Chapter

Reducing Compounds Roles in Oxidative Stress Relieving of Human Red Blood Cells

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Abstract

Oxidative stress is the consequence of an imbalance between pro-oxidant and antioxidant processes. Antioxidants that counteract reactive oxygen species do not all work the same way. Both resveratrol and the more powerful 4-hydroxytyrosol are excellent reducing agents. Polyphenol treatment (red wine polyphenols, resveratrol and catechin) is associated with a significant increase in anion permeability for chloride compared with control and 2.2′-azobis-2 amidinopropan dihydrochloride affected cells. Treatment with polyphenols was associated with a significant reduction in mean ± standard error of the mean membrane lipid peroxidation compared with control and 2.2′-azobis-2 amidinopropan dihydrochloride treatment. Hemolysis data are also obtained in the previously described conditions. 4-hydroxytyrosol is shown to significantly protect red blood cells from oxidative damage by 4-hydroxynonenal. But there are paradoxical effects like uric acid and creatinine. The obtained data evidence that both creatinine and uric acid levels have influence on the ratio of both malondialdehyde/protein and 4-hydroxynonenal/protein content on red blood cell ghosts, demonstrating their possible protective role against oxidative stress at low concentrations in blood and oxidizing power at higher concentrations. Finally, polyunsaturated fatty acids do not have all this reducing power.

Keywords: reactive oxygen species, resveratrol, 4-hydroxytyrosol, uric acid, creatinine, polyunsaturated fatty acids

1. Introduction

Chemical stress induced by the presence, in a living organism, of an excess of reactive chemical species, generally centered on oxygen (reactive oxygen species), secondary to an increased production of the same and / or to a reduced efficiency of the physiological systems of antioxidant defense. There are many antioxidants, among these there is resveratrol. The history of resveratrol (the *red wine* and pomegranate juice are rich in this substance, chemically it is a triidrostilbene "a derivative stilbenic", synthesized from the vine of red grapes.) 10 20 times more potent than Vitamin E and Glutathione is the most powerful of those produced by the body (prevents arteriosclerosis, cancer and recently even Alzheimer's and delays the appearance

of the characteristic signs of old age and can extend life by as much as 30%.). Another powerful antioxidant is hydroxytyrosol. The hydroxytyrosol (1- (2-hydroxy) ethyl-3,4-dihydroxybenzene) is classified as a phytochemical compound expressing strong antioxidant properties. The index ORAC (Oxygen Radical Absorbance Capacity, or the oxygen radical absorption capacity) for hydroxytyrosol totaled 40,000 μmolTE / g, about ten times greater than green tea at least twice compared to CoQ_{10}. In nature, hydroxytyrosol is found in high concentrations in olive vegetation water and also in lower concentrations in the olive leaf. Olives, leaves and olive pulp contain much higher amounts of hydroxytyrosol compared to olive oil. One study showed that a low dose of hydroxytyrosol in rats reduces the consequences of the side effects of oxidative stress induced by smoking [1]. Among the paradoxical antioxidants is creatinine. Creatinine (from the greek κρέας, Kreas, "flesh") is an intermediate of energy metabolism synthesized by the liver about (1 g / day) from arginine, S-adenosyl methionine and glycine and is used in mammalian muscles to regenerate ATP during the first seconds of muscle contraction. Recent reports showed that creatinine displays antioxidant activity which could explain its beneficial cellular effects. Some authors [2] have evaluated the ability of creatinine to protect human erythrocytes and lymphocytes against oxidative damage. Erythrocytes were challenged with model oxidants, 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) and hydrogen peroxide (H_2O_2) both in the presence and absence of creatinine. Incubation of erythrocytes with oxidants alone increased hemolysis, met-hemoglobin levels, lipid peroxidation [3] and protein carbonyl content. This was accompanied by decrease in glutathione cellular levels. Antioxidant enzymes and antioxidant power of the cell were compromised while the activity of membrane bound scavenger enzymes were lowered [2]. In a recent paper [4] authors demonstrated that hypertriglyceridemia affects Red Blood Cell (RBC) membrane oxidative status (increase of [malondialdehyde (MDA)] and [4-hydroxynonenal (HNE)] to membrane protein ratio) is increased by high levels of blood cholesterol, but selectively by hypertriglyceridemia [4, 5]. It is known that creatinine protects the erythrocytes by attenuating the AAPH and H_2O_2 induced alterations [5]. This protective effect was confirmed by electron microscopic analysis which showed that oxidant-induced cell damage was attenuated by creatinine. Thus, creatinine can function as a blood antioxidant, protecting cells from oxidative damage, genotoxicity and can potentially increase their lifespan. Another paradoxical antioxidant is uric acid. Uric acid is an organic molecule of natural origin. Urate is formed as a byproduct in living mammalian organisms higher in purine metabolism. It belongs to the group of oxypurines and is formed from a pyrimidine ring (α) condensed with an imidazole ring (β). Uric acid, despite being a major antioxidant in the human plasma, both correlates and predicts development of obesity, hypertension and cardiovascular disease, conditions associated with oxidative stress [6]. While one explanation for this paradox could be that a rise in uric acid concentration represents an attempted protective response by the host, we review the evidence that uric acid may function either as an antioxidant (primarily in plasma) or pro-oxidant (primarily within the cell). We suggest that it is the pro-oxidative effect of uric acid, that occurs in cardiovascular disease and may have a contributory role in the pathogenesis of this condition. Then finally there are the Polyunsaturated fatty acids. Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. This class includes many important compounds, such as essential fatty acids and those that give drying oils their characteristic property. Fatty acid composition and susceptibility to lipid peroxidation induced in vitro by (AAPH) were evaluated in human RBC membranes on days 30 and 180. n-3 PUFA treatment increased

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) concentrations in RBC membranes in a time-dependent manner in all of the n-3 PUFA groups. These modifications occurred with concomitant dose- and time-dependent increases in the membrane unsaturation index. After 30 d of treatment with n-3 PUFAs, significantly increased in RBC membranes of the intermediate- and high-dose groups. Because of the higher concentration of this antioxidant in these groups, the susceptibility of RBC membranes to peroxidation was decreased. However, after 180 d of treatment, to baseline values and AAPH-induced lipid peroxidation increased in a dose-dependent manner. These results show that high doses of dietary n-3 PUFAs, as well as long-time treatments, affect human RBC susceptibility to lipid peroxidation by changes in fatty acid composition content [7].

2. Study on powerful antioxidants

2.1 Study on resveratrol

The reducing agent treatment efficacy was observed by evaluation of anion permeability for chloride, lipid peroxidation and hemolysis in RBCs. Anion permeability for chloride is an indicator of membrane protein damage and is evaluated in RBCs by the specific absorption of methemoglobin (C_M) at 590 and 635 nm after treatment of heparinized blood with $NaNO_2$. The measurement of the membrane lipid degradation is obtained by the determination of MDA. The lipid peroxidation susceptibility is observed after the oxidative stress induced by AAPH. The hemolysis assays are conducted on blood samples in phosphate buffer saline. To evaluate in human RBC the *in vitro* effects of AAPH and the antioxidant activity of polyphenols from red wine (resveratrol, catechin and naturally red wine), authors start the research evaluating RBC anion permeability for chloride. **Figure 1** shows the values of membrane anion permeability for chloride in both groups (controls and AAPH-treated RBCs). The statistical analysis of data shows that AAPH *in vitro* treatment lowers the parameter in comparison to all controls. Furthermore, the reducing agent treatment (T2-T4) also significantly recovers the activity up to values higher than AAPH treatment. The recovery of anion permeability for chloride is always lower than control data. Anion permeability for chloride values were slightly higher after red wine and resveratrol treatments (as reducing agents) in both experimental groups (controls and AAPH-treated RBCs), corresponding to a preserved architecture and anion permeability for chloride of the human erythrocyte membranes. To confirm the efficacy of oxidant and reducing treatments on human RBC *in vitro* authors evaluated lipid peroxidation, hemolysis and RBC morphology. In **Figure 2**, MDA levels are described in membranes from several varieties of treated and untreated RBC with AAPH, under the action of reducing agents or without treatment. On the contrary, as described in **Figure 2**, AAPH-induced increases of MDA levels are partially recovered by natural reducing agents at levels almost comparable to control the group. C2-C4 results always slow down MDA levels in RBC membranes, but catechin is less effective. Red wine, among them, is almost as powerful as resveratrol alone in both groups (controls and AAPH-treated RBCs). As shown in **Figure 3** time courses of RBC hemolyses are described at several incubation times (60, 120, 180 and 240 min) either under the action of natural oxidative pathways or by treatment with radical donor AAPH (60 mM). This experiment shows also the natural reducing agents partially recover the effect of both oxidative and reducing agent treatments. As described in all experiments, AAPH-

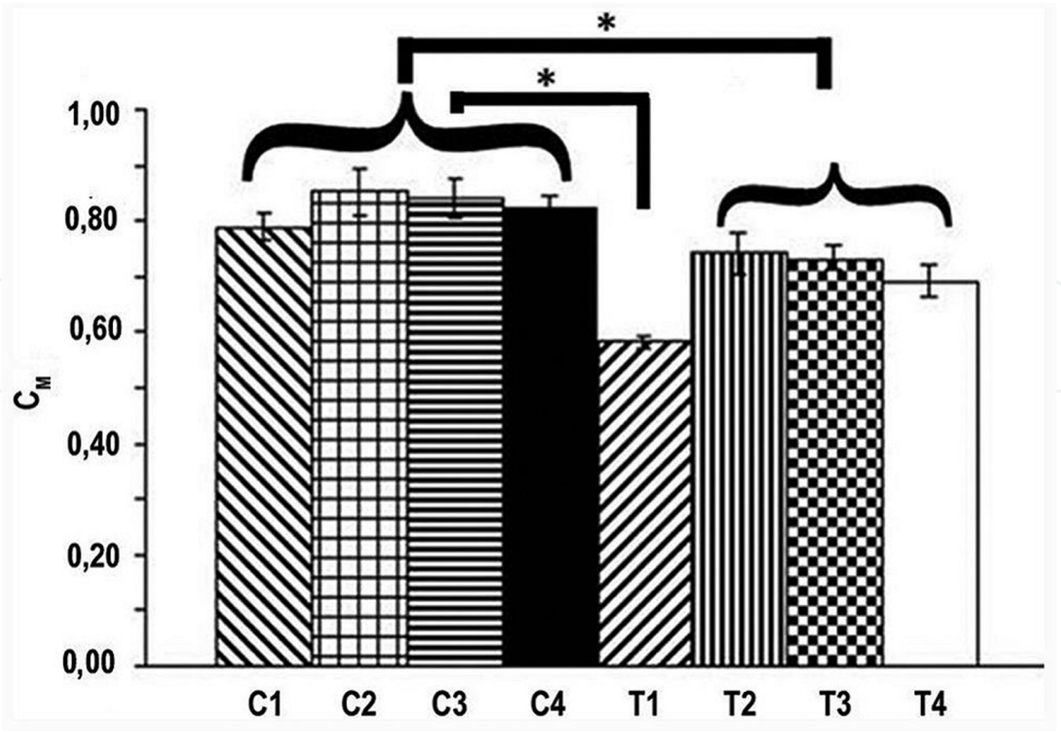

Figure 1.
*RBC membrane anion permeability for chloride. Data are mean ± standard error of the mean on 10 determinations. *P < 0.05 according to one-way analysis of variance and Bonferroni* post-hoc *test coupled data at each time were performed. C = control sample, T = treated sample [8] C1 = control; C2 = control + red wine 5, 2 mM (reducing power equivalents of gallic acid); C3 = control + resveratrol 5 μM; C4 = control + Catechin 50 μM; T1 = AAPH 60 mM; T2 = AAPH 60 mM + red wine 5,2 mM (reducing power equivalents of gallic acid); T3 = AAPH 60 mM + resveratrol 5 μM; T4 = AAPH 60 mM+ Catechin 50 μM.*

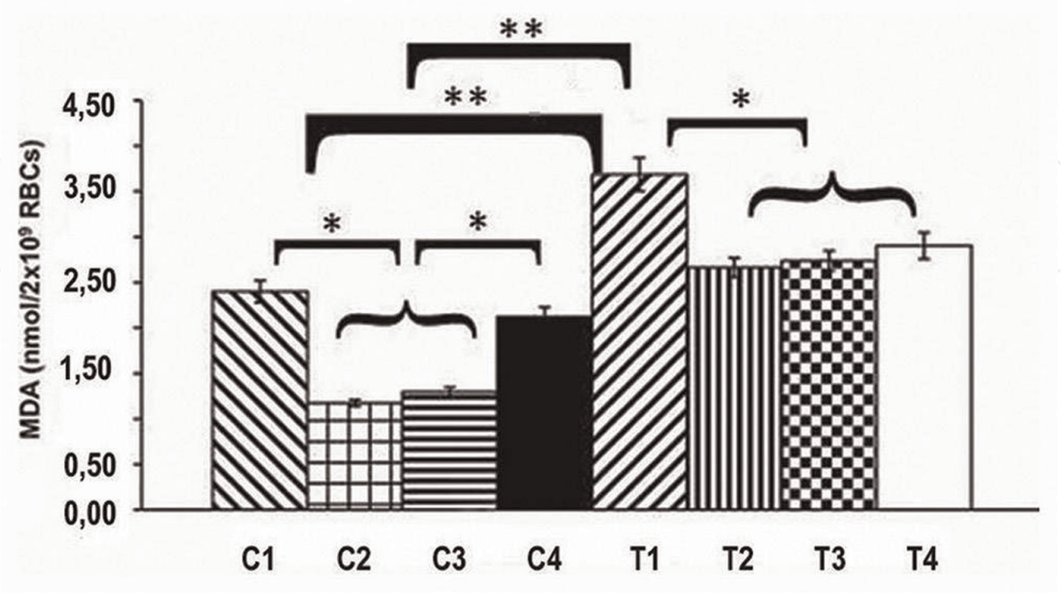

Figure 2.
*Effects of lipid peroxidation and induced by 2.2′-azobis-2 amidinopropan dihydrochloride (60 mM) (*t = 60 *min) on the malonyldialdehyde levels of human RBC membranes. Data are mean ± standard error of the mean on 10 determinations. Column labels are according to **P < 0.05 *and ***P < 0.01 *according to one-way analysis of variance and Bonferroni* post-hoc *test for coupled data at each time were performed (**Figure 1**).*

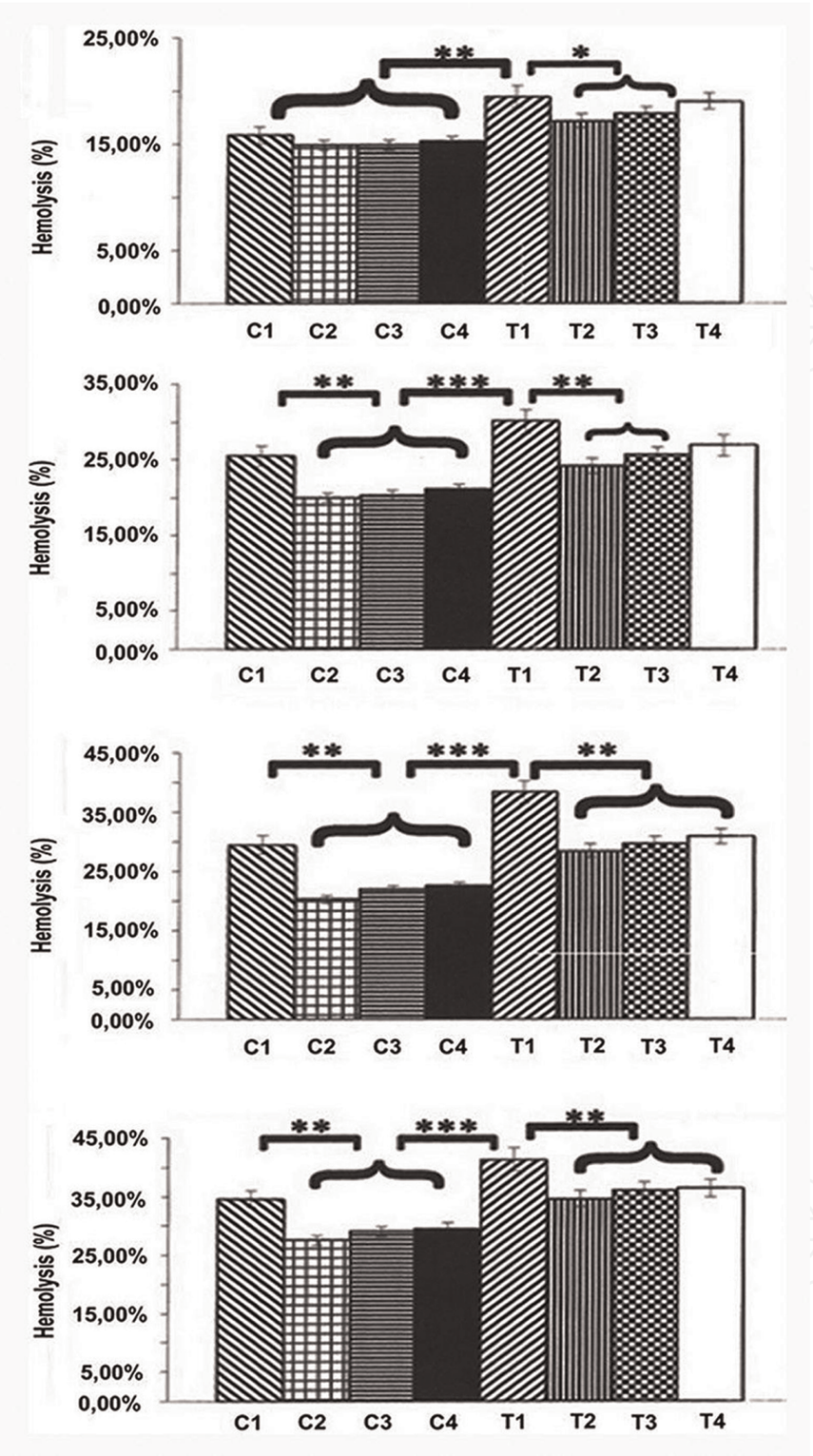

Figure 3.
*Effect of 2.2′-azobis-2 amidinopropan dihydrochloride-induced hemolysis in sportive human RBCs at several times. Data are mean ± standard error of the mean on 10 determinations. Column labels are according to incubation times a = (60 min); b = (120 min); c = (180 min); d = (240 min). *P < 0.05, ** P < 0.01 and *** P < 0,001 according to according to one-way analysis of variance and Bonferroni* post-hoc *test for coupled data at each time were performed (**Figure 1**). C = Control sample, T = Treated sample (Gallo et al., 2013) C1 = Control; C2 = Control + Red Wine 5, 2 mM (reducing power equivalents of gallic acid); C3 = Control +Resveratrol 5 μM; C4 = Control + Catechin 50 μM; T1 = AAPH 60 mM; T2 = AAPH 60 mM + Red Wine 5,2 mM (reducing power equivalents of gallic acid); T3 = AAPH 60 mM + Resveratrol 5 μM; T4 = AAPH 60 mM+ Catechin 50 μM. Of course C1 and T1 are a = (60 min). And so on, saying the others.*

induced hemolysis is more relevant than in other treated samples. As described in previous experiments (**Figures 1** and **2**) resveratrol and red wine polyphenol treatments are more efficient than catechin in preserving RBC membrane structural recovery at 60 and 120 min of incubation. In the present study, polyphenol (resveratrol and red wine) treatment is associated with an improvement in erythrocyte anion permeability for chloride-related with cell membrane derangements. The cell system exchange of Cl^- for HCO_3^- represents a target of membrane oxidative damage and is evaluated in RBCs by the specific C_M at 590 and 635 nm after treatment of heparinized blood with $NaNO_2$. According to our research, the in vitro action model of artificial agent AAPH on RBC plasma membranes shows that anion permeability for chloride (evaluated by C_M [9]) decreases after treatment and is partially recovered after administration of either resveratrol or red wine polyphenols extracts. Our data confirm those on LDL peroxidation [10] with AAPH by MDA, hemolysis and RBC morphology study from our laboratory [8, 11]. Moreover in the present work all previous results are compared, so that a relationship among them is evident and correlate each other, so that the action of red wine polyphenol extracts are demonstrated as effective reducing agents both alone and in mixture. MDA, hemolysis and RBC morphological study confirmed the resveratrol and red wine efficacy on improvement of RBC membrane integrity against in vitro oxidative stress damages. The described damages could be linked to the erythrocyte membrane framework, so a lack of adequate lipid organization can significantly influence ion permeability. The alteration of membrane fluidity consequent to lipid oxidation represented by MDA level increase can reflect the change of the structure and function of membrane lipids and proteins. The permeability is another important property of erythrocyte membrane. Depending on the exchanger ($Cl^- \leftrightarrow HCO_3^-$), that transports anions across the membrane, it is related to the framework of erythrocyte membrane. To sum up, the alteration of anion permeability can reflect the change of erythrocyte membrane protein framework. Moreover, our results could describe the overall effect of the three described alterations together.

2.2 Study on 4-hydroxytyrosol

The assay (ALDetectTM *Lipid Peroxidation* assay kit**)** is designed to measure either MDA alone (in hydrochloric acid) or MDA in combination with 4-hydroxyalkenals (in methanesulfonic acid).

So we have TOT = MDA + HNE and then TOT-MDA = HNE. The assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1) with MDA and 4-hydroxyalkenals to yield a stable chromophore with maximal absorbance at 586 nm at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm. For simultaneous determination of MDA and 4-hydroxyalkenals, one must use the procedure utilizing methanesulfonic acid (MSA) as the acid solvent. The procedure in which HCl is used will only detect MDA, since the 4-hydroxyalkenals do not form a chromophore with reagent R1 under those conditions. Interestingly, in addition to the 586 nm chromophore, 4-hydroxyalkenals gave a second chromophore with maximal absorbance at 505 nm and alkanals produced a single chromophore with maximal absorbance at 505 nm. The concentration of reducing agent is chosen according to literature data [12]. Reducing agent is provided from Sigma Chemical Co. (St. Louis, MO, USA) and used at the final concentration of 80 μM for 3 h at 37°C.In vitro 4-hydroxytyrosol treatment to evaluate the membrane lipids reducible by this phenol.

As described in **Figure 4a** and **b** the level of oxidation products of membrane lipids are referred as μM of MDA or HNE. MDA levels are always ten times those of HNE as referred in right-hand scale. In **Figure 4a** and **b** the scales are quite different (for instance: 0.00 /0.85 in A and 0.000/0.033 in B for MDA). We can also observe that MDA levels of pathological samples are slightly higher than control ones but HNE levels of pathological samples are four times those of control ones. After the 4-hydroxytyrosol action both lipid metabolites substantially decrease. The mean values of total membrane protein levels in our casuistry are described in **Figure 4**. It is evident that for the two dosed substances each group of data is significantly different ($P < 0.001$) from the other ones. In **Figure 5** are reported the total levels of MDA + HNE per milligram of total membrane proteins of RBC ghosts. As described the total oxidized lipid levels in RBC ghost membranes almost doubled in pathological samples in comparison to control ones. This shows that even if total membrane proteins dramatically increase in hypertriglyceridemic patients, also total oxidized lipids sufficiently increase in such samples so that ratio between patients RBC oxidized lipids to membrane proteins is always doubled. Such partial compensation of lipids in comparison to proteins from RBC membrane could be explained as an attempt of RBC metabolism to partially compensate ghost structure damages by oxidative stress. The ratio of total oxidized lipids to membrane proteins before and after 4-hydroxytyrosol treatment are referred in **Figure 5**. After 4-hydroxytyrosol treatment, the ratio of oxidized lipids to total membrane proteins decreases even more, but the values in our controls are always lower than in hypertriglyceridemic patients. The mean values of ratio of oxidized lipids (MDA + HNE) and membrane proteins in our casuistry are described in **Figure 5**. It is evident that the two groups of data are significantly different ($P < 0.001$). Neither Vitamin E nor Vitamin C are used because their ultraviolet–visible (UV–Vis) spectra superimpose to HNE determination method interfering with their evaluation. The structures most exposed to the damaging action of radicals are the lipid structures constituting the cellular and nuclear membranes that are subjected to destructuring. One of the most sensitive sites to damage caused by ROS is the plasma membrane, in particular the target is at the level of polyunsaturated fatty acids. The oxidation by ROS of lipids is called peroxidation. Alkenals are products of lipid peroxidation while hydroxytyrosol is a powerful reducing agent. This explains the decrease in the parameters of alkenals.

According to our data in RBC, ghosts are present both HNE and MDA products [13]. As demonstrated by higher levels of HNE in comparison to MDA, according to Sommerburg *et al*. [14] The RBC membrane preparations are obtained increasing the efficiency of Fe^{2+} removal with an higher concentration of EDTA (100 mM). Spectrophotometric method [15] to measures free HNE in human RBCs is optimized for the determination of alkenals in membranes. Other kinds of reducing agents, such as Vitamin E, are not tested because their UV–Vis spectra superimpose to N-methyl-2-phenylindole product spectra. In membranes of hypertriglyceridemic patients, the levels (triglycerides, total membrane proteins, total aldehydes and their ratio) are higher than in control samples. All the data decrease relevantly ($P < 0.001$) after the 4-hydroxytyrosol treatment, especially those of alkenals. In summary, lipid peroxidation is divided into two stages. Lipid Hydroperoxide is produced in the Early stage while Aldehydes (4-HNE, MDA) is produced in the Late stage. Lipid peroxidation consists of the radical peroxidation of a polyunsaturated fatty acid, with the formation of the lipoperoxide radical and its subsequent fragmentation into short-chain aliphatic and malondialdehyde compounds. The oxidation of arachidonic acid occurs by means of two enzymes: lipooxygenase and cyclooxygenase. The reaction sequence can take

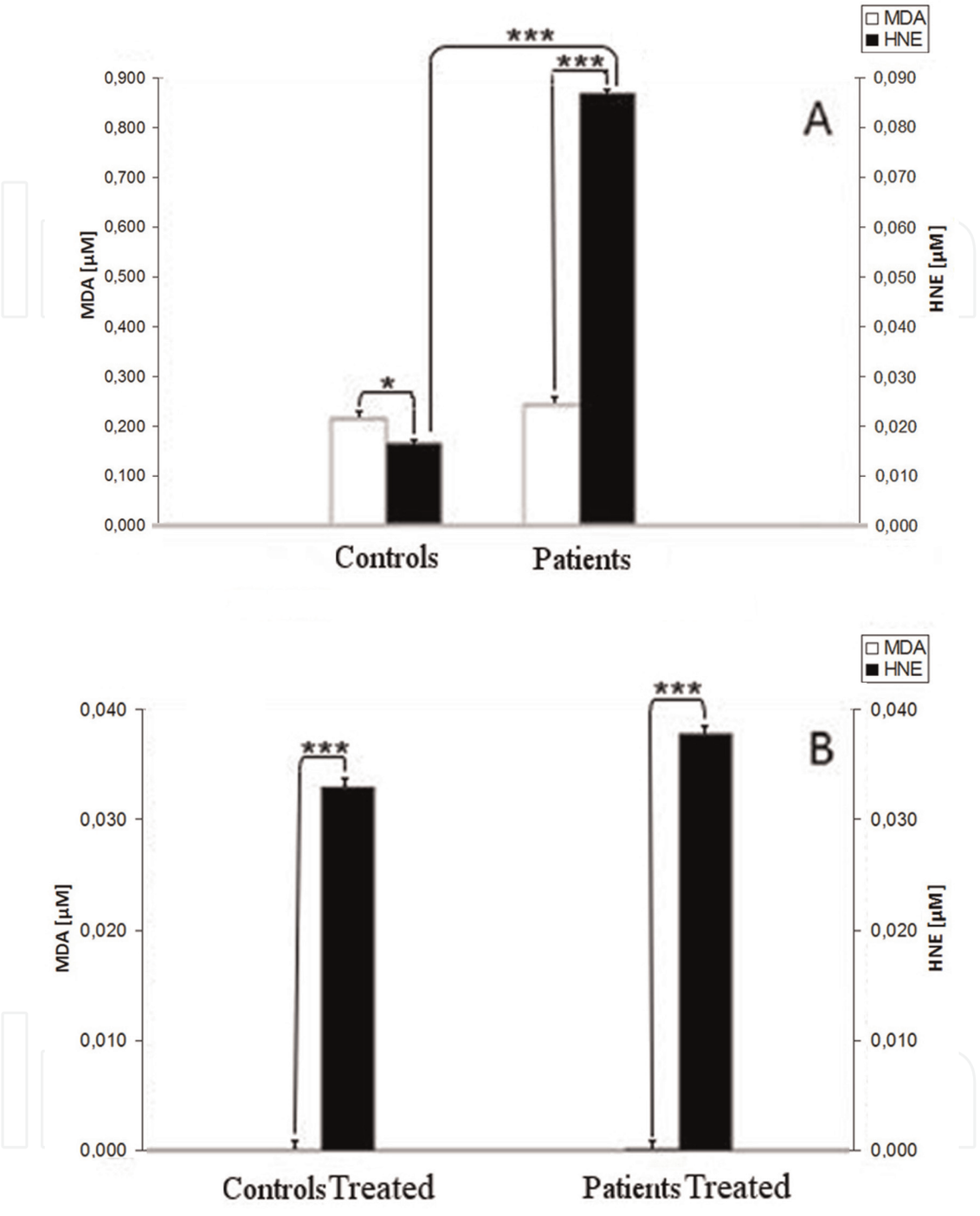

Figure 4.
Human RBC membrane concentration of MDA and HNE (µM absolute concentration in membrane samples) from controls and hypertriglyceridemic patients (A) and the same samples treated with 4-hydroxytyrosol (80 µM) (B). It is evident that for the two dosed substances each grouped data are significantly different for P < *0.001 by two ways ANOVA and Bonferroni* post hoc *test.* *P < *0.05;* **P < *0.01, and* ***P < *0.001.*

place under physiological conditions involving the arachidonic acid, contained in the membrane phospholipids, and is triggered by the superoxide anion, which is transformed into water producing the C-13 radical of arachidonic acid, which is followed by the radical in C-11. This, in the presence of oxygen, generates the radical peroxide or, by transferring the radical nature to another molecule, is transformed

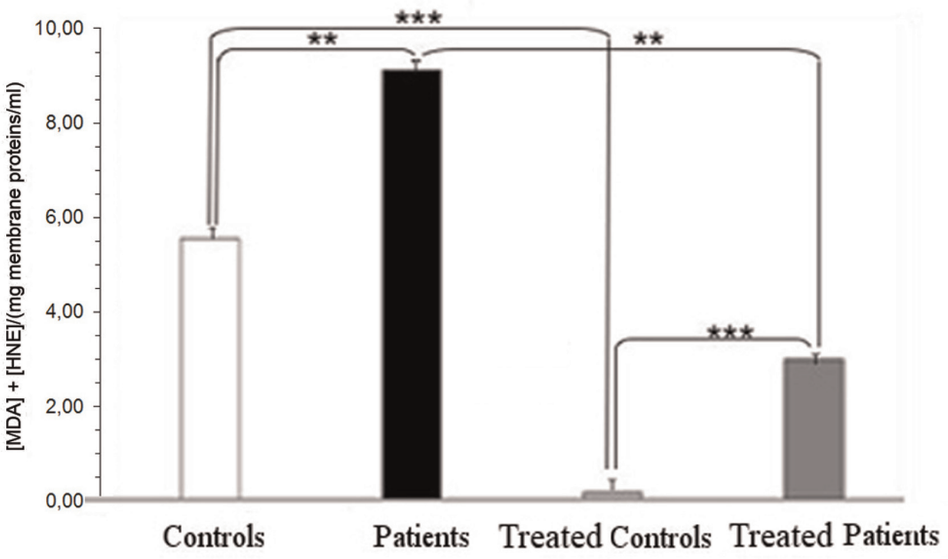

Figure 5.
Ratio of oxidized lipids (MDA + HNE) and membrane proteins. It is evident that all comparisons for the two dosed substances, each group of data is significantly different for $P < 0.001$ *by two-ways ANOVA and Bonferroni* post hoc *test.* $^{*}P < 0.05$; $^{**}P < 0.01$ *and* $^{***}P < 0.001$ *from the others.*

into stable hydroperoxide. The radical cycloperoxide, in the presence of oxygen and due to the effect of heat, can fragment, producing malondialdehyde (MDA) and aliphatic compounds of a radical nature. Peroxidation, like all chain reactions, consists of an "initiation", a "propagation" and a "termination" stage. The consequences of an intense peroxidation of biological membrane lipids are: loss of biological membrane fluidity, reduction of membrane potential, increased permeability to H^+ ions and other ions and rupture and release of cellular content to the outside.

3. Study on creatinine and uric acid

The studied population consisted of 10 patients with endogenous both hypercreatininemic hyperuricemic and ten normal subjects. The measurement of total proteins in RBC ghosts and HNE and MDA are conducted on blood samples of patients. The increase of MDA and HNE levels represent the elevated activities of oxidative stress in human body. It can be seen from **Figures 6** and **7** that the increase of protein concentration in the membrane of the blood samples is associated with the rise of MDA and HNE levels. From the ratio between either MDA levels (μM) or HNE levels and RBC membrane proteins (g/l), we deduced the following results on the basis of **Figures 8** and **9** in which we have divided samples into 3 groups according to their blood reactive oxygen species (ROS) levels. According to this kind of grouping, we can observe that both uric acid and creatinine levels have influence on the ratio either of MDA/protein or HNE/protein contents in RBC membranes, demonstrating their protective role against oxidative stress at low concentrations (lower than 5 mg/dL, for uric acid) and slight oxidizing power at high concentrations (higher than 1.1 mg/dL, for creatinine), as previously evaluated by Qasim and Mahmood [2] and even more powerful oxidant (HNE) could act similarly. In each plot, "a valley" was observed. In this valley, it is seen that there is a dramatically fall in μmole concentration of MDA per mg of RBC proteins which signifies low oxidative stress activity. Thus, it suggests

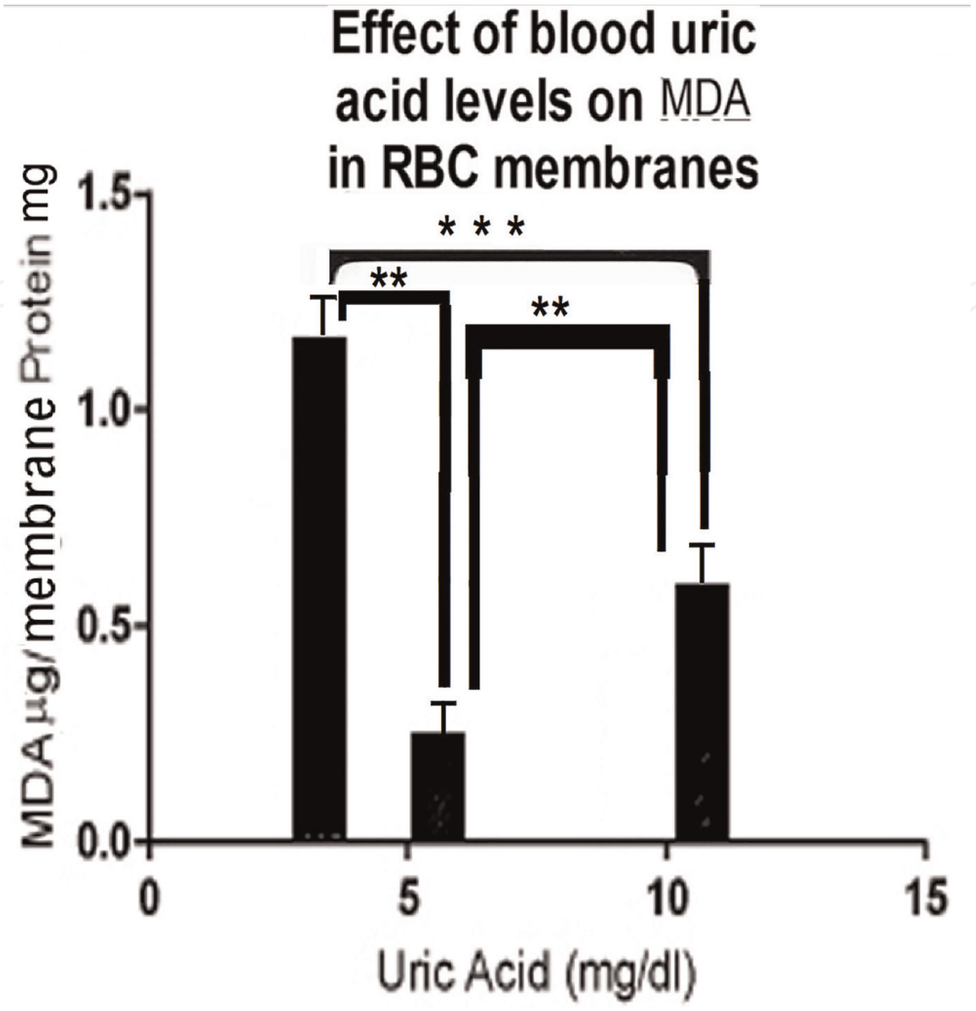

Figure 6.
Effect of blood uric acid levels on RBC membrane MDA. Each result is the main plus/minus standard error on the mean of ten independent experiments. Results are evaluated by one-way ANOVA and Bonferroni post-hoc test according to Graphpad prism 5.0. $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.*

that if the concentration of creatinine and uric acid is within medium range of uric acid concentration, each of these substances would strongly express their protective role toward oxidative stress. In **Figures 6–9** one can observe that the recovery of the curve is smooth and starts from the proximal area of Sample knot, suggesting that at this point both Creatinine and uric acid gradually loss their protective abilities as their concentration in the RBC membranes increase. The described results confirm the observation on MDA levels are similar for the action of uric acid and creatinine on ROS levels with almost parallel patterns [16–18]. An hypothetic explanation for this paradox could be that a rise in uric acid concentration represents an attempted protective response by the host [6]. Probably HNE data in the same experimental conditions should parallel the similar effects of ROS on the oxidation of longer chain lipids in human RBC membranes. The relationship between oxidation of long chain fatty acids and the concentration of both uric acid and creatinine blood levels according to their paradoxical action on oxidation of this kind of RBC constituents probably are attributable to multiple mechanisms of interaction of several constituents of ROSs mixture generated in human organisms with the molecular structures of RBC membranes as carbonylated proteins. This hypothesis shall be investigated in further

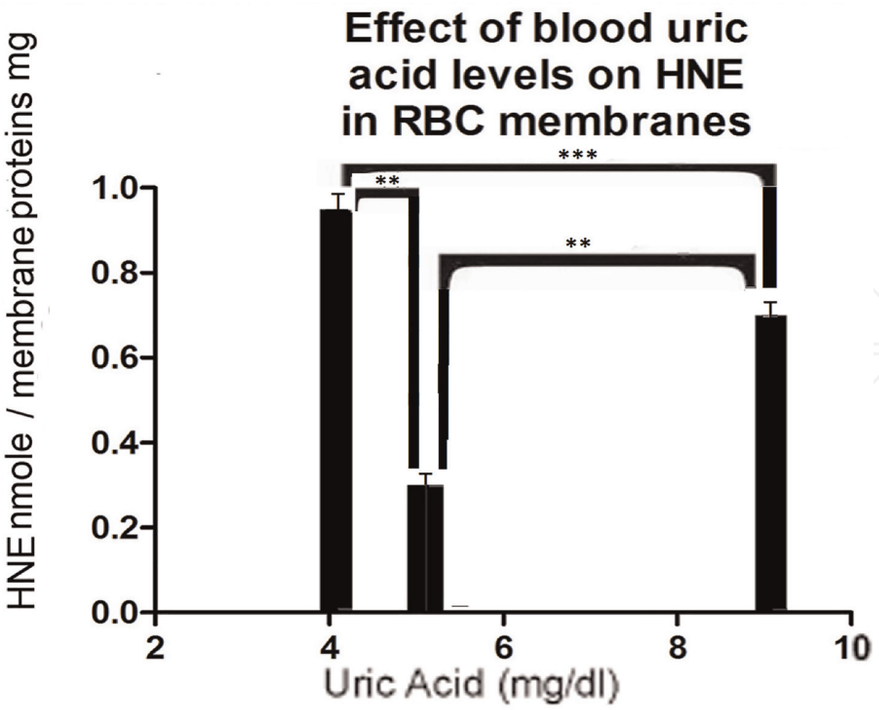

Figure 7.
Effect of blood uric acid levels on RBC membrane HNE. Each result is the mean plus/minus standard error on the main of ten independent experiments. Results are evaluated by one-way ANOVA and Bonferroni post-hoc test according to GraphPad prism 5.0. * $p < 0.05$, ** $p < 0.01$ *and* *** $p < 0.001$.

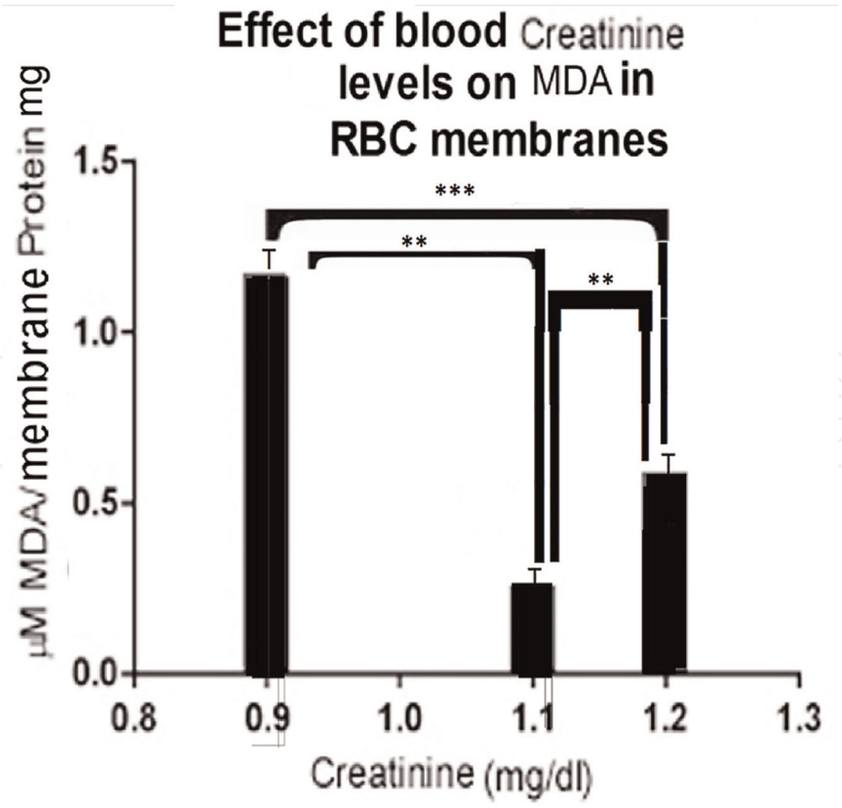

Figure 8.
Effect of blood creatinine levels on RBC membrane MDA. Each result is the mean plus/minus standard error on the main of ten independent experiments. Results are evaluated by one-way ANOVA and Bonferroni post-hoc test according to Graphpad prism 5.0. * $p < 0.05$ ** $p < 0.01$ *and* *** $p < 0.001$.

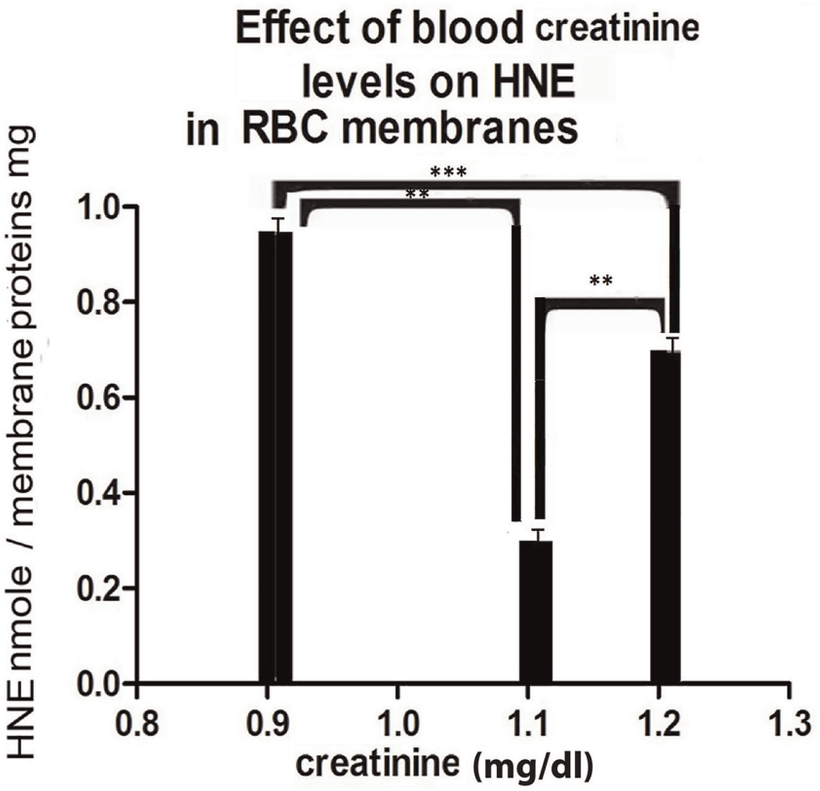

Figure 9.
Effect of blood creatinine levels on RBC membrane HNE. Each result is the mean plus/minus standard error on the main of ten independent experiments. Results are evaluated by one way ANOVA and Bonferroni post-hoc test according to GraphPad prism 5.0. * $p < 0.05$, ** $p < 0.01$ *and* *** $p < 0.001$.

research also on other structural components of membrane. At present, we have only data on total RBC membrane proteins.

The data from this research suggest that nitrogen metabolism, mainly creatinine, acts upon cellular lipid metabolism, as this chemical in itself is a reductant compound but at high intracellular concentrations it works as an oxidizing product, as described by Qasim and Mahmood [2]. Creatinine metabolism can interact with uric acid excretion by kidneys. Hyperuricemia damages kidneys where creatinine is excreted by humans. The interaction between concentrations of creatinine and uric acid, that is powerful scavenger of singlet oxygen, slows down the activity of oxidative stress in human erythrocyte membranes. The contemporaneous modulation of both creatinine and uric acid metabolism and their anatomical and functional consequences could modulate MDA and HNE levels. Only if the concentration of these 2 substances overcomes thresholds, they will begin to express their harmful both oxidative and reductive activities.

4. Study on PUFAs

Currently, in the literature, there are only partial discussions on the role of lipids and their oxidation products as intermediates of their membrane structural damage and/or on the protective role in the same structures [19, 20]. Elevated levels of triglycerides are associated with atherosclerosis and predispose to cardiovascular disease [21]. Oxidative stress, i.e., an altered balance between the production of free radicals

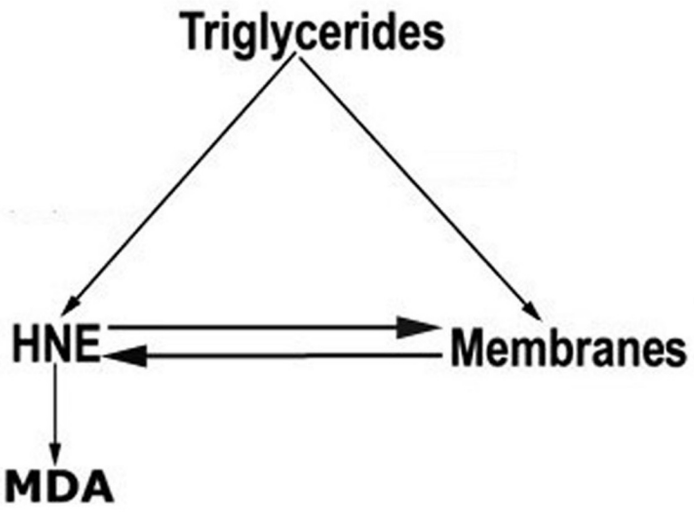

Figure 10.
Graphical resume of main metabolic steps of fatty acids in cell membrane.

and antioxidant defenses [22]. The peroxidation of n-3 and n-6 polyunsaturated fatty acids (PUFAs) and of their metabolites is a complex process. It is initiated by free oxygen radical-induced abstraction of a hydrogen atom from the lipid molecule followed by a series of nonenzymatic reactions that ultimately generate the reactive aldehyde species 4-hydroxyalkenals (HNE). Some authors show that high doses of dietary n-3 PUFAs, as well as long-time treatments, affect human RBC susceptibility to lipid peroxidation by changes in fatty acid composition content. According to experimental data, the accumulation of the alkenals in RBC membrane could be produced either by partial PUFA oxidation contained in glycerides and plasma glycerides and by glycerides into recycled plasma membrane in RBC neogenesis (**Figure 10**). According to these hypotheses, the increased charge of triglycerides in plasma forces its metabolism toward either incorporation in cell membrane of degradative oxidation. This last pathway induces increase of oxidative product such as alkenals and MDA. Furthermore, free alkenals can be dissolved in lipid membrane bilayer degrading their structures. This last process favors increased level of macromolecular assemblies in circulation that enhances microcirculation damage. Such last data could be studied in following works.

5. Conclusions

In the first study, it is evident that after *in vitro* oxidative damage of the membrane, red wine polyphenol extracts are as effective reducing agents also on C_M indicator of membrane protein damages. Consequently, both red wine and polyphenol extracts both alone and mixed among them efficiently relieve the effects of oxidative stress. Regarding the study on 4-hydroxytyrosol. All the data decrease relevantly ($P < 0.001$) after the 4-hydroxytyrosol treatment, especially those of alkenals. As for the creatinine and uric acid studies they this study suggest that at low concentrations of creatinine and uric acid there is oxidative stress in human erythrocyte membrane, but if creatinine and uric acid have slowed down to sufficient limits there is also oxidative stress, as demonstrated by MDA and HNE levels; only if the concentration of these two substances overwhelms a threshold. According to experimental data, the

accumulation of the alkenals in RBC membrane could be produced either by partial PUFA oxidation contained in glycerides and plasma glycerides and by glycerides into plasma membrane recycled RBC.

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Conflict of interest

There are no conflicts of interest.

Acronyms and abbreviations

ORAC	Oxygen Radical Absorbance Capacity, or the oxygen radical absorption capacity
AAPH	2, 2′-azobis (2-amidinopropane) dihydrochloride
RBC	red blood cell
MDA	malondialdehyde
HNE	4-hydroxynonenal
PUFAs	polyunsaturated fatty acids
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
ROS	reactive oxygen species
C_M	the visible absorbance spectrum method evaluating the absorption of methemoglobin

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