

## The protective effect of L-carnitine in peripheral blood human lymphocytes exposed to oxidative agents

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**Literature data indicate L-carnitine (LC), a trans-mitochondrial carrier of acetyl and long chain groups, as an agent possessing protective effects against oxidative stress in mammalian cells. However, the major factor involved in the protective mechanism is not known. The protection activity exerted by this agent against reactive oxygen species induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and *t*-butylhydroperoxide (*t*-butyl-OOH) treatment in isolated human peripheral blood lymphocytes (PBLs) has been studied. Human lymphocytes cells were isolated and pre-incubated with 5 mM LC before H<sub>2</sub>O<sub>2</sub> (100 µM) and *t*-butyl-OOH (400 µM) treatment. The protective effect of LC on treated PBLs was measured by single cell gel electrophoresis and the analysis of chromosomal aberrations. Results show that LC treated cells exhibited a significant decrease in the number of oxidative induced single-strand breaks and chromosomal aberrations.**

### Introduction

DNA suffers oxidative damage from free radicals produced in living cells by cellular metabolism and by exogenous agents.

This damage takes the form of single-strand breaks (SSBs), much less abundant double-strand breaks (DSBs), alkali labile sites and various species of oxidized purines and pyrimidines (1). Several agents efficiently remove endogenously produced free radicals before they damage DNA. Despite this antioxidant protection, significant oxidative damage occurs which is subjected to cellular repair processes. This damage, if not correctly repaired, can lead to genomic instability and increased risk of developing cancer.

Defect in DNA repair is illustrated by the occurrence of several human DNA repair disorders; for example, the inability to repair SSBs induced by alkylating agents has been shown in familial Alzheimer's disease (2). Altered response to oxidative DNA damage has been suggested as a causal factor in a neurodegenerative disease, ataxia telangiectasia (3).

Possible approaches to protect DNA from oxidative damage are to supply antioxidants or to increase the capacity of cells to cope with DNA damage by increasing, for example, their capacity to repair DNA damage. L-carnitine (LC) and its derivatives are known to protect against cell killing by oxygen radicals (4), protect the ischemic myocardium (5–7), improve

cardiac performance (5,7–11) and restore high-energy phosphate pools in myocardial cells (8–12). Several lines of evidence are present in the literature suggesting that LC could help cells to recover from SSBs induced by different types of DNA lesions (13) and the use of LC as a protection against oxygen free radicals (14).

In this work the effect of LC on the modulation of DNA damage induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or *t*-butylhydroperoxide (*t*-Butyl-OOH) has been studied in human peripheral blood lymphocytes (PBLs) and has been compared with mannitol, a well-known scavenger of the free hydroxyl radical (OH) (15). In order to dissect the mechanism by which LC prevents DNA damage, it was added to the cells before, during and after H<sub>2</sub>O<sub>2</sub> treatment. H<sub>2</sub>O<sub>2</sub> and *t*-butyl-OOH were chosen to induce DNA breaks. Both H<sub>2</sub>O<sub>2</sub> and *t*-butyl-OOH have been widely used as model compounds to investigate the effects of oxidative damage in cell cultures. The most significant lesion produced by both the peroxides appears to be the DNA SSB, either by production of the OH via Fenton type reactions or, possibly in the case of *t*-butyl-OOH, by lipid peroxidation and reaction involving calcium (16–18). In the present study, the alkaline version of the comet assay, a rapid and sensitive procedure for quantifying DNA lesions in mammalian cells (19,20) was employed to measure the frequency of SSBs and alkali-labile sites induced by H<sub>2</sub>O<sub>2</sub> or *t*-butyl-OOH immediately and at different recovery times in PBLs with and without LC treatment. In addition, the yield of induced chromosomal damage was evaluated.

### Materials and methods

#### Chemicals

H<sub>2</sub>O<sub>2</sub>, *t*-Butyl-OOH, mannitol (D-form), colcemid and 5-bromo-2-deoxyuridine (BrdUrd), were purchased from Sigma-Aldrich.

H<sub>2</sub>O<sub>2</sub> and *t*-butyl-OOH were diluted in distilled water at appropriate concentrations for treatment and were used freshly.

Mannitol was dissolved in distilled water at 1 M, and kept frozen at –20°C until use. It was used at a final concentration of 1 mM.

LC, kindly provided by Sigma-Tau, Italy, was dissolved in Ham's F10 and kept frozen at –20°C until use. It was used at a final concentration of 5 mM.

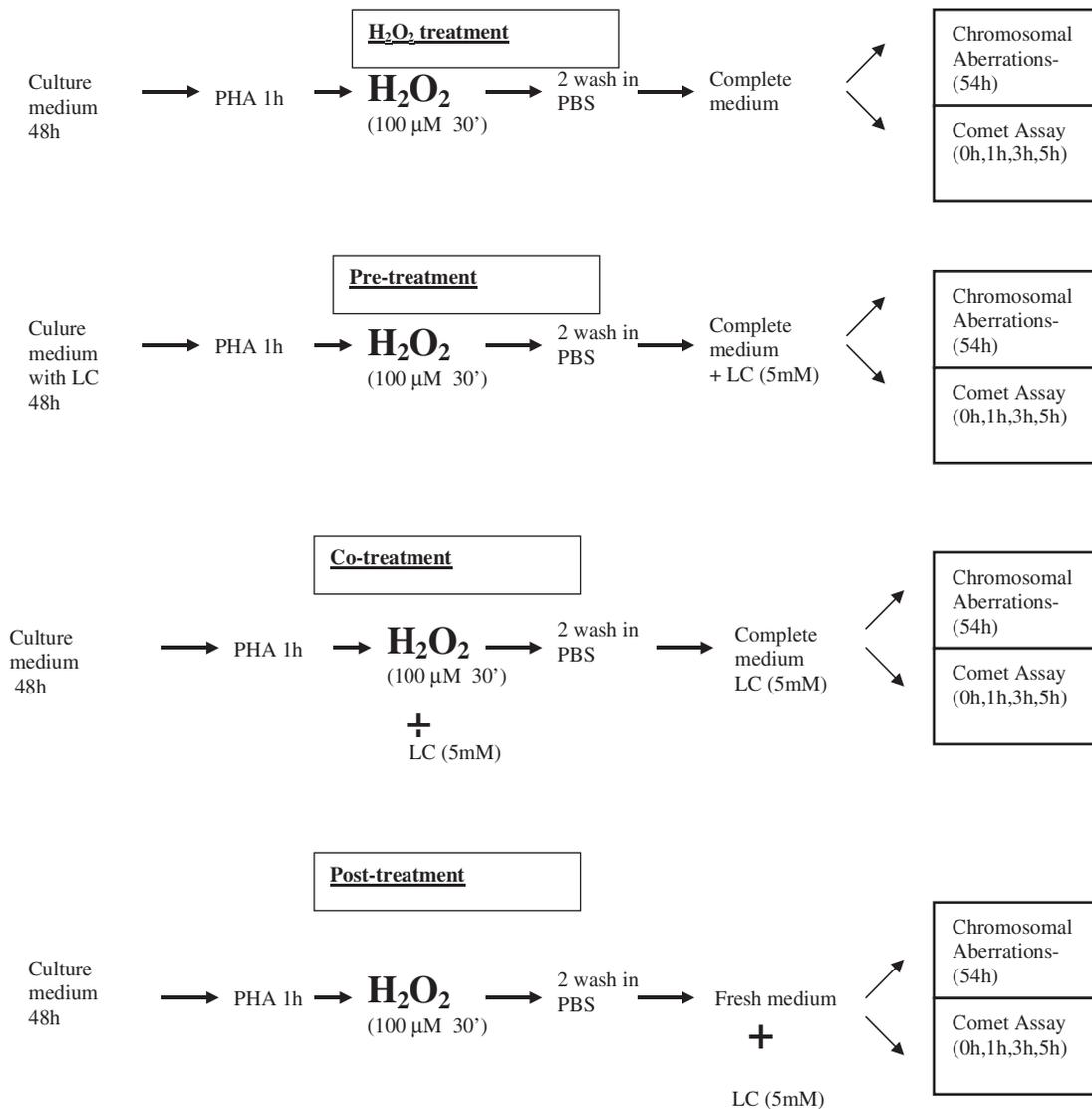
#### Lymphocyte isolation and treatment

Freshly obtained 'buffy coats' from healthy human donors were purchased at the Blood Donor Central of the Belcolle hospital, Viterbo, Italy. Three donors were used for each experiment.

Lymphocytes were separated from the concomitant erythrocytes and thrombocytes by centrifugation in Histopaque-1077 (Sigma-Aldrich) gradients and washed twice in Ham's F10 medium (Bio Whittaker). All steps were performed at room temperature.

Monocytes and other surface-active cells were removed by incubation in low-serum culture medium (Ham's F10 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% of heat-inactivated foetal bovine serum, all from Gibco RL, Life Technologies) for 2 h at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Afterwards, the medium with the free-floating G<sub>0</sub> lymphocytes was decanted into Falcon tubes and collected after 15 min centrifugation at 100 g at room temperature. The cells were

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**Fig. 1.** Experimental scheme with H<sub>2</sub>O<sub>2</sub>.

counted and  $2 \times 10^6$  cells/ml were kept for 48 h in fresh normal-serum medium, (the same as indicated earlier but with 10% inactivated foetal bovine serum) where needed supplemented with 5 mM LC according to the protocol scheme shown in Figure 1. All doses reported in the schemes were chosen based on pilot experiments. After 1 h phytohaemagglutinin (PHA, Murex) was added to complete medium at a concentration of 2% to stimulate cell growth.

#### Experiment performed with H<sub>2</sub>O<sub>2</sub> and LC

Three healthy donors were used for this experiment. Lymphocytes 1 h after PHA stimulation were washed twice and resuspended in fresh medium without serum and treated with H<sub>2</sub>O<sub>2</sub> (100 μM) for 30 min.

After treatment the cells were washed twice with phosphate-buffered saline (PBS) and each sample was divided equally between two flasks (one for comet assay and one for chromosomal aberrations) and incubated in complete medium. Some cultures were allowed to recover in the presence of BrdUrd for 54 h to determine the yield of chromosomal aberrations in first mitosis after treatment or were processed immediately and at different recovery time for studying the DNA repair kinetics with single cell gel electrophoresis (SCGE) analysis.

In Figure 1, pre-treated samples indicate cells supplemented with LC (5 mM) 48 h before H<sub>2</sub>O<sub>2</sub> (100 μM) treatment, during 30 min of exposure to H<sub>2</sub>O<sub>2</sub> and LC replaced immediately after.

Co-treated samples indicate lymphocytes supplemented with LC (5 mM) during and after H<sub>2</sub>O<sub>2</sub> treatment whereas post-treated samples indicate cells supplemented with LC (5 mM) only after H<sub>2</sub>O<sub>2</sub> treatment.

#### Experiment performed with *t*-butyl-OOH, LC and mannitol

Three healthy donors were used for the experiment with *t*-butyl-OOH and it was carried out according the protocol scheme shown in Figure 2. Briefly, human PBLs were isolated as described previously and suspended at  $2 \times 10^6$ /ml in Ham's F10 complete medium. Pre-treatment of PBLs with LC (5 mM) occurred for 48 h at 37°C in Ham's F10 complete medium supplemented with 10% inactivated foetal bovine serum and 2 mM glutamine. Before treatment with *t*-butyl-OOH, the cells were stimulated with PHA for 1 h, washed and resuspended in Ham's F10 complete medium without serum. Lymphocytes were exposed to *t*-butyl-OOH (400 μM) for 15 min and PBLs were again washed and resuspended in Ham's F10 complete medium plus 10% inactivated foetal bovine serum and 2 mM glutamine. Then they were either used immediately for comet assay or incubated at 37°C for various time periods for repair studies.

In experiments with mannitol lymphocytes were pre-treated 1 h before *t*-butyl-OOH exposure and left until sampling times.

#### SCGE analysis (comet assay)

The standard alkaline (pH > 13) SCGE, or comet assay, was performed according to the method developed by Singh and co-workers (19). SCGE analyses were carried out under non-UV fluorescent light as described by Tice *et al.* (21). Immediately after the treatment, cells were collected and processed for the assay. Briefly, 20 μl of cell suspension ( $2 \times 10^6$  cells) was mixed with 180 μl of 0.75% low melting point agarose in PBS at 37°C and was immediately pipetted on to a frosted glass microscope slide precoated with a layer of 1% normal melting point agarose, similarly prepared in PBS. Slides

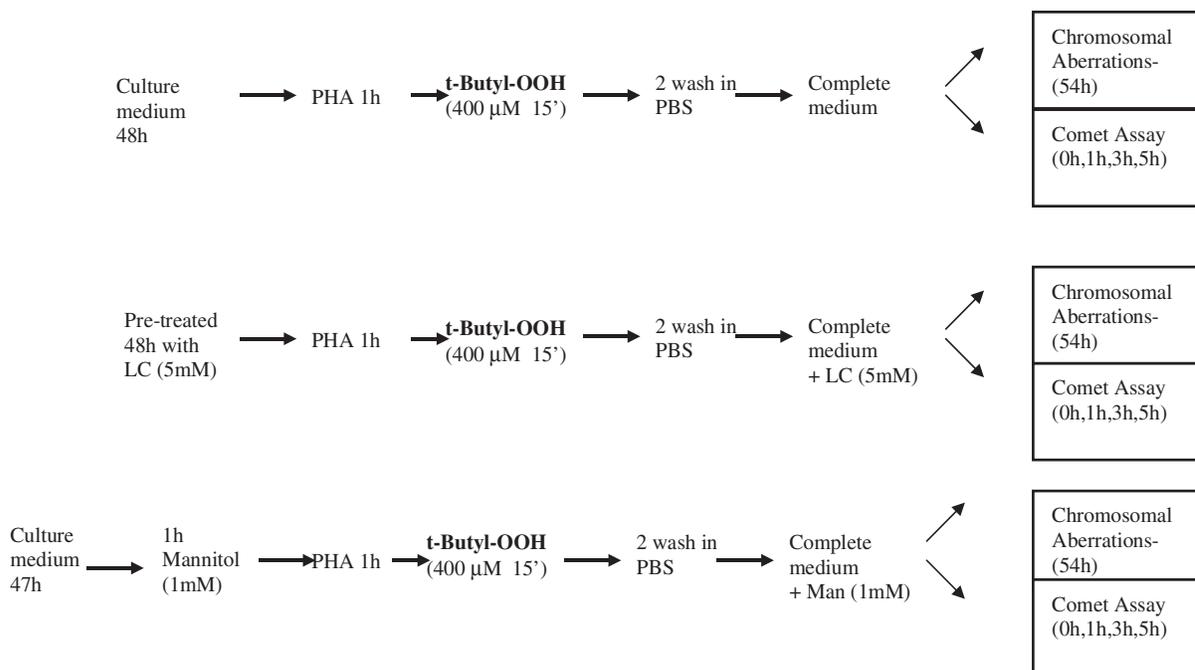


Fig. 2. Experimental scheme with *t*-butyl-OOH.

were then incubated in a lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, pH 10, with 1% Triton and 10% DMSO added fresh) for 50 min.

After lysis, slides were placed on a horizontal electrophoresis unit containing fresh buffer (1 mM EDTA and 300 mM NaOH, pH 13) and incubated for 15 min to allow unwinding of DNA. Electrophoresis was then conducted in fresh electrophoresis buffer for 20 min at 25 V and 300 mA at 4°C. Subsequently, the slides were gently washed in neutralization solution (0.4 M Tris-HCl, pH 7.5) for 5 min and fixed in 100% fresh methanol for 3 min. Slides were stained with 50  $\mu$ l ethidium bromide (20  $\mu$ g/ml) and covered with a coverslip. Stained nucleoids were examined at  $\times 400$  magnification with an automatic image analyser (Comet Assay III; Perceptive Instruments, UK) connected to a fluorescence microscope (Eclipse E400; Nikon). To evaluate the amount of DNA damage, computer generated tail moment values and percentage of DNA damage were used. From each donor and for each treatment 100 cells were evaluated after coding from two different slides.

#### Chromosomal aberrations

After  $H_2O_2$  or *t*-butyl-OOH treatment in presence or absence of LC (5 mM) the cells were incubated in fresh complete medium, containing LC where necessary, for a further 54 h. Colcemid (0.2  $\mu$ g/ml) was added 4 h before the cells were sampled. Chromosome preparations were obtained by a standard method, which included 10 min hypotonic (KCl, 5.6 g/l) treatment followed by fixation with methanol/acetic acid (3:1). Chromosomal aberrations were evaluated after FPG staining. Slides were coded and 100 BrdUrd unlabelled metaphases for each donor were analysed for each experimental point according to Savage (22). Gaps were not included in the total frequency of chromosomal aberrations.

#### Statistical analysis

For the Student evaluated comet assay analysis the significance of the effect of  $H_2O_2$  and *t*-butyl-OOH treatment against to untreated control's *t*-test. (\* $P < 0.05$ , \*\* $P < 0.001$ ).

The effects of the oxidizing agents under different experimental conditions (i.e. with and without LC) were compared using Student's *t*-test for paired samples.

Results are represented as the mean  $\pm$  standard error (SE) of three different samples. Control and treated cultures were compared by Student's *t*-test for the evaluation of induced chromosomal aberrations.

## Results

### Comet assay

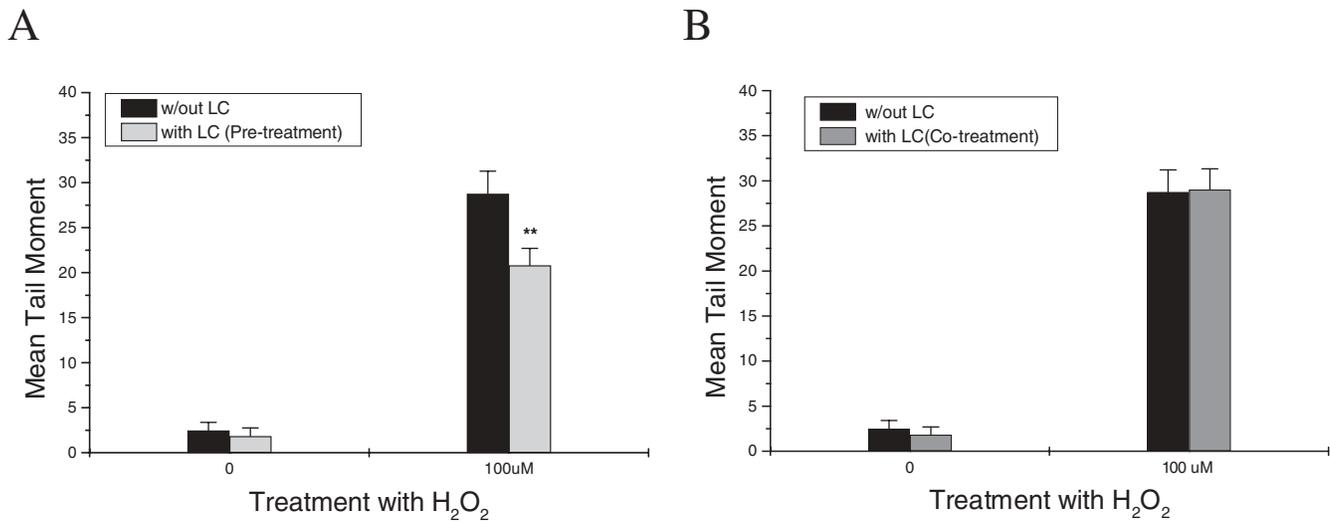
The induction and removal of oxygen radical-induced SSBs in PBLs were investigated following treatment with  $H_2O_2$

(100  $\mu$ M) or *t*-butyl-OOH (400  $\mu$ M) using the comet assay. This technique detects both SSBs and other DNA modifications, so called alkali-labile sites, which can be detected as SSBs, after denaturation in alkaline medium. Since real SSBs and alkali-labile sites cannot be distinguished in this assay, both types of DNA lesions will be referred to as SSBs.

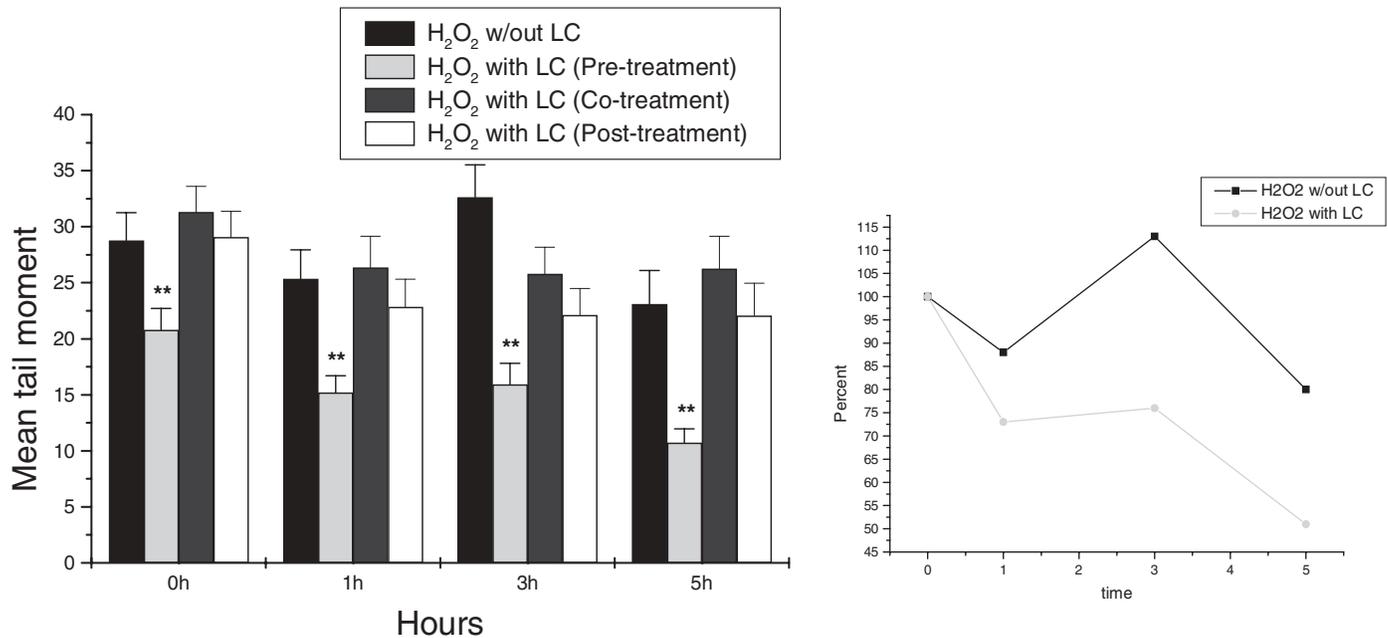
Since the data obtained from three donors were not significantly different, they were pooled.

Results concerning experiments performed with  $H_2O_2$  and LC, according to the scheme in Figure 1, are presented in Figure 3. Figure 3A and B show that treatment with  $H_2O_2$  increased numbers of SSBs in lymphocytes either LC supplemented (pre-treated and co-treated cells) or LC unsupplemented. Data in Figure 3A show a comparison between mean tail moment, measured immediately after  $H_2O_2$  treatment, in LC pre-treated lymphocytes culture and lymphocytes treated only with  $H_2O_2$ . A statistically significant difference was found between these two cell groups ( $P < 0.001$ ). Results in Figure 3B show a comparison between mean tail moment, measured immediately after  $H_2O_2$  treatment, in LC co-treated lymphocytes culture and lymphocytes treated only with  $H_2O_2$ . No difference was found between the samples. These results indicate that pre-treatment with LC for 48 h was necessary to increase the resistance to oxidative DNA damage.

Figure 4 represents a kinetic repair of DNA strand breaks in  $H_2O_2$  treated lymphocytes and in lymphocytes exposed to  $H_2O_2$  and supplemented with LC under different conditions according to the scheme in Figure 1. The kinetics of repair of DNA SSBs induced by  $H_2O_2$  in peripheral human lymphocytes was analysed and compared with cells pre-treated, co-treated and post-treated with LC. The kinetics of DNA repair was assessed at 0, 1, 3 and 5 h after  $H_2O_2$  treatment. Over the time, a slight reduction of mean tail moment in cells treated with  $H_2O_2$  without LC supplementation was observed and it was not statistically different compared with the data obtained in LC co-treated and post-treated cells.



**Fig. 3.** Single-cell gel electrophoresis analysis of DNA strand breaks at time 0 in PBLs treated with  $H_2O_2$  in presence or not of LC (5 mM). (A) Lymphocytes were supplemented with LC (5 mM) 48 h before  $H_2O_2$  exposure. (B) Lymphocytes were supplemented with LC at the same time of  $H_2O_2$  treatment. Values indicated the mean tail moment + error standard.  $*P < 0.001$  (treated versus control, Student's *t*-test).



**Fig. 4.** Repair of  $H_2O_2$  induced DNA damage in human lymphocytes: effect of LC (5 mM). Cells were incubated with LC (5 mM) 48 h before, during or after  $H_2O_2$  treatment (30 min at  $37^\circ C$ ) and analysed at different times. Average tail moment values were determined from 300 cells per experimental point immediately after  $H_2O_2$  treatment and 1, 3 or 5 h later. Insert: curve of repair kinetics with damage expressed as percentage of initial damage. Bars indicate standard error.  $*P < 0.05$ ;  $**P < 0.001$  (Student's *t*-test).

In contrast, the extent and the rate of DNA damage were statistically different between lymphocytes treated only with  $H_2O_2$  and LC pre-treated cells ( $P < 0.001$ ).

Similar results were obtained with cells treated with *t*-butyl-OOH. In this experiment performed according to the scheme in Figure 2, the oxidative damage induced by *t*-butyl-OOH in PBLs supplemented or not with LC was evaluated. Furthermore, mannitol pre-treated cells were compared with LC pre-treated samples (Figure 5) The addition of LC or mannitol alone had no effect on the level of SSBs, but both agents decreased the number of SSBs induced by *t*-butyl-OOH treatment ( $P < 0.001$ ). No difference in the repair of damage was seen between cells treated with *t*-butyl-OOH alone and

pre-treated with mannitol, whereas samples pre-treated with LC and exposed to *t*-butyl-OOH show a significant reduction in SSBs at each recovery time analysed (Figure 6).

#### Chromosomal aberrations

In order to investigate whether the apparent loss of  $H_2O_2$  or *t*-butyl-OOH induced SSBs by LC could reflect in chromosomal damage, the frequencies of chromosomal aberrations after  $H_2O_2$  or *t*-butyl-OOH treatment were determined in a separate set of experiments. PHA-stimulated parallel PBL cultures were recovered for 54 h after treatment and analysed for chromosomal aberrations in the first mitosis following PHA stimulation.

Figure 7 shows the effect of H<sub>2</sub>O<sub>2</sub> treatment on chromosomal aberrations in PBLs pre-treated or not with LC. There was no difference between control cells and cells treated with LC (5 mM) alone, while a significantly higher increase of abnormal cells and total chromatid aberrations was observed in PBLs treated with H<sub>2</sub>O<sub>2</sub> (100 μM) alone compared with LC treated ones.

Figure 8 shows similar results obtained with *t*-butyl-OOH.

## Discussion

The comet assay is a well-known assay to measure DNA SSBs immediately after their induction and to monitor their repair, but not always a direct correlation between primary DNA damage and its biological effect has been found. In the present work a correspondence between the reduction by LC of H<sub>2</sub>O<sub>2</sub> or *t*-butyl-OOH induced DNA damage and a reduction of induced chromosomal damage was observed. Since the

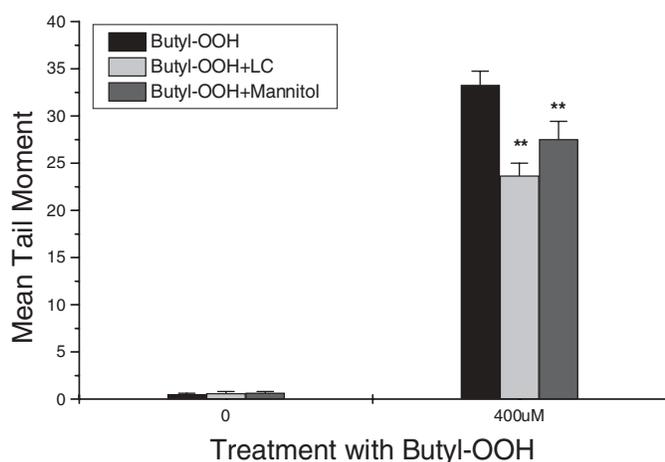
chromosomal aberrations induced by these chemical agents were only of the chromatid-type they must be the consequences of unrepaired SSBs that pass through DNA replication. The finding that LC pre-treatment reduces their frequency suggests that also the H<sub>2</sub>O<sub>2</sub> or *t*-butyl-OOH induced SSBs are reduced by LC.

H<sub>2</sub>O<sub>2</sub>, a well-defined oxidant agent, does not undergo any chemical reaction with DNA, it is envisaged that H<sub>2</sub>O<sub>2</sub> which crosses biological membranes can penetrate to the nucleus and react with transition metal ions to form •OH radicals. Attack of •OH radicals on the DNA at the sugar residue leads to fragmentation, base loss and strand breaks with a terminal sugar residue fragment (23,24). A direct rejoining repairs these frank breaks of DNA. In addition to this, •OH radicals can attack DNA bases and produce thymine glycol, 8-hydroxyguanine or 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Most of these oxidative DNA lesions are removed by base excision repair (BER), however some forms of oxidative base damage are repaired by systems that apparently recognize elements of DNA helical distortion rather than specific base damage (25).

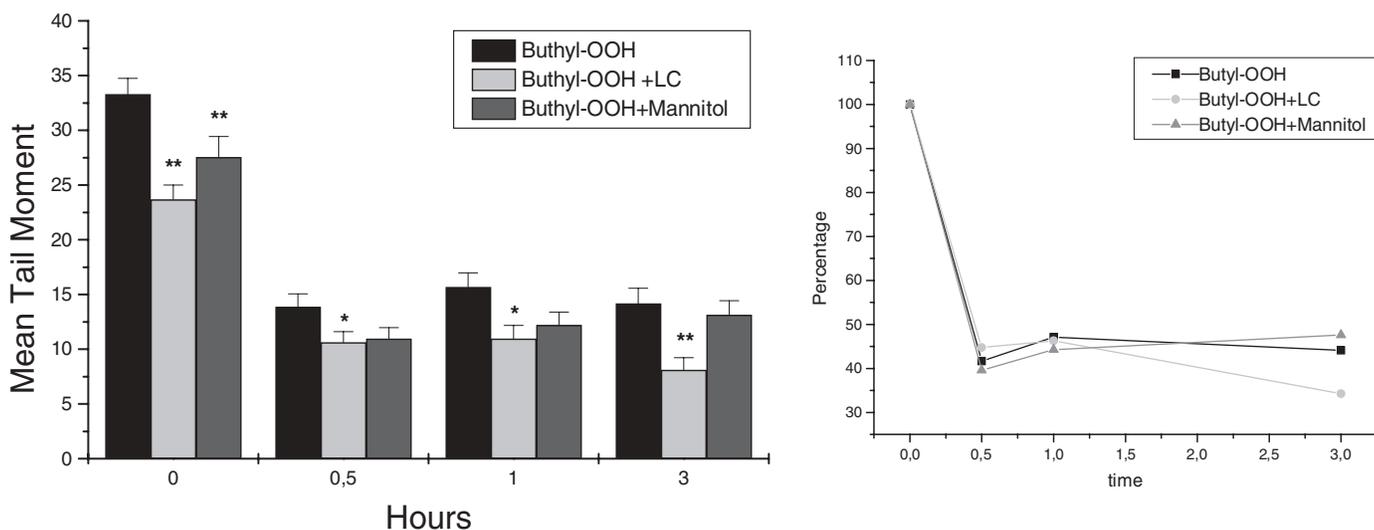
Reports on the ability of peripheral human lymphocytes to rejoin SSBs induced by reactive oxygen species are conflicting. Schraufstatter *et al.* (26) found no repair of strand breaks over 4 h of incubation following treatment with H<sub>2</sub>O<sub>2</sub>. Kaminskis and Li (27), using a xanthine/xanthine oxidase system to produce free radical damage, found that stimulated lymphocytes repair more quickly than unstimulated cells. Collins *et al.* (28) using the comet assay showed that repair in unstimulated lymphocytes is slow. This is because of the fact that the nucleotide pool is small in unstimulated lymphocytes. Literature data on the effect of LC supplementation *in vivo* and *in vitro* show that LC behaves as an antioxidant (4,29) and accelerates the disappearance of SSBs induced by oxygen radicals and alkylating agents (13).

SSBs measured by the comet assay can arise as direct products of DNA damage and as intermediates of BER oxidized pyrimidines and purines.

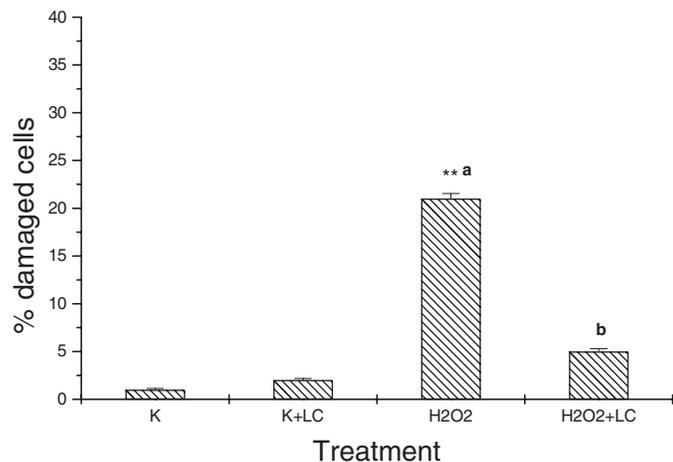
Cellular repair of oxidized bases consists of the removal of the base by glycosylase; breakage of the phosphodiester



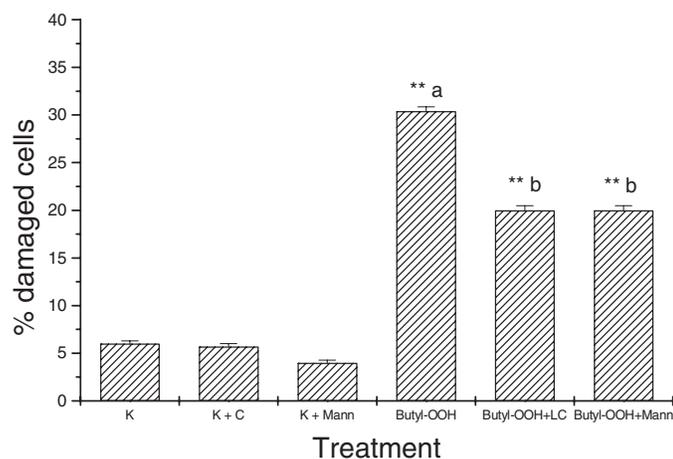
**Fig. 5.** Single-cell gel electrophoresis analysis of DNA strand breaks at time 0 in PBLs treated with *t*-butyl-OOH (400 μM) alone, in presence of LC (5 mM) or mannitol (1 mM). Values indicated the mean tail moment + error standard. \* $P < 0.001$  (treated versus control, Student's *t*-test).



**Fig. 6.** Repair of DNA damage induced by *t*-butyl-OOH in human lymphocytes: effect of LC or mannitol. Cells were incubated with LC 48 h or with mannitol 1 h before *t*-butyl-OOH treatment (15 min at 37°C) and analysed at different times. Average tail moment values were determined from 100 cells per experimental point immediately after *t*-butyl-OOH treatment and 0.5, 1 or 3 h later. Insert: curve of repair kinetics with damage expressed as per cent of initial damage. Bars indicate standard error. \* $P < 0.05$ ; \*\* $P < 0.001$  (Student's *t*-test).



**Fig. 7.** Effect of LC treatment (5 mM) on H<sub>2</sub>O<sub>2</sub> (100 μM) induction of chromosomal aberrations. The data are presented as the means ± SD of three studied donors. Values with superscripts a and b are significantly different from each other ( $P < 0.001$ ).



**Fig. 8.** Effect of LC treatment (5 mM) on *t*-butyl-OOH (400 μM) induction of chromosomal aberrations. The data are presented as the means ± SD of three studied donors. Values with superscripts a and b are significantly different from each other ( $P < 0.001$ ).

backbone at the base-less site by an AP endonuclease; removal of the sugar-phosphate and adjacent deoxyribonucleotides and polymerization of deoxyribonucleotides to fill the gap and finally ligation of the repair patch into the DNA (30). Thus, between incision and ligation, a break is present in the DNA. The AP sites also appear as breaks as they are alkali-labile sites.

Available evidence suggests that oxidized bases are repaired more slowly than frank SSBs (28). Our data show that pre-treatment of PBLs with LC affects the levels of frank SSBs induced by oxygen radicals. These findings suggest that an important factor in the protective mechanisms of LC could be represented by scavenging oxygen radicals directly or indirectly. In addition, the rate and the extent of disappearance of SSBs during post-treatment incubation times for LC pre-treated samples compared with the LC untreated, or co-treated and post-treated cells were significantly different. We can assume that such differences might be due to the enhanced repair of frank SSBs as a consequence of higher energy status in samples supplemented with LC for 48 h. Another possible explanation for such a difference could be

that LC does not enhance the repair of oxidized bases, as measured by the disappearance of SSBs, but prevents the creation of SSBs through a significant protective effect on the production of oxidized bases.

In fact, if LC was responsible for enhanced DNA repair, an increase in SSBs after treatment with H<sub>2</sub>O<sub>2</sub> associated with an increased rate of SSBs generation as intermediates in the repair of oxidized bases could be expected. No such effect has been seen (Figure 4).

The data obtained *in vitro* represent a possible strategy to reduce oxidative stress and protect human cells from the damage caused by reactive oxygen species using a natural compound like LC. As the DNA repair increased with the cellular energy status (31) one could argue that LC may also act by modulating the effect of oxidative stress and improving the production of energy by the cells. If a similar effect can be found in *in vivo* experiments LC could be of potential benefit in preventing degenerative diseases.

### Acknowledgements

We would like to thank Mr Angelo Schinoppi for expert technical assistance. Sigma-Tau (Italy) supported this work.

*Conflict of interest:* The study was funded by Sigma-Tau, manufacturer of L-carnitine.

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Received on September 8, 2005; revised on October 25, 2005;  
accepted on October 26, 2005