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Antioxidant and pro-oxidant effects of red wine and its fractions on Cu(II) induced LDL oxidation evaluated by absorbance and chemiluminescence measurements

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Abstract

Cu(II) mediated low density lipoprotein (LDL) oxidation has been followed by the changes in absorbance at 234 nm and the emitted low level chemiluminescence (CL). The similarity of the time profiles allows us to conclude that the emitted CL is due to the decomposition of a transient product, most likely a hydroperoxide. Red wine, as well as its fractions, afford a noticeable protection when added prior to the start of the LDL oxidation process. On the other hand, when they are added after the onset of the autocatalytic oxidation phase, red wine and its fractions behave as pro-oxidants. This is particularly evidenced by a strong burst of CL (enhancement of the light by a factor $\approx$ 20). This burst is reduced by metal chelators (EDTA and DFO) and can be associated to a sequence of reactions such as

$$XOH + Cu(II) \rightarrow XO^{\bullet} + H^{+} + Cu(I), CU(I) + LOOH \rightarrow \text{chemiluminescence}$$

where XOH is a phenolic compound and LOOH is a peroxide-like compound produced in the LDL oxidation.

Keywords: *Low-density lipoproteins, chemiluminescence, red win, copper*

Introduction

Low density lipoprotein (LDL) oxidation is one of the early events leading to atherogenic conditions [1–5]. The lipoprotein oxidation can, at least *in vitro*, be inhibited or delayed by antioxidants, such as α-tocopherol and reactive polyphenols [6–13]. The large amounts of these antioxidants in different beverages, such as tea or wines [14–19], is frequently mentioned as a rationale to explain the beneficial effects of their ingesta [7,20–25]. However, phenolic compounds can play a dual role, behaving as antioxidants or pro-oxidants [26–30]. This last behavior, that is also shown by α-tocopherol [31–35], has been associated to the capacity of phenols to recycle active metals, bringing them back to low oxidation states particularly prone to break peroxide bonds, leading to the formation of active free radicals [32,36–39]. Also, the capacity of phenol-derived radicals to react with lipid molecules, particularly in compartmentalized systems, can contribute to their pro-oxidation behavior [34,35,40].

Cu(II) promoted oxidation is the most frequently *in vitro* system employed to study the free radical mediated LDL oxidation, in spite of the complexity of the mechanism of the process [26,32,41–46]. In this system, the most frequently evaluated parameter is the induction time [17,26,32], associated to the depletion of the endogenous and added antioxidants [26,47–49], and/or to the production of enough amount of products (lipid–peroxides) able to lead to the autocatalytic phase of the process [50].

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Albertini and Abuja have shown that the effect of an additive upon the course of the Cu(II) mediated oxidation of LDL, depends on the compound considered and the time of its addition [26]. In particular, they have shown that, while 2,6-di-tert-butyl-4-methylphenol (BHT) behaves as an antioxidant, irrespective of the time of its addition, the behavior of Trolox is extremely, dependent, on the previous status of the lipoprotein. In fact, when added prior to the initiation of LDL oxidation, Trolox behaves as a strong antioxidant. On the other hand, when added at the beginning or during the autocatalytic phase of LDL oxidation promoted by Cu(II) addition, Trolox behaves as a prooxidant. This dual behavior was obtained when the rate of LDL oxidation was monitored by the change in absorbance at 234 nm as well as by the emitted chemiluminescence(CL).

Red wine antioxidants are constituted by several families of polyphenols with potential antioxidant capacities [7,10,12,17–19,51–54]. However, there is no clarity regarding which type of compounds contribute most to the observed antioxidant capacity. Kondo et al. have concluded that most of the antioxidant capacity in red wines is associated to the flavanol fraction [51], while Kerry and Abbey have reported that only 14% of the wine polyphenols were in the flavonols, and that anthocyanins contributed most to the lag time elicited by red wine in LDL oxidation [53]. Furthermore, there are no studies aimed at establishing if the antioxidant behavior of red wine and/or their fractions is, independent, of the LDL status at the moment of their addition.

In the present work we evaluated the effect of red wine on LDL oxidation when aliquots of the beverage are added at different stages of the oxidation process. The effect of several fractions of red wine, and of red wine itself, were evaluated from the time profiles of the absorbance changes and of the CL emitted in the Cu(II) promoted LDL oxidation. The source of the CL associated to the lipoprotein oxidation is also discussed.

Materials and methods

Isolation of polyphenolic fractions from red wine

A chilean Cabernet Sauvignon (Santa Emiliana, 2001) was used in all the experiments. Phenolic acids ("acidic fraction"), neutral phenolic molecules, e.g. catechins, procyanidins, flavonols ("neutral fraction") and anthocyanins ("aqueous fraction") of the wine were separated by an aqueous/ethyl acetate extraction method at pH 2 and pH 7, as described in the literature [12,55].

Free radical scavenging

The antioxidant content of red wine and its fractions was determined by the radical cation $ABTS^+$ (2,2′-azinobis(3-ethylbenzothizoline-6-sulfonic acid)). A solution of $ABTS^+$ was prepared by reacting ABTS (Sigma, 98%) (150 μM) dissolved in a phosphate buffer (100 mM, $pH = 7$) with potassium persulfate (75 mM). The bleaching of the blue–green $ABTS^+$ solution by reaction with the wine antioxidants at room temperature was evaluated by following the decay of the sample absorbance at 734 nm [56]. In absence of additives the absorbance of the radical cation remained unchanged by several hours.

LDL isolation

Human LDL (1.019–1.063 g/ml) were prepared by zonal centrifugation from plasma obtained from normolipidemic blood donors [57]. Prior to oxidation, EDTA in the LDL solution was removed with two passages through an Econo-Pac 10 G desalting column (Bio-Rad, Richmond, CA) hydrated and eluted with phosphate buffered saline (PBS). Protein concentrations in LDL preparations were determined by a modification of Lowry method using bovine serum albumin as a reference standard [58].

LDL oxidation

LDL (25 μg/ml) was oxidized in PBS in the presence of 5 μM $CuSO_4$. Wine, wine fractions and additives were added at different reaction times.

Conjugated diene formation. The formation of conjugated diene hydroperoxides from polyunsaturated fatty acids in LDL was followed at 234 nm using a Shimadzu UV-160 spectrophotometer thermostated at 37°C.

Chemiluminescence measurements. Low-level CL was measured at room temperature (23 ± 2°C) in a Beckman LS-6500 liquid scintillation counter operating in the "out of coincidence" mode.

Statistical. Statistical significance of the observed differences was established by the *t*-Student test for unmatched samples. Fischer test was applied to establish the similarity of the variances.

Metal content of wine samples

Metal (Fe and Cu) content of wine samples were evaluated employing a flame atomic absorption spectrometer (Perkin Elmer 2380).

Experimental results

Antioxidant capacity of red wine and its fractions

All the fractions of red wine, and red wine itself, show antioxidant activity, as evidenced by their capacity to bleach the $ABTS^+$ derived free radicals. Typical reactions profiles are shown in Figure 1. This figure shows that decay profiles are very similar for the whole wine and the three fractions considered. In fact, there is an instantaneous (less than 10 sec) decay that amounts to ca. 50–60% of the total decay, followed by

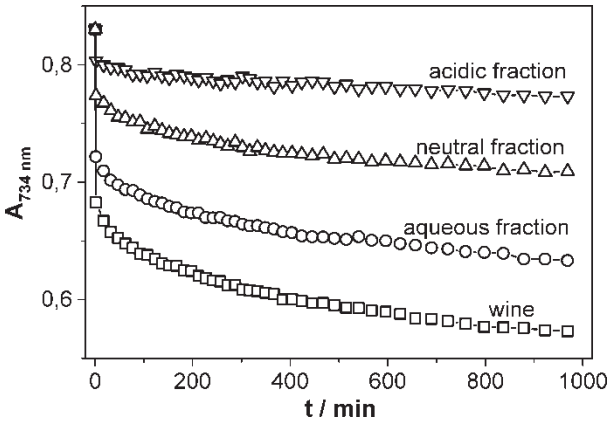

Figure 1. Kinetics of ABTS derived radical absorption bleaching by wine (0.33 μl/ml) and its fractions (aqueous: 0.33 μl/ml; neutral: 1.3 μl/ml; acidic: 1.3 μl/ml).

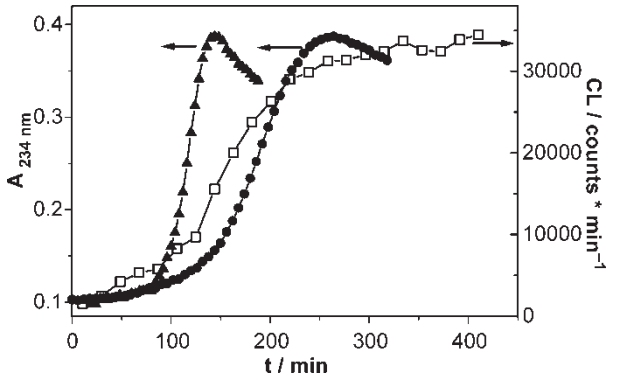

Figure 2. Kinetics of the LDL oxidation followed by absorption and CL. (•) Change in absorbance at 234 nm, 23°C; (▲) Change in absorbance at 234 nm, 37°C; (□) Chemiluminescence intensity, 26°C.

a considerably slower decay. This behavior is typical of complex mixtures and/or multifunctional compounds with more than one reaction site. The amount of antioxidants present in each sample, expressed in Trolox equivalents, was obtained from the changes in $ABTS^+$ radical absorbance after 15 min reaction time, and considering that each Trolox molecule is able to bleach two $ABTS^+$ derived radicals [56]. The results obtained, together with the contribution of each fraction to the value measured in the whole red wine, are included in Table I. These values closely resemble the fraction of phenolic groups present in each sample. In fact, Folin's assau gives the following percentages of phenols (referred to the wine): acidic fraction, 5%; neutral fraction, 9%; aqueous fraction, 70%.

Cu(II) mediated LDL oxidation

Effect of wine or its fractions when added before the start of the oxidation process. Cu(II) mediated LDL oxidation, as evaluated by the increase in absorbance, shows the typical sigmoidal profile of an autocatalytic process (Figure 2). A similar profile is obtained when the process is followed by the emitted CL. The longer lag time is due to the lower temperature employed in the CL experiments. However, it must be considered that, while the absorbance is directly related to product formation, the CL is a rate whose intensity must be related to the concentration of the emitting species [59–60]. It is interesting to note that the light intensity remains high for a long time, suggesting that CL is being emitted under conditions, where the formation of LDL oxidized products, measured by absorbance changes, is negligible.

The addition of antioxidants (Trolox, BHT) before Cu(II) addition delays the onset of the autocatalytic process [50]. The same occurs if wine or its fractions are added. Figure 3A shows that lag times are proportional to the volume of the added sample. Figure 3B shows that, in a microliter basis, wine is more efficient than the aqueous fraction, and that the last one is considerably more efficient than the acidic or the neutral fractions. The same conclusion can be reached from the CL measurements (Figure 3C). In Table II are given the expected contribution of each fraction to the lag time elicited by the whole wine.

The different response to the employed fractions mostly reflects differences in the amount of antioxidants present in each sample (Table I). The average efficiency of the antioxidants present in wine and its fractions in delaying the process, evaluated in the same batch of LDL, are also collected in Table II. This efficiency was derived from absorbance measurements and is defined as the delay time (in minutes) added by each micromolar unit of Trolox equivalents present in the considered fraction. The data suggest that the phenols present in the neutral and aqueous fraction, when tested, independently, are less efficient than those in the whole wine. However, only the difference between the wine and the aqueous fraction is statistically significant at the 0.95 confidence level. This could indicate a moderate degree of sinergism when the antioxidants are tested in the whole sample.

Table I. Contribution of each fraction to the total antioxidant capacity of red wine.

Sample	Trolox equivalents (a) (mM)	Contribution (%)
Wine	27.2 ± 2.5	100
neutral fraction	3.0 ± 0.6	11
acidic fraction	1.5 ± 0.4	6
aqueous fraction	19.2 ± 2.3	71

(a) Trolox equivalents determined by the ABTS assay. Values are (averages ± standard deviations) of 5, independent, determinations.

Effect of wine and its fractions when added during the oxidation process. BHT added to the LDL suspension precludes further oxidation of the sample, irrespective

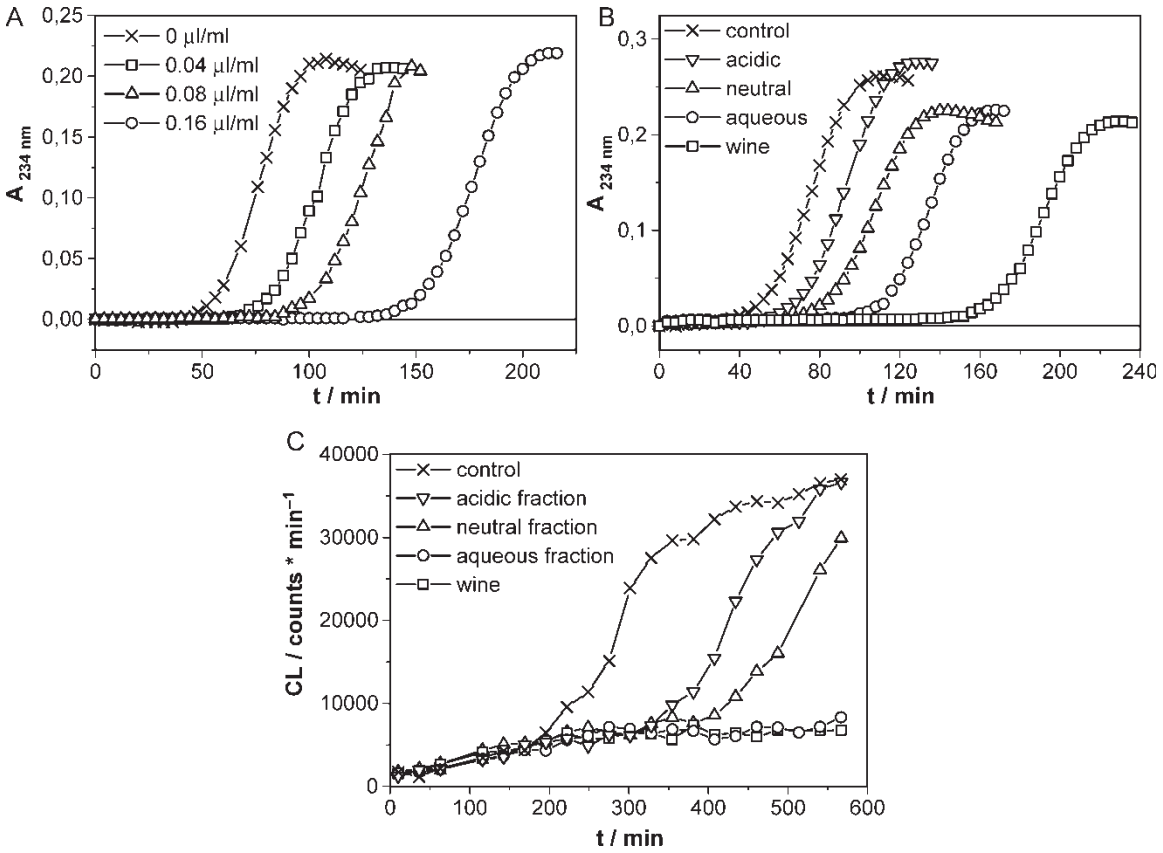

Figure 3. Effect of wine and its fraction on the kinetics of LDL oxidation. Wine samples were added prior to the start of the oxidative process. (A) Effect of different concentrations of the aqueous fraction. Oxidation evaluated by the change in absorbance at 234 nm. (B) Delaying of LDL oxidation in the presence of wine (0.08 µl/ml) and its fractions (aqueous: 0.08 µl/ml, neutral: 0.67 µl/ml, acidic: 0.33 µl/ml). Oxidation evaluated by the change in absorbance at 234 nm. (C) Delaying of LDL oxidation in the presence of wine (0.25 µl/ml) and its fractions (aqueous: 1.0 µl/ml, neutral: 1.0 µl/ml, acidic: 1.0 µl/ml). Oxidation evaluated by chemiluminescence.

of the time of its addition [26]. This is evidenced by the nearly constancy of the absorbance measured at 234 nm after BHT addition. On the other hand, wine and its fractions behave as pro-oxidants when added at late stages of the oxidation process. Typical results obtained from absorbance measurements at 234 nm are shown in Figure 4.

The effect of antioxidant addition at different stages of the oxidation process has also been measured by changes in CL. The addition of BHT at later stages of the oxidation process only produces a moderate decrease in the CL (Figure 5). This decrease is nearly, independent, of the additive concentration (micromolar range, data not shown), and is similar to that observed in the oxidation of biological samples, ranging from homogenates to purified proteins [59–61]. This behavior has been considered as evidence that the CL arises mainly from the decomposition of preformed

Table II. Efficiency of red wine and its fractions on delaying Cu(II) promoted LDL oxidation.

Sample	Efficiency (a) (min/µM)	Contribution (%)
Wine	43 ± 7	100
neutral fraction	28 ± 17	7
acidic fraction	46 ± 17	6
aqueous fraction	31 ± 9	51

(a) Efficiency given in minutes of LDL oxidation delay per Trolox microequivalent present in the testing sample. Values are (averages ± standard deviations) of 5–8 measurements.

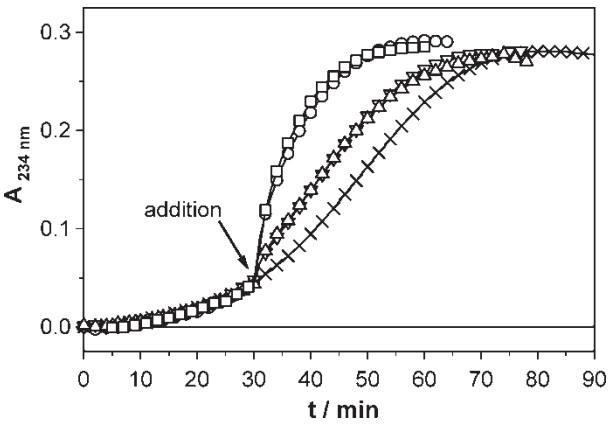

Figure 4. Pro-oxidant effect of wine and its fractions added at the start of the autocatalytic phase of LDL oxidation, detected by the change in absorbance at 234 nm. (×) Control; (□) wine, 0.08 µl/ml; (○) aqueous fraction, 0.08 µl/ml; (Δ) neutral fraction, 0.16 µl/ml; (▽) acidic fraction, 0.16 µl/ml.

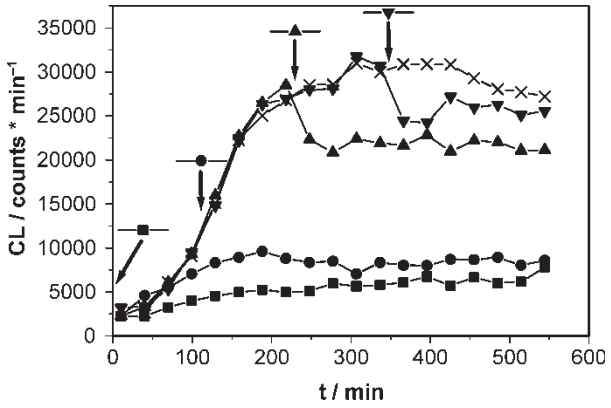

Figure 5. Effect of BHT (56 μM) addition upon the CL emitted in LDL oxidation. BHT was added at different times (indicated by arrows) in parallel experiments: (×) control; (■) BHT added prior to the oxidation; (●) BHT added at the onset of the autocatalytic phase (110 min.); (▲) BHT added near the end of the autocatalytic phase (230 min); (▼) BHT added at the end of the autocatalytic phase (360 min).

peroxides, and not from a Russel's like process [59]. In order to test this hypothesis, ebselen, a compound efficient in hydroperoxide removal, was added to the sample after its extensive oxidation. The obtained results are shown in Figure 6. The fast and significant decrease in CL observed after ebselen addition is compatible with a predominant role of preformed peroxides (or hydroperoxides) as precursors of the observed CL.

The effect of wine and its fractions upon the CL associated to the LDL oxidation also depends on the time elapsed prior to their addition. Typical results are shown in Figure 7. When added at the beginning of the process (Figure 3C) or prior to the onset of the autocatalytic phase (at 170 min, Figure 7), wine and its fractions notably delay the onset of the process.

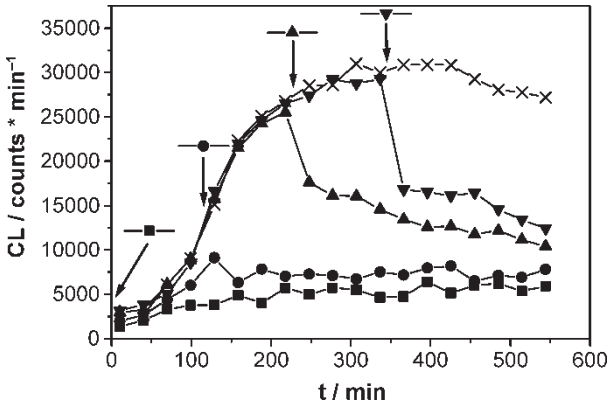

Figure 6. Effect of ebselen (23 μM) addition upon the CL emitted in LDL oxidation. Ebselen was added at different times (indicated by arrows) in parallel experiments. (×) control; (■) ebselen added prior to the oxidation; (●) ebselen added at the onset of the autocatalytic phase (110 min.); (▲) ebselen added near the end of the autocatalytic phase (230 min); (◆) ebselen added at the end of the autocatalytic phase (360 min).

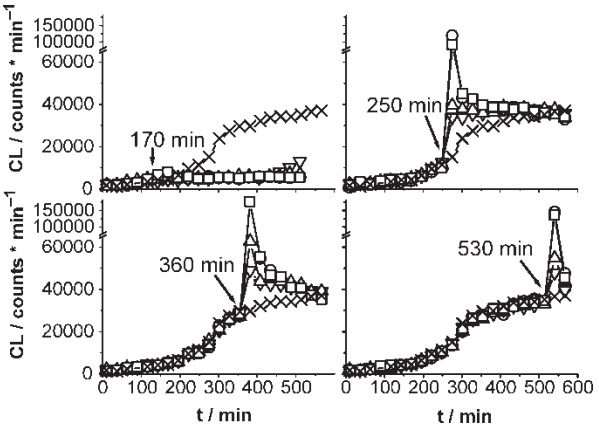

Figure 7. Effect of wine and its fractions on the CL emitted in LDL oxidation. Wine and its fractions were added at different times (indicated by arrows). Aliquots added: (□) 0.25 μl/ml wine; (○) 1.0 μl/m, aqueous fraction; (Δ) 1.0 μl/ml, neutral fraction; (▽) 1.0 μl/ml, acidic fraction. (×) Control sample.

On the other hand, when added at the beginning of the autocatalytic process (250 min), during the autocatalytic phase (360 min) or at the end of it (530 min) there is a concentration, dependent, burst of light that afterwards decays to the plateau level. It is interesting that the size of the burst follows an order

$$\text{wine} > \text{aqueous} > \text{neutral} > \text{acidic}$$

similar to their relative antioxidant capacities.

Assays aimed to assess the source of the CL burst

Presence of Transition Metals in Wine Samples. The amount of metals (Cu and Fe) present in wine samples was evaluated by atomic absorption spectroscopy. Cu was below the detection limit of the instrument (< 8 μM). Fe was detected in levels of ca. 70 μM. Since wine samples were diluted more than 10^3 times, the Fe ion concentration in the samples is in the nanomolar range. This concentration is considered to be too low to explain the CL burst shown in Figure 7. In fact, addition of nanomolar Fe(II) concentrations have no effect on the emitted CL, irrespective of the time of its addition. Only micromolar concentrations of Fe(II), added after the onset of the autocatalytic phase, produces a moderate increase in the emitted CL.

Influence of metal chelators EDTA and desferrioxamine on the wine pro-oxidant effect. The effect of the joint addition of wine and desferrioxamine (DFO) after the onset of the autocatalytic phase upon the emitted CL is shown in Figure 8. This figure shows the comparison of the light burst obtained with wine and with wine plus DFO. The addition of DFO reduces the size of the pulse but increases the time over which it is observed. Similar results were obtained when

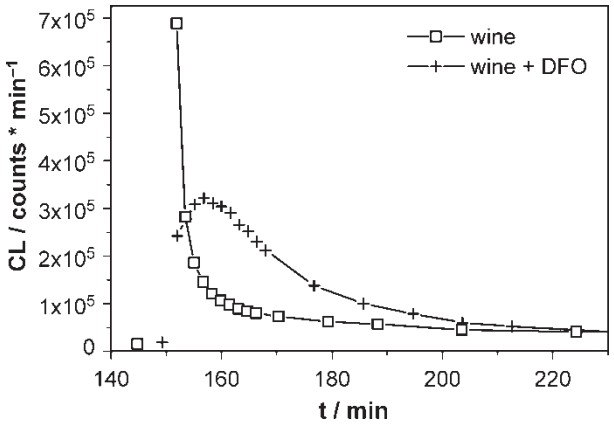

Figure 8. CL kinetics of the light burst caused by addition of wine (0.4 μl/ml) and wine (0.4 μl/ml) + DFO (60 μM) to LDL during the autocatalytic phase. Wine or (wine + DFO) were added after ca. 150 min. The CL prior to the addition was ca. 0.3×10^5 counts/min.

(wine + EDTA) were added. Figure 9 indicates that the size of the pulse resulting from the joint addition of EDTA and wine increases with the amount of added wine. It is interesting to note that, in the absence of wine, EDTA produces a moderate decrease in the size of the observed CL. The data obtained with DFO (data not shown) are qualitatively similar. However, in absence of red wine, the decrease elicited by EDTA is more noticeable than that produced by DFO addition. Similarly, in presence of wine, the burst of light is also smaller with EDTA than with DFO. This could be related to the low Cu(II) binding capacity of DFO. It is interesting to notice that, when added at the moment of the initial Cu(II) addition, both EDTA and DFO precludes completely the CL, both in presence and in absence of red wine.

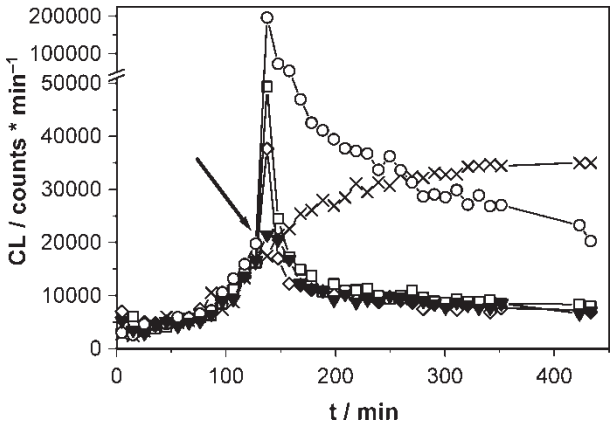

Figure 9. Effect of EDTA (30 μM) and EDTA plus wine upon the CL emitted along LDL oxidation. EDTA or EDTA plus wine were added during the autocatalytic phase (indicated by an arrow). (×) Control; (▼) EDTA; (◇) EDTA + 0.03 μl/ml wine; (□) EDTA + 0.08 μl/ml wine; (○) EDTA + 0.4 μl/ml wine.

Discussion

Amount of antioxidants in the fractions

The kinetics of the reaction of antioxidants with $ABTS^+$ radicals can provide a rough estimate of their average reactivity [62]. The results given in Figure 1 show that, the average quality of the antioxidants present in red wine and its fractions is rather similar, at least as derived from the fraction of the fast decay of the $ABTS^+$ radical absorbance following the addition of the wine sample. The data given in Table I show that, most of the antioxidants initially present in the red wine are very hydrophilic, and remain in the aqueous fraction. In fact, the antioxidant activity of this fraction amounts to ca. 71% of the total activity measured in the whole wine. The fact that the antioxidants present in the three fractions add to less (88%) than those present in wine could indicate minor chemical modifications during the fractionation process. In any case, Folin's assay of whole wine and its fractions indicate that only a small loss (ca. 16%) of phenolic groups takes place during the fractionation process.

Regarding the predominance of anthocyanins in determining the antioxidant activity of red wine, the present results are similar to those reported by Saint-Cricq de Gaulejac et al [17]. and Ghiselli et al [12]. The former authors reported that in red wines free anthocyanins constitute the most efficient fraction for superoxide trapping. With regards to the ability to trap peroxyl radicals, Ghiselli et al. reported that the aqueous fraction contains nearly 70% of the anti-oxidant capacity present in de-alcoholized wine [12].

Efficiency of the antioxidants present in the fractions on LDL protection

The data of Table II show that most of the red wine potential to protect LDL from its oxidation is related to the anthocyanins. This is in agreement with previous results [12], and the relative antioxidant capacity of the three fractions (Table I). The values of the efficiencies of the fractions (Table II) show that, in a Trolox equivalent basis, the average efficiency of wine and its fractions in delaying the onset of LDL oxidation are rather similar. This is not surprising since induction times, as measured in the present work, reflects only stoichiometric factors.

A noticeable feature of the data given in Table II is the fact that the contribution of the three fractions amounts only to ca. 60% of the effect elicited by the wine sample. A strong synergism between fractions seems improbable since the simultaneous addition of the three fractions gives a result very close to that of the aqueous fraction (data not shown). The effect of the presence of ethanol in the wine can also be ruled out, because the addition of 10% ethanol to a mixture of the fractions did not produce any significant change in the observed induction times (data not shown). A chemical modification during fractionation, evidenced also

(although to a lesser extent) in Table I could be the source of this discrepancy.

Pro-oxidant effect of wine and its fractions

Albertini and Abuja have reported that, contrary to BHT, Trolox behaves as a pro-oxidant when added at later times during LDL oxidation promoted by Cu(II) [26]. This behavior has been related to the capacity of Trolox to participate in the Cu(II)/Cu(I) redox cycling. Figures. 4 and 7 show that wine and its three fractions also behave as pro-oxidants when added after significant LDL oxidation. It is interesting to note that this pro-oxidant effect was also observed when wine or its fractions were added at the start of the Cu(II) mediated oxidation of aged LDL samples that have been maintained for several weeks at 4°C after EDTA removal (data not shown). If it is accepted that the difference between BHT and Trolox is mainly due to differences in their hydrophobicity, the present data are compatible with the relatively low hydrophobicity of the main antioxidants present in red wine samples. It is important to note that other phenols also present this dual behavior [27,28]. However, a noticeable difference between the present data and that reported by Albertini and Abuja employing Trolox is that these authors does not observe the burst of CL produced in the present work after addition of wine (or its fractions) when it is added at late stages of the Cu(II) mediated oxidation process [26]. In fact, Trolox (5 μM) addition after the onset of the autocatalytic phase produces, under our experimental conditions, only a brad peak of CL with an overshoot of less than a factor two over the maximum emission observed in absence of additives (data not shown).

The lack of protection afforded by different phenols added at late stages of the oxidation process, observed in the present and previous works [26–28], most probably implies that these compounds are not able to protect LDL directly from their Cu(II) mediated oxidation, and that they exert their protection by recycling the endogenous antioxidants. Furthermore, the fact that they even act as pro-oxidants could be due to an increased rate of free radical production. This is in agreement with the burst of light observed when wine or its fractions are added after the onset of the autocatalytic process. This enhanced oxidation could be due to

i) introduction of redox active metals with the wine samples;
ii) a role of the phenols in the Cu(II)/Cu(I) redox cycling; and/or
iii) attack of the lipids by the phenol-derived radicals.

In order to test these possibilities, we added chelators (EDTA, DFO) and low metal concentrations, and evaluated the amount of Fe and Cu ions present in the wine sample.

The concentrations of Fe and Cu in wine seem to be too low to have a significant pro-oxidant effect. After dilution of the wine sample, the Fe(II) concentration in the sample is below 70 nm. Addition of Fe(II) in a similar concentration do not produce any noticeable pro-oxidant effect. The addition of EDTA or DFO to the wine samples lead to a somewhat slower kinetics of the burst but show still a clear pro-oxidant effect immediately after the addition of the wine/chelator-mixture (Figure 8). On the longer time scale it can be observed an antioxidant effect, which is also present in the case of the addition of the chelators without wine (Figure 9). Therefore we conclude that there are two separate effects caused by the chelators and the wine, and that the chelators weekly affect the pro-oxidant effect of wine.

We consider that the increase in luminescence observed when the wine sample is added to the partially pre-oxidized LDL sample is compatible with a burst of free radicals associated to the sequence:[63,64]

$$\mathrm{Cu(II) + PhOH \rightarrow Cu(I) + PhO^{\star} + H^{+}}$$

$$\mathrm{Cu(I) + LOOH \rightarrow Cu(II) + LO^{\star} + HO^{-}}$$

followed by the CL emitted through the secondary reactions of the PhO* and/or LO* radicals [59–61,64]. The addition of EDTA (Figure 9) and DFO (in a smaller extent, data not shown) would reduce the rate of these processes as a consequence of their chelating capacity. The slower rate of the process is compatible with a less intense but longer lasting CL burst (Figure 8).

Origin of the CL associated to LDL oxidation

The fact that the CL correlates with product accumulation (Figure 2), the small effect in the CL of BHT addition under conditions that the oxidation process is totally inhibited (Figure 5), the burst of CL observed after polyphenol addition (Figure 7) and the strong reduction in CL afforded by ebselen addition (Figure 6), suggest that, as in other systems [59–61,64], the measured CL can be explained in terms of a mechanism comprising the formation of hydroperoxides followed by its chemiluminescent decomposition:

$$\mathrm{ROOH(+X^{\star}) \rightarrow products + light}$$

where the conversion of the precursor (ROOH) to the emitting species is partially promoted by free radicals (X*) and traces of transition metals. The partial protection by EDTA, added after the autocatalytic phase, is compatible with this interpretation.

With the present data it is difficult to decide if the light is associated to lipid or ApoB oxidation. A comparison of the time profiles of the oxidation process, measured by the increase in absorbance at 234 nm or by the CL in previous [26] and in

the present work (Figure 2), shows a close relationship between lipid peroxidation and CL. This could indicate that the CL is closely related to the lipid oxidation. However, it has to be taken into account that Trp oxidation in ApoB, a process known to produce CL [60,63], is an early event in LDL oxidation [64], and that oxidation of the pro tein takes place in a close relation to the oxidative modification of the lipoprotein in general [65].

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