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EFFECTS OF THE HETEROCYCLIC AMINE 2-AMINO-3,8-DIMETHYLIMIDAZO(4,5-F)QUINOXALINE (MEIQX) ON HUMAN LYMPHOCYTE DNA IN DIABETIC AND NON-DIABETIC POPULATION SUB-GROUPS: AN ASSESSMENT USING THE COMET ASSAY.

Susceptibility In Human Lymphocytes To Meiqx

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Abstract
Aims. 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) is one of the most abundant heterocyclic amines (HAs) in cooked food, and has been subjected to numerous toxicological procedures. Our aim was to assess the extent of DNA damage induced by MeIQx in lymphocytes in the alkaline Comet assay, and to compare various population sub-groups.
Materials and methods. The alkaline Comet assay was used to investigate lymphocytes from 63 individuals (diabetic and non-diabetic, different ethnicities, sexes, ages, diets, etc) in vitro.
Results. No statistically significant differences in susceptibility to DNA damage were observed between subject covariates using linear regression (multivariate analysis) techniques. However, using non-parametric data analysis, statistically significant differences were seen between groups above and below 45 years of age.
Conclusions. MeIQx produced highly statistically significant, yet relatively low levels of alkali labile sites, and single strand breaks in DNA in lymphocytes. There were no differences in susceptibility in population sub-groups to this dietary compound.

Keywords: Heterocyclic amines: 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline, MeIQx, Comet Assay, susceptibility.

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Rezumat
Scop. 2-amino-3,8dimetilimidazo (4,5-f) quinoxalină (MeIQx) este una dintre cele mai abundente amine heterociclice (HAs) din alimentele preparate şi a fost incriminată în numeroase investigaţii toxicologice. Scopul studiului de faţă a fost de a determina prin metoda alcalină Comet gradul de perturbare a ADN-ului din limfocite, perturbare indusă de MeIQx şi de a compara diferite subgrupe populaţionale.
Material şi metodă. S-a folosit metoda alcalină Comet pentru a investiga *in vitro* limfocitele de la 63 de indivizi (diabetici şi nediabetici, de diferite etnii, sexe, vârste, cu diferite diete, etc.)
Rezultate. Nu s-au observat diferenţe statistic semnificative de susceptibilitate a ADN-ului între subiecţi folosind tehnica regresiei liniare (analiza multivariată). Totuşi, folosind analiza datelor neparametrice, s-au observat diferenţe statistic semnificative între grupele de vârstă de peste şi sub 45 de ani.
Concluzii. MeIQx a determinat în limfocite un nivel foarte semnificativ statistic mai redus de locuri labile alcalin şi rupturi la o singură margine a ADN-ului. Nu au existat diferenţe de susceptibilitate la acest produs din dietă între subgrupele populaţionale.

Cuvinte cheie: amine heterociclice: 2-amino-3,8dimetilimidazo (4,5-f) quinoxalină (MeIQx), metoda Comet, susceptibilitate

INTRODUCTION

When protein rich foods are cooked, HAs are generated. One of the most abundant HAs in cooked food is 2-amino-3.8-dimethylimidazo (4,5-f)quinoxaline (MeIQx), and it is one of the main five HAs consumed within the Western diet (1). MeIQx is activated by N-oxidation to carcinogenic intermediates by cytochrome P4501A2 (CYP1A2) in the liver, and CYP1A1 and CYP1B1 in extrahepatic tissues (1). The endpoint of the reactive nitrenium ion binding to DNA occurs when

N-hydroxylamine metabolites are further activated by enzymatic esterification (1), but few toxicological data are available on the effect on DNA in humans (2). It has been possible to study the effect of macromolecular formation of

DNA adducts in both rodents and humans (2). MeIQx and other HAs are bioactivated at a low dose, and it is able to form both blood protein and colon DNA adducts, and this helps to explain how the compound results in tissue damage (2). However, human tissue cytosols and microsomal extracts have greater capacity to N-hydroxylate heterocyclic amines than rodents (2). MeIQx produced statistically significant DNA damage in the colons of male CD-1 mice up to 8 hours after injection, and also induced DNA damage in the stomach (3). This provides some evidence for the involvement of MeIQx in the

development of colon cancer (4), and possibly stomach cancer, although further work on the development of DNA adducts will assist in confirming links between MeIQx (and other HAs) and cancer. The numbers of DNA adducts obtained from both normal and tumour human tissue are higher than the level of adducts obtained from rats 48 hours after administration (5); approximately 4-5 times more adducts were formed in human colons in comparison with rats after administration of a human-equivalent dose (5). The derivatives of MeIQx will preferentially form DNA adducts with guanine bases, usually at the C-8 position (6, 7), allowing repair, misrepair or replication errors to arise during the mitotic cycle, resulting in mutations. MeIQx has also shown genotoxic effects in human liver slices, and its effect on unscheduled DNA synthesis (UDS) was considerably greater than for MeIQ, which is chemically similar (8).

A proportion of MeIQx is excreted via the urinary system, and it has also been reported (5) that more MeIQx is excreted in the urine of humans (range 20.2-58.6%) than it is in rats (10.9-11.8%). In comparison, another study (9) administered a regular meal (containing 1030ng MeIQx) of burger and salad to male volunteers for 2 days. No MeIQx was observed in the pre-meal urine, but the amount of non-metabolised MeIQx in 0-24 hr post-exposure urine corresponded to 1-6% of the original dose, confirming that an amount of HA does pass through the body unchanged (9). Large inter-individual variation in excretion rates was observed.

The present study has used human lymphocytes obtained from 63 diabetic and non-diabetic individuals, to assess the extent of DNA damage obtained by MeIQx in the alkaline Comet assay, and also to compare various sub-population groups, taking into account lifestyle and endogenous factors. This is to determine if there are differences in susceptibility in response to this dietary compound.

MATERIALS AND METHODS

Materials

The chemicals used in these experiments were purchased from the following suppliers: low (5517UB) and normal melting point agarose (15510-027) from GibcoBRL, Paisley, UK. Dimethyl sulfoxide (CAS-67-68-5), ethidium bromide (EtBr) (CAS-1239-45-8), hydrogen peroxide (CAS-7722-84-1), ethylenediaminetetraacetic acid (CAS-6381-92-6), Triton X-100 (CAS-9002-93-1) and 0.4% Trypan Blue (CAS-72-57-1) from Sigma Chemicals Co. Ltd., Poole, Dorset, UK. Ethanol (CAS-64-17-5), methanol (CAS-67-56-1); sodium hydroxide (CAS-1310-732) from BDHMerck, Poole, Dorset, UK. Lymphoprep from Axis shield, Oslo, Norway; foetal calf serum from SeraQ, Sussex, UK. 1x PBS tablets

from Oxoid, Hampshire, UK. 2-amino-3.8-dimethylimidazo(4,5-f)quinoxaline (CAS-77500-04-0) from TRC, Toronto, Canada.

Blood sampling
Venous blood samples were taken from healthy volunteers and from diabetic patients attending clinics at the department of Diabetes and Endocrinology, Bradford Royal Infirmary (UK). Samples were taken from subjects in the post-prandial (non-fasting) state: preliminary analysis indicated that this did not have any confounding effect on data obtained. Informed consent was obtained from all subjects and the study was approved by the local NHS Research Ethics Committee and the Sub-committee for Human Subjects at the University of Bradford. Lymphocytes were isolated using standard separation medium (Lymphoprep©) and immediately frozen down in FCS (10% DMSO) and transferred to long-term liquid nitrogen storage. All subjects were asked to complete a detailed questionnaire in order to identify variables of interest and to address potential confounding factors. Demographic, health and lifestyle factors were included.

The Comet assay
The alkaline Comet assay was used as previously described (10-12). Lymphocytes were quickly thawed and suspended in PBS, with or without MeIQx. Cells were treated with MeIQx at 37ºc for 30 minutes. Lysing time (was between 1 and 3 hours. Unwinding was for 30 minutes, with electrophoresis for 30 mins (25v, ~300mA). Coded slides were stained with EtBr post-neutralisation, and viewed (20x objective, 10x lens) using a fluorescent microscope (Leica, Weztler, Germany) equipped with appropriate filters. A charge coupled device (CCD) camera connected the microscope to an image analysis system (Kinetic Imaging, Liverpool, UK) incorporating the analysis software, Komet 4. The parameter for analysis was tail moment (tail length multiplied by intensity). At least 50 cells were scored from replicate slides, per dose, per subject. The dose range was determined when the viability of lymphocytes at all doses was above 90%, using Trypan blue exclusion (13).

Statistical analysis Two different statistical methods were used: firstly, the joint effect of individual covariates on the overall Comet scores (scored at 0, 20, 40, 60, 80 and 100 μM of MeIQx) was evaluated by multiple linear regression analysis of log-transformed data while accounting for within-subject correlation (multivariate analysis). To this purpose, the generalised estimating

equation (GEE) methodology was applied (Stata v.7, US). Secondly, the tail moment data violated the normality assumption, so non-parametrical data analysis (Mann Whitney) was also undertaken (SPSS v.11, US) to test between group (i.e., individual covariate) differences.

RESULTS

Using multivariate analysis, Table 1 shows group numbers, standard measurements (mean, standard deviation [SD], median and interquartile range [IQR]) of the between-subject distribution of Comet scores (mean tail moment) expressed as a mean calculated overall within-subject MeIQx levels. There was a statistically significantly estimated mean increase in DNA damage with MeIQx ($p<0.001$). Table I also details the joint effects of the single covariates considered in the study on the Comet score, in which no statistically significant differences were observed between the population co-variates studied (sex, race, age, diabetic/non-diabetic, smoking, alcohol and diet) except for age which was of borderline significance ($p=0.54$).

With non-parametrical data analysis, statistically significant differences were clearly seen with age (Fig. 1), with those older than 46 (n=32) by comparison with those less than 45 (n=31), showing consistently more DNA damage at the 20μM and 80μM dose ($p<0.001$***), 60μM dose ($p<0.01$**) and also at the 40μM and 100μM ($p<0.05$*) dose of MeIQx .

DISCUSSION

The increasing use of in-vitro genotoxicity tests such as the Comet assay allows assessment of DNA damage by agents such as MeIQx, which in animals produces a dissimilar response to humans and their tissues (5, 9).

The use of GEE linear regression has not shown any statistically significant differences between the population covariates when the tail moment values from all treatment levels, including the negative control, were combined in order to produce one overall value per subject group.

It was concluded (14) that there were significantly increased levels of DNA damage in males, but this current study has not observed any significant differences between the sexes, or between subjects' ethnicity. Differences observed between age (Figure I) support previous work (15, 16), showing DNA damage and lymphocyte mutations increase with age. Aging is accompanied by an increased production of free radicals (15), and greater DNA damage is seen in old subjects in comparison to young (17).The GEE analysis of differences

Factors & levels	No subjects (%)	Mea n	SD	Medi an	IQR	Predictors & contrasts	**p**	95% CL	P-value
Whole sample	63	7.62	3.22	7.16	5.27 – 9.63	Constant	1.033[(1)]	0.706 – 1.360	< 0.001
						MeIQx linear trend levels	0.012[(2)]	0.011 – 0.014	< 0.001
Sex									
- Male	33 (52.4)	7.69	3.42	6.86	5.48 – 9.80	Female vs Male	0.012	-0.186 – 0.210	0.905
- Female	30 (47.6)	7.54	3.03	7.22	5.01 – 9.09				
Race									
- Caucasian	33 (52.4)	7.91	3.42	7.26	5.52 – 9.63	Asians (and others) vs Caucasians	0.186	-0.077 – 0.448	0.166
- Asians (and others)	30 (47.6)	7.31	3.01	6.37	4.90 – 9.50				
Age at blood sample									
- ≤ 45 yrs	31 (49.2)	6.66	3.01	5.89	4.58 – 7.92	> 45 yrs vs ≤ 45 yrs	0.241	-0.004 – 0.486	0.054
- > 45 yrs	32 (50.8)	8.55	3.18	8.65	6.13 – 10.10				
Diabetic condition									
- Non-diabetic	27 (42.9)	7.35	3.19	6.11	5.27 – 9.63				
- Type 1 diabetes	15 (23.8)	7.49	2.95	7.24	4.92 – 10.24	Type 1 vs non-diabetic	-0.030	-0.297 – 0.237	0.827
- Type 2 diabetes	21 (33.3)	8.07	3.53	7.19	5.83 – 9.50	Type 2 vs non-diabetic	-0.005	-0.272 – 0.262	0.972
Smoking habit									
- Never/former	54 (85.7)	7.78	3.34	7.17	5.27 – 10.09	Current vs never/former	-0.169	-0.460 – 0.122	0.255
- Current	9 (14.3)	6.67	2.27	5.99	5.48 – 8.78				
Alcohol drinking									
- Never/former	34 (54.0)	7.16	3.10	6.03	4.90 – 9.50	Current vs never/former	0.045	-0.201 – 0.290	0.722
- Current	29 (66.0)	8.16	3.32	7.26	5.66 – 9.63				
Diet type									
- Western-type	42 (66.7)	8.13	3.08	8.19	5.66 – 9.80	Asian-like vs western-type	-0.222	-0.505 – 0.060	0.123
- Asian-type	21 (33.3)	6.60	3.32	5.60	4.42 – 7.19				

SD: standard deviation; IQR: inter-quartile range

β: regression coefficient point estimate; 95% CL: 95% confidence limits for β. Notes – (1) estimated expected log-transformed comet score value when all predictors are set to the lowest level. (2) Estimated expected variation in log-transformed comet score per unit increase in MeIQx

Table I - The study population distribution by individual covariates and the corresponding Comet scores expressed as mean and median values and their

index of variation, and the GEE level regression analysis of Comet assay results on MeIQx levels and between subject covariates.

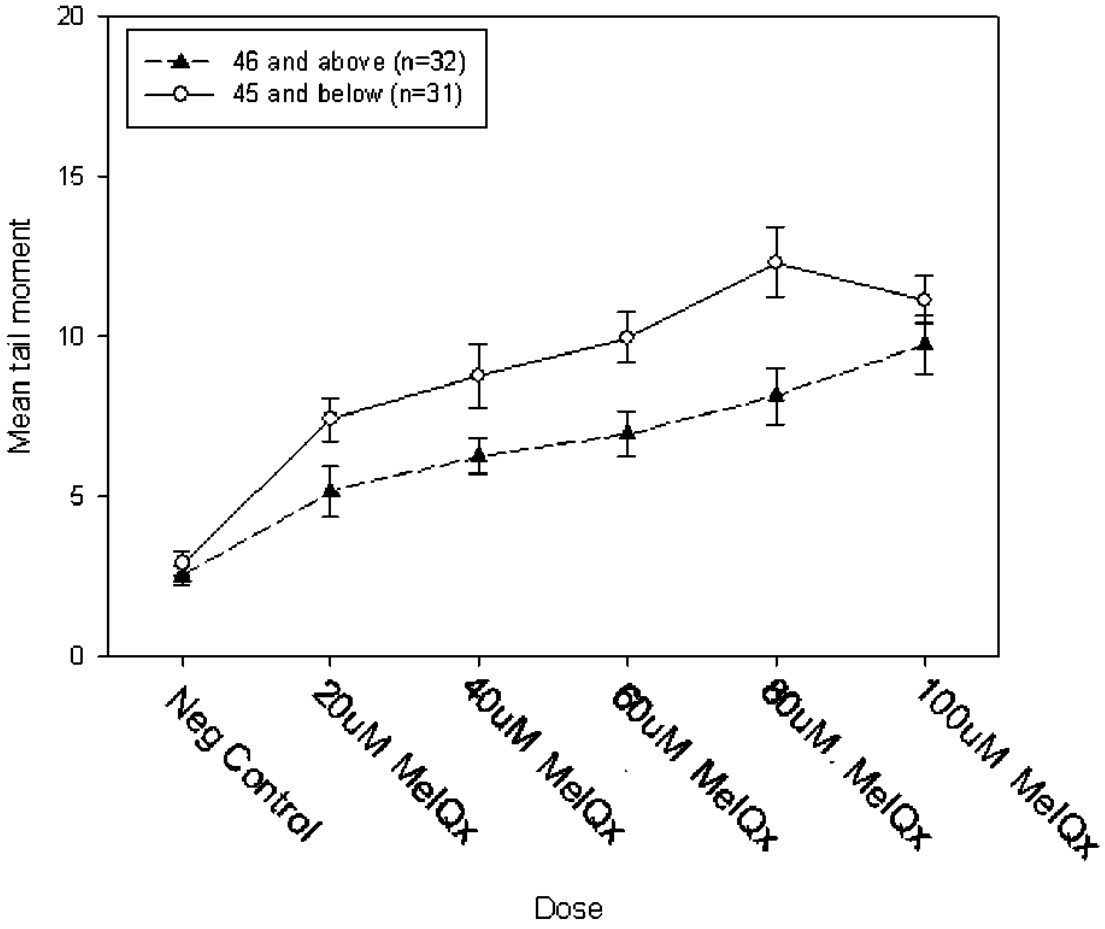

Fig. 1 - Differences in DNA damage induced in vitro in peripheral lymphocytes in those aged 45 and below (n=31), and those aged 46 and above (n=32). Significantly more DNA damage was observed in those aged over

in the present study in susceptibility between age showed that p=0.054, which although statistically non-significant, is worthy of noting, because of the dilution/loss of statistical power in this type of analysis. No differences were observed in DNA damage between diabetics and non-diabetics, which does not support previous studies (11, 18). A higher mean tail moment seen in those who drink alcohol supports previous findings (19), which showed that those who consume alcohol frequently (alcoholic) have higher levels of chromosome aberrations and micronuclei induction compared with abstainees and controls (19). Within an observed (20) control group, the difference between smokers and non-smokers was significant, and approximately the same level of difference was shown between diabetic smokers, who displayed a greater standard deviation. It was later shown (21) that smokers carry significant DNA damage in lymphocytes, and there are differences in repair capabilities, although individual variability of DNA repair makes it difficult to firmly conclude that smokers have a reduced DNA repair capacity. However previous research (22) reported a lack of difference between smokers and non-smokers, although it was suggested (no evidence provided) by Fenech (23) that the Comet assay was not suitable for detecting DNA damage arising from smoking. Cell factors such as signalling may contribute to endpoints observed; these are not always dependent upon smoking (23). No significant damage was observed between non-smokers and occasional smokers in this current study. No statistically significant differences in diet-type were seen, although overall the mean tail moment value for the Western-type of diet was higher, compared with those categorised as Asian-type, but the excretion of heterocyclic amines in vivo may be influenced by a number of different factors, including the quantity of cruciferous vegetables consumed (24). Although MeIQx has shown extensive damage in animal tissues (3), and human liver slices (8), the level of damage observed in the current study suggests that the genotoxic activity of the compound produces relatively low levels of alkali labile sites (ALS), or single strand breaks (SSB's) in DNA, the damage that the alkaline Comet assay detects. The human lymphocytes examined whilst having some metabolic activity, as shown in previous studies in this laboratory for the related heterocyclic amine, 2-amino - 3 - methylimidazo - (4, 5 f) quinoline (IQ) (25) do not appear to have activity levels as great as in liver cells (8). Overall, in vitro

treatment of human lymphocytes with MeIQx in the Comet assay was not able to differentiate differences in susceptibilities to this food mutagen in different population subgroups.

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