Chapter 6: Paper 1

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Hereditary tyrosinemia type 1 metabolites impair DNA excision repair pathways

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ABSTRACT

Hereditary tyrosinemia type 1 is an autosomal recessive metabolic disorder, which is caused by a defective fumarylacetoacetate hydrolase enzyme, and consequently metabolites such as succinylacetone and *p*-hydroxyphenylpyruvate accumulate. We used a modified comet assay to determine the effect of these metabolites on base- and nucleotide excision repair pathways. Our results indicate that the metabolites affected the repair mechanisms differently, since the metabolites had a bigger detrimental effect on BER than on NER.

1. Introduction

Hereditary tyrosinemia (HT1) is an autosomal recessive metabolic disorder (OMIM 276700) caused by a defective fumarylacetoacetate hydrolase enzyme (E.C.3.7.1.2) in the tyrosine catabolism [1,2]. Intermediate metabolites such as fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and to a lesser extent *p*-hydroxyphenylpyruvate (pHPPA), *p*-hydroxyphenylacetate (pHPAA) and *p*-hydroxyphenyllactate (pHPLA) accumulate intracellularly. Characteristic of HT1 is hepatocarcinoma (HCC) and somatic mosaicism [1,3].

The molecular mechanism of HCC and somatic mosaicism is largely unknown [4]. The accumulating metabolites most probably create an internal chemical milieu instigating the molecular events underlying the HCC and somatic mosaicism. In addition to the observation that the HT1 metabolite succinylacetone (SA) inhibits DNA ligase activity [5] and the suggestion that the accumulating metabolites most likely act as alkylating agents and/or disrupt sulfhydryl metabolism [6,7], we found that the DNA repair activity in liver cells is impaired by pHPPA [8].

The mechanism and extent by which DNA repair is affected by these metabolites is still unclear. We have used a modified comet assay [9,10] to assess the effect of HT1 metabolites on the first steps of the base- and nucleotide excision repair mechanisms, BER and NER. The primary substrate for BER is oxidized and alkylated DNA bases [11–13] and for NER large DNA adducts, caused by exposure to UV-light and chemicals such as benzo[a]pyrene [12]. For use in the modified comet assay these lesions were prepared by treating HepG2 cells with hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS) and benzo[a]pyrene (B[a]P) respectively. Repair of these lesions was investigated with protein extracts (PEs) prepared from cultured HepG2 cells exposed to the HT1 metabolites, SA and pHPPA.

2. Materials and methods

All reagents were obtained from Sigma (Johannesburg, South Africa), unless otherwise indicted.

2.1. HepG2 cells

HepG2 cells were cultured in a CO_2 incubator at 37 °C and 5% CO_2 in growth medium (Dulbecco's Modified Eagles Medium; Thermo Scientific) supplemented with 10% foetal bovine serum (FBS) (BioWhittaker), 1× Penicillin/Streptomycin (100 U Pen./ml; 100 μg Strep./ml; BioWhittaker) and 1× MEM non-essential amino acids (0.1 mM; BioWhittaker). They were harvested as follows: the cells were washed with 1× phosphate buffered saline (PBS), incubated with 1% trypsin (BioWhittaker) for 5 min, followed by the addition of 2 ml growth medium. The cells were then counted using trypan blue by mixing 25 μl trypan blue, 15 μl PBS and 10 μl cell suspension. Of this mixture 10 μl was added to the counting chamber of a haematocytometer and nine squares were counted. The volume of the cell suspension was adjusted with growth medium to 2.5×10^4 cells per 50 μl. To allow recuperation

Abbreviations: HMPA, high melting point agarose; LMPA, low melting point agarose; PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI PE, heat inactivated protein extract; Stdev, standard deviation; ND, not done.

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of the cells after the harvesting procedure they were incubated at 37 °C and 300 rpm for 2 h.

2.2. Preparation of substrate DNA

Recuperated HepG2 cells were treated with H_2O_2 to serve as substrate for BER of oxidized DNA bases. Cells were divided into two aliquots of 2×10^5 cells in 400 μl cell growth medium. The one aliquot were left untreated to serve as control and the other was exposed to hydrogen peroxide at a final concentration of 80 μM. Both cell suspensions were incubated for 1 h at 37 °C and 300 rpm. Meanwhile, frosted microscope slides were coated with 300 μl 1% high melting point agarose gel (HMPA). The slides were placed on a cool surface to allow it to set and remain cool until the sample was added. Fifty microliters of the untreated, or hydrogen peroxide-treated cell suspension were added to 130 μl 0.5% low melting point agarose (LMPA) and mixed briefly by pipetting. The cell–LMPA mixture (180 μl) was placed on one half of the pre-coated (HMPA) frosted microscope slide, and evenly distributed, taking care not to damage the thin layer of HMPA. For each of the untreated and H_2O_2 exposed cells, six microscope slides were coated. The LMPA-coated slides were allowed to solidify and were then placed overnight at 4 °C in cold lyses buffer (5 M NaCl, 0.4 M EDTA, 1% Triton X-100, 10% DMSO).

In parallel, recuperated HepG2 cells were treated with MMS to serve as substrate for BER of alkylated DNA bases. Twelve frosted microscope slides, previously coated with 300 μl of 1% HMPA, were each coated with 2.5×10^4 cells. This was done by diluting the recuperated cells to 2.5×10^4 cells per 50 μl cell growth medium. Fifty microliters of this diluted cell suspension were added to 130 μl 0.5% LMPA and mixed briefly by pipetting the mixture. The cell mixture (180 μl) was then evenly distributed on one half of the pre-coated (HMPA) frosted microscope slide. Thereafter the microscope slides were submerged in lyses buffer and left overnight at 4 °C. After lyses, the slides were washed for 15 min in PBS and then submerged in 20 μM MMS for 30 min at 4 °C. The microscope slides were then immersed in buffer B (45 mM HEPES, 0.25 mM EDTA, 2% glycerol, 0.3 mg/ml BSA, pH 7.8) for 15 min. These microscope slides were directly treated with protein extract.

To serve as substrate for NER, cells were treated with benzo[a]-pyrene as described by Plazar et al. [14]. Briefly, HepG2 cells were seeded in a six-well plate at a density of 2×10^5 per well one day prior to benzo[a]pyrene treatment. Two wells were seeded with cells, one as vehicle control and the other to be exposed to benzo[a]pyrene. After 24 h the cells were exposed to a final concentration of 75 μM benzo[a]pyrene, dissolved in DMSO, and were incubated for 24 h at 37 °C and 5% CO_2. The cells were then harvested and counted. The cell suspensions were diluted to 2.5×10^4 cells per 50 μl growth medium of which 50 μl was added to 130 μl 0.5% LMPA and mixed briefly. The cell mixture (180 μl) was evenly distributed on the frosted microscope slide pre-coated with HMPA and six microscope slides were coated for each of the different cell suspensions. These were left to solidify and were then placed overnight at 4 °C in cold lyses buffer.

2.3. Preparation of protein extracts

Three different protein extracts were prepared. Control protein extract was prepared from HepG2 cells cultured in standard growth medium. HepG2 cells were separately treated with either 50 μM SA or 100 μM pHPPA in growth medium for 48 h at 37 °C and 5% CO_2 to prepare metabolite treated protein extracts. These cells were harvested, counted and divided into aliquots of 5×10^6 cells. The aliquots were either used directly for protein extraction or frozen (−80 °C) as pellets until used. The method to prepare the respective protein extracts were described by Collins et al. [9] and Langie et al. [10]. In brief, each aliquot of 5×10^6 cells were resuspended in 50 μl buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 7.8), and snap frozen in liquid nitrogen. The frozen cell pellets were then left at room temperature to thaw. Whilst the cells were thawing, a 1% Triton X-100 in buffer A solution was prepared and 15 μl of this mixture were added per 50 μl of the thawed cells. The cell suspension were briefly vortexed and left on ice for 10 min. The cell suspensions were then centrifuged at 14,000*g* at 4 °C for 5 min and the supernatant transferred to a clean tube. The protein concentration was determined with the bicinchoninic acid assay according to specifications by the manufacturer (Thermo Scientific) and was adjusted to 1 mg/ml with 0.23% Triton X-100 in buffer A. After dilution the protein extracts were aliquoted and frozen at −80 °C until use.

2.4. DNA repair assay

The microscope slides that were coated with substrate cells were removed from the lyses solution and washed for 15 min with PBS followed by a washing step with buffer B for 15 min at room temperature. A working protein extract mixture was prepared for each of the different protein extracts. The working protein extract mixture included: 50 μl protein extract, 200 μl buffer B and 10 μl of a 65 μM ATP solution. A 50 μl aliquot of the working protein extract was evenly spread on the frosted microscope slide coated with substrate DNA imbedded in agarose. The microscope slides were then incubated for 10 min at 37 °C in a humidified atmosphere, whereafter they were placed in electrophoresis buffer (0.6 M NaOH, 50 mM EDTA) for 30 min at 4 °C. Electrophoresis was then done at 4 °C for 40 min at 30 V (200 mA). Following this, the slides were submerged for 15 min in the neutralizing buffer (0.5 M Tris–HCl, pH 7.5). The nucleoids were then stained in a 12.7 μM ethidium bromide solution for 30 min. Slides were finally washed for 5 min in double distilled water. An Olympus 1 × 70 fluorescence microscope (200×) was used to assess the slides. The Comet IV® software was used to capture the images and to determine the percentage of DNA in the comet head and tail of a minimum of 35 randomly selected comets.

2.5. Statistical methods

Descriptive statistics were calculated and the data presented graphically using boxplots. Since the distributional assumptions of analysis of variance (ANOVA) are violated (Fig. 1), we used the two-way heteroscedastic rank-based test, Brunner–Dette–Munk (BDM) [15] to investigate whether the control cases behave differently to protein treatment when compared to the exposed group (i.e. the existence of interaction). In case of such an interaction, the various protein treatments were compared separately for the controls and the exposed groups by using the one-way non-parametric rank-based permutation test. For post-hoc analysis, all groups were compared to control protein treatment using the modified method of Dunn [16] as described in [17].

Note that the Kruskal–Wallis non-parametric test assumes that groups have the same distributional shape, therefore all group variances should be similar. This is not true for the current study (Fig. 1). As an alternative, we used BDM [15], which is a heteroscedastic version of the Kruskal–Wallis that is robust against non-normality and heteroscedasticity. All statistical analyses were performed using R and SPSS.

3. Results

The effect of the different accumulating HT1 metabolites on the proteins involved in the initial steps of BER and NER was determined by exposing HepG2 cells to either 50 μM SA or 100 μM

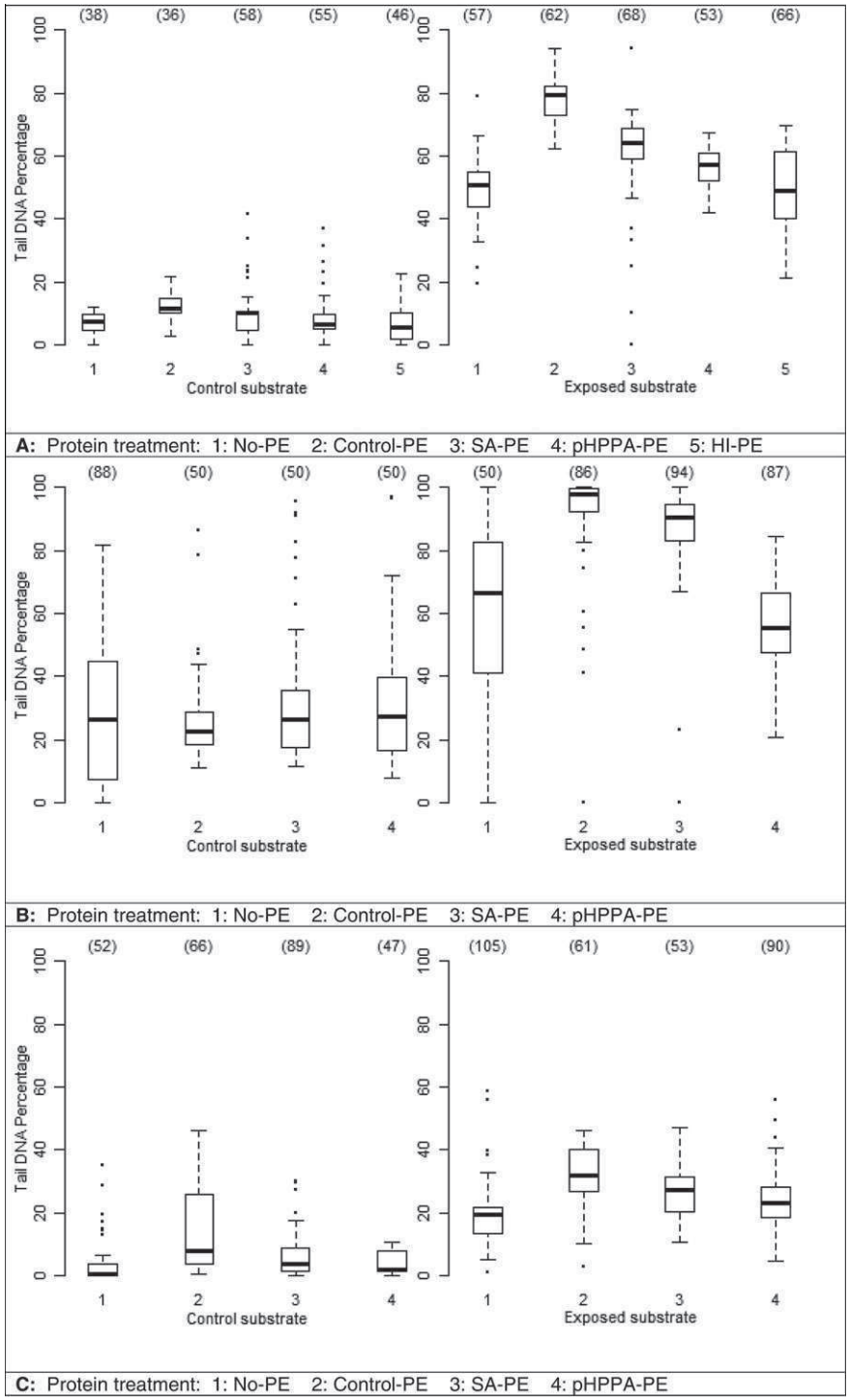

Fig. 1. Graphical representation of tail DNA percentages after different protein treatments. (A) BER (H_2O_2). (B) BER (MMS). (C) NER (B[a]P). The number of comets that were scored for each sample is given in brackets. PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI PE, heat inactivated protein extract.

pHPPA for 48 h. Protein extracts from these and untreated HepG2 cells were then applied to the appropriate agarose imbedded substrate DNA nucleoids, and the tail DNA percentages were determined. The extent of the increase in the percentage tail DNA measures the effectiveness of the proteins involved in the initial steps of BER and NER in their ability to recognize and excise the induced DNA lesions [9,10,18]. Control protein extract was assumed to have optimal repair capacity.

The tail DNA percentages for the three experiments are presented on the various factor levels, i.e. control vs. exposed groups and the different protein treatment levels, in Fig. 1 and Table 1.

In all the experiments, the control groups have lower tail DNA percentages (TDP) on expectation. For the BER experiments, the control groups reacted differently to the treatment when compared to the exposed groups. This interaction was statistically significant at a 5% level. No statistical interaction was observed for the

Table 1
Mean and median tail DNA percentages after treatment of nucleoids with protein extract. Standard deviations of tail DNA percentages are given in brackets. BER, base excision repair; NER, nucleotide excision repair; H_2O_2, hydrogen peroxide; MMS, methyl methanesulfonate; B[a]P, benzo[a]pyrene; PE, protein extract; CPE, control protein extract; SA-PE, succinylacetone exposed protein extract; pHPPA-PE, pHPPA exposed protein extract; HI PE, heat inactivated protein extract; Stdev, standard deviation; ND, not done.

		BER (H_2O_2)		BER (MMS)		NER (B[a]P)	
		Control substrate	Exposed substrate	Control substrate	Exposed substrate	Control substrate	Exposed substrate
−PE	Median	7.32	50.72	26.21	66.37	0.29	19.17
	Mean	6.85	50.10	26.85	60.95	3.90	19.10
	Stdev	(3.40)	(9.87)	(21.76)	(29.84)	(7.50)	(8.40)
+CPE	Median	11.76	79.20	22.60	97.90	7.75	32.03
	Mean	12.34	77.89	26.29	88.55	13.43	31.51
	Stdev	(3.90)	(7.07)	(14.72)	(24.65)	(13.74)	(9.39)
+SA-PE	Median	10.01	64.04	26.41	90.36	3.49	27.12
	Mean	9.77	61.33	32.70	87.12	5.76	26.41
	Stdev	(7.68)	(14.09)	(22.36)	(13.74)	(6.27)	(8.75)
+pHPPA-PE	Median	6.69	57.29	27.09	55.60	2.04	23.03
	Mean	8.59	56.14	32.94	56.65	4.09	24.20
	Stdev	(7.15)	(6.57)	(22.34)	(14.10)	(3.54)	(8.75)
+HI PE	Median	5.73	49.03	ND	ND	ND	ND
	Mean	6.78	49.67				
	Stdev	(5.37)	(12.00)				

NER (B[a]P) experiment using the two-way rank-based permutation test.

Subsequently, the effect of protein treatment was separately investigated for the controls and exposed groups. For all the experiments, except for the control group of the BER(MMS) experiment, a statistical significant protein treatment effect was observed (one-way BDM).

For the BER(H_2O_2) experiment we used heat inactivated protein extract, to asses whether the increase in TDP after the addition of PE is truly due to the addition of PE. The TDP for untreated exposed substrate nucleoids and heat inactivated PE treated substrate nucleoids should therefore be similar. The Dunn [16] post-hoc test comparing all the possible group comparisons was performed, which revealed that the TDP of untreated exposed substrate nucleoids and heat inactivated PE treated substrate nucleoids does not differ significantly. It is therefore clear that the addition of PE to H_2O_2-substrate nucleoids caused a change in the TDP, which is an indication of the ability of the proteins involved to recognize and excise the oxidized DNA lesions induced by H_2O_2.

Non-parametric post-hoc analysis comparing the various protein treatments to the control protein treatment (i.e. protein extract with optimal repair) revealed that all treatments were significantly different from the control protein treatment.

In specific, there was a significant increase in the TDP of H_2O_2 exposed nucleoids after treatment with control-PE, SA-PE and pHPPA-PE. The post-hoc analysis also indicated a significant decrease in the TDP after the treatment of H_2O_2 exposed nucleoids with protein extracts that were exposed to SA or pHPPA when compared to treatment with control-PE.

Also, a significant increase was measured between the TDP of MMS-substrate nucleoids not treated with PE and the control-PE treated MMS-substrate nucleoids (Fig. 1 and Table 1). Compared to the TDP of the MMS-substrate nucleoids treated with control-PE, the TDP of MMS-substrate nucleoids treated with SA-PE or pHPPA-PE decreased significantly. Furthermore, the TDP of MMS-substrate nucleoids not treated with PE and the nucleoids treated with pHPPA-PE did not differ significantly [16].

A significant increase was measured in the TDP of B[a]P exposed nucleoids after treatment with control-PE. On the other hand, a significant decrease was measured in the TDP of SA-PE and pHPPA-PE treated B[a]P exposed nucleoids, when compared to treatment with control-PE.

4. Discussion

The preservation of genomic integrity is particularly important for organism survival [11,13]. Different DNA repair mechanisms exist for this purpose, including direct reversal, mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination repair (HRR) and non-homologous end-joining (NHEJ) [12,13]. Of these repair mechanisms, BER and NER play an integral role in repairing single strand lesions. The fact that excision repair pathways operate in very similar ways but differ quite distinctively in the points of onset [11] allows investigation of the effect of genotoxic agents, such as HT1 metabolites, on both BER and NER in cultured cells.

The primary substrate of the BER pathway is oxidized and alkylated DNA bases [11–13]. In this study, hydrogen peroxide was used to induce DNA oxidation, which entails the formation of 8-oxo-G [19]. The very first step in BER is the hydrolysis of the *N*-glycosidic bond between the two linked bases (8-oxo-G paired with C) in order to remove the damaged base [13]. Human 8-oxo-guanine glycosylase 1 (hOGG1) is a type II glycosylase enzyme that specifically catalyses the removal of 8-oxo-guanine bases and cleaves the AP site to form a single strand break [20].

The decrease in TDP of H_2O_2-substrate nucleoids treated with SA-PE and pHPPA-PE compared to control-PE treated substrate nucleoids suggests that the base excision repair pathway were impaired by exposure of HepG2 cells to SA and even more so after exposure to pHPPA. It is therefore likely that these metabolites affect the capacity of hOGG1 to excise the 8-oxo-G DNA lesions. However, 8-oxo-G is not exclusively removed by hOGG1. There is a back-up mechanism that appears to require the *CSB* gene product, but whether this gene product is necessary for direct participation in the BER pathway or for the expression of DNA glycosylases, is unclear [21]. The very low level of initiation of the removal of 8-oxo-G by the pHPPA exposed protein extract, suggests that exposure to pHPPA might also affect this back-up pathway. The precise nature of these perturbing effects of SA and pHPPA, however, still needs to be clarified.

MMS is an extensively used [22,23] SN2-type DNA alkylating agent that methylates the ring nitrogens of purines, to generate 7-methylguanine and 3-methyladenine, which causes base mispairing and replication blocks [21,23]. MPG or *N*′-methylpurine DNA glycosylase is a type I DNA glycosylase, involved in the repair

of these alkylated DNA bases [13,21], by removing the modified base thus creating an AP site. The incision of the phosphodiester bond of the AP site is done by AP endonuclease (APE1), which interacts with and is stimulated by XRCC1 [13].

Our results showed that exposure of HepG2 cells to SA and pHPPA caused a decrease in the efficiency of PE from these cells to recognize and incise DNA lesions when compared to PE from unexposed HepG2 cells. The inter-dependant nature of the BER initiating proteins (MPG, APE1 and XRCC1), however, makes it difficult to conclude which of these proteins are the most adversely affected by SA and pHPPA.

NER primarily acts to repair large DNA adducts caused by exposure to chemicals such as benzo[a]pyrene (B[a]P) [12]. B[a]P is a polycyclic aromatic hydrocarbon that causes DNA damage by distorting the DNA structure when binding to it [10]. It needs to be bioactivated to form benzopyrene diol epoxide (BPDE), which is carcinogenic. B[a]P can be used to induce specific damage to the DNA in order to investigate transcription coupled NER (TC-NER). Langie and colleagues demonstrated a significant correlation between the removal of BPDE-adducts and the DNA repair capacity of cells, and suggested that increased tail moments and percentage fluorescence in the comet tail are indicative of the NER capacity of the protein extracts [10]. The modified comet assay therefore measures the effect on ERCC1 (excision repair cross complimenting) and associated proteins involved in specifically the initiating steps of NER. ERCC1 acts in an XPF-ERCC1 complex to excise the lesion and perform the 5′-incision, the 3′-incision is performed by XPG [13].

Our results have shown that the repair ability of protein extracts, prepared from cells exposed to either SA or pHPPA, was reduced. SA and pHPPA therefore affects the initiating proteins of NER, but do not debilitate them. Similar to the repair of alkylating DNA damage, the NER initiating proteins work in an integrated fashion, thereby making it difficult to distinguish which of the proteins are affected. It remains to be seen if exposure to SA and/or pHPPA has an effect on the expression level of these proteins or if it is a structural interaction impairing its function.

5. Conclusion

In conclusion, we have shown that some of the metabolites associated with HT1, namely SA and pHPPA have an impairing effect on both BER and NER. Our results indicated that exposure to these metabolites affected the repair mechanisms differently, since the metabolites had a bigger detrimental effect on the initiating proteins of BER than on those of NER. The harmful effect of the metabolites might therefore contribute to the development of HCC in HT1 patients. We furthermore observed that exposure to SA did not have as big an impact on repair proteins than exposure to pHPPA. This observation also confirms our previous suggestion that exposure to pHPPA has an effect on DNA repair [8]. Although, further investigation is still needed to define the mechanism by which SA and pHPPA affect the proteins initiating DNA repair, our results nonetheless sets the base for investigation into the effect of the HT1 accumulating metabolites, SA and pHPPA, on DNA repair.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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