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EFFECT OF TANNIC ACID ON SPONTANEOUS AND METHYL METHANESULFONATE-INDUCED MICRONUCLEI IN MICE

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ABSTRACT

The effect of tannic acid (TA) from Vetec Química Fina Ltda., Brazil, on the spontaneous and methyl methanesulfonate (MMS)-induced micronucleated polychromatic erythrocyte (mPC) frequency in mouse bone marrow was investigated. TA (500 mg/kg) only was administered by gavage to adult male and female BALB/c mice, 6 h before intraperitoneal injection of MMS (50 mg/kg), simultaneously with MMS and 6 h after MMS treatment. To estimate a dose-response activity, three TA doses (250, 500 and 750 mg/kg) were tested as a 6 h pre-treatment. Analysis of the mPC frequencies in the different groups showed that: 1) treatment with TA alone does not affect the spontaneous mPC incidence; 2) the frequency of MMS-induced mPC decreased only in some TA pre-treated males; 3) the decrease of mPC counts in TA pre-treated males was not dose-dependent. These results indicate that the TA-induced protection against genotoxicity must be taken with caution.

RESUMEN

El efecto del ácido tánico (AT) de la compañía Vetec Química Fina Ltda., Brasil, en la frecuencia de eritrocitos policromáticos micronucleados (EPm) espontáneos e inducidos por metilmetanosulfonato (MMS) fue investigado en la médula ósea de ratón. AT (500 mg/kg) fue administrado por una sonda intragástrica a ratones BALB/c machos y hembras adultas como tratamiento único, 6 h antes de una inyección intraperitoneal (ip) de MMS (50 mg/kg), al mismo tiempo del MMS y 6 h después del tratamiento con MMS. Para estimar la actividad de dosis respuesta, fueron probadas tres dosis de AT (250, 500 y 750 mg/kg) como pre-tratamiento de 6 h. Los análisis de la frecuencia de EPm en los diferentes grupos mostraron que: 1) un solo tratamiento de AT no afecta la incidencia de EPm espontáneos; 2) la frecuencia de EPm inducida por MMS disminuyó solamente en algunos machos pre-tratados con AT; 3) la disminución de la frecuencia de EPm en machos pre-tratados con AT no fue dependiente de la dosis. Estos resultados indican que la protección contra la genotoxicidad inducida por AT debe ser analizada con cuidado.

INTRODUCTION

Tannins are widespread compounds that occur in a variety of plants, including monocotyledons, dicotyledons and ferns. They are natural contents of beverages such as tea, coffee and wine and are also added as flavoring agents to beverages, ice cream, sweets, baked goods and liquors (Bichel and Bach 1968, Hartman and Shankel 1990). It is estimated that the human consumption of tannins is more than 1 g/day per person (Ramel *et al.* 1986).

Earlier studies suggested that tannic acid (TA) displays acute hepatotoxic effects (Wells *et al.* 1942) and can induce liver tumors in laboratory rodents (Korpássy and Mosonyi 1950, Kirby 1960, Bichel and Bach 1968). In spite of these observations, data are insufficient to clearly indicate that TA is carcinogenic in humans (Enomoto 1987). On the other hand, *Polygonum multiflorum* extract in which tannins were the major components significantly inhibited the tumorigenic activity of benzo[*a*]pyrene (BaP) in male rats (Horikawa *et al.* 1994) and polycyclic aromatic hydrocarbon-induced skin tumorigenesis in mice (Mukhtar *et al.* 1988).

Experimental evidences of the genotoxic activity of TA are

scarce and controversial. There are some indications that this compound has clastogenic activity in mammalian cells *in vivo* (Sharma *et al.* 1982) and *in vitro* (Stich and Dunn 1986), as well as a moderate mutagenic activity in somatic eye cells of *Drosophila melanogaster* (Szakmary and Knasmuller 1991). After addition of microsomal S9 mixture, TA exerted some mutagenic effect in the *Salmonella* test (Kuo *et al.* 1992).

In contrast to those findings, TA works as an antimutagenic factor in mutagenesis induced by 4-nitroquinoline-1-oxide (4NQO) or by UV radiation (UV) in bacteria (Shimoi *et al.* 1985). In cultured mammalian cells, TA decreases the frequencies of chromosome aberrations induced by UV, MMS or mitomycin C (MMC), but not those induced by X-rays or bleomycin (Sasaki *et al.* 1988). *In vivo* studies demonstrate that TA suppresses the mutagenic and clastogenic effects of MMC, ethyl nitrosourea (ENU) or 4NQO in mice (Sasaki *et al.* 1990, Imanishi *et al.* 1991), as well as those of aflatoxin B_1 in rats (Ito *et al.* 1989).

In a previous work, our group demonstrated that TA (Vetec) displays a significant co-mutagenic activity on the frequency of MMC-induced ring-X loss in *Drosophila melanogaster* . On the other hand, TA does not interfere with the genotoxic activity of MMS (Cunha *et al.* 1994).

The present study was aimed at determining the genotoxicity of TA from Vetec Química Fina Ltda., Brazil (Vetec) and its possible effect on the genotoxicity of the direct-acting mutagen MMS. For this objective we used the adult mouse bone marrow micronucleus (MN) test.

MATERIALS AND METHODS

Inbred BALB/c male and female mice, 12-16 weeks old, housed with food and water *ad libitum* in a room with controlled temperature and light, in the animal facilities at the Instituto de Biociências, Universidade Federal do Rio Grande do Sul, were used.

MMS, purchased from Sigma Chemical Company (St. Louis, MO), was dissolved in distilled water and administered by intraperitoneal (ip) injection. TA ($C_{76}H_{52}O_{46}$, CAS number 1401-55-4) from Vetec was dissolved in 5% sucrose and administered by gavage in a volume of 10 ml/kg of body weight.

In a first set of experiments 500 mg/kg of TA was administered to the mice as pre-, co- and post-treatment, respectively, 6 h before 50 mg/kg of MMS, together with it and 6 h after MMS treatment. The treatment schedule was based on Sasaki *et al.* (1990) and the MMS dose on Sato et al. (1990). In a subsequent confirmatory experiment, doses of 250, 500 and 1000 mg/kg of TA were tested 6 h before the MMS dose. Negative control groups received oral sucrose and ip water treatment. The effect of TA was measured in animals that received 500 mg/kg of TA by gavage and distilled water by ip injection. The MMS effect was measured in those mice that received oral sucrose and ip treatment of 50 mg/kg of MMS.

The mice were killed by cervical dislocation 24 h after the ip treatment. The bone marrow was removed and prepared as described by Salamone *et al.* (1980). Slides were coded, air-dried overnight, fixed with methanol and stained in May Grünwald-Giemsa solution. The frequency of polychromatic erythrocytes (PC) was obtained by counting 500 erythrocytes (PC + normochromatic erythrocytes) per slide, 1000 per animal. The mPC frequencies were determined by scoring 2000 PC per animal in the first experiment and 3000 in the subsequent experiment. The statistical analysis was performed with the data obtained in each 1000 PC, and expressed as average. The number and sex of the treated animals as well as the statistical test used are mentioned in **tables I** and **II**.

RESULTS AND DISCUSSION

The bone marrow mPC frequencies in mice treated with TA only (500 mg/kg) are not different from those of the negative control groups (**Tables I** and **II**). This result suggests that at this dose, TA from Vetec is not genotoxic to BALB/c mice as was detected by the MN test. In all situations MMS treatment increased the frequency of mPC significantly.

Our first experiment showed that TA reduced the mPC frequency induced by MMS in males only when administered as pre-treatment, but not simultaneously or post-treatment to MMS. None of those TA treatments had any effect in females (**Table I**). Similar results were reported by Sasaki *et al.* (1990) with TA (hydrolysate of Chinese gallotannin from Wako Pure Chemical Industries, Japan) in ddY male mice. The authors showed that 500 mg/kg of TA reduced the frequency of mPC induced by MMC, ethyl nitrosourea (ENU) and 4NQO, as well as the frequency of recessive color spots induced by ENU, only when administered as 6 h pre-treatment. The MMC-induced mPC frequency was also reduced by 6 h oral pre-treatment with green and black tea TAs (Imanishi *et al.* 1991). Ito *et al.* (1989) demonstrated that male rats given the water extract of green tea or a tannin mixture extracted from green tea as 24 h pre-treatment suppressed bone marrow chromosomal aberrations induced by aflatoxin B_1 (AFB_1). When the treatment was given only 2 h before or after the AFB_1 administration, the suppressing effect did not appear, suggesting an indirect action of the tea extracts.

In a second confirmatory and dose-response experiment the number of PC examined per animal was increased. The results obtained (**Table II**) confirmed the preceding observation -only in males does TA pre-treatment have an inhibitory effect on the clastogenicity induced by MMS, as pointed out by the results of the X^2 test. If we examine the individual data we can observe that the significance of the protection was developed in 2 to 4 animals in each TA pre-treated group. So, if we employ a Mann-Whitney test, the significance remains only in males dosed with 500 mg/kg, whereas in the other two tested doses the differences disappear. As discussed by Ashby and Tinwell (1995), irrespective of the statistical significance of the effect, a reproducible response has been elicited and possibly presents some biological significance. We can thus suggest that a 6 h pre-treatment of TA from Vetec, at the doses tested, induces

TABLE I. EFFECT OF TANNIC ACID, AS PRE-, CO- AND POST-TREATMENT ON THE FREQUENCY OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES INDUCED BY METHYL METHANESULFONATE

Treatment (mg/kg)	Number of animals	Sex	Number of analyzed PC	mPC/1000PC per animal[a]	mPC/1000PC Mean ± SD	PC/PC+NC ± SD
Controls						
negative	5	M	10,000	1.5; 1; 4.5; 3; 2.5	2.5 ± 1.40	0.38 ± 0.13
	5	F	10,000	1; 2; 1.5; 3.5; 1.5	1.9 ± 1.20	0.40 ± 0.10
TA (500)	5	M	10,000	0.5; 1.5; 2.5; 2; 3.5	2.0 ± 1.41	0.30 ± 0.12
	5	F	10,000	3; 2.5; 3; 2; 3	2.7 ± 1.11	0.39 ± 0.10
MMS (50)	5	M	10,000	31.5; 42.5; 30; 21; 26.5;	30.2 ± 8.10*	0.39 ± 0.09
	4	F	8,000	26.5; 28; 27; 29	27.6 ± 2.56*	0.37 ± 0.06
TA (500)+ MMS(50)						
pre-	5	M	10,000	18.5; 19; 25; 36.5; 19	23.6 ± 7.54*#	0.31 ± 0.12
	5	F	10,000	30; 21.5; 26.5; 37; 37	30.4 ± 6.51*	0.31 ± 0.16
co-	5	M	12,000	32; 28; 30; 24; 25	27.9 ± 3.70*	0.42 ± 0.14
	5	F	10,000	27; 30; 22; 22; 22	24.6 ± 3.67*	0.35 ± 0.08
post-	5	M	10,000	34.5; 19; 22; 44; 31	30.1 ± 10.03*	0.32 ± 0.05
	5	F	10,000	30; 30, 27.5; 23.5; 28	27.8 ± 3.42*	0.31 ± 0.10

MMS = methyl methanesulfonate; **mPC** = micronucleated PC; **NC** = normochromatic erythrocytes; **negative** = oral sucrose and ip water treatment; **PC** = polychromatic erythrocytes; **TA** = tannic acid; [a] the data represent the average of duplicate slides; * significantly higher than negative control group ($p < 0.01$); # significantly lower than MMS control group ($p < 0.05$) by X^2 test

TABLE II. EFFECT OF PRE-TREATMENT WITH DIFFERENT DOSES OF TANNIC ACID ON THE FREQUENCY OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES INDUCED BY METHYL METHANESULFONATE

Treatment (mg/kg)	Number of animals	Sex	Number of analyzed PC	mPC/1000PC per animal[a]	mPC/1000PC Mean ± SD	PC/PC+NC ± SD
Controls						
negative	3	M	9,000	4; 2.7; 1.7	2.8 ± 1.20	0.36 ± 0.11
	3	F	9,000	1.7; 1; 2	1.6 ± 0.73	0.43 ± 0.03
TA (500)	3	M	9,000	2; 3.7; 2.7	2.8 ± 0.88	0.33 ± 0.15
	3	F	9,000	1.3; 1.7; 1.7	1.6 ± 0.88	0.42 ± 0.11
MMS (50)	3	M	9,000	21.7; 22; 25	22.9 ± 2.15*	0.31 ± 0.03
	3	F	9,000	17.3; 27.7; 21.3	22.1 ± 5.53*	0.50 ± 0.06
TA+MMS (250)+(50)	5	M	15,000	7; 20.7; 17.3; 30.3; 13	17.7 ± 8.39*#	0.44 ± 0.14
	5	F	15,000	19; 24; 21; 10; 21.7	19.1 ± 6.05*	0.42 ± 0.14
(500)+(50)	5	M	15,000	11.3; 12.3; 17.3; 12.3; 34	17.5 ± 9.07*#z	0.38 ± 0.14
	5	F	15,000	22; 26.3; 11.3; 21.3; 21	20.4 ± 5.38*	0.47 ± 0.08
(700)+(50)	5	M	15,000	21.3; 10.7; 23.7; 22.7; 10.7	17.8 ± 6.66*#	0.36 ± 0.10
	5	F	15,000	26.7; 24.7; 22.7, 22.3; 26	24.5 ± 2.75*	0.50 ± 0.06

For abbreviations see Table I; [a] the data represent the average of duplicate slides; * significantly higher than negative control group ($p < 0.01$); # significantly lower than MMS control group ($p < 0.05$) by X^2 test; [z] significantly lower than MMS control group ($P < 0.05$) by Mann-Whitney test.

protection against MMS clastogenicity at least in some males.

TA (Aldrich Chemical Co.) inhibited the bacterial mutagenicity induced by nitropyrene (NP), but when S9 mix was added the inhibitory potency decreased. In presence of S9 mix some increase in mutation frequency was found when TA was tested alone (Kuo *et al.* 1992). Also in CHO cells, TA had significant protective effects on NP-induced cytotoxicity at low concentrations (at high concentrations, TA exerted highly toxic effects) and caused a significant reduction of the frequency of sister-chromatid exchanges. As TA has been shown to increase the glutation level and glutation-S-transferase activity in CHO cells, Kuo *et al.* (1992) suggest that the inhibitory effect can be due to its action on metabolic or detoxification enzymes.

It is also known that TA can inhibit mutagenesis by: a) inactivation of exogenous mutagens; b) inhibition of mutagen reactions with DNA (Conney 1982; Mukhtar *et al.* 1988); c) acting as an antioxidant (Hartman and Shankel 1990); d) enhancing the excision-repair activity (Shimoi *et al.* 1985, Sasaki *et al.* 1988 and 1990). However, these protective activities are in contrast with: a) the moderate mutagenic activity observed in both sexes and the synergistic effect of TA (Fluka-Switzerland) with direct-acting mutagens such as 4-NQO, MMS and cis-platinum, observed only in *D. melanogaster* males (Szakmary and Knasmuller 1991); b) the synergistic effect with X-rays in *Tradescantia*, results attributed to the inhibitory action of TAs (Fluka-Switzerland and Sigma Chemical Company) on the DNA repair process (Knasmuller *et al.* 1992); c) the co-mutagenic action on the frequency of ring-X loss when MMC-treated spermatozoa of *D. melanogaster* were used to fertilize oocytes previously exposed to different TA (Vetec) concentrations and the absence of this effect on the ge-notoxic activity of MMS (Cunha *et al.* 1994). The last responses are interpreted as a TA activation of uvrABC-type enzymes in the oocytes leading to an increase in double-strand breaks and to a lack of a TA effect on the activity of AP endonuclease.

In fact, TAs from different commercial sources (including Merck, Sigma, Aldrich and Fluka) have shown different behavior in alternative tannin assays (Makkar and Becker 1993). Those differences could be due to different proportions of tannin moieties in the preparations, influencing the response of the biological test systems. This fact may explain the controversial results on the mutagenic and/or antimutagenic action of TAs. Shimoi *et al.* (1985) found that besides TA, other tannins showed bio-antimutagenic effect. Horikawa *et al.* (1994) demonstrated that the ethyl acetate extract obtained from *P. multiflorum* strongly inhibited the mutagenicity of B[*a*]P in *Salmonella typhimurium* with S9 mix, similar to the TA (Wako) used as control. Knasmuller *et al.* (1992) found that the TA from Fluka seemed to have slightly higher mutagenic activity than that from Sigma.

Our results do not indicate the mechanism of TA modulation on MMS genotoxicity. The inhibition of clastogenic action of MMS observed by us only in pre-treated male mice suggest a sex restricted action of TA from Vetec. Even if co-mutagenic, the synergistic effect of TA with direct acting mutagens, including MMS, was detected also only in *D.melanogaster* males by the eye mosaic spots test (Szakmary and Knasmuller 1991). These results are taken as an indication of an antirecombinogenic activity of TA in females.

Taken together, all the above results suggest that TAs from different origins can act by preventing or increasing DNA lesions induced by different genotoxins. Consequently, these controversial findings indicate that the reported protective activity of tannins must be taken with caution and requires further evaluation.

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