

方法: 羟胺特异裂解天花粉蛋白唯一 Asn-Gly 肽键。制备性凝胶电泳获 HATf1 和 HATf2 二片段。免疫印迹确定天花粉蛋白上不同表位并筛选抗体。兔网织红无细胞系统测定天花粉蛋白及片段对蛋白合成的抑制活性。结果: HATf1 和 HATf2 纯度各达 96.6 % 和 80.5 %。HATf1 保留完整天

花粉蛋白的抑制活性。第 14 号和第 16 号抗天花粉蛋白单抗与二片段显示不同免疫反应性, 并用于封闭试验。第 14 号单抗能封闭天花粉蛋白及 HATf1 活性, 而第 16 号单抗则否。结论: 天花粉蛋白抑制蛋白质生物合成的活性部位位于 HATf1 侧, 近二部分交界。

Detection of DNA damage in peripheral lymphocytes by 7 compounds using comet assay¹

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KEY WORDS gel electrophoresis; single-stranded DNA; mutagenicity tests; hydrogen peroxide; ethyl methanesulfonate; dimethylnitrosamine; mitomycin C; benzo(a)pyrene; cyclophosphamide; 2-aminofluorene

AIM: To detect the DNA single strand breaks (SSB) in peripheral lymphocytes of mice, rats, and human induced by hydrogen peroxide (H_2O_2), ethyl methane sulphonate (EMS), dimethylnitrosamine (DMNA), mitomycin C (MMC), benzo(a) pyrene (BaP), cyclophosphamide (CP), and 2-aminofluorene (2-AF). **METHODS:** Alkaline single cell microgel electrophoresis assay *in vitro* (comet assay). **RESULTS:** All were positive with 2 exceptions: EMS ($0.97 \text{ mmol} \cdot \text{L}^{-1}$) in mice and MMC ($30 \mu\text{mol} \cdot \text{L}^{-1}$) in mice and human. The lowest concentrations detectable were H_2O_2 ($1 \mu\text{mol} \cdot \text{L}^{-1}$), EMS ($0.48 \text{ mmol} \cdot \text{L}^{-1}$), BaP ($5.0 \mu\text{mol} \cdot \text{L}^{-1}$), CP ($2.0 \text{ mmol} \cdot \text{L}^{-1}$), MMC ($10 \mu\text{mol} \cdot \text{L}^{-1}$), DMNA ($27.3 \text{ mmol} \cdot \text{L}^{-1}$), and 2-AF ($62.5 \mu\text{mol} \cdot \text{L}^{-1}$). CP, BaP, and 2-AF were positive only in the presence of metabolic activation system. **CONCLUSION:** H_2O_2 , DMNA, BaP, CP, and 2-AF induce SSB in

peripheral lymphocytes of mice, rats, and human detected by comet assay, whereas MMC induces SSB only in rats, and EMS in rats and human lymphocytes.

The single cell microgel electrophoresis assay (SCGE, comet assay) can quantitatively detect DNA single strand breaks (SSB) at the individual cell level in virtually any eukaryote cell population both *in vivo* and *in vitro*. With the advantage of need for extremely small numbers of cells per sample without radiation labeling, this sensitive, simple and cost-effective novel technique is promising in fast screening the genotoxicity of drug candidates^[1-7]. This study aimed to investigate 7 compounds with different genotoxic mechanisms for their potentialities to induce SSB in peripheral blood lymphocytes of mice, rats, and human using *in vitro* comet assay.

MATERIALS AND METHODS

Na_2 -edetic acid (Shanghai No 1 Reagent Factory), Tris, Me_2SO , trypan-blue (EMK), Triton X-100 (Farco), normal melting point agarose (NMA, Sigma Type I-A, mp = 88°C), low melting point agarose (LMA, Promega, mp = 64°C), ethidium bromide (Fluka), lymphocyte separation medium (LSM, Shanghai Huajing Biological Co), RPMI 1640 medium (Gibco BRL), fetal bovine sera (Sijiqing Institute of Biomaterials, Hangzhou).

Fully frosted slides ($25 \text{ mm} \times 75 \text{ mm}$), coverglass ($25 \text{ mm} \times 25 \text{ mm}$), horizontal electrophoresis unit (Shanghai DP

¹ Funded by the National Project of Doctor Research on Novel Pharmaceutical Technique, 1995-1996.

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Received 1996-09-26

Accepted 1997-04-04

Organic Glassware Factory). DY-A electrophoresis apparatus (Shanghai Institute of Biochemistry), binocular fluorescent microscope (Zeiss Axiolab, Germany).

Test compounds H_2O_2 (Shanghai Taopu Chemical Factory), CP (Shanghai No 12 Pharmaceutical Factory), EMS (E Merck), and MMC (Kyowa, Japan) were dissolved in PBS. BaP (Sigma), DMNA (Sigma), and 2-AF (Aldrich) were dissolved in Me_2SO . All the compounds were prepared immediately before use.

Isolation of lymphocytes Healthy human blood was provided by Shanghai Blood Center. Kunming mouse and SD rat blood samples were taken from the heart. Heparinized whole blood 1.5 mL was mixed with 1.5 mL normal saline, carefully added onto the top of 1.5 mL LSM (density 1.077, 1.085, 1.087 $kg \cdot L^{-1}$ for human, mouse, and rat lymphocytes, respectively) spun at $400 \times g$ for 30 min. The lymphocyte fraction was washed twice in 1.0 mL cold RPMI 1640.

Preparation of rat microsomal metabolic enzyme system (S_9 Mix): SD rats (δ , 180–200 g, Shanghai Experimental Animal Center, Chinese Academy of Sciences, Certificate No 005) were killed on d 5 after ip aroclor 1254 (500 $mg \cdot kg^{-1}$), the livers were washed with 3-fold volume of cold KCl 150 $mmol \cdot L^{-1}$ (containing PBS 10 $mmol \cdot L^{-1}$, pH 7.4) and homogenized in sterile condition. The mixture was spun at $9000 \times g$ for 10 min, the supernatant collected as S_9 was stored at $-70^\circ C$. The S_9 Mix was prepared by 1.5 mL S_9 , co-enzyme II 21.0 mg (70%), and G-6-P 7.6 mg, adjusted to 5.0 mL by sterile water.

SCGE protocol Cells ($1 \times 10^6/L$ PBS) were treated *in vitro* in a shaking water-bath at $37^\circ C$ for 1 h. Each test was performed with a negative (solvent) control, a positive control, and 2–3 concentrations of test compound. A 1% Me_2SO and 3% S_9 Mix were added when needed. Viability was determined by 0.4% trypan-blue staining before and immediately after incubation and calculating the survival rate compared to corresponding solvent controls. At the end of treatment, the cells were pelleted, washed with cold PBS, mixed with molten 0.5% LMA, and maintained at $37^\circ C$.

Microgel electrophoresis was done basically according to [3] with some modifications. Briefly, on the frosted slide, the first layer was 0.5% NMA (50 μL /slide), the second, mixture of cells and 0.5% LMA (50 μL /slide) and the third, LMA without cells. The cells were lysed in the cold fresh solution ($NaCl$ 2.5 $mol \cdot L^{-1}$, Na_2 -edetic acid 100 $mmol \cdot L^{-1}$, Tris 10 $mmol \cdot L^{-1}$, pH 10, with fresh 1% Triton X-100 and 10% Me_2SO added) for 1 h, then placed in fresh electrophoresis buffer (Na_2 -edetic acid 1, $NaOH$ 300 $mmol \cdot L^{-1}$). The DNA was allowed to unwind for 20 min and then subjected to electrophoresis at 25 V, 300 mA for 20 min. All steps were performed under deem red light. After the electrophoresis was completed, the slides were neutralized with Tris 0.4 $mol \cdot L^{-1}$ for 3×5 min, stained with ethidium bromide (20 $mg \cdot L^{-1}$, 35 μL /

slide), and observed at $400 \times$ magnification with an excitation filter of 515 nm–560 nm, and a barrier filter of 590 nm. The comet tail length (TL, μm) was measured using a calibrated scale in the ocular of the microscope from 100 randomly captured cells per slide, 3 slides per concentration. The calculated value of median TL in test minus control (T–C) was a direct reflection of induced damage. Positive results were defined as increases in the T–C $> 12.5 \mu m$ in reproduced experiments. The data obtained were transformed by natural log to normalize distributions and to generate data for a one tailed *t*-test.

RESULTS

In negative control groups, the cell viabilities in lymphocytes of mice, rats, and human were $92\% \pm 5\%$ ($n=14$), $89\% \pm 4\%$ ($n=11$), and $87\% \pm 6\%$ ($n=9$), respectively.

All were positive with 2 exceptions: EMS 0.97 $mmol \cdot L^{-1}$ in mice and MMC 30 $\mu mol \cdot L^{-1}$ in mice and human (Tab 1, Fig 1, Plate 2).

The lowest concentrations detectable were H_2O_2 1 $\mu mol \cdot L^{-1}$, EMS 0.48 $mmol \cdot L^{-1}$, BaP 5.0 $\mu mol \cdot L^{-1}$, CP 2.0 $mmol \cdot L^{-1}$, MMC 10 $\mu mol \cdot L^{-1}$, DMNA 27.3 $mmol \cdot L^{-1}$ and 2-AF 62.5 $\mu mol \cdot L^{-1}$. CP, BaP and 2-AF were positive only in the presence of metabolic activation system. The difference of DMNA (without S_9 Mix)- and CP (with S_9 Mix)-induced DNA damage to human lymphocytes was shown from the distribution of comet tail length (Fig 2).

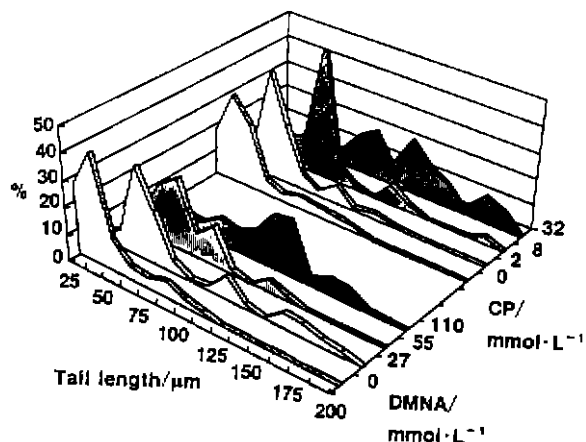


Fig 2. Distribution of comet tail length among human blood lymphocytes exposed to DMNA (without S_9 Mix), CP (with S_9 Mix). Data based on 100 cells per dose group.

DISCUSSION

The cell viability and TL in negative control

Tab 1. Comet assay of mouse, rat, and human lymphocytes exposed to 7 tested compounds.

Treatment		Mouse lymphocytes			Rat lymphocytes			Human lymphocytes		
		Viability* abs/rel (%)	Median TL μm^{b}	TL _{T-C} $\mu\text{m}^{\text{†}}$	Viability abs/rel (%)	Median TL μm	TL _{T-C} μm	Viability abs/rel (%)	Median TL μm	TL _{T-C} μm
PBS	10 $\mu\text{L}/\text{tube}$	89/100	27		84/100	25		88/100	33	
H ₂ O ₂	1 $\mu\text{mol}\cdot\text{L}^{-1}$	85/96	46	19	90/107	57	32	78/89	41	8
	3.3	83/93	65	38	80/95	88	63	74/84	57	24
	10	87/98	109	82	76/90	156	131	69/78	91	58
PBS	10 $\mu\text{L}/\text{tube}$	90/100	29		95/100	25		90/100	35	
EMS	0.24 $\text{mmol}\cdot\text{L}^{-1}$	82/91	32	3	87/92	35	10	80/89	35	0
	0.48	86/96	33	4	70/74	77	52	73/81	55	20
	0.97	76/84	41	12	81/85	56	31	70/78	84	49
H ₂ O ₂	10 $\mu\text{mol}\cdot\text{L}^{-1}$	80/89	77	48	76/87	84	49	82/91	95	60
PBS	5 $\mu\text{L}/\text{tube}$	96/100	28		91/100	26		84/100	34	
MMC	3.3 $\mu\text{mol}\cdot\text{L}^{-1}$	87/91	36	8	93/102	36	10	86/102	36	4
	10	80/83	32	4	85/93	39	13	78/93	40	6
	30	82/85	39	11	78/86	54	28	70/83	45	11
H ₂ O ₂	10 $\mu\text{mol}\cdot\text{L}^{-1}$	90/94	85	57	84/92	97	71	77/92	88	52
Me ₂ SO	5 $\mu\text{L}/\text{tube}$	89/100	25		85/100	29		93/100	29	
DMNA	27.3 $\text{mmol}\cdot\text{L}^{-1}$	84/94	42	17	79/93	33	4	88/95	40	11
	55	79/89	68	43	84/99	42	13	79/85	74	45
	109	67/75	83	58	85/100	53	24	70/75	136	107
H ₂ O ₂	10 $\mu\text{mol}\cdot\text{L}^{-1}$	80/90	91	66	80/94	62	33	85/91	81	52
PBS	10 $\mu\text{L}/\text{tube}$	86/100	28		85/100	31		90/100	31	
CP	2 $\text{mmol}\cdot\text{L}^{-1}$	89/103	44	16	90/106	42	11	84/93	50	19
	8	82/95	61	33	84/99	84	53	77/86	68	37
	32	79/92	101	73	77/90	99	68	79/88	125	94
PBS	5 $\mu\text{L}/\text{tube}$	96/100	30		97/100	27		89/100	35	
2-AF	15.6 $\mu\text{mol}\cdot\text{L}^{-1}$	79/82	35	5	90/93	36	9	81/91	41	6
	62.5	83/86	42	12	84/87	50	23	76/85	46	11
	250	70/73	78	48	76/78	141	114	84/84	51	16
CP	8 $\text{mmol}\cdot\text{L}^{-1}$	84/88	92	62	91/94	77	50	81/91	66	31
Me ₂ SO	5 $\mu\text{L}/\text{tube}$	97/100	25		89/100	24		94/100	28	
BaP	5.0 $\mu\text{mol}\cdot\text{L}^{-1}$	90/93	30	5	67/75	26	2	87/93	41	13
	45.0	85/88	50	25	84/94	57	33	80/85	48	20
	405.0	79/81	121	96	77/86	49	25	77/82	55	27
CP	8 $\text{mol}\cdot\text{L}^{-1}$	91/94	77	52	86/97	69	45	86/91	61	33

* Absolute No of viable cells in relation to total No of cells, as determined by trypan-blue exclusion; relative No of viable cells based on 100 % survival in the control.

^b Values were calculated from 100 cells per slide, 3 slides per determination.

[†] 'positive' when TL_{T-C} > 12.5 μm .

groups were in accordance with those (95.4 % \pm 2.3 %, 29 \pm 6 μm)⁽³⁾, showing that the experiment condition was stable. In most of the treated groups, the cell viabilities were > 75 %, indicating that the cytotoxicity level was low.

CP, BaP, and 2-AF were indirect mutagens, therefore were positive only in the presence of

metabolic activation system. The negative result of MMC may be due to that the mice and human lymphocytes were less sensitive to this compound.

The result was clear enough when TL was used as DNA damage indicator, although comet tail moment (TL \times fluorescent strength of comet tail) was more useful if image analysis system was available.

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ACKNOWLEDGMENT To Prof XU Wen-Si for his valuable direction in this project.

彗星试验检测 7 种化合物
对外周血淋巴细胞 DNA 的损伤¹

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关键词 凝胶电泳; 单链 DNA; 致突变试验; 过氧化氢; 甲磺酸乙酯; 二甲基亚硝胺; 丝裂霉素 C; 苯并(a)芘; 环磷酰胺; 2-氨基苄 **淋巴细胞 DNA 损伤**

目的: 检测过氧化氢(H₂O₂)、甲磺酸乙酯(EMS)、丝裂霉素 C (MMC)、二甲基亚硝胺(DMNA)、苯并(a)芘(BaP)、2-氨基苄(2-AF)和环磷酰胺(CP)诱发小鼠、大鼠及人外周血淋巴细胞 DNA 单链断裂。 **方法:** 体外单细胞微量凝胶碱性电泳试验(彗星试验)。 **结果:** 除 EMS 0.97 mmol·L⁻¹ 在小鼠淋巴细胞, MMC 30 μmol·L⁻¹ 在小鼠、人淋巴细胞中呈阴性外, 其余均为阳性。最低可检测浓度分别为 H₂O₂ 1 μmol·L⁻¹, EMS 0.48 mmol·L⁻¹, BaP 5.0 μmol·L⁻¹, CP 2.0 mmol·L⁻¹, MMC 10 μmol·L⁻¹, DMNA 27.3 mmol·L⁻¹, 2-AF 62.5 μmol·L⁻¹。 CP、BaP、2-AF 需经 S₉ Mix 代谢活化才显示毒性。 **结论:** 彗星试验检测出 MMC 诱导大鼠, EMS 诱导大鼠和人, 以及 H₂O₂、DMNA、BaP、CP 和 2-AF 诱导小鼠、大鼠和人外周血淋巴细胞 DNA 单链断裂损伤。

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