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Antioxidant-rich food supplement Lisosan G induces reversion of hepatic steatosis*)

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Summary

Antioxidant activity (AA) of Lisosan G, a food supplement obtained from wheat grains, was evaluated by using the LOX/RNO, ORAC and TEAC methods. Very high AA was found, associated with a high fraction of freely-extractable phytochemicals expected to be readily absorbed in the small intestine and to exert systemic healthy effects. So, a possible beneficial effect of Lisosan G against non-alcoholic hepatic steatosis induced in mice by 90 days of hyperlipidic diet was studied. Feeding Lisosan G to mice with hepatic steatosis caused rapid reversion of liver disorder: just after 7 days the liver resulted free of steatosis, showing triglyceride content and histological properties similar to control group. Contrarily, the switch of hyperlipidic diet towards a standard diet did not rapidly reverse hepatic steatosis, with a recovery observed only after 30 days. Our results shed a first insight on the therapeutic potential of Lisosan G against the hepatic steatosis disorder in animal species.

Keywords: antioxidant activity, hepatic steatosis, hyperlipidic diet, Lisosan G, oxidative stress

Abbreviations: AA = antioxidant activity; AAPH = 2,2'-azobis(2-amidinopropane); ABTS = 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate); fluorescein = 3',6'-dihydroxyspiro[isobenzofuran-1[3*H*], 9'[9*H*]-xanthen]-3-one; CYP2E1 = isoform 2E1 of hepatic cytochrome P450 oxidase system; HFD = high fat diet; LOX = lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12); ORAC = Oxygen Radical Absorbance Capacity; RNO = 4-nitroso-*N,N*-dimethylaniline; SD = standard diet; TEAC = Trolox Equivalent Antioxidant Capacity; Trolox = (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

In recent years the use of food supplements has become increasingly popular. So, nutraceutical value and therapeutic properties of these nutritional supplements have to be properly investigated, in particular by evaluating the effects on organs/tissues representing the main targets of their chemical components.

Lisosan G, a food supplement obtained from lysed fine bran and germ of organic wheat grains, has been recently investigated, showing interesting properties. It is characterized by a high content of polyunsaturated fatty acids, vitamins and oligoelements. Lisosan G has been demonstrated to not interfere with drug metabolizing enzymes and to possess a good radical scavenging activity against the non-physiological 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation (13). In addition, this nutritional supplement has been reported to protect rats against toxicity induced in liver by carbon tetrachloride (13) and in liver, kidney and testis by cisplatin, possibly by attenuating oxidative stress and by preserving antioxidant enzymes (14).

A goal of this study was to evaluate in some detail antioxidant activity (AA) of Lisosan G. For this purpose, hydrophilic and lipophilic antioxidant compounds, as well as insoluble-bound and free-soluble phenols, were extracted from Lisosan G and these extracts were then analyzed for AA and for their content in some antioxidant compounds. AA was evaluated using the recently developed lipoxygenase/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) method (22), based on the RNO bleaching reaction associated to linoleic hydroperoxidation catalyzed by soybean

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LOX-1 isoenzyme (23). With respect to the majority of AA assays, the peculiarity of this method is to use physiological radical species, to simultaneously detect different antioxidant mechanisms and to better highlight the synergistic effects among antioxidants (22); so, the new method may provide AA information more relevant from a biological point of view and potentially related to food health benefits. In addition to the LOX/RNO method, two well-known methodologies for AA assessment were used: the Trolox Equivalent Antioxidant Capacity (TEAC) assay (24) and the Oxygen Radical Absorbance Capacity (ORAC) method (19), measuring the scavenging capacity against ABTS radical cation and peroxyl radicals, respectively.

Hepatic steatosis is an emerging world-wide liver disorder in several animal species, which can progress to steatohepatitis and liver cirrhosis and interest several animal species (4, 8, 9). It was reported that steatosis is the most common liver affection in dairy cattle during lactation. It may lead to various disease like inflammation of reproductive organs, and displacement of the abomasum, a condition associated to an high mortality rates if not treated. Since steatosis is a well-known syndrome in peri-parturient dairy cattle, sheep, cats and horses, producer groups and practicing veterinarians have an increased interest in the pathogenesis and in the prevention of this disease. A recent study have demonstrated accelerated apoptosis of hepatocytes in dairy cows with fatty infiltration of the liver (27).

The mechanisms associated with liver steatosis are multifactorial and not fully understood (1), but redox imbalance and oxidative stress are considered mechanisms of hepatocellular injury in non-alcoholic steatosis. So, the use of antioxidants and hepatoprotective plant products as treatment strategy of non-alcoholic fatty liver diseases has become popular in the last decade (6). In the light of this, to move from *in vitro* assays to an *in vivo* investigation level, the possible bioactivity of Lisosan G was evaluated by studying its effects on high fat diet (HFD)-induced mouse hepatic steatosis, a disease potentially responsive to antioxidants. Really, liver may represent one of the first target organs of highly bioavailable antioxidants, so the individuation of an antioxidant-rich food supplement, acting against an emerging liver disease, is a reasoning goal.

Material and methods

Chemicals. Lisosan G is registered as nutritional supplement by the Italian Minister of Health and was supplied by Agrisan Company (Larciano, PT, Italy). HFD was purchased from Mucedula (Settimo Milanese, MI, Italy). All other chemicals and solvents were of standard brands available on the market and were of the highest commercially available purity.

Extraction of hydrophilic, lipophilic and phenolic compounds. Hydrophilic extracts were prepared as described in Laus et al. (11) by extracting Lisosan G with deionized water at a (w/v) ratio equal to 1 g/5 mL in an ice-water bath for 1 h.

Lipophilic compounds were extracted according to the procedure described in Panfili et al. (21), modified as in Laus et al. (11). Briefly, Lisosan G (2 g) was saponified with 60% (w/v) KOH under nitrogen at 70°C for 45 min and then the suspension was extracted 4 times with (9 : 1, v/v) *n*-hexane/ethyl acetate. The organic phases were evaporated to dryness under vacuum at 40°C and the dry residue was reconstituted in 2 mL of ethanol.

Insoluble-bound and free-soluble phenolic compounds were extracted according to the procedure described in Sosulski et al. (26), modified as reported in Laus et al. (11). Briefly, free-soluble compounds were obtained by two extractions of Lisosan G (1 g) with 10 mL of 80% (v/v) ethanol at room temperature; bound phenolic compounds were released by alkaline hydrolysis with 20 mL of 2 M NaOH at room temperature for 1 h under nitrogen. The extracts and hydrolysates were purified by two extractions with *n*-hexane and three extractions with ethyl acetate. The ethyl acetate fractions were evaporated to dryness under vacuum at 40°C and the dry residues were reconstituted in 1.5 (free) or 2 (bound) mL of water.

Spectrophotometric determination of total phenolic, flavonoid and protein contents. Total phenolic, flavonoid and protein contents of hydrophilic and phenolic extracts were determined by means of a PerkinElmer Lambda 45 UV/Vis spectrophotometer (PerkinElmer, Wellesley, MA, USA), according to the methods described by Singleton et al. (25), Dewanto et al. (3) and Waddell and Hill (29) respectively, modified as reported in Laus et al. (11).

Determination of Antioxidant Activity (AA) by means of the LOX/RNO, TEAC and ORAC methods. The LOX/RNO reaction was spectrophotometrically monitored, as described in Pastore et al. (22, 23), by measuring the RNO absorbance decrease at 440 nm and 25°C. The (%) decrease of the rate of RNO bleaching measured in the presence of extract, with respect to the rate of the control reaction, was measured. AA values were obtained by means of a dose--response curve obtained using (±)-6-hydroxy-2,5,7,8--tetramethylchromane-2-carboxylic acid (Trolox) as standard antioxidant.

The TEAC protocol, reported in Re et al. (24) and modified as in Pastore et al. (22), was used. The $ABTS^{\bullet+}$ radical cation was produced by ABTS oxidation with potassium persulfate solution. Absorbance of a diluted solution of $ABTS^{\bullet+}$ was measured at 734 nm and 25°C (A_{734}) after 5 or 2.5 min of incubation with hydrophilic/phenolic or lipophilic extracts respectively. The (%) decrease of A_{734} measured after extract incubation, with respect to A_{734} of the uninhibited radical cation solution, was calculated; AA was determined by means of a proper concentration-response curve obtained with Trolox.

The ORAC protocol, described in Ou et al. (19) and modified as in Pastore et al. (22), was applied. Fluorescence intensity decay due to 3',6'-dihydroxyspiro[isobenzofuran--1[3*H*], 9'[9*H*]-xanthen]-3-one (fluorescein) oxidation by peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) (AAPH) thermal decomposition was continuously

monitored at 37°C at excitation and emission wavelengths of 485 and 515 nm respectively, by means of a Perkin Elmer LS 55 spectrofluorimeter. The area under the fluorescence decay kinetic curve was calculated. AA was quantified using a calibration curve obtained with Trolox.

Animals and experimental protocol. The experimental protocol was performed in compliance with the relevant laws and institutional guidelines and was approved by the Ethic Committee for Animal Care. Thirty six male mice of 25-30 g were used. Twenty-one mice were fed with a HFD for 90 days (HFD group). Fifteen were used as control and fed with standard diet (SD group). After 90 days three animals were sacrificed from HFD group to check the grade of steatosis. The other HFD animals were divided in two groups each of nine animals: a group was fed with SD (HFD/SD) and the other only with Lisosan G (HFD/LIS). After 7, 15 and 30 days three animals for each group were sacrificed to analyze liver.

Histological examinations. Livers from mice of the SD, HFD, HFD/SD and HFD/LIS groups were removed at different times of diet changes and immediately fixed in 10% formalin. Tissues were subjected to standard histological techniques, including dehydration, embedding in paraffin, and cutting into 5-µm-thick sections, followed by staining with hematoxylin and eosin. Histological analyses of the liver were performed under light microscope Zeiss, Observer Z.1. Three mice for each thesis were analyzed.

Determination of hydroxylation of aniline and triglyceride content. The hydroxylation rate of aniline to 4-aminophenol, marker of the isoform 2E1 of hepatic cytochrome P450 oxidase system (CYP2E1) was determined in liver microsomes using the method of Ko et al. (7). Triglyceride content was measured in liver homogenates using a kit (Giesse Diagnostics, Rome, Italy).

Results and discussion

Antioxidant activity (AA) of Lisosan G extracts as evaluated by the LOX/RNO, TEAC and ORAC methods. A first goal of this study was to study in some detail AA of Lisosan G. As shown in Table 1, the LOX/RNO method measured the highest AA values for the hydrophilic component; this resulted the most active fraction also according to the TEAC and ORAC protocols. With regards to phenolic extracts, all three methods showed much higher AA values for the insoluble-bound component than the free-soluble one, in agreement with many literature data reporting insoluble-bound phenols as the major phenolic component in cereal whole grains (12). Interestingly, only the LOX/RNO method revealed remarkable AA values for the lipophilic extract. The comparison among these different AA assays shows the peculiarity of the LOX/RNO method to measure much higher AA values for all antioxidant components. This result fits well with previous data obtained for wheat grains (10, 11, 22) and it is expected, given the ability of the new method to simultaneously detect several important antioxidant functions and synergistic interactions among antioxidant compounds (22). On the whole, these results indicate a very high total AA of Lisosan G; compared with other wheat species, it resulted from about 3- to 10-fold higher, depending on the cereal species and AA assay (10, 11).

Tab. 1. Antioxidant activity (AA), evaluated by means of the LOX/RNO, TEAC and ORAC methods, of hydrophilic, lipophilic and phenolic extracts from Lisosan G

Extract	AA (µmol Trolox eq./g dry weight)		
	LOX/RNO	TEAC	ORAC
Hydrophilic extract	1576 ± 27	54 ± 4	195 ± 19
Free-soluble phenolic extract	83 ± 2	8.5 ± 1.7	27.2 ± 1.5
Insoluble-bound phenolic extract	1294 ± 24	33.5 ± 1.8	74.6 ± 3.8
Lipophilic extract	258 ± 2	3.7 ± 0.8	1.1 ± 0.1

Explanation: Data are reported as mean value ± standard error (n = 4)

Consistently with the high AA values, the hydrophilic extract from Lisosan G showed very high water-soluble flavonoid concentration and protein content, about 50- and 7-fold, respectively, higher than that measured in durum wheat whole flour (11). In agreement with published data for cereal whole grains (2), total phenolic content was higher in the insoluble-bound extract than in the soluble one. Interestingly, the ratio insoluble/soluble phenols was lower than that obtained for wheat: only 2.2, *i.e.* about 4-fold lower than that measured for example in durum wheat (11). This result, together with the very high AA of hydrophilic fraction, strongly suggests that the type of processing of wheat grains to obtain Lisosan G may significantly allow the release of a lot of cell wall-linked phytochemicals. To better highlight this aspect, the ratio between the sum of AA values obtained for the hydrophilic (H), lipophilic (L) and free-soluble phenolic (FSP) fractions, and AA values relative to the insoluble-bound phenolic component (IBP), was calculated. For the LOX/RNO, TEAC and ORAC methods, $AA_{(H+L+FSP)}/AA_{(IBP)}$ ratios calculated from data of Table 1 were about 1.5, 2 and 3, respectively. Interestingly, these values resulted about 5-, 2- and 2-fold, respectively, higher than that obtained for durum wheat grains (8, 10, 11). This may have physiological relevance. In fact, free phenolic compounds (aglycones) and some glucosides can be readily absorbed in the small intestine (16); this also occurs in the case of hydrophilic and lipophilic antioxidants, like vitamins C and E, respectively (28). These antioxidants are expected to rapidly exert healthy systemic effects in the body. On the contrary, some phenolic compounds in the esterified form or bound to cell wall polymers are very poorly absorbed or not absorbed at all at the small intestine, while they may be released mostly by colonic microflora digestion (16). Since absorption occurs in the colon less readily than in the small intestine because of a smaller exchange area and a lower density of transport systems, bound phenols are absorbed less

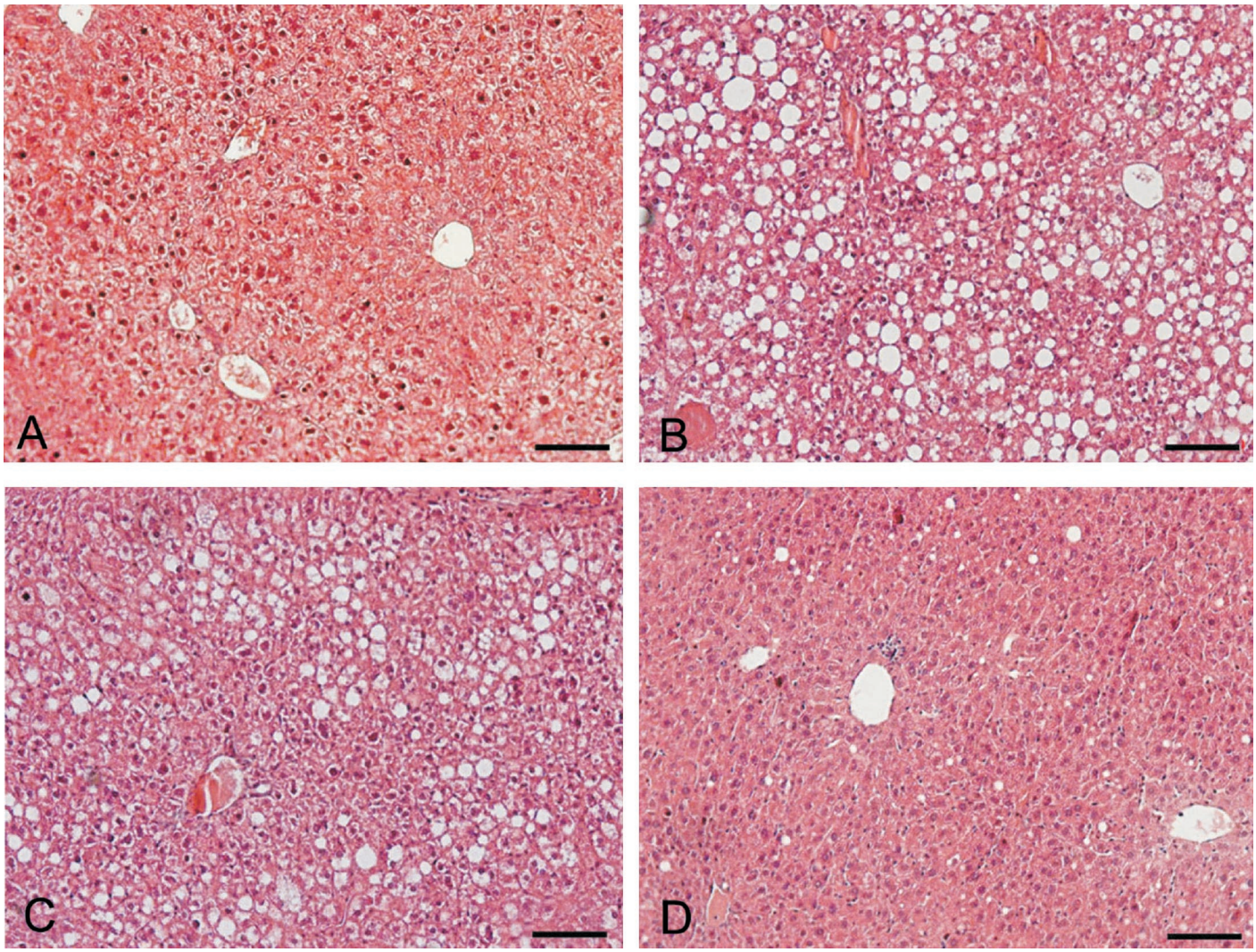

Fig. 1. Histological analyses of hepatic architecture in response to diet changes. Sections (5 µm thick) of liver obtained from: mice fed with standard diet (SD) (A); mice fed with high fat diet (HFD) for 90 days (B); HFD group followed by a recovery of 7 days with SD (HFD/SD group) (C); HFD group followed by a recovery of 7 days with Lisosan G (HFD/LIS group) (D). Bar = 100 µm

rapidly and less efficiently than the free and soluble esterified ones. As a consequence, a major local activity of bound phenols is expected in the terminal intestine, while a delayed, lower though continuous, systemic activity is expected (16, 28). On the whole, the very high AA of Lisosan G, together with the high $AA_{(H+L+FSP)}/AA_{(IBP)}$ ratio, may be indicative of a greater and more ready antioxidant bioavailability.

This suggests a possible use of Lisosan G in the treatment of diseases when high doses of easily absorbed antioxidants may be beneficial. First of all, liver diseases, being liver one of the first target organs of bioavailable phytochemical action. So, in this study the effect of Lisosan G on the hepatic steatosis, an emerging world-wide liver disorder in animal species, was evaluated in mice.

Effect of Lisosan G on hepatic steatosis induced in mice by high fat diet. Steatosis was induced by feeding mice with HFD for 90 days. In Fig. 1 and 2 the histological alterations and recovery occurring in mice livers in response to diet changes are showed. Treated mice (HDF group) clearly showed dietary-induced hepatic steatosis after 90 days of HFD: excess lipids resulting in macrovesicular and microvesicular steatosis were evident in hepatocyte cells of HFD group (Fig. 1B) when compared to SD group (Fig. 1A). After 90 days, HFD was stopped and mice were divided in two groups, fed with either SD (HFD/SD) or Lisosan G (HFD/LIS), in order to follow the recovery of normal liver morphology. Animals were then analyzed at 7, 15 and 30 days from the beginning of the new diet. After 7 days the HFD/SD group showed only a slight recovery, being lipid accumulation still predominant in liver tissue cells (Fig. 1C); on the contrary, the liver histology in HFD/LIS group already returned fairly similar to SD mice, in which steatosis was rarely present in hepatocyte cells (Fig. 1D and A). After 15 days, differences between the HFD/LIS and HFD/SD groups were still evident (Fig. 2A and B). Finally, after 30 days from the replacement of HFD, histological analyses demonstrated the complete recovery of normal liver morphology in both groups of mice (Fig. 2C and D).

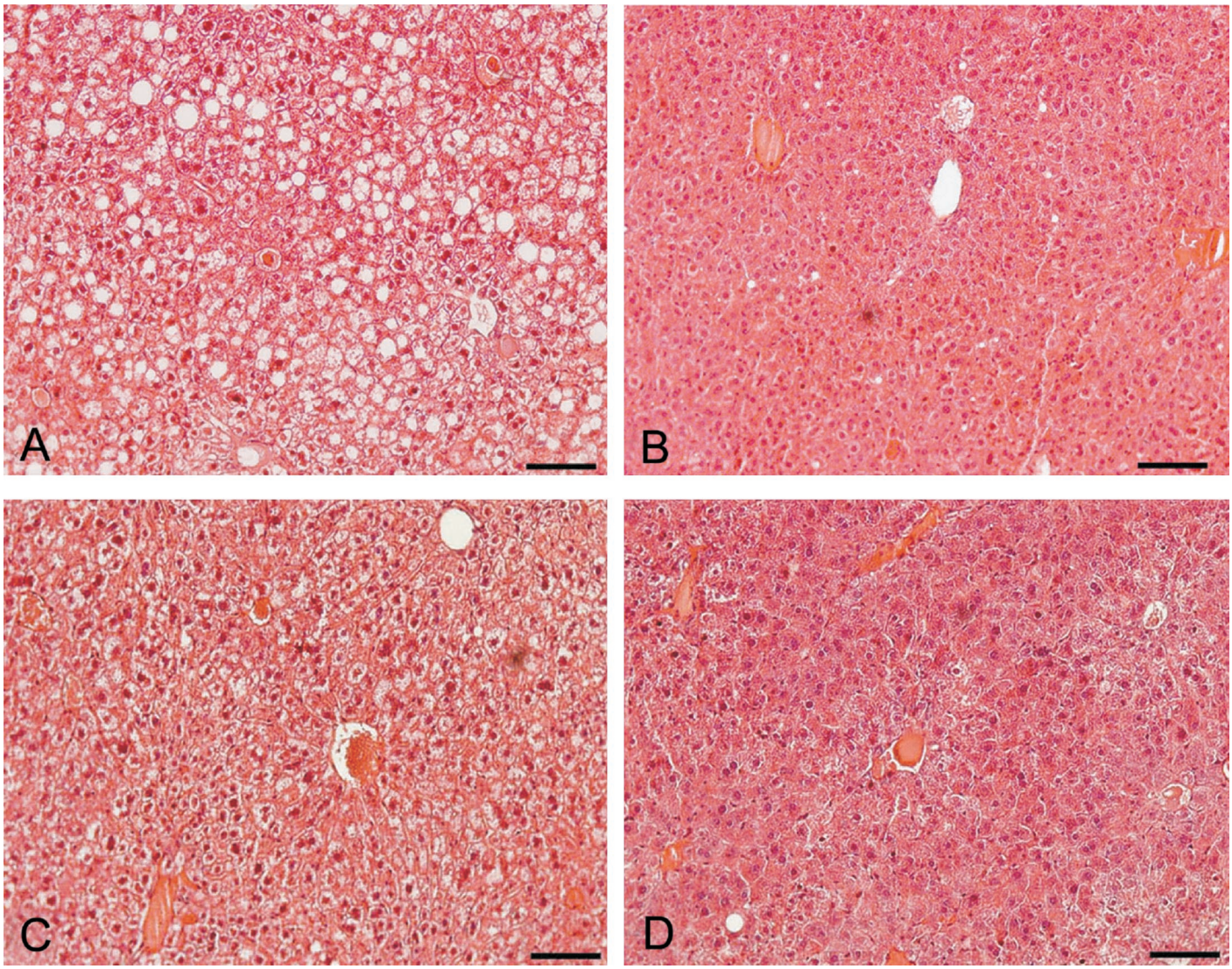

Fig. 2. Histological analyses of hepatic architecture in mice in response to diet changes. Sections of liver from: HFD/SD group after 15 days (A) and 30 days (C) of SD; HFD/LIS group after 15 (B) and 30 days (D) of Lisosan G diet. Bar = 100 µm

After 90 days of HFD the triglyceride content in liver was double that of SD group [21 ± 4 and 10 ± 3 (standard deviation, n = 3) mg triglycerides/g tissue, respectively]. Consistently with histological observations, following the new diet with Lisosan G, liver triglyceride content returned to SD group levels quickly after 7 days. On the contrary, the hepatic triglycerides in the HFD/SD group were still about 2.2- and 1.9--fold higher than that of SD group at 7 and 15 days respectively, and returned to control level only after 30 days. No significant differences were observed in animal weight between HFD/SD and HFD/LIS groups.

These results indicate that Lisosan G feed preparation is able to rapidly free mice liver from steatosis, returning it to control levels both in terms of histological properties and triglyceride content after only 7 days. By contrast, HFD/SD animals did not improve in this short period: after 15 days their liver still showed fat and steatosis and it became comparable to control group only after 30 days.

It is known that nutritionally-induced obesity in animal models may cause changes in drug metabolizing enzymes and transporters (5). In particular, in mice fed with a HFD for 16 weeks the CYP2E1 enzyme was induced in response to elevated liver concentrations of ketones and fatty acids (17). For this reason, the activity of the aniline hydroxylase associated to CYP2E1 was measured in both SD and HFD groups. In the adopted experimental condition, the HFD for 90 days was found to not modulate CYP2E1, being the values 1.5 ± 0.2 and 1.7 ± 0.3 nmol/min/mg prot. for SD and HFD groups, respectively. This result is in agreement with Ghose et al. (5), suggesting that the duration of the HFD was not sufficient to induce this enzyme and that the adopted HFD induced only a light steatosis. Consistently, the body weight of mice, as well total plasma cholesterol content, resulted not changed between SD and HFD groups (data not shown).

Although molecular mechanisms of beneficial effects of Lisosan G in mice hepatic steatosis have still

to be investigated, an important role of its antioxidant properties may be suggested, although other mechanisms cannot be excluded. Many studies have reported a protective effect of plant extracts or dietary components against liver disorders associated to their antioxidant action. A long-term apricot feeding was reported to exert beneficial effects on carbon tetra chloride-induced liver steatosis and damage in rats due to its antioxidant components and its strong radical--scavenging ability (20). Flavonoids from *Litsea coreana* Levl. have been shown to improve liver injury and protect rats from liver fibrosis, due to their ability to regulate the lipid metabolism, as well as to their AA (6). Geniposide, a glucoside extracted from *Gardenia jasminoides* Ellis, has been demonstrated to exert a protective effect against hepatic steatosis in HFD-fed rats, with a mechanism associated with its antioxidant action (15). Pycnogenols, a family of flavonoids isolated from French maritime pine bark, have been recently reported to prevent the progression of non--alcoholic steatohepatites in rats by acting as antioxidants (18).

In this study the property of Lisosan G, a bioavailable antioxidant-rich food supplement, to induce a rapid recovery of mice hepatic steatosis in terms of histological properties and tryglyceride content is shown for the first time. This sheds a first insight on the potential therapeutic application of Lisosan G against this emerging hepatic disorder. Proper investigations are now necessary to evaluate if the inclusion of Lisosan G in the diet of animals can have a hepatoprotective effect.

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