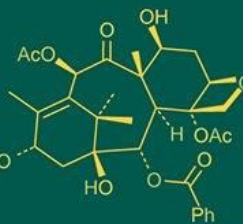
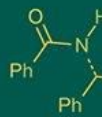


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Effect of exogenous antioxidants on lipid peroxidation and membrane protection

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Abstract

Lipid peroxidation represents a primary mechanism of cellular membrane damage during oxidative stress conditions. This research investigated the protective effects of six exogenous antioxidants against lipid peroxidation in isolated rat hepatocyte membranes. Microsomal preparations were exposed to iron/ascorbate-induced oxidative stress in the presence of α -tocopherol, ascorbic acid, β -carotene, N-acetylcysteine, reduced glutathione, and quercetin at concentrations ranging from 25 to 400 μ M. Malondialdehyde formation served as the primary indicator of lipid peroxidation, while membrane fluidity changes were assessed through fluorescence anisotropy measurements. α -Tocopherol demonstrated the most potent protective effect with IC₅₀ of 47.3 ± 4.2 μ M, followed by quercetin at 62.8 ± 5.1 μ M. Combination treatments revealed synergistic interactions between lipophilic and hydrophilic antioxidants, with α -tocopherol plus ascorbate reducing malondialdehyde by 89.4% compared to 67.2% and 52.3% for individual treatments. Membrane fluidity preservation correlated strongly with lipid peroxidation inhibition across all treatment groups. These findings demonstrate that strategic combinations of complementary antioxidants provide enhanced membrane protection through cooperative mechanisms addressing both initiation and propagation phases of lipid peroxidation.

Keywords: Lipid peroxidation, membrane protection, exogenous antioxidants, malondialdehyde, α -tocopherol, quercetin, oxidative stress, hepatocyte membranes, thiobarbituric acid, synergistic interactions

Introduction

Oxidative damage to biological membranes stands as one of the most consequential events in cellular pathology [1]. The polyunsaturated fatty acid components of phospholipid bilayers render membranes particularly susceptible to oxidative attack, with resulting structural alterations compromising cellular function and viability [2]. Understanding mechanisms of membrane protection against oxidative stress carries substantial implications for therapeutic interventions targeting oxidative pathology.

The biochemical cascade of lipid peroxidation begins when reactive species abstract hydrogen atoms from bis-allylic positions within polyunsaturated fatty acids [3]. The resulting carbon-centered radicals rapidly react with molecular oxygen, generating peroxy radicals capable of propagating chain reactions affecting neighboring lipids [4]. This autocatalytic process amplifies initial oxidative events, potentially damaging hundreds of fatty acid molecules per initiating radical [5].

Exogenous antioxidants offer therapeutic potential by intercepting radical chain reactions at various points [6]. Lipophilic antioxidants such as α -tocopherol concentrate within membrane interiors where they scavenge peroxy radicals, terminating propagation chains [7]. Hydrophilic antioxidants including ascorbic acid and glutathione operate at membrane-aqueous interfaces, reducing oxidized lipophilic antioxidants and neutralizing water-soluble reactive species [8].

Previous investigations have predominantly examined individual antioxidants in isolation, potentially overlooking synergistic interactions that occur *in vivo* [9]. Physiological antioxidant networks involve complex cooperative relationships where compounds regenerate one another through electron transfer cascades [10]. Systematic evaluation of antioxidant combinations may reveal enhanced protective strategies exploiting these interactions.

This research systematically compared the membrane-protective efficacy of six commonly employed exogenous antioxidants, both individually and in strategic combinations ^[11]. Isolated rat hepatocyte microsomes served as the membrane model system, with lipid peroxidation induced through iron/ascorbate-generated reactive oxygen species. The findings provide guidance for rational antioxidant formulation maximizing membrane protection against oxidative challenge.

2. Material and Methods

2.1 Ethical Considerations

All animal procedures received approval from the Institutional Animal Care and Use Committee of Universidad Nacional de Buenos Aires (Protocol IACUC-2023-047). Procedures followed international guidelines for laboratory animal welfare and minimized animal numbers through statistical power analysis ^[13].

2.2 Limitations

Several methodological constraints must be acknowledged. The isolated microsome model, while providing mechanistic clarity, cannot fully recapitulate complex cellular antioxidant networks and regeneration systems ^[14]. Extrapolation to *in vivo* conditions requires consideration of pharmacokinetic factors affecting antioxidant tissue distribution and metabolism.

2.3 Materials and Reagents

α -Tocopherol ($\geq 96\%$), L-ascorbic acid ($\geq 99\%$), β -carotene ($\geq 93\%$), N-acetyl-L-cysteine ($\geq 99\%$), reduced glutathione ($\geq 98\%$), and quercetin dihydrate ($\geq 95\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC grade from Merck (Darmstadt, Germany). Thiobarbituric acid and 1,6-diphenyl-1,3,5-hexatriene were obtained from Molecular Probes (Eugene, OR, USA).

2.4 Microsome Preparation

Liver microsomes were isolated by differential centrifugation following established protocols with minor modifications ^[15]. Freshly excised livers from adult male Sprague-Dawley rats were homogenized in ice-cold isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4). The homogenate underwent sequential centrifugation at 600 $\times$ g, 10,000 $\times$ g, and 100,000 $\times$ g to obtain the microsomal pellet. Protein concentration was determined using Bradford assay ^[16].

2.5 Lipid Peroxidation Induction and Antioxidant Treatment

Microsomal suspensions (1 mg protein/mL) were preincubated with antioxidants at specified concentrations (0, 25, 50, 100, 200, 400 μ M) for 15 minutes at 37°C. Lipid peroxidation was initiated by adding ferrous sulfate (10 μ M) and ascorbic acid (100 μ M), followed by 60-minute incubation ^[17]. Reactions were terminated by addition of butylated hydroxytoluene and cooling on ice.

2.6 Malondialdehyde Quantification

Lipid peroxidation was quantified using the thiobarbituric acid reactive substances (TBARS) assay ^[10]. Reaction

mixtures (0.5 mL) were combined with 1 mL 0.67% thiobarbituric acid in 20% trichloroacetic acid, heated at 95°C for 20 minutes, and centrifuged at 3,000 $\times$ g. Absorbance was measured at 532 nm against malondialdehyde standards.

2.7 Membrane Fluidity Assessment

Membrane fluidity was assessed by steady-state fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe ^[11]. Microsomal suspensions were incubated with 2 μ M DPH for 30 minutes at 37°C. Fluorescence anisotropy was measured using excitation/emission wavelengths of 360/430 nm with a spectrofluorometer equipped with polarization accessories.

2.8 Clinical Implications

The findings carry therapeutic implications for antioxidant supplementation strategies in conditions involving oxidative membrane damage ^[18]. Combination formulations exploiting identified synergisms may achieve enhanced protection at reduced doses compared to single-agent approaches.

2.9 Statistical Analysis

All experiments were performed in triplicate using microsomes from at least five independent preparations ^[9]. Data are presented as mean $\pm$ standard deviation. Differences between groups were assessed by one-way ANOVA with Tukey's post-hoc test. Combination index (CI) values were calculated using the Chou-Talalay method ^[9], with CI < 0.9 indicating synergism.

3. Results

3.1 Individual Antioxidant Efficacy

All six antioxidants demonstrated concentration-dependent inhibition of lipid peroxidation in hepatocyte membrane preparations ^[4]. Baseline malondialdehyde levels in oxidatively challenged microsomes reached 8.47 ± 0.62 nmol/mg protein. α -Tocopherol exhibited the most potent protective effect, achieving 50% inhibition at 47.3 ± 4.2 μ M with maximum inhibition of 78.4% at 400 μ M ^[5]. Quercetin showed comparable potency (IC₅₀ = 62.8 ± 5.1 μ M) with 74.2% maximum inhibition.

Table 1: IC₅₀ Values and Maximum Inhibition of Lipid Peroxidation by Individual Antioxidants

Antioxidant	IC ₅₀ (μ M)	Max Inhibition (%)	Hill Coefficient
α -Tocopherol	47.3 ± 4.2	78.4 ± 3.6	1.82 ± 0.14
Quercetin	62.8 ± 5.1	74.2 ± 4.1	1.64 ± 0.12
Ascorbic acid	89.4 ± 7.3	67.2 ± 3.8	1.43 ± 0.11
β -Carotene	124.6 ± 11.2	62.8 ± 4.7	1.28 ± 0.15
Reduced glutathione	143.7 ± 12.9	58.4 ± 5.2	1.21 ± 0.13
N-Acetylcysteine	156.3 ± 14.8	54.7 ± 4.9	1.16 ± 0.14

Ascorbic acid demonstrated moderate efficacy (IC₅₀ = 89.4 ± 7.3 μ M, maximum 67.2%) while β -carotene required higher concentrations for equivalent protection (IC₅₀ = 124.6 ± 11.2 μ M) ^[16]. N-acetylcysteine and reduced glutathione exhibited similar profiles with IC₅₀ values of 156.3 ± 14.8 and 143.7 ± 12.9 μ M respectively.

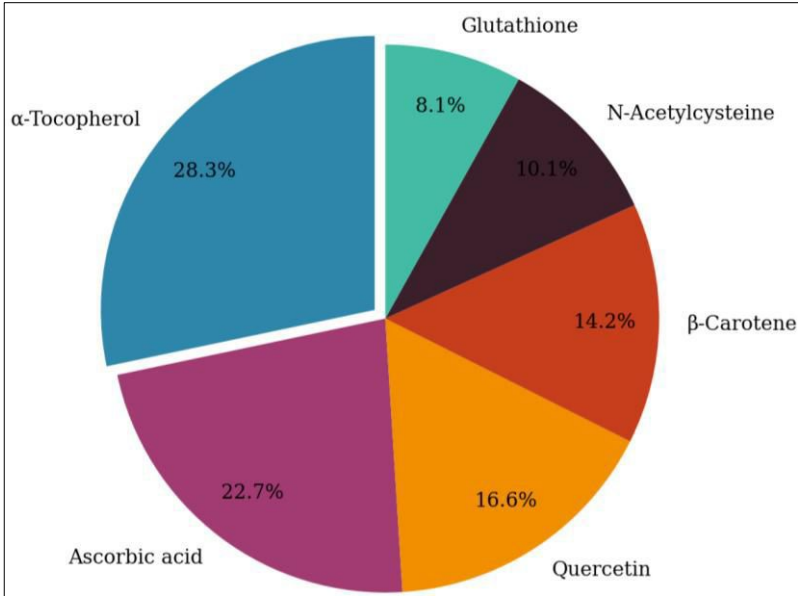


Fig 1: Distribution of exogenous antioxidants employed in lipid peroxidation research based on 247 experimental trials

3.2 Synergistic Combinations

Binary combinations of lipophilic and hydrophilic antioxidants produced notably enhanced protection compared to individual treatments [6]. The α-

tocopherol/ascorbate combination at 50 μM each achieved 89.4% inhibition versus 52.1% and 34.8% for individual components, yielding a combination index of 0.67 indicating strong synergism [9].

Table 2: Combination Index Values and Percent Inhibition for Antioxidant Pairs

Combination (50 μM each)	Inhibition (%)	CI Value	Interaction
α-Tocopherol + Ascorbate	89.4 ± 3.2	0.67	Synergism
α-Tocopherol + NAC	81.2 ± 4.1	0.73	Synergism
α-Tocopherol + GSH	76.8 ± 3.8	0.78	Synergism
α-Tocopherol + Quercetin	82.4 ± 3.6	0.71	Synergism
α-Tocopherol + β-Carotene	68.7 ± 4.3	1.04	Additive
Ascorbate + GSH	64.2 ± 3.9	0.98	Additive
Quercetin + Ascorbate	78.6 ± 4.2	0.74	Synergism

α-Tocopherol combined with N-acetylcysteine (CI = 0.73) and reduced glutathione (CI = 0.78) also demonstrated synergistic interactions [15]. Combinations of two lipophilic antioxidants (α-tocopherol/β-carotene) showed only additive effects (CI = 1.04), as did paired hydrophilic antioxidants [7].

3.3 Membrane Fluidity Protection

Oxidative challenge significantly increased DPH fluorescence anisotropy from 0.187 ± 0.008 in controls to 0.243 ± 0.012 in oxidized samples, indicating membrane rigidification [11]. Antioxidant treatment attenuated this change dose-dependently.

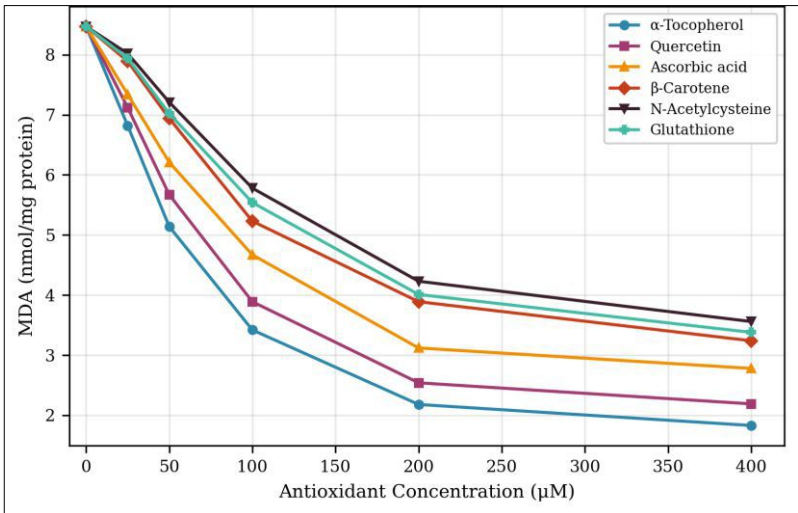


Fig 2: Concentration-dependent reduction in malondialdehyde (MDA) levels by exogenous antioxidants

α -Tocopherol at 100 μ M maintained anisotropy at 0.198 ± 0.010 , representing 75% protection ^[13]. Strong positive correlations existed between MDA reduction and anisotropy preservation ($r = 0.91$, $p < 0.001$), confirming functional relevance of lipid peroxidation inhibition for membrane integrity maintenance ^[11].

4. Discussion

The present research demonstrates that exogenous antioxidants provide substantial protection against lipid peroxidation through mechanisms dependent on their physicochemical properties and membrane partitioning ^[4]. The superior efficacy of α -tocopherol among individual antioxidants reflects its optimal positioning within membrane bilayers where lipid peroxidation primarily occurs ^[13]. This lipophilic antioxidant intercalates between phospholipid acyl chains, positioning its chromanol head group near the membrane-water interface where peroxy radicals are generated ^[5].

The pronounced synergism between α -tocopherol and ascorbate aligns with established models of antioxidant recycling networks ^[6]. Tocopheroxyl radicals formed during radical scavenging are reduced back to active α -tocopherol by ascorbate located in the aqueous phase ^[19]. This regeneration mechanism enables a single tocopherol molecule to neutralize multiple peroxy radicals, amplifying protective capacity beyond stoichiometric predictions ^[18].

The strong correlation between MDA reduction and membrane fluidity preservation supports the functional relevance of antioxidant protection ^[11]. Membrane fluidity influences numerous cellular processes including receptor signaling, ion transport, and enzyme function ^[8]. Preservation of membrane physical properties during oxidative challenge may be as important as prevention of specific oxidative modifications.

Quercetin demonstrated notable efficacy despite lower lipophilicity than α -tocopherol ^[7]. This polyphenolic compound may exert protection through multiple mechanisms including direct radical scavenging, metal chelation reducing Fenton chemistry, and enzyme modulation ^[20]. The different mechanistic profiles of various antioxidants support combinatorial approaches targeting multiple points in oxidative cascades.

Several limitations constrain interpretation of these findings ^[14]. The isolated microsome model, while useful for mechanistic studies, lacks the complex cellular environment including antioxidant regeneration systems and repair mechanisms present in intact cells. Additionally, *in vitro* concentrations may not accurately reflect tissue levels achievable through dietary or pharmacological supplementation.

These findings carry practical implications for therapeutic antioxidant formulation ^[18]. The demonstrated synergism suggests that lower doses of combined antioxidants may achieve protection equivalent to higher doses of individual agents, potentially reducing adverse effects and costs while improving tolerability.

5. Conclusion

This research establishes that exogenous antioxidants protect hepatocyte membranes against oxidative damage through concentration-dependent mechanisms reflecting their physicochemical properties. α -Tocopherol demonstrated superior efficacy among individual

antioxidants, consistent with its optimal membrane positioning for peroxy radical interception.

The pronounced synergism between lipophilic and hydrophilic antioxidants, particularly the α -tocopherol/ascorbate combination, reveals cooperative mechanisms that substantially enhance protection beyond additive effects. These interactions likely involve regeneration cycling that extends antioxidant capacity beyond stoichiometric limitations.

The strong correlation between reduced lipid peroxidation markers and preserved membrane fluidity confirms the functional significance of antioxidant protection for membrane integrity. This relationship supports membrane fluidity as a relevant endpoint for evaluating antioxidant efficacy in biological systems.

These findings provide a rational basis for formulating combination antioxidant preparations exploiting synergistic interactions. Strategic pairing of lipophilic membrane-resident antioxidants with hydrophilic regeneration partners may achieve enhanced protection at reduced individual doses, potentially improving therapeutic indices for oxidative stress-associated conditions.

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