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Mutagenicity of the peroxisome proliferators clofibrate, Wyeth 14,643 and di-2-ethylhexyl phthalate in the lacZ plasmid-based transgenic mouse mutation assay

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Abstract

Background: Peroxisome proliferators are considered rodent carcinogens that are putative human non-carcinogens based on the presumed absence of direct genetic toxicity in rodent and human cells and the resistance of human cells to the induction of peroxisomes by peroxisome proliferators. The highly sensitive *lacZ* plasmid-based transgenic mouse mutation assay was employed to investigate the mutagenicity of several peroxisome proliferators based on several lines of evidence suggesting that these agents may in fact exert a genotoxic effect.

Methods: Male and female *lacZ*-plasmid based transgenic mice were treated at 4 months of age with 6 doses of 2,333 mg di-2-ethylhexyl phthalate (DHEP), 200 mg Wyeth-14,643, or 90 mg clofibrate per kg of bodyweight, respectively, over a two-week period. Control animals were treated with the respective vehicles only (35% propyl glycol for DEHP and Wyeth-14,643 treatment controls and sterile water for clofibrate treatment controls).

The mutant frequency in liver, kidney and spleen DNA was determined as the proportion of retrieved mutant and wild-type *lacZ* plasmids expressed in *Escherichia Coli C* host cells employing a positive selection system for mutant plasmids.

Results: Exposure to DEHP or Wyeth-14,643 significantly increased the mutant frequency in liver, but not in kidney or spleen, of both female and male mice. Treatment with clofibrate did not lead to an increased mutant frequency in any of the organs studied.

Conclusion: The results indicate that some peroxisome proliferators display an organ-specific mutagenicity in *lacZ* plasmid-based transgenic mice consistent with historical observations of organ- and compound-specific carcinogenicity.

Background

Peroxisome proliferators are a chemically and structurally diverse class of chemical compounds that include phthalate esters such as di-2-ethylhexyl phthalate (DEHP), hypolipidemic drugs, analgesics, uricosuric drugs, and environmental pollutants. Because of the commercial value of many agents that do induce peroxisome proliferation, research efforts have focused on the mechanism of toxicity/carcinogenicity of these chemicals with the goal of elucidating a species-specific response. This group of chemicals has been found to induce liver cancer in rats and mice, most likely due to oxidative stress from

peroxisome proliferation and/or increased hepatocellular proliferation [1]. It has often been claimed that humans are refractory to the adverse effects of peroxisome proliferators based on data that indicated little peroxisome proliferation in human liver and no induction in cultured hepatocytes as well as the observation that no increase in liver cancer was evident in patients treated with hypolipidemic fibrate drugs.

It has been purported that there are several strong lines of evidence supporting the conclusion that peroxisome proliferation-induced rodent liver carcinogenesis is not relevant to humans [2]. Specifically, Galloway et al. [3] reported that direct genetic toxicity has been eliminated as a common mechanism of carcinogenic action for peroxisome proliferators in general, based on the absence of chromosome aberrations in human lymphocytes and CHO cells following exposure to nafenopin or methyl clofenapate at dose levels up to those that are toxic. Moreover, Roberts et al. [2] argue that the resistance of human hepatocyes to the induction of peroxisomes by peroxisome proliferators [4-6] as well as the resistance of human hepatocytes to peroxisome proliferator-mediated induction of replication and suppression of apoptosis [4-6] preclude the possibility that peroxisome proliferators are relevant to human carcinogenesis. It should be realized, however, that the classification of carcinogens as either genotoxic or non-genotoxic is primarily based on the sensitivity and specificity of the experimental procedure(s) used to assess whether a particular carcinogen induces DNA addition products (DNA adducts) and/or DNA mutations. Notwithstanding the sensitive techniques at our disposal, the (recognized) inherent limitations of any technique may obfuscate the true genotoxic nature of a carcinogen.

The development of transgenic rodent mutation models has provided the means to determine gene mutations from virtually any tissue in a relatively quick way. A recent study by Shane et al. [7] indicated that WY-14,643 is mutagenic in a *lacI* bacteriophage lambda-based mouse mutation assay, causing predominantly G -> T and G -> C transversion mutations. Subsequently, Deutsch et al. [8] provided evidence for the genotoxic nature of WY-14,643 when it was shown that exposure of human myeloid leukemia K562 cells to this peroxisome proliferator caused extensive DNA damage as measured by the Comet assay.

These findings indicate that peroxisome proliferators may pose a cancer risk to rodents, and perhaps humans, through genotoxic mechanisms. To investigate whether (rodent) peroxisome proliferators should indeed be considered as genotoxic agents, *lacZ* plasmid-based transgenic mice were exposed to either clofibrate, DEHP, or WY-14,643 and mutant frequencies were assayed in various organs at 21 days following the last exposure. DEHP and WY-14,643 were shown to significantly elevate the mutant frequency in both male and female liver DNA while clofibrate, at the dose level studied, was apparently non-mutagenic in male and female liver.

Methods

Animals and exposures

Male and female *lacZ*-plasmid transgenic mice were treated at 4 months of age with 6 doses of 2,333 mg DEHP, 200 mg WY-14,643, or 90 mg clofibrate per kg of bodyweight, respectively, over a two-week period. Control animals were treated with the respective vehicles only (35% propyl glycol for DEHP and WY-14,643 treatment controls and sterile water for clofibrate treatment controls). Twenty-one days after the last treatment, animals were sacrificed and organs were flash-frozen in liquid nitrogen and stored at -80°C until used for DNA isolation.

DNA isolation

Organs were homogenized in 9 ml lysis buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA) using a Brinkmann homogenizer for 10 sec. SDS (Invitrogen, Carlsbad, CA) and proteinase K (Invitrogen) were added to final concentrations of 1% and 0.5 mg/ml, respectively. The mixture was incubated for 16 hours at 45°C. Subsequently, the mixture was extracted once with an equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol at a ratio of 25/24/1 (Invitrogen). The aqueous phase was gently mixed with one fourth volume of 8 M potassium acetate and extracted with an equal volume of chloroform/isoamyl alcohol (24/1) prior to ethanol precipitation. Genomic DNA was digested with 40 units HindIII (New England BioLabs, Beverly, MA) in the presence of paramagnetic beads (Dynabeads M450 sheep anti-mouse IgG, Dynal, Lake Success, NY) that were precoated with a *lacZ/lacI* fusion protein (Leven, Bogart, GA). Following two wash steps using 250 μl 1X binding buffer, plasmid DNA was eluted from the beads with isopropyl-thio-β-galactoside (IPTG). The retrieved plasmids were circularized with T4 DNA ligase (Invitrogen) and electroporated into *E. coli C* (*ΔlacZ*, *galE* $^{-}$) host cells. To determine the number of transformants, a small aliquot (0.1%) of the transformed cells was plated in top-agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Gold Biotechnology, St. Louis, MO). The majority (99.9%) of the transformed cells was plated in top-agar containing phenyl-β-galactoside (P-gal; Sigma), allowing growth of only those cells harboring plasmids with a mutated *lacZ* reporter gene. Mutant frequencies were calculated as the ratio between the number of colonies on selective (P-gal) plates vs. the number of colonies

on permissive (X-gal) plates times the dilution factor (1,000 ×).

Statistical analysis

Variability in the mutant frequency data was examined according to a binomial variance statistic as described [9,10]. The binomial variance statistic is referenced to a chi-square distribution with *df* equal to the number of organs minus one. Significant excess variability is noted if the statistic is larger than the upper 5% point from this reference distribution. If at any level the binomial variance statistic fails to identify excess variability, the individual observations are used to calculate a pooled mutant frequency as a more precise estimate of the true mutant frequency π. Comparisons between organs were performed using a two-sided paired *t*-test.

Results

The average (± SD × 10^{-5}) spontaneous mutant frequency for liver, kidney, and spleen were 6.69 ± 0.60 (n = 4), 5.60 ± 0.42 (n = 4), and 6.16 ± 1.06 (n = 4), respectively for female mice and 6.37 ± 1.11 (n = 4), 4.59 ± 0.40 (n = 4), and 5.98 ± 0.79 (n = 4), respectively for male mice. The spontaneous mutant frequencies in kidney, liver and spleen were not significantly different between female and male mice. Moreover, the average spontaneous mutant frequency in spleen was not significantly different from the average spontaneous mutant frequency in liver and only slightly, albeit not significantly, higher from the average spontaneous mutant frequency in kidney (Figure 1). It should be noted, however, that despite similar spontaneous mutant frequencies the proportion of no-change and size-change mutants may vary widely between organs. A previous study on spontaneous mutant frequencies in *lacZ* plasmid-based transgenic mice indicated that the proportion of size-change mutants in tissues with a relatively high cell turnover (liver, lung and spleen) is significantly higher as compared to tissues with a relatively low cell turnover (brain and kidney) [11].

In order to understand the mechanism by which peroxisome proliferators attribute to rodent hepatocarcinogenesis the possible role of genetic toxicity should not be dismissed if just one peroxisome proliferator is shown to be unequivocally inactive as a genetic toxin. Based on recent findings that WY-14,643 is mutagenic [7,12], possibly mediated through oxidative stress [8], this study aimed to determine whether other peroxisome proliferators are mutagenic as well. To address this issue, *lacZ* plasmid-based transgenic mice were treated with WY-14,643, DEHP, or clofibrate and mutant frequencies were measured in liver, the common target organ in rodents for these three compounds. Mutant frequencies in kidney and spleen were also measured after exposure to DEHP (both organs) or WY-14,643 (kidney only).

The number of animals per group and the number of CFUs per tissue determine the statistical power that can be achieved at a given false positive rate. Since the number of CFUs that can be analyzed in a single experiment on a single plate can be as high as 2 × 10^6, it is not necessary to consider the number of experiments per sample. A previous analysis of the *lacZ* mutant frequency for sources of statistical variability showed that excess variability was not evidenced from rescue-to-rescue (within-organ) [10]. In general, by comparing the data as two binomial proportions using a one-sided test with significance level α = 0.05 and power 0.90, it has been estimated that in order to detect an approximate 50% increase above an average background mutant frequency of 5.5 to 7.0 × 10^{-5} using 4 animals per treatment group, approximately 1 × 10^6 CFUs per group need to be analzyed.

The results shown in Figure 1 clearly indicate that the six-dose exposure to DEHP or WY-14,643 over a two week time period significantly increased the mutant frequency in liver of both female and male mice by approximately 40%. In contrast, treatment with clofibrate did not lead to an increased mutant frequency in liver of either female or male mice. The mutant frequencies in kidney or spleen were not significantly different between female and male control mice and DEHP-exposed mice. Moreover, treatment with WY-14,643 did not lead to a significant increase in the mutant frequency of either female or male kidney. The absence of a carcinogen-induced increase in the mutant frequencies of kidney and spleen can not be attributed to a lack of sensitivity of the *lacZ* plasmid-based mice as outlined above and thus confirms the organ-specific mutagenic nature of DEHP and WY-14,643.

Discussion

Transgenic rodent gene mutation models provide the means to determine gene mutations in the DNA from any tissue in a relatively quick way. The *lacZ* plasmid-based transgenic mouse mutation assay is somewhat unique among other, commercially available, models (e.g., MutaMouse™ and Big Blue™), by virtue of its ability to accurately quantify both point mutations and large deletions including those which originate in the *lacZ* plasmid concatamer and extend into the 3' flanking genomic region [13]. It should be noted that to date there is no single, agreed-upon protocol for conducting mutagenicity assays with transgenic rodents although several aspects have been agreed upon by the Transgenic Mutation Assays Workgroup of the International Workshop on Genotoxicity Procedures [14,15]. In this study, the duration of treatment (two weeks), the number of treatments (six), as well as the manifestation time of 21 days (i.e., the time between the last treatment and the time of sampling) were chosen based on the rationale that multiple treatments generally produce a larger response than single treatments

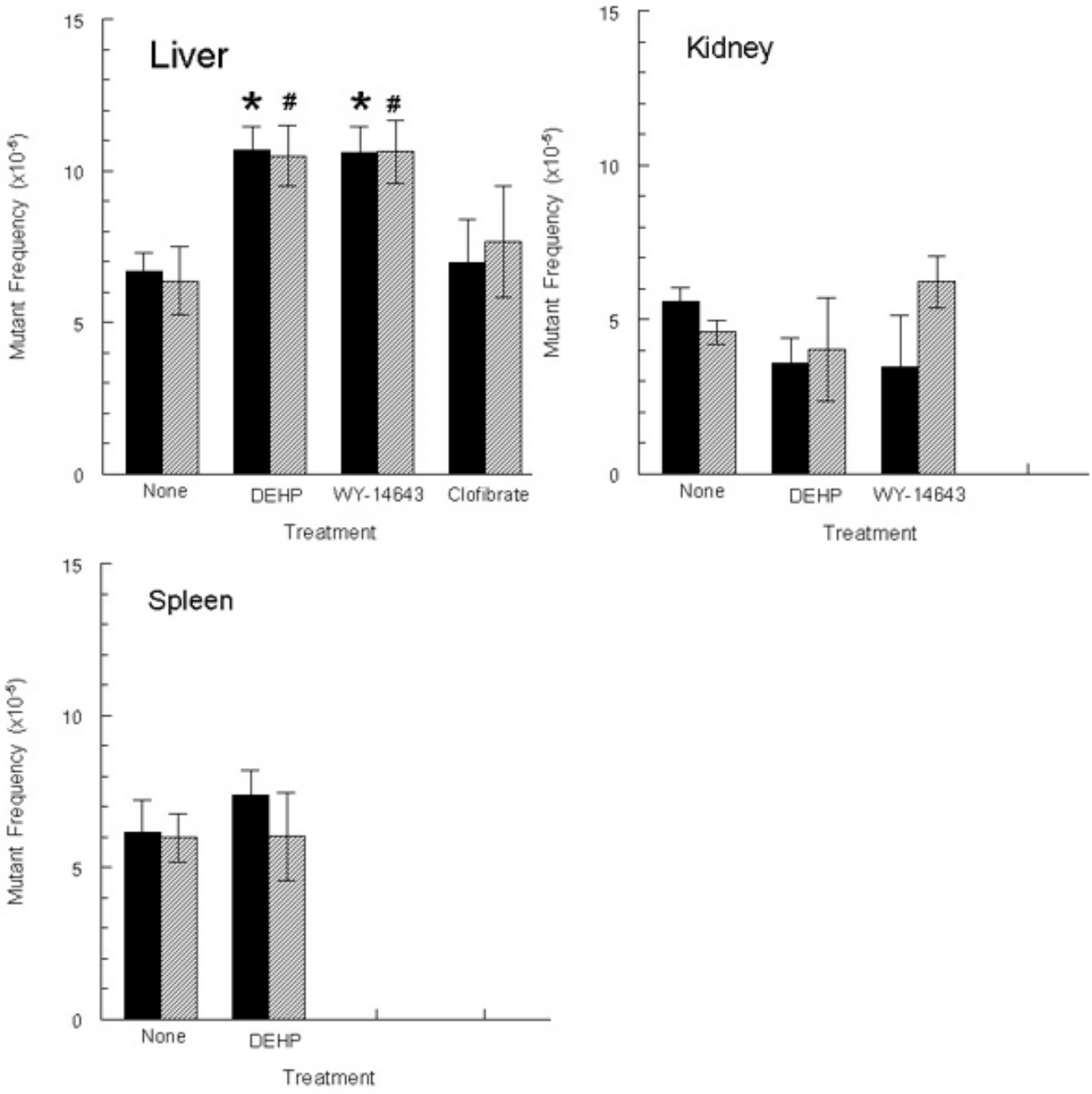

Figure 1
Transgenic *lacZ* mutant frequencies in organs exposed to peroxisome proliferators. Female (solid bars) and male (hatched bars) mice were treated with six doses of 2,333 mg DEHP, 200 mg WY-14,643, or 90 mg clofibrate per kg of bodyweight, respectively, over a two-week period. Twenty-one days after the last treatment, animals were sacrificed and organs were frozen at -80°C until used for DNA isolation. The binomial variance statistic is referenced to a λ^2 distribution with *df* equal to the number of organs minus one. Comparisons between organs were performed using a two-sided paired *t*-test. Mutant frequencies in liver exposed to the peroxisome proliferators DEHP or WY-14,643 were significantly different from mutant frequencies in unexposed livers at $P < 0.01$ (*) or $P < 0.025$ (#). All other comparisons were not significant.

Table 1: Mutagenicity of peroxisome proliferators in various models

Chemical	Rats[a]	Mice[a]	Genotox[b]	*lacZ*	P53$^{+/-}$	RasH2	TgAC-dermal	TgAC-oral	XPA$^{-/-}$	XPA/p53	Neonatal	SHE
Clofibrate	+	-	-	-(IP)[c]	-(G)	+/Eq.(G)	+		In.		-	
WY-14,643	+	+	-	+(IP)	-(diet)		-	Eq.(diet)	+			+
DEHP	+	+	-	+(IP)	Eq.	+(diet)	-	-(diet)	-	-	-	

[a]Results from standard long-term (18–24 months) bioassay. [b]Primarily related to results in the Ames assay as a reasonable indicator of reactivity of the chemical or a metabolite with DNA. [c]IP = intraperitoneal; G = gavage; In = Inadequate; Eq = equivocal.

[15] and sampling time should not exceed 2–4 weeks. The latter requirement is based on recent findings that the mutant frequency may actually decline, even in tissues characterized by a long turnover time of the cell population [11,16]. The observation that carcinogen-induced mutant frequencies do not necessarily increase and then remain constant over time indicates that cell proliferation is not the only factor determining whether, and to what extent, mutations are evident in a particular tissue as was previously surmised (Bielas and Heddle, 2000). Although in the present study there was no statistically significant difference in the average spontaneous mutant frequencies of liver, kidney, and spleen, it has been demonstrated previously, using a greater number of animals, that the average spontaneous mutant frequency in liver is significantly higher as compared to the average spontaneous mutant frequencies in kidney and spleen [10].

DEHP was recently downgraded by a working group of International Agency for Research on Cancer (IARC) from "a possible or reasonably anticipated human carcinogen" to "not classifiable as to its carcinogenicity to humans" (group 3) based on the assertion that DEHP produces liver tumors in rats and mice by a mechanism involving peroxisome proliferation, which was judged to be not relevant to humans [17]. Similarly, clofibrate has been classified as not classifiable as to its carcinogenicity in humans [18] while Wyeth 14,643 has not been evaluated yet. Both DEHP and Wyeth 14,643 are typically considered rodent carcinogens that are putative human non-carcinogens based on their mechanism of action while clofibrate is considered a rodent carcinogen that is a putative human non-carcinogen based on epidemiological data.

Oral repeat-dose studies in rats and mice have consistently shown that the primary target organ of these chemicals is the liver [19]. However, in contrast to DEHP and WY-14,643, clofibrate produced hepatocellular carcinomas in rats only while no increase in the incidence of tumors was reported in mice [19]. If peroxisome proliferation alone is predictive of liver cancer outcome, then similar levels of peroxisome proliferation should lead to similar incidences of liver tumors in experimental animals. However, this is not always the case. For example, at doses of DEHP and WY-14,643 that produce similar levels of peroxisome proliferation in rat liver, WY-14,643 produced an earlier and much greater liver tumor response than did DEHP [20]. Additionally, clofibrate induces peroxisome proliferation in both rats and mice [21] but only produced hepatocellular carcinomas in rats [19]. Thus, peroxisome proliferation alone does not provide an adequate mechanistic explanation for the different, species-specific, carcinogenic potencies of DEHP, WY-14,643 and clofibrate in rodent liver. Indeed, based on the relationship between the observed mutagenic potential of DEHP, WY-14,643 and clofibrate in female and male *lacZ* plasmid-based transgenic mice and their reported carcinogenic potencies in rodent liver, the classification of peroxisome proliferators as non-genotoxic may need to be reconsidered.

Several studies from a government/industry/academic partnership, coordinated by the International Life Sciences Institute (ILSI), have recently evaluated several alternative models for their sensitivity in identifying carcinogens, including clofibrate, WY-14,643 and DEHP. Among these models were the TgAC transgenic mouse model (employing both dermal and oral administration) [22], the Tg-rasH2 transgenic model [23], the heterozygous p53 gene knockout model [24], the homozygous XPA and the homozygous XPA-heterozygous p53 gene knockout models [25], and the neonatal mouse model [26]. The chemicals were also evaluated in the *in vitro* SHE assay. The data presented in Table 1 indicates that each of these peroxisome proliferators produced positive or equivocal results in 1 or more of the aforementioned models, and each of the models, except for the neonatal mouse model, gave positive or equivocal results with 1 or more of these three chemicals. The data presented in Figure 1 and Table 1 indicates that, based on the observed mutant frequencies, the *lacZ* transgenic mouse mutation assay correctly predicted the organ-specific carcinogenic potential of these three peroxisome proliferators in mice. In contrast, the other eight alternative models were unable to unambiguously predict the carcinogenic action of clofibrate, WY-14,643 and DEHP and in most instances did not yield any potential information on the organ-specific

action of these peroxisome proliferators (Table 1). The variable results obtained with peroxisome proliferators may be related to the mechanism(s) by which they are producing tumors. These chemicals act by producing a strongly oxidative damaging milieu and, as such, may exert a genotoxic effect which, in combination with a concurrent increase in hepatocellular proliferation, would ultimately lead to hepatocellular carcinogenesis.

Several lines of evidence support the notion that peroxisome proliferators may indeed be exerting a genotoxic effect, albeit not in all exposed tissues or organs. First, WY-14-643, a more potent carcinogen than DEHP, was more active in inducing chromosome aberrations in a study of morphologic transformation in Syrian hamster embryo (SHE) cells [27]. Second, several peroxisome proliferators, including DEHP, WY-14,643, clofibrate and simfibrate were shown to produce DNA damage *in vitro* as well as *in vivo* [12,28,29]. Third, male B6C3F1 Big Blue transgenic mice which were fed a diet containing WY-14,643 displayed a significant increase in their liver mutant frequency, as compared to untreated control mice [12].

Conclusions

The organ-specific mutagenicity of two peroxisome proliferators, DEHP and WY-14,643, has been unambiguously established using the *lacZ* plasmid-based transgenic mouse mutation model. Moreover, exposure to clofibrate did not result in an elevated mutant frequency in mouse liver, consistent with the historical observation that this compound does not increase the incidence of tumors in mice. These results suggest that peroxisome proliferation may not be the only necessary factor in the carcinogenicity of DEHP and WY-14,643, and perhaps other peroxisome proliferators as well, thereby contending the prevailing opinion that peroxisome proliferators do not pose a carcinogenic risk to humans because of species differences in peroxisome proliferation. The elucidation of the mechanistic action(s) of peroxisome proliferators including oxidative damage, perturbation of growth control (enhancement of cell proliferation and suppression of apoptosis) as well as mutagenicity and carcinogenicity should greatly aid our ability to determine whether these agents do indeed pose a cancer risk to humans.

Author's contributions

METIB carried out all aspects of the study.

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