

## **α-Lipoic acid has anti-inflammatory and anti-oxidative properties: an experimental study in rats with carrageenan-induced acute and cotton pellet-induced chronic inflammations**

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### **Abstract**

α-Lipoic acid (ALA) has been termed the ‘ideal’ antioxidant, a readily absorbed and bioavailable compound capable of scavenging a number of free radicals, and it has been used for treating diseases in which oxidative stress plays a major role. The present study was designed to gain a better understanding for the positive effects of ALA on the models of acute and chronic inflammation in rats, and also determine its anti-oxidative potency. In an acute model, three doses of ALA (50, 100 and 200 mg/kg) and one dose of indomethacin (25 mg/kg) or diclofenac (25 mg/kg) were administered to rats by oral administration. The paw volumes of the animals were calculated plethysmometrically, and 0·1 ml of 1% carrageenan (CAR) was injected into the hind paw of each animal 1 h after oral drug administration. The change in paw volume was detected as five replicates every 60 min by plethysmometry. In particular, we investigated the activities of catalase, superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), inducible NO synthase (iNOS) and myeloperoxidase (MPx), and the amounts of lipid peroxidation (LPO) or total GSH in the paw tissues of CAR-injected rats. We showed that ALA exhibited anti-inflammatory effects on both acute and chronic inflammations, and a strongly anti-oxidative potency on linoleic acid oxidation. Moreover, the administration of CAR induced oedema in the paws. ALA significantly inhibited the ability of CAR to induce: (1) the degree of acute inflammation, (2) the rise in MPx activity, (3) the increases of GST and iNOS activities and the amount of LPO and (4) the decreases of GPx, GR and SOD activities and the amount of GSH. In conclusion, these results suggest that the anti-inflammatory properties of ALA, which has a strong anti-oxidative potency, could be related to its positive effects on the antioxidant system in a variety of tissues in rats.

**Key words:** α-Lipoic acid; Cotton pellets; Chronic inflammation; Carrageenan; Acute inflammation; Myeloperoxidase; Antioxidant enzymes; Inducible nitric oxide synthase

Inflammatory diseases such as rheumatoid arthritis, asthma and hepatitis are the major causes of morbidity in humans. Today, it is well known that chronic inflammation also leads to the development of cancer, CVD and neurodegenerative diseases. Oedema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability and/or the mediators

that increase blood flow. Several experimental models of paw oedema have been described. Carrageenan (CAR)-induced paw oedema is widely used for determining the acute phase of inflammation. Histamine, 5-hydroxytryptamine and bradykinin are the first detectable mediators in the early phase of CAR-induced inflammation, whereas PG are detectable in the late phase of inflammation<sup>(1–5)</sup>.

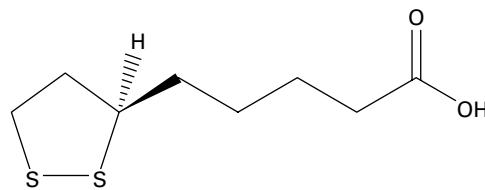
**Abbreviations:** ALA, α-lipoic acid; CAR, carrageenan; CAT, catalase; DIC, diclofenac; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; IND, indomethacin; iNOS, inducible nitric oxide synthase; LPO, lipid peroxidation; MPx, myeloperoxidase; NOS, nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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It is well known that the acute inflammatory process, in which vascular permeability increases and leucocyte migration occurs, involves inflammation mediators including neutrophil-derived active oxygen species and free radicals, such as  $H_2O_2$  and superoxide, and the hydroxyl radicals, such as NO, PG and cytokines<sup>(3–5)</sup>. Polymorphonuclear leucocytes that are the first cells to arrive at the inflammatory site in the body release free oxygen radicals and free hydroxyl radicals<sup>(3–7)</sup>. On the other hand, in many physiological functions of the body and the pathogenesis of inflammation, ion exchanges between intracellular and extracellular areas have been identified. It has been reported that increased intracellular Ca ion stimulates inflammation events and Ca channel blocker drugs diminish these events<sup>(3–7)</sup>. However, the chronic inflammation model, induced by subcutaneous implantation of foreign bodies, is used commonly in experiments<sup>(3,5,8,9)</sup>. Chronic inflammation starts with the occurrence of proliferative cells<sup>(9)</sup>. These cells can be spread or can form a granuloma tissue. Inflammatory granuloma tissue is a typical example of chronic inflammation. On the other hand, granuloma formation is initialised by an antigen (for example, a cotton pellet). The cotton pellet stimulates the immune system, antibodies and production of IL. This stimulation results with the proliferation of lymphocytes and the formation of granulation tissue over the pellets<sup>(8,10)</sup>. Non-steroidal anti-inflammatory drugs and ulcers reduce granuloma tissue that is formed with cellular reactions by inhibiting granulocyte infiltration, while glucocorticoids do it by inhibiting fibrosis and macrophage function<sup>(5,8–10)</sup>.

Reactive oxygen species (ROS) including free radicals such as hydroxyl radicals ( $OH^-$ ) or superoxide anions ( $O_2^-$ ) and non-free radical species such as  $H_2O_2$  constantly cause lipid peroxidation (LPO) in tissues by attacking unsaturated fatty acids. Oxidative stress also plays an important role in the pathogenesis of many other diseases such as respiratory distress syndrome, rheumatoid arthritis, local or systemic inflammatory disorders, sepsis, diabetes, atherosclerosis and cancer<sup>(2,10–18)</sup>. On the other hand, aerobic organisms possess antioxidant defence systems including enzymatic and non-enzymatic antioxidants to combat the toxic effects of ROS. These antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), GSH, glutathione reductase (GR), tocopherols, vitamin C,  $\beta$ -carotene, vitamin A and flavonoids. Myeloperoxidase (MPx), found in azurophilic granules of mammalian neutrophils and in human monocytes, is involved in microbial killing and inflammatory tissue damage, and neutrophil infiltration into gastric mucosal tissues is a critical process in the pathogenesis of gastric lesions induced by non-steroidal anti-inflammatory drugs and ulcers<sup>(8,19)</sup>. NO, enzymatically produced from NO synthase (NOS), first discovered in the vascular endothelium, plays many important roles in health and diseases. NOS is present in several isoforms in a large number of cells besides endothelial cells, such as the constitutive



**Fig. 1.** Chemical structure of  $\alpha$ -lipoic acid.

endothelial NOS and neuronal NOS under physiological conditions and inducible NO synthase (iNOS), which is induced by cytokines under pathophysiological conditions<sup>(4,11,16–18)</sup>.

$\alpha$ -Lipoic acid (ALA, Fig. 1) is a naturally occurring essential co-enzyme in mitochondrial multienzyme complexes catalysing the oxidative decarboxylation of  $\alpha$ -keto acids such as pyruvate,  $\alpha$ -ketoglutarate and branched-chain  $\alpha$ -keto acid<sup>(20–22)</sup>. ALA has been shown to combat oxidative stress by quenching a variety of intracellular ROS<sup>(21,22)</sup>. In addition to ROS scavenging, ALA has also been shown to be involved in the recycling of other cellular antioxidants including vitamins C and E, and GSH<sup>(23)</sup>. ALA has been demonstrated to be effective in preventing pathology in various experimental models in which ROS have been implicated, such as ischaemia–reperfusion injury<sup>(15)</sup>, diabetes<sup>(24)</sup>, hypertension<sup>(25)</sup>, radiation injury<sup>(26)</sup> and HIV activation<sup>(27)</sup>. On the other hand, in cancer cells, ROS also play a crucial role in cell growth and apoptosis regulation. ALA and its reduced form, dihydrolipoic acid, have been shown to inhibit proliferation and induce apoptosis of several cancer and transformed cell lines, while being less active towards normal non-transformed cells<sup>(28)</sup>.

In the present study, we aimed to evaluate: (a) the reducing effects of ALA on acute (CAR induced) and chronic phases (cotton pellet granule) of inflammation, (b) the changes of LPO and antioxidant levels (SOD, GSH, CAT, glutathione S-transferase (GST), GPx and GR), and MPx and iNOS activities, as markers of acute inflammation, in paw tissues of rats and (c) *in vitro* antioxidant potency of ALA.

## Materials and methods

### Animals

A total of seventy-eight male Wistar rats, weighing 180–190 g, were provided from the Experimental Animal Laboratory of Ataturk University, the Experimental Animal Teaching and Research Center. The animals were housed in groups of five or six per cage for at least 5 d under controlled conditions of constant temperature/humidity and exposed to a 12 h light–dark cycle<sup>(29)</sup>. Animals for acute inflammation experiments were assigned randomly to the control groups receiving either CAR (0.1 ml of 1% CAR per animal) or water and to the experimental groups receiving CAR plus ALA at doses of 50, 100 and 200 mg/kg, indomethacin (IND) at a dose of 25 mg/kg

and diclofenac (DIC) at a dose of 25 mg/kg. Besides, the animals were grouped similarly to evaluate chronic inflammation via the cotton pellet-induced granuloma test. The experiments were conducted according to the ethical norms approved by the Ethics Committee of the Experimental Animal Teaching and Research Center (no: B.30.2.ATA.0.70/144-4).

### Chemicals

All chemicals for laboratory experiments were purchased from Sigma Chemical (Munich, Germany). Other compounds were obtained from various sources: ALA was obtained from Life Time (Istanbul, Turkey), DIC (Myadren 50 mg Tablet) was obtained from Fako (Istanbul, Turkey) and IND (Endosetin capsule) was obtained from Nobel (Istanbul, Turkey).

### Carrageenan-induced paw oedema in rats

In this type of experiment, the effects of ALA and positive controls (IND and DIC) on CAR-induced paw oedema in rats were investigated<sup>(8)</sup>. The animals were divided into seven groups, each consisting of six rats (Table 1 and Fig. 2). ALA, CAR, IND and DIC solutions were prepared by dissolving in distilled water. Briefly, three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) and DIC (25 mg/kg) were administered orally to rats with the aid of gavages. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after the last dose. The change in paw volume was detected as five replicates every 60 min by plethysmometry. The anti-inflammatory potency of ALA was determined via comparison with the result obtained from the animals receiving equal volumes of positive controls (IND and DIC), CAR (control) and distilled water (negative

control). The last group of animals (seventh group) was used for antioxidant enzyme research and is called the healthy group (no drug or CAR administration). The anti-inflammatory effects of these drugs were calculated by the following equation:

$$\text{Anti-inflammatory activity (inhibition \%)} = (1 - D/C) \times 100,$$

where  $D$  represents the percentage difference in paw volume after the administration of drugs to the rats, and  $C$  represents the percentage difference of volume in the control groups.

### Biochemical analysis of carrageenan-induced paw oedema in rats

**Preparation of tissues homogenates.** After the plethysmometric analyses, the activities of CAT, SOD, GST, GPx, GR, iNOS and MPx, and the amounts of LPO or total GSH in paw tissues of rat oedema were determined. To prepare the tissue homogenates, paw oedema tissues of each rat groups ( $n=6$ ) were ground with liquid N<sub>2</sub> in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 ml of an appropriate buffer. The mixtures were homogenised on ice using an ultra-turrax homogeniser for 15 min. Homogenates were filtered and centrifuged by using a refrigerated centrifuge at 4°C. Then, these supernatants were used for the determination of the enzymatic activities. All the assays were carried out at room temperature as three parallel measurements.

**Glutathione S-transferase activity.** Total GST activity was determined as described by Habig & Jakoby<sup>(30)</sup>. Briefly, the enzyme activity was assayed spectrophotometrically at 340 nm in a 4 ml cuvette containing 0.1 M-PBS (pH 6.5), 30 mM-GSH, 30 mM-1-chloro-2,6-dinitrobenzene and tissue homogenate. Enzyme activity was expressed as nmol/min per mg protein.

**Table 1.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on carrageenan (CAR)-induced paw oedema (fourth hour) in rats† (Mean values with their standard errors,  $n=6$ )

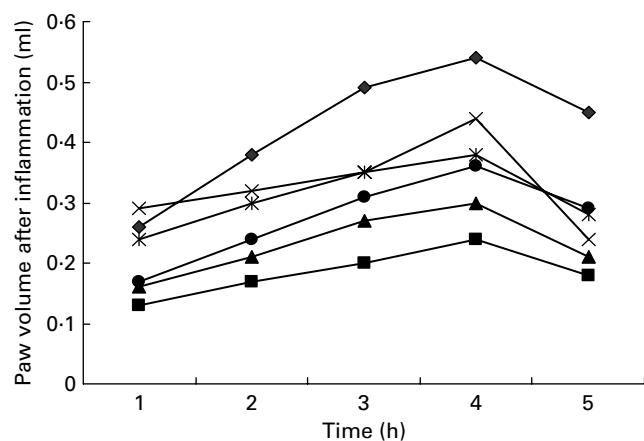
Treatment	<i>n</i>	Dose (mg/kg body wt)	Paw volume before inflammation (ml)	Difference between paw volumes (ml) at fourth hour		Anti-inflammatory effect (inhibition %)‡
				Mean	SEM	
CAR + ALA	6	50	1.08	0.44	0.08	18.5
CAR + ALA	6	100	1.1	0.38*	0.01	29.6
CAR + ALA	6	200	1.09	0.32**	0.03	40.7
CAR + DIC	6	25	1.02	0.30**	0.02	44.4
CAR + IND	6	25	1.09	0.24**	0.02	55.5
Control (CAR)	6	—	1.04	0.54	0.05	—

*n*, Number of animals.

Mean values were significantly different when compared to that of the control group: \* $P<0.05$ , \*\* $P<0.01$ .

† Three doses of ALA (50, 100 and 200 mg/kg), and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. Three doses of the ALA-treated group and one dose of the IND- and DIC-treated groups were compared with the CAR group. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration.

‡ Inhibition % in paw volumes in relation to control (carrageenan) group.



**Fig. 2.** Effects of three doses of  $\alpha$ -lipoic acid (ALA) and one dose of indomethacin (IND) or diclofenac (DIC) on carrageenan (CAR)-injected rats, with time-dependent increase in the rat's paw volume after CAR injection. Three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration. —◆—, CAR; —■—, CAR + IND; —▲—, CAR + DIC; —×—, CAR + ALA (50 mg/kg); —\*—, CAR + ALA (100 mg/kg); —●—, CAR + ALA (200 mg/kg).

**Glutathione peroxidase activity.** GPx activity was determined according to a previously reported method<sup>(31)</sup>. The absorbance at 340 nm was recorded for 5 min. The activity was determined by measuring the amount of oxidised NADPH as  $\mu\text{mol}/\text{min}$  per mg tissue.

**Glutathione reductase activity.** GR activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm<sup>(32)</sup>. Results are expressed as the amount of enzymes required to catalyse the oxidation of 1  $\mu\text{mol}$  of NADPH/min per mg of tissue.

**Catalase activity.** Decomposition of  $\text{H}_2\text{O}_2$  in the presence of a CAT was measured at 240 nm<sup>(33)</sup>. CAT activity was defined as the amount of enzymes required to decompose 1 nmol of  $\text{H}_2\text{O}_2/\text{min}$ , at 25°C and pH 7.8. Results are expressed as mmol/min per mg of tissue.

**Superoxide dismutase activity.** As outlined by Sun *et al.*<sup>(34)</sup>, SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with nitro blue tetrazolium to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction and is expressed as mmol/min per mg of tissue.

**Inducible nitric oxide synthase activity.** NOS activity was measured spectrophotometrically using the oxidation of oxyhaemoglobin to methaemoglobin by NO as described previously<sup>(35,36)</sup>. The absorption difference between 401 and 421 nm was continuously monitored with a dual wavelength recording spectrophotometer (Thermo Spectronic-HEMOS  $\beta$ , Cambridge, UK) at 37°C. For total NOS assay, the incubation medium contained 1.6  $\mu\text{M}$ -oxyhaemoglobin, 200  $\mu\text{M}$ -CaCl<sub>2</sub>, 1 mM-MgCl<sub>2</sub>, 100  $\mu\text{M}$ -L-arginine,

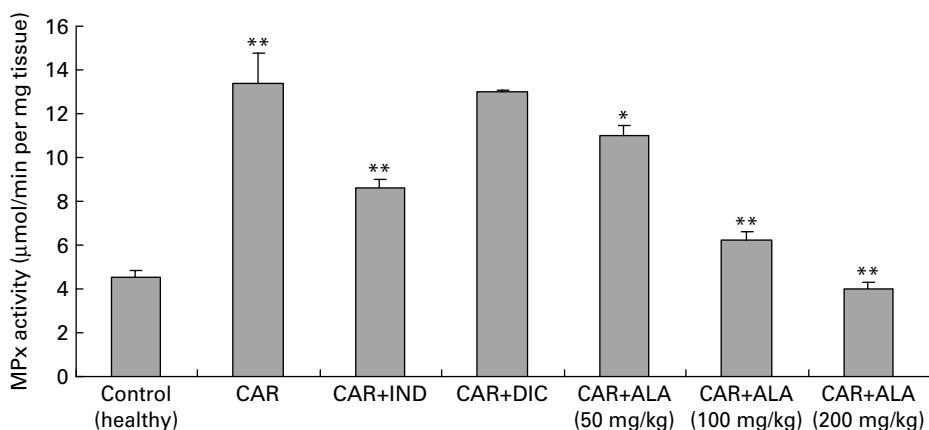
100  $\mu\text{M}$ -NADPH, 40 mM-potassium phosphate (pH 7.2), 1 mM-NG-nitro-L-arginine and 10% (v/v) tissue extract with 50 mM-L-valine to inhibit arginase. For the cNOS (constitutive) assay, 1 mM-glycol diethyl diamine tetra-acetic acid was added to the above-mentioned incubation medium without NG-nitro-L-arginine. Oxyhaemoglobin oxidation was confirmed as being caused by NO synthesis. iNOS activity was calculated by subtracting the cNOS activity from the total NOS activity.

**Myeloperoxidase activity.** MPx activity was measured according to the modified method of Bradley *et al.*<sup>(37)</sup>. The homogenised samples were frozen and thawed three times, and centrifuged at 1500 g for 10 min at 4°C. MPx activity in the supernatant was determined by adding 100 ml of the supernatant to 1.9 ml of a 10 mmol/l phosphate buffer (pH 6.0) and 1 ml of 1.5 mmol/l o-dianisidine dihydrochloride containing 0.0005% (w/v)  $\text{H}_2\text{O}_2$ . The changes in absorbance at 450 nm of each sample were recorded on a UV-visible spectrophotometer (Fig. 3). MPx activity in tissues was expressed as  $\mu\text{mol}/\text{min}$  per mg tissue.

**Total GSH determination.** The amount of GSH in the paw oedema was measured according to the method of Sedlak & Lindsay<sup>(38)</sup>. The paw oedema was collected by scraping, then it was weighed and homogenised in 2 ml of a 50 mM-Tris-HCl buffer containing 20 mM-EDTA and 0.2 M-sucrose at a pH of 7.5. The homogenate was centrifuged. After centrifugation at 4200 rpm for 40 min at 4°C, the supernatant was used to determine GSH using 5,5'-dithiobis(2-nitrobenzoic acid). Absorbance was measured at 412 nm using a spectrophotometer. The results of the GSH level in the paws were expressed as nmol/mg tissue.

#### Determination of lipid peroxidation or malonaldehyde formation

The levels of LPO were determined by estimating malonaldehyde using the thiobarbituric acid test<sup>(39)</sup>. Briefly, the rat stomachs were promptly excised and rinsed with cold saline. To minimise the possibility of interference of Hb with free radicals, any blood adhering to the mucosa was carefully removed. The paw tissues were scraped, weighed and homogenised in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added with a solution containing 0.2 ml of 80 g/l sodium laurylsulphate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 ml of *n*-butanol-pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The supernatant was measured at 532 nm. The standard curve was obtained using 1,1,3,3-tetramethoxypropane. The recovery was over 90%. All the samples were measured in duplicate. The results were expressed as nmol malonaldehyde/g of wet tissue.



**Fig. 3.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in the activity of myeloperoxidase (MPx) enzyme in carrageenan (CAR)-injected rat paws (fifth hour). Three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration. (The same experiment has been performed three times, and then data determined are expressed as means with standard errors of three parallel measurements. Three doses of ALA-treated group and one dose of IND- and DIC-treated groups were compared with the CAR group. The CAR group was compared with the healthy group.) Mean values were significantly different: \* $P<0.05$ , \*\* $P<0.01$ .

#### Cotton pellet-induced granuloma test in rats

In this series of experiments, the effect of ALA on the proliferative phase of inflammation was investigated using the cotton pellet test<sup>(8)</sup>. Animals were divided into six groups each consisting of six rats (Table 2). Three groups of animals received ALA at doses of 50, 100 and 200 mg/kg body weight (wt) with the aids of gavages, while the other groups of animals received DIC at a dose of 25 mg/kg body wt and IND at a dose of 25 mg/kg body wt orally, and the control group received an equal volume of distilled water. Thirty minutes after administration of the drugs, the animals were anaesthetised using 25 mg/kg body wt sodium thiopental. Under sterilised conditions, cotton pellets of 7 (SEM 1) mg were implanted subcutaneously in the interscapular area. ALA, DIC and IND were administered once a day for 7 d at the doses

indicated earlier. On the eighth day, the animals were killed via high-dose anaesthesia, and the cotton pellets with the granuloma tissue about them were removed. The wet weights of these pellets were measured, and the anti-proliferative effects of ALA, DIC and IND were determined by comparison with the control group.

#### Total antioxidant activity assays

The total antioxidant activities of ALA were determined by the thiocyanate method<sup>(3,40)</sup> and compared with known antioxidants, trolox and ascorbic acid. Briefly, each sample (1 mg) in 1 ml distilled water was mixed with 5 ml linoleic acid emulsion (0.02 M, pH 7.0) and 5 ml of the phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.5608 g of linoleic acid

**Table 2.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on cotton pellet-induced granuloma test in rats†

(Mean values with their standard errors)

Treatment	n	Dose (mg/kg body wt)	Cotton pellet weight (mg)		Granuloma (wt/mg)		Anti-proliferative effect (%)‡
			Mean	SEM	Mean	SEM	
ALA	6	50	7	1	146**	3.8	67.7
ALA	6	100	7	1	141**	3.8	68.9
ALA	6	200	7	1	133**	4.1	70.6
DIC	6	25	7	1	108**	4.9	76.1
IND	6	25	7	1	73**	5.71	83.8
Control	6	—	7	1	452	9.6	—

n, Number of animals.

Mean values were significantly different when compared with that of the control group: \*\* $P<0.01$ .

† Three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. At 30 min after administration of the drugs, cotton pellets were implanted subcutaneously in the interscapular area under anaesthesia. ALA, DIC and IND were administered once a day for 7 d. On the eighth day, the cotton pellets with the granuloma tissue about them were removed. Three doses of the ALA-treated group, and one dose of the IND- or DIC-treated groups were compared with the control group.

‡ Inhibition in granuloma weights in relation to the control (untreated) group.

with 0.5608 g of Tween-20 as an emulsifier and 100 ml of the phosphate buffer, and then the mixture was homogenised. The reaction mixture was incubated at 37°C. Aliquots of 0.1 ml were taken at different intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding 4.7 ml ethanol (75%), 0.1 ml ammonium thiocyanate (30%), 0.1 ml sample solution and 0.1 ml ferrous chloride (0.02 M, in 3.5% HCl). The mixture was allowed to stand for 3 min and then the peroxide value was determined by reading the absorbance at 500 nm using the UV-visible spectrophotometer (Thermo Spectronic-HELIOS β). A control was performed with linoleic acid but without the extracts. Inhibition % was calculated using the following formula:

$$\text{Inhibition \%} = (1 - (\text{absorbance of sample at } 500 \text{ nm} / \text{absorbance of control at } 500 \text{ nm})) \times 100.$$

Trolox and ascorbic acid solutions prepared in the conditions mentioned earlier were used as positive controls.

### Statistical analyses

Data of enzyme activities and inflammation were subjected to one-way ANOVA, with the presence of negative and positive controls, by using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). Differences among the groups were attained using the least significant difference option, and significance was declared at  $P < 0.05$ , 0.01 and 0.005.

## Results

### *Carageenan-induced paw oedema*

Intraplantar injection of CAR in rats led to a quadratic and time-dependent increase in paw volume (Fig. 2).

**Table 3.** Effects of α-lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in the activities of some glutathione metabolism enzymes (glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR)) in CAR-injected paws (fifth hour) of rats† (Mean values with their standard errors of three parallel measurements)

Treatment‡	Dose (mg/kg body wt)	GST activity (nmol/min per mg tissue)		GPx activity (μmol/min per mg tissue)		GR activity (μmol/min per mg tissue)	
		Mean	SEM	Mean	SEM	Mean	SEM
CAR + ALA	50	15.12	0.35	21.49	0.07	19.07	0.26
CAR + ALA	100	14.53*	0.56	22.65*	0.49	18.61	0.31
CAR + ALA	200	12.90**	0.19	23.67**	0.45	18.40	0.15
CAR + DIC	25	14.47*	0.08	22.84*	0.19	20.17*	0.37
CAR + IND	25	17.74**	0.43	23.22**	0.47	20.79*	0.13
CAR	–	15.74**	0.51	21.44	0.12	18.8**	0.05
Control (healthy)	–	13.90	0.11	22.33	0.63	29.31	0.26

n, Number of animals.

Mean values were significantly different when compared to that of control group: \* $P < 0.05$ , \*\* $P < 0.01$ .

† Three doses of ALA (50, 100 and 200 mg/kg), and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. Three doses of the ALA-treated group, and one dose of the IND- or DIC-treated groups were compared with the CAR group. The CAR group was compared with the healthy group. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration.

‡ The same experiment was performed three times and then data determined are expressed as means with their standard errors of three parallel measurements.

The increase in paw volume was observed at 1 h and was maximal at 4 h after the administration. However, CAR-induced paw oedema was reduced in a dose-dependent manner by treatment with ALA at 1, 2, 3, 4 and 5 h after an injection of CAR (Fig. 2). At doses of 100 and 200 mg/kg, ALA significantly ( $P < 0.05$  and 0.01) reduced CAR-induced paw oedema by 29.6 and 40.7%, respectively, at the fourth hour (Table 1). At doses of 25 mg/kg, treating DIC and IND also significantly reduced the oedema by 44.4 and 55.5%, respectively. The paw volume increased by 0.54 ml in the control group relative to the baseline values, while increases of 0.44, 0.38, 0.32, 0.24 and 0.30 ml were observed in the groups administered with 50, 100 and 200 mg/kg of ALA, IND and DIC, respectively. The present results show that IND, DIC, and 100 and 200 mg/kg of ALA (Fig. 2) had significant anti-inflammatory effects against the paw oedema caused by CAR. The anti-inflammatory effect of ALA was also weaker than that of DIC and IND, which inhibit PG synthesis by cyclo-oxygenase.

### Comparison of enzymatic activities and lipid peroxidation or GSH levels in rat paw tissues

In order to explore the effects of antioxidant defences on the inflammation process in all paw tissues, the activities of antioxidant enzymes (GST, GPx, GR SOD and CAT) and the amounts of GSH and LPO were evaluated. The results are presented in Tables 3 and 4 with Figs 4 and 5. Table 3 shows that the activities of GPx, GR and SOD for CAR-injected groups were lower, and GST and CAT activities were higher than those for the healthy rat group. However, compared with GST, GPx and SOD levels in ALA-administered paw tissues, the opposite results were found for the activities of these enzymes in CAR-injected tissues. In contrast to CAR-injected paws,

**Table 4.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in the activity of catalase (CAT) and superoxide dismutase (SOD) enzymes in CAR-injected paws (fifth hour) of rats† (Mean values with their standard errors of three parallel measurements)

Treatment‡	Dose (mg/kg body wt)	CAT activity (mmol/min per mg tissue)		SOD activity (mmol/min per mg tissue)	
		Mean	SEM	Mean	SEM
CAR + ALA	50	133.0**	2.9	125.4*	0.33
CAR + ALA	100	166.0**	2.9	129.1*	0.34
CAR + ALA	200	194.0**	3.2	136.2**	0.75
CAR + DIC	25	88.4**	1.2	128.6*	0.61
CAR + IND	25	91.5**	1.5	119.8	0.68
CAR	—	117.6**	1.5	121.1**	1.07
Control (healthy)	—	91.0	1.1	132.3	0.72

n, Number of animals.

Mean values were significantly different when compared to that of the control group: \*P<0.05, \*\*P<0.01.

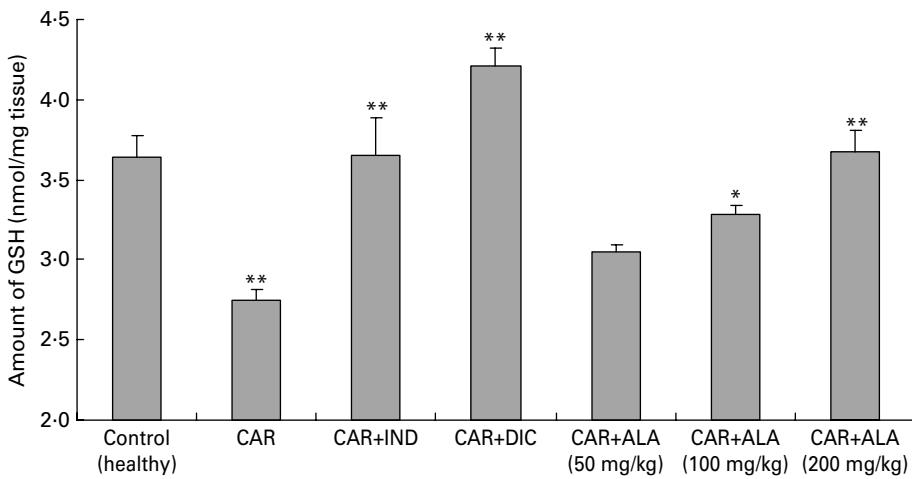
† Three doses of ALA (50, 100 and 200 mg/kg), and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. Three doses of the ALA-treated group and one dose of the IND- or DIC-treated groups were compared with the CAR group. The CAR group was compared with the healthy group. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration.

‡ The same experiment was performed three times.

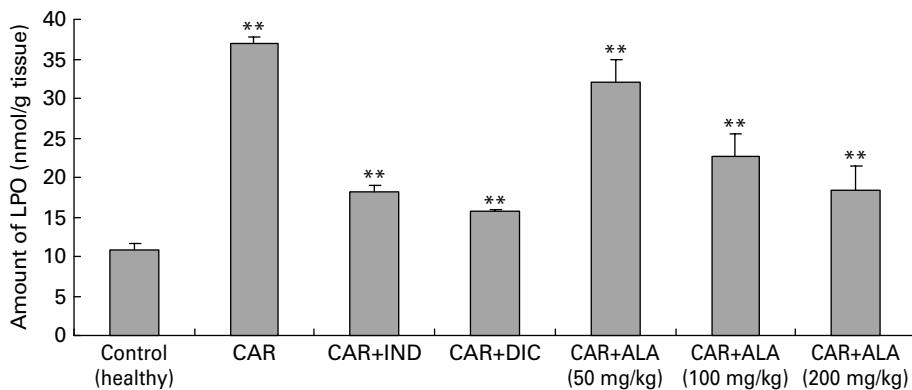
DIC and IND increased the activities of GPx and GR ( $P<0.05$ ) and decreased CAT activity ( $P<0.05$ ). Table 3 shows that the activity of GST was decreased by the administration of 50 and 100 mg ALA per kg ( $P<0.05$ ), while the activity of GR was reduced during CAR oedema and this reduction was not affected by all the doses of ALA ( $P>0.05$ ). Similarly, DIC decreased GST activity but increased GR activity ( $P<0.05$ ). However, IND increased both the activity of GST elevated by CAR further and the activity of GR alleviated by CAR. The activity of GPx was 22.33 in healthy paws. This score was decreased by CAR

to 21.44 ( $P>0.05$ ), but it was increased by 50 and 100 mg/kg doses of ALA ( $P<0.05$  and  $P<0.01$ ), DIC ( $P<0.05$ ) and IND ( $P>0.01$ ). However, compared with the activities of the CAT and SOD enzymes in ALA-, IND- and DIC-administered paw tissues, different results were found. The injected CAR increased CAT activity, while decreased SOD activity in paw tissues. In contrast to CAR-injected paws, all the doses of ALA and DIC increased SOD activity ( $P<0.05$ ), while DIC and IND decreased CAT activity ( $P<0.05$ ). On the other hand, CAT activity was elevated significantly ( $P<0.01$ ) by all the doses of ALA further (Table 4). Likewise, both IND with DIC and all the doses of ALA had an increasing effect ( $P<0.05$ ) on the level of GSH alleviated by CAR (Fig. 4), and in contrast to CAR-injected paws (Fig. 5), all the doses of ALA and DIC decreased the amount of LPO ( $P<0.01$ ).

In the present study, changes in MPx activity of paw tissues were widely used as an index of neutrophil infiltration in gastric and inflammatory tissue damage. MPx activity was determined by using an appropriate method. It is observed from Fig. 3 that the administration of CAR significantly ( $P<0.01$ ) increased the MPx activity. In contrast to CAR injection, the administration of the 50 ( $P<0.05$ ), 100 and 200 ( $P<0.01$ ) mg/kg doses of ALA and 25 mg/kg dose of IND reduced the level of MPx; however, DIC did not indicate any effect. Separately, iNOS activity in the rat paw tissues was also given in the Table 5. The intraplantar injection of CAR increased significantly iNOS activities ( $P<0.01$ ) in paw homogenates at the fifth hour. The activity of iNOS was 0.14 in healthy paws. This score was elevated by CAR to 0.36 ( $P<0.05$ ), but it was alleviated by CAR + DIC, CAR + IND and CAR + ALA (50, 100 and 200 mg/kg doses) to 0.18, 0.22, 0.17, 0.13 and 0.11, respectively ( $P<0.01$ ).



**Fig. 4.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in level the of GSH in carrageenan (CAR)-injected rat paws (fifth hour). Three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration. (The same experiment has been performed three times and then data determined are expressed as means with standard errors of three parallel measurements. Three doses of the ALA-treated group, and one dose of the IND- and DIC-treated groups were compared with the CAR group. The CAR group was compared with the healthy group.) Mean values were significantly different: \*P<0.05, \*\*P<0.01.



**Fig. 5.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in the amount of lipid peroxidation (LPO) in carrageenan (CAR)-injected rat paws (fifth hour). Three doses of ALA (50, 100 and 200 mg/kg) and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration. (The same experiment has been performed three times and then the data determined are expressed as means with standard errors of three parallel measurements. Three doses of the ALA-treated group, and one dose of the IND- and DIC-treated groups were compared with the CAR group. The CAR group was compared with the healthy group.) Mean values were significantly different: \*\* $P < 0.01$ .

#### Cotton pellet-induced granuloma test

As shown in Table 2, the mean weight of the cotton pellets in the ALA (50, 100 and 200 mg/kg body wt) groups was 146 (SEM 3.8), 141 (SEM 3.8) and 136 (SEM 4.1) mg, respectively, while it was 108 (SEM 4.9) and 73 (SEM 5.71) mg in the DIC (25 mg/kg body wt) and IND (25 mg/kg body wt), respectively. According to these results, the anti-proliferative effect of ALA (50, 100 and 200 mg/kg body wt) groups was shown to be 67.7, 68.9 and 70.6%, respectively, and of DIC was 76.1% and of IND was an 83.8% response. The present results show that ALA in all the doses also has significant ( $P < 0.01$ ) anti-inflammatory effects on the chronic inflammation process.

**Table 5.** Effects of  $\alpha$ -lipoic acid (ALA), indomethacin (IND) and diclofenac (DIC) on changes in the activity of inducible nitric oxide synthase (iNOS) enzyme in CAR-injected paws (fifth hour) of rats  
(Mean values with their standard errors of three parallel measurements)

Treatment‡	Dose (mg/kg body wt)	iNOS (nmol/min per mg tissue)	
		Mean	SEM
CAR + ALA	50	0.17**	0.02
CAR + ALA	100	0.13**	0.05
CAR + ALA	200	0.11**	0.03
CAR + DIC	25	0.18**	0.03
CAR + IND	25	0.22**	0.01
CAR	–	0.36**	0.06
Control (healthy)	–	0.14	0.03

n, Number of animals.

Mean values were significantly different when compared to that of control group:  
\*\* $P < 0.01$ .

† Three doses of ALA (50, 100 and 200 mg/kg), and one dose of IND (25 mg/kg) or DIC (25 mg/kg) were administered to rats by oral administration. Three doses of the ALA-treated group, and one dose of the DIC- and IND-treated groups were compared with the CAR group. The CAR group was compared with the healthy group. The paw volumes of the animals were calculated plethysmometrically, and 0.1 ml of 1% CAR was injected into the hind paw of each animal 1 h after oral drug administration.

‡ The same experiment was performed three times.

#### In vitro antioxidant activity of $\alpha$ -lipoic acid

ROS had negative effects on the pathogenesis of inflammation and gastric ulcer. Because of this, antioxidants may be effective on the pathogenesis of these diseases. Therefore, in the present study, the *in vitro* antioxidant action of ALA was also determined using thiocyanate methods and compared to the positive controls, trolox and ascorbic acid (Table 6). ALA in 5 mg acted as a powerful antioxidant by inhibiting the peroxidation of linoleic acid with 79.1%. In particular, in 1 and 2.5 mg, ALA showed weak antioxidant potential.

#### Discussion

Inflammatory processes are the physiological response of the organism to different stimulants such as trauma, infections or immunological mechanisms. The arachidonic acid cascade is highly activated during inflammation, resulting in the formation of eicosanoids, and it is mediated by cyclo-oxygenase and 5-lipoxygenase enzymes. These complex inflammatory reactions involve the release of a wide variety of inflammatory mediators, i.e. PG, thromboxanes and leukotrienes. Although drugs with a central mechanism of anti-pyretic and anti-inflammatory actions are known to down-regulate fever and inflammation, the role of the antioxidant mechanism pathway in mediating the action of such agents is still unknown.

In the present study, the anti-inflammatory effects of the ALA, a highly effective antioxidant agent, were investigated. The effect of ALA on the acute phase of inflammation was observed in the CAR-induced paw oedema test, and its anti-inflammatory potency was compared to DIC and IND. In our experiment, the mean paw volume of the control group reached its peak at the fourth hour. The mean paw volume in the groups receiving CAR, ALA, DIC and IND started to decrease at the fourth hour. At doses of 50, 100 and 200 mg/kg, ALA significantly

**Table 6.** Antioxidant activity of three doses of  $\alpha$ -lipoic acid (ALA) and one dose of positive controls (Trolox and ascorbic acid) on linoleic acid oxidation, *in vitro*

(Mean values with their standard errors)

Samples	Total antioxidant activity		
	Absorbance (forty-fourth hour, 500 nm)	SEM	Inhibition (%)
ALA (1 mg)	0.527*	0.58	34.9
ALA (2.5 mg)	0.333*	0.25	58.9
ALA (5 mg)	0.169**	0.31	79.1
Trolox (1 mg)	0.057**	0.01	93.0
Ascorbic acid (1 mg)	0.694	0.02	14.3
Control	0.810	0.04	—

Mean values were significantly different when compared to that of the control group: \* $P<0.05$ , \*\* $P<0.01$ .

( $P<0.01$ ) reduced CAR-induced paw oedema by 18.5, 29.6 and 40.7%, respectively, at the fourth hour (Table 1). DIC and IND, which significantly reduced the increase in paw volume by 44.4 and 55.5%, respectively, showed a greater effect in this model of inflammation.

CAR injection into the rat paw provokes a local, acute inflammatory reaction which is a suitable method for evaluating anti-inflammatory agents<sup>(2–5,8)</sup>. It appears that the delayed onset of CAR-induced oedema has been linked to inflammation mediators such as NO, PG, cytokines and free radicals, such as H<sub>2</sub>O<sub>2</sub>, superoxide, and hydroxyl radical, as well as to the release of other neutrophil-derived mediators<sup>(2–5,16,17)</sup>. In order to explore the effects of antioxidant defences on the acute inflammation process in all paw tissues, the levels of antioxidant parameters were evaluated.

The GST (*E.C. 2.5.1.18*) are a multigene family of isoenzymes responsible for detoxification of xenobiotics in aerobic organisms. Endogenous substrates include the toxic products of tissue damage, including the hydroperoxide products of oxidative damage (including lipid peroxides), and aromatic xenobiotics. The present study determined that GST activity was inhibited by both ALA and DIC in CAR-induced paw tissues in which CAR significantly increased (Table 3). El Midaoui *et al.*<sup>(41)</sup> reported that high-glucose feeding rapidly induced hypertension and that the plasma and aorta GST activities of rats were decreased by ALA administration.

Although oxygen intermediates are highly reactive and potentially cytotoxic, their formation is not typically deleterious to the cells, because these ROS are neutralised by endogenous enzymes and free radical scavengers. First, SOD catalysing the dismutation of superoxide anions (O<sub>2</sub><sup>–</sup>) to H<sub>2</sub>O<sub>2</sub> and GSH-related enzymes preserving GSH status plays an important role in the antioxidant defence system by ensuring the degradation of these species into less harmful compounds<sup>(2,3,14,16,42)</sup>. From the present study (Table 4), we have established that ALA and DIC

increase SOD activity, which was inhibited by CAR in rat paw tissues. The similar results were reported by Selvakumar *et al.*<sup>(43)</sup> in cyclophosphamide-induced rat sperm and by Kokilavani *et al.*<sup>(44)</sup> in arsenic-induced toxicity of rats. Separately, Khattab<sup>(45)</sup> reported the importance of superoxide anion in CAR-induced acute inflammation and highlighted that the potential anti-inflammatory effects of SOD mimetic are mediated via the superoxide anion scavenging in acute inflammation. SOD destroys the highly reactive radical superoxide by converting it into the less reactive peroxide (H<sub>2</sub>O<sub>2</sub>), which can be destroyed by a CAT reaction. CAT is a highly reactive enzyme, reacting with H<sub>2</sub>O<sub>2</sub> to form water and molecular oxygen and can form methanol, ethanol, formic acid or phenols via donating hydrogen. In the present study (Table 4), we established that IND and DIC decreased, but all the doses of ALA increased further CAT activity, which was increased by CAR in rat paw tissues. On the other hand, it was reported by Selvakumar *et al.*<sup>(43)</sup> that ALA has an increasing effect on the activity of the CAT enzyme in rats.

Separately, similar to SOD, we showed that GPx activity was elevated by one dose of DIC and IND, or 100 and 200 mg/kg doses of ALA, which was alleviated by CAR in rat paw tissues (Table 3). Decrease in the activity of GPx during CAR administration indicates the reduction in the levels of GSH and increase in the levels of peroxides. Moreover, the fact that arsenic decreased Se levels in rats<sup>(31)</sup>, which acts as a cofactor for GPx, might be a reason for the decrease in the activity of GPx.

On the other hand, Chen *et al.*<sup>(46)</sup> suggested that CAT stimulated the expression of mRNA and protein for cyclo-oxygenase-2 in rat aortic smooth muscle cells, despite not affecting the expression of either mRNA or protein for cyclo-oxygenase-1. That is, CAT exerted a biphasic effect on PG synthesis and enhanced PG production by external administrations in small concentrations. This suggested that at small concentrations, CAT administration may cause inflammation as reflected by increased cyclo-oxygenase-2 activity. One of the factors causing the formation of CAR-induced acute inflammation process is possibly an augmentation of CAT activity, which was ascertained in the results of the present experiment.

Reduced GSH having pleiotropic roles, including the maintenance of cells in a reduced state, serves as an electron donor for certain anti-oxidative enzymes (e.g. GPx) and the formation of conjugates with some harmful endogenous and xenobiotic compounds via catalysis of GST. The anti-inflammatory effects of ALA can be supported by GSH levels in CAR-induced rat paw tissues. Furthermore, treatment with ALA enhanced GSH level which had been decreased by CAR in the rat paws (Fig. 4). In other studies, it was shown that ALA increased GSH levels in arsenic-induced toxicity of rats<sup>(44)</sup>, cyclophosphamide-induced rat sperm<sup>(43)</sup>, ischaemia-reperfusion injury of rats<sup>(15)</sup> and 2,4,6-trinitrobenzene sulphonic acid-induced gut inflammation of rats<sup>(36)</sup>.

Since ALA raises intracellular GSH levels, it has been used for treating diseases in which oxidative stress plays a major role. On the other hand, GR indirectly participates in the protection of cells against oxidative stress. The enzymatic activities of GR have been investigated in various tissues under physiological and pathological conditions<sup>(47)</sup>. Table 3 shows that GR activity was activated by both IND and DIC in CAR-induced paw tissues in which CAR significantly inhibited. However, ALA has no effect on the GR activity.

A primary measure of oxidative damage in tissues is LPO, where thiobarbituric acid is measured as the index of LPO. The injection of the inflammation-inducing agent, CAR, considerably increases the levels of LPO<sup>(48)</sup>. In the present investigation, there is a significant increase in the levels of LPO in the paws of CAR-injected rats (Fig. 5). The increase in the levels of LPO is due to the increased release of Fe that is believed to be involved in the Fenton type of reaction. This 'free Fe' is considered as a potent enhancer of ROS formation, as exemplified by the reduction of H<sub>2</sub>O<sub>2</sub> with the generation of highly aggressive hydroxyl radicals. However, administrations of CAR plus ALA, IND and DIC significantly ( $P < 0.01$ ) alleviated the amount of LPO. Similar results related to effects of ALA on LPO have been reported by the literature<sup>(15,43,44)</sup>. In the present study, ALA pre-treatment significantly reversed the increase of lipid peroxide and the altered antioxidant status. The increase in LPO reported here may be the result of increased production of free radicals and/or a decrease in antioxidant status. ALA scavenges the singlet oxygen, H<sub>2</sub>O<sub>2</sub> and hydroxyl radicals, and also chelates the ferrous ions involved in the production of hydroxyl radicals<sup>(21,22)</sup>.

MPx is an enzyme found primarily in azurophilic granules of neutrophils, which is used as a marker for tissue neutrophil content, and its inhibition implicates the presence of anti-inflammatory activity<sup>(37)</sup>. In the present study, we determined that MPx activity significantly ( $P < 0.05$ ) increased in the paw at 5 h after CAR injection when compared with healthy rats (Fig. 3). Moreover, MPx activity was alleviated by 50, 100 and 200 mg/kg doses of ALA more than IND. On the other hand, DIC had no effect on the activity of MPx in CAR-injected rat paws. It was reported that the release of MPx from inflammatory cells is another indicator to evaluate the degree of inflammation, and anti-inflammatory drugs may also exert their effects via the inhibition of MPx pathways<sup>(41,47,49)</sup>. A variety of anti-inflammatory drugs depress the increases in MPx activity in the case of inflammation<sup>(49)</sup>. Our data show that ALA and IND significantly decreased MPx activity (Fig. 4). The effect of DIC on MPx activity is inconsistent<sup>(50)</sup> because of variations in different tissues. Each molecule of ALA contains two atoms of sulphur (Fig. 1). Because of its high sulphur content, ALA can inactivate MPx activity in rat paws. The decrease in MPx activity in response to ALA could be linked to the sulphur

present in its chemical structure. Interestingly, most of the non-steroidal anti-inflammatory drugs and other drugs contain a thiol group (e.g. D-penicillamine and tiopronin) and are widely used in the treatment of rheumatoid arthritis<sup>(51)</sup> and related diseases. It was shown that ALA treatment was effective in improving tissue LPO, and MPx activity increased and decreased GSH levels in 2,4,6-trinitrobenzene sulphonic acid-induced gut inflammation and ischaemia-reperfusion injury in rats<sup>(15,52)</sup>.

A growing body of evidence suggests that sustained production of NO resulting from up-regulation of the inducible isoform of NOS after CAR injection may cause an inflammatory response in rat paws. It is well established that iNOS levels under normal physiological conditions are low; however, the administration of CAR causes the induction of iNOS<sup>(53,54)</sup>.

In the present study, it was demonstrated that CAR administration in rats resulted in significant increases in iNOS levels in paws which were attenuated by ALA pre-treatment (Table 5). Similarly, it was reported that ALA alleviated the activity of iNOS<sup>(24)</sup>. In addition to ROS, excessive production of reactive nitrogenous species, especially NO through the activation of the constitutive as well as the expression of iNOS, has been shown to play an important role in various models of inflammation<sup>(3,4,11)</sup>. It has been also widely accepted that NO produced by iNOS is cytotoxic<sup>(3,18,55,56)</sup>. In view of these results, it can be suggested that ALA is a cytoprotective compound.

The molecular regulation of iNOS expression resulting in excessive amounts of NO released in paws is a complex event and requires the transcription factor NF-κB. It has been shown that down-regulation of NF-κB with antioxidants resulted in decreases in the amount of NO released in the circulation, a treatment effect attributed to the reduction in iNOS expression<sup>(57)</sup>. Another explanation for the reduction in NO levels in paws might be due to the direct scavenging effect of NO by the sulphydryl groups of ALA<sup>(23)</sup>. It has been demonstrated that oxidative stress up-regulates and antioxidants down-regulate the pleiotropic transcription NF-κB, a DNA-binding protein that induces the expression of cytokines and vascular adhesion molecules<sup>(57)</sup>. The ability of ALA to inhibit the activation of NF-κB has been demonstrated with human T-cells exposed to TNF-α or phorbol-12-myristate-13-acetate or cultured aortic endothelial cells challenged with advanced glycation end products. Cho *et al.*<sup>(58)</sup> showed that ALA decreased activations of NF-κB and pro-inflammatory chemical mediators, such as IL-4 and IL-5 in BAL fluids and ovalbumin-specific IgE. Similarly, Zhang & Frei<sup>(59)</sup> reported that ALA may also affect NF-κB-dependent expression of many other inflammatory genes, such as IL-1 and IL-6, a tissue factor and TNF-α in numerous cell types. These investigations suggest that the functional mechanism underlying the therapeutic efficacy of ALA as an antioxidant on initiation and progression of various inflammatory diseases may be related to inhibition of NF-κB activation.

through decreased oxidative stress and subsequent reduction in pro-inflammatory chemical mediators.

In the chronic inflammation experiment, ALA significantly inhibited the weight of the cotton pellets inserted under the skin of rats. The effects of ALA, IND and DIC on cotton pellet-induced granuloma in rats are shown in Table 2. At doses of 50, 100 and 200 mg/kg, ALA as well as DIC and IND (25 mg/kg) inhibited markedly ( $P<0.01$ ) granuloma formation surrounding the pellets compared with the control group. ALA (200 mg/kg) produced a maximum inhibition of 70·6%, while 50 and 100 mg/kg of ALA produced 67·7 and 68·9% inhibition in granuloma weight, respectively. IND and DIC significantly decreased the weight of implanted cotton pellets 83·8 and 76·1% in comparison with the control group, respectively.

It is known that inflammatory granuloma is a typical response of a chronic inflammatory process, and it has been established that the dry weight of the pellets is well correlated with granulomatous tissue. The cotton pellet granuloma method is frequently used to investigate the chronic phase of inflammation<sup>(2–5,8,60)</sup>. The anti-proliferative potency of IND and DIC was higher than that of ALA in the cotton pellet-induced granuloma test. Little data have been reported about mediators of chronic inflammation. However, the chronic inflammation model, induced by subcutaneous implantation of foreign bodies, is used commonly in experiments. Chronic inflammation starts with the occurrence of proliferative cells. These cells can be spread or can form a granuloma tissue. Inflammatory granuloma tissue is a typical example of chronic inflammation<sup>(60)</sup>. Granuloma formation is initialised by an antigen (for example, a cotton pellet). The cotton pellet stimulates the immune system, antibodies and the production of IL. This stimulation results with the proliferation of lymphocytes and the formation of granulation tissue over the pellets. Non-steroidal anti-inflammatory drugs and ulcers reduce granuloma tissue that is formed with cellular reactions by inhibiting granulocyte infiltration, while glucocorticoids do it by inhibiting fibrosis and macrophage functions.

It was reported that ALA on initiation and progression of various inflammatory diseases might be related to the inhibition of NF- $\kappa$ B activation through decreased oxidative stress and subsequent reduction in pro-inflammatory chemical mediators<sup>(58,59)</sup>. Antioxidants have various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging<sup>(3,5,17)</sup>. In the present study, ALA (1, 2·5 and 5 mg/ml), ascorbic acid (1 mg/ml) and Trolox (1 mg/ml) after 44 h incubation with linoleic acid emulsion are also summarised as inhibition (%) in Table 6. Trolox and ascorbic acid were used as positive controls for the hydrophilic antioxidants. In general, the oxidation of linoleic acid was inhibited by ALA, ascorbic acid and Trolox, as observed when compared with the control.

However, the antioxidant activities of all the doses of ALA and Trolox except for ascorbic acid in comparison with the control were statistically significant ( $P<0.05$  and 0·01). The 1, 2·5 and 5 mg/ml doses of ALA exhibited potent antioxidant activities with 34·9, 58·9 and 79·1% inhibition of linoleic acid peroxidation, respectively. However, the antioxidant activities of ALA were lower than that of Trolox. The present results showed that ALA has a strong anti-oxidative potential (Table 6). Given this, it must be considered that the antioxidant potential of ALA might play an important role in the prevention of inflammation processes in rats.

In conclusion, the current results showed that tested agents (ALA, IND and DIC) reduced paw volume, at a smaller magnitude in response to ALA than IND and DIC. The level of antioxidant system enzymes (GST, GPx, GR, SOD and CAT, MPx, iNOS) and levels of GSH or LPO were adversely affected by oedema induction. ALA ameliorated the adverse effects of oedema on these enzymes. In addition, ALA exhibited an anti-inflammatory effect on the cotton pellet-induced chronic inflammation in rats. The anti-inflammatory properties of ALA which has a strong anti-oxidative potency could be related to its positive effects on the antioxidant system in a variety of rat tissues. In the light of the present results, we can suggest that ALA, an over-the counter drug with no side effects, possesses a dual beneficial effect, such that it might improve the prognosis of acute and chronic inflammatory diseases when used alone and/or combined with a known anti-inflammatory agent.

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### References

1. Albayrak A, Uyanik H, Odabasoglu F, et al. (2010) The effects of diabetes and/or polymicrobial sepsis on the status of anti-oxidant enzymes and pro-inflammatory cytokines on heart, liver, and lung of ovariectomized rats. *J Surg Res* (In the Press).
2. Halici Z, Dengiz GO, Odabasoglu F, et al. (2007) Amiodarone has anti-inflammatory and anti-oxidative properties: an experimental study in rats with carrageenan-induced paw edema. *Eur J Pharmacol* **566**, 215–221.

3. Tanas S, Odabasoglu F, Halici Z, et al. (2010) Evaluation of anti-inflammatory and antioxidant activities of *Peltigera rufescens* lichen species on acute and chronic inflammation models. *J Nat Med* **64**, 42–49.
4. Salvemini D, Wang ZQ, Wyatt P, et al. (1996) Nitric oxide: a keymediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br J Pharmacol* **118**, 829–838.
5. Odabasoglu F, Halici Z, Cakir A, et al. (2008) Beneficial effects of vegetable oils (corn, olive and sunflower oils) and  $\alpha$ -tocopherol on anti-inflammatory and gastrointestinal profiles of indomethacin in rats. *Eur J Pharmacol* **591**, 300–306.
6. Jain NK, Kulkarni SK & Singh A (2001) Role of cysteinyl leukotrienes in nociceptive and inflammatory conditions in experimental animals. *Eur J Pharmacol* **415**, 85–94.
7. Kumtepe Y, Odabasoglu F, Karaca M, et al. (2010) Protective effects of telmisartan on ischemia/reperfusion injury of rat ovary: biochemical and histopathologic evaluation. *Fertil Steril* **93**, 1299–1307.
8. Suleyman H, Odabasoglu F, Aslan A, et al. (2003) Antiinflammatory and antilulcer effects of aqueous extract of *Lobaria pulmonaria*. *Phytomedicine* **10**, 552–557.
9. Hosseinzadeh H, Ramezani M & Salmani GH (2000) Antinociceptive, anti-inflammatory and acute toxicity of *Zataria multiflora* Boiss. extracts in mice and rats. *J Ethnopharmacol* **73**, 379–385.
10. Kapu SD, Ngwai YB, Kayode O, et al. (2001) Anti-inflammatory, analgesic antilymphocytic activities of the aqueous extract of *Crinum giganteum*. *J Ethnopharmacol* **78**, 7–13.
11. Handy RLC & More PK (1998) A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hind paw oedema and NOS activity. *Br J Pharmacol* **123**, 1119–1126.
12. Odabasoglu F, Karakus B, Bayir Y, et al. (2006) Effects of methanol extract of *Lobaria pulmonaria* on glutathione S-transferase activity and gastric damages in rats. Abstracts from XIXth International Symposium on Medicinal Chemistry. *Drug Future* **31**, 168.
13. Moreira PI, Harris PLR, Zhu XW, et al. (2007) Lipoic acid and n-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer disease patient fibroblasts. *J Alzheimers Dis* **12**, 195–206.
14. Cadirci E, Altunkaynak BZ, Halici Z, et al. (2009) Alpha-lipoic acid as a potential target for the treatment of lung injury caused by cecal ligation and puncture-induced sepsis model in rats. *Shock* **33**, 479–484.
15. Sehirli O, Sener E, Cetinel U, et al. (2008) Alpha-lipoic acid protects against ischemia–reperfusion injury in rats. *Clin Exp Pharmacol* **35**, 249–255.
16. Karaca M, Odabasoglu F, Kumtepe Y, et al. (2009) Protective effects of erythropoietin on ischemia/reperfusion injury of rat ovary. *Eur J Obstet Gynecol Reprod Biol* **144**, 157–162.
17. Dengiz GO, Odabasoglu F, Halici Z, et al. (2007) Gastroprotective and antioxidant effects of montelukast on indomethacin-induced gastric ulcer in rats. *J Pharmacol Sci* **105**, 94–102.
18. Nishida K, Ohta Y & Ishiguro I (1998) Contribution of NO synthases to neutrophil infiltration in the gastric mucosal lesions in rats with water immersion restraint stress. *FEBS Lett* **425**, 243–248.
19. Cadirci E, Oral A, Odabasoglu F, et al. (2010) Atorvastatin reduces tissue damage in rat ovaries subjected to torsion and detorsion: biochemical and histopathologic evaluation. *Naunyn Schmiedebergs Arch Pharmacol* **381**, 455–466.
20. Huong DTT & Ide T (2008) Dietary lipoic acid-dependent changes in the activity and mRNA levels of hepatic lipogenic enzymes in rats. *Br J Nutr* **100**, 79–87.
21. Bilska A & Wlodex L (2005) Lipoic acid – the drug of future? *Pharmacol Rep* **57**, 570–577.
22. Odabasoglu F (2006) Alfa-lipoik asit (Alpha lipoic-acid). *Pharma Sark* **1**, 12–15.
23. Biewenga GP, Haene GR & Bast A (1997) The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol* **29**, 315–331.
24. Hurdag C, Ozkara H, Citci S, et al. (2005) The effects of alpha-lipoic acid on nitric oxide synthetase dispersion in penile function in streptozotocin-induced diabetic rats. *Int J Tissue React* **27**, 145–150.
25. Malinska D & Winiarska K (2005) Lipoic acid: characteristics and therapeutic application. *Postepy Hig Med Dosw* **59**, 535–543.
26. Demir U, Demir T & Ilhan N (2005) The protective effect of  $\alpha$ -lipoic acid against oxidative damage in rabbit conjunctiva and cornea exposed to ultraviolet radiation. *Ophthalmologica* **219**, 49–53.
27. Patrick L (2000) Nutrients and HIV: part three – N-acetylcysteine, alpha-lipoic acid, L-glutamine, and L-carnitine. *Altern Med Rev* **5**, 290–305.
28. Wenzel U, Nickel A & Daniel H (2005)  $\alpha$ -Lipoic acid induces apoptosis in human colon cancer cells by increasing mitochondrial respiration with a concomitant  $O_2^-$ -generation. *Apoptosis* **10**, 359–368.
29. Canadian Council on Animal Care (1993) *Guide to the Care and Use of Experimental Animals*, vol. I, 2nd ed. Ottawa, ON: Canadian Council on Animal Care/Bradda Printing Services, Inc.
30. Habig WH & Jakoby WB (1981) Assays for differentiation glutathione-S-transferase. *Method Enzymol* **77**, 398–405.
31. Lawrence RA & Burk RF (1976) Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* **71**, 952–958.
32. Carlberg I & Mannervik B (1985) Glutathione reductase. *Method Enzymol* **113**, 484–490.
33. Aebi H (1984) Catalase. *Method Enzymol* **105**, 121–126.
34. Sun Y, Larry WO & Ying L (1988) A simple method for clinical assay of superoxide dismutase. *Clin Chem* **34**, 497–500.
35. Knowles RG, Salter M, Brooks SL, et al. (1990) Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem Biophys Res Commun* **172**, 1042–1048.
36. Feelisch M & Noack EA (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139**, 19–30.
37. Bradley PP, Priebat DA, Christensen RD, et al. (1982) Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78**, 206–209.
38. Sedlak J & Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25**, 192–205.
39. Ohkawa H, Ohishi H & Yagi K (1979) Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**, 351–358.
40. Odabasoglu F, Aslan A, Cakir A, et al. (2005) Antioxidant activity, reducing power and total phenolic content of some lichen species. *Fitoterapia* **76**, 216–219.
41. El Midaoui A, Elimadi A, Wu L, et al. (2003) Lipoic acid prevents hypertension, hyperglycemia, and the increase in heart mitochondrial superoxide production. *Am J Hypertens* **16**, 173–179.
42. Halici Z, Karaca M, Keles ON, et al. (2008) Protective effects of amlodipine on ischemia–reperfusion injury of rat ovary: biochemical and histopathologic evaluation. *Fertil Steril* **90**, 2408–2415.

43. Selvakumar E, Prahalathan C, Sudharsan PT, *et al.* (2006) Chemoprotective effect of lipoic acid against cyclophosphamide-induced changes in the rat sperm. *Toxicology* **217**, 71–78.
44. Kokilavani V, Devi MA, Sivarajan K, *et al.* (2005) Combined efficacies of DL- $\alpha$ -lipoic acid and meso 2,3-dimercaptosuccinic acid against arsenic induced toxicity in antioxidant systems of rats. *Toxicol Lett* **160**, 1–7.
45. Khattab M (2006) Tempol, a membrane-permeable radical scavenger, attenuates peroxynitrite- and superoxide anion-enhanced carrageenan-induced paw edema and hyperalgesia: a key role for superoxide anion. *Eur J Pharmacol* **548**, 167–173.
46. Chen G, Kamal M, Hannon R, *et al.* (1998) Regulation of cyclo-oxygenase gene expression in rat smooth muscle cells by catalase. *Biochem Pharmacol* **55**, 1621–1631.
47. Shacter E, Lopez RL & Pati S (1991) Inhibition of myeloperoxidase–H<sub>2</sub>O<sub>2</sub>–Cl-system of neutrophils by indometacin and other non-steroidal anti-inflammatory drugs. *Biochem Pharmacol* **41**, 975–984.
48. Govindarajan R, Vijayakumar M, Rao CV, *et al.* (2007) Antiinflammatory and antioxidant activities of *Desmodium gangeticum* fractions in carrageenan-induced inflamed rats. *Phytother Res* **21**, 975–979.
49. Paine IMM, Ximenes VF, Fonseca LM, *et al.* (2005) Effect of therapeutic plasma concentrations of non-steroidal anti-inflammatory drugs on the production of reactive oxygen species by activated rat neutrophils. *Braz J Med Biol Res* **38**, 543–551.
50. Matsui H, Murata Y, Kobayashi F, *et al.* (2001) Diclofenac-induced gastric mucosal fluorescence in rats. *Digest Dis Sci* **46**, 338–344.
51. Bonta IL, Parnham MJ, Vincent JE, *et al.* (1980) 5 Anti-rheumatic drugs: present deadlock and new vistas. In *Progress in Medicinal Chemistry*, pp. 185–273 [GP Ellis and GB West, editors]. North-Holland/Elsevier: Amsterdam.
52. Kolgazi M, Jahovic, Yüksel M, *et al.* (2006)  $\alpha$ -Lipoic acid modulates gut inflammation induced by trinitrobenzene sulfonic acid in rats. *J Gastroen Hepatol* **22**, 1859–1865.
53. Cuzzocrea S, Bruscoli S, Crisafulli C, *et al.* (2007) Estrogen receptor antagonist fulvestrant inhibits the anti-inflammatory effect of glucocorticoids. *Mol Pharmacol* **71**, 132–144.
54. Hurdag C, Uyaner I, Gurel E, *et al.* (2008) The effect of alpha lipoic acid on NOS dispersion in the lung of streptozotocin-induced diabetic rats. *J Diabetes Complications* **22**, 56–61.
55. Odabasoglu F, Suleyman H, Aslan A, *et al.* (2005) The effects of diffractaic acid on MPx and NOS enzymes activities in indomethacin-induced ulcer in rats. *Adv Mol Med* **1**, 402–403.
56. Jean YH, Chen WF, Duh CY, *et al.* (2008) Inducible nitric oxide synthase and cyclooxygenase-2 participate in anti-inflammatory and analgesic effects of the natural marine compound lemnalol from Formosan soft coral *Lemnalia cervicorni*. *Eur J Pharmacol* **578**, 323–331.
57. van den Berg R, Haenen GR, van den Berg H, *et al.* (2001) Transcription factor NF- $\kappa$ B as a potential biomarker for oxidative stress. *Br J Nutr* **86**, S121–S127.
58. Cho YS, Lee J, Lee TH, *et al.* (2004)  $\alpha$ -Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *J Allergy Clin Immunol* **114**, 429–435.
59. Zhang WJ & Frei B (2001)  $\alpha$ -Lipoic acid inhibits TNF- $\alpha$ -induced NF- $\kappa$ B activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J* **15**, 2423–2432.
60. Olajide OA, Makinde JM & Awe SO (1999) Effects of the aqueous extract *Bridella ferruginea* stem bark on carrageenan induced oedema and granuloma tissue formation in rats and mice. *J Ethnopharmacol* **66**, 113–117.