

ORIGINAL ARTICLE

Assessment of urinary mutagens presence in a population of non smokers

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Key words

Ames test • Urinary mutagenicity • Lifestyle behaviours

Summary

The paper presents the early results of a study involving a group of 312 non smoking and not professionally exposed subjects (144 males and 168 females) in order to evaluate the probable presence of urinary mutagens possibly derived from aspecific exposures. Urine samples were assayed by the Ames test on the YG1024 Salmonella typhimurium strain in the presence of S9 mix with plate incorporation method with preincubation. At the moment of sample collection, the subjects were invited to fill a questionnaire on their main characteristics and lifestyle.

On the basis of laboratory data analysis, it emerged that, on 288 samples with a valuable mutagenic activity, 20 urinary extracts (8 of which were males and 12 were females) showed mutagenicity levels twice as much as spontaneous revertants. Diet and indoor exposure to passive smoking, fireplace and cooking fume exposure seemed to play a major role among the lifestyle behaviours investigated in generating positive

mutagenic response with a statistically significant difference between positive and negative samples induction (Chi square, $P = 0.0057$ and $P = 0.0168$ respectively). After correction of induced revertants by means of creatinine excretion determination, it appeared that females, who had the higher mean urinary mutagenic activity, showed a mutagenicity level twice as much as men (364 ± 491 revertants/mmol creatinine for males against 605 ± 868 revertants/mmol creatinine in females, Mann-Whitney U-test, $z = -3.97$, $P < 0.0001$) possibly in consequence of their greater cooking fumes exposure.

The study, that carefully evaluated the characteristics of involved subjects, reveals the presence, even though modest, of mutagens in urine of an apparently not significantly exposed population. In addition, standardization of method leads to suppose little feasible a confounding influence of considered features. Moreover, it would be therefore rather interesting to study the effect of low exposure time persistence.

Introduction

The Ames *Salmonella*/microsome mutagenicity assay (Ames test) is a widely accepted short-term bacterial assay for identifying substances producing genetic damage and likely to be genotoxic carcinogens. The test employs several histidine dependent *Salmonella* strains, each carrying a different mutation in one of various genes of histidine operon. The *Salmonella* strains are grown on a minimal media agar plate containing traces of histidine: only those bacteria that revert to histidine independence (*his*⁺) are able to form colonies. The number of spontaneous revertant colonies per plate is quite stable. When bacteria come together with urinary extracts containing a mutagen, the number of revertant induced colonies per plate is increased, usually in a dose-associated manner. The test had been widely used to monitor damage induced by specific genotoxic agents (present in occupational activity, tobacco-smoke, food, etc.) that are able to develop metabolites eliminated with urine.

In all our previous studies [1-5], conducted with the plate test with preincubation test method on YG1024 *Salmonella typhimurium* strain, we could confirm that strong sources of exposures reveal a clear mutagenic

response in urine samples. In particular in the paper on occupationally exposed coke oven workers [6], we demonstrated the presence of mutagenicity (in some cases as apparent mutagenic response), even in a population, included as control group, lacking of evident exposure. This result was considered as expression of environmental mutagens other than traditional risk factors and it persuaded to look for a confirmation of the hypothesis. The aim of the present study is therefore to evaluate the presence of urinary mutagens level on a population of healthy non smoker subjects, without a known mutagenic exposure, with the Ames test on YG1024 *Salmonella typhimurium* strain in presence of S9 mix, in order to identify environmental risk conditions and what possible factors could mainly influence them.

For that reason, considering a very low and uncertain exposure, features affecting assay outcome, already named by Gatehouse [7], were considered with attention and in great part reduced. First of all, YG1024 *Salmonella typhimurium* strain had been chosen because it has been demonstrated more sensitive in revealing the presence of Polycyclic Aromatic Hydrocarbons (PAHs), aromatic and heterocyclic amines derived mutagenicity than the previous TA98 from which it is derived [8]. It has been

successfully applied in the investigation of the presence of PAH urinary mutagenic metabolites that may be found in patients topically treated with coal tar [9] or occupationally exposed subjects like coke oven workers [6], but also to mutagens originated from diet [1] and smoke [2-5]. In all of these studies, we were able to show a sound urinary mutagenicity and, when comparing a population of exposed workers with controls, we could also found a good statistically significant difference between the two groups [6]. In addition to the employ of a *Salmonella* strain that eliminate the potential mistake of applying low sensitivity bacteria, we standardized:

- protein concentration in S9 microsomal fraction;
- dimethylsulfoxide (DMSO) purity;
- bacterial concentration in overnight cultures checked for each assays;
- histidine and biotin content in culture medium and top agar.

Materials and methods

SUBJECTS

Individuals (n = 312) recruited for the study (in the period October 2002-June 2005) were all non smokers: 144 males and 168 females (mean age 42; range 20-65). At the moment of urine collection, participants, after giving written informed consent, were asked to complete a questionnaire concerning their main data and their behaviour (diet, passive smoking, drug intake, etc.) in the previous 24 hours. They were municipal workers referring to Preventive Medicine Service of Padua University for periodic check-ups. The study was approved by Ethics Committee of the School of Medicine, University of Padua.

SAMPLE COLLECTION

Urine samples (at least 150 ml) were collected in polyethylene containers in the morning on wakening and stored in the dark at -20°C until analysis.

REAGENTS

In order to standardize the method we utilized dimethylsulfoxide of high grade purity (99.9% from Merck); we controlled amino acids content in culture medium and top agar carefully dispensing the media amount in the plate; we employed S9 microsomal fraction from a single batch to maintain protein concentration constant. Additionally, we regularly checked bacterial concentration in overnight cultures for each assays.

URINARY MUTAGENICITY

After being thawed and filtered, urine samples were concentrated in glass columns (1.5 X 10 cm) packed with washed XAD-2 resin (4 g/100 ml urine) [10] and eluted with 20 ml of acetone. Extracts were dried under a nitrogen stream, resuspended in DMSO (250 ml urine/ml) and placed in the dark at -20°C. Sample mutagenic activity was determined using the plate incorporation preincubation technique on the YG1024 *Salmonella*

typhimurium strain in the presence of Aroclor 1254-induced rat liver S9 (500 µl/plate) (Moltox, Boone, NC, USA) [11]. For all assays overnight bacterial culture concentration was determined. Mutagenic activity was expressed as positive sample, when at least one of the tested doses was able to double the number of revertants with respect to spontaneous ones, and as the slope of the linear portion of the dose-response curve calculated by the linear regression method from at least two urine extract doses different from zero, expressed as number of revertants/ml urine. Urinary mutagenicity was adjusted by urinary creatinine, determined spectrophotometrically at 520 nm using a commercial kit (Boehringer, Mannheim, Germany) based on the reaction of creatinine with picrate in alkaline medium, allowing to express it as number of revertants/mmol of creatinine.

Comparisons of urinary mutagenicity levels were carried out using the Mann-Whitney U-test and were performed with Stat View Statistical software Version 5.0.1 (SAS Institute Inc., USA).

RESULTS

Mean overnight bacterial culture concentration was $3.03 \pm 1.42 \times 10^9$ microorganisms/ml. The mean \pm SD of spontaneous revertants in all experiments (n= 64) was 81.8 ± 10.5 . 2-aminofluorene (0.2 µg/plate) was used as a positive control (599.5 ± 114).

The study population was initially composed by 312 non smoking healthy subjects but, as 7.7% of examined urinary samples (n = 24) exhibited a toxic action that prevents bacterial growth in all tested doses, we could verify the urinary mutagenic activity of remaining 288 volunteers. As reported in Table I, females were 154 (53.5%) and males were 134 (46.5%). The mean age was 43 ± 9 years old (range 20 - 65 years).

Table II reports data on urinary mutagenicity of all volunteers taking part in the study investigated using the plate incorporation preincubation technique on the YG1024 *Salmonella typhimurium* strain in the presence of metabolizing system S9. Twenty-three out of 288 (8%) urinary extracts showed a clear mutagenic activity revealed by the double number of induced as regards

Tab. I. Characteristics of the population.

| | | |
|--|---------------|-------------|
| Number of subjects | 312 | |
| Toxic samples ^a | 24/312 (7.7%) | |
| Subject with detectable urinary mutagenicity | 288 | |
| Gender | Female | 154 (53.5%) |
| | Male | 134 (46.5%) |
| Age (years) | Mean \pm SD | 43 ± 9 |
| | Range | 20-65 |
| ^a Urine samples that did not permit bacterial growth in all tested doses. | | |

Tab. II. Mutagenic activity assayed on YG1024 *Salmonella typhimurium* strain with metabolic activation in urinary extracts of study population.

| | Males | Females | All |
|--|--------------------|---------------------|----------------|
| Urinary mutagenicity | | | |
| (net rev/ml) ^a | 2.07 ± 1.65 | 1.89 ± 1.41 | 1.97 ± 1.52 |
| (net rev/mmmole creatinine) ^b | 364 ± 491* | 605 ± 868* | 493 ± 727 |
| Positive samples ^c | 9/134 ** (9.1%) | 14/154 ** (6.7%) | 23/288 (8%) |
| Urinary mutagenicity of positive samples | | | |
| (net rev/ml) ^a | 3.52 ± 0.25° | 4.0 ± 1.0° | 3.73 ± 1.08 |
| (net rev/mmmole creatinine) ^b | 745 ± 895°° | 799 ± 417°° | 778 ± 629 |

^a Calculated by the linear regression method on the slope of the linear portion of the dose-response curve.
^b Urinary mutagenicity adjusted for creatinine excretion.
^c At least one dose tested doubling the number of spontaneous revertants.
* Mann-Whitney U-test, $z = -3.97$, $P < 0.0001$
** Chi square = 0.550; $P = 0.4584$
° Mann-Whitney U-test, $z = -0.756$, $P = 0.4497$
°° Mann-Whitney U-test, $z = -1.386$, $P = 0.1658$

to spontaneous revertants in at least one of the tested doses. The majority (14 out of 23) of positive samples belonged to women. The volunteers mean urinary mutagenicity was rather low, as expected in healthy subjects without a specific exposure, with a value of 1.97 ± 1.52 revertants/ml and 493 ± 727 revertants/mmmole

creatinine after adjustment for urinary dilution. The analysis of urinary mutagenicity in relation to gender showed that, on the whole, women had the higher levels of urinary mutagens with a statistically significant difference (364 ± 491 revertants/mmmole creatinine for males against 605 ± 868 revertants/mmmole creatinine in

Tab. III. Lifestyle behaviours in the 24 hours before urine collection investigated by means of a self administered questionnaire.

| Behaviours investigated | Males N (%) | Females N (%) | N (%) |
|---|-------------|---------------|------------|
| Place of residence | | | |
| City-suburbs | 93 (69.4) | 117 (76.0) | 210 (72.9) |
| Country | 41 (30.6) | 37 (24.0) | 78 (27.1) |
| Traffic exposure | | | |
| Intense | 59 (44.0) | 79 (51.3) | 138 (47.9) |
| Moderate | 75 (56.0) | 75 (48.7) | 150 (52.1) |
| Diet ^a | | | |
| increasing urinary mutagenic capacity | 58 (43.3) | 52 (34.0) | 110 (38.3) |
| not increasing urinary mutagenic capacity | 76 (56.7) | 101 (66.0) | 177 (61.7) |
| Passive smoke exposure | | | |
| Yes | 16 (11.9) | 9 (5.8) | 25 (8.7) |
| No | 118 (88.1) | 145 (94.2) | 263 (91.3) |
| Fireplace exposure | | | |
| Yes | 9 (6.7) | 5 (3.2) | 14 (4.9) |
| No | 125 (93.3) | 149 (96.6) | 274 (95.1) |
| Cooking smoke exposure | | | |
| Yes | 9 (6.7) | 24 (15.6) | 33 (11.5) |
| No | 125 (93.3) | 130 (84.4) | 255 (88.5) |
| Outdoor work ^b | | | |
| Yes | 34 (25.4) | 4 (2.6) | 38 (13.2) |
| No | 100 (74.6) | 150 (97.4) | 250 (86.8) |
| Outdoor hobbies ^b | | | |
| Yes | 3 (2.2) | 0 (0) | 3 (1.0) |
| No | 131 (97.8) | 154 (100) | 285 (99.0) |

^a Data available only on 287 subjects.

^b For a period of time longer than 4 hours.

females, Mann-Whitney U-test, $z = -3.97$, $P < 0.0001$). When comparing the frequency of positive sample and the levels of urinary mutagenicity of positive samples between male and female subjects no statistically significant difference could be found.

Information about lifestyle behaviours in the 24 hours before urine collection is summarized in Table III: all activities that could influence urinary mutagenicity were taken into account. The 72.9% of the subjects lived in an urban (city or suburbs) area but only the 47.9% reported to be exposed to intense traffic. The 38.3% of the population declared to have consumed food (baked or pan-fried) that can increase urinary mutagenic activity. Passive smoke exposure concerned only 25 individuals (8.7%) while 11.5% of study population, for mainly women, stated they were exposed to cooking smoke. Thirty-eight subjects, mostly men, spent outdoor more than 4 hours for working activities and only 3 men did it for hobby (about 1% of total population). A modest percentage of volunteers (4.9%) reported to be exposed to fireplace smoke.

When considering lifestyle behaviours according to age and gender (Tabs. IV, V), it appears that, although both groups had a similar mean age, about half of male population was between 41 and 50 years old, while the majority of women was younger (31-40 years old). Women showed the greater urban exposure, accounting for the higher percentage of subjects living in an urban

area (76.0% against 69.4% in men) and exposed to intense traffic (51.3% against 45.5% in men). Besides that, as we expected, female population was more exposed to cooking smoke as compared to men (15.6% *versus* 6.7%). The prevailing factor that influenced urinary mutagenicity in male population was diet: 43.3% of men reported the consumption of baked or pan-fried food in respect of the 34.0% of women. Men were also more exposed (11.9%) to passive smoking than females (5.8%). Environmental exposure due to outdoor occupational/recreational activities seemed to concern mainly male group (27.6% in men against 2.6% in women). Barely the minority of subjects involved in the study declared to be exposed to fireplace smoke and men were more numerous (6.7%) than women (3.2%).

The frequencies of positive samples according to lifestyle behaviours are reported in Table VI. Individuals expressing a positive mutagenic activity were mainly resident in an urban area (8.1% of people living in city or suburbs). The other main considered features able to increase urinary mutagenicity present in the population with positive urinary sample were diet (13.6%) and indoor exposure to passive smoking or fireplace and cooking smoke exposure in the 24 hours before the sample collection (14.9%). The comparison between frequencies analyzed by Chi square test showed a statistically significant difference in lifestyle behaviours of subjects

Tab. IV. Lifestyle behaviours according to age in male population.

| Age | N (%) | Urban residence N (%) | Intense traffic exposure N (%) | Outdoor exposure ^a N (%) | Fireplace N (%) | Cooking smoke exposure N (%) | Diet N (%) | Passive smoking exposure N (%) |
|-------------------|-----------|--------------------------|--------------------------------------|--|--------------------|------------------------------------|------------|--------------------------------------|
| Up to 30 | 10 (7.5) | 7 | 2 | 2 | 1 | 0 | 5 | 3 |
| 31-40 | 28 (20.9) | 16 | 8 | 7 | 4 | 3 | 12 | 5 |
| 41-50 | 62 (46.2) | 42 | 28 | 17 | 1 | 4 | 26 | 5 |
| 51- Over 60 | 34 (25.4) | 28 | 21 | 11 | 3 | 2 | 15 | 3 |
| All 27- 62 (44±8) | 134 (100) | 93 (69.4) | 61 (45.5) | 37 (27.6) | 9 (6.7) | 9 (6.7) | 58 (43.3) | 16 (11.9) |

^a Outdoor permanence for more than 4 hours.

Tab. V. Lifestyle behaviours according to age in female population.

| Age | N (%) | Urban residence N (%) | Intense traffic exposure N (%) | Outdoor exposure ^a N (%) | Fireplace N (%) | Cooking smoke exposure N (%) | Diet N (%) | Passive smoking exposure N (%) |
|-------------------|-----------|--------------------------|--------------------------------------|--|--------------------|------------------------------------|------------|--------------------------------------|
| Up to 30 | 24 (15.6) | 174 | 11 | 2 | 2 | 4 | 8 | 3 |
| 31-40 | 52 (33.8) | 41 | 25 | 1 | 1 | 8 | 18 | 3 |
| 41-50 | 49 (31.8) | 35 | 29 | 1 | 1 | 6 | 14 | 1 |
| 51- Over 60 | 29 (18.8) | 27 | 14 | 0 | 1 | 6 | 12 | 2 |
| All 20- 65 (40±9) | 154 (100) | 117 (76.0) | 79 (51.3) | 5 (2.6) | 5 (3.2) | 24 (15.6) | 52 (34.0) | 9 (5.8) |

^a Outdoor permanence for more than 4 hours.

Tab. VI. Comparison between frequencies of positive and negative samples according to the behaviours considered.

| Behaviours investigated ^a | | Positive samples N (%) | Negative samples N (%) | All N | Chi square test | |
|--------------------------------------|---|---------------------------|---------------------------|-------|-----------------|--------|
| | | | | | χ^2 | P |
| Residence | 0 | 6 (7.7) | 72 (92.3) | 78 | 0.013 | 0.9107 |
| | 1 | 17 (8.1) | 193 (91.9) | 210 | | |
| Traffic exposure | 0 | 13 (8.7) | 137 (91.3) | 150 | 0.197 | 0.6569 |
| | 1 | 10 (7.2) | 128 (92.8) | 138 | | |
| Diet | 0 | 8 (4.5) | 169 (95.5) | 177 | 7.649 | 0.0057 |
| | 1 | 15 (13.6) | 95 (86.4) | 110 | | |
| Indoor exposure ^b | 0 | 13 (5.9) | 208 (94.1) | 221 | 5.722 | 0.0168 |
| | 1 | 10 (14.9) | 57 (85.1) | 67 | | |
| Outdoor exposure ^c | 0 | 21 (8.5) | 227 (91.5) | 248 | 0.564 | 0.4528 |
| | 1 | 2 (5.0) | 38 (95.0) | 40 | | |

^a Score 0 for conditions that can not increase urinary mutagenicity and score 1 for conditions known to increase urinary mutagenicity.

^b Global exposure to passive smoking, fireplace exposure and cooking smoke exposure.

^c Outdoor permanence for more than 4 hours.

with positive and negative samples exclusively for diet ($P = 0.0057$) and indoor exposure ($P = 0.0168$), suggesting a possible main role of these factors in inducing the mutagenic response.

Discussion and conclusions

The study considered urinary mutagenicity in healthy non smoking subjects lacking of any specific exposure by means of the sensitive YG1024 *Salmonella typhimurium* strain using plate incorporation with preincubation technique in presence of metabolizing system in order to achieve the maximum sensitivity. Since it is well known that many features can exert an influence on urinary mutagenic activity, people enrolled in the study completed a questionnaire involving their characteristics and their lifestyle habits in the 24 hours before sample collection. The most recurrent sources of exposure, that could increase mutagenicity, were residence in an urban area (72.9%), intense traffic (47.9%) and diet (38.3%). Men declared to stay outdoor for working/recreational activity more frequently (27.6%) as regards to women (2.6%). On the contrary, female population, as expected, stated a higher (15.6%) exposure to cooking smoke than men (6.7%). We would highlight that up to now, no study determined urinary mutagenicity exclusively in subjects without a specific (occupational or caused by voluptuary habits like smoke and consumption of grilled meat) exposure known to enhance it. Previous investigations [12, 13] only compared exposed and control population without carefully taking into account possible confounding environmental and lifestyle sources of exposure. Using less sensitive strain and technique (plate incorporation test on TA98), other researchers determined a

mutagens' content ranging from 0.5 revertants/ml [14] to 2.3 revertants/ml [15]. With the fluctuation technique, Hagmar et al. [16] found a mean mutagenicity of 451 revertants/mmol creatinine, but they did not control the smoking status. The only available data on YG1024 with non exposed subjects, reporting very high urinary mutagenicity levels but involving only 5 non smoking control [17], exhibit poor significance.

In addition, we chose a bacterial strain that is peculiarly affected by the presence of PAHs, aromatic and heterocyclic amines urinary mutagenic metabolites increasing assay sensibility, while the standardization of bacterial overnight culture density and media protein content enhance specificity.

In conclusion, the present study shows the presence, even of low levels, of mutagens (with a mean value of 1.97 revertants/ml) in urine of apparently non exposed subjects assayed by plate incorporation test on YG1024 *Salmonella typhimurium* strain. This mutagenic activity is higher in women than in men with a statistically significant difference.

Excluding interfering features, we can hypothesize that the presence of low mutagenic activity could be ascribed to exposure to factors depending on urban environment, lifestyle behaviour and daily life activities, as cooking fumes. In this case, we can not undervalue that this kind of exposure is more continuative, albeit of low intensity, and it could however represent a long term risk factor. Additionally the presence of mutagens in overnight urine acquires particular value taking into account that they remain in strict contact with bladder mucosa for several hours. It would be, therefore, proper to consider the possibility of carrying out a longitudinal study in order to verify if time constant exposure could induce an advancing revertants rise.

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