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Effects of lipoic acid supplementation on rat brain tissue: An FTIR spectroscopic and neural network study

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Abstract

The unfortunate increase in exposure to free radicals justifies antioxidant supplementation. Therefore, in the current study, the effect of exogenously administered lipoic acid, a natural amphipathic antioxidant, on rat brain tissue was investigated via Fourier transform infrared spectroscopy in order to understand its interactions with biological molecules. The results suggest that lipoic acid slightly disorders the acyl chains of phospholipids as observed from the frequency of the CH_2 stretching vibrations while it strengthens the hydrogen bonding of the interfacial region of phospholipids as indicated by the C=O stretching band. Moreover, lipoic acid seems to cause an increase in the quantity of proteins, without affecting the protein secondary structure revealed by neural network predictions based on FTIR data. These slight variations in the lipid structure and the unaltered protein secondary structure may suggest that lipoic acid is non-toxic and thus support the usage of lipoic acid as an antioxidant supplement.

Keywords: Lipoic acid; Brain tissue; FTIR spectroscopy; Lipid order/disorder; Hydrogen bonding; Neural networks; Protein secondary structure

1. Introduction

α-Lipoic acid (LA) is a natural molecule consisting of a five-membered cyclic disulphide and hydrocarbon tail ending with a carboxylic acid group (Fig. 1). Hence, lipoic acid is a predominantly lipophilic molecule having an amphipathic character due to its carboxylic acid group attached to the ring structure. Lipoic acid is present in our diet mainly in animal foods such as meat and liver and at low or undetectable levels in plant foods such as potato (Kataoka, 1998; Lachman, Hamouz, Orsak, & Pivec, 2000). However, lipoic acid is also considered beneficial when used as a food supplement as its antioxidant function has been previously reported and several studies have revealed its protective effects in cases such as aging, diabetes mellitus and vascular and neurodegenerative diseases all in which free radicals are involved (Hagen et al., 1999; Packer, Kraemer, & Rimbach, 2001; Packer, Tristchler, & Wessel, 1997; Wollin & Jones, 2003; Yilmaz, Ozkan, Yildirim, Ozturk, & Ersan, 2002). Studies are generally dealing with the biological consequences of lipoic acid administration in cases associated with oxidative stress or the differences between the antioxidant activities of lipoic acid and its derivatives (Arivazhagan, Thilakavathy, Ramanathan, Kumaran, & Panneerselvam, 2002; Matsugo et al., 1997; Packer et al., 1997; Yilmaz et al., 2002). There are also some studies on the spectroscopic and chromatographic analysis of the structural and quantitative aspects of lipoic acid and its derivatives in solution (Chen et al., 2005; Schepkin, Kawabata, Tritschler, & Packer, 1996). However, structural information on the exact mode of interaction of lipoic acid with molecules of biological systems is lacking, despite the fact that investigation of the interactions of antioxidative drugs with biomolecules is important in understanding the mechanism of their action.

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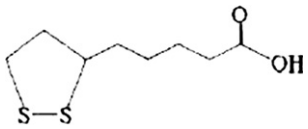

Fig. 1. The chemical structure of α-lipoic acid.

Antioxidant defense and repair systems are a fundamental part of the body's effort to overcome the damage inflicted by free radicals. The brain is especially exposed to free radicals, because it generates more free radicals per gram of tissue than does any other organ due to its high and constant oxygen consumption to supply the energy it needs. Its exposure is further enhanced due to its relatively high levels of polyunsaturated fatty acids, which are particularly good substrates for free radical-mediated reactions (Pamplona et al., 1999; Reiter, 1995).

In the current study, the effects of exogenously administered α-lipoic acid on the protein quantity, membrane acyl chain order, and state of intramolecular hydrogen bonding of the interfacial region of lipids of rat brain tissues were investigated which, to the best of our knowledge, have not previously been reported. For these purposes, we used Fourier transform infrared (FTIR) spectroscopy, which is a non-perturbing rapid technique giving information on several biomolecules, such as proteins and lipids, simultaneously by monitoring the different functional groups belonging to these biomolecules. It is widely accepted that FTIR spectroscopy is a highly sensitive tool capable of providing strong insight on structural and functional alterations induced by various factors (Cakmak, Togan, & Severcan, 2006; Takahashi, French, & Wong, 1991; Toyran, Lasch, Naumann, Turan, & Severcan, 2006; Toyran, Zorlu, Donmez, Oge, & Severcan, 2004).

2. Materials and methods

2.1. Chemicals

DL-α-Lipoic acid and olive oil were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA), while ethanol was acquired from Riedel-de Haën (Sigma–Aldrich Laborchemikalien GmbH, Seelze, Germany) and KBr was obtained from Merck (Merck, Darmstadt, Germany). The chemicals were used without further purification.

2.2. Experimental protocol for lipoic acid treatment

After the approval by the Medical School Ethics Committee at Fırat University, a total of eleven 10–12 week old male Wistar rats (250–300 g), which received proper care in compliance with the Ethics Committee, were housed in cages with *ad libitum* rat chow and water in a room with a 12:12 light:dark cycle. The animals were randomly divided into two groups. The lipoic acid group ($n = 6$) received 12 mg/kg lipoic acid dissolved in 2:8 ethanol:olive oil every other day via intraperitoneal (i.p.) injection. The control group ($n = 5$) was injected (i.p.) ethanol:olive oil every other day. The injections were continued for 7 weeks, after which the decapitation was performed. The brain tissues were stored at −80 °C until sample preparation for FTIR spectroscopic studies.

2.3. Sample preparation

The brain tissue samples were first dried in a LABCONCO freeze dryer (FreeZone®, Model 77520) overnight in order to remove water. The dried samples were ground with liquid nitrogen with an agate mortar and pestle. The powdered brain samples were thoroughly mixed with completely dried potassium bromide at a ratio of 1 mg sample to 100 mg KBr. Afterwards, this mixture was dried again in a freeze dryer for 2 h to completely remove any trace of unbound water. Then, the samples were pressed into KBr pellets by subjecting the dehydrated mixture to a pressure of ~85 kg/cm^2 for ~8 min.

2.4. Spectroscopic analysis

Infrared spectra were recorded with a BOMEM MB157 FTIR spectrometer (Michelson Series, Canada) equipped with a deuterated triglycine sulfate (DTGS) detector. The spectrometer was continuously purged with dry air to eliminate atmospheric water vapour and carbon dioxide (CO_2) interference. Pellets were scanned at room temperature in the 3600–445 cm^{-1} spectral range. To improve the signal-to-noise ratio for each spectrum, 200 interferograms with a spectral resolution of 4 cm^{-1} were averaged. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra automatically. Each sample was scanned under the same conditions with three different pellets, all of which gave identical spectra. These replicates were averaged and these averaged spectra for each sample were then used for further data and statistical analysis.

All the spectral analyses following the FTIR studies were performed with Win-Bomem Easy for Microsoft Windows Version 3.04 (Galactic Industries, USA). Deconvolution process was applied only to the C–H stretching region (3050–2800 cm^{-1}), with a gamma factor set for 0.7 for each of the spectra, in order to increase the resolution of the overlapping bands.

The variations in the frequencies and band areas were determined accurately from the original baseline-corrected spectra belonging to the corresponding control and treated groups. The full spectral range was interactively baseline corrected with respect to the 4000, 1950 and 900 cm^{-1} basepoints, where there are no absorption bands. The spectra shown in the figures were normalized in specific regions only to show the spectral variations visually.

2.5. Protein secondary structure predictions using neural networks

Neural networks were first trained using FTIR spectra of 18 water soluble proteins recorded in water (Severcan, Severcan, & Haris, 2001). The secondary structures of these proteins were known from X-ray crystallography. Amide I band, namely absorption values from 1600 cm^{-1} to 1700 cm^{-1}, was preprocessed before applying to the neural networks. Preprocessing involves normalization and discrete cosine transformation (DCT) of the amide I band of the FTIR spectra. To improve the training of the neural networks, the size of the training data set was increased by interpolating the available FTIR spectra.

The NNs were trained using Bayesian regularization. For each structure parameter, a separate NN was trained whose number of inputs, i.e. the number of DCT coefficients, and number of hidden neurons were optimized. The trained NNs have standard error of prediction values of 4.19% for α-helix, 3.49% for β-sheet and 3.15% for turns. The secondary structure parameters of the new proteins were predicted by applying to the inputs of the trained NNs the preprocessed FTIR data. It should be mentioned that there can be vast differences in the infrared spectra between dissolved and lyophilized proteins. Therefore, the results are not really an accurate determination of secondary structure. However, in this study since the main point of interest was the relative changes between the secondary structure contents of two identical preparations, this does not introduce any problem. The details of the training and testing algorithm can be found in Severcan, Haris, and Severcan (2004).

2.6. Statistical analysis

The differences between the control and lipoic acid treated group were calculated by means of the non-parametric Mann–Whitney U test with the Minitab Statistical Software Release13.0 program. Significance was accepted at $p < 0.05^{*}$.

3. Results

The effect of lipoic acid treatment on rat brain tissue was investigated with FTIR spectroscopy by monitoring different functional groups. Fig. 2 shows a representative infrared spectrum obtained from untreated rat brain tissue in the 3600–445 cm^{-1} frequency range. The main absorption bands labeled in this figure belonging to lipids, proteins, carbohydrates and nucleic acids were defined in detail in Table 1 according to the literature. The spectra were analyzed in two major regions; 3600–2800 cm^{-1} and 1800–445 cm^{-1} (Cakmak et al., 2006; Dovbeshko, Gridina, Kruglova, & Pashchuk, 2000; Fukuyama, Yoshida, Yanagisawa, & Shimizu, 1999; Garidel, 2002; Hendler, Barnett, Dracheva, Bose, & Levin, 2003; Jung, 2000; Mendelsohn & Mantsch, 1986; Perromat, Melin, Lorin, & Deleris, 2003; Takahashi et al., 1991; Toyran et al., 2006, 2004). No curve fitting procedure was applied since the bands were clearly resolved (Cakmak et al., 2006; Dogan, Siyakus, & Severcan, 2006; McCrae et al., 2001).

It should be emphasized that, all the spectra presented in the figures were normalized with respect to specific selected bands but these spectra were used only for illustrative purposes. However, each original baseline-corrected spectrum

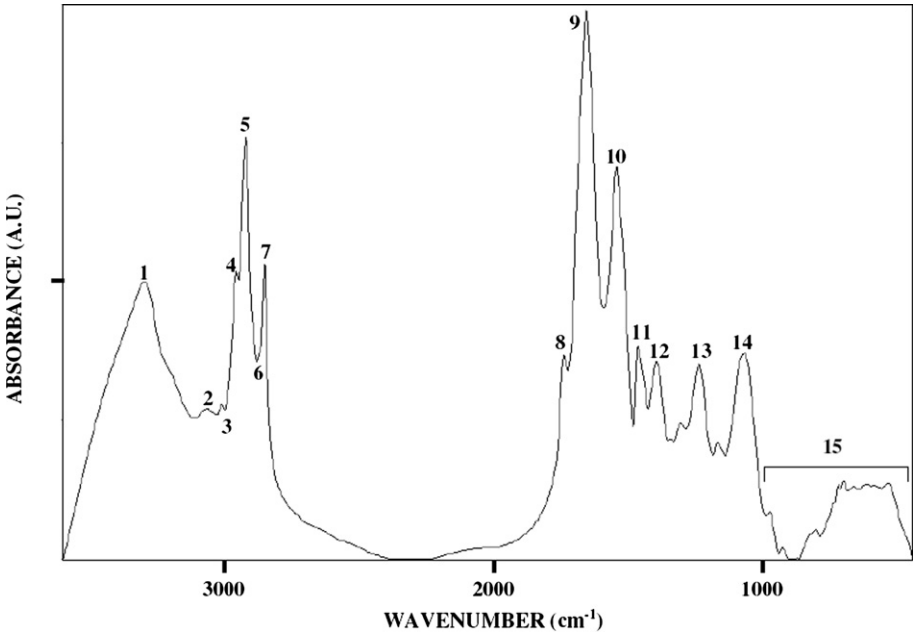

Fig. 2. Representative FTIR spectrum of the lyophilized control rat brain in the 3600–445 cm^{-1} range.

Table 1
General band assignments of the FTIR spectra of brain tissue in the 3600–445 cm^{-1} spectral range according to the literature (Cakmak et al., 2006; Dovbeshko et al., 2000; Fukuyama et al., 1999; Garidel, 2002; Hendler et al., 2003; Jung, 2000; Mendelsohn & Mantsch, 1986; Perromat et al., 2003; Takahashi et al., 1991; Toyran et al., 2006, 2004)

Peak number	Wavenumber (cm^{-1})	Definition of the assignment
1	3304	Amide A: mainly N–H stretching of proteins
2	3066	Amide B: N–H stretching of proteins
3	3012	Olefinic HC=CH stretch: lipids
4	2958	CH_3 asymmetric stretch: mainly lipids
5	2922	CH_2 antisymmetric stretch: mainly lipids
6	2872	CH_3 symmetric stretch: mainly proteins
7	2851	CH_2 symmetric stretch: mainly lipids
8	1739	Carbonyl C=O stretch: lipids
9	1653	Amide I: C=O stretching of proteins
10	1548	Amide II: N–H bending and C–N stretching of proteins
11	1467	CH_2 bending stretch: mainly lipids
12	1399	COO^- symmetric stretch: fatty acids and amino acids
13	1237	PO_2^- asymmetric stretch: mainly phospholipids
14	1072	PO_2^- symmetric stretch: mainly nucleic acids; HO–C–H stretch: carbohydrates
15	1000–445	Fingerprinting region: mainly nucleic acids

belonging to the corresponding control and treated groups was considered separately during the accurate measurement of the spectral parameters. The mean values of the spectral parameters and the statistical significances of the differences between these parameters of specific bands were presented in Table 2.

Fig. 3 shows the FTIR spectrum of the control and lipoic acid treated rat brain tissue in the 3600–3030 cm^{-1} range. The spectra were normalized with respect to the CH_2 antisymmetric stretching mode (2922 cm^{-1}). The band at 3304 cm^{-1} corresponds to the amide A stretching mode that can generally be associated with N–H and intermolecular O–H molecules. The numerical calculations revealed that lipoic acid led to a significant increase ($p < 0.05^*$) in the peak area of the amide A band from 47.3 ± 11.3 in the control group to 73.5 ± 4.1 in the lipoic acid treated group (Table 2).

Table 2
Values of some of the spectral parameters of the control and lipoic acid treated rat brain samples

Frequency (cm^{-1})	Control	Lipoic acid
	Band area	
3304	47.3 ± 11.3	$73.5 \pm 4.1^*\uparrow$
	Band frequency	
2922	2922.1 ± 0.2	$2922.8 \pm 0.2^*\uparrow$
2851	2851.2 ± 0.1	$2851.8 \pm 0.1^*\uparrow$
1739	1740.5 ± 0.1	$1738.5 \pm 0.3^{**}\downarrow$
Amide I (1700–1600)	*NN predictions*	
α-Helix	54.5 ± 6.9	59.7 ± 4.4 NS
β-Sheet	19.1 ± 5.9	14.7 ± 2.1 NS
Turns	14.8 ± 0.9	15.0 ± 0.6 NS
	Band frequency	
1237	1237.4 ± 0.1	1237.2 ± 0.9 NS

Values are presented as means ± SD. Comparison was performed by the Mann–Whitney U test. Significance was accepted at $p < 0.05^*$ and $p < 0.01^{**}$. The upward arrow indicates an increase while the downward arrow indicates a decrease with respect to the control group. NS implies that the variation is not significant.

Fig. 4 shows the deconvolved FTIR spectrum of the control and lipoic acid treated rat brain tissue in the 3000–2800 cm^{-1} range. The spectra were normalized with respect to the CH_2 antisymmetric stretching mode (2922 cm^{-1}). The absorbance bands at 2922 cm^{-1} and 2851 cm^{-1}, which correspond respectively to the antisymmetric and symmetric stretching vibrations of methylene ($-CH_2$) groups, mainly monitor lipids. Lipoic acid led to an increase in the frequency values of these CH_2 stretching vibrations. The statistical analysis showed that the frequency values of the CH_2 antisymmetric and symmetric stretching vibrations increased slightly but significantly from 2922.1 ± 0.2 cm^{-1} to 2922.8 ± 0.2 cm^{-1} and from 2851.2 ± 0.1 cm^{-1} to 2851.8 ± 0.1 cm^{-1}, respectively ($p < 0.05^*$) (Table 2).

Fig. 5 shows the FTIR spectrum of the control and lipoic acid treated rat brain tissue in the 1770–1720 cm^{-1} range. The spectra were normalized with respect to the C=O stretching mode (1739 cm^{-1}). This region is commonly coupled with the infrared band of the stretching mode of the C=O bond present in the interfacial region of phospholipids. As also seen from the figure, lipoic acid led to a shift to lower values in the frequency of the C=O stretching mode from 1740.5 ± 0.1 cm^{-1} in the control group to 1738.5 ± 0.3 cm^{-1} in the lipoic acid treated group. Although this variation was small, it was statistically significant ($p < 0.01^{**}$) (Table 2).

The amide I band region, corresponding to absorption values between 1600 and 1700 cm^{-1}, was analyzed using neural network predictions based on FTIR data. As seen from Table 2, lipoic acid induced slight but not significant alterations in the protein secondary structure ($p > 0.05$ NS). The spectral analysis also revealed that lipoic acid did not cause any significant variations in the phosphate asymmetric stretching vibration (1237 cm^{-1}) (Table 2)

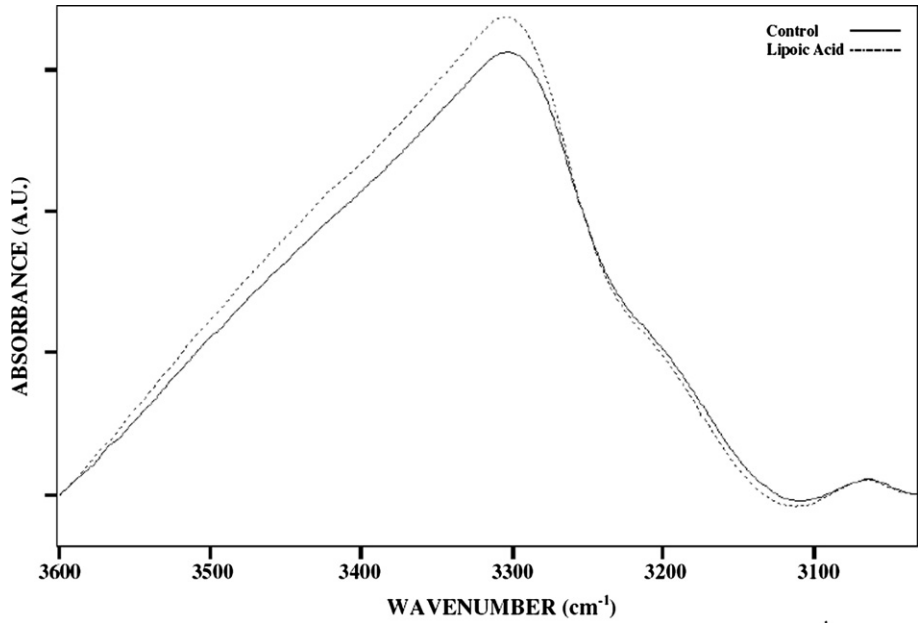

Fig. 3. FTIR spectra of the lyophilized control (solid line) and lipoic acid (dotted line) treated rat brain samples in the 3600–3030 cm^{-1} range. The spectra were normalized with respect to the band at 2922 cm^{-1}.

and the rest of the bands in the 1800–445 cm^{-1} spectral region (data not shown).

4. Discussion

At the present time, there is an unfortunate increase in exposure to free radicals due to many environmental, lifestyle, and pathological situations. Therefore, the endogenous antioxidant defenses may not always be enough to cope with such additional exposure to free radicals. Thus, it seems reasonable to propose that exogenous antioxidants could be very effective in diminishing the cumulative effects of oxidative stress. Many studies have presented evidence for the benefits of antioxidant rich nutrition and antioxidant supplements (Willcox, Ash, & Catignani, 2004).

The current study was carried out specifically to investigate the effect of the exogenous supplement of lipoic acid on rat brain tissue. The value of lipoic acid as an

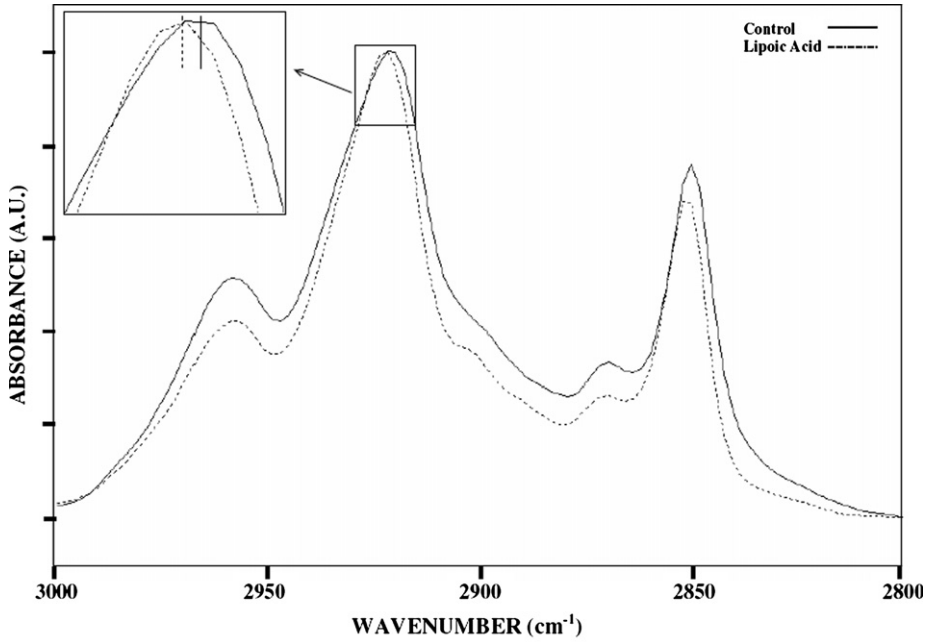

Fig. 4. Deconvoluted FTIR spectra of the lyophilized control (solid line) and lipoic acid (dotted line) treated rat brain samples in the 3000–2800 cm^{-1} range. The spectra were normalized with respect to the band at 2922 cm^{-1}.

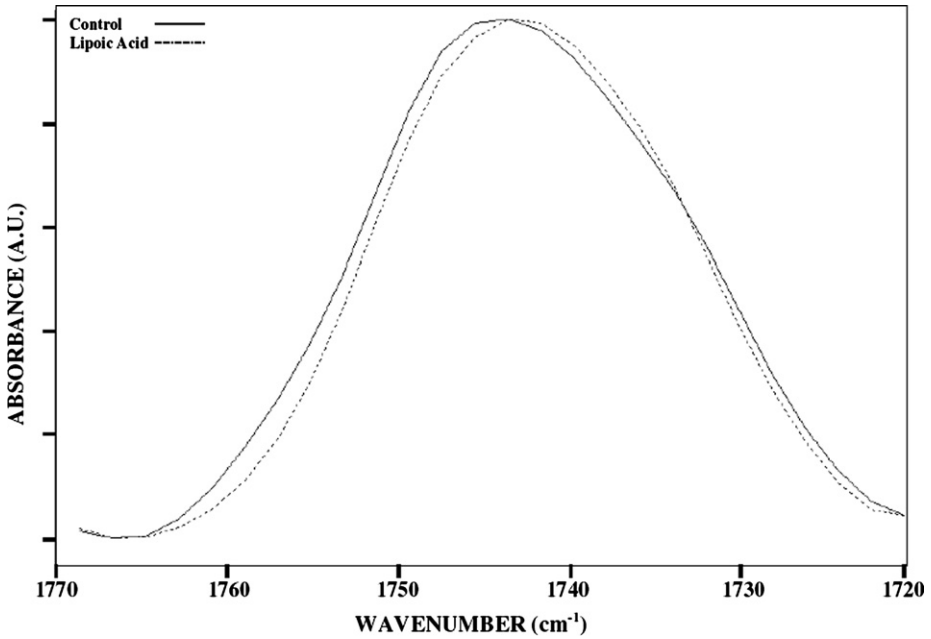

Fig. 5. FTIR spectra of the lyophilized control (solid line) and lipoic acid (dotted line) treated rat brain samples in the 1770–1720 cm^{-1} range. The spectra were normalized with respect to the band at 1739 cm^{-1}.

antioxidant lies in its distinct advantage of being easily transported to the brain by readily passing the blood brain barrier (Packer et al., 1997; Seaton, Jenner, & Marsden, 1996). Lipoic acid was given alone in the absence of any condition inducing oxidative stress, in order to gain insight on the molecular mode of interaction of lipoic acid with biological molecules. The dose used (12 mg/kg) in the present study was chosen because it was in the range of the therapeutic doses used in previous studies (200–1800 mg/day in humans) (Packer et al., 2001; Wollin & Jones, 2003).

In the present study, special care was given in order to prepare the pellets at the same thickness by taking the same amount of sample and applying the same pressure. Furthermore, the spectra of each sample were collected in triplicates, finally to observe that they were identical. Therefore, in the present study it is possible to directly relate the intensity and/or more accurately the area of the absorption bands to the concentration of the corresponding functional groups (Cakmak et al., 2006; Dogan et al., 2006; Kačuráková & Wilson, 2001).

The band viewed at about 3304 cm^{-1} is defined as the amide A band mainly due to N–H stretching of proteins with negligible contribution from O–H stretching of intermolecular hydrogen bonding since unbound water was removed from the system (Cakmak et al., 2006; Jung, 2000). The significant increase in the peak area of the amide A band set off by lipoic acid (Fig. 3) means that it led to an increase in the protein quantity of the system. This could be a supportive sign of the protective effect of lipoic acid, since it was previously suggested that free radical damage can cause a reduction in protein synthesis (Makrides, 1983). Such an antioxidative action of lipoic acid is further supported by the observation that lipoic acid treatment increased the protein content that was considerably lower in the brain of aged rats (Arivazhagan et al., 2002).

It is known that the CH_2 antisymmetric (2922 cm^{-1}) and symmetric (2851 cm^{-1}) stretching vibrations give valuable information about the state-of-order of the hydrocarbon tails in lipids because they are good monitors of the changes in acyl chains (Mendelsohn & Mantsch, 1986; Toyran et al., 2004). The significant increase in the frequencies ($p < 0.05^{*}$) of these bands with lipoic acid treatment (Fig. 4) indicates that lipoic acid functions by disordering the lipid system by increasing the number of methylene gauche conformers.

The C=O stretching band (1739 cm^{-1}) is strongly associated with lipids implying that any shift in the frequency of this band can be directly correlated with an alteration in the state of intramolecular hydrogen bonding of the interfacial region of the phospholipid structure with water molecules and/or some functional groups of other molecules (Takahashi et al., 1991). In the current study, it has been observed that lipoic acid treatment caused a significant decrease ($p < 0.01^{**}$) in the frequency of the C=O stretching band (Fig. 5), indicating a lipoic acid-induced strengthening in the hydrogen bonding of the carbonyl groups of phospholipids. Both the CH_2 and C=O stretching bands seem to become more symmetric in the lipoic acid treated samples. Especially the shoulders of the C=O band seem to disappear. Such a loss of several distinct environments could support the interpretation of decreased ordering observed in the CH_2 stretching bands.

There are various techniques, such as curve fitting, partial least squares analysis, factor analysis, and neural networks (NN), found in the literature to predict the secondary structure of proteins from their infrared spectra. In this work, a neural network technique that is able to

provide predictions better than the previous results was used (Severcan et al., 2004). These NN predictions based on FTIR data presented that lipoic acid induced slight but not significant alterations in the secondary structure of the proteins of the system (Table 2).

It is possible that the hydrophobic part of lipoic acid enters the non-polar part of the membrane, weakens the van der Waals interactions between the acyl chains, and consequently leads to a disordering in the system by increasing the number of methylene gauche conformers (Fig. 1). In other words, the decrease in the van der Waals interactions may be the reason of the disordering effect of lipoic acid observed in the C–H stretching region of lipids. Moreover, the decrease in frequency observed in the C=O stretching vibration (1739 cm^{-1}), might indicate that the O–H group of lipoic acid interacts with phospholipids by hydrogen bonding with the carbonyl groups, and without interacting with the phosphate groups.

The results of the current study have provided insight on the lipoic acid–membrane interaction and suggested for the first time that lipoic acid induces a disordering effect on the state-of-order of lipids. From a physiological aspect, this disordering induced by lipoic acid might be advantageous because the interaction of antioxidants with lipid radicals is more efficient when membrane lipids are more disordered. Moreover, it is worth emphasis that macromolecular characteristics and lipid order/disorder are fundamental factors related with several physiological disorders in an indirect manner because they are strongly correlated with ion channel kinetics and functioning (Awayda, Shao, Guo, Zeidel, & Hill, 2004). In addition, the unaltered protein secondary structure and nucleic acid structure (data not shown) further supports the usage of lipoic acid as an antioxidant supplement. However, despite the numerous studies on lipoic acid, there is no consensus on the optimum dosage, dose frequency, and form of administration of lipoic acid (Wollin & Jones, 2003). Therefore, it should always be kept in mind that optimal care should be taken when using the commercially available lipoic acid supplements and the unwise use of supplements should be avoided.

In addition, this study also clearly demonstrated that FTIR spectroscopy, which can be a rapid and sensitive tool for studying biomolecules simultaneously, reveals even subtle variations in spectral parameters (Fukuyama et al., 1999; Toyran et al., 2006, 2004).

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