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Comparison of Genomic Damage Caused by 5-Nitrofurantoin in Young and Adult Mice Using the In vivo Micronucleus Assay

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The antibiotic 5-nitrofurantoin (5-NF) has been used widely for the treatment of urosepsis in children during the last 20 years. Recent experimentation suggests the need for reevaluating its genotoxic potential. Because of possible differences in the metabolism and clearance of 5-NF in young and adult animals, we conducted a study to determine whether micronuclei caused by 5-NF were age-related. The in vivo micronucleus (MN) assay was performed on 3- and 8-week-old mice given single intraperitoneal injections of 5, 10, and 50 mg/kg 5-NF. Blood samples from the tail vein were taken before injection (baseline) and at 48, 96, 168, and 336 hr (2 weeks) after the treatment. One thousand reticulocytes were analyzed for micronuclei from each animal. Compared to similar baseline values for young and adult mice, 5-NF caused a significant increase in MN frequency in both age groups. The mean MN frequency in the young animals was higher than in the adult animals for each dose and sampling time. MN frequencies remained significantly elevated in young animals even 2 weeks after exposure to 5-NF. The results of the study confirm the genotoxic potential of 5-NF in young and adult animals, and indicate that young animals are more sensitive to the genotoxic effects of 5-NF than adult mice and that the response in young mice persists for a significantly longer time. These findings may be related to poorly developed mechanisms of xenobiotic detoxification and renal elimination in young animals. Environ. Mol. Mutagen. 46:59–63, 2005. © 2005 Wiley-Liss, Inc.

Key words: 5-nitrofurantoin; micronucleus assay; development; mice

INTRODUCTION

5-Nitrofurantoin (5-NF) has been used in the prophylaxis and therapy of urinary infections in adults and children, and in cattle, pigs, and poultry for more than 50 years. Bacterial mutagenicity tests, sex-linked recessive and dominant lethal tests in Drosophila, and in vitro chromosome aberration and sister-chromatid exchange (SCE) assays indicate that 5-NF is genotoxic ([Fonatsch et al., 1977; Ni et al., 1987; Slapšyte et al., 1996] reviewed in Thompson [1986]). The alkaline elution assay also indicates that 5-NF produces DNA damage in the rodent liver, kidney, lung, spleen, and bone marrow, with cross-links being produced in the liver [Parodi et al., 1983]. In two-year feeding studies, 5-NF increased the incidence of tubular adenomas, benign and mixed tumors, and granulosa-cell tumors of the ovary in female mice [National Toxicology Program, 1989]. According to published data [Slapšyte et al., 2002], genotoxic effects are detected in children after long-term treatment with 5-NF. An increased risk of transitional cell carcinoma was reported in patients with a history of treatment with this compound [Steineck et al., 1995].

As the in vivo mutagenicity of 5-NF has not been adequately investigated, the genotoxic potential of this drug has not yet been fully established. In addition, given the exposure of children as well as adults to this drug, dosing regimens that compare responses in young and adult animals are warranted. The in vivo micronucleus (MN) assay is the method of choice for this purpose because it is well validated, detects both the clastogenic and aneugenic activities of test agents, and can be used for repeated sampling of developing animals, as it requires only small sampling volumes.

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TABLE I. Descriptive Statistics for MN Frequencies After Single Doses of 5-NF. N = 8 Animals per Data Point, 1,000 Reticulocytes per Animal

	Sampling time									
	Baseline		48 (hr)		96 (hr)		168 (hr)		336 (hr)	
Dose (mg/kg)	MN (%)	S.D.	MN (%)	S.D.	MN (%)	S.D.	MN (%)	S.D.	MN (%)	S.D.
3 weeks of age at treatment										
5	0.1	0.10	0.62	0.17	0.58	0.13	0.38	0.10	0.34	0.05
10	0.09	0.08	0.72	0.13	0.67	0.14	0.60	0.25	0.38	0.11
50	0.11	0.09	0.65	0.10	0.63	0.19	0.53	0.15	0.50	0.14
8 weeks of age at treatment										
5	0.07	0.05	0.40	0.08	0.41	0.13	0.25	0.05	0.11	0.11
10	0.12	0.04	0.46	0.08	0.44	0.09	0.40	0.12	0.18	0.08
50	0.12	0.04	0.42	0.14	0.52	0.08	0.48	0.08	0.26	0.05

In a previous study, we demonstrated that exposure of adult mice to 5-NF results in increased reticulocyte MN frequencies. Results from the present study confirmed earlier findings of the pilot study [Fucic et al., 2003]. In this present study, we have extended our observations by comparing the induction and persistence of micronuclei in adult and young mice treated with a single dose of 5-NF.

MATERIALS AND METHODS

BALB/C mice were received in 2000 with a certificate of Health Report from Charles River Laboratories (Iffa Credo, Lyon) for Specific Pathogen Free (SPF) animals. The physical facilities and equipment for the accomodation and care of the animals were in accordance with the provisions of EEC Council Directive 86/609. Mice were given irradiated feed pellets (Mucedola, Italy) and water *ad libitum*. Eight-week-old (adult) and three-week-old (young) animals were divided into four groups. Since no qualitative sex-related difference in MN was expected [Morita et al., 1997], each group consisted of 8 animals (4 males and 4 females). After sampling to establish baseline MN frequencies, three groups received single interperitoneal injections of 5, 10, or 50 mg/kg 5-NF (Sigma, St. Louis, MO). The drug doses were based on doses recommended in human medical practice (DRUGDEX Product Indeks). Carboxymethyl cellulose was used as vehicle. The fourth group, which served as a positive control, received a single injection of 75 mg/kg cyclophosphamide (Sigma).

MN assays were performed on the animals before they were administered 5-NF and cyclophosphamide to establish baseline MN frequencies and again 48, 96, 168, and 336 hr (14 days) after the dosing. Peripheral blood (5 µl per sample) was collected from the tail vein. The blood was smeared onto an acridine-orange coated slide (Toyobo, Osaka, Japan) covered with a cover slip, and analyzed for micronuclei according to the procedures given by Hayashi et al. [1990]. Micronuclei were evaluated in 1,000 reticulocytes per animal according to the recommendations of the Collaborative Study Group of the Micronucleus Test [Morita et al., 1997].

Analysis of variance (ANOVA) for repeated measures was used for comparison of the responses as a function of dose at each sampling time and their interactions. Doses and groups were analyzed as fixed effects. Once a significant difference was detected, we used the Scheffe post-hoc multiple comparison test [Sokal and Rohlf, 1995] to determine which doses, groups, or interactions were significant. We set the level of statistical significance at 5% ($P \leq 0.05$). The analysis and graphs were made with STATISTICA 6.0 [Sokal and Rohlf, 1995].

TABLE II. Results of ANOVA with Repeated Measures on In Vivo MN Data from Mice Treated with 5-NF

	Sum of squares	Mean of square	*F*	*P*
Between subjects				
Intercept	2695.177	2695.177	1044.245	0.00000
Group	107.101	107.101	41.497	0.00000
Dose	30.172	15.086	5.845	0.00701
Within subjects				
Time	509.118	127.279	99.666	0.00000
Time group	32.392	8.98	6.341	0.00011

RESULTS

All animals survived the 2-week experiment period. The effect of gender was tested by a two-way ANOVA. In accordance with our expectation [Morita et al., 1997], there was no effect of sex for both the age groups, and the data were reported without regard to gender. The positive control, cyclophosphamide, produced a 7.1% MN frequency at 48 hr after the treatment. The MN frequencies at other sampling times were similar to the baseline frequency.

Descriptive statistics for the results of the 5-NF experiments are given in Table I. Table II shows the results of ANOVA with repeated measurement. The differences between the groups were significant. The MN frequencies were significantly higher in young animals than in adults (Fig. 1). There were also significant differences between the doses. Scheffe's post-hoc multiple comparison test indicated that the MN frequencies produced by the 5 and 10 mg/kg and the 5 and 50 mg/kg doses were significantly different ($P = 0.011$ and $P = 0.014$). There were significant differences in the MN frequencies at the different sampling times (Table II), except between the 48 and 96 hr sampling times. The interaction between group and time was significant (Table II, Fig. 1), suggesting differences in the distribution and pattern of the rate of elimination of genomic damage in young and adult animals (Fig. 1, Fig. 2).

MN frequency reached baseline values 2 weeks after injection in adult animals, whereas MN frequency in young

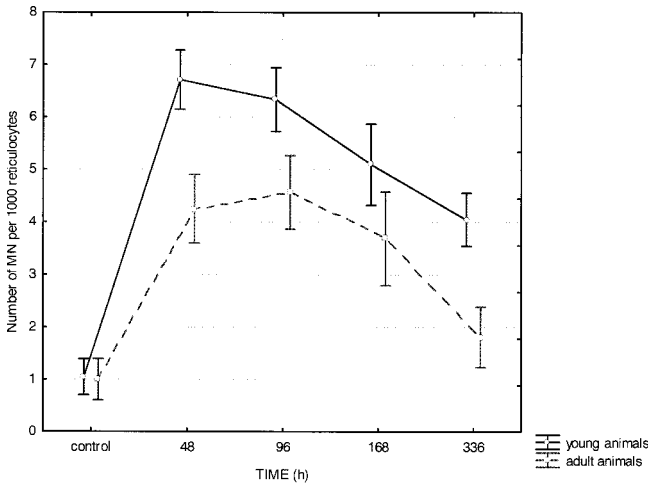

Fig. 1. Mean values and 95% confidence intervals of MN frequency for young and adult mice exposed to single doses of 5-NF (all doses combined), and sampled for up to 336 hr after the treatment.

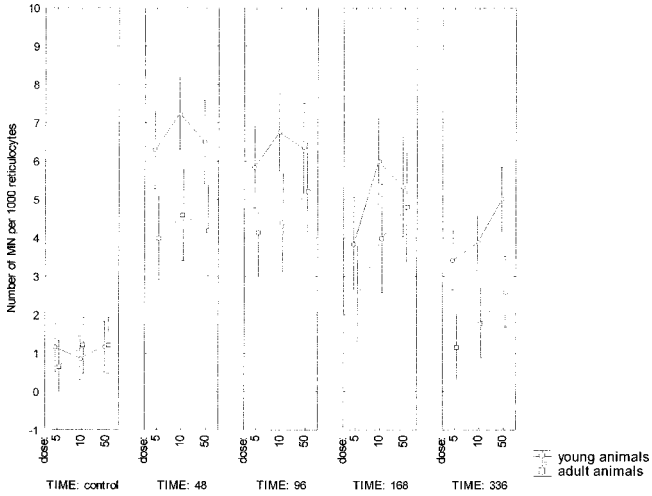

Fig. 2. Mean values and 95% confidence intervals of MN frequency for young and adult mice treated with 5, 10, and 50 mg/kg of 5-NF, and sampled for up to 336 hr after the treatment.

animals remained increased up to 336 hr (2 weeks) after injection with 5-NF, the latest time sampled. For both age groups, the highest deviations from the baseline values were detected 48 hr and 96 hr after injection (Fig. 1).

DISCUSSION

The severity of genomic damage caused by xenobiotics and drugs depends on the pharmacokinetics of the substance, which often differs between children and adults because of physiological differences, the maturity of enzyme systems, and the efficiency of drug clearance. As a consequence, chemical substances in children often have a significantly longer half-life than in adults [Ginsberg et al., 2002]. Data about the activity or concentrations of several enzymes, such as hepatic cytochromes, and protective substances in the body, such as gluthatione, are very limited for 1- to 5-year-old children [Alcorn et al., 2002].

Urinary tract infection is one of the most common forms of infectious diseases among children. 5-NF is very commonly used in long-term prophylaxis for preventing recurrences of infections, and the treatments may last for over 12 months. In addition, X-rays in the form of cytourethrography are usually involved in the diagnosis of patients. Therefore, despite the relatively low doses received, patients with urinary tract infections are in practice exposed to a combination of drugs and radiation.

We investigated whether young animals are more sensitive to the genotoxic effects of 5-NF than adult animals. Our results indicated that 5-NF produced significantly higher reticulocyte MN frequencies in young mice than in adults, at all doses tested and at all sampling periods evaluated. The MN frequencies reached their peak in both animal groups between 48 and 96 hr after drug administration. In addition, dose- and age-related effects were seen in the rate of decrease in MN frequency after the peak. The elimination of micronuclei was slower for mice treated with 50 mg/kg 5-NF than it was for mice treated with lower doses. This difference could be due to the greater persistence of the genotoxic agent at the high dose. Two weeks after the injection of 5-NF, the MN frequencies in adult animals reached baseline levels. However, in spite of data indicating that 5-NF is rapidly eliminated from animals, the MN frequencies in young animals remained increased 2 weeks after the treatment. This pattern of MN frequency persistence in adult and young animals is similar to that described for 5-NF-induced DNA damage by Parodi et al. [1983].

5-NF metabolism under aerobic conditions results in the production of the nitroaromatic anion and reactive forms of oxygen, that is, the superoxide anion, hydrogen peroxide, and the hydroxyl radical [Michielis and Remacle, 1988; Martinez et al., 1995; Gram, 1997; Arancibia et al., 2003]. In the next step, 5-NF is converted into a basic molecule [National Toxicology Program, 1989] that can bind to proteins. Most of the administered NF-type antibiotics are excreted within hours, but their protein-bound metabolites are highly stable and are excreted in 4–9 days [Leitner et al., 2001]. In vitro studies of 5-NF metabolism in hepatocytes showed that cytotoxicity was accompanied by lipid peroxidation [Klee et al., 1994].

Peroxidated lipids, formed by reactive oxygen species, are potent genotoxic agents. The last step in the metabolism of peroxidated lipids is the formation of reactive aldehydes, so called second toxic messengers. These are stable compounds with clastogenic and aneugenic potential. The most relevant reactive aldehydes, malonaldehyde and 4-hydroxynonenal (HNE), have a high affinity

for proteins, but can also cause DNA fragmentation, stimulate cell proliferation, increase the frequency of SCE, and disrupt microtubules [Olivero et al., 1990; Eckl et al., 1993; Wiseman and Halliwell 1996; Neely et al., 1999; Yang et al., 2003]. The potential of 5-NF to disrupt microtubules *via* HNE could cause mitotic spindle dysfunction and, along with direct DNA damage, could result in the induction of micronuclei. As an adduct, HNE continues to be biologically active, but it also has the potential to change the macromolecule to which it is bound and to influence both cell proliferation and apoptosis [Esterbauer et al., 1991; Žarković 2001].

Under anaerobic conditions, 5-NF is metabolized to a nitroso and/or hydroxylamine form [Leskovac and Popovic, 1980], which has the potential to interact with DNA [Ni et al., 1987]. It was also reported that the predominant 5-NF metabolite found in urine is 5-nitro-2-furoic acid [Olivard et al., 1962]. Protein-protein disulfide formation, which modifies the action of enzymes and which has been detected after the administration of NF [Bandow et al. 2003], could also be caused by HNE.

5-NF-related cytogenetic damage, therefore, potentially could be caused by a number of different products of 5-NF metabolism. As described earlier, these include modified bases, such as 8-hydroxyguanine [Laval, 1996], transitional metabolites, and aldehydes. To our knowledge, protein-bound metabolites and reactive oxygen species appear to be the most relevant products with respect to 5-NF genotoxic activity. The protein-bound metabolites of 5-NF require further investigation in pharmacokinetic studies [Leitner et al., 2001].

Differences in tissue oxygenation levels may be the key in determining the pathway for 5-NF metabolism in vivo [Minchin et al., 1986]. Differences in metabolism may be expected during the developmental stages of different organs and tissues. Consequently, these age-related differences could determine the type and the severity of genomic damage caused by 5-NF. It is possible that metabolites could be active in young animals for longer periods of time, which might account for the increased MN frequencies that we observed 2 weeks after 5-NF administration. This hypothesis is consistent with the observation that the frequency of SCE in children is related to the duration of 5-NF therapy, indicating that the effects of 5-NF on SCE could be cumulative [Slapšyte et al., 2002].

However, there is relatively little direct information on the comparative metabolism of 5-NF in children and adults, or in young and adult animal models. In contrast to adult rats in which 5-NF is rapidly excreted, excretion in young rats (5- to 15- day-old) is slower because of the immature renal tubules and higher reabsorption of the drug [Braunlich et al.,1978; Wierzba et al., 1982]. A similar observation was reported in children under the age of two in whom the excretion of 5-NF was half the rate of adults [Wierzba et al., 1984]. This observation may be particularly significant for lactating women who take 5-NF, since the drug is actively transported into human milk in which it achieves concentrations greatly exceeding those in the serum [Gerk et al., 2001].

Glutathione has multiple functions, including the protection of cells from oxidative stress and reactive oxygen intermediates. It has been shown that healthy neonates and infants have significantly lower plasma glutathione levels [Ono et al., 2001]. In rats, gluthatione transferase activity decreases significantly at 20–25 days (age), and after 50 days, it becomes stable and reaches adult concentrations [Lundquist et al., 1995]. Further research should establish whether there is a similar enzyme profile in children.

Vitamin E deficiency is a significant factor in the elimination of 5-NF, as it modifies 5-NF deposition and elimination because of the decreased renal clearance of both 5-NF and metabolites [Statham et al., 1985]. Repeated infections, lower social status, and malnutrition are frequently interrelated, and vitamin deficiency could be a modifying factor for 5-NF metabolism in children.

The incidence of bladder cancer is increasing [Johansson et al., 1997]. Transitional cell carcinoma, related to 5-NF exposure in childhood [Steineck et al., 1995], accounts for 90% of all bladder cancers and has a long latency period [Kamat and Lamm, 1999]. Mechanisms for the induction of transitional cell carcinoma may involve direct DNA damage or agents that increase cell proliferation [Cohen, 1998]. It may be significant that 5-NF may play a role in both mechanisms, DNA damage *via* oxygen radicals and cell proliferation *via* reactive aldehydes.

5-NF has been used extensively in the treatment of urinary infections. In 1986, for example, 9,300 kg of 5-NF were purchased in the US alone. Our results indicate that 5-NF may have increased genotoxicity in young animals, making its use in young children problematic. The application of drugs for use in children should take into account age-dependent differences in drug absorption, renal elimination, drug-protein binding, and metabolism. Considering the potential risks involved, it may be prudent that drug development studies incorporate age-modelling performed at the very beginning of the preclinical and clinical studies. Such drug development processes would include in vivo genotoxicology studies in young animals, studies of pediatric pharmacology and pharmacokinetics, and genotoxicology follow-ups during preclinical and clinical trials as critical parameters in the evaluation of the risk-benefit ratio for a drug.

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