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Hvdroquinone: Assessment of genotoxic potential in the *in vivo* alkaline comet assay

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ABSTRACT

Hydroquinone (HQ) exposure is common as it is a natural component of plant-based foods and is used in some fingernail polishes, hair dyes, and skin lighteners. Industrially it is used as an antioxidant, polymerization inhibitor, and reducing agent. The current study was undertaken to determine whether HQ may cause DNA damage in an *in vivo* comet assay in F344 rats. DNA strand breaks were assessed in the duodenum as a direct tissue contact site, the testes, and the liver and kidneys, which were tumor sites in bioassays. Rats were exposed to HQ by gavage at 0, 105, 210, or 420 mg/kg/day. At all dose levels, mean % tail intensity and tail moment values for all tissues in animals given HQ were similar to the control. There were no statistically significant increases in tail intensity in any tissue following HQ treatment of male and female rat and data for all animals fell within the available historical control ranges for each tissue. There was no evidence of induction of DNA damage in cells isolated from duodenum, kidney or liver of male and female rats or in the testes of male rats following exposure to HQ at a dose levels up to 420 mg/kg/day, which caused acute renal necrosis.

1. Introduction

Exposure to hydroquinone (1,4-benzenediol, HQ) is ubiquitous as it is a common, naturally occurring antioxidant in plants used for food and beverages. 4-Hydroxyphenyl-β—D-glucopyranoside (β-arbutin), which hydrolyses to HQ when consumed, is present in plant-based foods [1,2]. Dietary exposure to HQ is expected to increase if recommendations for people to move to a more plant-based diet are followed as some common fruits have been found to raise HQ blood levels in people [1].

HQ has a large number of industrial uses, primarily as an antioxidant in the manufacture of rubber, a polymerization inhibitor for vinyl and acrylic monomers, chemical intermediate, and as a reducing agent in photographic development. HQ is also used as a polymerization inhibitor in some acrylics used to coat fingernails, and some hair dyes and skin lighteners.

The National Toxicology Program (NTP) reported that HQ produced an increased incidence in renal tubule adenomas in male F344 rats but not in females, and liver adenomas and thyroid gland follicular cell hyperplasia in male and female B6C3F$_1$ mice [3,4]. Findings of mononuclear cell leukemia in female F344 rats and liver adenomas in B6C3F$_1$ mice were inconsistent between those studies [2].

Reexamination of renal pathology in rats included in the NTP HQ bioassay by Hard et al. [5] showed that the renal adenomas in male rats colocalized with the more severe forms of chronic progressive nephropathy (CPN). McGregor [6] concluded that it is likely that the mode of carcinogenic action of hydroquinone is exacerbation of this natural disease process. The absence of kidney tumors in female rats, which do not develop the severe chronic progressive nephropathy seen in male rats, supports McGregor's conclusion.

Further studies by Hard et al. [7] involving NTP cancer bioassays of 24 other chemicals using F344 rats showed clear evidence of a qualitative and statistically significant associations between advanced stages of CPN severity and the development of low-grade renal tubule tumors and atypical renal cell hyperplasia similar to that seen in male F344 rats following exposure to HQ.

Numerous genotoxicity studies have been conducted with HQ and have been reviewed by Kari [3] IPCS [8] IARC [9] OECD [10], and McGregor [6]. In general, negative results for gene mutation were obtained when tested in *Salmonella typhimurium,* and frequently positive results for chromosomal effects were reported *in vivo* following intraperitoneal injection. However, negative or even protective results were obtained when tested by oral administration [3,11].

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Matsumoto et al. [12] explored the relationship between HQ-induced mutagenicity and tumorigenicity in a lacZ transgenic mutation assay. Male Muta™ mice were given HQ by oral at dose levels of 0, 25, 50, 100, or 200 mg/kg bw/day for 28 days. HQ administration was associated with a decrease in body weight gain for all treatment groups. There were no significant differences in mutant frequencies in the liver, stomach, lungs, or kidneys related to HQ exposures compared to controls. According to the authors the results suggest that a mutagenic mechanism is not responsible for HQ-induced carcinogenesis.

The chemical evaluation process under REACH Regulation (EC) No. 1907/2006 includes a process referred to as the Community Rolling Action Plan (CoRAP). As part of CoRAP, HQ was further studied primarily due its wide use and results seen during *in vitro* studies and *in vivo* studies with parenteral administration.

The aim of this study was to investigate the potential for HQ to cause DNA damage when administered to male and female F334 rats with the results to be used to further refine human risk assessment regarding mutagenicity and carcinogenicity as part of the European Union Community Rolling Action Plan.

2. Materials and methods

The comet assay design met the requirements of the OECD Guideline 489 (2016), the European Food Safety Agency report "*Minimum criteria for the acceptance of in vivo alkaline Comet Assay Reports*" [13], Tice et al. [14], Hartmann et al. [15], and ECHA guidance [16]. The design criteria included dosing rats by oral administration and assessing DNA damage in the liver, kidney, testes, and duodenum. The liver and kidney samples represented target organs in prior rodent bioassays. The liver and gastrointestinal tract are major sites for HQ metabolism. The duodenum represented a tissue directly exposed to HQ and the testes was intended to represent exposure to gonadal tissue.

The assay was conducted in conformance with the United Kingdom and OECD Good Laboratory Practice principles. The study design was approved in advance and its subsequent conduct overseen by the Animal Welfare and Ethical Review Body (AWERB) of the test facility and was in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.1. Test chemical and control substances

The test material, sourced from Solvay (St Fons, France), was 99.9 % HQ (CAS Number 123−31-9), when analyzed by high pressure liquid chromatography using an Agilent Series 1100 HPLC System (Agilent Technologies, Inc., Santa Clara, CA) with a thermostatted column. HQ was dissolved in purified water prior to each dosing occasion. Homogeneity and concentration of the dosing formulations were confirmed at 1 and 50 mg/mL, spanning the expected test concentration range. Test solutions were stored at 15–25 °C, protected from light and used for animal dosing within 2.5 h of preparation. Analyses demonstrated the stability of HQ during the administration period.

The animals in the vehicle control group were dosed with purified water. The positive control, ethyl methanesulfonate (EMS), which was sourced from Sigma-Aldrich Chemical Co. (Poole, UK), was freshly prepared in purified water at a concentration of 15 mg/mL and dosed at 150 mg/kg bodyweight. EMS was selected as the positive control as this is the standard control used in the laboratory and forms the basis of the laboratory's historical control data (HCD). All other chemicals were sourced from Sigma-Aldrich, or an equivalent supplier, unless stated otherwise.

2.2. Animals

The animals used in these studies were Fischer F344 rats obtained from Harlan UK Ltd. (Oxon, UK). Rats were 6–9 weeks of age and weighed 140–221 g (males) and 85–154 g (females) at the start of dosing in a dose range finding study and the main comet assay. Fischer F344 rats were selected for this study because most of the database for HQ was obtained in this strain and this strain of rats has a greater susceptibility to HQ-related effects on renal tubules [17,18]. In addition, a small number of Sprague Dawley rats were treated as concurrent vehicle and positive controls due to the lack of laboratory historical control data in Fischer F344 rats. Animals were housed in wire topped, solid bottomed cages, with a maximum of three animals of the same sex per cage. For periods of time not exceeding 24 h, rats scheduled for urinalysis were housed individually in urine collection cages. The animals were housed in rooms provided with 15–20 air changes/hour at temperatures in the range of 20–24 °C, 45–65 % relative humidity, and a 12 -h light/dark cycle.

2.3. Dose administration and tissue sampling

Groups for the comet assay consisted of six males and six females dosed with control (purified water) or HQ (at 105, 210 or 420 mg HQ/kg body weight/day) at 10 mL/kg at 0 h (day 1) and either 23.5 h (control and HQ animals sampled for duodenum, kidney and liver) or 22 h (control and HQ animals sampled for testes). In addition, groups of positive control animals (n = 3/sex) received a dose of EMS at 150 mg/kg body weight/day at 21 h (positive control animals sampled for duodenum, kidney and liver) or at 0 and 21 h (positive control animals sampled for testes). All groups of animals were terminated for tissue sampling at 24 h (Day 2). Due to a technical error in processing the kidneys, a second group of six male and six females were dosed with 0, 105, 210, or 420 mg/kg HQ and processed as described above for the comet assay. The kidneys from these animals were also assessed for histopathology and increased the number of animals available for histopathology to 12.

Final dose administration and sample times were determined by consideration of available toxicokinetic data for HQ and the general recommendations of OECD 489. Duodenum, kidney, and liver were sampled from males and females 30 min after the final dose administration. The sampling time for the somatic tissues was selected based on peak plasma time (T_{max}) reported in previous toxicokinetic studies [19,20], and is consistent with recent IWGT recommendations [21] and OECD 489 (2016). The somatic tissues of the positive control-treated animals were sampled three hours after a single administration, following the sample time used for the laboratory's historical control data. Gonad analysis was performed on a separate group of animals to allow a different sampling time for this tissue. In the absence of specific kinetic information for gonad exposure the lower limit (2 h) of the default sampling time stated in OECD Test Guideline 489 (2−6 h) was used based on the assumption there would be a delay in peak gonad exposure compared to somatic tissues.

Tissue selection for comet analysis was based on the known properties of HQ. The kidney was selected as it is the primary target organ for F344 rats treated with HQ and a site in which tumors have been reported. The liver was selected as it is the primary site for metabolism of HQ and liver adenomas have been reported in HQ-treated mice. The duodenum was selected as a key site of contact following oral administration instead of the (fore)stomach to include possible enterohepatic recirculation of HQ metabolites. Male testes were included in this comet assay to examine the possible interaction of HQ with germ cells [22].

Selection of HQ doses was determined from data obtained in a dose-range finding study. Small groups of male and female F344 rats (n = 2 or 3/sex) were dosed with HQ at 300, 420, 600 or 1000 mg/kg body weight/day using the same route, dose volume (except 600 and 1000 mg/kg body weight/day doses, which were dosed at 20 mL/kg due to solubility limitations) and dose administration times as described above for the comet assay. No clinical signs of toxicity were observed at 300 mg/kg/day. At 420 mg/kg/day, only mild, transient signs were observed (piloerection solely in male animals approximately 1−2 h after the second administration). More severe signs (including tremors, decreased activity and hunched posture) were observed at the higher

doses with animals reported to be moribund or mortality observed. From these results 420 mg/kg/day was considered to be an appropriate estimate of the maximum tolerated dose (MTD) and was therefore selected as the maximum dose for the comet assay. Two lower doses of 210 and 105 mg/kg/day were also selected.

2.4. Evaluation of systemic toxicity

Body weights were recorded during the study set-up, then on Day 1 (prior to dosing) and on Day 2 prior to necropsy. Post-dose observations of animal condition were recorded prior to each dose administration, immediately after each dose administration, 0.5, 1, 2, and 4 h after the first dose and immediately prior to necropsy.

A battery of clinical chemistry endpoints was examined to assess possible systemic effects. A 0.6 mL terminal blood sample was taken from the abdominal aorta into lithium heparin tubes, mixed thoroughly, cooled, and then centrifuged (2300*g*, 4 °C, 10 min) to separate plasma. Clinical chemistry assays included the following analytes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transferase, sodium, potassium, calcium, inorganic phosphorus, chloride, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol, glucose, urea, total bilirubin, and creatinine.

Urine was collected over approximately 24 h following the first dose administration from vehicle control and high dose (420 mg/kg/day) F344 rats (with *ad libitum* access to food and water). Urine collected during this period was observed for unusual coloration.

Histopathologic examinations were conducted on samples of the duodenum, liver, kidneys and testes from the vehicle and HQ-treated rats. Samples of the duodenum, liver and kidneys were fixed in neutral buffered formalin. The right testis, with epididymis, was preserved in modified Davidson's fixative. No histopathology samples were preserved for the positive control animals. Tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy.

2.5. Preparation of cell suspensions

The duodenum samples were washed thoroughly in Merchant's solution (0.5 mM NaEDTA, 10 % DMSO in phosphate buffered saline, pH 7.4) and placed into fresh buffer. Each sample was vortexed in Merchant's solution for approximately 15 s. The tissue was removed from the Merchant's solution and the inner surface gently scraped (released material discarded) using the back of a scalpel blade. The tissue was vortexed in Merchant's solution for a further 15 s prior to gently scraping the inside of the duodenum with the back of a scalpel blade. The kidney samples were cut into small pieces and washed thoroughly in Merchant's solution. The pieces were then pushed through bolting cloth (pore size of 150 μm) with approximately 4 mL of Merchant's solution to produce single cell suspensions. The liver samples were washed thoroughly in Merchant's solution and placed in fresh buffer. The samples were cut into small pieces in Merchant's solution and the pieces of liver were then pushed through bolting cloth (pore size of 150 μm) with approximately 4 mL of Merchant's solution to produce single cell suspensions. The left testes were finely minced using a scalpel blade and tweezers and filtered through bolting cloth (pore size of 150 μm) with ice cold Merchant's solution to produce single cell suspensions. All cell suspensions were held on ice prior to slide preparation.

2.6. Slide preparation

Three slides were prepared per single cell suspension per tissue. Slides were dipped in molten normal melting point agarose (NMA) such that all the clear area of the slide and at least part of the frosted area was coated. Thirty (30) μL of each single cell suspension was added to 300 μL of 0.7 % low melting point agarose (LMA) at approximately 37 °C. 100 μL of cell suspension/agarose mix was placed on to each slide. The slides were then coverslipped and allowed to gel on ice. Once gelled, the coverslips were removed and all slides placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH adjusted to pH 10 with NaOH, 1 % Triton X-100, 10 % DMSO) overnight at 2−8 °C, protected from light.

Following lysis, slides were washed in purified water for 5 min, transferred to electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 2−8 °C and the DNA unwound for 20 min (duodenum) or 30 min (kidney, liver and testes). At the end of the unwinding period the slides were electrophoresed in the same buffer at 0.7 V/cm for 20 min (duodenum) or 40 min (kidney, liver, and testes). Different unwinding and electrophoresis times were used to optimize detection of % tail DNA as determined by experience in the laboratory, as discussed by Azqueta et al. [23]. At the end of the electrophoresis period, slides were neutralized in 0.4 M Tris, pH 7.0 (3 × 5-minute washes). After neutralization the slides were dried and stored at room temperature prior to comet scoring. Prior to scoring, the slides were stained with 100 μL of 2 μg/mL ethidium bromide and coverslipped.

2.7. Slide analysis

Scoring was carried out using fluorescence microscopy at 400X magnification. All slides were coded during analysis to remove scorer bias. Measurements of Olive tail moment and tail intensity (%DNA in tail) were obtained from 150 cells/animal/tissue. In the majority of tissues (and animals), this was achieved by scoring 50 cells from each of three slides. In some instances, only two slides per tissues/animal were scorable (either due to an absence of cells on one slide or due to irregular cell morphology on one slide) and so 75 cells were scored from each of the remaining two slides. The number of 'hedgehogs' (a morphology indicative of highly damaged cells often associated with severe cytotoxicity, necrosis or apoptosis) observed during comet scoring was recorded for each slide. To avoid the risk of false positive results 'hedgehogs' were not used for comet analysis.

The following criteria were used for analysis of slides: only clearly defined non-overlapping cells were scored, hedgehogs were not scored, and cells with unusual staining artefacts were not scored.

2.8. Data evaluation

The experimental unit of exposure for *in vivo* studies is the animal, and all analysis was based on individual animal response. Values obtained from each parameter were treated as follows: the median value per slide was calculated, the mean of the slide medians was calculated to give the mean animal value, and the mean of the animal means and standard error of the mean was calculated for each group. Median tail intensity data were used for statistical analysis. The positive control groups were compared to the vehicle control groups using a two-sample t test. The test was interpreted with one-sided risk for increased response with increasing dose. The vehicle control group and the treated groups were analyzed separately using one-way analysis of variance (ANOVA). An overall dose response test was performed along with Dunnett's test for pairwise comparisons of each treated group with the vehicle control. For all tissues, the tests were interpreted with a one-sided risk. Levene's test for equality of variances between the groups was performed and where this showed evidence of heterogeneity ($p \leq 0.01$), the data was rank transformed prior to analysis.

High levels of 'hedgehogs' indicated the nuclear complex had been significantly fragmented and was considered evidence of excessive DNA damage. Such damage may be due to the cytotoxic nature of the treatment or due to excessive mechanical disruption during cell isolation, which had the potential to interfere with comet analysis.

The data were considered valid if the following criteria were met: there was a marked increase in group mean positive control values compared to the concurrent vehicle control and the high dose was determined to be a MTD, the maximum recommended dose or the

maximum practicable dose. Data from control F344 rats were compared to data from control Sprague Dawley (data not shown). As the values were considered comparable it was considered acceptable to use the laboratory's historical control data in Sprague Dawley rats for data evaluation.

HQ was considered to induce DNA damage if: (i) at least one of the test doses exhibited a statistically significant increase in tail intensity, in any tissue, compared with the concurrent vehicle control and, (ii) the increase in tail intensity was dose-related in any tissue. HQ was considered positive in this assay if both of the above criteria were met but negative in this assay if neither of the above criteria were met.

3. Results

3.1. Systemic toxicity: assessment of the MTD for evidence of systemic HQ exposure

No obvious clinical signs of toxicity were observed in animals following treatments with vehicle, EMS, or HQ (105, 210 or 420 mg/kg/day) during the comet assay. As was observed in the range-finding study, clinical responses to HQ were subtle at 420 mg/kg with signs of twitching (tremors) and vocalization reported for one female rat. Similar signs were observed in male rats during the range-finding study at the same dose level and were previously reported by Topping et al. [24]. As seen in the range-finding study increasing the dose of HQ by about 50 % to 600 mg/kg results in much more serious effects and mortality. No treatment related mortality was observed in the current study.

Urine collected from all high dose animals was reported to be darker in color than the concurrent control group. This was also reported in other studies [24]. The darker urine color was attributed to metabolites of HQ and was considered evidence of renal exposure following oral gavage dosing.

Body weight gain was reduced for both male (3.3 g, n = 18) and female (4.0 g, n = 12) animals given 420 mg/kg HQ when compared to the male (8.4 g, n = 18) and female (6.2 g, n = 12) negative control animals. The reduction in weight gain was greater for males (38 %) *vs.* females (16 %). No consistent reduction in weight gain was observed at lower HQ dose levels.

There were no macroscopic findings considered to be related to administration of HQ. Microscopically, there were findings in the liver and kidneys, but none in the duodenum and testes. Clinical chemistry results showed some increases and some decreases in results; however, except for those discussed below there was no correlation with the animals' clinical status.

A decrease in glycogen vacuolation, which indicates increased utilization of glycogen stored in the liver, was observed in the liver of male rats given 210 or 420 mg/kg/day and in female rats from all HQ-treated groups. The reduction in stored glycogen is possibly due to increased metabolism in the liver or systemically, rather than a pathologic change. In the liver, increased hepatocyte mitosis was present in rats from all HQ-treated groups, with a generally dose-related effect. The increased hepatocyte mitosis seen in rats from all HQ-treated groups is also considered an early indication of increased hepatocyte metabolism. Focal necrosis, characterized by an area of necrotic hepatocytes, with some inflammation, and by scattered eosinophilic and/or shrunken hepatocytes, often with pyknotic nuclei, was observed in one of six male rats given 420 mg/kg/day HQ. The AST and ALT values for individual male and female rats in the HQ-dosed groups were elevated above control levels. The only statistically significant differences ($p < 0.05$) were observed for the mean values for the high dose groups (male and female ALT and AST).

In the kidneys, acute renal tubular necrosis was present in males and females given 210 or 420 mg/kg/day. Renal changes were of greater severity and/or incidence in males and correlated with increased urea and/or creatinine in these animals. The changes in the renal tubules were characterized by hypereosinophilia of the mid cortical band corresponding with the S3 segment of the proximal tubules with occasional shrunken hypereosinophilic tubular cells with pyknotic nuclei at the minimal severity grade. At the higher severity grades, there was loss of cellular detail, cell swelling, and hypoeosinophilia, karyolysis and destruction of tubular cells in the S3 segment of the proximal convoluted tubule, with intralumenal debris. The incidence of histopathologic lesions in the kidneys from 12 animals per sex dosed with HQ are shown in Table 1. The 105 mg/kg dose level was a NOEL for acute renal tubular necrosis. At the 210 mg/kg HQ/day dose level, only 1 of 12 males and 1 of 12 females were affected with minimal changes. At 420 mg/kg HQ/day, 9 of 12 females and 11 of 12 males showed renal tubular necrosis, which was not only more frequent in male rats but was also graded at a higher severity level. Creatinine values for individual male and female rats in the high dose group were elevated and urea nitrogen was elevated for some male high dose rats. These clinical chemistry changes are consistent with the renal tubule changes present microscopically.

The MTD (420 mg/kg/day) was considered acceptable based on the severity of the observations seen at higher doses in the range-finder experiment and the dose-related decrease in body weight gain and histopathologic changes observed in the groups of males and females dosed with HQ contrasted with the concurrent F344 vehicle control groups. The changes observed more specifically in the kidneys and liver provided evidence of adequate systemic exposure of the target tissues to HQ.

3.2. Comet assay assessment

There was no dose-related increase in % hedgehogs in the duodenum, kidney, liver or testes cells following treatment with HQ, demonstrating that treatment with HQ did not cause excessive DNA damage that could have interfered with comet assay analysis (Tables 2–5). Group mean vehicle control values were comparable to laboratory historical control data/in-house data for each tissue and there was a statistically significant increase in tail intensity in the positive control groups compared to the concurrent vehicle controls. Consequently, all data were accepted as valid for comet evaluation.

HQ did not induce a statistically significant increase in % tail intensity in either the duodenum (Table 2), liver (Table 3) or kidney (Table 4) in male and female rats or in the testes (Table 5) of male rats. Furthermore, except for the % tail intensity in the liver for male rats (Table 3), there was no evidence of any statistically significant linear trend in the data. In general, the individual animal % tail intensities for all tissues fell within the laboratory's historical control data. There were some exceptions, and these are discussed in more detail below.

In female rats treated with HQ, the group mean tail intensities for the duodenum were slightly elevated compared to the concurrent vehicle control, although at all dose levels this was less than two-fold and was not significantly different to the concurrent vehicle controls (Table 2). For each dose group the increase in group mean tail intensity was attributed to a single animal that had a tail intensity that exceeded the 95 % reference ranges of the historical control data; however, all animals were within the observed range of the historical control data (Table 6). Furthermore, all other HQ-treated animals were highly comparable to the concurrent vehicle control and within the 95 %

Table 1
Incidence of Acute Renal Tubular Necrosis in F344 Rats Given Hydroquinone by Gavage.

Dose Level (mg/kg/day)	Males				Females			
	0	105	210	420	0	105	210	420
No. examined	12	12	12	12	12	12	12	12
Severity Grade 0	12	12	11	1	12	12	11	3
1	0	0	1	4	0	0	1	6
2	0	0	0	3	0	0	0	1
3	0	0	0	4	0	0	0	2

Table 2
Comet Assay Results for the Duodenum of Male and Female F344 Rats Given HQ.

Group / Dose	Total comets scored	Males					Females					HCD Min-Max % Tail Intensity [95 % Range]
		Tail Intensity		Tail Moment		Hedgehogs (%)	Tail Intensity		Tail Moment		Hedgehogs (%)	
		Mean	SEM	Mean	SEM	Mean	Mean	SEM	Mean	SEM	Mean	
Vehicle	900	0.78	0.16	0.09	0.02	11.64	1.77	0.59	0.19	0.07	21.90	
HQ 105 mg/kg/day	900	0.93	0.15	0.10	0.01	11.10	3.02	0.94	0.36	0.11	22.82	
HQ 210 mg/kg/day	900	0.86	0.16	0.10	0.02	11.02	3.26	1.10	0.39	0.14	19.05	0.07–9.85 [0.38–5.27]
HQ 420 mg/kg/day	900	0.88	0.21	0.11	0.03	10.81	2.97	1.02	0.34	0.12	21.40	
EMS 150 mg/kg/day	450	14.11**	1.14	1.86	0.17	13.40	11.41***	0.37	1.37	0.08	26.51	2.58–35.10 [5.99–28.59]
Statistics		SR, A					S, A					

SEM Standard error of the means.
** $p < 0.01$, *** $p < 0.001$.
HCD: Historical Control Data.
Statistics:
S Two-sample *t*-test (Vehicle *vs.* EMS).
SR Two-sample *t*-test (Vehicle *vs.* EMS), using rank-transformed data.
A ANOVA, Dose Response and Dunnett's (Vehicle *vs.* HQ groups).

Table 3
Comet Assay Results for the Liver of Male and Female F344 Rats Given HQ.

Group / Dose	Total comets scored	Males					Females					HCD Min-Max % Tail Intensity [95 % Range]
		Tail Intensity		Tail Moment		Hedgehogs (%)	Tail Intensity		Tail Moment		Hedgehogs (%)	
		Mean	SEM	Mean	SEM	Mean	Mean	SEM	Mean	SEM	Mean	
Vehicle	900	0.28	0.08	0.04	0.01	3.48	0.72	0.17	0.10	0.02	4.71	
HQ 105 mg/kg/day	900	0.45	0.10	0.06	0.01	3.22	0.90	0.24	0.13	0.03	5.60	
HQ 210 mg/kg/day	900	0.42	0.16	0.05	0.02	2.24	1.02	0.42	0.13	0.05	4.43	0.01–9.52 [0.05–5.06]
HQ 420 mg/kg/day	900	0.64	0.17	0.09	0.02	3.56	1.34	0.47	0.18	0.06	5.12	
EMS 150 mg/kg/day	450	20.45***	2.04	3.26	0.45	5.31	21.52**	1.31	3.55	0.33	5.89	5.02–84.19 [10.65-62.51]
Statistics		DR, S, A					SR, A					

SEM Standard error of the means.
** $p < 0.01$, *** $p < 0.001$.
HCD: Historical Control Data.
Statistics:
DR Significant Dose Response test (Vehicle and HQ groups).
S Two-sample *t*-test (Vehicle *vs.* EMS).
SR Two-sample *t*-test (Vehicle *vs.* EMS), using rank-transformed data.
A ANOVA, Dose Response and Dunnett's (Vehicle *vs.* HQ groups).

reference ranges of the historical control data. The female HQ duodenum data across all dose levels showed a normal degree of variation and there were no HQ-related increases in DNA damage.

For the assessment of the liver, there were four animals (2 males and 2 females) in the two highest dose groups with slightly higher tail intensities than the concurrent control group (Table 7). In males, these high animals resulted in a significant dose-response test (Table 3). However, all animals (including the four mentioned above) were considered to be generally comparable with concurrent vehicle controls and fell within the 95 % reference ranges of the laboratory's historical control data. The male and female liver comet data were therefore within the normal biological variation of the assay and there was no HQ-induced DNA damage.

4. Discussion and conclusion

The *in vivo* alkaline comet assay was conducted as part of the European Union Community Rolling Action Plan (CoRAP) to clarify concerns for human health based on the possible carcinogenicity of HQ due to genetic damage. The initial requirement included exposing rats to HQ orally and assessing genotoxicity in contact tissue, target sites of metabolism, target organs and gonads in either a transgenic rodent somatic and germ cell mutation assay (TGR), or an *in vivo* comet assay. Both of these assays have been completed according to recent OECD test

Table 4
Comet Assay Results for the Kidneys of Male and Female F344 Rats Given HQ.

Group / Dose	Total comets scored	Males					Females					HCD Min-Max % Tail Intensity [95 % Range]
		Tail Intensity		Tail Moment		Hedgehogs (%)	Tail Intensity		Tail Moment		Hedgehogs (%)	
		Mean	SEM	Mean	SEM	Mean	Mean	SEM	Mean	SEM	Mean	
Vehicle	900	1.94	0.46	0.22	0.05	11.55	1.02	0.23	0.12	0.04	14.50	
HQ 105 mg/kg/day	900	1.49	0.29	0.18	0.03	12.10	1.65	0.74	0.18	0.08	17.91	
HQ 210 mg/kg/day	900	1.38	0.38	0.17	0.06	11.00	0.63	0.22	0.08	0.03	17.08	0.48–6.63 [0.82–6.36]
HQ 420 mg/kg/day	900	1.67	0.35	0.19	0.04	12.59	0.84	0.27	0.11	0.03	17.24	
EMS 150 mg/kg/day	450	22.18***	0.35	3.50	0.07	13.87	18.26**	1.95	2.80	0.39	18.00	10.16–41.90 [NA]
Statistics		S, A					SR, A					

SEM Standard error of the means.
**p < 0.01, *** p < 0.001.
HCD: Historical Control Data.
NA - Not applicable, insufficient datasets to calculate a relevant reference range.
Statistics:
S Two-sample *t*-test (Vehicle *vs.* EMS).
SR Two-sample *t*-test (Vehicle *vs.* EMS), using rank-transformed data.
A ANOVA, Dose Response and Dunnett's (Vehicle *vs.* HQ groups).

Table 5
Comet Assay Results for the Testes of Male F344 Rats Given HQ.

Group / Dose	Total comets scored	Tail Intensity		Tail Moment		Hedgehogs (%)	HCD Min-Max % Tail Intensity [95 % Range]
		Mean	SEM	Mean	SEM	Mean	
Vehicle	900	0.16	0.02	0.01	0.00	0.65	
HQ 105 mg/kg/day	900	0.19	0.03	0.01	0.00	0.89	
HQ 210 mg/kg/day	900	0.17	0.02	0.01	0.00	0.47	0.04–0.35 [NA]
HQ 420 mg/kg/day	900	0.19	0.04	0.01	0.00	0.65	
EMS 150 mg/kg/day	450	10.71**	0.91	1.09	0.14	11.86	12.85–15.55 [NA]
Statistics		SR, A					

SEM Standard error of the means.
**p < 0.01.
HCD: Historical Control Data.
NA - Not applicable, insufficient datasets to calculate a relevant reference range.
Statistics:
SR Two-sample *t*-test (Vehicle *vs.* EMS), R Rank-Transformed Data.
A ANOVA, Dose Response and Dunnett's (Vehicle *vs.* HQ groups).

guidelines and have shown no evidence of gene mutation or DNA strand breaks in two different rodent species at MTD levels and with examination of target tissues.

Matsumoto et al. [12] completed a *lacZ* transgenic mutation assay (OECD Test Guideline 488) in male Muta™ mice by administering HQ orally at dose levels of 0, 25, 50, 100, or 200 mg/kg bw/day for 28 days, which was an MTD that decreased body weight gain in all HQ treatment groups. They found no significant differences in mutant frequencies in the liver, stomach, lung, thyroid, or kidney between HQ-treated mice and controls. Matsumoto et al. [12] did not include gonadal tissue in their analysis.

Our assay similarly found that HQ given orally with two administrations over 24 h at an MTD level of 420 mg/kg bw/day did not induce DNA strand breaks in male or female rat duodenum, liver or kidneys or in male testes. The study was also intended to address further concerns regarding potential germ cell mutagenesis, using analysis of gonadal cells as a surrogate to germ cells [22]. Exposure of the testes following HQ oral administration was demonstrated in an earlier toxicokinetics study that showed ^{14}C label, although at minor levels (much lower than in liver and kidneys), in testes of male F344 rats at 48 h following a single oral dose of 350 mg/kg [25]. In the present study no DNA strand breaks were detected in rat testes following two consecutive oral administrations at 420 mg/kg, indicating HQ (or its metabolites) has no significant interaction with the genetic material of gonadal cells, and thus no obvious potential germ cell mutagenic effect.

These new results add to a large number of existing genotoxicity data, and contrast with previously reported positive effects including induction of micronuclei, chromosome aberrations, and chromosome loss, particularly following intraperitoneal administration of HQ, or when studied *in vitro*. Understanding the differences in genotoxicity results is important because HQ exposure through food sources is common and may increase with recommendations to improve the

Table 6
Individual Animal Comet Results for the Duodenum of Female F344 Rats Given HQ.

Group / Dose	Animal ID	Median %Tail Intensity	
		Mean	Standard deviation
Vehicle	201	1.39	1.13
	202	2.36	0.08
	203	0.37	0.25
	204	0.50	0.19
	205	4.32	0.66
	206	1.69	1.36
HQ 105 mg/kg/day	213	0.56	0.42
	214	3.55	0.4
	215	7.30	1.42
	216	1.97	2.6
	217	2.61	1.73
	218	2.16	0.67
HQ 210 mg/kg/day	219	7.43	2.73
	220	4.38	0.51
	221	3.13	0.77
	222	3.95	1.26
	223	0.39	0.3
	224	0.31	0.16
HQ 420 mg/kg/day	225	1.48	0.95
	226	7.54	1.78
	227	1.02	0.84
	228	4.02	1.67
	229	1.29	0.71
	230	2.45	0.12
EMS 150 mg/kg	231	10.84	2.3
	232	12.10	0.86
	233	11.30	4.16

Table 7
Individual Animal Comet Results for the Liver of F344 Rats Given HQ.

Group / Dose	Male			Female		
	Animal ID	Median % Tail Intensity Mean	SD	Animal ID	Median % Tail Intensity Mean	SD
Vehicle	1	0.37	0.16	201	0.25	0.08
	2	0.25	0.13	202	1.22	0.36
	3	0.10	0.08	203	1.00	0.57
	4	0.23	0.08	204	0.51	0.31
	5	0.63	0.2	205	1.06	0.48
	6	0.12	0.06	206	0.27	0.1
HQ 105 mg/kg/day	13	0.68	0.42	213	0.44	0.07
	14	0.55	0.49	214	1.09	0.56
	15	0.36	0.22	215	1.58	1.02
	16	0.72	0.83	216	1.54	0.31
	17	0.13	0.05	217	0.59	0.24
	18	0.25	0.28	218	0.15	0.05
HQ 210 mg/kg/day	19	0.35	0.16	219	0.33	0.13
	20	0.41	0.49	220	0.62	0.16
	21	0.34	0.22	221	0.16	0.14
	22	1.19	1.49	222	0.91	0.28
	23	0.20	0.06	223	2.96	0.29
	24	0.03	0.02	224	1.17	0.18
HQ 420 mg/kg/day	25	1.34	0.95	225	0.11	0.01
	26	0.14	0.02	226	0.62	0.17
	27	0.41	0.31	227	0.73	0.26
	28	0.53	0.48	228	3.40	1.78
	29	0.60	0.47	229	1.58	0.36
	30	0.80	0.53	230	1.59	0.79
EMS 150 mg/kg	31	23.96	1.99	231	20.69	3.07
	32	20.51	1.28	232	19.79	2.91
	33	16.89	9.29	233	24.09	3.36

human diet with larger intakes of fruits and vegetables. In addition, a better understanding of these results for HQ may aid in assessing the risks associated with non-compliance with the European Union Cosmetic Regulation 1223/2009 affecting consumer exposure to previously allowed skin preparations [26].

HQ has been extensively studied for genotoxicity and the results of these studies have been reviewed multiple times [2,3,6,8–10,27,28]. Generally, HQ is considered not to cause gene mutations in *Salmonella typhimurium* assays and Kari [3] provides an example of a negative Ames/*Salmonella typhimurium* assay with and without metabolic activation where the purity and stability of the HQ used for testing is documented. There is some apparent gene mutagenicity in mammalian cells as summarized by [3] and [9], although a closer evaluation of the mouse lymphoma data (summarized in [3]) using the global evaluation factor as described by OECD test guideline 490 (2016), suggests that the positive effects in this assay occur at concentrations that induce excessive toxicity.

HQ is widely reported to induce chromosome damage and/or loss both *in vitro* in several cell types, or *in vivo* in mice. IARC [9] concluded that hydroquinone induced micronuclei and chromosomal aberrations in mouse bone marrow in several studies but not sister chromatid exchanges in a single study. IARC also reported that hyperploidy and chromosome loss (as demonstrated by centromere-positive micronuclei), but not polyploidy, were also found in mouse bone marrow. These effects are consistent with HQ having an aneugenic effect. Many of these studies were reviewed by McGregor [6], who concluded that at least a portion, if not all, of the chromosomal effects observed in these studies were caused by interference by HQ or its metabolites with chromosomal segregation, probably due to interaction with mitotic spindle proteins, which is a non-DNA reactive mechanism for aneugenicity. McGregor [6] also noted that the majority of the positive findings *in vivo* were in studies that used intraperitoneal injection, a physiologically irrelevant route of exposure that is no longer recommended for routine safety evaluation. Of the five studies reviewed by McGregor [6] that used the oral route, four were clearly negative for genotoxicity.

Jurica et al. [29] reported low levels of DNA damage in white blood cells from rats exposed to 200 mg HQ/kg bw orally by gavage for 14 or 28 consecutive days. Peripheral blood samples were taken 24 h after the final dose administration and processed through the alkaline comet assay. The authors describe the extent of DNA damage observed (<10 % DNA in the comet tail) as being low genotoxicity; however, the biological relevance of these findings is highly questionable and most likely reflects natural variation in background DNA damage observed in the authors' laboratory. They report a statistically significant decrease in tail length, tail intensity, and tail moment in male rats treated with 200 mg HQ/kg bw over 14 days compared to the control animals (which received 14 gavage administrations of bidistilled water), but an increase in tail length and tail moment (but not tail intensity) in male rats administered the same dose over a 28 day period. No statistically significant DNA damage (in any parameter measured) was observed in female rats treated with 200 mg HQ/kg bw over 14 days but a significant decrease in tail length (but not tail moment or tail intensity) was observed after 28 days administration. The authors do not report historical vehicle control data for the three comet parameters measured; however, examination of the 14 and 28 day data reported for male and female bidistilled water control animals reveals a wide range of background values that generally encompass the values presented for HQ-treated animals. Overall, the data presented by Jurica et al. [29] display no evidence that HQ induces DNA damage (as detected by the comet assay) in the white blood cells of rats treated with HQ over 14 or 28 days.

Consistent with McGregor's observations, English et al. [30] reported that oral administration of HQ for 6 weeks at a nephrotoxic dose (50 mg/kg) did not produce covalent DNA adducts in the kidneys of rats when tested in a ^{32}P- post labelling assay.

The results of the current *in vivo* alkaline comet study demonstrate

HQ did not cause DNA damage at much higher exposure levels in rats. There was also no evidence of gene mutations in the TGR assay in mice. Both of these assays are sensitive to gene mutations, with the comet assay also able to detect chromosome damage that is expressed as DNA strand breaks. However, neither assay is sensitive to chromosome loss and therefore these findings add further weight of evidence to the arguments of McGregor [6], that HQ genotoxicity is expressed predominately through an aneugenic (*i.e.* chromosome loss) mechanism when tested *in vitro* and following ip administration.

Aneugenic chemicals can be expected to cause malignant neoplasms, reproductive failures, and developmental abnormalities. Kari [3] reported that HQ exposure provided some evidence of renal cell adenomas in male F344 rats, liver adenomas in female mice, and a reduction in hepatocellular carcinomas in male mice compared to the control group. Reproductive and developmental toxicity studies with HQ have only resulted in minor effects in fetuses at maternally toxic dose levels. Thus, the pattern of effects following HQ oral administration is consistent with HQ not directly causing DNA-damaging activity. An assessment of the weight-of-evidence of the strongest studies suggests that no potential aneugenic activity of HQ is expressed following oral exposure.

Substances that cause aneugenicity usually demonstrate non-linear dose relationships and exert their activity through mechanisms other than direct interaction with DNA. Consequently, it is widely accepted that safe thresholds may be established for an aneugen [16,31,32]. Such thresholds are the most likely explanation for the discordant results for micronucleus formation between the ip and oral studies.

The current knowledge of the toxicokinetics of HQ and differences seen depending on the route of exposure adds further weight of evidence for a threshold for HQ genotoxicity. In some of the earlier studies, HQ was given by intraperitoneal injection, which previously was the default route for chemicals of unknown toxicokinetics. However, Divincenzo et al. [33] demonstrated that radiolabeled HQ was readily absorbed orally and widely distributed in the tissues of rats; therefore, the oral route is also relevant, especially when considering potential human exposure through food consumption. Furthermore, current guidance in the OECD test guidelines for *in vivo* genotoxicity assessment states that intraperitoneal injection is generally not recommended since it is not typically a relevant route of human exposure [34].

Since the early work of Divincenzo et al. [33], the toxicokinetics, metabolism, and distribution of HQ by oral, dermal, intratracheal, intravascular, and intraperitoneal routes have been extensively investigated and modeled in several key studies [19,20,35–40]. This body of work demonstrates that total HQ (free and bound) peaks within 30 min of oral gavage administration in rats and a human volunteer and then declines exponentially. Parent HQ is rapidly metabolized primarily to glucuronide and sulfate metabolites whether after oral, dermal, intraperitoneal, or intratracheal administration with up to 99 % of the HQ excreted by the kidneys. Metabolism of HQ occurs in the gastrointestinal and liver after oral absorption with a high rate of first pass metabolism and by the liver after other routes of exposure. A significant route difference is a higher rate of HQ metabolism to glutathione metabolites, which are considered responsible for nephrotoxicity, when HQ is administered by intraperitoneal (ip) injection *versus* oral administration. Significant species, rat strain, and gender differences are seen in urinary enzyme levels, urine cytology, and blood urea nitrogen level after exposure of $B6CF_1$ mice when HQ is given orally in the range of 200–400 mg/kg. Pharmacokinetic modeling predicted that consistent with the observed nephrotoxicity, male F344 rats form more glutathione conjugates than SD rats at equivalent dose levels, which provides a partial explanation for the greater degree of nephrotoxicity reported in the F334 rats and associated kidney adenomas. Comparative toxicity studies and modeling of oral and ip routes for HQ exposure also find that ip administration of HQ results in greater amounts of glutathione conjugates than comparable doses given orally, which is consistent with observed *in vivo* test results measuring HQ protein adducts [19,38].

The difference in genotoxicity between the ip and oral studies are a clear reflection of the toxicokinetic differences between the ip and oral routes of exposure. The questionable relevance of micronucleus studies of HQ conducted by ip administration is provided by O'Donoghue et al. [11] who showed that large oral doses (0.8 % in the diet for 6 days) can be fed to mice repeatedly resulting in a reduction in the background incidence of micronuclei in addition to protection against potassium bromate-induced micronuclei.

In conclusion, in two recent OECD guideline studies, HQ produced neither gene mutations nor DNA strand breaks when administered orally up to the MTD to mice and rats. These results add to existing data and provide further evidence that genotoxicity is not directly involved in the development of tumors in rodents following oral HQ exposure. The absence of DNA damage in the testes in the comet assay is consistent with developmental toxicity studies in which HQ was concluded to not be a reproductive or developmental toxicant. Existing *in vitro* data and *in vivo* studies administering HQ by intraperitoneal injection frequently report results consistent with aneugenicity. The negative TGR and comet assay results show that HQ is not causing direct DNA reactivity in experimentally induced target tissues, oral absorption sites, or the major site of HQ metabolism. If HQ were to be considered a probable aneugen without DNA reactivity after oral exposure, the mechanism of toxicity would be considered an effect with a threshold for safe exposure. While existing HQ genotoxicity studies by the oral route do not directly address aneugenicity through a technique that determines its interaction with the kinetochore, large oral doses (1152 mg/kg/day) can be given to rats without inducing micronuclei, a potential endpoint for aneugenicity. This suggests that if HQ were an aneugen following oral administration, the threshold for an aneugenic effect would be above the chronic dose level for nephrotoxicity (50 mg/kg/day).

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

John O'Donoghue is a consultant toxicologist who prepared this manuscript for the Hydroquinone Consortium.

Carol Beevers is a consultant genetic toxicologist, who assisted in the preparation of this manuscript and during her previous employment at Covance Laboratories Ltd, Harrogate, UK, was the study director responsible for the conduct of the GLP comet assay described in this manuscript.

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