Slow oxidation of high density lipoproteins as studied by EPR spectroscopy

Stojanović, Nataša; Krilov, Dubravka; Herak, Janko N.

Slow oxidation of high density lipoproteins as studied by EPR spectroscopy

NATAŠA STOJANOVIĆ[1], DUBRAVKA KRILOV[2*] & JANKO N. HERAK[1]

[1]*Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia*;

[2]*Medical School, University of Zagreb, Zagreb, Croatia*

*Correspondence: Dubravka Krilov, Department of Physics and Biophysics, University of Zagreb, Medical School, Šalata 3b, 10000 Zagreb, Croatia. Tel: +385 1 4566 951. Fax: +385 1 4590 276. E-mail: krilov@mef.hr

Abstract

There is relatively little information on the role of HDL oxidation in atherogenesis although there are indications that oxidation might affect atheroprotective activities of HDL. Recently we reported the study on LDL oxidation initiated and sustained by traces of the transition metal ions under conditions which favor slow oxidation. Here we report the results of the analogous study on the oxidation of the two HDL subclasses. The oxidation process was monitored by measuring the time dependence of oxygen consumption and concentration of the spin-trapped free radicals using EPR spectroscopy. In both HDL_2 and HDL_3 subclasses, the dependence of the oxidation process on the copper/lipoprotein molar ratio is different from that in LDL dispersions. Comparison of the kinetic profiles of HDL_2 and HDL_3 oxidation revealed that under all studied experimental conditions HDL_2 was more susceptible to copper-induced oxidation than HDL_3.

Keywords: *high density lipoprotein, slow oxidation, copper ions, EPR spectroscopy*

Introduction

Numerous epidemiological and clinical studies have demonstrated inverse and independent relationship between high density lipoprotein cholesterol (HDL-C) level and the risk for cardiovascular disease [1]. However, it is not known whether this relationship is causal [2,3]. The association between low HDL-C level and an increased risk for coronary artery disease has been explained by antiatherogenic activities of HDL, including its role in reverse cholesterol transport (RCT), its antioxidant and anti-inflammatory properties [4,5]. High density lipoprotein is a class of plasma lipoproteins characterized by high density and small size. HDL particles are heterogeneous in physicochemical properties, intravascular metabolism and biological activity [3,6]. It has been suggested that the concentration of various HDL subclasses, not the total HDL-C concentration, and the kinetics of HDL metabolism are important determinants of the HDL anti-atherogenic activities and the risk of atherosclerosis, respectively [3,4]. It is now quite generally accepted that oxidatively modified low density lipoprotein (LDL) is implicated in the initiation and progression of atherosclerosis, a chronic inflammatory disease [7]. The role of HDL in inflammation is more complex. In its basal state, HDL is anti-inflammatory, but during acute inflammation HDL become pro-oxidant [8]. HDL can also undergo oxidative modification *in vivo* and HDL lipids are equally or even more susceptible to oxidation than those of LDL [9-12]. Compared to LDL, relatively little is known about the role of HDL oxidation in atherogenesis. There is also little information on relative susceptibility to

oxidation of different HDL subclassess. Oxidative modification of HDL might have important consequences concerning the efficiency of HDL in promoting cholesterol efflux from the peripheral cells (RTC) and in inhibiting LDL oxidation [11,13]. Therefore, studying the mechanisms of the HDL oxidation might have potential pathophysiological significance.

Like with LDL, oxidation of HDL has been studied *in vitro* using variety of initiators including H_2O_2, myeloperoxidase-derived oxidants, copper, lipooxygenase and peroxyl radicals [11,14]. Copper ions are commonly used since metal ions may contribute to lipoprotein oxidation at least in the late stages of atherosclerotic lesion development [11,15]. In our earlier studies, we demonstrated that copper could also initiate oxidation of LDL in a closed system with a defined and limited amount of oxygen and a with very small concentration of copper ions (the Cu^{2+} ion to LDL molar ratio was within the range of 0.01 to 0.2) [16,17]. The main features of the temporal behavior of several oxidation markers (oxygen consumption, free radical generation, lipid hydroperoxide formation and α-tocopherol concentration) were explained in terms of the tocopherol-mediated peroxidation (TMP), initiated by traces of transition metal ions associated with LDL particles [18,19]. The developed probabilistic kinetic model could simulate slow and mild LDL oxidation under unfavorable oxidizing conditions that could be physiologically relevant. There is also evidence that TMP might be relevant for HDL oxidation [20-22]. That promted us to investigate the application of the probabilistic kinetic model

based on TMP to study copper-initiated oxidation of the HLD_2 and HDL_3 subclasses.

Dynamics of copper-induced HDL oxidation carried out under unfavourable oxidizing conditions was followed by measuring oxygen consumption and concentration of trapped free radicals in a closed system. The study on LDL oxidation has demonstrated that oxygen consumption is the best indicator of the oxidation progress in lipoprotein dispersions [17].

Materials and methods

Chemicals

2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), diphenyl-picryl-hydrazyl (DPPH), a 3-carbamoyl-2,2,5,5-tetramethyl-3-pyroline-1-yloxy (CTPO) spin probe and a *N*-*t*-buthyl-α-phenylnitrone (PBN) spin trap were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Potassium bromide (KBr) p.a. and copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_20$) p.a. were obtained from Merk (Darmstadt, Germany). All other chemicals for buffer preparation (NaCl, KCl, Na_2HPO_4, KH_2PO_4) and lipoprotein isolation were of the highest purity grade available and were purchased from Sigma-Aldrich. Aqueous solutions for all experiments were prepared using ultrapure water obtained from a SG Ultra Clear water purification system (SG Wasseraufbereitung und Regenerierstation GmbH; Barsbüttel, Germany).

HDL isolation and oxidation

HDL_2 (*d*=1.063-1.125 g/ml) and HDL_3 (*d*=1.125-1.210 g/ml) subclasses were isolated from plasma of healthy female blood donors in presence of ethylenediaminetetraacetic acid (EDTA) (1 mg/ml) by sequential ultracentrifugation using Beckman Optima™ LE-80K Ultracentrifuge equipped with a 70.1 Ti rotor (Beckman Instruments, Palo Alto, CA, USA) [23]. Before copper oxidation, isolated HDL_2 and HDL_3 subfractions were dialyzed at 4°C for 24 hours against 0.15 M phosphate-buffered saline (PBS), pH 7.4, to remove salts and EDTA. Subsequently, HDL preparations were concentrated using Minicon®-B15 concentrators (Millipore Corporation, Bedford, MA, USA) and then adjusted to the desired concentration with buffer (PBS) or buffered solutions of the spin probe (CTPO) or spin trap (PBN). The lipoprotein concentrations were assessed by determination of total dry mass of the preparations, after removal of all salts by dialysis, assuming the relative molecular mass of 360 000 for HDL_2 and 175 000 for HDL_3. The purity of the HDL preparations was checked by lipoprotein electrophoresis kit (Lipidophor All in 12; Immuno AG, Vienna, Austria).

Prior to oxidation at 37°C, freshly prepared $CuCl_2$ solution (2-4 μmol/L) was added to the HDL_2 and HDL_3 dispersions (28-114 μmol/L). Some HDL samples were oxidized without adding any $CuCl_2$. Oxidation was carried out in tightly closed quartz capillaries (Drummond Scientific Company; Broomal, PA, USA) to prevent oxygen diffusion from the atmosphere into the samples.

The Cu^{2+} ion to HDL molar ratio was within the range of 0.02 to 0.14.

Free radical detection

Oxidation-iduced free radicals in the HDL_2 and HDL_3 samples were recorded with the electron paramagnetic resonance (EPR) spectroscopy using PBN dissolved in PBS as a spin trap. PBN was added to the HDL samples prior to incubation at final concentration of 50 mM. The EPR spectra were recorded at 37°C on a Varian E-109 spectrometer, equipped with a variable temperature control unit. The quantitative estimate of the free radical content in the HDL samples was done by comparing the HDL spectra with the spectra of known concentration of the stable radical (TEMPO) after double integration.

Measurement of oxygen consumption

Oxygen concentration in the HDL_2 and HDL_3 samples was measured by EPR oxymetry using a CTPO spin probe [24]. EPR spectrum of CTPO added to the HDL dispersions immediately before incubation at 37°C is sensitive to the concentration of oxygen in an aqueous phase of the sample [16].

Results and discussion

To investigate the effect of copper content on the dynamics of HDL oxidation, samples of HDL_2 and HDL_3 subclasses with varying copper concentration were prepared. In these experiments, the highest molar ratio of copper to HDL was 0.14, the value much lower than in most other studies of copper-induced HDL

oxidation [21,22,25]. It has been demonstrated that transition metal ions are ubiquitous in the contamination of *in vitro* systems [26]. It means that traces of the transition metal ions were present even in the HDL samples with no copper ions purposely added.

The change of oxygen concentration in the aqueous phase of the HDL samples with the incubation time is shown in Figure 1. The results are typical of six independent experiments performed with the samples of different donors. The measurements were made for the HDL samples (≈ 56 μmol/L) with no copper ions added (triangles), with 2 μmol/L (squares) and 4 μmol/L of copper ions added (circles). The data for HDL_2 are shown in panel A and those for HDL_3 in panel B. The curves presented in the figure were calculated by the polynomial fitting procedure. The experimental data demonstrate that in both HDL subclasses the oxidation measured by the rate of oxygen consumption is accelerated with the increase of the copper content. Since oxygen is consumed in the lipid peroxidation chain reactions, these results confirm the involvement of the copper in both initiation and propagation of the HDL oxidation. Figure 1 also demonstrates that the oxidation process is faster in HDL_2 than in HDL_3 samples. If samples of equal molar or mass concentration are compared, the rate of HDL_2 oxidation is higher than the rate of HDL_3 oxidation within the studied range of copper concentration. That difference is somewhat less pronounced in the samples without purposely added copper, presumably due to much lower intrinsic concentration of the metal ions.

Similar differences between the two subclasses of HDL were observed by measuring concentration of induced free radicals. The increase in concentration of the trapped free radicals with time of incubation is presented in Figure 2. In both panels the curves for the samples with no added (triangles) and those with 4 μmol/L added copper (circles) are presented. Panel A refers to HDL_2, and panel B to HDL_3. The presence of PBN-spin adducts in EPR spectra was detected only after a significant amount of oxygen was consumed, as could be observed by comparing Figure 1 and Figure 2. Similar behaviour was observed for LDL oxidation [16]. The trapped free radicals could be located in the lipid or protein domain of the HDL particles. In our earlier study of LDL oxidation [16] about 90% of spin adducts were found to be protein-bound. Since the protein/lipid ratio in HDL is higher than in LDL and because HDL-associated apolipoproteins AI and AII are oxidized by lipid hydroperoxides during mild oxidation of HDL [14], it is probable that the percentage of the protein-bound trapped radicals in HDL is even higher. No attempt was made to prove that.

The dependence of the rate of oxygen consumption on HDL concentration is shown in Figure 3. Panel A shows the data for HDL_2 (56 μmol/L - circles and 28 μmol/L - squares) and panel B the data for HDL_3, (114 μmol/L - circles and 29 μmol/L - squares). Contrary to our expectations based on the model of LDL oxidation [19], oxygen was consumed faster in the samples of lower HDL concentration for both HDL subclasses. Such a behaviour was observed in four independent experiments performed with the HDL samples of different donors.

However, the free radical accumulation did not behave in the same way, as shown in Figure 4. For both HDL subclasses the rate of free radical accumulation was faster in the samples of higher HDL concentration. In that figure panel A shows the data for HDL_2 (56 μmol/L - circles and 28 μmol/L - squares) and panel B for HDL_3 (114 μmol/L - circles and 57 μmol/L - squares). Apparently different behaviour of the two oxidation markers during slow and mild oxidation of the HDL could be the result of interparticle interaction, i.e. due to transfer of the oxidation products from one particle to another. It is quite probable that most of the free radicals carrying lipid peroxidation chain reactions are stabilized in the protein domain of the HDL particle or end up as non-radical products in the recombination reactions. If so, the total number of PBN-trapped free radicals is expected to be higher in the samples of higher HDL concentration. On the other hand, in the dispersions of higher HDL concentration the formed lipid hydroperoxyl radicals have higher probability to directly react with the surface proteins on the neighbouring HDL particles, thus avoiding reaction with oxygen. In accordance with this line of reasoning is the observation that in mildly oxidized HDL the apolipoproteins dissociate more easily from the particles than in native HDL [14].

To conclude, it is obvious that the copper-induced oxidation of HDL is a rather complex process and that our theoretical model of LDL oxidation [19] is not appropriate for the description of the kinetics of the HDL oxidation. The probabilistic kinetic model is based on the concept of LDL particles as distinctive compartments with only weak inter-particle interaction between the

oxidation bursts. To model the oxidation of the HDL dispersions, the intra-particle and inter-particle events should be taken into account at the same level of calculation.

Acknowledgement

This work is supported by the Ministry of Science, Education and Sports of the Republic of Croatia.

References

[1] Gordon DJ, Rifkind BM. High-density lipoproteins: the clinical implications of recent studies. N Engl J Med 1989;321:1311-1316.

[2] Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ, Fogelman AM. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. J Lipid Res 2004;45:993-1007.

[3] Assmann G, Gotto, AM. HDL cholesterol and protective factors in atherosclerosis. Circulation 2004;109 (suppl III): III-8-III-14.

[4] Von Eckardstein A, Nofer J-R, Assmann G. High densty lipoproteins and atherosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2001;21:13-27.

[5] Nofer J-R, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A. HDL and arteriosclerosis: beyond reverse cholesterol transport. Atherosclerosis 2002;161:1-16.

[6] Kontush A, Chantepie S, Chapman MJ. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. Arterioscler Thromb Vasc Biol 2003;23:1881-1888.

[7] Witzum, JL, Steinberg D. Role of oxidized low density lipoprotein in atherogensis. J Clin Invest 1991;88:1785-1792.

[8] Van Lenten BJ, Navab M, Shih D, Fogelman AM, Lusis AJ. The role of high-density lipoproteins in oxidation and inflammation. Trends Cardiovasc Med 2001;11:155-161.

[9] Bowry W, Stanley KK, Stocker R. High density lipoprotein is the major carrier of lipid hydroperoxides in fasted human plasma. Proc Natl Acad Sci USA 1992;89:10316-10320.

[10] Nin X, Zammit V, Upston JM, Dean RT, Stocker R. Co-existence of oxidized lipids and α-tocopherol in all lipoprotein fractions isolated from advanced human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1999;19:1708-1718.

[11] Francis GA. High density lipoprotein oxidation: in vitro susceptibility and potential in vivo consequences. Biochim Biophys Acta 2000;1483:217-235.

[12] Pennathur S, Bergt C, Shao B, Byun J, Kassim SY, Singh P, Green PS, McDonald TO, Brunzell J, Chait A, Oram JF, O'Brien K, Geary RL, Heinecke JW. Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. J Biol Chem 2004;279:42977-42983.

[13] Bergt C, Pennathur S, Fu S, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. Proc Natl Acad Sci USA 2004;101:13032-13037.

[14] Pankhurst G, Wang XL, Wilcken DE, Baernthaler G, Panzenböck U, Raftery M, Stocker R. Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein. J Lipid Res 2003;44:349-355.

[15] Gaut JP, Heinecke JW. Mechanisms for oxidizing low-density lipoprotein. Insights from patterns of oxidation products in the artery wall and from mouse models of atherosclerosis. Trends Cardiovasc Med 2001;11:103-112.

[16] Herak JN, Krilov D, Stojanović N, Marincel J. Very slow autoxidation of low-density lipoprotein spares α-tocopherol. Chem Phys Lipids 1998;94:63-70.

[17] Krilov D, Stojanović N, Herak JN. Probabilistic kinetic model of slow oxidation of low-density lipoprotein. II. Experiments. Chem Phys Lipids 2004; 129:75-84.

[18] Bowry VW, Stocker R. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. J Am Chem Soc 1993;115:6029-6044.

[19] Herak JN, Stojanović N, Krilov D. Probabilistic kinetic model of slow oxidation of low-density lipoprotein. I. Theory. Chem Phys Lipids 2004;129:63-74.

[20] Garner B, Witting PK, Waldeck AR, Christison JK, Raftery M, Stocker R. Oxidation of high density lipoproteins I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by α-tocopherol. J Biol Chem 1998;273:6080-6087.

[21] Raveh O, Pinchuk I, Schnitzer E, Fainaru M, Schaffer Z, Lichtenberg D. Kinetic analysis of copper-induced peroxidation of HDL, autoaccelerated and tocopherol-mediated peroxidation. Free Radical Biol Med 2000;29:131-146.

[22] Laureaux C, Thérond P, Bonnefont-Rousselot D, Troupel SE, Legrand A, Delattre, J. α-tocopherol enrichment of high-density lipoproteins: stabilization of hydroperoxides produced during copper oxidation. Free Radical Biol Med 1997;22:185-194.

[23] Havel RJ, Eder HA, Brandon JH. The distribution and chemical composition of centrifugally separated lipoproteins in human serum. J Clin Invest 1995;34:1345-1353.

[24] Lai CS, Hopwood LE, Hyde JS, Lukiewicz S. ESR study of 0_2 uptake by Chinese hamster ovary cells during the cell cycle. Proc Natl Acad Sci 1982;79: 1160-1170.

[25] Thomas MJ, Chen Q, Zabalawi M, Anderson R, Wilson M, Weinberg R, Sorci-Thomas MG, Rudel LL. Is the oxidation of high-density lipoprotein lipids different than the oxidation of low-density lipoproteina lipids? Biochemistry 2001;40:1719-1724.

[26] Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combination to avoid. Radiat Res 1996;145:532-541.

Figure captions

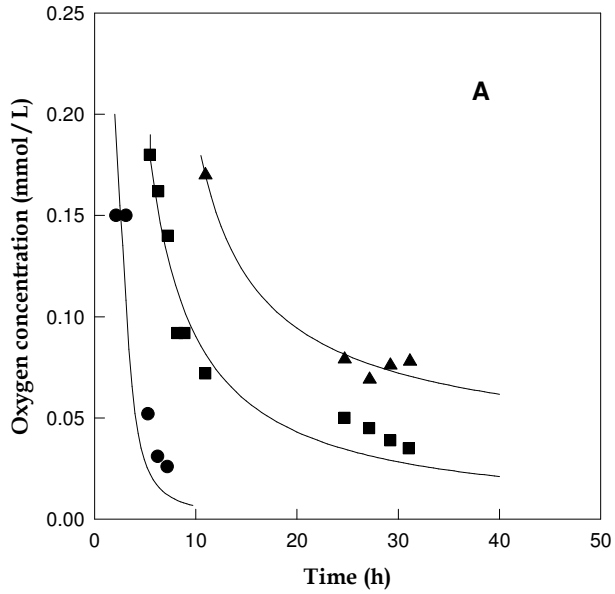
Figure 1 Panel A: Time dependence of oxygen consumption in HDL_2 (56 μmol/L) without added Cu^{2+} ions (▲) and with added 4 μmol/L (●) and 2 μmol/L (■) of copper. Panel B: Time dependence of oxygen consumption in HDL_3 (57 μmol/L) with added 2 μmol/L (■) and 4 μmol/L (●) of copper ions, respectively. Data shown represent a typical experiment of four different HDL preparations.

Figure 2 Accumulation of PBN-trapped free radicals with time of incubation. Panel A: HDL_2 (56 μmol/L) without the added Cu^{2+} ions (■) and with Cu^{2+} ions added (4 μmol/L (●)). Panel B: HDL_3 (57 μmol/L) without added Cu^{2+} ions (■) and with Cu^{2+} ions added (4 μmol/L (●)).

Figure 3 Representative data on the effect of copper (2 μmol/L) on the rate of oxygen consumption in the HDL_2 samples of two different concentration: 28 μmol/L (■) and 56 μmol/L (●) (Panel A) and on the rate of oxygen consumption in the HDL_3 samples of two different concentration: 29 μmol/L (■) and 114 μmol/L (●) (Panel B).

Figure 4 The time dependence of PBN-trapped free radicals in the HDL_2 samples of concentrations: 28 μmol/L (■) and 56 μmol/L (●) (Panel A) and HDL_3 samples of concentrations: 57 μmol/L (■) and 114 μmol/L (●) (Panel B). The copper content in the samples was 4 μmol/L.

Figure 1


A
Oxygen concentration (mmol / L)
Time (h)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50

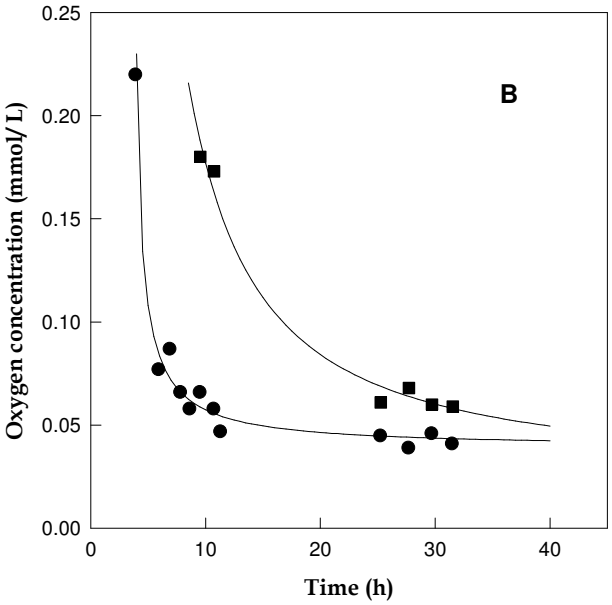

B
Oxygen concentration (mmol/ L)
Time (h)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40

Figure 2

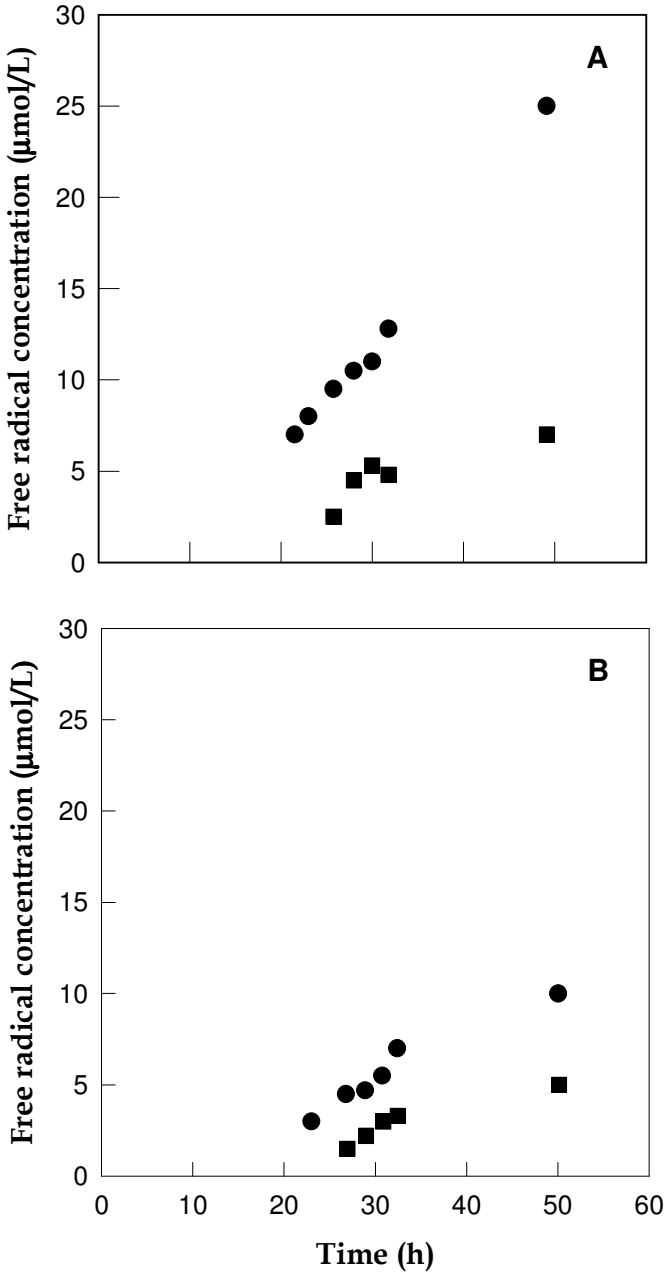
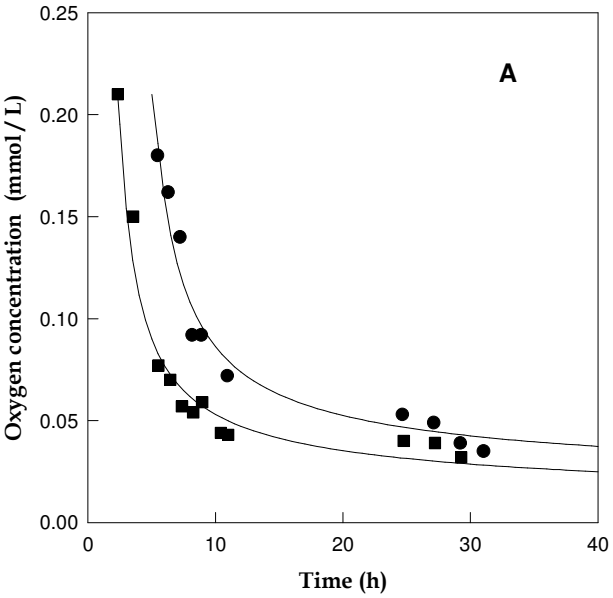

A
B
Free radical concentration (µmol/L)
Free radical concentration (µmol/L)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
Time (h)

Figure 3


A
Oxygen concentration (mmol / L)
Time (h)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40

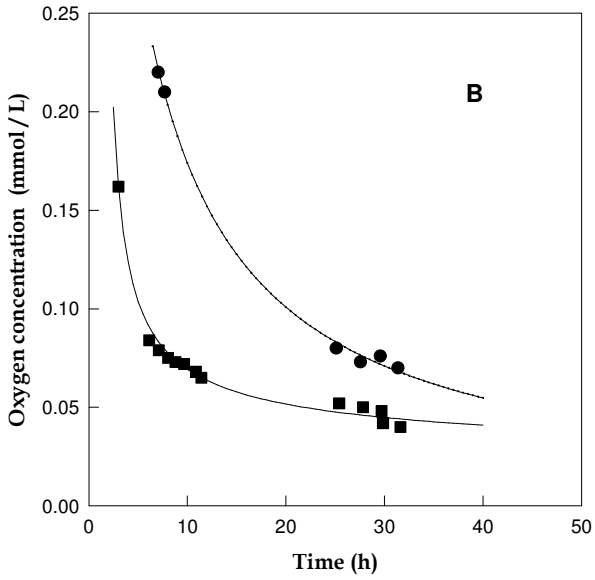
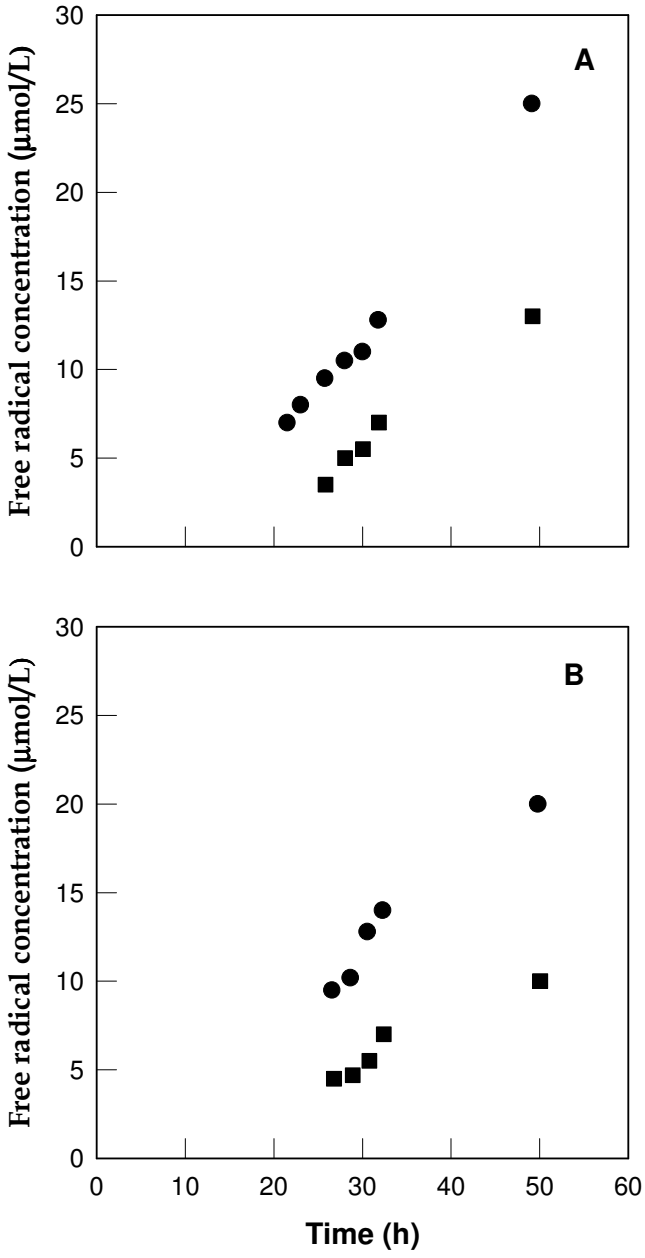

B
Oxygen concentration (mmol / L)
Time (h)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50

Figure 4


A
B
Free radical concentration (µmol/L)
Free radical concentration (µmol/L)
0
5
10
15
20
25
30
0
10
20
30
40
50
60
Time (h)