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The prolongation of the lifespan of rats by repeated oral administration of [60] fullerene

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ABSTRACT

Countless studies showed that [60]fullerene (C_{60}) and derivatives could have many potential biomedical applications. However, while several independent research groups showed that C_{60} has no acute or sub-acute toxicity in various experimental models, more than 25 years after its discovery the *in vivo* fate and the chronic effects of this fullerene remain unknown. If the potential of C_{60} and derivatives in the biomedical field have to be fulfilled these issues must be addressed. Here we show that oral administration of C_{60} dissolved in olive oil (0.8 mg/ml) at reiterated doses (1.7 mg/kg of body weight) to rats not only does not entail chronic toxicity but it almost doubles their lifespan. The effects of C_{60}-olive oil solutions in an experimental model of CCl_4 intoxication in rat strongly suggest that the effect on lifespan is mainly due to the attenuation of age-associated increases in oxidative stress. Pharmacokinetic studies show that dissolved C_{60} is absorbed by the gastro-intestinal tract and eliminated in a few tens of hours. These results of importance in the fields of medicine and toxicology should open the way for the many possible -and waited for- biomedical applications of C_{60} including cancer therapy, neurodegenerative disorders, and ageing.

1. Introduction

Since 1993 countless studies showed that [60]fullerene (C_{60}) and derivatives exhibit paramount potentialities in several fields of biology and medicine [1] mainly including specific DNA cleavage, imaging [2], UV and radioprotection [3], antiviral, antioxidant, and anti-amyloid activities [1,4–7], allergic response [8] and angiogenesis [9] inhibitions, immune stimulating and antitumour effects [10,11], enhancing effect on neurite outgrowth [12], gene delivery [13], and even hair-growing activity [14]. However, although several independent research groups confirmed the innocuousness of pristine C_{60} [15–17] the toxicity of this fullerene is still a matter of debate [18,19]. As recently demonstrated, this is mainly due to the lack of characterisation of the tested materials [15–19]. Nevertheless, the metabolic fate and the *in vivo* chronic effects of C_{60} itself still remain unknown. In order to fulfil the potential of C_{60} and derivatives in the biomedical field these issues must be addressed.

Aqueous suspensions were previously used to investigate the acute and sub-acute toxicities as well as the *in vivo* antioxidant properties of pristine C_{60} [20,21]. But, such suspensions are not appropriate for determining toxicity at reiterated doses, because fullerene is active only in soluble form [21] and because the extremely slow dissolution of C_{60} in biological media prevents controlling accurately the active fraction [21]. This may be the reason for which the chronic toxicity of C_{60} has never been investigated to our knowledge.

C_{60} is soluble in lipid droplets inside living cells [21] as well as in fats in general [22,23]. Moreover, C_{60} can freely cross membrane barriers as observed experimentally [21] and recently modelled by computer simulations [24]. Thus, C_{60} interactions with living systems as well as its toxicity should be determined using soluble forms.

Recently, liposomes were used as carriers to study the biodistribution of unmodified C_{60} in rats after tail vein administration [25]. But, as C_{60} was not detected in blood due to its rapid clearance by tissue-filtration, such formulation was not appropriate for characterizing its pharmacokinetics [25].

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While C_{60} solubility in vegetable oils [22,23] is not high enough to study its acute toxicity according to institutional recommendations (European Medicines Agency, Evaluation of Medicines for Human Use, 2004) [26], such solutions should be quite appropriate for studying its chronic toxicity at reiterated doses [27].

As the *in vivo* behaviour of soluble forms of C_{60}, including absorption, biodistribution, and elimination was unknown, we determined the *in vivo* fate of C_{60} dissolved in olive oil before studying its chronic effects at reiterated doses.

Oily solutions cannot be administered intravenously because of possible vessel obstruction, so we characterized the pharmacokinetics of C_{60} dissolved in olive oil (0.8 mg/ml) after oral gavage (o.g.) and intra-peritoneal (i.p.) administration to rats (4 mg/kg of body weight (mg/kg bw)).

Finally, as C_{60} is known to be a powerfull antioxidant [5,6,21], we checked the effects of C_{60}-olive oil solutions on oxidative stress in a classical model of CCl_4 intoxication in rats [28,29]. Although the oxidative stress involved in CCl_4 intoxication is unlikely to occur during physiopathological conditions, CCl_4 intoxication in rats provides an important model for elucidation of the mechanism of action of hepatotoxic effects such as fatty degeneration (steatosis), fibrosis, hepatocellular death, and carcinogenicity involving oxidative stress [28,29].

2. Materials and methods

2.1. C_{60}-olive oil solution preparation

Virgin olive oil is obtained from a *Chemlali Boughrara* cultivar from Tunisia planted in the Sahel area. C_{60} (purity 99.98%) was obtained from SES Research Corporation (USA) and used without further purification.

Fifty mg of C_{60} were dissolved in 10 ml of olive oil by stirring for 2 weeks at ambient temperature in the dark. The resulting mixture was centrifuged at 5.000 g for 1 h and the supernatant was filtered through a Millipore filter with 0.25 μm porosity.

2.2. Pharmacokinetics and biodistribution studies

All experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of Paris XI University.

2.2.1. Pharmacokinetics

Pharmacokinetic studies were carried out with male Wistar rats (weighing 200–220 g). Rats were housed in individual cages and maintained in an air-conditioned room (22–25 °C) on a 12 h light/dark cycle with water and food available. The rats were acclimated for 7 days before treatment.

After sodium pentobarbital (20 mg/kg bw in 1.0 ml/kg bw) anaesthesia, a catheter was introduced into the rat right jugular vein, positioned subcutaneously with the tip in the inter-scapular region. The prepared rats were then allowed to recover for 24 h, and the blood catheters were flushed with 0.9% NaCl solution containing 20 IU/ml of heparin to avoid possible clot obstruction.

Before C_{60} administration, the rats were fasted overnight but with access to water. The same single dose of C_{60} (4 mg/kg bw) was delivered orally, through a gavage needle, or intra-peritoneally to two groups of three rats. Blood (0.20 ml) was withdrawn *via* the canular prior to dosing ($t = 0$) and at 15, 30, 60 min and then at 2, 4, 8, 10, 12, 24 and 48 h post-dosing. Antithrombin heparin (20 IU/ml) was added in each blood sample. After each blood collection 0.20 ml of sterile 0.9% NaCl solution were injected to the animal, to avoid hypovolemia. The rats were sacrificed 48 h after C_{60} administration for organ collection (livers, spleens, and brains). Urines were collected at 24 h and 48 h after C_{60} administration then frozen at −20 °C until analysis.

2.2.2. Biodistribution

For biodistribution studies, 4 groups of 3 rats (weighing 200 ± 20 g) were treated daily for 7 days either by i.p. administration (2 groups) or oral gavage (2 groups) with the same dose (4 mg/kg bw) of the same C_{60}-oil solution (0.8 mg/ml). At day 1 (D_1), and D_8, one group of orally treated and one group of i.p. treated animals were sacrificed for blood and organ collection. Urines were collected daily, then frozen under the same conditions as for pharmacokinetic studies.

2.3. Chronic toxicity and effects of C_{60} on survival of rats

The rats were housed three per cage and acclimated for 14 days, before dosing. Three groups of 6 rats (10 months old, weighing 465 ± 31 g) were administered daily for one week, then weekly until the end of the second month and then every two weeks until the end of the 7th month, by gavages with 1 ml of water or olive oil or C_{60} dissolved in olive oil (0.8 mg/ml), respectively.

The rats were weighed before each dosing. Routine observations following official recommendations [27] were made on all animals inside and outside the cage once a day throughout the study for signs of departure from normal activity, morbidity and mortality.

2.4. Effects of C_{60}-olive oil solutions on oxidative stress

Sixty rats randomly divided into 10 groups of 6 rats were pre-treated daily for 7 days by oral gavages (og groups) or by i.p. injection (ip groups). Groups A (GAog and GAip), received 1 ml of water. Groups B and C (GBog, GCog and GBip, GCip) were pre-treated with 1 ml of olive oil while groups D and E (GDog, GEog and GDip, GEip) were pre-treated with 1 ml of C_{60}-olive oil.

Twenty-four hours before sacrifice, groups GA, GC and GE were i.p. injected with a single dose of CCl_4 (1 ml/kg bw) while GB and GD, used as controls, were administered with a 0.9% NaCl aqueous solution under the same conditions.

2.5. Chromatographic analyses, sample preparation and method validation

2.5.1. Chromatographic analyses

Chromatographic analyses of C_{60} in blood, urine, liver, spleen and brain were performed as described previously [30] with the following modifications.

HPLC separations were performed using a P4000 multi-solvent delivery system coupled with a UV6000LP photodiode array detector (Thermo Separation Products, Les Ulis, France). Instrument monitoring and data acquisition were performed using ChromQuest Software from the same origin. Peak identifications were based on their UV–Visible spectra and the traces were recorded at 330 nm. Separations were carried out with a Hypersil 120-5 ODS, 5 μm cartridge (Macherey–Nagel, Hoerdt, France) protected with a 4.0 mm × 10 mm pre-column packed with the same stationary phase.

For liver and spleen samples, separations were performed at 25 °C with a flow rate set at 0.8 ml/min and a mobile phase composed of a mixture of toluene and methanol (35/65, v/v).

For whole blood, urine, and brain samples, separations were performed with 20% of toluene and 80% of methanol for the first 5 min, at which time the toluene was increased to 60% for 10 min and then hold constant for the remaining 7 min of each sample run. At least 10 column volumes of the initial composition were flushed through the column prior to injecting the sample.

2.5.2. Sample preparation

For whole blood, one hundred μl of sample were diluted in 400 μl of 0.1 M sodium dodecyl sulfate (SDS). After adding 0.5 ml of acetonitrile and shaking for 5 min, C_{60} was extracted by adding 5 ml of toluene containing 0.2 μg/ml of C_{70} used as internal standard (IS) to the mixture and shaking for 24 h in the dark. After centrifugation (2000 g for 15 min), the supernatant was evaporated under a stream of nitrogen. Then the residue was dissolved in 0.1 ml of toluene and diluted in acetonitrile (50/50, v/v) before injection of 100 μl into the chromatograph.

For urine, 1.0 ml of sample were mixed with 0.2 ml of acetonitrile and then loaded into a Sep-pak plus C_{18} cartridge (Waters, St Quentin en Yvelines, France) prealably conditioned with 5 ml of a mixture of water/acetonitrile (10/2, v/v). After washing the C_{18} cartridge with 5 ml of acetonitrile, the retained compounds were eluted with 2 ml of toluene containing 0.2 μg/ml of C_{70} and evaporated under a stream of nitrogen. The residue was then dissolved in 0.1 ml of toluene and diluted in acetonitrile (1/1, v/v) before injection of 100 μl into the chromatograph.

For organs, about 1.0 g of liver (right lobe) or brain or 0.2 g of spleen were accurately weighed and then homogenized with 5 ml of 0.1 M SDS and 5 ml of acetonitrile. After shaking for 5 min, 20 ml of toluene containing 2.0 μg/ml of IS were added and the mixture was shaken for 24 h in the dark. After centrifugation (2000 g for 15 min), the supernatant was evaporated under a stream of nitrogen. Then the residue was dissolved in 1 ml of toluene for liver and spleen samples or 0.2 ml of toluene for brain samples, and diluted in acetonitrile (50/50, v/v) before injection of 100 μl into the chromatograph. Samples exceeding the limit of linearity were reanalyzed after appropriate dilution.

2.5.3. Method validation

For the calibration and the validation of the method, we used whole blood, urine, and organ samples of untreated rats spiked with C_{60}-olive oil solutions (19/1, v/v or m/m).

The linearity of the method was checked between 0.01 and 1.0 μg/ml under gradient elution ($y = 0.5963x + 0.0006$; $n = 6$; where y is the peak area in AU min and x is the concentration of the injected solution in μg/ml; the relative standard deviations (RSDs, $n = 5$) for the slope and the intercepts were 6.4% and 4.3%, respectively). The limit of detection for a signal to noise ratio equal to 3 was 0.001 μg/ml.

Under isocratic conditions, the linearity of the method was checked between 0.01 and 10.0 μg/ml ($y = 0.597x + 0.0098$; $n = 7$; where y is the peak area in AU.min and x is the concentration of the injected solution in μg/ml; the RSDs ($n = 5$) for the slope and the intercepts were 5.2% and 3.9%, respectively). The limit of detection for

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a signal to noise ratio equal to 3 was 0.002 μg/ml. The between run (BWR) and between day (BWD) precisions were determined ($n = 6$) for the lowest and the highest level of each curve of calibration.

Under gradient elution conditions the RSDs were 7.2% and 10.5% for the BWR and 5.3% and 8.4% for the lowest levels and the highest levels, respectively. Under isocratic conditions, the RSDs were 5.6% and 8.5% for the BWR and 3.3% and 6.4% for the lowest levels and the highest levels, respectively.

The recovery of the method was determined for each kind of sample at two levels ($n = 3$, for each level). For whole blood, urine, and brain samples the recoveries were determined at 0.01 and 0.05 μg/ml or μg/g, respectively and they were 94.3 ± 4.9% and 93.8 ± 5.1% and 98.1 ± 2.5% and 96.9 ± 3.5%, respectively. For liver samples the levels were 0.2 and 30 μg/g and the recoveries were 97.3 ± 2.8% and 99.1 ± 2.2%, respectively. For spleen samples the levels were 2.0 and 200 μg/g and the recoveries and between run precision were 95.3 ± 4.2% and 96.1 ± 3.2%, respectively.

2.6. Biochemical tests and pathological examinations

Tissue and blood sampling, serum alanine amino-transferase (ALT) activity, and oxidized glutathione/total glutathione (GSSG/TGSH) ratio, where TGSH is the sum of reduced (GSH) and oxidized glutathione (GSSG), were performed as previously described [30].

Superoxide-dismutase (SOD) and catalase (CAT) activities were determined as previously described [31,32].

Hepatic microsomal fractions were used for measuring the cytochrome P450 2E1 (CYP2E1) specific oxidative activity such as p-nitrophenol hydroxylase. The hepatic microsomal fractions were prepared by differential centrifugation, as described previously [33] and were stored at −80 °C until required. The hydroxylation of p-nitrophenol to 4-nitrocatechol was determined by HPLC as described previously [46]. Microsomal protein concentration was determined by the Bradford method [34], using bovine serum albumin as a standard.

Pathological examinations and optical microscopy analyses were blindly performed by a pathologist ignoring all protocol procedures as well as the purpose of the study. The reparation and staining protocols of organ pieces for optical and transmission electron microscopy (TEM) were performed as described previously [21].

2.7. Pharmacokinetic analysis

Pharmacokinetic analysis of the individual observed rat plasma data obtained after oral and i.p. routes was performed using the WinNonLin® software (Pharsight Corporation, Mountain View, California). A non-compartmental approach was used to calculate the main pharmacokinetic parameters.

The maximal plasma concentration (C_{max}) and the time (T_{max}) to reach C_{max} were obtained directly from experimental observations. The terminal elimination rate constant (λ_z) was calculated by linear regression analysis of the natural logarithm of the last experimental concentrations and the terminal half-life ($t_{1/2}$) was calculated by dividing Ln2 by λ_z. The area under the plasma concentration-time curve from zero to infinity (AUC_0^∞) was the addition of AUC from zero to the last experimental concentration (C_T), calculated by the trapezoidal rule, and of AUC from C_T to infinity, calculated by dividing C_T by λ_z. The area under the first moment curve from zero to infinity ($AUMC_0^\infty$) was the addition of AUMC from zero to the last experimental concentration (C_T), calculated by the trapezoidal rule, and of AUMC from C_T to infinity, calculated by $[((C_T.\ T)/\lambda_z) + (C_T/\lambda_z^2)]$. The mean residence time (MRT) was calculated by dividing $AUMC_0^\infty$ by AUC_0^∞. The apparent plasma clearance (Cl/F) was calculated by dividing the dose by AUC_0^∞, and the apparent volume of distribution (Vd/F) was calculated by dividing the dose by $(AUC_0^\infty . \lambda_z)$.

2.8. Statistics

The normality of data distribution was tested by Shapiro–Wilk test. Data are presented as the mean and standard deviation in the case of normal distributions or as the median and the range. Comparisons with control were performed by using Student test, according to the homogeneity of variances determined by Fisher test, or by Mann–Whitney test. A value of $P < 0.05$ was considered statistically significant.

The survival distributions for C_{60}-olive oil-treated and control rats were estimated by the non-parametric Kaplan–Meier estimator and compared by a log-rank estimated test.

3. Results

3.1. C_{60}-olive oil preparation

The composition and quality characteristics of olive oil were determined as previously described following analytical methods described in the EEC 2568/91 and EEC 1429/92 European Union Regulations [35].

The resulting C_{60}-olive oil solution is purple and contains 0.80 ± 0.02 mg/ml ($n = 6$) as determined by HPLC [30] after appropriate dilution in the mobile phase. The chromatographic profile and the extracted spectra of these solutions are similar to those obtained with a control C_{60}-toluene equimolar solution.

The stability of both oily and control solutions stored at ambient temperature and in the dark was checked monthly during 48 months. No change was recorded under our chromatographic conditions.

3.2. Pharmacokinetics and biodistribution

3.2.1. Pharmacokinetics

Fig. 1represents the evolution of whole blood C_{60} concentrations *versus* time following single dose o.g. and i.p. administration of the same dose of C_{60} dissolved in olive oil.

Table 1summarizes the main pharmacokinetic parameters. The maximal concentrations (C_{max}) are reached 4 and 8 h after i.p. and o.g. administrations, respectively.

The apparent volume of distribution (Vd/F) of C_{60} after i.p. administration is higher than the blood volume in rats [36], indicating that C_{60} is well distributed in tissues. The value of Vd/F after o.g. is less significant because the administered dose cannot be ponderated by the C_{60} bioavailability, which is unknown (Table 1).

The elimination process is slower after i.p. administration than after o.g., as illustrated by the elimination half-lifes and the mean residence times of C_{60} (Table 1).

3.2.2. Biodistribution

At day 1 (D_1) after administration, C_{60} contents in livers and spleens represent 0.14% and 0.18% of the administered dose by the oral route, respectively, and 4.73% and 1.55% by the i.p. route, respectively (Table 2).

After 7 successive days of administration (D_8), C_{60} contents in livers and spleens correspond to 0.39% and 0.51% of the total administered dose by the oral route, respectively, and 5.54% and 2.39% by the i.p. route, respectively (Table 2).

At D_1 and D_8 C_{60} content in brains represents less than 0.01% of the administred dose after o.g. while these values are higher than 0.12% after i.p. administration (Table 2).

Microscopic examination at D_8 of the spleen reticulo-endothelial system (RES), where the highest concentrations are

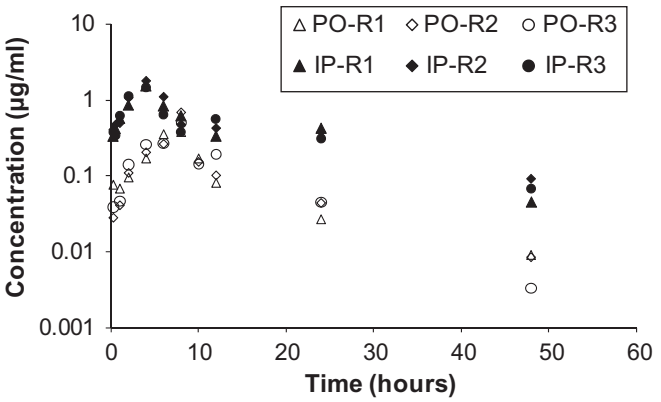

Fig. 1. Blood concentrations after oral and intra-peritoneal administrations. Individual rat whole blood C_{60} concentrations *versus* time plot following single dose (4 mg/kg bw) of C_{60} dissolved in olive oil (0.8 mg/ml), administered by intra-peritoneal (IP) or oral routes (PO: *per os*) (R1, R2, R3 = rat 1, rat 2 and rat 3).

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Table 1
Pharmacokinetic parameters. Obtained in rats after oral ($n = 3$) or intra-peritoneal (IP, $n = 3$) administration of C_{60} dissolved in olive oil.

	Oral value ± SD (RSD %)	IP value ± SD (RSD %)
$t_{½}$ (h)	9.3 ± 2.7 (28.6%)^{NS}	13.9 ± 2.9 (20.8%)
C_{max} (μg/ml)	0.52 ± 0.16 (30.7%)***	1.47 ± 0.15 (10.2%)
T_{max} (h)	8.0 ± 0.1	4.0 ± 0.1
AUC$_0^\infty$ (μg h ml^{-1})	4.37 ± 0.60 (0.14%)***	21.21 ± 1.50 (7.1%)
Cl/F (ml h^{-1})	185.5 ± 27.5 (14.8%)***	37.8 ± 2.6 (6.9%)
Vd/F (L)	2.56 ± 1.09 (42.6%)*	0.75 ± 0.12 (15.5%)
MRT (h)	12.6 ± 0.9 (7.1%)*	18.0 ± 1.9 (10.6%)

Statistical differences were evaluated by *T*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS: non-significant.

observed, shows the presence of some C_{60} aggregates that are larger and more numerous after i.p. administration (Fig. 2c and d) than after o.g. (Fig. 2a, b): thus C_{60} concentrations reached the limit of solubility in spleens. In contrast there are no observable deposits inside the livers in all cases indicating that C_{60} concentrations in these organs are not high enough to trigger precipitation.

While transmission electron microscopy (TEM) at D_8 after i.p. administration shows numerous spleen macrophages laden C_{60} crystals (Fig. 2e) only some C_{60} crystals were observed inside liver macrophages and very rare crystals in lung (Fig. 2f) and kidney cells (Fig. 2g).

3.3. Chronic toxicity and effects of C_{60} on lifespan of rats

Fig. 3 shows the animal survival and growth. After five months of treatment (M_{15}) one rat treated with water only exhibited some palpable tumours in the abdomen region. Due to the rapid development of tumours (about 4 cm of diameter) this rat died at M_{17}. As rats are known to be sensitive to gavages, we decided to stop the treatment for all rats and to observe their behaviour and overall survival.

All remaining animals survived with no apparent sign of behavioural trouble until M_{25} (Fig. 3a). At the end of M_{25} the animals of the control groups showed signs of ulcerative dermatitis with ageing while C_{60}-treated animals remained normal. As the growths of all surviving animals showed no significant difference until M_{30} (Fig. 3b) indicating that the treatment did not alter their food intake, we continued observing their survival.

At M_{38} all water-treated control rats were dead (Fig. 3a). This agrees with the expected lifespan of this animal species that is thirty to thirty six months. At this time 67% of olive-oil-treated rats and 100% of C_{60}-treated rats were still alive.

The survival distributions for C_{60}-olive oil-treated rats and controls were estimated by the non-parametric Kaplan–Meier estimator (Fig. 3) and compared by a log-rank estimated test. The estimated median lifespan (EML) for the C_{60}-treated rats was 42 months while the EMLs for control rats and olive oil-treated rats were 22 and 26 months, respectively. These are increases of 18 and 90% for the olive-oil and C_{60}-treated rats, respectively, as compared to controls.

The log-rank test leads to χ^2 values (one degree of freedom) of 7.009, 11.302, and 10.454, when we compare water-treated and olive oil-treated rats, water-treated and C_{60}-treated rats, and olive oil-treated and C_{60}-treated rats, respectively. This means that olive oil extends the lifespan of rats with respect to water with a probability of 0.99 while C_{60}-olive oil extends the lifespan of C_{60}-treated rats with a probability of 0.999 and 0.995 with respect to water and olive oil treatments, respectively.

Table 2
Biodistribution of C_{60}. C_{60} concentrations in whole blood (WB), liver, spleen and brain of rats daily treated with a single dose of C_{60} dissolved in olive oil (4 mg/kg body weight) by oral gavages or i.p. route (IP, Mean ± SD, $n = 3$) (Lw = liver weight; Sw = spleen weight; Bw = brain weight; TAD = total administered dose).

	Oral (D_1)	Oral (D_8)	IP (D_1)	IP (D_8)
WB (C_{60}, μg/ml)	0.03 ± 0.01	0.18 ± 0.06	0.36 ± 0.06	0.56 ± 0.17
Liver (C_{60}, μg/g)	0.21 ± 0.04	2.92 ± 0.82	4.91 ± 1.52	31 ± 12
Lw (g)	5.2 ± 0.6	7.5 ± 0.8	7.7 ± 0.8	10.0 ± 1.1
C_{60} (%/TAD)	0.14	0.39	4.73	5.54
Spleen (C_{60}, μg/g)	2.99 ± 1.37	51 ± 14	23 ± 6	191 ± 40
Sw (g)	0.48 ± 0.10	0.56 ± 0.13	0.54 ± 0.17	0.70 ± 1.3
C_{60} (%/TAD)	0.18	0.51	1.55	2.39
Brain (C_{60}, μg/g)	0.013 ± 0.003	0.20 ± 0.08	0.54 ± 0.17	3.78 ± 1.25
Bw (g)	1.81 ± 0.03	1.83 ± 0.03	1.85 ± 0.05	1.82 ± 0.04
C_{60} (%/TAD)	0.003	0.007	0.125	0.123

3.4. Effect of C_{60}-olive oil solutions on oxidative stress

CCl_4 toxicity with respect to rats is well known [26,27] 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 inter-strain variability. In addition, to avoid errors due to inter-individual and inter-season variability, a CCl_4-treated control group was included in each experiment.

3.4.1. Animal behaviour and pathological examinations

A few minutes after CCl_4 injection, the animals showed inactivity, lethargy, and pilo-erection. For both GA groups (pre-treated with water only) these symptoms persisted during a period of 24 h until the animals were sacrificed for pathological examination. In contrast, for the animals pre-treated with olive oil or with C_{60}-oil (GC and GD groups) these symptoms completely disappeared about 5 h after CCl_4 intoxication.

After abdomen incision, 24 h after 0.9% NaCl administration, the livers of the control groups GB_{og} and GD_{og} orally treated with olive oil only or C_{60}-oil, respectively, exhibited normal morphology with brown colour more pronounced for GD_{og} animals than for GB_{og} ones (Fig. 4).

The livers of i.p. treated control groups GB_{ip} and GD_{ip} also exhibited normal morphology, nevertheless they showed large deposits of fat due to the accumulation of the administered lipids (Fig. 4). The brown colour of the GD_{ip} livers was more intense than that of the orally treated rats (GD_{og}).

As compared to spleens of GB animals treated with olive oil only, spleens of GD animals treated with C_{60}-olive oil exhibited a darker colour while those of GD_{ip} were hypertrophic (enlarged). Stronger effects in the appearances were observed in previous studies after administration of high doses of suspended C_{60} crystals to rodents, without any organ damage or toxic effect [20,21].

Twenty-four hours after CCl_4 administration, the livers of GA animals pre-treated with water were pale and looked mottled while their lobes were adherent in most cases (5 of 6 rats). In contrast the livers of GC and GE groups pre-treated with olive oil or C_{60}-oil, respectively, exhibited normal morphology with the same features as those observed for GB and GD control groups (Fig. 4).

At the microscopic scale, the liver sections of both GB and GD control groups treated with olive oil only or C_{60}-oil revealed normal parenchymal architecture without any inflammation or fibrosis. These liver sections only showed hepatocytes with clear cytoplasm due to lipid accumulation (Fig. 4). This phenomenon was more abundant in liver cells of i.p. treated animals than in those of orally treated animals. In these groups, C_{60} deposits were detected only in spleens as brown and diffuse clusters inside macrophages with higher abundances for i.p. treated rats than for orally treated ones (Fig. 2).

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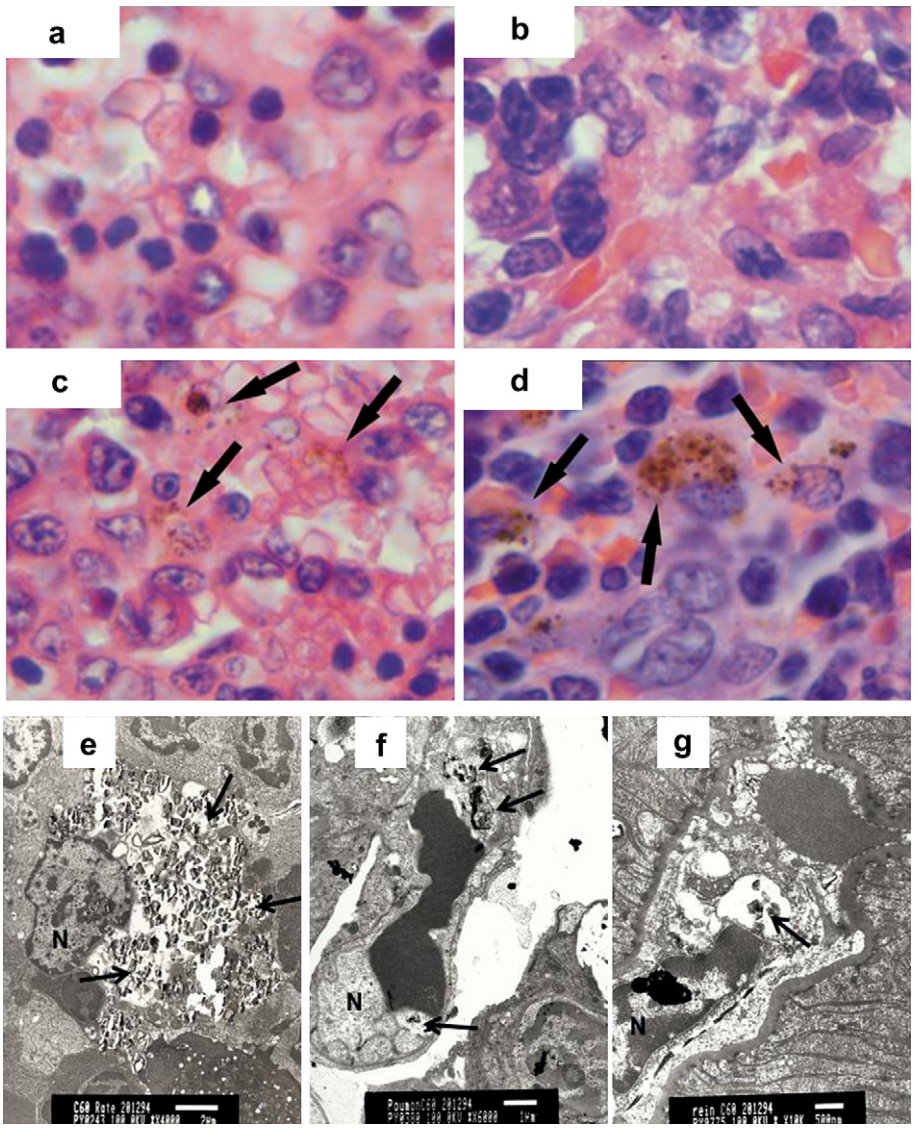

Fig. 2. Optical and electronic microscopies. Biodistibution studies after 7 successive days of C_{60}-olive oil treatment (4 mg/kg bw). Optical microscopy of spleen sections (Hematoxylin-Eosin staining, magnification = 1000×): (a) oral and (b) i.p. treatment with olive oil only; (c) oral and (d) i.p. treatment with C_{60}-olive oil. The arrows indicate C_{60} crystals-containing macrophages with specific brown colour. Transmission electron microscopy: compared to (e) spleen macrophages, TEM micrographs show only a few C_{60} crystals inside (f) lung and (g) kidney macrophages.

At the same time the liver sections of GA and GC animals co-treated with water and CCl_4 or with olive oil and CCl_4, respectively, showed important damage including many inflammatory areas as well as large necrotic areas with ballooning necrotic cells associated with an important steatosis (Fig. 4). In contrast, microscopic examination of the liver sections of GE animals co-treated with C_{60}-olive oil and CCl_4, revealed few necrotic areas with some ballooning cells without apoptosis limited to some cords of hepatocytes (Fig. 4).

3.4.2. Biochemical tests

3.4.2.1. C_{60} effects on CCl_4 induced liver damage. Circulating levels of alanine amino-transferase activity (ALT), used as a biochemical marker of liver injury [29], confirmed liver-protection by C_{60}.

Twenty-four hours after CCl_4 injection, the increase of ALT for GA and GC animals (pre-treated with water or olive oil) can reach more than 14 times and 12 times, respectively, the normal activity observed for GB control group (Fig. 5). In contrast, in the E_{og} and E_{ip} groups pre-treated with C_{60}-oil, the median of ALT activity was only about 5 and 1.2 times higher, respectively, than that observed in the control groups.

3.4.2.2. C_{60} effects on the endogenous antioxidant systems: glutathione, superoxidismutase and catalase activities

3.4.2.2.1. Glutathione system. The increase of the GSSG/TGSH ratio, used as a gauge for the circulating redox equilibrium [21,29], in the GA and GB groups pre-treated with water and olive oil can reach about 10 and 13 times respectively the GSSG/TGSH of the control group (Fig. 5) thus reflecting the intensity of the oxidative stress induced by the metabolism of CCl_4.

Oral pre-treatment with C_{60}-oil significantly prevents the increase of the GSSG/TGSH ratio in the GD_{og} group. As compared to the control group, the increase of GSSG/TGSH in the GD_{og} group was about 4 times higher only.

In the GD_{ip} group i.p. pre-treated with C_{60}-oil, the GSSG/TGSH was even significantly lower than in the control group. As the liver C_{60} content is significantly higher after i.p. administration than after o.g. (Table 2), this result confirms the dose–effect relationship.

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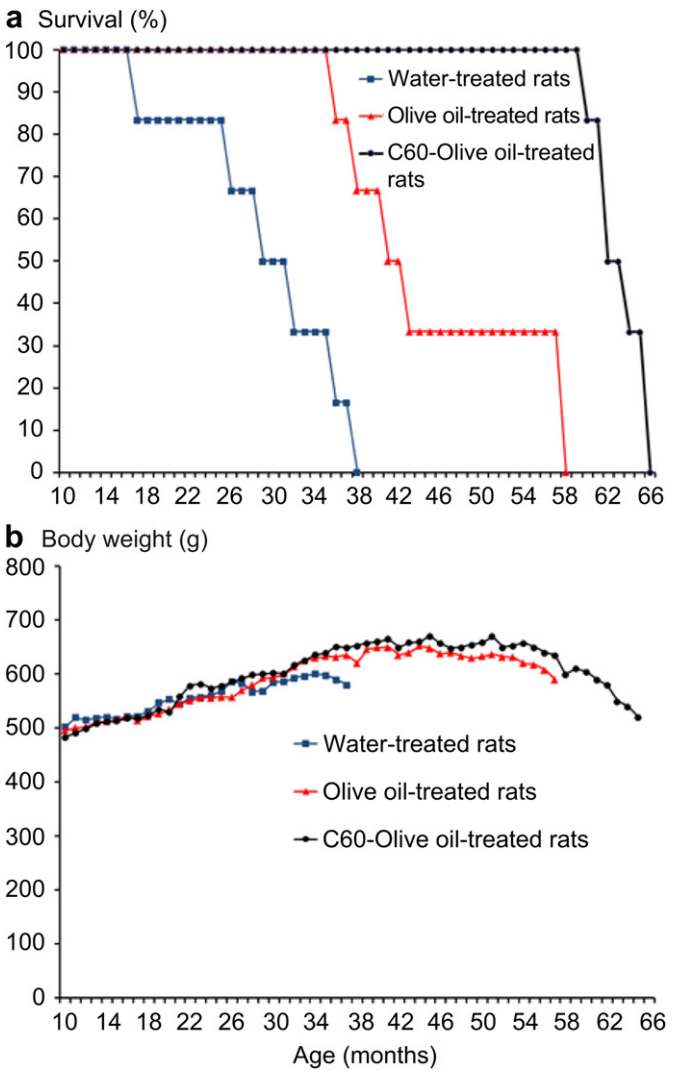

Fig. 3. Animal survival and growth. Chronic effects of C_{60} in rats. (a) Survival and (b) growth of surviving animals, after treatment (oral gavages) at reiterated doses (1.7 mg/kg bw) with water, olive-oil or C_{60}-olive oil.

It is worthnoting that in the GB animals treated by C_{60}-oil without CCl_4 intoxication the GSSG/TGSH ratio was significantly decreased (about twice as less) as compared to the control group.

3.4.2.2.2. Superoxidismutase (SOD) and catalase (CAT) activities. Animals of GA and GC groups pre-treated with water or olive oil by oral or i.p. routes adjusted to CCl_4 intoxication by increasing the CAT and SOD enzymatic activities in erythrocytes and livers (Fig. 6).

C_{60}-oil pre-treatment led to a significant attenuation of the increase of these activities. In addition this attenuation was more expressed in liver where fullerene accumulates than in blood (Table 2).

3.4.2.3. Effects of C_{60} on CCl_4 metabolism. The microsomal cytochrome P450 (CYP2E1) activity determined after microsome extraction from livers of orally treated animals shows that rats pre-treated by water or olive oil adjusted to CCl_4 intoxication by enhancing the biosynthesis of CYP2E1 (Fig. 7).

C_{60}-pretreatment significantly attenuated the increase of CYP2E1 activity after CCl_4 intoxication without inhibitory effect on this enzyme.

4. Discussion

4.1. C_{60}-olive oil solution preparation

It is well known that C_{60} and derivatives are prone to aggregate even in their best solvents [37]. The C_{60}-olive oil solution used in this study can be considered as free of C_{60} aggregates because: 1 – its colour is purple that is characteristic of C_{60} solutions while the colour of C_{60} aggregate-containing solutions are rather brown, which is true even for water-soluble derivatives [3]; 2 – it is freely and instantaneously soluble in toluene in contrast to C_{60} aggregate-containing solutions, which slowly dissolve even in the best solvents of C_{60}. Besides, the concentrations of C_{60} in olive oil as determined by HPLC agree with those previously published by other authors [22].

The stability of C_{60}-olive oil solution determined under our experimental conditions agrees with recently published results showing that the addition of [60]fullerene significantly hampers the peroxide formation thus increasing the stability of the tested oils [38].

4.2. Pharmacokinetics and biodistribution

4.2.1. Pharmacokinetics

The results of this pharmacokinetic study show for the first time that C_{60} is absorbed by the gastro-intestinal tract (Fig. 1).

In the case of oily solutions, the drug release rate is controlled by the partition coefficient of the drug between the oily vehicle and the tissue fluid and lipophilic drugs may be released concurrently with the disappearance of the oily vehicle from the injection site [39]. Thus, in the case of i.p. administration, the delay of 4 h for reaching C_{max} (Table 1) can be attributed to the affinity of C_{60} for the oily phase.

In the case of highly hydrophobic drugs (Log $P > 5$) it is well known that the absorption of the molecules by the gastro-intestinal tract occurs *via* the mesenteric lymphatic system after association with developing lipoproteins in the enterocytes rather than *via* the portal blood [40]. Therefore, as the octanol/water partition coefficient of C_{60} is estimated to be 6.67 [41], the absorption of C_{60} occurs *via* the mesenteric lymphatic system rather than *via* the portal blood. The longer delay for reaching C_{max} after o.g. (Table 1) can then be assigned to the fact that the flow rate of the mesenteric lymph in the *lamina propria* underlying the enterocytes is some 500 times lower than that of the portal blood [40].

C_{max} and the area under the curve (AUC) after o.g. are about 3 times and 5 times lower, respectively, than after i.p. administration. Although i.p. administration does not allow assessing the absolute bioavailability, AUCs comparison suggests that a significant percentage of the orally administered dose is absorbed by the gastro-intestinal tract.

The elimination half-lives indicate that C_{60} is completely eliminated from blood 97 h after administration irrespective of the route of administration. The difference in the elimination half-lives could be attributed to some precipitation of C_{60} in the injection site followed by a slow dissolution of C_{60} crystals in the surrounding tissue fluid. Consequently, the AUC after i.p. administration represents the soluble fraction only. Nevertheless, the precipitated fraction is likely very weak because the total elimination is only slightly delayed. The precipitation phenomenon is unlikely to occur for the oral route where the absorbed dose is about 5 times smaller and where C_{60} is carried by lipoproteins.

The elimination process follows a non-urinary route because unmodified C_{60} was not detected in urine samples taken up 48 h after administration. Previous investigations showed that C_{60} is mainly eliminated through the bile ducts [21] as it has been

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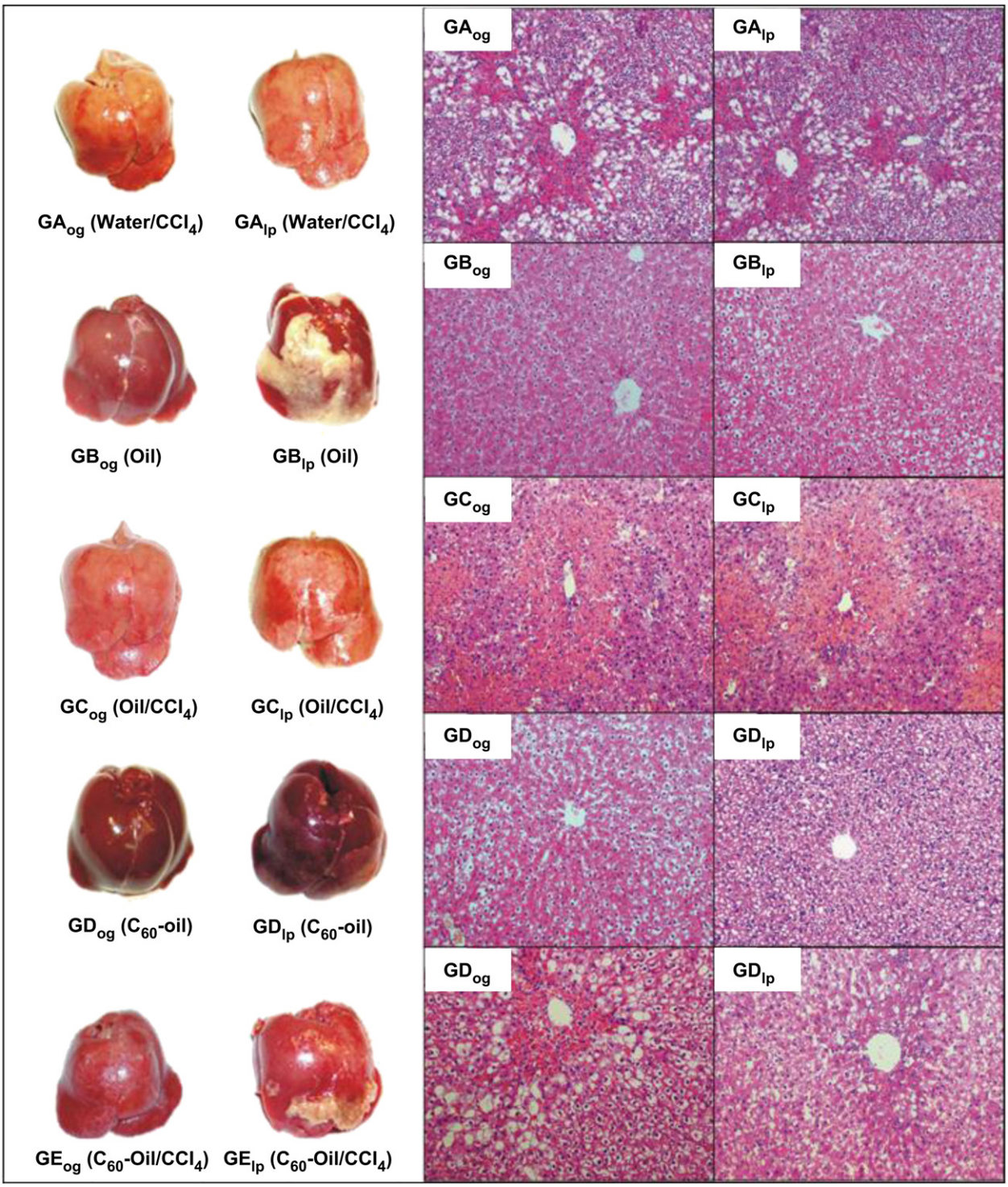

Fig. 4. Macroscopic and microscopic (Hematoxylin-Eosin staining, magnification = 100×) effects of C_{60}-oil pretreatment (4 mg/kg bw, during 7 successive days) on CCl_4 intoxication in rat livers. The animals were pre-treated by (GA_{og}-GE_{og}) oral gavages or (GA_{ip}, GE_{ip}) i.p. injection: (GA) water/CCl_4; (GB) oil/NaCl; (GC) oil/CCl_4; (GD) C_{60}-oil/NaCl; (GE) C_{60}-oil/CCl_4.

recently confirmed [25]. Besides, a small increase in C_{60} concentrations at 12 and 24 h after i.p. administration (Fig. 1) suggests the presence of an enterohepatic circulation [40]. Furthermore, it has been already shown that C_{60} reacts inside the liver cells with vitamin A following a Diels–Alder like reaction both in mice and in rats [21,42]. These two routes may be sufficient for C_{60} elimination, nevertheless, we have to look for other possible biotransformations and elimination routes, all the more so as the fate of the addition product is not known.

4.2.2. Biodistribution studies

As C_{60} and some of its derivatives mainly accumulate in the livers and spleens of rodents [15,21,42] we studied the biodistribution of C_{60} in these organs. To investigate its effects at

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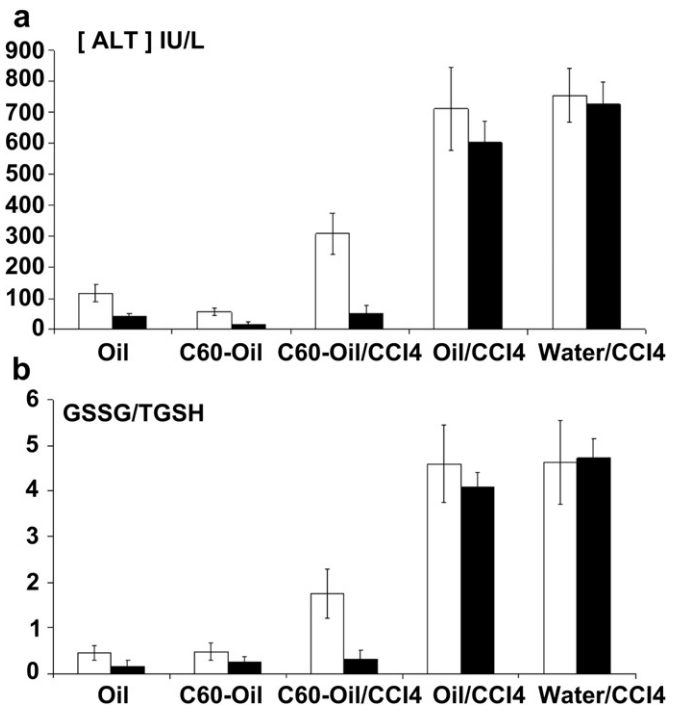

Fig. 5. Effect of C_{60} pre-treatment (4 mg/kg bw, 7 successive days) on (a) serum ALT activity and (b) circulating levels of oxidized glutathione/total glutathione ratio of rats intoxicated by CCl_4 administration. (GSSG) oxidized and (GSH) reduced glutathione, TGSH: total glutathione (GSSG + GSH). (White bars) orally pre-treated rats and (black bars) i.p. pre-treated rats. Data are the mean ± SD for six rats.

reiterated doses we also studied the accumulation of C_{60} in these organs after 7 successive days of administration.

The differences in C_{60} contents in livers and spleens (Table 2) can be obviously assigned to the differences in the absorbed doses. However, the delay of elimination which is somewhat larger after i.p. administration could also play a non negligible role. The presence of C_{60} crystals inside the cells after i.p. administration (Fig. 2) supports the hypothesis according to which the precipitation of part of the administered C_{60} in the injection site may contribute to the observed delay of elimination after i.p. administration. Nevertheless, the weakness of organ concentrations notably at D_8 after 7 daily successive administrations of C_{60} dissolved in olive oil clearly shows that C_{60} molecules are eliminated from the organs in a few hours after both oral and i.p. administrations.

Previous results obtained after i.p. administration of large doses of micronized C_{60} [21] or intratracheally instilled C_{60} suspensions [43] showed that the clearance of C_{60} from organs can take several days. These longer delays of eliminations are likely due to the slow dissolution of C_{60} crystals inside the organ RESs [21,43]. In the case of tail vein administration [25] it is difficult to compare the data because C_{60} was complexed with liposomes. The scarceness of C_{60} crystals inside lung and kidney cells (Fig. 2) confirms the difference in behaviour of C_{60}-liposome complexes, which mainly accumulates in lungs after tail vein administration [25].

As C_{60} contents in lungs and kidneys are likely weaker than those in livers and spleens, we only focused on C_{60} content in brains because the issue of its translocation to the brain is still a matter of debate [25,43].

Whereas C_{60} particles were not detected in the brain after intratracheal instillation [43], the presence of significant amounts in the brain 24 hours after both oral and i.p. administrations under our experimental conditions (Table 2) confirms that solubilized C_{60} can cross the blood–brain barrier [25].

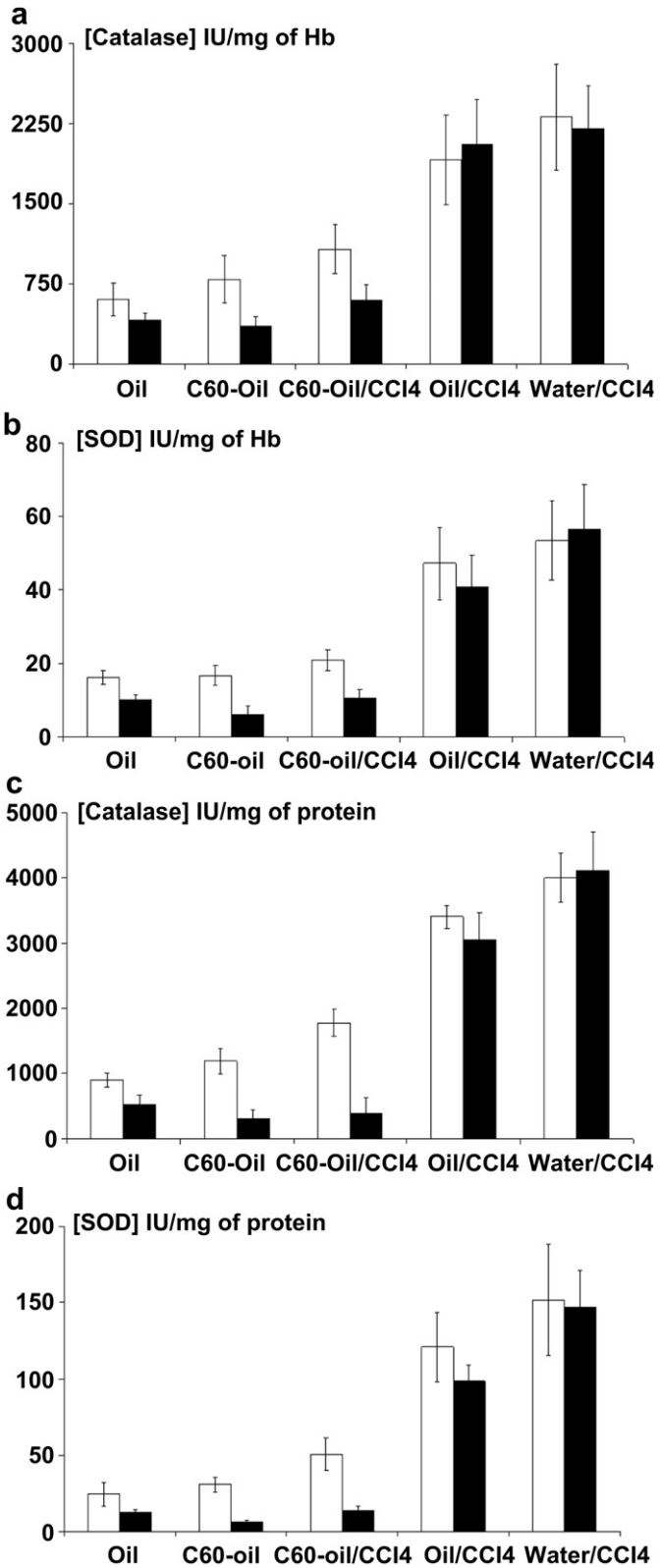

Fig. 6. Effect of C_{60} pre-treatment (4 mg/kg bw, 7 successive days) on catalase and superoxide-dismutase (SOD) activities of rats intoxicated by CCl_4 administration. (a, b) erythrocyte activities and (c, d) liver activities. (White bars) orally pre-treated rats and (black bars) i.p. pre-treated rats. Data are the mean ± SD for six rats.

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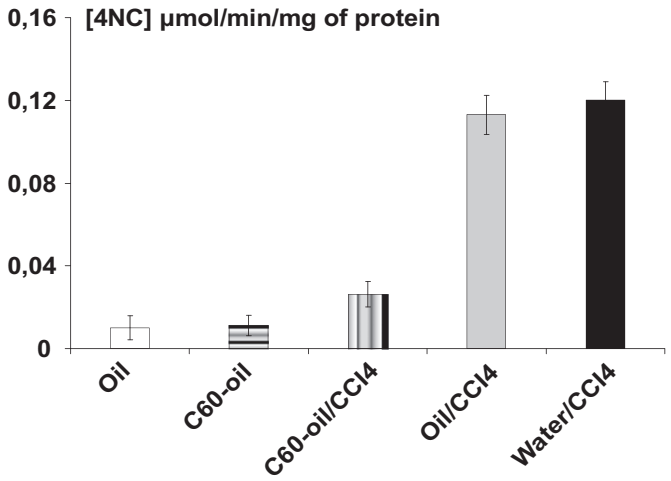

Fig. 7. Effect of C_{60} pre-treatment by oral gavages (4 mg/kg bw, 7 successive days) on the hepatic microsomal cytochrome CYP2E1 activity of rats intoxicated by CCl_4 administration. The oil and C_{60}-oil groups refer to control rats without CCl_4 administration. Data are the mean ± SD for six rats.

A complete biodistribution study including intestine, skin, bone and fatty tissue is in progress in our laboratory.

4.3. Chronic toxicity and effects of C_{60} on survival of treated rats

As C_{60} is absorbed after o.g. we designed a protocol to study its chronic toxicity according to the general guidelines of US FDA [27] with some modifications. C_{60} has no acute or sub-acute toxicity in rodents [5,15] as it was further confirmed in various experimental models [16–19]. As it can act as an antioxidant (5, 6, 21), we investigated its chronic toxicity concomitantly with its effects on the survival of rats.

Ten-month old male rats (M_{10}) were chosen instead of young rats as officially recommended [27], in order to avoid possible compensatory effects that can occur during early development [44]. As biodistribution studies after daily gavages showed that C_{60} accumulates in livers and spleens, in order to avoid the negative effects of prolonged olive oil administration such as obesity, excessive steatosis, liver lipid degeneration, and insulin resistance [45], we treated the rats daily only during 7 days and weekly during the first two months, then every two weeks until one control rat died.

Our results show that while olive oil treatment can lead to an increase of 18% of lifespan of treated rats, C_{60}-olive oil can increase it up to 90%, as compared to controls. The effects of olive-oil on health and ageing are well known [46], and its effect as a function of dose has been thoroughly discussed [45]. But, what is noteworthy is that at M_{38} all C_{60}-treated rats were still alive. Thus, based on previous investigations [44], C_{60} should be the most efficient ever material for extending lifespan.

Significantly weaker similar effects have already been reported in several experimental models but for different hydrosoluble C_{60}-derivatives [44,47]. The effects of C_{60}-derivatives on ageing were attributed to the antioxidant properties and the attenuation of age-associated increases in oxidative stress [4,44].

Actually, the free-radical scavenging effect remains valid for a number of C_{60}-derivatives with different addends [3,4,44,47–49], which indicates that this property is related to the C_{60} moiety. Indeed C_{60} itself is a powerful antioxidant as demonstrated in different experimental models [5,6,21].

As our results show that C_{60} is more efficient than its derivatives [44,47], they confirm that the effects of C_{60}-derivatives on ageing are mainly due to the C_{60} moiety, as it has been postulated previously [4].

This is the first investigation of the *in vivo* chronic effects of a soluble form of C_{60}. The absorbed doses are very low and their efficacy on oxidative stress can be questioned. We already showed that C_{60} is a powerful antioxidant in a classical *in vivo* experimental model in rats [21]. But we then used an aqueous suspension and the most efficient doses were about 2500 times higher (2 g/kg bw), which are considerably higher than those observed for its derivatives as well as those used for most biomedical applications. In addition there was a latency period (14 days after administration) to reach the maximum efficiency. It was stated that there was no correlation between the degree of protection and the number of C_{60} clusters observed in the livers, suggesting that C_{60} is active only in a soluble form that is when its double bounds are accessible [21].

To check this hypothesis we studied the effects of C_{60}-olive oil solutions in the same experimental model.

4.4. Effects of C_{60}-olive oil solutions on oxidative stress

As we wrote before, four possible mechanisms for C_{60}-liver protection were proposed [21]: (1) C_{60} can scavenge large numbers of free radicals [5,6,21]; (2) it can act as a decomposition catalyst for $O_2^{\bullet -}/H_2O_2$, as it has been postulated for its tris-malonic acid derivatives [4] or (3) as a cytochrome P450 inhibitor as it has been reported for some fullerene derivatives [21]; and (4) it can inactivate Kupffer cells (liver resident macrophages) through accumulation and overloading with a large number of C_{60} aggregates [2].

Biodistribution studies (Table 2) show that C_{60}-liver concentrations after seven successive daily o.g. or i.p. administrations of 4 mg/kg bw of C_{60}-olive oil solution are nearly 7 times lower or 1.5 times higher, respectively, than those observed in previous studies at 14 days, after i.p. injection of large doses (2 g/kg bw) of C_{60}, when the optimum hepatoprotective effect was obtained [21]. Thus, in order to study the effects of C_{60}-oil solutions on CCl_4 toxicity, we pre-treated the animals daily for 7 days by i.p. or o.g. administrations before CCl_4 treatment.

Pathological examinations show that even at very low doses, 500 times lower than that used previously [21], C_{60}-olive oil solutions effectively protects the livers against CCl_4 toxicity. These results are in agreement with those reported for very low doses of water solution of hydrated C_{60} fullerene in other experimental models [5,6].

The number of necrotic areas observed in the liver sections of GE_{ip} animals was significantly lower than that observed in the GE_{og} group (Fig. 4). As C_{60} concentrations in the livers of GE_{ip} animals are probably about 10 times higher than those of the livers of GE_{og} group (Table 2), these results confirm the dose–effect relationship reported previously [21].

The results obtained for liver injury biomarkers (Fig. 5) are even better than those obtained after administration of a large dose (2 g/kg bw) of C_{60} suspended in aqueous medium [21]. These results confirm the hypothesis according to which this fullerene is active against oxidative stress only in soluble form [21]. In addition, while the median of ALT in the GD_{og} animals orally treated with C_{60}-oil was equivalent to that of the control group, the activity of this enzyme was even lower in the GD_{ip} animals. These results strengthen the dose–effect relationship and confirm that C_{60} is a powerful liver-protective agent.

As to the involved mechanism, since the doses used in these experiments are very low and since there are no excessive C_{60} deposits inside Kupffer cells (hepatic macrophages), the hypothesis according to which C_{60} can inactivate these phagocytes by overloading them [21] must be discarded.

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The initial liver damage after CCl_4 administration is mediated through its metabolism by cytochrome P450 2E1 (CYP 2E1) resulting in the formation of the trichloromethyl radical $CCl_3^{\bullet}$ [28,29]. This radical can also react with oxygen to form a highly reactive species, the trichloromethyl peroxy radical $CCl_3OO^{\bullet}$ which can rapidly initiate the chain reaction of lipid peroxidation [28,29]. As C_{60} is able to scavenge *in vitro* a large number of radicals per molecule [50] including $CCl_3^{\bullet}$ and $CCl_3OO^{\bullet}$ [51] and because this property can be involved in the mechanism of protection against CCl_4 toxicity, we explored the effects of C_{60} on the antioxidant systems that play a critical role in the defence against oxidative stress, including the circulating levels of reduced (GSH) and oxidised forms (GSSG) of glutathione as well as catalase (CAT) and superoxide-dismutase (SOD) activities in erythrocytes and livers [29].

The results obtained for the glutathione system (Fig. 5) confirm the antioxidant effect of C_{60} and even its modulating effect on the intracellular redox status even in the absence of CCl_4 [21]. The results obtained for the antioxidant enzyme activities (Fig. 6) also confirm the antioxidant effect of C_{60} against CCl_4 toxicity.

The suppression of CYP2E1 activity can result in a reduction in the level of the resulting CCl_4 reactive metabolites, and, correspondingly, a decrease of tissue injuries. Therefore, a possible mechanism for the liver-protection by C_{60} may involve CYP2E1 inhibition. As C_{60} is insoluble in the usual biological systems used to study the *in vitro* activity of this enzyme, we used the hepatic microsomal fractions of orally treated rats to assay the CYP2E1-specific oxidative activity p-nitrophenol hydroxylase [33] in order to check this hypothesis. C_{60}-pretreatment significantly attenuated the increase of CYP2E1 activity after CCl_4 intoxication without inhibitory effect on this enzyme (Fig. 7). The absence of inhibitory effects is reflected in the presence of a residual activity significantly higher than that of the control group. Besides, the activity of the control group treated with C_{60} only is not significantly different from the control group treated with water only. Therefore the hypothesis of CYP2EI inhibition by C_{60} must also be discarded.

The results of the present study, notably the prevention of GSH depletion, rather suggest that the protective effect involves a free-radical scavenging mechanism.

It has been recently reported that administration of C_{60} suspended (not dissolved) in corn oil by oral gavage can increase the hepatic level of 8-oxodG whereas corn oil per se generated more genotoxicity than the particles [52]. Surprisingly, the authors did not conclude that C_{60} may prevent the genotoxicity of the used vehicle.

It is to be stressed that dissolved C_{60} appears hundred times more active than when it is in suspension [21]. In fact the action of soluble C_{60} is immediate while that of suspended C_{60} is delayed because it has to be dissolved to act. In all cases, based on C_{60}-liver content (Table 2), these results prove that this fullerene is active at the nano molar level. However, the involved mechanism remains to be established.

For the time being the hypothesis of free-radical scavenging of $CCl_3O^{\bullet}$ or $CCl_3COO^{\bullet}$ remains possible. C_{60} could act as a free-radical scavenger as it has been widely demonstrated *in vitro*, but up to now no resulting C_{60} adduct has been observed *in vivo*. The only *in vivo* reaction ever observed for C_{60} is a Diels–Alder like reaction with retinol and retinyl-esters inside the liver cells [42]. We are presently trying to detect some C_{60}-adducts resulting from a possible radical addition.

C_{60} could also act as a superoxide-dismutase mimetic as it has been modelized *in silico* and experimented *in vitro* for one of its water-soluble derivatives [4]. However, our results show that this is unlikely for pristine C_{60} because the increase of H_2O_2 concentration due to such activity should induce a correlated increase of catalase activity, which is not the case under our conditions (Fig. 6). Alternatively, C_{60} could act as superoxide/catalase mimetics *in vitro* [4], but this is not the case *in vivo*.

Another possible mechanism has been also proposed for water solutions of hydrated C_{60} fullerene [6]. It suggests that the structured water layer around C_{60} can be able to deactivate hydroxyl radicals by allowing recombination to hydrogen peroxide. Once again, this mechanism remains to be confirmed by means of other experiments.

5. Conclusion

The effect of pristine C_{60} on lifespan emphasizes the absence of chronic toxicity. These results obtained with a small sample of animals with an exploratory protocol ask for a more extensive studies to optimize the intestinal absorption of C_{60} as well as the different parameters of the administration protocol: dose, posology, and treatment duration. In the present case, the treatment was stopped when a control rat died at M_{17}, which proves that the effects of the C_{60} treatment are long-lasting as the estimated median lifespan for C_{60}-treated rats is 42 months. It can be thought that a longer treatment could have generated even longer lifespans. Anyway, this work should open the road towards the development of the considerable potential of C_{60} in the biomedical field, including cancer therapy, neurodegenerative disorders and ageing. Furthermore, in the field of ageing, as C_{60} can be administered orally and as it is now produced in tons, it is no longer necessary to resort to its water-soluble derivatives, which are difficult to purify and in contrast to pristine C_{60} may be toxic.

Acknowledgements

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