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Toxic and genotoxic effects of oral administration of furan in mouse liver

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In this study, the effects induced in mouse liver by repeated oral exposure to furan were investigated. To this aim, the compound was given for 28 days by daily gavage to male B6C3F1 mice at 2, 4, 8 and 15 mg/kg body weight (b.w.)/day. Twenty-four hours after last administration, animals were sacrificed, liver was excised and the following parameters were evaluated: histological alterations, apoptosis, cell proliferation, polyploidy, overall DNA methylation, gene expression and DNA damage by the immunofluorescence detection of foci of phosphorylated histone H2AX (γ-H2AX) and by alkaline comet assays, using both standard and modified protocols for the detection of DNA cross links. Liver DNA damage by comet assays was also evaluated in mice receiving furan as a single acute oral dose (15, 100 or 250 mg/kg b.w.). Microscopic analysis of liver sections indicated that repeated oral administration of furan was moderately toxic, producing mild histological alterations with necrotic figures, apoptosis and limited regenerative cell proliferation. The flow cytometric analysis of DNA content in single-cell suspensions of liver cells showed a statistically significant increase in polyploid (8N) cells at the highest dose. No treatment-related changes in overall DNA methylation, γ-H2AX foci, DNA strand breaks and cross links were observed at the end of the 4-week exposure period. However, several genes involved in DNA damage response, beyond stress and liver toxicity, were overexpressed in mice treated with the highest furan dose (15 mg/kg b.w./day). Acute administration of furan induced evident liver toxicity at the highest dose (250 mg/kg b.w.), which was associated with a significant increase of DNA damage in the alkaline comet assay and with a distinct decrease in γ-ray-induced DNA migration. Overall, the results obtained suggest that the contribution of genotoxicity to the mechanism of furan carcinogenicity in mouse liver should not be dismissed.

Introduction

Furan is a heterocyclic compound widely used in the chemical manufacturing industry as an intermediate in the synthesis of a variety of polymers. Recent surveys of canned and jarred foods have shown that furan may also be present in a variety of food items that undergo heat treatment, including baby food, at concentrations that may exceed 100 μg/kg of food. Based on data available, upper dietary exposure levels in the order of one to few micrograms of furan per kilogram body weight (b.w.) can be calculated, with relatively higher figures for babies consuming canned food (1,2).

National Toxicology Program (NTP) 2-year long-term bioassays in Fisher 344/N rats and B6C3F1 mice have shown that furan is a potent carcinogen in both species (3). In rats, daily administration of a low furan dose (2 mg/kg b.w.) induced cholangiomas in nearly 100% of animals of both sexes; at higher doses (4 and 8 mg/kg b.w.), hepatocellular adenomas and carcinomas and mononuclear cell leukaemia were also significantly increased. In mice, daily administration of 8 and 15 mg furan/kg b.w. was associated with high incidences of hepatocellular adenomas and carcinomas in males and females. In both species, numerous non-neoplastic liver lesions were also observed.

Considering the high carcinogenic potency of furan, a relatively small margin exists between doses carcinogenic in rodents and human exposure levels associated with the presence of furan in food. Whether this difference is sufficiently large to be acceptable, or it may imply a health risk, cannot be established at present. In fact, uncertainties on the carcinogenic mode of action of furan prevent a reliable assessment of risk at low doses (4). In particular, the role of genotoxicity in the mode of action of furan needs to be elucidated in order to establish whether a tolerable human exposure level can be set for furan, similarly to other non-genotoxic carcinogens (5). Information on furan genotoxicity is largely based on the results of studies *in vitro* with cultured mammalian cells, in which both furan and its main metabolite *cis*-2-butene-1,4-dial tested positive (3,6,7). On the other hand, limited and contrasting information is available on the genotoxic potential of furan *in vivo*. When given by intraperitoneal (i.p.) injection, furan increased the incidence of structural chromosomal aberrations, but not sister chromatid exchanges, in bone marrow cells of mice (3); in another study, using a sensitive flow cytometric method, no induction of micronuclei was observed in mouse polychromatic erythrocytes following i.p. or subcutaneous injection of furan (8). In liver, the main target of furan carcinogenicity, no induction of unscheduled DNA synthesis (UDS) was observed in rats and mice treated with a single oral dose of furan (3,9).

In order to establish the plausibility of a genotoxic mode of action, the weight of the experimental evidence has to be critically evaluated based on its relative relevance (10); in this respect, the evidence of genotoxicity in the target organ for

tumour induction, especially if elicited under exposure conditions similar to those associated with cancer onset, can be considered of the highest relevance and adds confidence to the hypothesis of a genotoxic mode of action. In case of furan, the available information on genotoxicity in liver is limited to negative UDS assays following single oral administration (3,9). These assays, however, have been considered unsuitable to demonstrate the absence of genotoxicity of furan *in vivo* because they are basically insensitive to mutagenicity resulting from misrepair or non-repair (4). No information is available on the genotoxic effects elicited by furan when administered under the same treatment conditions applied in long-term studies.

Thus, in order to provide information useful for the elucidation of the mode of action and for the characterization of risk, an investigation was undertaken to assess the genotoxic effects induced *in vivo* in B6C3F1 mice by repeated oral administration of furan, using the same dose levels applied in the NTP carcinogenicity bioassay. The analysis of end points related to genotoxicity and genetic stability of liver cells was integrated with markers of tissue damage and cellular toxicity, in order to achieve a comprehensive picture of the effects elicited by subacute administration of carcinogenic doses of furan in mouse liver.

Materials and methods

Animals

Male B6C3F1 mice aged 5–6 weeks were obtained from Harlan s.r.l. (Udine, Italy). Animals were housed in a room with a barrier system under standard environmental conditions (22 ± 2°C, 55 ± 15% relative humidity, on a 12 h light–dark cycle), with drinking water and laboratory rodent diet *ad libitum*. Experiments were carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health on the *in vivo* experimentation.

Chemicals

Furan (C_4H_4O, CAS 110-00-9) was purchased from Sigma–Aldrich Italia, Milan, Italy. The product tested (cat. no. 18,592-2) was >99% pure, stabilized with 0.025% of butylated hydroxytoluene. Fresh solutions of furan in corn oil (CAS 8001-30-7, from Sigma-Aldrich Italia) were prepared before each experiment. Precautions were used in order to minimize evaporative losses during furan handling. For the preparation of test solutions, a known volume of furan was added to a weighted amount of corn oil, the solution weighted and adjusted to the final concentration adding the required amount of corn oil. Solutions of furan were quickly aliquoted in filled amber vials, one for each day of treatment, which were tightly sealed and stored at 4°C. The actual furan concentrations of test solutions were routinely checked by head-space capillary gas chromatography with flame ionization detector. The correlation coefficient of nominal and actual furan concentrations in corn oil solutions was >0.99.

Treatments of animals

After arrival, mice with similar average body weight were assigned to treatment groups and acclimatized 1 week in standard conditions. Health status of animals was controlled daily. Body weight was recorded at the beginning of treatment and then weekly. In acute experiments, three furan doses, 15, 100 and 250 mg/kg b.w., were given once by gavage 3 h before sacrifice to mice (five to six per experimental group). In repeat dose experiments, four furan dose levels, 2, 4, 8 and 15 mg/kg b.w., were administered by gavage 5 days/week for 28 days, and animals were sacrificed by cervical dislocation 24 h after last administration. The delivered volume was 10 ml/kg b.w. in all cases.

Methyl methanesulphonate (Sigma–Aldrich Italia), dissolved in dimethylsulphoxide (DMSO) and administered by i.p. injection at 80 mg/kg b.w., was used as positive control in genotoxicity assays. Carbon tetrachloride (Sigma–Aldrich Italia), dissolved in corn oil and given by i.p. at 40 mg/kg b.w. 24 h before sacrifice, served as positive control in the analysis of chemically induced liver lesions. Irradiation *in vivo* with 4 Gy of γ-rays was used as positive control for γ-H2AX (see below).

For the assessment of liver cell proliferation, satellite groups of treated and control mice were given i.p. injections of 5-bromo-2-deoxyuridine (BrdU) (Sigma–Aldrich Italia), 100 mg/kg b.w. in 0.9% NaCl, both 24 and 2 h before the sacrifice.

Preparation of biological samples

At sacrifice, liver was excised, weighted and dissected. Left lobes were minced to obtain a monodispersed cell suspension used for comet assays, flow cytometry, immunodetection of anti-phospho-histone and determination of overall DNA methylation. Caudate lobes were fixed, embedded in paraffin and sectioned for histological analyses, BrdU incorporation and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays. Right lobes were stored in a RNA stabilizing solution (RNA later; Ambion) at −80°C for subsequent RNA extraction and gene expression analysis (see below).

Histopathology

Five-micrometre-thick liver sections were deparaffinized by heating at 60°C, followed by washing in xylene and rehydrated through a graded series of ethanol. After redistilled water washings, sections were stained with haematoxylin and eosin, washed in tap water, then in redistilled water and mounted. Samples were examined at a light microscope (Optiphot; Nikon).

BrdU incorporation

Liver sections were prepared and deparaffinized as described above. Before the incubation with anti-BrdU antibody (Boehringer, Mannheim, Germany) and secondary anti-mouse-AP antibody (Dako, Glostrup, Denmark), sections were trypsinized for 30 min in a humid chamber and DNA breaks were induced by heating the sections for 10 min at 100°C in pH 6 citrate buffer. Staining was accomplished using the Fast Red Substrate System (Dako), which yields a red reaction product at the site of the target antigen. The immunostained sections were lightly counterstained with haematoxylin for microscopic examination. BrdU incorporation was quantitated by assessing a total of 1000 cells per tissue section and calculating the percentage of positive nuclei (red nuclei) under a light microscope at ×400 magnification.

TUNEL assay

For the evaluation of apoptosis, a commercial kit for TUNEL assay (Roche, Indianapolis, IN, USA) was used, according to the manufacturer's instructions. Tissue sections, deparaffined as described above, were permeabilized by incubation with 20 μg/ml proteinase K (Sigma–Aldrich Italia) for 20 min at 37°C in a humid chamber and treated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase, followed by the fluorescein isothiocyanate (FITC)-labelling solution. A 4′,6-diamidino-2-phenylindole (DAPI) solution in Vectashield antifade (Vector Laboratories, Burlingame, CA, USA) was used to counterstain nuclei for the analysis of FITC-labelled nuclei. Sections were examined in a fluorescence microscope at ×400 magnification. DNase-I-induced DNA strand breaks (3 U/ml; Sigma–Aldrich Italia) served as positive control.

Immunofluorescence detection of γ-H2AX

Foci of phosphorylated histone H2AX (γ-H2AX) were detected and quantitated in hepatocytes. Freshly separated hepatocytes were harvested by cytospin centrifugation at 20 g for 5 min, fixed for 10 min in 4% formaldehyde and maintained in phosphate-buffered saline (PBS) at 4°C until immunofluorescence labelling. To this aim, cells were permeabilized with 0.5% Triton X-100 for 10 min. After a blocking step in 10% bovine serum albumin, slides were immunostained with a mouse monoclonal anti-phospho-histone H2AX antibody (Ser139, clone JBW301; Upstate Biotechnology, Charlottesville, VA, USA) and diluted 1:1000 in 3% goat serum/PBS for 2 h in a humid chamber at room temperature. Samples were washed two to three times for 10 min in 0.02% Tween 20 in PBS, incubated with Alexa fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR, USA) and diluted 1:1000 in 3% goat serum/PBS for 45 min at 37°C in a humid chamber. After further washes, nuclear DNA was stained with 1 μg/ml DAPI. Slides were mounted in Vectashield (Vector Laboratories) and analysed under a fluorescence microscope. Based on the number of γ-H2AX foci, cells were classified in one of three classes: 0 signals, 1–9 signals or ≥10 signals. Two hundred cells were analysed for each animal.

DNA methylation

Overall, DNA methylation was determined according to a published method based on the incorporation of [^{3}H]methyl groups by a bacterial CpG methylase in genomic DNA (11). In this assay, the ability of DNA to incorporate [^{3}H]methyl groups *in vitro* is inversely related to endogenous methylation. Briefly, DNA (0.5 μg per sample) was incubated with 2 μCi [^{3}H]methyl *S*-adenosylmethionine (Perkin-Elmer Italia, Monza, Italy) in the presence of 3 U SssI CpG methylase (New England BioLabs), 3 μl NEN buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 at 25°C) and sterile distilled water to a total reaction volume of 30 μl. The reaction mixture was incubated at 30°C for 1 h. All samples were tested in triplicate. Methylated DNA was isolated on Whatman DE81 ion-exchange paper filter (Carlo Erba, Milan, Italy) and washed three times with 15 ml of 0.5 M phosphate buffer

(pH 7.4), once with 2 ml 70% ethanol and once with 2 ml 100% ethanol. The filter was air-dried at room temperature and placed in vial with 10 ml of aqueous counting scintillation fluid. Reaction mixtures with all components except DNA served as blank. The radioactivity incorporated in samples was determined with a Packard TRI-CARB1600 TR beta-counter.

Flow cytometric analysis of DNA content in liver cells

Monodispersed liver cells were fixed in 70% ethanol and stored at −20°C. For analysis, cells were treated with a detergent solution (0.1 M citric acid and 0.5% Tween 20) for 10 min at room temperature and pipetted to obtain a nuclear suspension that was stained with a solution containing 50 μg/ml propidium iodide (PI, Sigma-Aldrich Italia) and 40 μg/ml RNAse (Sigma). Flow cytometry was performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). The excitation wavelength was 488 nM. Red fluorescence was collected using a long pass red filter (LP620). A minimum of 10 000 cells per sample were analysed. Cell Quest software (Becton Dickinson) was used for acquisition and analysis. After exclusion of doublets and aggregates, regions were set on DNA histograms to evaluate the percentage of nuclei with 2N, 4N or 8N DNA content.

Comet assay

For comet assays, freshly isolated liver cells were resuspended in PBS at 10×10^6 cells/ml. The assay was performed essentially as described by Singh *et al*. (12) with minor modifications. Briefly, cells were suspended in 0.7% (w/v) low-melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA) and sandwiched between a lower layer of 1% (w/v) normal-melting point agarose (Bio-Rad Laboratories) and a upper layer of 0.7% (w/v) low-melting point agarose on microscope slides (Carlo Erba). The slides were then immersed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA and 10 mM Tris, pH 10) containing 10% DMSO (Carlo Erba) and 1% Triton X-100 (Sigma) overnight at 4°C. At lysis completion, the slides were placed in a horizontal electrophoresis tank with fresh alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH $\geq$ 13) and left in the solution for 25 min at 4°C to allow the DNA to unwind and the alkali-labile sites to express. Electrophoresis was carried out at 4°C for 25 min, 27 V (1 V/cm) and 300 mA, using a Hoefer power supply (Pharmacia Biotec Inc., San Francisco, CA, USA). After electrophoresis, slides were immersed in 0.3 M sodium acetate in ethanol for 30 min. Slides were then dehydrated in absolute ethanol for 2 h and immersed for 5 min in 70% ethanol. Slides were air-dried at room temperature. Immediately before scoring, slides were stained with 12 μg/ml ethidium bromide (Sigma) and examined, at ×200 magnification, with an Olympus fluorescence microscope. Slides were analysed by a computerized image analysis system (Delta Sistemi, Rome, Italy). One hundred cells were scored for each experimental point from two different slides. To evaluate the amount of DNA damage, the percentage of total DNA in the tail (% DNA) was used.

In view of the chemical structure of the main furan metabolite, cross links could be involved as primary lesion induced by furan administration. To probe this hypothesis, aliquots of hepatocyte suspensions were also assayed following a modified comet protocol designed to highlight the presence of DNA cross links (13). Briefly, slides prepared as for standard protocol were irradiated with 4 Gy Ce^{137}-γ-rays (dose rate 0.891 Gy/min) just before the lysis step. All the following steps were the same as for the standard protocol.

RNA purification and gene expression analysis

Total RNA was isolated from frozen tissue with the PaxGene Blood RNA System (Qiagen, Hilden, Germany) and purified with commercial reagents (Rneasy Mini Kit; Qiagen). The quality of all RNA samples was checked by electrophoresis on an Agilent 2100 BioAnalyzer using an RNA 6000 Nano LabChip® (Agilent Technologies, Boeblingen, Germany).

The analysis of gene expression of selected toxicity and DNA damage response genes was carried using RT^2 Profiler PCR arrays (SABiosciences, Frederick, MD, USA): each array contained a panel of 96 primer sets for a thoroughly researched set of 84 relevant pathway-focused genes (stress and toxicity response or DNA damage response), plus 5 housekeeping genes and 7 RNA and polymerase chain reaction (PCR) quality controls. RNA samples were converted into first strand complementary DNA, the template for the PCR, using the RT^2 First Strand Kit (SABiosciences). Then, the template was mixed with the RT^2 qPCR Master Mix and aliquoted into each well of the same plate containing pre-dispensed gene-specific primer sets. Real time PCR was performed using a two-step cycling program: 10 min at 95°C, required to activate the HotStart DNA polymerase, and 40 cycles of 15 s at 95°C and 1 min at 60°C. A threshold of 2-fold change in gene expression was defined to identify up-regulated and down-regulated genes.

Statistics

Mean frequencies of the study parameters in different experimental groups were compared by two-tailed Student's *t*-test. The limit for statistical significance was set at $P = 0.05$.

Results

Subacute (28-day) oral administration

Mortality, clinical signs and body weight. A preliminary range-finding experiment was performed administering five furan doses, i.e. those tested in the NTP long-term bioassay (8 and 15 mg/kg b.w.) and three lower ones (1, 2 and 4 mg/kg b.w.), to groups of three mice, 5 days a week for 28 days. No mortality or signs of overt toxicity related to furan administration were recorded during treatment period. Based on the lack of adverse effects, the dose levels of 2, 4, 8 and 15 mg/kg b.w. were selected for subsequent repeat dose main experiments with six animals per dose (eight vehicle controls). Data on body weights, body weight gain and organ weight at the end of 28 days of oral administration of furan in the preliminary and in the main experiments are summarized in Table I. Body weight and body weight gain were not significantly affected by treatment. Compared to mice receiving the vehicle alone, slightly increased relative liver weights ($P < 0.05$) were recorded in mice treated with 4 mg furan/kg b.w./day and above.

Histopathology, cell proliferation and apoptosis. Histopathological examination of liver tissue showed that the two higher furan concentrations (Figure 1) induced patchy areas of necrosis in the parenchyma. They were surrounded by hepatocytes showing eosinophilic cytoplasm and hyperchromatic nuclei, with densely packed chromatin, suggesting regenerative hyperplasia.

Cell proliferation was evaluated measuring nuclear BrdU incorporation. BrdU-positive cells were clearly identified after staining with Fast Red (Figure 2). Red nuclei, which incorporated BudR during S phase, were relatively rare in liver sections of mice treated with corn oil or with the lower furan concentrations (1 and 2 mg/kg b.w.); on the other hand, a significant, although not dose related, increase of labelled macrophages and hepatocytes was observed at the two higher furan doses (8 and 15 mg/kg b.w.) (Figure 3).

An increased incidence of apoptotic cells, evidenced by the TUNEL assay, was only observed in liver sections of mice treated with the highest furan dose. Apoptotic cells were mainly clustered around blood vessels and bile ducts (Figure 4).

DNA damage (comet assay and γ-H2AX). Comet assay did not highlight any treatment-related induction of DNA strand breaks in mouse liver cells following repeated oral administration of furan (Table II). The experiment was repeated at the concentrations of 8 and 15 mg/kg b.w., applying both the standard and the radiation-modified protocols. In the repeat experiment, the negative findings of the first experiment were confirmed (data not shown); moreover, no effect of furan pretreatment on γ-rays induced DNA migration was observed (Table II).

No difference in the frequency of foci of phosphorylated histone H2AX was observed comparing hepatocytes samples from control and furan-treated mice, while a clear-cut increase of labelled foci was observed following in the positive control mouse treated with γ-rays (Figure 5).

Flow cytometry and DNA methylation. The flow cytometric analysis of DNA content showed a small but significant increase of 8N cells in liver cells of mice treated with the highest furan dose (5.23 ± 0.51 versus 7.98 ± 0.79 in control and treated mice, respectively; $n = 6$; $P < 0.05$). Representative histograms of the distribution of DNA content in liver cells from untreated and 15 mg/kg treated mice are shown in Figure 6.

DNA samples extracted from liver parenchymal cells were analysed in order to assess the effect on DNA methylation of repeated exposure to carcinogenic doses of furan. Data on overall DNA methylation in hepatocytes from subacutely treated mice are summarized in Table III. The results obtained indicate that, in the range of doses applied, repeated oral administration of furan does not result in a significant change in overall DNA methylation in liver at the end of the 4-week treatment period.

Gene expression analysis. The relative expression of a set of stress and toxicity response genes was examined by quantitative real-time PCR. Genes significantly (>2-fold) up- or down-regulated in furan-treated mice are listed in Table V. Several genes involved in cell proliferation (*Ccnd1* and *p21*), macrophage response (*Ccl4* and *Cxcl10*), heat shock (*Hspa8* and *Hspb1*) and oxidative stress/reactive oxygen species (ROS) production (*Gstm1*, *Gstm3*, *Cyp4a10* and *Cyp4a14*) were over-expressed in liver of treated mice compared to controls, while several inflammatory interleukines (*Il1a*, *Il1b* and *Il18*) were down-regulated (Table IV).

Among the 84 analysed genes implicated in DNA damage response pathways, over-expression of genes involved in cell cycle arrest (*Chck1* and *Chck2*), homologous recombination (HR) (*Rad51c* and *Rad51l1*), base excision repair (BER) (*XRCC1*) and trans-lesion synthesis (TLS) (*Pol k*) was observed in furan-treated mice compared to controls (Table V).

Table I. Body and organ weights of B6C3F1 mice after 28 days of furan administration

Furan dose (mg/kg b.w.)	Mice	Final body weight	Body weight gains	Liver	
	n	(g) Mean ± SE	(g) Mean ± SE	Weight (g) Mean ± SE	Hepatic index[a] Mean ± SE
0	11	29.7 ± 0.6	4.6 ± 0.4	1.45 ± 0.06	4.88 ± 0.1
2	7[b]	29.4 ± 0.5	5.7 ± 1.0	1.35 ± 0.07	4.59 ± 0.2
4	9	30.2 ± 0.8	5.9 ± 0.5	1.63 ± 0.07	5.40 ± 0.2*
8	9	28.4 ± 0.9	3.7 ± 0.5	1.53 ± 0.07	5.40 ± 0.2*
15	9	28.3 ± 0.4	3.4 ± 0.7	1.53 ± 0.03	5.41 ± 0.1*

Cumulative data from two experiments.
[a]Organ weight (g)/body weight (g) × 100.
[b]Two mice from the preliminary experiment died within the first 2 weeks due to intubation error.
*$P < 0.05$ (Student's *t*-test).

Single-dose oral administration

General toxicity and histopathology. Acute oral administration of high furan doses (100–250 mg/kg b.w.) elicited signs of overt toxicity. No deaths were observed in the 3 h after administration preceding sacrifice. Microscopic examination of haematoxylin- and eosin-stained liver sections showed diffuse alteration of liver parenchyma with areas of necrosis and steatosis in surrounding hepatocytes, associated with the highest administered dose (Figure 1).

Comet assays. The results of standard alkaline comet assays on liver cells isolated 3 h after acute oral administration of furan are summarized in Table VI. Data show a statistically significant increase in DNA damage parameter (percentage of DNA in the

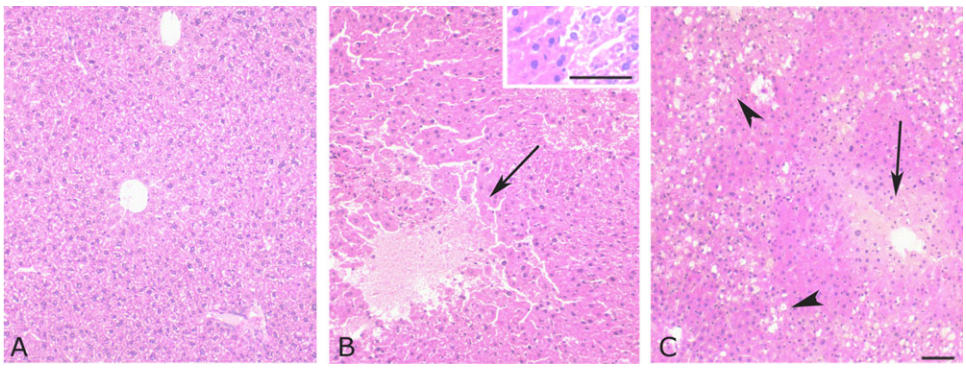

Fig. 1. Histopathological analysis of liver parenchyma. Mice were treated for 28 days with repeated oral administrations of low furan doses (subacute treatment, **B**) or with one single oral administration of high furan doses (acute treatment, **C**). Sections from liver tissue were stained with haematoxylin and eosin. With respect to control (**A**), the two higher furan concentrations in the subacute treatment (B) caused discrete areas of necrosis (arrow), surrounded by hepatocytes with eosinophilic cytoplasm and hyperchromatic nuclei. Inset: detail of hyperplastic hepatocytes, on the left, compared to normal cells, on the right hand side. In the acute treatment (C), the highest dose induced a diffuse alteration of the liver structure, with multiple areas of necrosis (arrow) and evident steatosis in surrounding hepatocytes (arrowheads); bar = 50 µm.

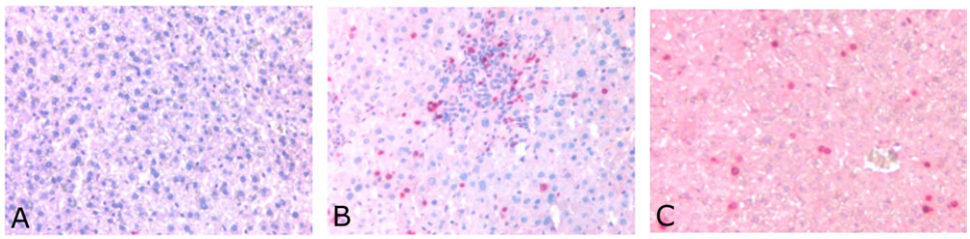

Fig. 2. Immunohistochemical analysis of liver parenchyma to evaluate BrdU incorporation in cell nuclei. Liver sections of mice treated for 28 days with repeated oral administrations of furan (8 mg/kg b.w., **B** or 15 mg/kg b.w., **C**) and tissue sections of control group mice administered with corn oil (**A**) are represented. A significant increase of Fast Red-labelled macrophages (ellipsed cells) and hepatocytes (round cells) was observed in sections of mice treated with 8 mg/kg b.w. (B) and 15 mg/kg b.w. (C) compared to the section of the control liver (A), where no labelled cells can be observed.

tail of comet), which may indicate the presence of DNA strand breaks and/or alkali-labile sites produced by the treatment, or repair intermediates of furan-induced DNA lesions.

When the radiation-modified comet protocol was applied, a significant decrease of γ-ray-induced DNA migration was observed in cells from furan-treated mice compared to untreated mice (Table VI). This decrease is better evidenced in Figure 7, in which representative distribution histograms of percentage of tail DNA values of liver cells from mice treated with different concentrations of furan are reported. These histograms clearly show a decrease of γ-ray-induced DNA migration dependent on furan dose, indicating the involvement of DNA cross link in furan-induced DNA damage following acute *in vivo* exposure.

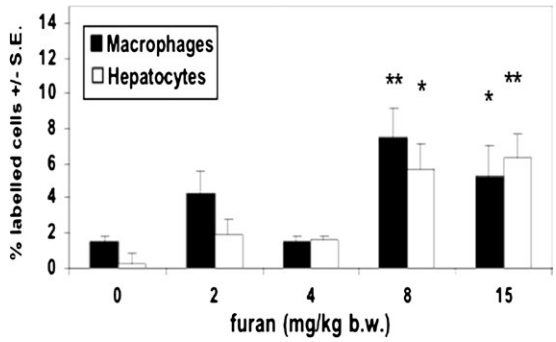

Fig. 3. Graphic representation of the BrdU-labelled cell frequencies (%), quantitated by assessing a total of 1000 cells per tissue section, on the livers of treated and control mice after 28 days oral administration of furan or corn oil, respectively. $N = 4$: number of mice in both control group and group treated with furan 15 mg/kg b.w.; $N = 3$: number of mice treated with furan 2, 4 and 8 mg/kg b.w. Statistical evaluation by Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

Discussion

The results of long-term studies show that rodents' liver is the main target of both furan toxicity and carcinogenicity. In the NTP bioassay on B6C3F1 mice and Fisher 344 rats, multiple non-neoplastic lesions were detected in all treated animals, in addition to benign and malignant tumours (3). Subsequent studies were performed to elucidate the toxic effects elicited in mouse liver by the administration of carcinogenic doses of furan. In these studies, repeated (3 weeks) oral administration of 8 and 15 mg furan/kg b.w. to female B6C3F1 mice elevated serum levels of liver-related enzymes and induced mild necrosis and dose-dependent increases in liver cell proliferation and apoptosis (9,14). These effects were prevented by the co-administration of aminobenzotriazole, an irreversible inhibitor of cytochrome P450 (14), highlighting the central role of cytochrome P450-mediated metabolism in furan-induced hepatotoxicity. These findings, and the observed dose–response relationship for cell proliferation and tumourigenicity (15), lead to hypothesize that furan carcinogenicity may be the result of compensatory cell proliferation consequent to cytotoxicity.

A causative role for induced cell proliferation in mouse liver carcinogenicity is well established (16), and it is believed to play a key role in the hepatocarcinogenicity of non-genotoxic agents such as chloroform, carbon tetrachloride and thioacetamide (17,18,19). Cell proliferation is a necessary component of tumour development in multistage carcinogenesis, and cell proliferation secondary to sustained cytotoxicity can raise tumour incidence increasing the likelihood of spontaneous mutations or promoting spontaneously initiated cells (20). In the case of furan, the primary toxic insult triggering compensatory cell proliferation, and eventually neoplastic transformation, has not been identified. Data point to the involvement of cytochrome P450-mediated metabolism, which

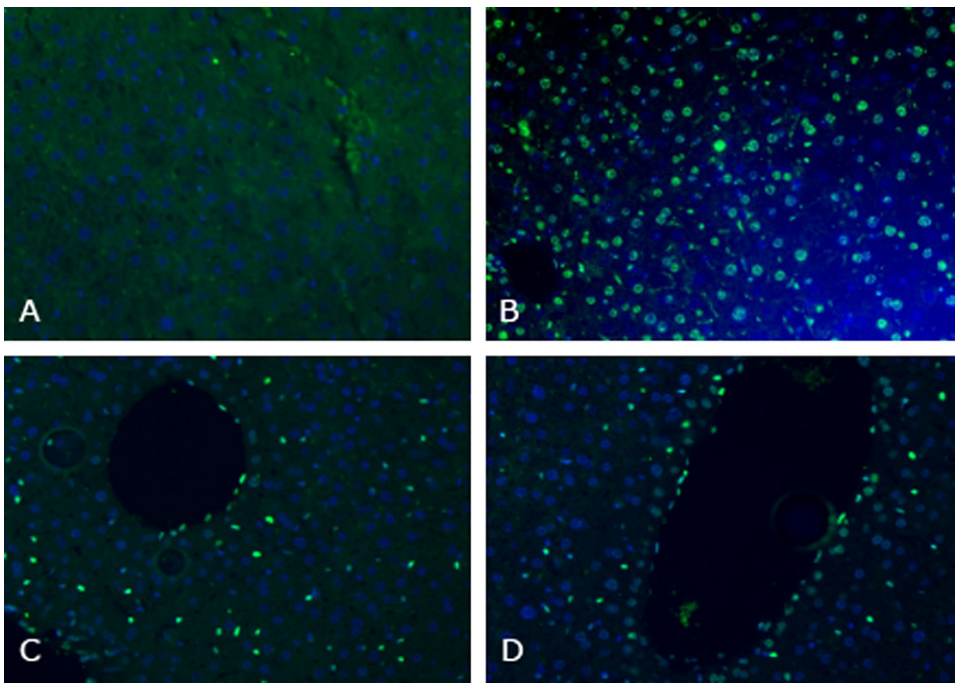

Fig. 4. Apoptotic FITC-labelled cells, highlighted by the TUNEL assay reaction on liver sections of mice treated 28 days with low furan doses. An increase in apoptosis induction on liver sections of animals administered with the highest furan dose, 15 mg/kg b.w. (**C** and **D**), can be observed, compared to the apoptotic cell frequencies induced in the control liver (**A**). Cell apoptosis increase at 15 mg/kg b.w. was mainly evident around the liver vessels. The positive control (**B**) shows FITC-labelled DNase-I-induced strand breaks.

forms electrophilic intermediates capable of covalent interaction with nucleophilic targets of DNA and proteins (21). An early study suggested that furan metabolites bind extensively to protein but not to DNA (22), but the significance of this study was questioned because of possible technical flaws (4). Thus, it is conceivable that binding of furan metabolites to DNA may participate both in cancer initiation, inducing stable genetic alterations, and in promotion, inducing lethal damage. Indeed, the observation that furan exposure is associated with a distinct increase in apoptosis has been interpreted as the response to an increased level of lethal DNA damage (14). An indirect mechanism, related to adenosine triphosphate depletion, has also been invoked to explain furan-mediated double-strand breaks in liver cells *in vitro*, and possibly *in vivo* (23). However, in view of the genotoxicity of furan and its main metabolite *cis*-1,4-butendial in several *in vitro* systems (see Introduction), the possible involvement of DNA damaging activity in the mode of action of furan should receive consideration.

Table II. Results of alkaline comet assay in liver cells of mice after 28 days of treatment with furan

Standard comet assay			Modified comet assay	
Dose	No. of animals	Mean % tail DNA ± SE	No. of animals	Mean % tail DNA ± SE
0	8	1.28 ± 0.10	6	39.87 ± 1.71
2	6	1.14 ± 0.24		—
4	6	1.61 ± 0.19		—
8	6	1.24 ± 0.17	6	39.53 ± 2.00
15	6	1.37 ± 0.20	6	43.47 ± 1.47

Standard assay and protocol modified to detect DNA cross links.

In this work, the evaluation of histological alterations, cell proliferation and apoptosis in liver of male B6C3F1 mice basically confirmed the results reported by others in female mice, supporting the conclusion that no major gender differences exist in furan susceptibility in B6C3F1 mice (3). After 28

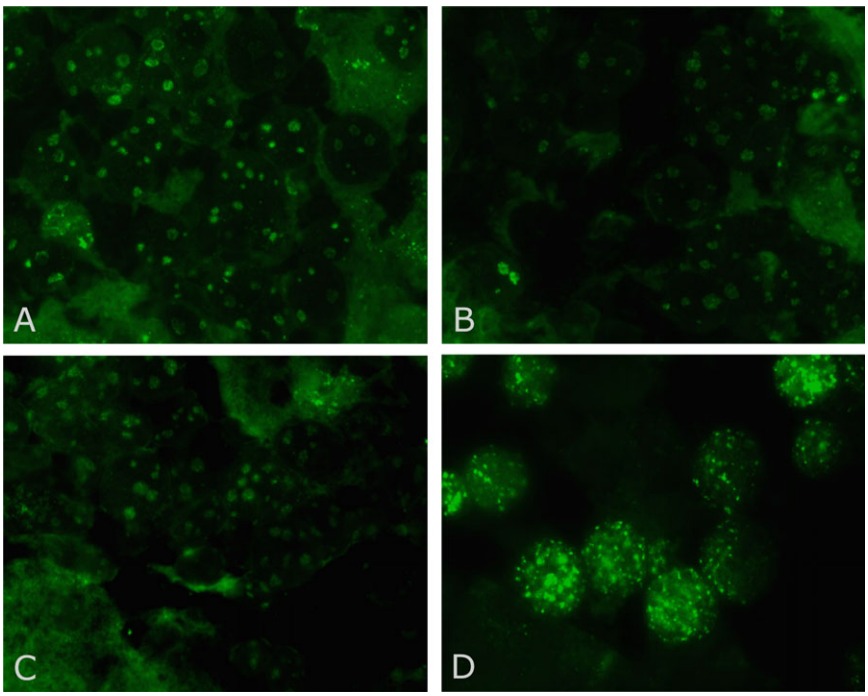

Fig. 5. Immunofluorescent detection of γ-H2AX foci in hepatocytes of mice after 28 days of treatment with furan. A positive control (**A**) obtained from the liver after *in vivo* treatment of a mouse with γ-rays (4 Gy) highlights a relevant amount of FITC-labelled H2AX phosphorylated foci. No differences in FITC-labelled signals were observed between samples of cells from mice treated at the two highest furan doses, 8 and 15 mg/kg b.w. (**C** and **D**, respectively) and the negative control was treated with the solvent alone (**B**).

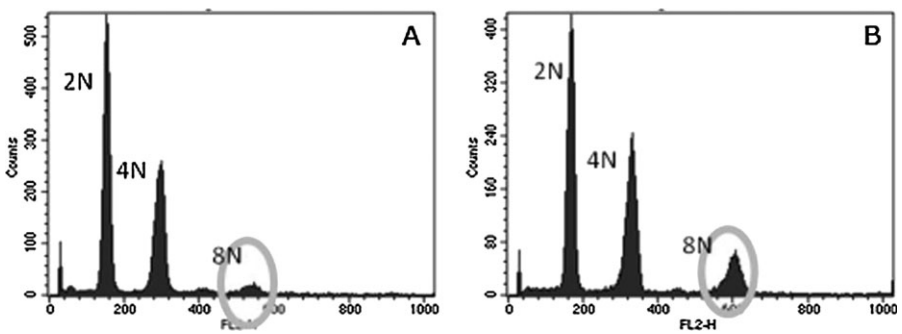

Fig. 6. Representative flow cytometric histograms of liver cells from solvent mice (**A**) or mice treated with 15 mg/kg b.w. furan for 28 days (**B**).

Table III. Effect of subacute administration of furan on global DNA methylation in liver DNA of B6C3F1 mice

	Incorporation of radioactivity (d.p.m./μg DNA)—individual animal data				
	Control	2 mg/kg b.w.	4 mg/kg b.w.	8 mg/kg b.w.	15 mg/kg b.w.
	3979	3676	4034	3817	2545
	2781	3475	3517	2714	3018
	2593	3121	3215	3500	3517
	3212	2912	4232	2917	
	2851	2517	3418	3303	
	2896		3004	2884	
	2193		2812		
	3214				
Mean	2964	3006	3464	3189	3026
SD	526	458	519	423	486

Treatment: 15 mg/kg b.w./day for 28 days.

days of oral administration of furan, a minimal increase in relative liver weight, usually interpreted as the result of the balance between apoptosis and cell proliferation (24), was observed. Histopathological analysis showed occasional necrotic areas surrounded by hepatocytes with highly eosinophilic cytoplasm and hyperchromatic nuclei. Scattered apoptotic nuclei were present and were mainly localized around blood vessels. BrdU incorporation confirmed a small but significant increase of hepatocytes proliferation. Gene expression analysis highlighted the over-expression of several stress and toxic response genes related to oxidative and metabolic stress, heat shock proliferation, inflammation, necrosis and apoptosis. Taken together, these results suggest that daily administration with hepatocarcinogenic furan doses elicited mild toxicity in mouse liver, with compensatory cell proliferation to replace damaged cells eliminated by apoptosis or necrosis.

Table IV. Genes related to 'stress and toxicity response' significantly up- or down-regulated in furan-treated mice compared to control animals

Gene	Description	Molecular function/biological process	Fold up- or down-regulation Test sample/control sample
Up-regulated			
Ccl4	Chemokine (C-C motif) ligand 4	Inflammation, macrophage response	2.44
Cxcl10	Chemokine (C-X-C motif) ligand 10	Inflammation, macrophage response	2.14
Ccnd1	Cyclin D1	Cell proliferation	2.09
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21)	Cell proliferation	5.45
Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 5	Metabolism, hepatotoxicity	3.23
Cyp4a10	Cytochrome P450, family 4, subfamily a, polypeptide 10	PPARa-dependent metabolism fatty acid, ROS production	4.28
Cyp4a14	Cytochrome P450, family 4, subfamily a, polypeptide 14	PPARa-dependent metabolism fatty acid, ROS production	12.17
Gstm1	Glutathione *S*-transferase, mu 1	Oxidative stress	2.98
Gstm3	Glutathione *S*-transferase, mu 3	Oxidative stress	2.77
Hspa8	Heat shock protein 8	Heat shock	2.93
Hspb1	Heat shock protein 1	Heat shock	5.15
Nfkbia	NF-kb inhibitor, alpha	Inflammation	2.86
Down-regulated			
Cyp2c29	Cytochrome P450, family 2, subfamily c, polypeptide 29	Metabolism	−4.33
Egr1	Early growth response 1	Cell proliferation	−4.56
Ephx2	Epoxide hydrolase 2, cytoplasmic	Metabolic stress	−4.93
Il1a	Interleukin 1 alpha	Inflammation	−2.53
Il1b	Interleukin 1 beta	Inflammation	−3.97
Il18	Interleukin 18	Inflammation	−2.07
Ung	Uracil DNA glycosylase	DNA damage response	−2.13

Treatment: 15 mg/kg b.w./day for 28 days. PPARa, peroxisome proliferator-activated receptor alpha; ROS, reactive oxygen species.

Table V. Genes related to 'DNA damage repair pathways' significantly up- or down-regulated in furan-treated mice compared to control animals

Gene	Description	Molecular function/biological process	Fold up- or down-regulation Test sample/control sample
Up-regulated			
Chaf1a	Chromatin assembly factor 1, subunit A (p150)	Nucleosome assembly, DNA synthesis and repair	2.41
Chek1	Checkpoint kinase 1 homologue (*Schizosaccharomyces pombe*)	Cell cycle arrest, DNA damage response	2.56
Chek2	CHK2 checkpoint homologue	Cell cycle arrest, DNA damage response	2.38
Lig1	Ligase I, DNA, adenosine triphosphate-dependent	DNA synthesis and repair	2.08
Pol e	Polymerase (DNA directed), epsilon	DNA replication	2.17
Pol k	Polymerase (DNA directed), kappa	Trans-lesion synthesis, DNA damage response	2.30
Rad51c	Rad51 homologue c (*Saccharomyces cerevisiae*)	Homologous recombinational repair, DNA damage response	2.81
Rad51l1	RAD51-like 1 (*Saccharomyces cerevisiae*)	Homologous recombinational repair, DNA damage response	4.52
Xrcc1	X-ray repair complementing defective repair in Chinese hamster cells 1	BER, DNA repair	3.85

Treatment: 15 mg/kg b.w./day for 28 days.

In addition to cell proliferation, repeated furan administration also significantly increased the incidence of polyploid or endoreduplicated liver cells with an 8N DNA content. Polyploidy is a normal physiological process in the liver associated with differentiation, increased age and metabolic load. On the other hand, the onset of hepatocyte polyploidy may reflect injurious process in the liver and it has been shown to be induced in regenerating liver and during pathophysiological states (25). Furthermore, premature polyploidy has been reported in DNA repair-deficient mice and has been associated with accumulating DNA damage (26).

A preliminary investigation on the effect of furan exposure on DNA methylation was also undertaken. Modulation of gene expression and gene silencing by changes in methylation pattern are known to be involved in chronic liver diseases and hepatocarcinogenesis (27,28,29). In this work, the assessment of overall DNA methylation did not show treatment-related changes. No change in overall DNA methylation was also observed in a recent study on mice exposed 6 months to the hepatocarcinogen 2-acetylaminofluorene, despite the occurrence of more specific epigenetic alterations (30). Thus, further work is required to elucidate whether furan exposure is associated with changes in DNA or histone methylation at relevant target genes.

Table VI. Results of alkaline comet assay in liver cells of mice after acute treatment with furan

Standard comet assay			Modified comet assay	
Dose	No. of animals	Mean % tail DNA ± SE	No. of animals	Mean % tail DNA ± SE
0	11	1.40 ± 0.19	6	34.00 ± 1.55
15	10	1.43 ± 0.27	5	39.99 ± 1.63
100	10	1.44 ± 0.28	5	38.19 ± 2.67
250	10	3.49 ± 0.97**	5	28.65 ± 0.95*

Standard assay and protocol modified to detect DNA cross links.
*$P < 0.05$; **$P < 0.01$.

The assessment of genotoxicity highlighted a significant increase of DNA damage in liver of mice receiving furan as a high, toxic acute oral dose. The same *in vivo* treatment proved to decrease DNA migration in liver cells following *in vitro* exposure to ionizing radiation, pointing to the presence of DNA–DNA cross links (13). On the other hand, no treatment-related effects were observed in mice receiving carcinogenic furan doses for 28 days. In particular, neither the detection and quantitation of foci of phosphorylated histone H2AX (γ-H2AX), a marker of double-strand DNA breaks (31), nor the alkaline comet assay, which identifies single-strand breaks and alkaline labile lesions such as abasic sites, revealed any excess of DNA damage in liver cells of treated animals. However, these findings should be interpreted with caution before excluding any significant genotoxicity associated with low-dose furan exposure. In fact, both the end points analysed have shown some limitations in the detection of furan-induced DNA damage. Concerning γ-H2AX, in a previous study on the effects of furan on mouse spleen, it was shown that a treatment-related increase of γ-H2AX foci was only detectable when spleen cells were stimulated to grow *in vitro* (32). Thus, it is possible that the relatively low incidence of proliferation in liver cells prevented the expression of primary DNA damage in double-strand breaks visualized by γ-H2AX foci. Similarly, the results of comet assays should be evaluated keeping in mind that negative results with both standard alkaline and radiation-modified comet protocols are also reported in the previously cited study in mouse spleen, despite other biomarkers highlighted significant DNA damage. In this respect, it should also be mentioned that a recent study with mammalian cells demonstrated that *cis*-2-butene-1,4-dial, the hypothesized key genotoxic furan metabolite, is only borderline active in comet

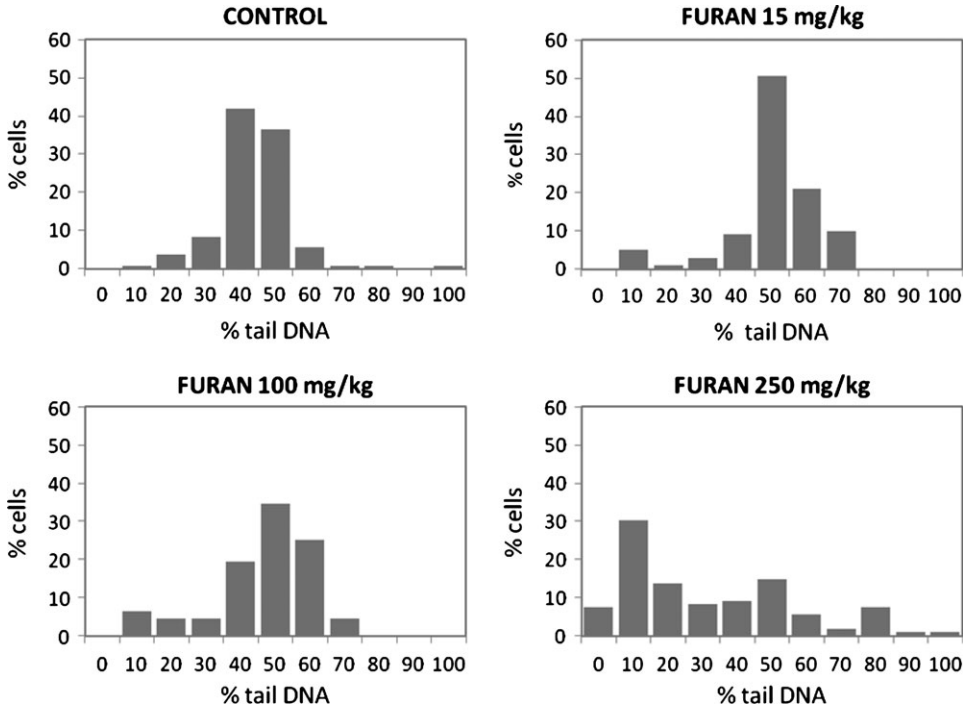

Fig. 7. Alkaline comet assay to detect DNA cross links: representative distribution histograms of percentage of tail DNA of liver cells from mice treated with different concentrations of furan.

assays (6). Thus, it is conceivable that comet assays lack the sensitivity required to demonstrate the induction of DNA damage in liver cells following repeated exposure to relatively low furan doses, only detecting the massive formation of cross links following acute high-dose exposure. Indeed, gene expression analysis highlighted the over-expression of several genes involved in the response to DNA damage in liver of mice treated for 28 days with a non-toxic carcinogenic furan dose: in particular, *XRCC1*, which plays a key role in BER, *Rad51* and *Pol k*, involved in HR and TLS, respectively. This pattern fits with the induction of cross links, non-coding lesions stalling replicative fork that trigger HR and TLS (33), beyond BER (34). Thus, even though the over-expression of DNA repair genes only provides an indirect evidence of genotoxicity, the induction of DNA damage under the repeated exposure regimen applied cannot be ruled out.

In conclusion, the results obtained demonstrate that furan is genotoxic in mouse liver when administered at a high toxic dose. No direct evidence of genotoxicity was observed in liver of mice treated for 28 days with lower furan doses, which were hepatocarcinogenic following chronic administration; however, under these treatment conditions, over-expression of some DNA damage-related genes was observed. Despite the final demonstration of furan genotoxicity in the target organ under exposure conditions associated with hepatocarcinogenesis is still lacking, overall these results suggest that the contribution of genotoxicity in the mode of action of furan should not be dismissed, even though non-genotoxic mechanism(s), such as compensatory cell proliferation, are likely to contribute as well.

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