Genotoxicity of instant coffee: possible involvement of phenolic compounds

Maria Paula Duarte a,b, António Laires a,b, Jorge Gaspar a,c, Daniela Leão a, José Santos Oliveira b, José Rueff a,*

a Department of Genetics, Faculty of Medical Sciences, New University of Lisbon, R. da Junqueira 96, P-1349-008 Lisbon, Portugal
b Faculty of Sciences and Technology, New University of Lisbon, P-2825-114 Caparica, Lisbon, Portugal
c ULHT, Campo Grande, 376, 1700 Lisbon, Portugal

Received 21 January 1999; received in revised form 31 March 1999; accepted 6 April 1999

Abstract

Instant coffee exhibits direct genotoxic activity in the tester strains TA 98, 100, 102, 104 and YG 1024. In the Ames tester strain TA 100, the presence of S9 mix, S100 mix, S9 mix without cofactors led to a significant decrease of the genotoxicity observed. The decrease observed in the presence of S9 mix seems to be highly correlated with the catalase content of S9 mix. The genotoxicity of instant coffee detected in strain TA 100 was dependent on the pH, with higher genotoxic effects at pH values above neutrality. Also, dependent on the pH was the ability of some phenolic molecules present in coffee promoting the degradation of deoxyribose in the presence of Fe3+/EDTA. These results suggest that apart from other molecules present in instant coffee responsible for their genotoxicity in several short term assays, phenolic molecules could also be implicated in the genotoxicity of coffee, via reactive oxygen species arising from its auto-oxidation.

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Keywords: Coffee; Mutagenicity; Polyphenol; Oxygen species

1. Introduction

The mutagenic effects of instant coffee have been studied in several short term assays. These studies showed that instant coffee induces mutations in several strains of Salmonella typhimurium [1,2] as well as in Escherichia coli WP2 uvrA/pKM101 and K12 [3]. The mutagenic effects of coffee have also been observed in Chinese hamster lung cells [4], in human lymphocytes [5] and in hamster ovary cells [6]. In the Ames test (strain TA100) mutagenic activity is only detected after roasting [3,7] and is considerably reduced or even totally eliminated by the addition of mammalian liver enzymes [1,2]. When tested in vivo, however, coffee has shown poor evidences of genotoxic effects [8–10].

IARC evaluated coffee consumption as possibly carcinogenic to the human urinary bladder [11].

Coffee and some of its constituents also exhibit the ability to enhance [12–14] or to decrease [15–18] the genotoxic effects of other compounds.
The identification of the major mutagens of coffee has been the subject of several studies. Coffee is a complex mixture composed of thousands of different molecules, some of them with genotoxic activity. Methylglyoxal and H$_2$O$_2$ seem to be responsible for most of the mutagenicity of instant coffee [19]. Fujita et al. [19,20] showed that methylglyoxal added to a solution of hydrogen peroxide considerably enhances the mutagenicity of this solution and concluded that the interaction between these two substances was responsible for most of the mutagenic effects of coffee. The formation of H$_2$O$_2$ in instant coffee increases continuously after preparation, indicating the presence of a hydrogen peroxide generating system [15,19]. This H$_2$O$_2$ generation system might be formed as a consequence of roasting, since green coffee beans were found to have a low capacity to generate H$_2$O$_2$ [19]. During roasting, most of the chlorogenic acid present in green coffee beans decomposes giving rise to a wide range of other phenols like caffeic acid, catechol, pyrogallol or hydroquinone [21] (Fig. 1), and the auto-oxidation of these polyphenols, which occurs mainly at pH values above neutrality [22], could be the source of H$_2$O$_2$ present in the roasted coffee beans.

2. Material and methods

2.1. Chemicals

Methylglyoxal, catalase, superoxide dismutase (SOD) and deoxyribose were from Sigma (St. Louis,

Table 1
Specific mutagenic activity (rev/mg) of instant coffee in the strains, TA 98, 100, 102, 104 and YG 1024

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plate incorporation assay</th>
<th>Pre-incubation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without S9 mix</td>
<td>With S9 mix</td>
</tr>
<tr>
<td>TA98</td>
<td>1.92 ± 0.91</td>
<td>negative</td>
</tr>
<tr>
<td>TA100</td>
<td>5.02 ± 0.70</td>
<td>negative</td>
</tr>
<tr>
<td>TA102</td>
<td>15.05 ± 0.61</td>
<td>9.83 ± 0.57</td>
</tr>
<tr>
<td>TA104</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>YG1024</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

Negative—absence of dose–response curves and absence of spontaneous rate doubling at all doses tested.

Fig. 1. Decomposition of chlorogenic acid during coffee roasting [21].
MO, USA), caffeic acid, catechol, hydroquinone and
chlorogenic acid were from Aldrich (Steinheim, Ger-
mained) and pyrogallol and thiobarbituric acid (TBA)
were from Merck (Darmstadt, Germany). Bacto agar
was supplied by Difco (Detroit, MI, USA) and nutri-
ent broth (NB) by Oxoid (Hampshire, England).

2.2. Rat liver enzymes

Preparation of S9 (microsomal fraction of rat
liver, induced with Aroclor 1254) was carried out as
described by Maron and Ames [23]. The cytosolic
fraction S100 was obtained after the centrifugation

![Graph showing mutagenic activity of instant coffee detected with *S. typhimurium* TA 100 (pre-incubation assay) with (*) and without (■) S9 mix and with S9 mix without cofactors (●), S100 mix (+) and albumin mix (♦). Bars: standard deviation.]
of S9 at 100,000 × g for 60 min. The protein content of S9 was determined according to Lowry et al. [24]. Catalase and SOD activities were determined according to Wheeler et al. [25] and Marklund and Marklund [26], respectively. S9 mix (10% in S9) was prepared as described by Maron and Ames [23] and S100 mix (10% in S100) was prepared like S9 mix using S100 instead of S9.

2.3. Preparation of coffee samples

Instant coffee (Nescafé®, Nestlé) was purchased at a local market and the samples were prepared dissolving 6.25 g of instant coffee in 50 ml of distilled boiling water. The solutions were centrifuged at 1500 × g for 15 min and the supernatant was separated in aliquots and frozen at −20°C.

2.4. Ames assay

The strains of S. typhimurium TA 98, TA 100, TA 102 and TA 104 were kindly provided by Professor B.N. Ames (Berkeley, CA, USA) and the strain YG 1024 was kindly provided by Professor Take-
Mutagenicity testing were carried out according to the plate incorporation assay or the pre-incubation assay described by Maron and Ames [23]. In the pre-incubation assay, the test mixtures composed of the coffee solution (volumes from 0 up to 320 μl), 100 μl of the tester strains and 500 μl of S9 mix, S100 mix, S9 mix without cofactors, albumin mix or phosphate buffer 0.1 M, pH 7.4, 6.3 or 8.1, were incubated at 37°C for 30 min. The genotoxicity induced by 15 mg of instant coffee in the Ames tester strain TA 100 was also assayed in the presence of different amounts of S9 mix, catalase (530 U/ml) and SOD (67.9 U/ml). Albumin mix was prepared as S9 mix using a 35-mg/ml calf serum albumin solution instead of S9. The mutagenicity of pyrogallol and the effect of methylglyoxal (1.5 μg/plate) on the mutagenicity of pyrogallol were tested in the Ames tester strain TA 100 using the pre-incubation assay. At least three independent experiments were performed for each assay. Specific mutagenic activities were determined from the slope as the least square-line of the linear portion of the dose–response curve.

2.5. Deoxyribose degradation assay

Deoxyribose degradation was assayed according to Gaspar et al. [27] by incubating for 2 h at 37°C, 1.2 ml of a reaction mixture composed of potassium phosphate buffer pH 6.3, 7.4 and 8.1 (15 mM), the phenols studied (chlorogenic acid, hydroquinone, catechol, pyrogallol and caffeic acid) at a concentration of 100 μM, deoxyribose (2.8 mM), FeCl₂ (20 μM) and EDTA (100 μM). Hydrogen peroxide was used as a positive control (1.42 mM) for the hydroxyl radical generation. Deoxyribose degradation was measured by the TBA method using 1 ml of trichloroacetic acid (2.8%) and 1 ml of TBA (1%) in 0.05 M NaOH. The mixture was incubated at 100°C for 15 min, cooled and the absorbance was measured at 532 nm. For each assay, three independent experi-

![Fig. 5. Production of TBA reactive products arising from deoxyribose degradation by chlorogenic acid (Chl ac.), caffeic acid (Caf. ac.), catechol (Cat.), hydroquinone (HQ) and pyrogallol (Pir.) at pH 6.3, 7.4 and 8.1. Bars: standard deviation.](image-url)
ments were performed. Negative controls were performed and subtracted in each experiment.

3. Results

The protein content of S9 was 35 mg/ml and the enzymatic activities of catalase and SOD were 5300 and 679 U/ml, respectively.

Table 1 shows the specific mutagenic activity of instant coffee, determined from the slope of the least-squares line of the linear portion of the dose–response curves, obtained using the plate incorporation assay or the pre-incubation assay, with different strains of *S. typhimurium* in the presence and in the absence of S9 mix. The values show that the pre-incubation assay was more efficient in detecting the mutagenic activity of instant coffee than the plate incorporation assay. The addition of S9 mix led to a significant decrease or to a complete abolition of the genotoxic effects detected in all the strains except in TA 102.

In order to elucidate what constituents of S9 could be involved in this genotoxic deactivation, further experiments were undertaken using S9 mix prepared without cofactors (NADP and glucose 6-P). Additionally, similar experiments were performed using S100 mix and albumin mix. These experiments showed that the genotoxicity of instant coffee in the Ames tester strain TA 100, using the pre-incubation assay, shows a typical dose–response curve up to 15 mg coffee/plate followed by toxicity above that value without S9 mix or in the presence of albumin mix, whereas a dose–response with no apparent toxic effects could be found up to 40 mg/plate in the presence of S9 mix with or without cofactors or S100 mix (Fig. 2 and Table 1).

Since the results obtained suggested a detoxification by cytosolic enzymes present in S9 mix, the effect of several amounts of S9 mix, catalase and SOD in the genotoxicity induced by 15 mg of coffee in the strain TA 100 was also studied. The results show that while SOD has no effect in the mutagenic activity of instant coffee, catalase reduces the genotoxicity of this beverage and seems to be the main factor responsible for the deactivation observed in the presence of S9 mix (Fig. 3).

Fig. 4 depicts the effect of the pH in the mutagenic activity of instant coffee in strain TA 100. The results show that the genotoxicity of this beverage was dependent on the pH, being higher at pH values above the neutrality.

All the phenolic molecules assayed showed, in the presence of Fe³⁺-EDTA, the ability to promote the degradation of deoxyribose. Pyrogallol presented the highest effects on the deoxyribose degradation. This activity was more pronounced at pH values above neutrality (Fig. 5) and was drastically reduced by active catalase but not by heat inactivated catalase (data not shown).

Fig. 6 depicts the effect of methylglyoxal on the mutagenicity of pyrogallol in strain TA 100. The results show that the mutagenicity of pyrogallol was

![Fig. 6. Mutagenic activity of pyrogallol without methylglyoxal (○) and with 1.5 μg/plate of methylglyoxal (■) in strain TA 100 (pre-incubation assay). Bars: standard deviation.](image-url)
significantly increased in the presence of methyl-
glioxal.

4. Discussion

Instant coffee showed a direct-acting genotoxic activity in all the strains used in the Ames test with pre-incubation. When the experiments were carried out in the presence of S9 mix this genotoxicity was significantly reduced (strains TA 98 and TA 100), completely abolished (strains TA 104 and YG 1024) or very slightly reduced (strain TA 102) (Table 1). The different results obtained in the presence of S9 mix and the positive responses obtained in strains with different sensibilities to different genotoxic compounds, suggest that molecules present in instant coffee may exert DNA lesion by different mechanisms and/or that there are different classes of genotoxicants present in this complex mixture. This latter hypothesis may be illustrated by the fact that S9 which contains 5300 U/ml catalase and 679 U/ml SOD does not inhibit the response in TA 102 whereas it does so in TA 100 which responds to different kinds of mutagens including reactive oxygen species.

Since the strain YG 1024 is highly sensitive to heterocyclic aromatic amines [28,29], the absence of genotoxic response in this strain when the assay was performed in the presence of S9 mix (Table 1) seems to exclude the involvement of this kind of compounds in the genotoxic activity induced by instant coffee, in the experimental conditions used.

The genotoxicity of instant coffee in strain TA 100 was not affected by the presence of a solution of albumin at the same protein concentration than S9 mix (Fig. 2). This result is in agreement with the findings of Aeschabacher et al. [2] and Friederich et al. [30] and shows that the deactivation performed by S9 mix is not the result of a non-specific binding of the active compounds to proteins, but instead a specific enzymatic inactivation. Furthermore, this enzymatic inactivation does not seem to depend on cytochrome P450 but instead from the activity of cytosolic enzymes, since S9 mix prepared in the presence or in the absence of the cofactors needed for the activity of cytochrome P450, as well as S100 mix exhibited similar effects (Fig. 2).

The study of the effects of two cytosolic enzymes, namely catalase and SOD, in the genotoxicity of instant coffee in strain TA 100 showed that catalase, but not SOD, exhibits a clear detoxificant activity. The comparison between the effect of S9 mix and of a catalase solution with the same catalase activity seems to indicate that this enzyme, was the main factor responsible for the decrease in the genotoxicity of instant coffee observed in presence of S9 mix (Fig. 3). The results obtained suggest the involvement of H2O2 in the mutagenic activity of instant coffee. Since it has been reported that green coffee beans did not show significant ability to produce hydrogen peroxide [19], of the molecules present in coffee after roasting, phenolic molecules arising from the decomposition of chlorogenic acid (Fig. 1) could be responsible for the formation of this compound in roasted coffee. It is known that phenolic molecules, especially at pH values above neutrality, can deprotonate and in this form react with O2 giving rise to superoxide anions and subsequently to hydrogen peroxide [22]. Several results obtained in this work seem to support the hypothesis that the genotoxicity of coffee might be related to the H2O2 arising from the auto-oxidation of phenolic molecules. (1) Several phenolic compounds present in roasted coffee, namely caffeic acid, catechol, hydroquinone, pyrogallol and chlorogenic acid (see Fig. 1) [21] showed, in presence of Fe3+/EDTA, the ability to promote the degradation of deoxyribose, specially at pH values above neutrality (Fig. 5). This activity should be mediated by hydrogen peroxide since it was specifically inhibited by catalase. (2) As the auto-oxidation of phenols occurs preferentially at pH values above neutrality, if the genotoxic activity of instant coffee was related to the H2O2 arising from this process, than it should be higher at alkaline pH values. In fact, when the Ames assay was performed in strain TA 100, at different pH values it was observed that the genotoxicity of this beverage increased as the pH value increased (Fig. 4). (3) The mutagenicity of pyrogallol in strain TA 100, was enhanced by the presence of methylglyoxal (Fig. 6) suggesting the same kind of interaction that was described for methylglyoxal and hydrogen peroxide on the mutagenic effects of coffee [19,20].

Since the genotoxicity of instant coffee seems to be associated with the presence of reactive oxygen
species, namely hydrogen peroxide, arising from the auto-oxidation of phenolic compounds, which occurs preferentially at pH values above neutrality, we cannot exclude that in conditions favourable to the fast oxidation of these kind of compounds, namely, the alkaline pH of the human intestine, the formation of a significant amount of reactive oxygen species occurs, in a way that the mechanisms of cellular defence against oxidative damage can be transiently overtaken, making possible the occurrence of genetic injury mediated by this kind of species.

Acknowledgements

This work was supported in part by JNICT (Project PRAXIS/PSAU/C/67-96).

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