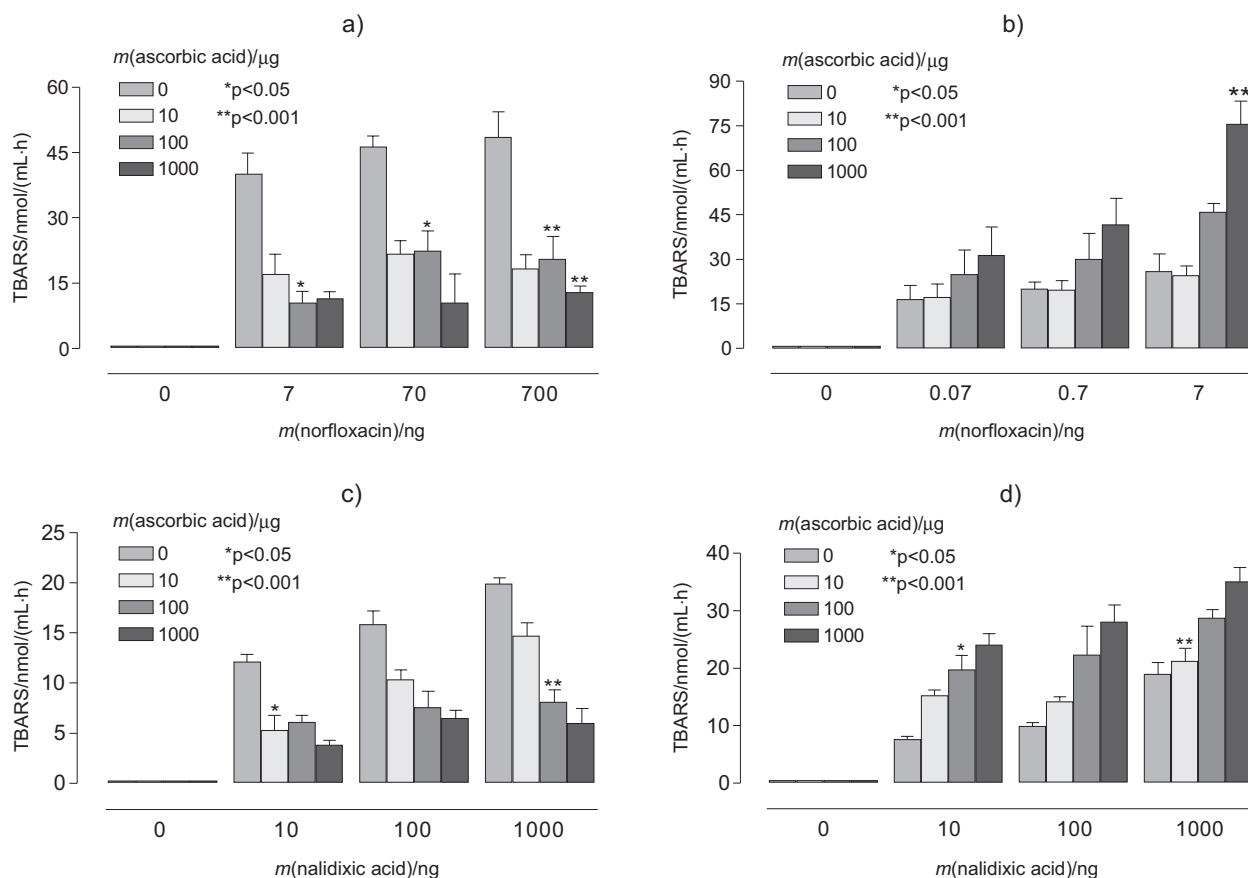

Fig. 2. Antioxidant activity of ascorbic acid (AA) against lipoperoxidation (TBARS) induced in *S. typhimurium* TA102 by: (a) norfloxacin (NOR) in a cation-free medium, (b) norfloxacin (NOR) in Fe^{3+} solution, (c) nalidixic acid (NLX) in a cation–free medium, (d) nalidixic acid (NLX) in Fe^{3+} solution; TBARS, (nmol/(mL·h)). Each point represents the average of three independent assays

Conclusions

The role of vitamin C (AA) *in vivo* has been studied widely, but there are still many contradictory results. Carr and Frei (*10*) also reported that AA increases alkoxyl and hydroxyl ion production when free metal ions are present, but the effect of these reactions *in vivo* needs further study. Some *in vivo* studies failed to demonstrate prooxidation activity by the measurement of DNA damage in human volunteers, perhaps because sufficient free iron was not available in those patients (*28*). Nevertheless, vitamin C in commercial formulae, combined with minerals, must be used with care on patients with infectious diseases during quinolone treatment. On the other hand, low doses of AA (1 mg daily) reduce quinolone-induced mutations, so the consumption of fruits and vegetables rich in AA, avoiding free iron ions, may be a good alternative for decreasing the oxidative damage generated by these therapeutic drugs.

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