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[60]Fullerene is a Powerful Antioxidant in Vivo with No Acute or Subacute Toxicity

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ABSTRACT

In the present work, we report the effects of C_{60}-pretreatments on acute carbon tetrachloride intoxication in rats, a classical model for studying free-radical-mediated liver injury. Our results show that aqueous C_{60} suspensions prepared without using any polar organic solvent not only have no acute or subacute toxicity in rodents but they also protect their livers in a dose-dependent manner against free-radical damage. To be sure, according to histopathological examinations and biological tests, pristine C_{60} can be considered as a powerful liver-protective agent.

Thanks to its spherical molecules with 30 carbon double bonds, [60]fullerene or C_{60}[1] can react easily with free radicals: it is a very efficient free-radical scavenger,[2] which labels this molecule as a "radical sponge". Because pristine C_{60} is soluble in only a limited number of solvents, such as toluene or dichlorobenzene, it was necessary to resort to water-soluble C_{60}-derivatives as free-radical scavengers in several biological systems,[3,4] thus demonstrating robust antioxidant properties.[5] Administration of a carboxylated C_{60}-derivative has been patented recently as a method for increasing metazoan's lifespan in order to illustrate these findings.[6] Surprisingly, despite a large number of experiments performed by several teams from different countries showing that [60]fullerene has no acute or subacute toxicity in several biological systems, international headlines reported recently that some aqueous dispersion of this fullerene might be very toxic in living systems such as bacteria, algae, and fishes by inducing oxidative stress.[7−10] According to the latter authors, C_{60} derivatives are less toxic than pristine C_{60}, which is contrary to all previous studies.[11−14]

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In previous experiments, we administered intraperitoneally (ip) micronized C_{60} into Swiss mice.[15] Despite the large amounts injected (2.5−5.0 g/kg of body weight), C_{60} did not show lethal, acute,[15] or subacute[16] toxicity with respect to this animal species. Nevertheless, in early stages, C_{60} induced hypertrophy and hyperplasia of hepatic stellate cells (HSCs: liver resident nonparenchymal cells also referred to as fat-storing or perisinusoidal cells, lipocytes, and Ito cells) where it mainly accumulates.[15] HSCs play a central role in the production of extracellular matrix in both normal and fibrotic liver.[17] The phenomena of hypertrophy and hyperplasia of HSCs, showing the activation of these cells, usually occurs under different pathological conditions leading to liver fibrosis.[17] Indeed, the activation of HSCs irremediably leads to their transformation into myofibroblast-like cells (MFC).[17] This phenomenon is now recognized as the main event in hepatic fibrogenesis, which occurs through the amplification of extracellular matrix production by these cells.[18] Furthermore, the transformation of HSCs into MFCs can be modulated by oxidative stress-related products[18] as reflected in rat intoxication with CCl_4, a well-known in vivo free radical initiator.[19,20]

In the case of C_{60}, however, despite HSC activation, the mouse liver structure remained normal and no fibrosis, either sinusoidal or portal, developed.[15] Fifty-six days after C_{60}-treatment, HSCs decreased in number without any transfor-

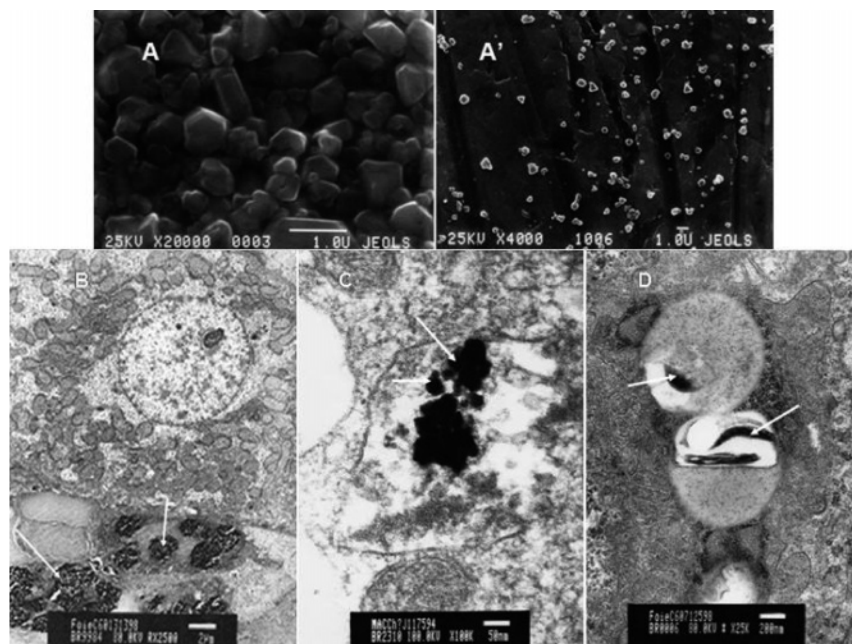

Figure 1. Electron microscopy. (A) SEM micrographs of the aqueous suspension of mC_{60} used in this study; (A') dilution of A; (B−D) TEM micrographs of liver sections of C_{60}-treated rats; (B and C) C_{60} clusters (arrows) inside the liver cells; (D) dissolution of C_{60} inside lipid droplets.

mation into MFCs.[16] This surprising phenomenon of inhibition of HSCs transformation into MFCs may be attributed to the free-radical-scavenging properties of C_{60}. To check this hypothesis, we investigated the effects of C_{60} against the radical-related toxicity of carbon tetrachloride in rats, which provides an important model for elucidation of the mechanism of action of hepatotoxic effects such as fatty degeneration (steatosis), fibrosis, hepatocellular death, and carcinogenicity.[18,20] This work allowed us to check the harmlessness of C_{60} to another rodent species as well.

Kinetics of C_{60} Accumulation in the Livers. Before studying the effects of C_{60} on CCl_4 acute toxicity, we determined the kinetics of accumulation and excretion as well as the distribution of this fullerene in liver rats.

Forty two rats housed in individual metabolic cages were treated with an intraperitoneal (ip) injection of an aqueous suspension of micronized C_{60} (0.5 g/kg of body weight (bwt)) (Figure 1A). Every day of the first week after the treatment and then every week until D_{21}, three rats were sacrificed for histopathological examination, biochemical tests, and C_{60} determination in livers.

Figure 2A shows that the maximum of accumulation, which reached about 24% of the injected amounts, occurred during the first week following injection. At D_{14} and D_{21}, the medians of the concentrations decreased to 5% and 1% of the values measured at D_7, respectively, indicating that C_{60} can be eliminated and/or transformed by the rat livers.

The fecal elimination of unmodified C_{60} is illustrated in Figure 2B. Maximum elimination occurred at D_7; however, the total of the maximum amount eliminated during the first 2 weeks represents less than 14% of the maximum amount contained within the livers on the same time. Thus, the main part of injected fullerene has probably been transformed in the livers.

The chromatographic profile of a hepatic extract of a C_{60}-treated rat, obtained under the same conditions as for mouse livers,[21] is represented in Figure 2C. This chromatogram shows some additional peaks eluting between those corresponding to the retinyl-esters and the peak of C_{60}, which are absent in hepatic extracts obtained from the control rats. The two main additional peaks correspond to C_{60}-retinol monoadducts formed in the liver following a Diels−Alder-like reaction because (1) they exhibit the same spectral features (Figure 2D) and they have the same retention characteristics as those of the C_{60}-retinol monoadduct we isolated previously from mouse livers;[21] and (2) like vitamin A, they are sensitive to air-oxidation and heating, indeed, they can be converted into unmodified C_{60} after heating at 50 °C for 10 min. These results suggest a possible excretion of C_{60} via the retinol catabolism. To confirm this new pathway of xenobiotic detoxication, it is necessary for one to isolate and quantify the C_{60}-retinol end products from feces and/or urine.

Distribution of C_{60} in the Liver. After anaesthesia and abdomen incision, the livers of all C_{60}-treated animals exhibited normal morphology, irrespective of the injected amounts. The liver colors depended on the individuals, the amount of C_{60} injected, and the processing times, which indicates that the distribution of fullerene in this organ was not uniform in every case. After injection with large amounts of C_{60} (2 g/kg of body weight), two-thirds (4/6) of the treated rats exhibited livers with brown and homogeneous colors but with intensities ranging from very dark brown from D_1 to D_7 (Figure 3B) to pale brown at D_{14}. In the remaining third, the livers exhibited only some irregular brown spots, visible on the hepatic lobes (Figure 3D). For the rats treated with a lower amount of C_{60} (0.5 g/kg), 50% of the individuals (3/6) showed mottled brown livers from D_1 to D_7, whereas

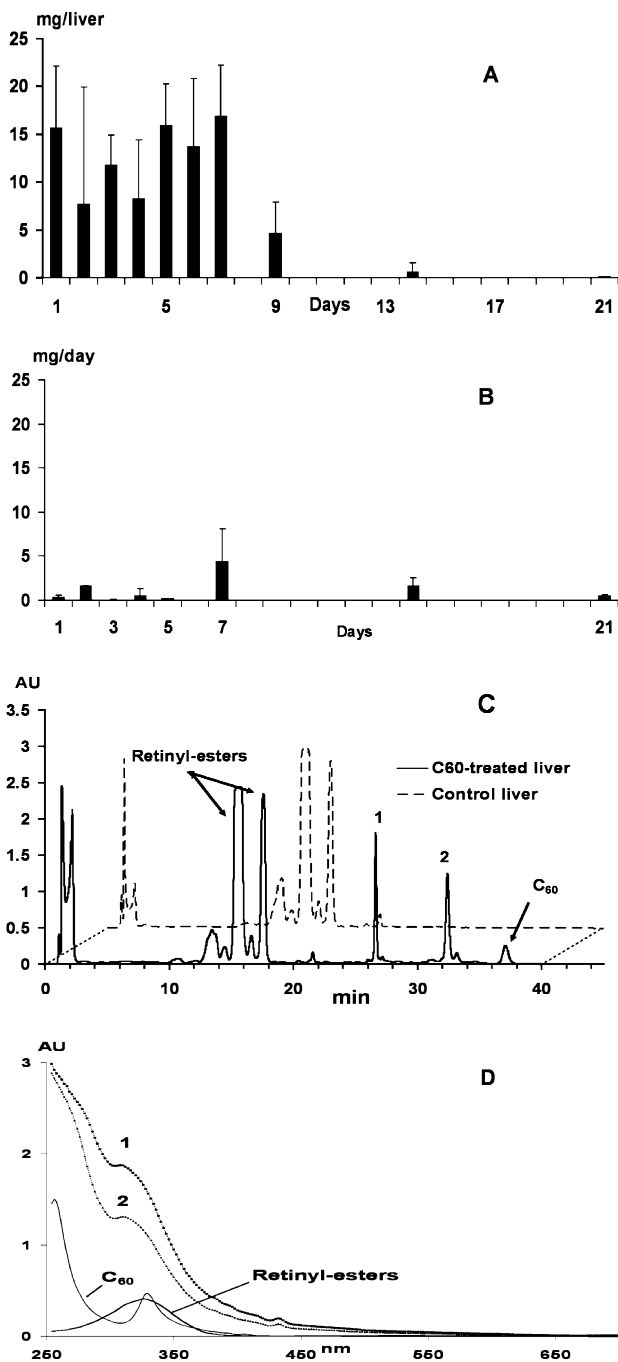

Figure 2. Some aspects of C_{60} pharmacokinetics after administration of a single dose (0.5 g/kg of body weight) of an aqueous suspension of micronized C_{60}: (A) kinetics of accumulation in the livers; (B) kinetics of fecal elimination; (C) chromatographic profiles of hepatic extracts showing additional peaks (1 and 2) in C_{60}-treated rats; (D) spectra extracted from C (data are the mean ± SD for three rats).

the others kept livers with normal pigmentation. From D_{14} to D_{21}, all of the animals exhibited livers with normal color (Figure 3A). As for the animals that received the weakest amount of C_{60} (0.25 g/kg), the liver pigmentation remained normal in most cases.

Microscopic examinations of the liver sections revealed a normal parenchymal architecture without inflammation or fibrosis (Figure 3E). In contrast to what was observed for Swiss mice,[15] C_{60} did not induce hypertrophy or hyperplasia of HSC. The rat reticulo-endothelial system was able to dam up the flow of C_{60} particles, whereas the mouse one was not. Those hepatocytes with a clear aspect were lipid-rich and resembled those of animals treated only with the vehicle. The abundance of C_{60} particles in the liver sections was well correlated with the intensity of the brown pigmentation of the organs. In the normal-colored livers, the C_{60} particles were very small and rare. In the mottled livers, the C_{60} particles were more numerous but their abundance depended on the area examined. Finally, in the livers exhibiting a uniform brown color, the C_{60}-containing macrophages were abundant and showed a slight hypertrophy without strong macrophagic activity. They had a mottled ochre color with black spots and were localized mostly in the periportal areas (Figure 3F).

Characteristic C_{60} particles[22] were detected by TEM in all of the liver sections essentially inside Kupffer cells (Figure 1B) and some hepatocytes of the capsule as well as inside rare HSCs.

The aqueous suspension used in the present study contains C_{60} crystals with relatively large sizes ranging from 60 to 1650 nm (Figure 1A), which can explain the intraindividual variations concerning the C_{60} distribution and the coloration of the organs. However, inside the liver cells, most of the aggregates contained C_{60} crystals with an average size lower than 50 nm (Figure 1C) and the dissolution of the fullerene inside lipid droplets was sometimes observed (Figure 1D), indicating that this fullerene is absorbed well by the organs.

These results show for the first time that C_{60} does not present any acute or subacute toxicity in rats up to 2 g/kg bwt, as in the case of mice. The normal circulating levels of serum alanine aminotransferase (ALT) activity detected in these animals, which is indicative of parenchymal cell damage, confirmed the absence of cellular lesions in all instances (Figure 4). Finally, these results show that it is necessary to inject large doses of C_{60} (2 g/kg bwt) in order to obtain sufficient and reproducible accumulation in the livers.

Effects of CCl_4 on Rats. CCl_4 toxicity with respect to rats is well-known, nevertheless, we systematically studied the effects of this halo-alkane on the animals we used in our experiments in order to avoid misinterpretations due to interstrain variability. In addition, to avoid errors due to interindividual and interseason variability, a CCl_4-treated control group was included in each experiment.

The animals treated with doses equivalent to/or higher than 1.0 mL/kg showed inactivity, lethargy, and pilo-erection. These symptoms persisted during a period of 24 h until the animals were sacrificed for histopathological examination. After abdomen incision, the livers were pale and looked mottled and their lobes were adherent in most cases (Figure 3C). At the microscopic scale, these livers showed important damage; many inflammatory areas as well as large necrotic areas with ballooning necrotic cells were associated with an important steatosis (Figure 3G). In a few cases, there were also some apoptotic lesions. The increase of ALT activity as a function of CCl_4 dose can reach more than 70 times the normal activity (Figure 4).

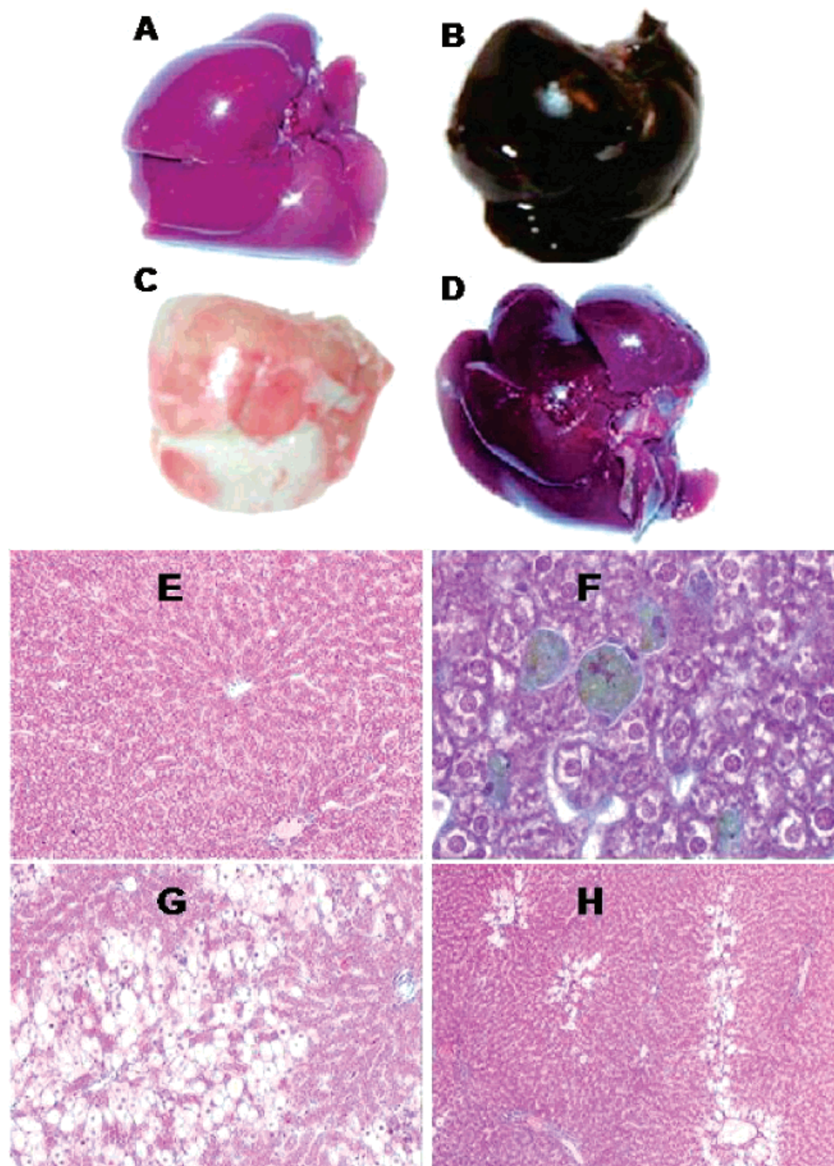

Figure 3. Macroscopic and microscopic effects of C_{60} on rat livers.; (A) control liver; (B) liver after 7 days of pretreatment with C_{60} (2.0 g/kg of body weight); (C) liver of a rat intoxicated with CCl_4 (1 mL/kg of body weight); (D) liver after 14 days of pretreatment with C_{60} before CCl_4-treatment. Trichrome staining of liver sections (magnification = 100x) from: (E) C_{60} treated rat; (F) magnification of E; (G) CCl_4 treated rat; (H) an example of C_{60} pretreated rat before CCl_4 treatment showing a few necrotic areas limited to some cords of hepatocytes. The liver sections of the other C_{60} pretreated rats (5/6) showed only a slight steatosis.

To obtain obvious and reproducible histopathological and biochemical lesions, the results of the present study, which are in agreement with those reported previously,[23] show that it is necessary to inject a dose equivalent to/or higher than 1 mL/kg of CCl_4.

Effects of C_{60} on CCl_4 Acute Toxicity as a Function of the Pretreatment Time. In a first experiment, we investigated the effects of C_{60} on CCl_4 intoxication as a function of the pretreatment time. Rats were divided randomly into six groups of six. At D_0, groups 1 and 3 received the vehicle only, whereas groups 2 and 4−6 received an ip injection of C_{60} (2.0 g/kg bwt). At D_3, group 3 was subjected to CCl_4 injection (1.0 mL/kg bwt). At D_7, group 4 was also subjected to the same dose of CCl_4. At the end of the experiment, D_{14}, groups 4−6 were injected with the same dose of CCl_4, whereas groups 1 and 2 received an ip injection of 0.9% NaCl (1.0 mL/kg bwt). Twenty-four hours after CCl_4 or NaCl treatment, each group of animals was sacrificed for histopathological examination and biochemical tests.

After CCl_4 administration (1.0 mL/kg), the C_{60}-pretreated rats (2 g/kg) exhibited the same symptoms of lethargy and pilo-erection as those observed for the group treated with CCl_4 only. However, in contrast to the group treated with CCl_4 only, the symptoms of the cotreated rats disappeared after 3−4 h after CCl_4 intoxication. Twenty-four hours after CCl_4 intoxication, the livers of all rats pretreated with C_{60} during various periods, 3, 7, or 14 days, exhibited normal morphology with the same features as those observed for the animals treated with C_{60} only. In five of the six rats per group, with the exception of a slight steatosis, the microscopic examinations performed at D_4, D_8, and D_{15} revealed neither necrotic ballooning cells nor apoptosis. For the three

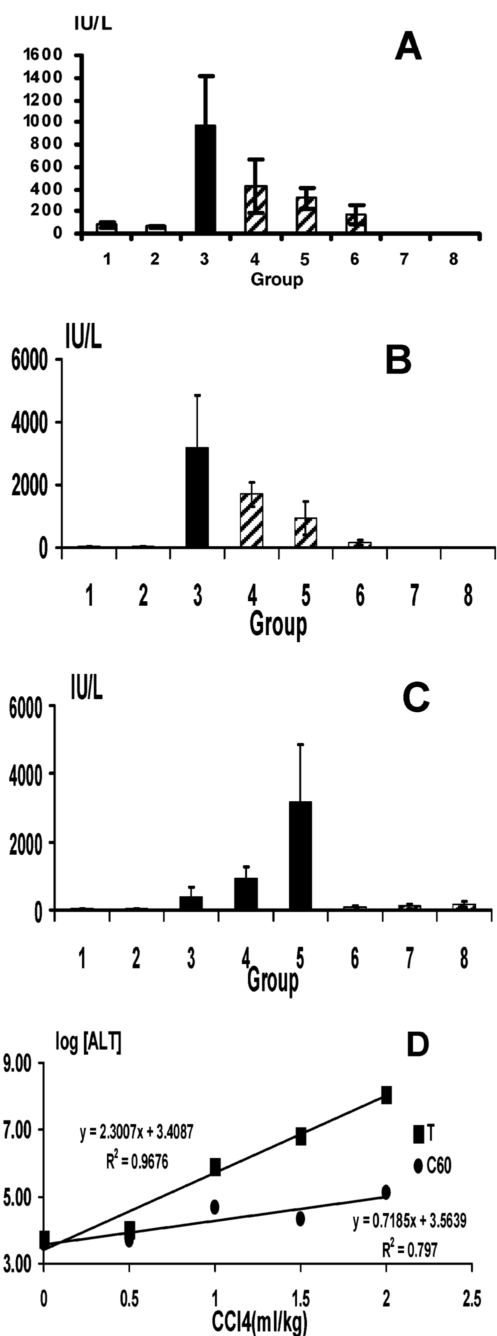

Figure 4. Effect of C_{60} pretreatment on serum ALT activity ((n = 6), groups 1 and 2 were used as controls and received only the vehicle or C_{60}, respectively. The black bars represent the groups treated with only CCl_4 and the dotted bars represent the co-treated groups). (A) as a function of the pretreatment time with a single dose of C_{60} (2.0 g/kg body weight; G_4, 3 days; G_5, 7 days; and G_6, 14 days) before injection of a single dose of CCl_4 (1.0 mL/ kg body weight). (B) as a function of the dose of C60: 14 days of pretreatment with increasing C_{60} doses (0.25, 0.5, and 2.0 g/kg body weight; for groups 3, 4, and 5, respectively) before injection of a single dose of CCl_4 (2.0 mL/kg body weight). (C) as a function of the dose of CCl_4: 14 days of pretreatment with a single dose of C_{60} (2.0 g/kg body weight) before injection of increasing CCl_4 doses (0.50, 1.0, and 2.0 mL/kg of body weight) for groups 6, 7, and 8, respectively. Groups 3−5 received only CCl_4 at the same increasing doses. (D) dose−effect relationship.

remaining rats (1 per group), we observed a few necrotic areas limited to some cords of hepatocytes (Figure 3H).

For most of the animals sacrificed on D_3 or D_8, C_{60} was detected as ochre and diffuse clusters, mostly inside macrophages and occasionally inside HSC. For those rats sacrificed on D_{15}, C_{60} was detected only as very small particles inside the Kupffer cells.

The determination of ALT activity in sera confirmed the protection of the livers by C_{60}. In the group treated with CCl_4 only, the median of ALT activity was about 12 times higher than that observed in the group treated with the vehicle only. In the groups pretreated with C_{60} for 3, 7, and 14 days, the medians were, respectively, 5.5, 4.1, and 1.5 times higher than that of the control group (Figure 4A).

Antioxidant Effect of C_{60}. The initial liver damage after CCl_4 administration is mediated through its metabolisation by cytochrome P450 II E1 resulting in the formation of the trichlormethyl radical $CCl_3^•$. This radical can also react with oxygen to form a highly reactive species, trichloromethylperoxy radical $CCl_3OO^•$, which can rapidly initiate the chain reaction of lipid peroxidation.[20] C_{60} is able to scavenge a large number of radicals per molecule[2] including $CCl_3^•$ and $CCl_3OO^•$.[24] Because this property can be involved in the mechanism of protection against CCl_4 toxicity, we explored the effects of C_{60} on the status of glutathione, vitamin E, and vitamin C, which are antioxidant systems that play a critical role in the defense against oxidative stress.[18] Because C_{60} can react with vitamin A in the liver, we also studied its effect on circulating vitamin A, which is a probe for vitamin A liver storage.[25]

With respect to vitamin C, in the group treated with CCl_4 only, the median of circulating vitamin C increased dramatically, reaching 8 times the medians of the other groups (Figure 5). Therefore, rats adjusted to CCl_4 intoxication by enhancing the biosynthesis of ascorbate, in agreement with what was observed by other authors.[26] In the rats pretreated with C_{60}, the circulating levels of vitamin C remained normal, thus indicating the absence of oxidative stress in the hydrophilic compartment. Determination of the contents of reduced glutathione (GSH) and of its oxidized form (GSSG) within the liver confirmed the results obtained for vitamin C. In the group treated with CCl_4 only, the GSSG/(GSH+GSSG) ratio (GR) used as a gauge for the intracellular redox equilibrium was more than two times higher than that of the control group (Figure 5). The GR of the control group and that of the cotreated (C_{60} + CCl_4) group were nearly equivalent, whereas this ratio was decreased significantly in the group treated with C_{60} only, suggesting that this fullerene can modulate the intracellular redox status even in the absence of CCl_4.

Analysis of the results obtained for the circulating levels of vitamin E confirmed the protective effect for the lipophilic compartment (Figure 5). Indeed, statistical analysis showed that (i) the median of vitamin E was decreased significantly in the CCl_4-treated group in comparison with that of the controls and (ii) there were no differences between the control group and the two groups treated with C_{60} or with C_{60} + CCl_4.

With regard to the circulating levels of vitamin A (Figure 5), statistical analysis showed that there were significant

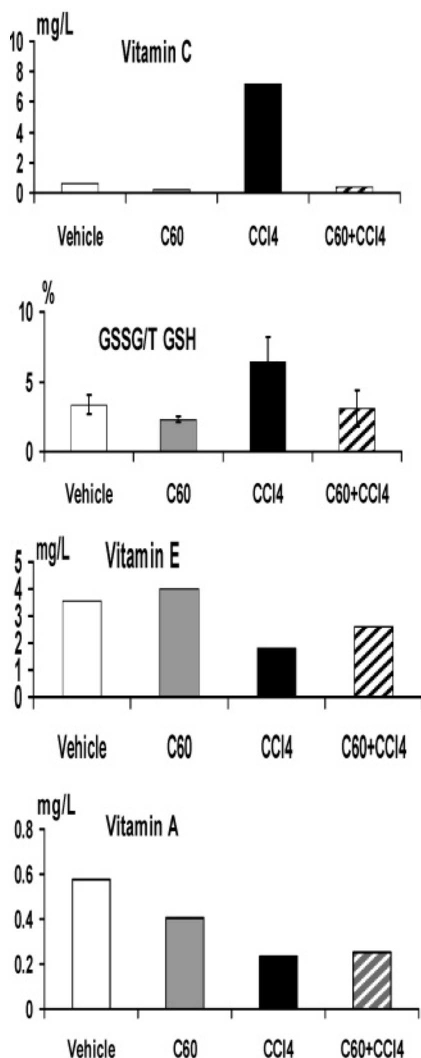

Figure 5. Effect of C_{60} pretreatment (2.0 g/kg body weight) on the antioxidant status of rats intoxicated with CCl_4 (1.0 mL/kg). Data are the median or the mean ± SD for six rats. T GSH: total glutathione (GSSG + GSH).

decreases in those groups treated with C_{60} and/or CCl_4 in comparison with that of the control group, whereas there was no difference between the two groups treated with C_{60}. The decrease of vitamin A concentration in the group treated with CCl_4 is due to the liver depletion usually observed in the chemically mediated liver fibrosis.[17] In the group treated with C_{60}, the decrease of vitamin A concentration may be attributed to a liver depletion consecutive with the reaction with fullerene, leading to either its immobilization and/or to the elimination of the whole adduct after vitamin A-adduct metabolization. These hypotheses must be confirmed by determining the retinol content of the liver as well as by the identification of the "postulated" end products. In the cotreated group (C_{60} + CCl_4), both the postulated mechanisms for the CCl_4 and the C_{60}-treated groups could explain the decrease of vitamin A concentration. However, the median in the cotreated group was not decreased significantly in comparison to those of the groups treated with CCl_4 and C_{60}. Thus, there was no addition of the effects of C_{60} and CCl_4. This phenomenon is probably due to the protective effect of C_{60}.

Taken together, these results show for the first time that C_{60}-pretreatment obviously protects the liver against oxidative stress. Nevertheless, the results obtained for ALT activity (Figure 4A) were not quite those we expected to observe after the kinetic study (Figure 2A) according to which the best protection should have occurred after 7 days of pretreatment when maximum C_{60} accumulation is reached. Because the dose of C_{60} used in the kinetic study was 4 times lower than that used in the present study, we determined the concentration of C_{60} in the livers and we observed that the accumulation kinetics was not dose-dependent. As a matter of fact, in the present experiment, the median concentration of C_{60} in the livers on D_8 (14.0 mg/g, range: 9.0−30.0 mg/g) was significantly higher than that on D_{15} (0.98 mg/g, limits: 0.05−2.60 mg/g). Thus, this apparent independence of the effects of C_{60} on CCl_4 toxicity with respect to its liver content suggests that fullerene is active only when in solution or when modified by vitamin A. Incidentally, it is well known that C_{60} and its derivatives are prone to aggregate and that fullerene must be in solution in order to scavenge free radicals, that is to say when its unsaturated bonds are accessible.[27] Furthermore, except in dichlorobenzene and chloronaphthalene, C_{60} is difficult to dissolve even in its usual solvents, which may explain the latency period between the accumulation maximum in the liver and the best protection and subsequently the apparent independence with respect to the liver content. Indeed, at the microscopic level, there was no correlation between the degree of protection and the number of C_{60} clusters in the livers, indicating that the fullerene is active only in a soluble form. This is why pristine C_{60} cannot be used as such in an in vitro biological system. This is why until now authors have chemically transformed C_{60} into water-soluble derivatives in order to perform such in vitro studies. Obviously, these derivatives are quite different.

Dose−Response Relationship. To confirm the last hypothesis and to check the dose−response relationship, in a first experiment we pretreated rats with increasing doses of C_{60} (0.25, 0.50, and 2.00 g/kg) for 14 days before intoxication with a single dose of CCl_4 (2 mL/kg). Thirty-six rats were divided randomly into six groups of six. At D_0, groups 1 and 3 received the vehicle only, while the other groups received an ip injection of C_{60} at increasing doses: 0.25 g/kg for group 4, 0.50 g/kg for group 5, and 2.00 g/kg for groups 2 and 6. At D_{14}, groups 3−6 were subjected to CCl_4 injection (2.0 mL/kg), while groups 1 and 2 received an ip injection of 0.9% NaCl (2.0 mL/kg).

Twenty-four hours after CCl_4 injection, most rats (four per group) pretreated with C_{60} with doses equivalent/or higher than 0.5 g/kg, exhibited livers with the same aspect and morphology as those observed in the groups treated with the equivalent doses of C_{60} only. The livers of the other cotreated rats showed the same features as those observed in the group treated with CCl_4 only. In the group pretreated with the lowest dose of C_{60} (0.25 g/kg), microscopic examination revealed several necrotic areas together with some spread

steatosis. In the other groups pretreated with C_{60} (0.5 or 2.0 g/kg), there was also spread steatosis; however, despite the large dose of CCl_4, the necrotic areas were very rare. Actually, in these groups necrosis was observed only in one-third of the rats (two per group). C_{60} particles were not visible in every case; they were obvious only in the group having received the largest dose (2 g/kg), particularly inside Kupffer cells as well as inside some hepatocytes. In the group treated with CCl_4 only, ALT activity increased dramatically, reaching more than 50 times the basal activity (Figure 4B), and it was equally shown that pretreatment with C_{60} prevented this effect in a dose-dependent manner. As a matter of fact, in the groups pretreated with 0.5 and 2.0 g/kg, the ALT activities were only 9 and 3 times higher than the baseline activity, respectively.

To confirm the dose-dependence of the protective effect, in a second experiment we studied the effect of the pretreatment for 14 days with a single dose of C_{60} (2 g/kg) on the intoxication with increasing doses of CCl_4 (0.50, 1.0, and 2.0 mL/kg). Thirty-six other rats were divided randomly into six groups of six. At D_0, groups 2 and 6−8 received an ip injection of C_{60} (2 g/kg) while groups 2 and 3−5 received the vehicle only. At D_{14}, all of the groups were subjected to an ip injection of CCl_4 at increasing doses: 0.50 mL/kg for groups 3 and 6, 1.0 mL/kg for groups 4 and 7, and 2.0 mL/kg for the two remaining groups. Twenty-four hours after CCl_4 treatment, all of the animals were sacrificed for histopathological examination and biochemical tests.

The ALT activities are represented in Figure 4C. The protection by C_{60} is particularly obvious for the intoxication by CCl_4 doses superior or equal to 1.0 mL/kg. In the groups treated with the highest doses of CCl_4 only (1.0 and 2.0 mL/kg), ALT activities can reach 22 to 77 times the basal activity, respectively, while the C_{60}-pretreated equivalent groups were, respectively, 1.8 to 4 times higher. Statistical analysis confirmed the dose−effect relationship; the slopes of the curves "log [effect: (ALT)] = f (dose)" were significantly different (Figure 4D).

Recently, it has been reported that this fullerene might be very toxic in some living systems such as bacteria, algae, and fishes by inducing oxidative stress.[8,9] However, the aqueous suspension used in such studies has been obtained after dissolution of C_{60} in THF.[8,9] Because it is well established that complete removal of solvent from C_{60} is achieved by heating the sample to 450 K under a pressure of $<10^{-6}$ mmHg for at least 50 h,[28] the experimental conditions used by the authors were obviously inadequate for complete removal of THF from the final aqueous nano-C_{60} suspension.[8,9] Indeed, according to the authors "This suspension consisted of stable 30- to 100-nm aggregates in which the fullerenes facing the water were most likely partially modified but the central core of the aggregate contained unmodified fullerenes."[8] However, as far as the reactivity of a given compound is concerned in biology as well as in chemistry, the surface is obviously more important than the core. Therefore, the toxic effects of such dispersions, discovered in brain fishes,[8] while the livers and gills of these animals remained insensitive to the toxicant, can obviously be attributed to the residual THF adsorbed on the C_{60} aggregates. Such brain-selective action is typical for the inhaled narcotics, and it is well known that THF has a narcotizing effect accompanied with a more pronounced toxic effect as compared to usual narcotics.

As a matter of fact, a Russian team already studied the biological effects of a well described "aqueous colloidal solution of fullerenes" on several biological systems. In contrast, the authors reported a lot of positive effects without any toxicity.[29,30]

The very first example of a negative effect linked to surface modification of C_{60} has been reported in 1996, when the authors studied the effects of a C_{60}−PVP solution on mouse embryos.[31] Incidentally, a few years later it was shown that C_{60} can react with PVP to give highly stable charge-transfer complexes.[32]

The present study confirms and greatly strengthens the absence of toxicity observed in several biological systems from bacteria[33] and fungal[34] to human keratinocytes[35] and leukocytes[22] through drosophila,[36] mice,[15,16,37] and guinea pigs.[38] More recently, a Chinese team confirmed the absence of toxicity up to 226 $\mu g/cm^2$ of C_{60} in a model system using alveolar macrophages.[39] By the same way, a Japanese team confirmed the positive effects of C_{60}-derivatives on human skin keratinocytes.[40]

In contrast to the contention of the above international headlines,[7−10] our results demonstrate clearly that C_{60} protects the liver in a dose-dependent manner against CCl_4 acute toxicity by modulating the oxidative stress generated through the metabolization of this halo-alcane. The mechanism of this protection could be attributed to the ability of C_{60} to scavenge large numbers of radicals. The antioxidation process has been well established in vitro in a model experiment using C_{60}-containing-liposomes;[41] however, it will be necessary to isolate and identify, from the treated livers, the fullerene transformation byproducts in order to confirm this hypothesis in vivo. Alternatively, C_{60} can also act as a decomposition catalyst for O_2^-/H_2O_2 as it has been postulated for its tris-malonic acid derivative[42] or as cytochrome P 450 inhibitor as it has been established for one of its derivatives.[43] The mechanism of this protection including the effects of C_{60} on Kupffer cells activation is under investigation in our laboratory.

It is worth noting that this liver-protective effect can explain the absence of toxic effect of nano-C_{60} aggregates on liver and gill fishes observed by other authors.[8] The absence of protection in the brain[8] could then be attributed to the fact that, under such experimental conditions, C_{60} cannot cross the brain barrier, whereas THF can.

It has to be emphasized that in contrast to the "nano-C_{60} aggregated water-soluble fullerene species" used by other authors,[8,9] the aqueous suspensions of micronized C_{60} (100 mg/mL) used in the present study were prepared by mechanical-milling in liquid media without adding any polar organic solvent (Supporting Information). In addition, fullerenes are found commonly in nature, not only in meteorite impact structures[44] or in the rocks of the Shunga district in Karelia[45] and of other geological beds[46] but also

in a 10 000-year-old ice core in which the presence of carbon nanotubes and fullerene nanocrystals[47] reflects combustion products similar to contemporary airborne carbon nanocrystal aggregates.[48] This natural abundance supports the absence of toxicity our experimental work confirms, and we feel that it cannot be said that the C_{60} discovered in dinosaur eggs[49] was the origin of the mass extinction of these animals, or was it?

Note Added after ASAP Publication. A minor text correction was made to the Dose−Response Relationship paragraph in the version published ASAP November 22, 2005; the corrected version was published ASAP December 5, 2005.

Supporting Information Available: Preparation of aqueous suspensions of micronized C_{60}, details of the experiments conducted, HPLC, biochemical tests, and microscopy and statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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