

Mutagenicity Induced by Hyperthermia, Hot Mate Infusion, and Hot Caffeine in *Saccharomyces cerevisiae*

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ABSTRACT

Mate, an infusion containing caffeine (3 g/liter), is drunk hot by most Uruguayan, North Argentinian, and South Brazilian people. This beverage has been recently associated with esophageal cancer in Brazil and Uruguay. To test the mutagenic and lethal effects of mate infusion, caffeine, hyperthermia, and their combinations, we used *Saccharomyces cerevisiae* as an eucaryotic model system measuring *lys* to *LYS* reversions. We showed that mate infusion was not mutagenic, whereas caffeine at the same concentration contained in mate, produced a 5-fold increase in the spontaneous mutation rate. The highest observed mutagenic rate corresponded to hyperthermia (54°C at 60 min). Hot caffeine also produced a time-dependent mutagenic effect, whereas hot mate infusion determined a significantly lower mutagenic effect than hot caffeine. The differential lethality produced by the tested agents plays an important role in the expression of the induced mutagenic damage. Caffeine and mate infusion could decrease the mutagenic effect of hyperthermia through the channeling of part of the induced DNA lesions into an error-free repair pathway.

INTRODUCTION

The intake of beverages containing caffeine such as coffee and tea is associated or correlated with cancer as suggested by epidemiological studies (see Ref. 1 for review). Recently, hot mate infusion drinking has been associated with esophageal cancer in Brazil and Uruguay (Refs. 2-4; see Ref. 5 for a review). In addition, in South America the highest rate of this type of neoplasia is reported in Uruguay (4). Mate is an infusion of the herb *Ilex paraguayensis* which contains caffeine (6). Interestingly, its concentration is the highest one found among other tested drinks containing caffeine (7). It is drunk habitually hot and in large quantities (approximately 1 liter/day in 2 separate intakes) by most Uruguayan, North Argentinian, and South Brazilian people.

Studies on the mutagenic, carcinogenic, and teratogenic effects of caffeine have given contradictory results (8-10). On the other hand, investigations *in vitro* using different cell lines have been performed in relation to the lethal and mutagenic effects of hyperthermia (11-13) and several epidemiological studies found drinking temperatures important for esophageal cancer both for mate and tea (5).

We investigated the putative mutagenic effect of either hot mate or hot caffeine as a first step to elucidate their role in esophageal carcinogenesis. To characterize mutagenic effects, an auxotrophic haploid strain of *Saccharomyces cerevisiae* was used as a model system (14, 15). We showed that mate infusion was not mutagenic whereas caffeine at the same concentration contained in the mate infusion displayed a moderate mutagenic effect. Both hot mate infusion and hot caffeine solution were highly mutagenic. This effect depended on exposure times. Moreover, hyperthermia as a single agent induced the highest observed mutagenic rate as well as a dramatic lethality. These findings are in favor of the hypothesis that carcinogenicity of the hot mate beverage is not due to caffeine or other mate components itself, but to hyperthermia alone.

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MATERIALS AND METHODS

Yeast Strain

We used an auxotrophic haploid strain of *Saccharomyces cerevisiae* SC7K *lys2-3* (16, 17).

Culture Conditions

Cells were grown in liquid nutrient medium (YED) containing 5 g/liter of yeast extract and 10 g/liter of dextrose at 30°C in a shaker until the stationary phase of growth was attained. To determine mutagenesis, OM² was used as follows: 6.7 g/liter of yeast nitrogen base, 20 g/liter of dextrose, and 20 g/liter of agar. To score survival, OM + lysine (50 µg/ml) and/or YED solidified by 20 g/liter of agar were used (15, 18). All products were from Difco (Detroit, MI).

Treatments

To investigate if the putative mutagenic effect of hot mate was due to its caffeine content or to the elevated temperature (hyperthermia), the cells were exposed to the mate infusion or to the caffeine solution (at the same concentration contained in mate Ref. 6), either at 30°C or at 54°C. The latter is the average temperature of a common mate intake. In addition, cell samples were exposed to hyperthermia (HT, 54°C) as a single agent. All treatments were performed in YED for 0-90 min. Cells were sampled in the early stationary phase of growth (cell density 5×10^7 cells/ml, 20% budding cells), washed, and resuspended according to the desired treatment.

Mate Infusion Treatment. Cell samples were resuspended in the mate infusion, prepared as is usual in the common drink, and added to YED. The concentration of mate corresponded to the habitual drink (6). This treatment was performed for 0-90 min in two conditions: (a) at 30°C (M); and (b) at 54°C (M + HT).

Caffeine Treatment. Cell samples were exposed to caffeine (Sigma, St. Louis, MO) diluted in YED at a concentration of 3 g/liter. This is the average caffeine concentration contained in a mate infusion (6). Caffeine treatment was performed at 30°C (C) and at 54°C (C + HT), during the same interval used in the mate treatment.

Hyperthermia Treatment. Cell samples were resuspended in YED and submitted for 0-90 min to 54°C. The exposure to hyperthermia was performed in a thermostated water bath shaker. The time constant of the warm up period was half-time = 3 s (19).

Mutation Frequency, Mutation Yield, and Survival. Reversion to prototrophy was performed according to the method described in the literature (15, 20). The main experimental details were as follows: to determine the number of revertants (*lys* to *LYS*), 1.6 ml of control or treated cells were plated on OM (8 plates/point: 0.2 ml/plate) whereas survivors, appropriately diluted according to the expected yield of viable colonies, were scored on OM + lysine and on YEDA (4 plates/point incubated over 5 days at 30°C). The OM plates were incubated for 15 days at 30°C. No selection of population fractions was performed.

Mutation yield is defined as

$$Y(t) = \frac{Nm(t)}{N_0}$$

and mutation frequency as

$$F(t) = \frac{Nm(t)}{Ns}$$

² The abbreviations used are: OM, omission medium; HT, hyperthermia treatment; C, caffeine treatment; Y, mutation yield; F, mutation frequency; M, mate infusion.

where N_m is the number of induced revertants/ml, N_0 is the total number of treated cells/ml, N_s is the number of survivors per ml and t is the exposure time to the used agents (15, 20). Survival fraction (S), defined as $S(t) = N_s/N_0$, was determined as usual and is described elsewhere (16).

Confidence intervals (95%) were calculated according to binomial distribution.

RESULTS

Fig. 1 shows mutagenicity (reversion *lys* to *LYS*) produced by M and C treatments. It is clear that mate infusion induced a very low mutation induction, whereas caffeine induced a 5-fold increase in the spontaneous mutation yield (Y) value ($Y = 3.2 \times 10^{-5}$ for $t = 60$ min versus 6.5×10^{-6} for $t = 0$).

Fig. 2 illustrates the Y as a function of exposure times for HT, M + HT, and C + HT treatments. The maximal Y values attained after either M + HT or C + HT treatments corresponded to shorter exposure times as compared with HT treatment, and both were significantly lower as compared to those of HT.

Fig. 3 shows F as a function of exposure time for the combined treatments (M + HT and C + HT) as well as for HT as a single agent. It can be seen that all three maximum corresponded to the same exposure time ($t = 60$ min). Fig. 4 shows the survival fractions (S) as a function of exposure times to the used single agents and their combinations. The corresponding D_0 values of the exponential components are given in Table 1 (D_0 values are the exposure times for $S = 0.37 \times S_0$, where S_0 is the ordinate at $t = 0$). It is noteworthy that M and C produced similar and very low lethality, whereas HT produced a dramatic exponential inactivation of the population. In combination with either C or M, hyperthermia produced a less than additive lethal effect.

DISCUSSION

Given the fact that drinking hot beverages containing caffeine (*i.e.*, coffee, tea, and mate) is a worldwide habit, we investigated the pu-

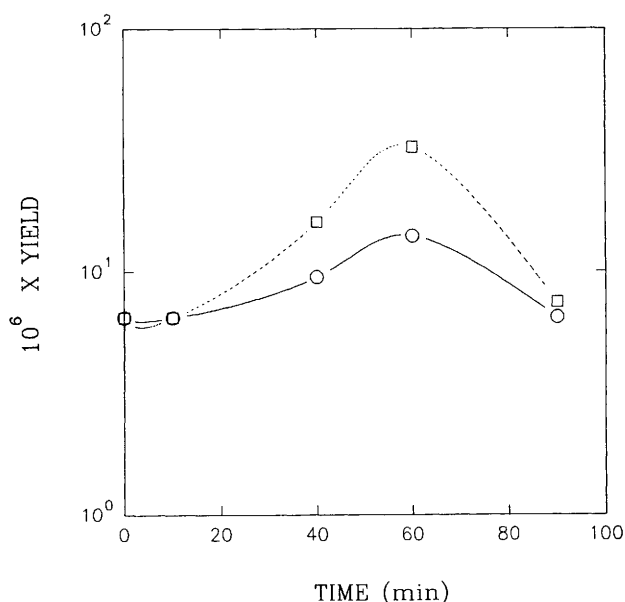


Fig. 1. Mutation yield $Y(t)$ as a function of exposure times to: (○) mate infusion and (□) caffeine 3 g/liter. Mean values of at least three experiments. The curves were fitted using spline interpolation routines for smoothing points in the plots. Binomial confidence intervals correspond to the size of the symbols.

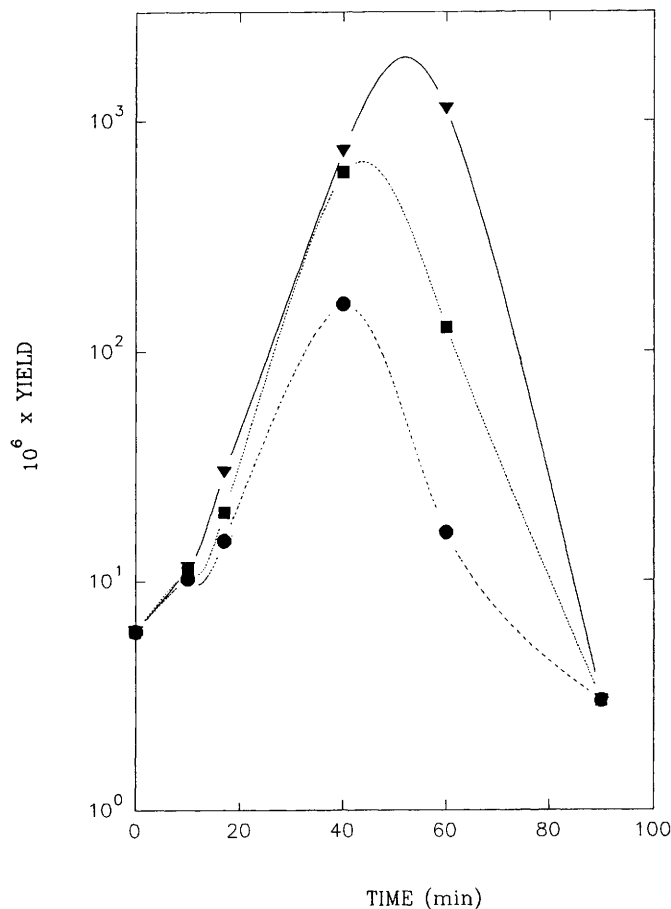


Fig. 2. Mutation yield $Y(t)$ as a function of exposure times to: (▼) hyperthermia; (●) hyperthermia plus mate infusion and (■) hyperthermia plus caffeine. Note that Y values are multiplied by 10^6 . Data from one model experiment are represented. The curves were fitted using spline interpolation routines for smoothing points in the plots. Binomial confidence intervals correspond to the size of the symbols.

tative mutagenic effect of either hot mate infusion or hot caffeine as a first step to elucidate the role of these hot drinks in esophageal carcinogenesis.

The observed mutagenicity induced by hyperthermia (Figs. 2 and 3) is in accordance with previous results that demonstrate a clear genotoxic effect of hyperthermia in yeast and in other cell lines (11, 21–25). In fact, using the same yeast population, we have recently demonstrated that mutation frequency to 48°C is an exponential function of exposure times in the range of 2 to 40 min. The observed mutagenic effect to this temperature was statistically significant giving values of 2.5×10^{-5} to 2×10^{-4} for survival levels of 5×10^{-1} to 1×10^{-2} , respectively (21). Furthermore, an interrelationship between forward mutation rate and exposure time to 52°C was described in yeast cells by Schenberg-Frascino and Moustacchi (11). Since hyperthermia produces intra- and intermolecular bond disruption, it could induce potentially mutagenic and lethal events through alleatory effects at different targets such as DNA, enzymes involved in DNA metabolism, membrane lipids, and proteins. It is striking that the mutant frequency corresponding to 60 min of hyperthermia is approximately 1, with a surviving fraction $S(t) = 3 \times 10^{-4}$. This value is in accordance with the predictions of the mathematical models proposed by Haynes *et al.* (20).

It is noteworthy that caffeine in combination with hyperthermia produced a decrease in mutagenicity induced by HT as single agent (Figs. 2 and 3). This observation could be due to two factors. One is the higher lethality of the HT + C combination (Fig. 4), since mutation yield [$Y(t)$] is the product of the mutation probability ($Pmut$) times the survival probability [$S(t)$]: $Y(t) = Pmut \times S(t)$ (15, 20, 26,

27). In fact, caffeine produces double-strand breaks (28, 29) most probably through the inhibition of the decatenation activity of topoisomerase II as shown *in vitro* (29). Moreover, this drug intercalates into DNA and disturbs chromatin structure, and polynucleotides pool through interference with precursors of phosphoribosyl 1-pyrophosphate metabolism (30). The second factor is the inhibition of error-prone repair. It is known that caffeine inhibits at least a component of repair which takes place during late S and G₂ (18, 31–33). By this mechanism caffeine could indirectly increase the alternative probability of error-free repair in a minor fraction of the population. The existence of alternative pathways with different mutation probabilities which act on lesions produced by different DNA-damaging agents and share some common steps has been demonstrated and partially characterized in these cells (16, 34–36).

Regarding the effects of mate in combination with hyperthermia, it seems clear that part of the decrease in the mutation yield, as compared to HT, could be explained by the same mechanisms invoked in the case of C + HT treatment since the average caffeine concentration contained in the mate infusion was used. However, hot mate infusion produced a significantly mutation decrease in relation to C + HT combination (Figs. 2 and 3). This fact could be due to the mate content in B group vitamins (Table 2), which are known precursors of redox coenzymes needed in error-free excision repair (37–39).

Since caffeine, either alone or in combination with HT, simulated M or M + HT lethality, respectively, it is most probably that the lethal effect of mate infusion was due to its caffeine content (Fig. 4). In fact, mate and caffeine (at the same concentration contained in the mate infusion) produced low and similar lethal effects. The high resistance observed to M and C, as single agents, could be explained by the fact that these cells were treated in stationary phase of growth (80% in G₁

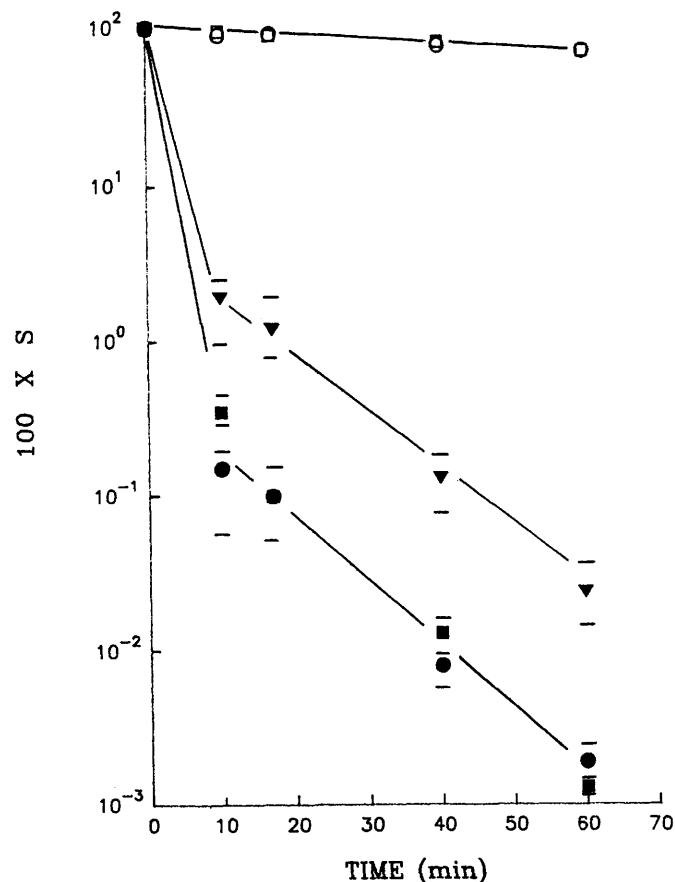


Fig. 4. Survival curves as a function of exposure times to the following agents: (○) mate infusion; (□) caffeine; (▼) hyperthermia; (●) hyperthermia plus mate infusion; (■) hyperthermia plus caffeine. Mean values of at least six experiments.

Table 1 D_{0} values (min) upon different treatments

Treatment	D_{0}^{a}	D_{0}^{b}
M	170.0	
C	170.0	
HT	2.4	12.2
M + HT	1.2	11.3
C + HT	1.2	11.3

^a D_{0} corresponding to the first exponential component of survival curve.

^b D_{0} corresponding to the second exponential component of survival curve.

Table 2 Caffeine and vitamins contents in mate infusion^a

Content	Amount (mg/10 g)
Thiamine	1.86
Riboflavine	2.3
Niacine	4.85
Ascorbic acid	36.4
Caffeine ^b	300.0

^a From "La yerba mate" (Features of Brazilian Production-Servicio de Propaganda e Expansao Comercial, SEPRO. Brazilian Government, 1963); see also Ref. 5.

^b Ref. 6.

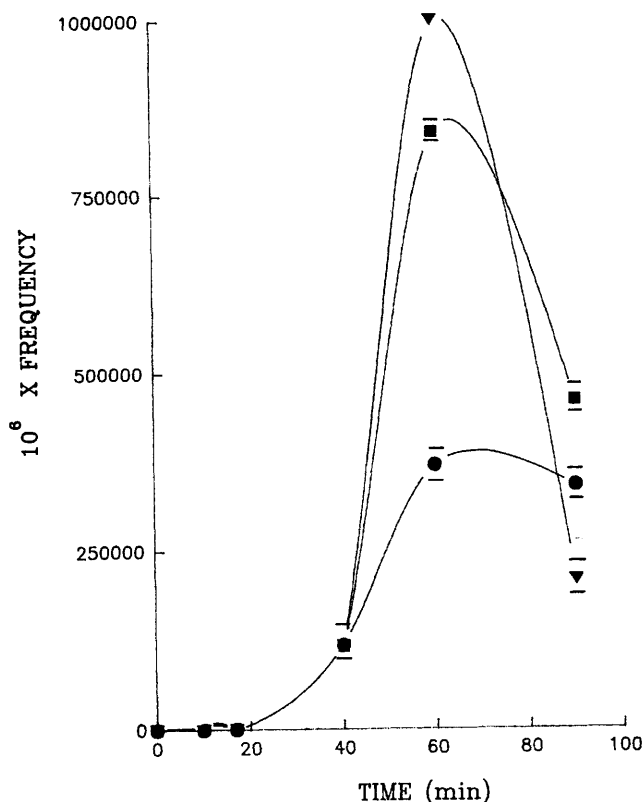


Fig. 3. Mutation frequency $F(t)$ as a function of exposure times to: (▼) hyperthermia; (●) hyperthermia plus mate infusion; and (■) hyperthermia plus caffeine. Note that F values are multiplied by 10^6 . Data from one model experiment are represented. The curves were fitted using spline interpolation routines for smoothing points in the plots. When the binomial confidence intervals are not represented, they correspond to the size of the symbols.

phase) and caffeine acts mainly during the S and G₃ phases (30). In combination with HT, either M or C induced higher lethality than HT alone. The corresponding survival curves presented approximately the same slopes as that of HT (Fig. 4; Table 1). It is noteworthy that a less than additive inactivation was obtained by the combined treatment. This fact could indicate a common target for inactivation by both agents (DNA and/or enzymes involved in DNA metabolism) (28, 29, 40). The potentiating effects of post-treatments with caffeine or hyperthermia on mutagen-induced cell killing and chromosome damage have been described (41–43). Epidemiological studies suggest a correlation between the intake of hot mate infusion and the incidence of

esophageal cancer in our region. Moreover, in Paraguay, where mate is mainly drunk cold, esophageal cancer incidence is lower than in Southern Brazil, Uruguay, and Argentina (3). In addition, several studies in other countries and regions such as Japan, northern Iran, the ex-Soviet Union, and Puerto Rico suggest that inhabitants of high risk areas drink larger quantities of hot tea than those of low risk areas (5, 44-47).

The aforementioned epidemiological data suggest that mate *per se* is not carcinogenic whereas the elevated temperature at which it is drunk could produce cancer. In our experimental conditions using yeast cells hyperthermia was highly lethal. A much lower but still significant rate of cell death and subsequent cell regeneration could also be induced in the esophageal mucosa by drinking of hot caffeine containing beverages. According to current views (48) the increased cell proliferation is a potential promotional effect.

Our experiments showed lethal and mutagenic events induced either by hot caffeine containing beverages or by hyperthermia in yeast cells, and no data on the effect of these agents are reported for mammalian cells. Therefore, we do not know yet if in the hyperthermia-related induction of esophageal cancer in human, lethal, or mutagenic events are more relevant. Additional experiments at the molecular level using mammalian cells are planned in our laboratory to further elucidate the role of these agents in the induction of neoplasia in human beings.

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