

## Beneficial effect of Lisosan G on cultured human microvascular endothelial cells exposed to oxidised low density lipoprotein

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**Background & objectives:** Nutritional compounds which display anti-inflammatory and antioxidant effects have specific applications in preventing oxidative stress and endothelial dysfunction. In this study we evaluated the effect of Lisosan G (powder of *Triticum sativum* grains) on human microvascular endothelial cells (HMEC-1) exposed to oxidized low density lipoprotein (ox-LDL).

**Methods:** The protective effects of Lisosan G were evaluated on human microvascular endothelial cells exposed to ox-LDL. Intercellular adhesion molecular-1 (ICAM-1), endothelin-1 (ET-1), and interleukin-6 (IL-6) concentrations and the expression of the respective genes were evaluated in response to incubation with ox-LDL, after co-incubation with ox-LDL and Lisosan G or exposed to Lisosan G alone. The analysis of LOX-1 gene was performed with RT-PCR semi quantitative method. The degree of oxidation induced in relation to control, was established by measurement of malondialdehyde (MDA) production.

**Results:** The incubation with ox-LDL induced a significant increase in ICAM-1, IL-6 and ET-1 levels compared to the basal condition ( $P<0.01$ ,  $P<0.05$ , and  $P<0.01$ , respectively), while in presence of Lisosan G, ICAM-1 levels showed a significant reduction both compared to the cultures treated with ox-LDL and control ( $P<0.01$ ). IL-6 levels did not show any difference; ET-1 levels showed a partial reduction after co-treatment with Lisosan G, and also with Lisosan G alone, reduced the concentration below control ( $P<0.01$ ). The modulation of these markers was confirmed by RT-PCR analysis. An association between MDA formation and the three markers production was observed. Semi-quantitative analysis of *LOX-1* gene expression showed a significant up-regulation only after ox-LDL exposure.

**Interpretation & conclusions:** The results demonstrate that Lisosan G may have an important role in the prevention of microcirculatory dysfunction.

**Key words** Adhesion molecules - endothelins - endothelium - inflammation - lipoproteins - oxidative stress

Oxidant by-products of normal metabolism cause extensive damage to DNA, protein, and lipid. Many clinical and experimental studies support a direct role of microcirculatory dysfunction in the genesis

of target organ damage during oxidative stress and hypercholesterolaemia<sup>1</sup>. Due to a chronically enhanced oxidative stress, in diabetes and hypertension oxidised low density lipoprotein (ox-LDL) is formed in large

quantity, and is also characterized by abnormalities in microcirculatory function<sup>2</sup>.

The influence of both native and oxidized LDL on the release of potent vasoactive substances - such as nitric oxide (NO) and endothelin-1 (ET-1) by the endothelium is potentially relevant both in initiating and maintaining abnormal tissue perfusion<sup>3</sup>. OxLDL can induce NO synthesis also by acting as an inflammatory stimulus<sup>4</sup>, that is, by triggering interleukin-6 (IL-6)-mediated inducible nitric oxide synthase (iNOS) synthesis and activation<sup>4</sup>. Intracellularly generated oxygen-derived free radicals<sup>5</sup> such as superoxide anions (O<sup>2-</sup>), hydroxyl radicals (OH<sup>·</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are shown to play a role in endothelial dysfunction in hypertension and atherosclerosis promoting intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells and ICAM-1 dependent adhesion of monocytes<sup>6</sup>. Modulation of the expression of these adhesion molecules by free radicals may, therefore, be an important mechanism in initiating atherosclerosis<sup>7</sup>.

ET-1 is a potent vasoconstrictor peptide synthesized by endothelial cells which plays a significant role in the regulation of vascular function and structure because it is a strong regulator of mitogenesis and able to stimulate the macrophages to synthesize monocytes chemoattractant protein<sup>8</sup>. Information on the mechanisms through which native and Ox-LDL interact with the endothelium is limited to large vessels; in these cells, the acute effect of Ox-LDL appears to be largely, though not entirely, determined by the interaction with the lectin-like Ox-LDL receptor (LOX<sub>1</sub>-R)<sup>9</sup>. However, other mechanisms could be involved, since both modified apolipoprotein B and lipid moieties such as isoprostanes present in oxidized LDL can interact with other receptors present in endothelial cells, such as the scavenger receptor or the thromboxane A<sub>2</sub> receptor<sup>10</sup> whose role in modulating the synthesis of vasoactive products is unknown.

Nutritional compounds which display anti-inflammatory and antioxidant effects have specific applications in preventing oxidative stress induced injury which characterizes their pathogenesis<sup>11</sup>. Polyphenolic constituents of red wine known for its anti-atherogenic properties, are thought to be beneficial in reducing the incidence of cardiovascular diseases (CVD) for example, resveratrol appears to be a natural antioxidant that enhances cholesterol efflux. These properties make it a potential natural antioxidant

that could be used to prevent and treat cardiovascular disease<sup>12</sup>. Resveratrol restored endothelial function in type 2 diabetes by inhibiting TNF $\alpha$ -induced activation of NAD(P)H oxidase and preserving eNOS phosphorylation, suggesting the potential for new treatment approaches to promote vascular health in metabolic diseases<sup>13</sup>. Antioxidant defenses against this damage include ascorbate, tocopherol, and carotenoids.

In our laboratory, it has been shown that the powder of grain of *Triticum sativum* (Lisosan G), used as nutritional supplement, did not interfere with the phase 1 and 2 drug metabolizing enzymes and had hepatoprotective effects in rats intoxicated by carbon-tetrachloride<sup>14</sup>. In addition, it has shown a radical scavenger activity may be due to the presence of antioxidant molecules in this compound. Lisosan G is a good source of vitamins, oligoelements, polyunsaturated fatty acids. On the basis of these results, it was suggested that Lisosan G might be co-administrated during drug therapy<sup>14</sup>. Recently we have shown a protective effect of Lisosan G against the cisplatin induced toxicity. It has been suggested that the protective effect of Lisosan G could be associated mainly with the attenuation of the oxidative stress and the preservation in antioxidant enzymes<sup>15</sup>.

The present study was undertaken to evaluate the protective effects of Lisosan G on human microvascular endothelial cells (HMEC-1) exposed to ox-LDL. We also compared the protective mechanism of Lisosan G in copper-dependent LDL oxidation.

### Material & Methods

The study was performed in the laboratories of the Institute of Agricultural Biology and Biotechnology, CNR, Pisa, Italy.

*Cell cultures:* HMEC-1 was kindly provided by Prof. E W Ades (Center for Disease Control, Atlanta, USA). Primary cells were immortalized by transfection with PBR-322-based plasmid containing the coding region for the simian virus 40 large T<sup>16</sup>. Cells were cultured in M-199 medium (Sigma Life Sciences, USA) supplemented with 10 per cent (v/v) foetal bovine serum, 1 per cent glutamine, 1 per cent antibiotics, 1 per cent endothelial growth factor (EGF; Sigma Chemical Co., USA) and 0.5 per cent hydrocortisone, in a 95 per cent O<sub>2</sub>, 5 per cent CO<sub>2</sub> atmosphere and maintained at a density of 6x10<sup>5</sup> cells/ml by sub-culturing, every 4-5 days, a 1:2-1:3 dilution of cell suspension in fresh medium (cell viability was >95% as determined by trypan blue exclusion).

For all experiments, cells (at the 7<sup>th</sup>-8<sup>th</sup> passage) were plated in 60 x 15 mm dishes and allowed to reach 70 per cent confluency. In order to minimize interferences with the assays, 48 h before any treatment cells were incubated with M 199 medium without phenol red and supplemented with antibiotics only.

To evaluate the effect of Lisosan G was a gift by Agrisan Company, Pistoia, Italy on cells viability, HMEC-1 were incubated for 24 h at different doses (0, 0.07, 0.7, 1.4 mg/ml) of Lisosan G. The highest dose showing no effect on cell viability was used in the experiments (cell viability was >95% as determined by trypan blue exclusion). The powder of Lisosan G was sonicated in water and after centrifugation the supernatant was added to the medium 1 h before the treatment with ox-LDL.

ICAM-1, ET-1, and IL-6 concentration in the medium and the expression of the respective genes of HMEC-1 were measured in response to incubation with 200 µg/ml of ox-LDL or with co-incubation of Lisosan G, or with Lisosan G alone.

*LDL isolation and oxidized LDL preparation:* To isolate LDL, blood (40 ml) was collected in tubes containing EDTA (1 mg/ml) from 10 healthy volunteers recruited from laboratory staff of our institute and immediately centrifuged at 1207 g at 4°C. All the aliquots were pooled and LDL ( $d=1.019-1.063$ ) was isolated by ultracentrifugation at 158000 g, at 4°C (OPTIMA L-90 K, Beckman, CA, USA) in NaBr solutions with EDTA to avoid oxidation during isolation. All solutions were degassed, and samples were kept at 4°C in a dark environment. To remove EDTA, the LDL fraction was gel-filtered on a Sephadex column (Econo-Pac 10 DG columns; Bio-Rad, CA, USA) and eluted in 1 M PBS buffer (pH 7.4).

The oxidation of LDL solution (200 µg/ml) was performed after incubation with 10 µM CuSO<sub>4</sub> in M-199 medium at 37°C 24 h. After the challenge, CuSO<sub>4</sub> was removed by double filtration (Centricon Plus-20; Millipore Corp, Bradford, MA, USA). Immediately Ox-LDL solution was used for 24-h incubation of 70 per cent confluent monolayers of HMEC-1 placed in 6-well dishes containing 3 ml of medium and approximately 10<sup>5</sup> cell each.

The extent of oxidation was estimated by measuring the concentration of malondialdehyde (MDA) (as thiobarbituric acid-reactive substances, expressed as nmole MDA per mg LDL protein)<sup>17</sup>.

Medium concentrations of ET-1 and interleukin-6 (IL-6) were evaluated by two different kit of ELISA technique employing a solid-phase sandwich, enzyme-linked immuno-sorbent assay (Cayman, Ann Arbor MI, USA; Biosource International, Inc. Camarillo, California, USA). Medium concentration of ICAM-1 was evaluated with ELISA method (Bender MedSystem Vienna, Austria, Europe).

All analysis was done in duplicate, and concentrations were normalized to 10<sup>5</sup> cells by counting.

*Reverse transcription polymerase chain reaction (RT-PCR) for LOX-1 gene:* RNA extraction was performed with Micro-to-Midi Total RNA Purification System (Invitrogen Life technologies, USA). After removing medium, an RNA lysis solution was added to dishes, and distributed to the entire surface. Cell lysates were then transferred to tubes, and passed through a 21-gauge needle attached to a 1 ml syringe, 3-4 times. After adding 70 per cent ethanol, samples were applied to an RNA Spin Cartridge, and centrifuged at 12,000 g for 15 sec at 25°C. After washing with buffers, RNase-free water was added to the columns to collect RNA. In a 0.5-ml tube, 1 µl random primer (50 ng/µl) (Random primers are oligodeoxyribonucleotides, mostly hexamers, suitable for DNA synthesis), 1 µl RNA (10 pg/µl), and 2 µl of 10 mM dNTP Mix (Invitrogen Life technology, USA) was adjusted to 12 µl with diethylpyrocarbonate (DEPC) water. RNA was denaturated by incubating the solution at 65°C for 5 min and then placed on ice. The 5X cDNA synthesis Mix was prepared on ice: 4 µl of buffer, 1 µl of 0.1 M DTT, 1 µl water, 1 µl Ribonuclease (RNase) inhibitor (RNaseOUT) (40 U/µl) and 1 µl ThermoScript RT (15 U/µl) (Invitrogen Life Tecnology, USA). RT-PCR condition and primers utilized in our study are previously reported<sup>18-20</sup>.

*Determination of peroxidation products - LDL solutions:* LDL aliquots (50 µg protein/ml) were incubated with Cu<sup>2+</sup> at 37 °C using a Perkin Elmer spectrophotometer (PE-Lambda Bio2, England) equipped with a Peltier-thermostatted 8-position cuvette holder. Cu<sup>++</sup>-mediated LDL oxidation was monitored at 234 nm every 1.5 min for 2 h at 37 °C by the kinetic profiles of conjugated dienes (CD). It was characterized in terms of lag time (t lag), which reflects the protective effect of endogenous antioxidants and formation and breakdown of reactive intermediates of hydroperoxide<sup>18,19</sup>. For three experiment conducted, t lag was calculated graphically from the intercept

of lines drawn through the linear portions of the lag phase and the propagation phase.  $\text{Cu}^{2+}$ -mediated LDL oxidation was initiated in a buffer solution  $\text{PH} = 7.4$ , with  $0.2 \text{ mM CuSO}_4$ . The kinetic profiles of conjugated dienes were characterized by the respective  $t_{\text{lag}}$ . Lisosan-G concentrations analyzed were: 20, 40, 60, 80  $\mu\text{g/ml}$ . These concentrations were compared with a solution of vitamin C  $0.2 \mu\text{g/ml}$ .

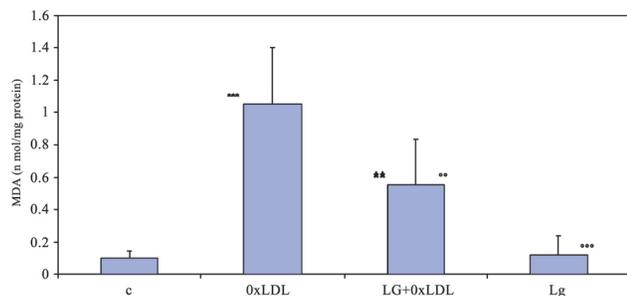
**Statistical analysis:** One-way ANOVA with Dunnet's post hoc test was used to evaluate the modulation of endothelial vasoactive products in HMEC-1 cells treated with ox-LDL or ox-LDL plus Lisosan G or only Lisosan-G. The same analysis was performed to establish difference among  $\text{Cu}^{++}$ -mediated LDL oxidation and with or without Lisosan G concentrations or in presence of vitamin C. Linear regression analysis was conducted to establish the association between each markers and MDA production.

## Results

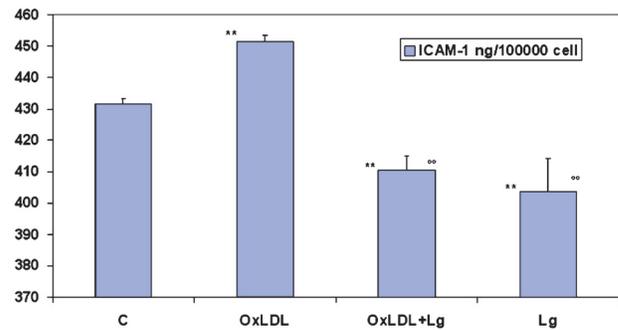
In a preliminary study, HMEC-1 were treated with different doses of Lisosan G (0, 0.07, 0.7, and 1.4  $\text{mg/ml}$ ) to find the optimal experimental dose to avoid cytotoxicity (data not shown). There was no difference among the doses, except a slight reduction of cells viability at the higher dose, consequently  $0.7 \text{ mg/ml}$  of Lisosan G was used in further experiments.

The induced oxidative stress by ox-LDL ( $200 \mu\text{g/ml}$ ) in HMEC-1 cells, was quantified by MDA formation ( $1.05 \text{ nmole MDA/mg protein}$ ). The MDA production was significantly higher than control ( $P < 0.001$ ) in the cell treated with ox-LDL (Fig. 1).

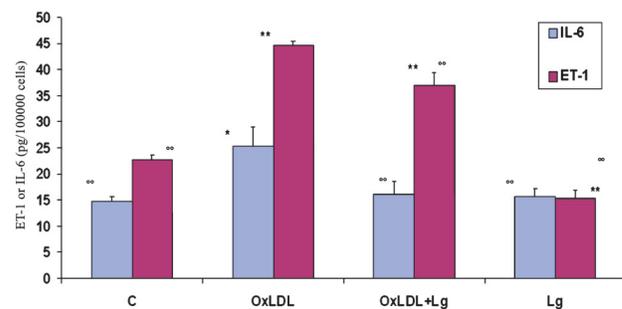
The incubation with ox-LDL induced an increment of ICAM-1 medium concentration compared to the basal condition ( $P < 0.01$ ), while in presence of Lisosan G a significant reduction was observed compared to



**Fig. 1.** MDA production in presence or absence of ox-LDL and Lisosan G. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared to control; °° $P < 0.01$ , °°° $P < 0.01$  compared to ox-LDL.



**Fig. 2.** Effect of oxLDL on endothelial ICAM-1 production. Lisosan G shows a significant reduction of ICAM-1 medium concentration (ELISA method). \*\* $P < 0.01$  compared to control; °° $P < 0.01$  compared to ox-LDL incubation.

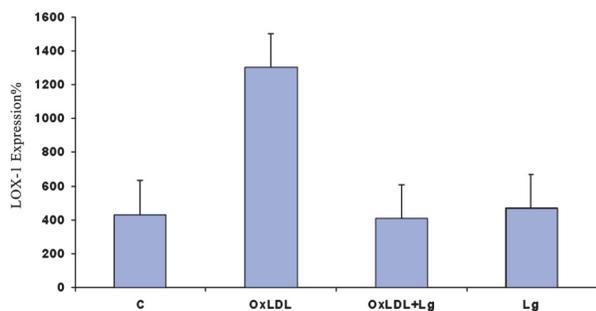


**Fig. 3.** Effect of ox-LDL on endothelial IL-6 and ET-1 production. These markers were significantly reduced after the incubation with Lisosan G. \* $P < 0.05$ , \*\* $P < 0.01$  compared to control; °° $P < 0.01$  compared to ox-LDL incubation.

cultures treated with ox-LDL and control ( $P < 0.01$ ) (Fig. 2).

IL-6 medium concentration increased after treatment with ox-LDL compared to basal condition ( $P < 0.05$ ), while the treatment with Lisosan G incubated with ox-LDL or alone did not show any difference suggesting a protective effect (Fig. 3). The analysis of ET-1 medium concentration showed a significant increase after ox-LDL treatment compared to control ( $P < 0.01$ ), but reduced after co-treatment with Lisosan-G (Fig. 3). The treatment with Lisosan G alone reduced the concentration below the basal condition (Fig. 3).

Semi-quantitative analysis of *LOX-1* gene expression showed a significant upregulation only after ox-LDL exposure (Fig. 4). Linear regression analysis conducted between MDA concentration and production of ICAM-1, IL-6 and ET-1 in the group of cells exposed to ox-LDL with or without Lisosan-G, showed an association especially in the first two cases,



**Fig. 4.** Semi-quantitative RT-PCR analysis of *LOX-1* gene after endothelial incubation with ox-LDL and Lisosan G.

respectively ( $P < 0.01$ ,  $R^2 = 0.28$ ;  $P < 0.01$ ,  $R^2 = 0.0055$ ;  $P < 0.06$ ,  $R^2 = 0.40$ ).

The analysis of the effect of Lisosan G on  $\text{Cu}^{++}$ -mediated LDL oxidation is reported in Fig. 5.

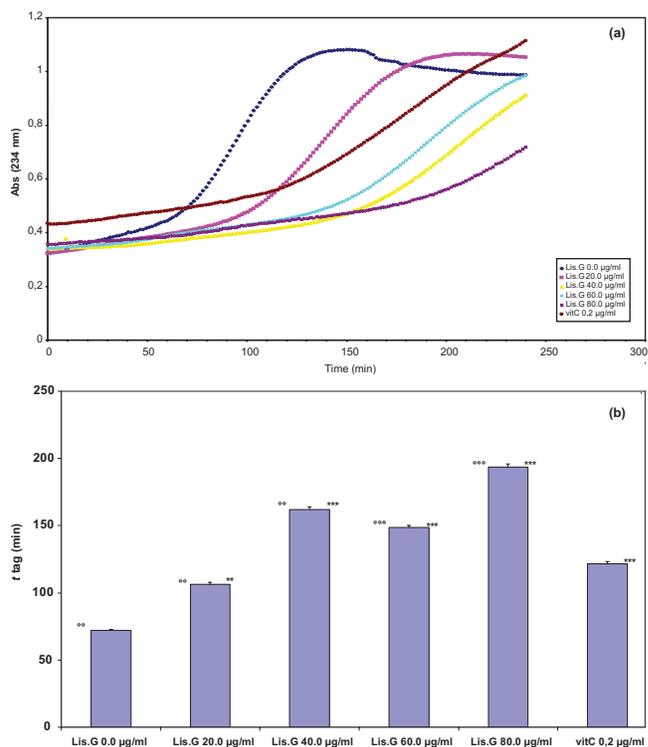
The calculated  $t$  lag, shows a significant increment in relation with the dose of Lisosan G. The effect of vitamin C (0.2  $\mu\text{g}/\text{ml}$ ), used as a positive control, showed a significant higher  $t$  lag respect to Lisosan (G 20  $\mu\text{g}/\text{ml}$ ), but significantly reduced respect to the other levels of Lisosan G.

### Discussion

It is now well established that adhesion of monocytes to the endothelium is one of the earliest cellular events in atherogenesis. Several mechanisms promote cell-cell interaction between monocytes and endothelial cells. Our study demonstrated that ICAM-1 was basically expressed by HMEC-1 cells. Further, the inflammation process initiated by ox-LDL on cultured HMEC-1 cells has been shown to promote significantly the expression of adhesion molecule ICAM-1<sup>20</sup>. In our study, ox-LDL induced significantly the expression of ET-1 (2-fold the control), IL-6 and *LOX-1* (3-fold) compared to control. Similar results have been shown earlier<sup>21,23</sup>.

The exposure to Lisosan-G not only reduced the effect of ox-LDL on ICAM-1 production, but also showed a significant reduction in ICAM-1 compared to control. This suggests the role of this natural compound to prevent the early events of atherosclerosis or microcirculatory dysfunction. Lisosan G co-incubated with ox-LDL partially reduced the increment of ET-1 suggesting that this effect seems not to be linked exclusively to the oxidation, but also to the presence of LDL, as described earlier by Lubrano *et al*<sup>21</sup>.

Except for ET-1, since the regulation of ICAM-1, IL-6 expression by ox-LDL appears to be the result of the redox activity of ox-LDL induced by oxygen free radicals present in the cellular environment. As a result, the rescue of oxygen free radicals by Lisosan G, during the 1 h pre-exposure period, could reduce the extension of the cellular oxidative stress induced by ox-LDL. Consequently, the levels of ICAM-1 and IL-6 in the medium were decreased at control level or below. The decrease in oxidative stress induced by Lisosan G was also shown by the significant reduction of MDA concentration, that is directly associated especially with the production of ICAM-1 and IL-6. These results were confirmed by PCR analysis for ICAM-1, IL-6 expression, and prepo-ET-1 mRNA. As previously reported the ET-1 biosynthesis is mediated by membrane-bound  $\text{Zn}^{2+}$ - metalloproteases, endothelin-converting enzymes (ECE-1), which catalyze *in vitro* and *in vivo* the biotransformation of the inactive big endothelin to the biologically active ET-1<sup>24</sup>. Elevated levels of this hormone have been implicated in the



**Fig. 5 (a).** Kinetic curves of  $\text{Cu}^{++}$ -induced LDL oxidation absence and in the presence of increasing doses of Lisosan G or in presence of vitamin C. **(b)**  $t$  lag increase in relation with the enhancement of Lisosan G dose.  $t$  lag of vitamin C was confronted.  $P^{**} < 0.01$ ,  $*** < 0.001$  compared to control;  $^{\circ\circ} P < 0.01$  compared to  $t$  lag of vitamin C X 0.2  $\mu\text{g}/\text{ml}$ ;  $^{\circ\circ\circ} P < 0.01$  compared to  $t$  lag of vitamin C 0.2  $\mu\text{g}/\text{ml}$ .

development of various pathophysiological disorders including hypertension, atherosclerosis and congestive heart failure<sup>25</sup>.

LOX-1 was initially identified as the major receptor for ox-LDL in endothelial cells and was later found to have an inducible expression in macrophages and smooth muscle cell<sup>26,27</sup>. LOX-1 activation by ox-LDL binding stimulates intracellular signalling, gene expression and production of superoxide radicals<sup>28,29</sup>. *In vivo*, its expression is enhanced in pro-atherogenic settings including hypertension, hyperlipidaemia, diabetes, and atherosclerosis.

It has been shown that soluble form of LOX-1 correlated with oxidative stress markers in stable coronary artery disease<sup>30</sup>. Our results showed that Lisosan G can inhibit the upregulation of LOX1 in endothelial cells. Lisosan G showed a strong ability to inhibit Cu<sup>++</sup>-induced lipid peroxidation in humans LDL suggesting strong antioxidant properties.

In conclusion, our results demonstrate that Lisosan G may be considered a potential candidate in the prevention of atherosclerosis and microcirculatory dysfunction reducing the oxidative/inflammatory process. Further, Lisosan G could be an important food supplement to prevent the damaging effect induced by oxidative stress on endothelial cells. Additional *in vivo* studies need to be carried out to confirm the beneficial effects of Lisosan G against endothelial cells damage.

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