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Effect of alpha-lipoic acid on rat kidney and liver microsomes subjected to oxidative stress induced by ascorbate-Fe^{++}

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Abstract

Metabolic changes during kidney and liver disease can induce increased production of oxygen radicals that play an important role in the progression of kidney and liver damage and in the appearance of important comorbidities. In the study reported here, the effect of alpha-lipoic acid (ALA) on the chemiluminescence (CL) and TBARS determination of microsomes isolated from rat kidney and liver was analyzed. Oxidative stress in microsomes was induced by subjecting samples (1 mg of protein) to an ascorbate-Fe++-dependent (0.4 mM)-Fe++ (2.15 µM) prooxidant system at 37 °C for 120 min. Oxidative damage was quantified by two methods, TBARS and CL: CL was determined using a Packard 1900 TR liquid scintillation counter (Meriden CT, USA). CL was expressed as cpm (counts per minute) was read every 10 minutes to establish the course of peroxidation as a function of time. Likewise, the total value of cpm (sum of the readings) was used to compare the inhibitory effect of ALA using different concentrations corresponding to 0.05; 0.15 and 0.25 µg of ALA per mg of microsomal protein. Controls were run simultaneously without the addition of ascorbate-Fe++. It was observed that the CL was lower in the kidney and liver microsomes obtained from the ALA group than in the control group (without ALA). In addition, -ALA was found to be concentration-dependent (0.05, 0.15 and 0.25 µg of ALA per mg of solution) reduced in CL, measured as total cpm. After that it was done the determination of lipid peroxidation products (Thiobarbituric Acid Reactive Substances—TBARS). TBARS were measured in the microsomal samples. Lipoperoxidation inhibition depended on the concentration of ALA in the incubation mixture. The results show that for all the concentrations tested, a protective effect on the induced oxidative damage was found. CL and TBARS level analyzes indicate that ALA may act as an antioxidant protecting rat kidney and liver microsomes from lipoperoxidative damage.

Keywords: Alpha-lipoic acid; Chemiluminescence; Microsomes; Lipid peroxidation; Liver; Kidney; TBARS

1. Introduction

Oxidative stress is caused by over expression of reactive oxygen species (ROS), including hydroxyl radicals, superoxide anion, and hydrogen peroxide (H2O2), [1,2]and is considered the imbalance between reactive oxygen species (ROS) formation and cellular antioxidant capacity which is a result of enhanced ROS generation and/or dysfunction of the antioxidant system. ROS can be generated from several cellular sources, including nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase and the mitochondrial electron transport chain [3].However, the production of ROS in concentrations greater than those that antioxidant systems are capable of neutralizing, causes oxidative damage to the cells [4], degenerative processes, and disease induction. In this work was the oxidative stress in kidney and liver microsomes was induced by subjecting samples (1 mg of protein) to an ascorbate-Fe^{++}-(inductor) dependent (0.4 mM)-Fe++ (2.15 µM) prooxidant system at 37 °C for 120 min. In aqueous media, ascorbic acid is an antioxidant at

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concentration levels of the order of 1 mM and acts as a prooxidant at low concentrations (0.4 mM), especially in the presence of metal ions. Ascorbic acid can chelate Fe +3 and reduce it to Fe +2, which is more active in the initiation and homolytic decomposition of hydroperoxides.

ROS have also been associated with cellular aging: the theory of progressive membrane aging emphasizes the damage produced to membrane fatty acids in the following aspects: a) membrane fatty acids are found in the primary site of production of ROS (mitochondrial and microsomal membranes) and are so close to them that no antioxidant defense system is capable of preventing their peroxidation; b) lipid peroxidation constitutes an autocatalytic chain reaction that, once started, will continue until it is eventually stopped by antioxidant mechanisms; c) many of the products of lipid peroxidation such as malonaldehyde (MDA alquenales y 2,4-alcadienales are themselves very reactive molecules and are therefore highly damaging agents on other molecules [5].

One of the most important antioxidants is alpha-lipoic acid (ALA). It is a disulfide compound that can quench free radicals and indirectly recirculate cellular antioxidants. ALA is used as a cofactor of the mitochondrial respiratory enzymes pyruvate dehydrogenase and ketoglutarate dehydrogenase [6]. Over the years, ALA has gained a considerable attention as a food additive with beneficial effects both in the treatment or management of several aliments [7, 8, 9] are primarily related with its antioxidant activity, but ALA has also demonstrated interesting cardiovascular, cognitive, anti-ageing, detoxifying, anti-inflammatory, anti-cancer, and neuroprotective properties [8] ALA was able to reduce oxidative stress in the heart and other organs, including the liver and kidney [10]. Both ALA and its reduced form DHLA have a determinant role in oxidative metabolism [11]. For instance, it has been shown that ALA and DHLA have several positive health benefits, including as biological antioxidants, metal chelators and detoxification agents, being also able to reduce the oxidized forms of other antioxidant agents, including glutathione, vitamins C and E, and to modulate various signaling pathways, such as insulin and nuclear factor kappa B [12]. It has also been used forage-associated cardiovascular, cognitive, and neuromuscular deficits [13, 14], to reform endothelial dysfunction [15], to decrease oxidative stress [16] and to inhibit the formation of atherosclerosis plaque [17].

The production of ROS in the liver and kidney has been implicated as a common factor in the etiology of a number of diseases [18, 19]. Since free radicals are extremely reactive and can be combined unspecifically with different cellular components [20] when they are in excess they can act with membranes (lipid peroxidation), nucleic acids and proteins, having studied with greater emphasis the attack of ROS caused on lipids, an effect that leads to lipoperoxidation: the oxidation of the α-methylene bonds of unsaturated fatty acids that results in the formation of lipoperoxides and hydroperoxides and finally the fragmentation of the lipid molecule [9].

As biomembranes are rich in polyunsaturated fatty acids, these reactions lead to disintegration of the membrane structures and ultimately irreversible cell damage (Lawrence 2021). TBARS was used to quantify the degree of lipid peroxidation in rat kidney and liver microsomes [21].

The present study was designed to determine whether rat kidney and liver microsomes could be a target for non-enzymatic lipid peroxidation, as well as to establish the level of protection of said membranes incubated with ALA. The degradative process was followed by TBARS and by chemiluminescence determination [22]. The measurement of the light emission of a chemical reaction is very useful analytically because, under appropriate experimental conditions, the light output is directly related to the analytical concentration, which allows an accurate and sensitive quantitative analysis. In addition, the emission of light is generally represented by a steady state kinetics, which simplifies the handling of the sample and the measurement procedures. The 2-thiobarbituric acid assay (TBARS) is one of the oldest and most frequently used methods to evaluate lipid oxidation in membranes. Malonaldehyde (MDA) is an oxidation product of polyunsaturated acids present in membranes (PUFAs) that reacts with 2-thiobarbituric acid to produce a colored complex. Other aldehydes such as alkenals and 2,4-alkadienals also react, so it is more appropriate to use the name thiobarbituric acid reactive substances (TBARS) [23]. Chemiluminescence and TBARS have been widely used as an indicator of the formation of ROS in whole cells and organs, allowing the study of a number of pathophysiological conditions related to oxidative stress.

2. Material and methods

2.1. Materials

Female Wistar AH/HOK were obtained from Laboratory Animal Facility, Faculty of Veterinary Science, National University of La Plata. BSA (Fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. Alpha lipoic acid

was kindly supplied by Craveri Lab S.A., Arengreen 830/Miranda 5237 C1405CYH. L (+) ascorbic acid was from Merck Laboratories. All other reagents and chemicals were of analytical grade from Sigma.

2.2. Alpha-lipoic acid solution preparation

A 200 mg tablet of alpha-lipoic acid was diluted it in 20ml of distilled water. Then 1ml of the stock solution was taken and diluted in 10ml of distilled water, obtaining a concentration 2.1mg/ml of ALA. The following concentrations were used for the experiments: 0.05mg /ml, 0.15mg/ml and 0.25mg/ml.

2.3. Animals and preparation of kidney and liver microsomes

Seven-weeks-old female Wistar AH/HOK rats (n =6), weighing 120 g to 137 g were used. All rats were fed with commercial rat chow and water ad libitum. The rats were sacrificed by cervical dislocation and kidneys and livers were rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30% (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4 using a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 1,000 x g, the pellets were discarded, and the resulting supernatant was centrifuged at 20,000 x g for 10 min. After centrifugation, 5 ml of the resulting supernatant were passed through a Sepharose 4 B column (1.6cm x 12 cm) equilibrated and diluted with 10 mM Tris-HCl buffer pH7.4 0.01% Na N3. The microsomal fraction appeared in the void volume (12-20 ml) and the cytosol was discarded. All operations were performed at 4 °C and under dim light. The quality of this microsomal preparation is compositionally similar with respect to concentrations and activities of certain microsomal enzymes to that obtained by ultracentrifugation [24].

2.4. Non-enzimatic lipid peroxidation of rat kidney and liver microsomes

The microsomes (1 mg of microsome protein) with addition of alpha-lipoic acid solution (0.05, 0.15 and 0.25µg- alpha-lipoic acid group) were incubated at 37 °C with 0.01M phosphate buffer pH 7.4, 0.4mM ascorbate, final vol. 1 ml. Chemiluminescence and peroxidation were initiated by adding ascorbate-Fe^{++} to microsomes (1 mg protein) (ascorbate-Fe^{++} group) (Wright et al. 1979). To carry out the experiments, three groups were established: 1) control group (only organelles), 2) ascorbate-Fe^{++} group (organelles + inducer) and 3) ALA group (organelles + inducer + ALA). The microsomes (1 mg of microsome protein) with addition of alpha-lipoic acid solution (0.05, 0.15 and 0.25µg- alpha-lipoic acid group) were incubated at 37 °C with 0.01M phosphate buffer pH 7.4, 0.4mM ascorbate, final vol. 1 ml. The phosphate buffer is contaminated with enough iron to provide the necessary ferrous or ferric iron (final concentration in the incubation mixture was 2.15 µM) for peroxidation [25]. Simultaneously, preparations were made with microsomes, which lacked ascorbate (control group). Light emission from the membranes was determined over a period of 120 min, chemiluminescence was recorded as cpm every 10 min, and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per minute on a Packard 1900 TR liquid scintillation analyzer with chemiluminescence software.

2.5. Measurement of lipid peroxidation

Levels of MDA were measured using the thiobarbituric acid (TBA) fluorometric assay (Esterbauer and Cheeseman 1990). Stock TCA-TBA-HCL reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid. 1.0 ml of the kidney or liver microsome samples were combined with 2.0 ml of TCA-TBA-HCL and mixed well. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 min. The absorbance of the sample was determined at 535 nm against a blank containing all reagents except lipid. The concentration of malondialdehyde in the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Data were expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein.

2.6. Protein determination

Proteins were determined by the method of Lowry et al. [26] using BSA as a standard.

2.7. Statistical analysis

Data were expressed as means ± S.D. of six independent determinations. The statistical analysis used was the Student's t-test. The 0.05 level was selected as the point of least statistical significance. The statistical criterion of significance was selected at different p values and was indicated in each case.

3. Results and discussion

3.1. Light emission of rat kidney microsomes during peroxidation

The incubation of rat kidney microsomes in the presence of ascorbate-Fe^{++} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence) when the control and ascorbate-Fe^{++} groups were compared. The values were 418.33 ± 21.06 in the control group while 736 ± 75.51 in the ascorbate-Fe^{++} group, showing a significance value of $p < 0.0005$. After incubation of microsomes in an ascorbate-Fe++ system at 37 °C for 120 min in the presence of increasing amounts of of alpha-lipoic acid solution (0.05, 0.15 and 0.25µg- alpha-lipoic acid group) per mg of protein, the cpm originated from light emission was lower (concentration dependent) in the alpha-lipoic acid group than in the control group. The values were 582.67 ± 53.54 with the addition of 0.05 µg alpha-lipoic acid /mg protein; 554.67 ± 26.23 with the addition of 0.15 µg alpha-lipoic acid /mg protein and finally 499.00 ± 21.35 cpm with the addition of 0.25 µg alpha-lipoic acid /mg protein. The significance values were $p < 0.005$ with the 0.05 dose, while for the 0.15 and 0.25 doses a highly significant value corresponding to $p < 0.0005$ was obtained (Figure 1).

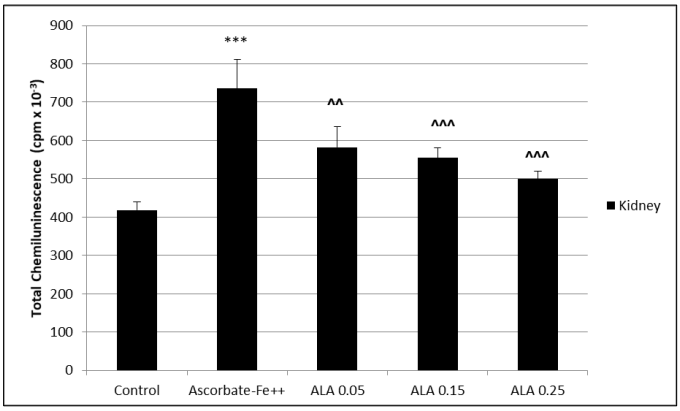

Figure 1 Light emission of rat kidney microsomes during peroxidation with different concentration of ALA. The results are expressed as the mean ± S.D. of six independent experiments. Statistically significant differences between control vs ascorbate-Fe++ group are indicated by *** $p<0.0005$ and ascorbate-Fe++ vs ALA groups are indicated by ^^$p<0.005$, ^^^$p<0.0005$

3.2. Light emission from rat liver microsomes during peroxidation

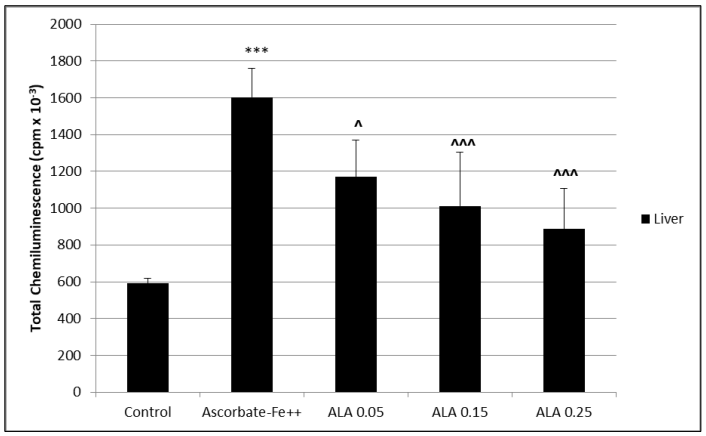

Figure 2 Light emission of rat liver microsomes during peroxidation with different concentration of ALA. The results are expressed as the mean ± S.D. of six independent experiments. Statistically significant differences between control vs ascorbate-Fe++ group are indicated by *** $p<0.0005$ and ascorbate-Fe^{++} vs ALA groups are indicated by ^$p<0.05$, ^^^$p<0.0005$

The incubation of rat liver microsomes in the presence of ascorbate-Fe^{++} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence) when the control and ascorbate-Fe^{++} groups were compared. The values were 591.00 ± 25.47 in the control group while 1603.33 ± 156.08 in the ascorbate-Fe^{++} group, showing a

significance value of $p < 0.0005$. After incubation of microsomes in an ascorbate-Fe^{++} system at 37 °C for 120 min in the presence of increasing amounts of of alpha-lipoic acid solution (0.05, 0.15 and 0.25µg- alpha-lipoic acid group) per mg of protein. The values were 1170.33 ± 199.56 with the addition of 0.05 µg alpha-lipoic acid /mg protein; 1012.00 ± 291.02 with the addition of 0.15 µg alpha-lipoic acid /mg protein and finally 886.67 ± 219.34 cpm with the addition of 0.25 µg alpha-lipoic acid /mg protein. The significance values were $p < 0.05$ with the 0.05 dose, while for the 0.15 and 0.25 doses a highly significant value corresponding to $p < 0.0005$ was obtained (Figure 2).

3.3. Comparative percentage of light emission inhibition of rat kidney and liver microsomes

After incubation of rat kidney and liver microsomes in an Ascorbate-Fe^{++} system (120 min at 37°C), it was observed that the percentage of inhibition cpm/mg of protein from light emission (chemiluminescence) was lower in kidney microsomes than liver microsomes during the addition of alpha-lipoic acid. Thus, the percentage of peroxidation inhibition produced by alpha-lipoic acid was 44.70% in liver microsomes and 32.20% in kidney microsomes for the 0.25 µg dose of alpha-lipoic acid (Figure 3).

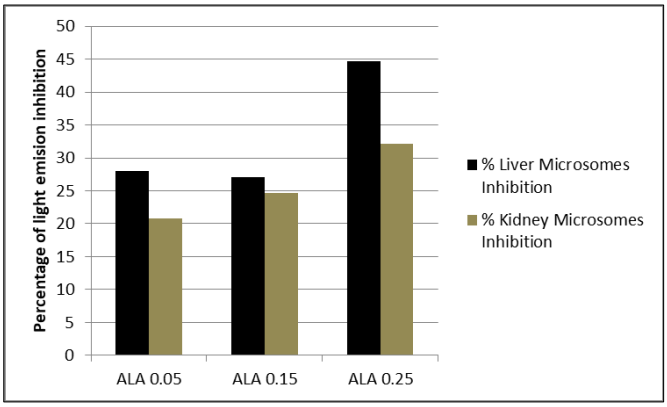

Figure 3 Percentage of inhibition of light emission in kidney and liver microsomes by ALA

3.4. Determination of Lipid Peroxidation Products (Thiobarbituric Acid Reactive Substances- TBARS)

TBARS assay was used to quantify the degree of lipid peroxidation in rat kidney and liver microsomes. Statistically very significant levels of MDA were found ($p<0.0005$) when comparing the control vs. ascorbate-Fe^{++} groups which indicates that in the presence of ascorbate-Fe^{++} (inducer) the membranes were peroxidized.

In addition, when the ascorbate-Fe^{++} groups were compared with the ALA group in increasing concentrations of the acid: 0.05; 0.15 and 0.25 µg ALA/mg Pt MDA levels were found to be statistically significantly lower than in the ascorbate Fe^{++}group. In conclusion, there was a significant increase in the level of MDA in the ascorbate Fe^{++} group that was restored after treatment with increasing concentrations of ALA in both kidney microsomes (Figure 4) and liver microsomes (Figure 5).

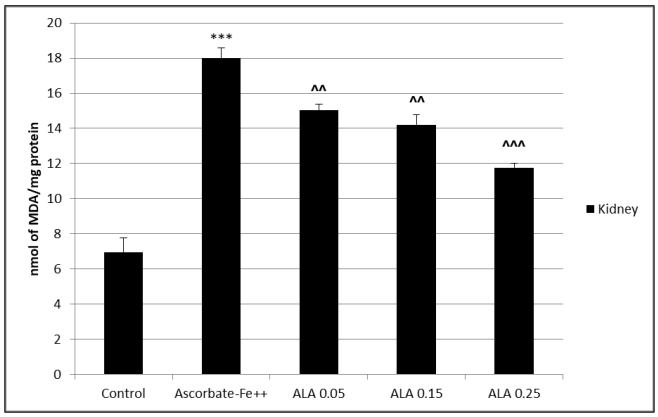

Figure 4 Determination of Lipid Peroxidation Products (Thiobarbituric Acid Reactive Substances—TBARS) in kidney microsomes: effect of ALA. Statistically significant differences between control vs ascorbate-Fe^{++} group are indicated by *** $p<0.0005$ and ascorbate-Fe^{++} vs ALA groups are indicated by ^^$p<0.005$,^^^$p<0.0005$

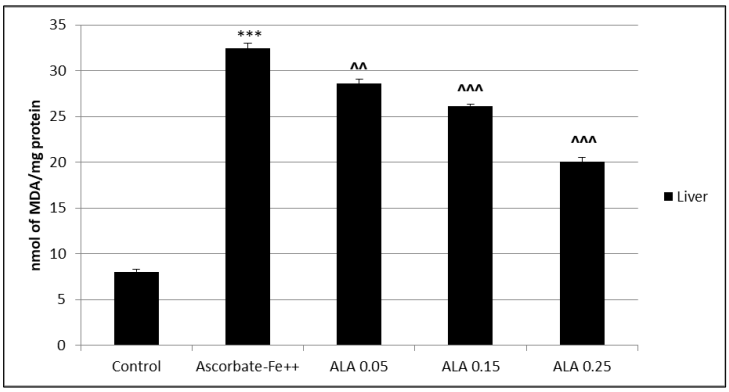

Figure 5 Determination of Lipid Peroxidation Products (Thiobarbituric Acid Reactive Substances—TBARS) in liver microsomes: effect of ALA. Statistically significant differences between control vs ascorbate-Fe++ group are indicated by *** p<0.0005 and ascorbate-Fe++ vs ALA groups are indicated by ^^p<0.005,^^^p<0.0005

During cellular respiration, univalent reduction of oxygen occurs [27] and therefore most of the ROS formed under physiological conditions are different forms of oxygen radicals: superoxide anion, hydroxyls and singlet oxygen. The balance between the generation of free radicals and their neutralization is controlled by complex mechanisms that include: a) the maintenance of the spatial separation between the sites of radical production and the biomolecules vulnerable to them, b) the enzymatic mechanisms of radical trapping: superoxide dismutase, catalase and glutathione peroxidase and c) the presence of substances with antioxidant properties: vitamin C, vitamin E and glutathione [28].

ALA metabolites have been shown to have anti-inflammatory and antioxidant effects [29]. It has been shown that ALA is able to exert a significant antioxidant effect through a scavenger activity on free radicals [30], as well as its capability of LA to chelate metals [31]. Thus ALA is unique among natural antioxidants in its ability to fulfill a lot of requirements, making it a potentially highly effective therapeutic agent for a number of pathological conditions implicated with oxidative damage [32].

In this study we analyzed the in vitro antioxidant effect of ALA on non-enzymatic peroxidation in rat kidney and liver microsomes in order to find an inhibitory dose that offers greater protection caused by ROS. It is important to know how biological compounds with antioxidant properties contribute to the protection of specialized membranes against deleterious effects produced by reactive oxygen species and other free radicals. With respect to previous studies carried out in our Laboratory referring to the effect of ALA on rat liver and kidney mitochondria, [33] we observed that the cpm values obtained in liver microsomes in the presence of ascorbate-Fe $^{++}$ and in the presence of the same concentrations of ALA (0.05, 0.15 and 0.25 µg ALA/mg Pt) were higher in relation to the values obtained in liver mitochondria subjected to the same experimental conditions. Similarly, the antioxidant effect of ALA was greater in liver microsomes, with highly significant differences being obtained at values of 0.15 and 0.25 µg ALA/mg Pt. When we analyzed the % inhibition of non-enzymatic peroxidation, we observed that the highest % in mitochondria was 25.71%, while in microsomes the value was 44.20% at a dose of 0.25 µg ALA/mg Pt. Taking into account since CL and TBARS assays have been used as an indicator of ROS formation, we observed that the protective effect of ALA increases correlatively with the decrease in cpm, and MDA levels with greater protection in liver microsomes being observed. In the same way, when we compared the cpm values obtained in kidney microsomes in the presence of ascorbate-Fe^{++} and in the presence of the same concentrations of ALA (0.05, 0.15 and 0.25 µg ALA/mg Pt), we observed that they were higher in mitochondria (Mariana Gavazza et al. 2021). In relation to the values obtained in kidney microsomes subjected to the same experimental conditions was observed that the antioxidant effect of ALA was greater in kidney mitochondria, obtaining highly significant differences at values of 0.15 and 0.25 µg ALA/mg Pt.

When we analyzed the % inhibition of non-enzymatic peroxidation, we observed that the highest % in mitochondria was 35.70% at a dose of 0.15 µg ALA/mg Pt, while in microsomes the value was 32.20% at a dose of 0.25 µg ALA/mg Pt. Taking into account that CL and TBARS assays has been used as an indicator of ROS formation, we observed that the protective effect of ALA increases correlatively with the decrease in cpm, and MDA levels being observed in this case in which protection was greater in rat kidney mitochondria. Given the results obtained, in this work we observed that the protective effect of ALA was greater in liver microsomes than in rat kidney microsomes.

4. Conclusion

Based on these studies, it can be concluded that ALA is an ideal antioxidant in the prevention of oxidative stress development in kidney and liver microsomes because it alleviates non enzymatic lipid peroxidation. However, further research is needed to explain the mechanism of action of ALA in oxidation-stressed in this organelles.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors have no conflict of interest to declare.

Statement of ethical approval

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. The Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences, UNLP, approved all procedures accommodation and experimentation under File 600-009500/12-000 Resolution 38.

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