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IN-VITRO MUTAGENIC, GENOTOXIC AND ANEUGENIC POTENTIAL OF OMEPRAZOLE

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ABSTRACT: The aim of this investigation was to re-evaluate the *in-vitro* mutagenic, genotoxic, and aneugenic potential of omeprazole through the Ames test, comet (SCGE), and cytokinesis-block micronucleus assays (CBMN), as previous reports on the mutagenic and genotoxic effects of omeprazole are scarce and inconclusive. The results of this study indicated a negative mutagenic effect through the Ames test. Comet assay endpoints such as tail length, tail intensity, and tail moment showed a mild genotoxic effect with dose-dependence. The CBMN assay endpoints were implemented according to the requirements for *in-vitro* evaluation of genotoxicity after 4 h of exposure, with or without metabolic activation. Endpoints, such as binucleated cells with micronuclei (BNMN), total micronuclei (MN), arrested metaphasis (AM), nuclear buds (NB), nucleoplasmic bridges (NPB), and necrotic cells (NC), showed a dose-dependent effect. The number of apoptotic cells (AP) showed a statistically significant increase compare to the control value but was without dose-dependence. This *in-vitro* study suggests omeprazole exhibits mild genotoxic and aneugenic effects.

INTRODUCTION: Omeprazole (CAS # 73590-58-6) is one of the most widely used proton pump inhibitors (PPI) for the treatment of various gastrointestinal disorders. The mutagenicity, carcinogenicity and genotoxicity of this drug have been an important research goal for many investigators. Although this drug has consistently shown negative results in the Ames test and gene mutations on mouse lymphoma thymidine kinase (TK) locus [1-3], the outcomes of many other assays have been variable, making it challenging to draw conclusions surrounding its genotoxicity [4-9]. Additionally, computer prediction modeling has revealed omeprazole to be a potential genotoxicant [10].

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The long-term carcinogenic assays performed on male rats exhibited strongly positive results [1, 8, 11]. Also, the micronucleus rate in human lymphocytes increases after *in-vitro* exposure [7], as well as after *in-vivo* exposure in patients treated with omeprazole [12]. It has not been possible to formulate a positive genotoxic agreement for this drug on the basis of the above-mentioned results. Finally, recent epidemiological meta-analysis investigations have revealed that proton pump inhibitors increase carcinogenic risks in humans, increasing the risk for gastric, esophageal, and pancreatic cancers [13-20].

The current literature on the carcinogenic and genotoxic effects of omeprazole suggests that the majority of the available data are inconclusive for reliable assessment of the potential genotoxic or carcinogenic risk to humans. However, the testing guidelines for the above-mentioned assays have undergone significant changes during the last twenty years. It has to test omeprazole according to the present guidelines when safety of long-term use

in humans is doubtful or not ascertained, if some evidence suggests genotoxicity and carcinogenicity activity in animals and humans [8, 21, 22].

The aim of this investigation was to re-evaluate the *in-vitro* mutagenic, genotoxic, and aneugenic potential of omeprazole a variety of endpoints, including the Ames test, Comet (SCGE), and Cytokinesis-block micronucleus (CBMN) assays on human peripheral blood lymphocytes. The combination of three in vitro tests (Ames, Comet, and CBMN) allows for the detection of a wide range of DNA damage and a more precise risk assessment.

MATERIALS AND METHODS:

Ames test: The mutagenic potential of omeprazole was evaluated, using TA1535, TA1537, TA102, TA98, and TA100 Salmonella tester strains, with and without metabolic activation (±S9). A preliminary test with TA98 and TA100 only was done a week before the main test. All salmonella tester strains, S9-mix, and sterile petri dishes with minimal glucose agar ready for use were obtained from Moltox (USA). Eight different concentrations 0.5, 1, 5, 10, 20, 100, 1000, and 5000 µg/plate of omeprazole, negative and positive controls (obtained from Sigma-Aldrich, see **Table 1** and **Table 2**) were plated in triplicate with overnight cultures of salmonella tester strains and (Histidine/ Biotine Top agar, Moltox) using the plate incorporation method [23-25].

In sterile test tubes, 100 µL of each omeprazole dilution solubilized in DMSO (Sigma-Aldrich), vehicle control (DMSO), or positive control was added to 500 µL of phosphate buffer (Moltox) without metabolic activation –S9, or 500 µL phosphate buffer with metabolic activation of +S9 (Moltox). After incubation at 37 ºC for 20 min, 2.0 mL of top agar 42-45 ºC was added to each tube.

Mixtures of top agar and omeprazole samples were then poured onto petri dishes with minimal agar. When top agar hardened, plates were inverted and incubated in the dark at 37 ºC for 48-72 h. Results from the preliminary and main test are presented in **Table 1** and **Table 2**. The mean and standard deviation of the number of revertants per plate were counted automatically using a colony counter, Sorcerer 2.2, from Perceptive Instruments, UK.

The test was considered positive if a two-fold increase in the colony numbers with TA98, TA102, and TA100, or a threefold increase in colony number with TA1535 and TA1537 with or without dose-response was detected [24, 25].

Comet Assay: An alkaline (pH > 13) Single Cell Gel Electrophoresis (SCGE) Assay or Comet Assay was used to measure the genotoxic potential of omeprazole. The alkaline comet assay is predominately used to detect DNA single-strand breaks (SSB), double-strand breaks, alkali-labile sites (DSB), DNA cross-links, and SSB associated with incomplete excision repair, commonly observed with the use of chemotherapeutic drugs [26-29]. Therefore, SCGE was utilized to determine whether omeprazole exhibited genotoxic activity, potentially playing a role in carcinogenesis. Peripheral blood lymphocytes were isolated from whole blood immediately before use by processing with Ficoll-Paque Plus (Healthcare, Sweden). Six different concentrations of omeprazole 0.5, 1, 4, 8, 16, and 32 µg/mL (dissolved in DMSO) were added tolymphocyte suspensions for a 3 h exposure. Hydrogen peroxide 0.1 M) was used as a positive control (15 min exposure), as depicted in **Table 3** (preliminary) and **Table 4** (main results). Cell viability (dead and live before exposure) was measured by means of Trypan Blue (Sigma) visualization test on a hemocytometer. In this study, cell viability was calculated to be within the frame of 97-99%. After exposure to different concentrations of omeprazole, the lymphocyte suspensions were mixed with low melting agarose and embedded on precoated slides with normal melting agarose. When the first layer was solidified, the coverslip was removed, and a second layer of low-melting agarose was spread on the slides. Subsequently, slides were treated and processed according to the protocols described in literature [27, 29]. Evaluation of this assay included imaging of 100 cells (2 slides × 50 cells) per dose by means of fluorescence microscopy (Nikon eclipse 80i) collected from two independent researchers, and analyzed automatically with Comet Assay IV ™ (Perceptive Instruments, UK). Tail length, tail intensity, and tail moment are the three parameters widely regarded as the most informative measures of DNA damage in the Comet assay.

To test the reproducibility of the results, two different experiments were carried out, presented on **Table 3** (preliminary) and **Table 4** (main results), as well as **Fig. 1**, with or without metabolic activation (±S9).

Cytokinesis-Block Micronucleus Assay: The cytokinesis-block micronucleus assay (CBMN) was employed to assess the genotoxic effects associated with exposure of omeprazole to the human peripheral lymphocytes [30, 31]. The CBMN assay biomarkers [32] evaluated include the proliferative index, binucleated cells with micronuclei (BNMN), total micronuclei (MN), arrested metaphase (AM), nuclear buds (NB), nuclear bridges (NPB), apoptotic (AP), and necrotic cells (NC). Blood samples were taken from three healthy, non-smoking male donors, aged 20, 22, and 23 years. The initial cultures included 4.5 mL of RPMI 1640, enriched with 15% of heat-inactivated fetal calf serum (Invitrogen), and 1% phytohemagglutinin (PHA, Gibco). To each tube was added 0.5 mL whole blood. The choice of omeprazole concentrations (0.1, 0.4, 0.8, 3.2, 6.4, 12.8 µg/mL) was based on the already proposed results [5, 7].

Bleomycin, taking into account its established clastogenic/aneugenic effects, served as a positive control [30-32]. Concentrations of this classical "radiomimetic" included 2, 4, and 8 µg/ml. The cultures were then incubated at 37 ºC in a humidified atmosphere of 5% CO_2. The set of duplicate cultures were treated with omeprazole for 4 h, starting from 40 to 44 hours, as recommended for *in-vitro* investigations [36]. Cytochalasin B, 6 µg/ml was added on 44 h after initiation of the cultures together with fresh medium. At 72 h, all samples were centrifuged, and suspensions were treated with 3mL cold 4 ºC 0.75 M KCl during cells pellet vortexing [37-40] followed immediate centrifugation. The first fixation of cell suspensions was done with 5 mL-cold methanol: glacial acetic acid (3:1) and subsequently 50 µL of formaldehyde was added per culture for 1 h. The next two fixations were done without formaldehyde.

Finally, cell suspensions were spread onto clean slides and stained with KaryoMAX Giemsa Stain (Gibco) for 15 min at pH 6.8 for all endpoint visualization. Slides were coded to ensure blinding and 1000 (2 × 500) binucleated cells were evaluated per donor and dose implemented from two different scorers. Nuclear division index (NDI) was calculated from 500 cells using the formula:

$$[(1 \times MI) + (2 \times M2) + (3 \times M3) + (4 \times M4)] / N$$

Where M1-M4 represents the number of cells with one to four nuclei and N is the total number of intact cells scored [31]. In order to test reproducibility experiment was independently repeated separately with and without metabolic activation (±S9). The mean values from these experiments are shown in **Table 5** and **6**, as well as **Fig. 2** for NDI, BNMN, Arrested Metaphases (AM), nuclear buds (NB), nuclear bridges (NPB), and necrotic cells (NC).

The Institutional Review Board at the University of Findlay, OH, USA, approved the study, the protocol, and the consent form.

Statistics: The fold increase of different tester strains from the Ames test was calculated by dividing the colony numbers of the specific strain treated with positive controls by the colony numbers of the same strain in the solvent-treated controls. Data for individual strains are reported in **Table 1** and **2**. The mean and standard deviation of the number of revertants per plate were counted automatically using a colony counter, Sorcerer 2.2, from Perceptive Instruments, UK.

Comet assay results were analyzed automatically with Comet Assay IV™ software developed by Perceptive Instruments, UK.

Analysis of omeprazole genotoxicity with cytokinesis-block micronucleus test was based on 21 samples (3 each at dosages 0, 0.5, 1, 4, 8, 16, and 32 µg/ml), and analysis for Bleomycin was based on 12 samples (3 each at dosages 0, 2, 4 and 8 µg/ml) after 4 h exposure, both with and without metabolic activation. Data were analyzed using SPSS version 25 (2017, Armonk, NY: IBM Corp). Pearson correlation analysis was performed on NDI, BNMN, MN, AM, NB, NPB, AP, and NC for finding their association (if any) with genetic damage versus control. In order to increase robustness, parametric tests (*e.g.,* Student's t-test) were also performed. For all predictors of DNA damage, linear regression analysis and Analysis of Variance (ANOVA) were performed. A value of $p \leq 0.05$ was set for statistical significance.

Additionally, the criteria for a positive response were the demonstration of a significant, reproducible, and concentration-dependent increase in the number of the comet and CBMN endpoints relative to the number of the same endpoints in the solvent controls.

RESULTS:

Ames Test: Our data from experiments performed within the frame of the Ames test (preliminary with TA98 and TA100 only, and the main test with all strains) with or without S9 mix confirmed that omeprazole is non-mutagenic.

TABLE 1: AMES TEST PRELIMINARY RESULTS (±S9 METABOLIC ACTIVATION)

Concentration of drugs µg/plate		Revertant colonies (means ± SD	
		Base-pair substitution (BPS)	Frameshift (FS)
		TA100	TA98
-S9	Negative Control	86.3±8.7	28.0±3.6
	Omeprazole		
-S9	0.5	86.0±7.8	27.0±5.9
-S9	1	88.3±11.1	28.0±2.4
-S9	5	91.7±6.5	26.3±3.8
-S9	10	91.3±7.4	27.3±3.3
-S9	20	84.0±6.7	24.7±2.9
-S9	100	77.0±4.8	22.7±3.1
-S9	1000	53.7±9.9	15.3±3.7
-S9	5000	17.0±8.3	0.0
	Positive Control	SA	4-NPHD
-S9	0.5	93.3±13.5	103.7±16.3***
-S9	5.0	517.3±84.7***	506.7±20.5***
+S9	Negative Control	92.0±9.4	34.0±3.7
	Omeprazole		
+S9	0.5	96.3±7.9	30.7±2.0
+S9	1	98.0±10.6	35.3±5.4
+S9	5	89.3±7.8	27.7±4.5
+S9	10	95.3±5.4	24.3±2.7
+S9	20	89.8±7.1	22.0±2.9
+S9	100	72.7±7.4	26.7±3.4
+S9	1000	45.0±7.5	21.7±2.5
+S9	5000	5.0±1.6	1.3±1.2
	Positive Control	SA	4-NPHD
+S9	0.5	159.3±35.9**	145.3±14.6***
+S9	5.0	507.0±12.6***	491.0±24.9***

Statistical differences from negative control: *$p<0.05$; **$p<0.01$; ***$p< 0.001$. Negative control and omeprazole dissolved in DMSO. Positive control carcinogens: SA (sodium azide, dissolved in sterile water); 4-NPHD (4-nitro-o-phenylendiamine, dissolved in DMSO)

At the same time, results from positive controls showed statistically significant increased numbers of revertant colonies with or without metabolic activation for all Salmonella strains (TA98, TA100, TA102, TA1535, and TA1537). The fold increase of positive controls compare to solvent controls indicated dose-response and good reproducibility between preliminary and main experiments **Table 1** and **2**.

Comet Assay: The results of the alkaline comet SCGE assay on isolated human lymphocytes suggest that omeprazole acts as genotoxicant **Table 3-4**. Tail length, tail intensity, and tail moment suggest a dose-dependent increase compare to negative controls with or without metabolic activation. DNA damage (tail length) is noted to begin at a concentration of 4 µg/ml for preliminary and main experiments without activation (-S9), as well as from 1 µg/ml for both (+S9) experiments.

This result was confirmed in the second (main) experiment. The maximum DNA damage is reached at 16 µg/ml for the preliminary experiment, as well as for the main experiment without metabolic activation (-S9). Maximal damage is seen at a similar concentration (16 µg/ml) for other endpoints investigated (Tail intensity or Tail moment). The highest dose of omeprazole tested (32 µg/mL) was cytotoxic.

Omeprazole dose-dependency is demonstrated on **Fig. 1** preliminary (A, C, E), as well as main results (B, D, F) with and without metabolic activation for Tail length, Tail intensity, and Tail moment.

Hydrogen peroxide (positive control), induced statistically significant increases of DNA damage compared to negative control and omeprazole DNA damage from both experiments with this assay.

TABLE 2: AMES TEST MAIN RESULTS (±S9 METABOLIC ACTIVATION)

Concentration of drugs µg/plate		Revertant colonies (means ± SD)				
		Base-pair substitution (BPS)		BPS, FS	Frameshift (FS)	
		TA 1535	TA 100	TA 102	TA 1537	TA 98
-S9	Negative Control	10.3±2.3	81.0±4.9	230±28.9	9.7±2.3	32.3±2.6
	Omeprazole					
-S9	0.5	10.7±3.6	91.0±4.5	231±20.7	9.0±1.6	28.3±3.4
-S9	1	10.3±1.2	98.0±9.4	223±10.6	12.0±2.9	26.7±4.0
-S9	5	9.7±2.5	86.6±5.3	228±20.0	9.0±2.2	29.3±2.5
-S9	10	9.3±1.3	86.7±4.2	219±26.6	11.7±2.6	29.3±5.7
-S9	20	8.7±2.0	81.3±5.3	219±15.2	8.3±1.7	25.6±1.7
-S9	100	10.0±1.6	80.7±7.9	213±24.4	10.7±1.2	25.7±6.1
-S9	1000	5.0±1.7	51.7±7.4	148±17.6	6.0±0.8	20.7±4.5
-S9	5000	0.7±0.5	12.0±2.4	0.3±0.5	2.3±1.2	0.0±0.0
	Positive Control	SA	SA	MMC	9-AA	4-NPHD
-S9	0.5	94.0±6.6***	78.3±8.3	659±60.3***	107.3±9.7***	96.7±10.6***
-S9	5.0	374.7±81.7***	479.7±44.3***	1104±159.4***	583.7±40.7***	492.0±32.9***
+S9	Negative Control	12.3±1.7	92.0±7.4	336±25.0	13.3±2.3	32.7±2.5
	Omeprazole					
+S9	0.5	11.0±2.1	91.7±3.7	324±18.1	12.7±3.8	31.3±3.4
+S9	1	10.7±1.7	95.3±9.5	309±28.9	10.3±2.5	30.3±4.9
+S9	5	9.7±2.5	89.6±1.3	334±21.8	10.7±1.7	28.3±2.0
+S9	10	10.3±1.3	87.3±7.0	320±30.0	13.3±3.8	31.3±2.7
+S9	20	8.3±1.2	86.3±2.5	344±27.5	10.0±2.9	29.0±2.4
+S9	100	5.0±1.6	87.3±9.8	311±25.7	9.0±4.3	33.7±2.5
+S9	1000	6.0±1.7	49.0±12.2	178±20.9	11.7±1.3	23.0±3.3
+S9	5000	3.0±0.8	1.3±1.8	0.0±0.0	8.0±1.6	2.7±1.7
	Positive Control	2-AAN	2-AAN	2-AAN	2-AAN	2-AAN
+S9	0.5	40.0±5.0***	152.0±8.9***	677±73.4***	42.3±5.1***	144.7±9.3***
+S9	5.0	162.3±34.7***	482.0±65.7***	1847±170.5***	181.0±35.5***	471.0±37.8***

Statistical differences from negative control: *p<0.05; **p<0.01; ***p< 0.001. Negative control and omeprazole dissolved in DMSO. Positive control carcinogens: SA (sodium azide, dissolved in sterile water); MMC (mitomycin, dissolved in DMSO); 2-NF (2-nitrofluorene, dissolved in DMSO); 9-AA (9-aminoacridine, dissolved in DMSO); 2-AA (2-aminoacridine, dissolved in DMSO); 4-NPHD (4-nitro-o-phenylendiamine, dissolved in DMSO).

TABLE 3: COMET ASSAY PRELIMINARY RESULTS (±S9)

Group µg/ml		Tail Length ± SD	Tail Intensity ± SD	Tail Moment ± SD
-S9	Control	38.3±7.0	1.5±1.0	0.8±0.2
-S9	0.1 M H_2O_2	116.3±21.7***	36.6±8.7***	12.2±5.1***
	Omeprazole			
-S9	0.5 µg/ml	42.5±6.4	2.1±0.9	1.2±0.5
-S9	1 µg/ml	44.3±8.1	3.9±1.2	3.0±0.9*
-S9	4 µg/ml	61.3±18.5*	5.2±2.1**	3.7±1.1*
-S9	8 µg/ml	66.0±12.1**	7.1±1.1***	3.5±1.3**
-S9	16 µg/ml	71.0±19.3**	7.5±1.5***	2.2±0.9*
-S9	32 µg/ml	61.5±15.5**	6.8±1.3***	2.5±1.1*
+S9	Control	49.4±8.0	2.3±0.7	1.2±0.6
+S9	0.1 M H_2O_2	166.3±29.5***	43.3±11.5***	19.3±6.7***
	Omeprazole			
+S9	0.5 µg/ml	41.2±10.1	2.5±1.0	1.4±1.4
+S9	1 µg/ml	59.3±12.3*	7.3±1.2**	2.0±0.7
+S9	4 µg/ml	68.4±9.9**	10.1±2.3***	4.2±1.4**
+S9	8 µg/ml	75.1±17.2***	12.2±3.5***	4.7±1.5***
+S9	16 µg/ml	79.4±14.1***	9.9±2.1***	3.9±1.4**
+S9	32 µg/ml	71.3±16.5***	8.1±3.3***	3.4±0.9**

Statistical difference from negative control: Student's t-test: *p<0.05; **p<0.01; ***p<0.001. Negative control and omeprazole dissolved in DMSO.

TABLE 4: COMET ASSAY MAIN RESULTS (±S9)

Group μg/ml		Tail Length ± SD	Tail Intensity ± SD	Tail Moment ± SD
-S9	Control	33.5±9.3	1.7±1.1	0.3±0.2
-S9	0.1 M H_2O_2	130.3±35.9***	31.4±5.2***	14.6±1.65***
	Omeprazole			
-S9	0.5 μg/ml	35.1±6.6	1.9±1.0	0.5±0.2
-S9	1 μg/ml	47.6±7.1	5.2±1.3	2.0±0.4*
-S9	4 μg/ml	52.6±7.6*	6.4±1.6**	1.7±0.5**
-S9	8 μg/ml	47.8±0.6**	6.3±2.6***	2.0±1.0**
-S9	16 μg/ml	68.0±9.8**	7.4±1.8***	3.3±1.2***
-S9	32 μg/ml	87.3±7.3**	8.5±3.8***	4.1±1.1*
+S9	Control	40.5±8.1	2.8±0.8	0.5±0.3
+S9	0.1 M H_2O_2	123.1±26.7***	26.2±8.1***	16.1±3.7***
	Omeprazole			
+S9	0.5 μg/ml	38.4±8.3	3.8±1.0	1.1±0.5
+S9	1 μg/ml	54.0±11.1*	6.5±2.1**	1.8±1.0
+S9	4 μg/ml	67.8±14.1**	8.0±2.8***	2.9±0.8**
+S9	8 μg/ml	72.1±10.6***	8.9±2.2***	3.5±1.2***
+S9	16 μg/ml	73.8±14.2***	11.0±4.2***	2.7±1.1**
+S9	32 μg/ml	66.2±15.3***	7.1±3.5***	2.8±1.2**

Statistical difference from negative control: Student's t-test *$p<0.05$; **$p<0.01$; ***$p<0.001$. Negative control and omeprazole dissolved in DMSO.

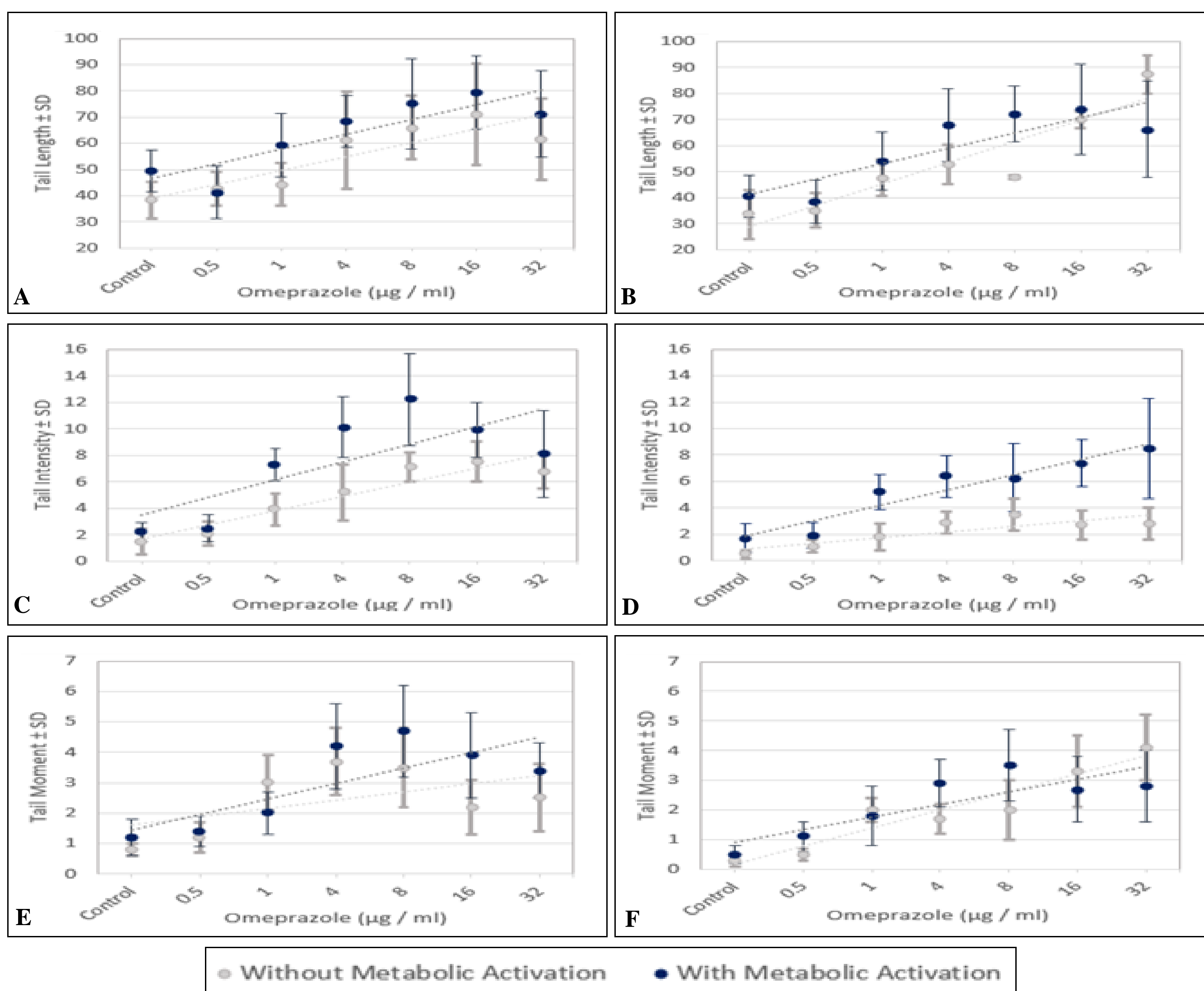

FIG. 1: DOSE RELATED EFFECTS ON COMET ASSAY PRELIMINARY (A, C, E) AND MAIN (B, D, F) EXPERIMENTS

Cytokinesis-Block Micronucleus Assay: The results obtained from three independent lymphocyte cultures from three donors were summarized and presented in tables 5 and 6 (without and with metabolic activation ±S9). Analysis of omeprazole genotoxicity was based on 21 samples (3 each at dosages 0, 0.5, 1, 4, 8, 16, and 32 µg/ml) and analysis for bleomycin was based on 12 samples (3 each at dosages 0, 2, 4 and 8 µg/ml) after 4 hours exposure, both with and without activation. **Table 5** showed the results from the experiment after 4 hours of exposure to omeprazole without metabolic activation (-S9). A positive, statistically significant result for BNMN was obtained after omeprazole concentration of 3.2, 6.4, and 12.8 µg/ml. A dose-dependent increase in BNMN was established in **Fig. 2**.

TABLE 5: INDUCTION OF CBMN ASSAY ENDPOINTS (-S9 METABOLIC ACTIVATION)

Drug µg/mL	NDI	BNMN Mean ± SD	MN Mean ± SD	AM Mean ± SD	NB Mean ± SD	NPB Mean ± SD	AP Mean ± SD	NC Mean ± SD
Control	2.3±0.2	8.3±0.9	9.7±0.9	9.0±2.2	3.3±1.9	1.7±0.5	2.3±0.5	2.7±0.9
Bleomycin								
2	1.7±0.05*	23.0±5.5**	29.7±6.9**	10.7±1.2	7.3±0.5*	7.3±1.3**	7.3±2.0*	14.7±4.1**
4	1.6±0.05**	38.7±7.4***	51.3±9.3***	14.0±0.8*	8.3±0.5**	5.7±2.6*	6.7±2.8*	17.7±2.9***
8	1.5±0.08**	46.7±14.5***	54.3±14.6***	15.0±2.2*	9.3±0.9**	8.3±2.3**	8.7±2.3**	27.0±5.7***
R	-0.888×××	0.823××	0.800××	0.889×××	0.657×	0.633×	0.601×	0.872×××
R^2	0.789	0.677	0.640	0.790	0.432	0.401	0.361	0.760
p-value	0.000	0.001	0.002	0.000	0.020	0.027	0.039	0.000
Omeprazole								
0.1	2.1±0.08	9.0±0.8	7.7±0.9	7.7±1.2	4.7±2.0	2.0±0.8	2.0±0.8	4.7±2.1
0.4	2.0±0.08	8.0±0.8	8.3±0.5	9.0±0.8	3.7±0.5	2.7±1.2	1.7±0.5	6.0±1.6*
0.8	1.9±0.05*	8.7±1.3	10.0±0.8	12.0±3.5	8.3±0.9*	5.3±2.9*	7.0±1.6*	11.0±2.8**
3.2	1.8±0.05*	11.0±2.4*	12.7±2.6*	15.3±3.7*	13.7±6.1**	7.3±2.0**	6.7±0.5**	14.7±4.9***
6.4	1.7±0.05**	15.7±2.9**	17.3±1.7**	15.7±1.7**	9.0±1.4**	6.7±3.8*	1.7±2.3	15.3±2.6***
12.8	1.5±0.3***	13.7±3.7**	15.7±4.9**	17.0±0.8***	11.0±1.4**	10.0±5.9**	6.7±0.5**	29.7±2.6***
R	-0.755×××	0.629×××	0.695×××	0.721×××	0.495×	0.610××	0.350	0.912×××
R^2	0.570	0.396	0.483	0.520	0.245	0.372	0.123	0.832
p-value	0.000	0.002	0.000	0.000	0.023	0.003	0.120	0.000

Statistical differences from negative control: Student's t-test *p<0.05; **p<0.01; ***p<0.001; R - Pearson's Correlation; R^2 – Coefficient of determination; ×p<0.05; ××p<0.01; ×××p<0.001; NDI- nuclear division index; BNMN-binucleated cells with micronuclei; MN total micronuclei; AM-arrested metaphases; NB-nuclear buds; NPB-nucleoplasmic bridges; AP-apoptotic cells; NC-necrotic cells. Positive control, negative control and omeprazole dissolved in DMSO.

TABLE 6: INDUCTION OF CBMN ASSAY ENDPOINTS (+S9 METABOLIC ACTIVATION)

Drug µg/mL	NDI	BNMN Mean ± SD	MN Mean ± SD	AM Mean ± SD	NB Mean ± SD	NPB Mean ± SD	AP Mean ± SD	NC Mean ± SD
Control	2.2±0.09	6.3±0.5	8.0±0.8	10.0±0.8	3.0±0.8	2.0±0.8	2.3±0.5	2.7±0.5
Bleomycin								
2	1.6±0.05*	26.7±4.6**	36.0±4.9**	12.3±2.0	10.3±2.0*	4.3±1.2*	11.0±2.9**	15.7±5.8**
4	1.6±0.05**	42.7±6.1***	53.7±10.3***	17.0±0.8*	9.3±0.9**	7.0±0.8*	9.3±1.9*	19.0±7.3***
8	1.5±0.1**	50.0±5.1***	57.0±12.7***	19.3±1.2**	11.0±1.4**	9.7±1.2*	12.7±1.9*	23.3±6.5***
R	-0.788×××	0.895×××	0.802××	0.906×××	0.685×	0.926×××	0.709×	0.719××
R^2	0.621	0.801	0.643	0.821	0.469	0.857	0.503	0.517
p-value	0.002	0.000	0.002	0.000	0.014	0.000	0.010	0.008
Omeprazole								
0.1	2.1±0.08	7.3±0.5	8.7±0.5	7.7±1.2	3.3±2.0	1.0±0.8	3.0±0.8	4.0±1.4
0.4	2.0±0.05	7.3±0.9	8.0±0.8	8.7±1.7	4.0±0.8	2.0±0.8	3.7±0.9	4.7±1.7
0.8	1.9±0.09*	9.7±1.2*	9.7±1.2	10.0±0.8	6.7±1.2**	5.0±2.2*	5.7±0.5*	11.7±2.5***
3.2	1.8±0.05*	10.3±1.2*	12.3±1.7*	16.0±0.8**	13.0±2.9***	8.0±2.1**	7.0±0.8**	16.0±3.5***
6.4	1.8±0.09**	13.3±2.0**	15.0±1.6**	16.3±1.7**	10.3±1.9***	10.0±2.2**	4.7±1.7*	19.0±4.3***
12.8	1.7±0.05***	16.3±1.2***	17.3±1.2***	19.0±1.6***	10.7±0.5***	8.0±2.2**	3.3±2.0	19.0±3.3***
R	-0.750×××	0.905×××	0.906×××	0.832×××	0.623××	0.652××	0.013	0.750×××
R^2	0.563	0.819	0.821	0.692	0.388	0.425	0.000	0.563
p-value	0.000	0.000	0.000	0.000	0.003	0.001	0.954	0.000

Statistical differences from negative control: Student's t-test *p<0.05; **p<0.01; ***p<0.001; Pearson's Correlation; R^2- Coefficient of determination; ×p<0.05; ××p<0.01; ×××p<0.001; NDI- nuclear division index; BNMN-binucleated cells with micronuclei; MN total micronuclei; AM-arrested metaphases; NB-nuclear buds; NPB-nucleoplasmic bridges; AP-apoptotic cells; NC-necrotic cells. Positive control, negative control and omeprazole dissolved in DMSO.

Similarly, mild dose-dependences were obtained for arrested metaphases, nuclear buds, nuclear plasmic bridges, and necrotic cells. The remaining endpoint, such as apoptotic cells, showed a statistically significant increase, but without dose-dependency **Table 5**.

These results were confirmed in the second experiment, **Table 6** with metabolic activation (+S9), where all endpoints, including arrested metaphases (AM-a marker for aneugenicity), showed good dose-dependencies.

Fig. 2 illustrates the genotoxic and aneugenic effects of omeprazole. Additionally, **Table 5** and **Table 6** confirmed that bleomycin produced strong dose-dependent cytotoxic and genotoxic effects for all investigated endpoints, as well as weak aneugenic effects. Comparison of CBMN endpoints after omeprazole exposure to those after bleomycin exposure revealed statistically significant higher genotoxicity for bleomycin, measured by BNMN, MN, and NC as almost equal aneugenic effect for both chemicals, measured through arrested metaphases.

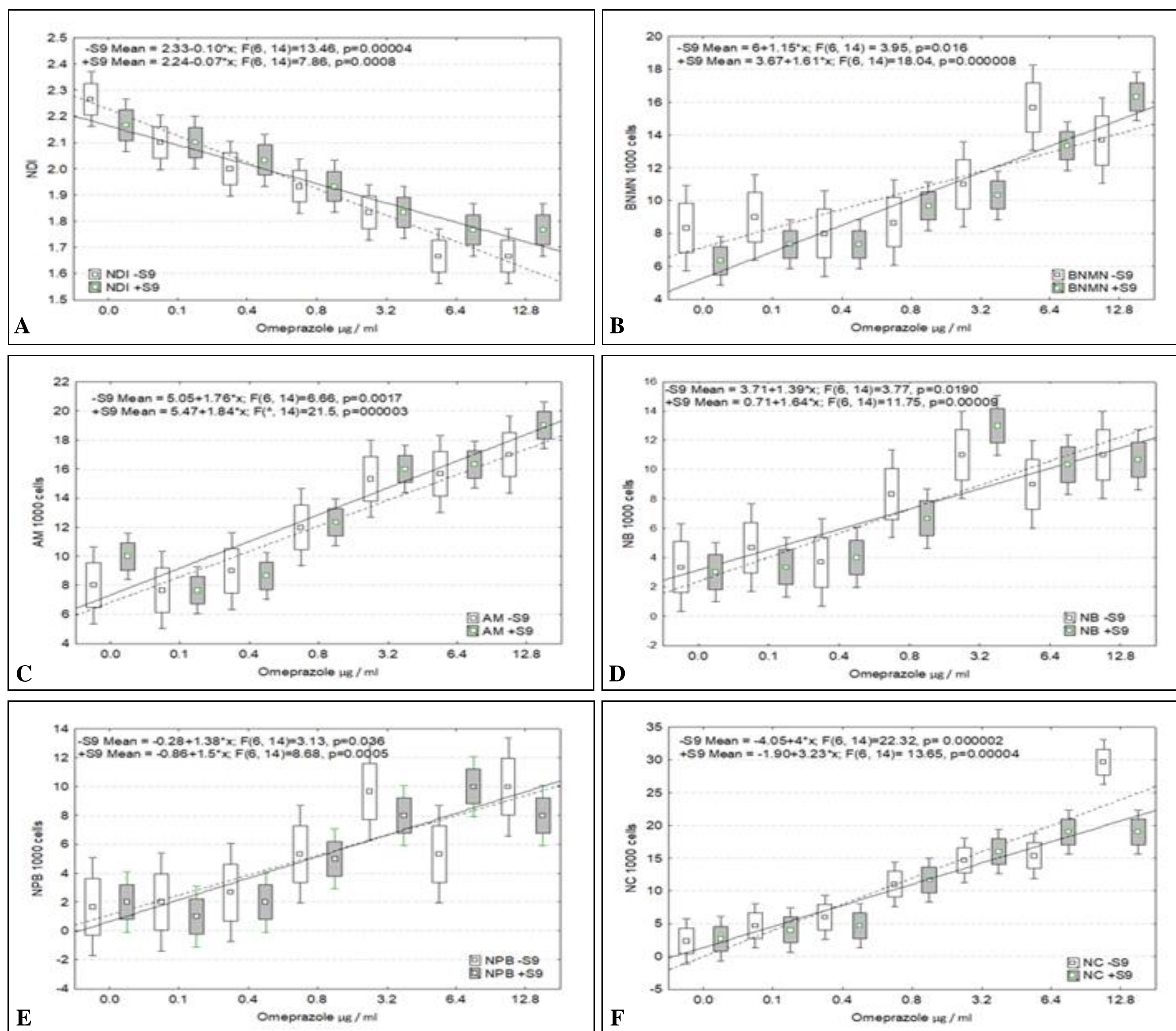

FIG. 2: DOSE RELATED INCREASE OF CBMN ASSAY ENDPOINTS (±S9)

DISCUSSION: The present work has been designed to investigate the potential *in-vitro* mutagenic, genotoxic, and aneugenic effects of omeprazole. Data reported in the literature on the mutagenicity of omeprazole are negative, taking into account all available results through the Ames test, as well as the mouse lymphoma (TK) locus [1-3]. The result reported herewith and without metabolic activation are in support of that.

Additionally, this study investigated genotoxic effects through alkaline Comet assay after human lymphocytes exposure to omeprazole. All endpoints of the Comet assay showed moderate, statistically significant levels of increased DNA damage effects, as well as dose-dependencies which is in support of omeprazole genotoxicity.We did not find any supporting results in the literature with omeprazole genotoxicity evaluation through Comet assay.

It is well known that results from the CBMN assay have shown significant differences between labs [34-41]. There are well-established 72 hours' culture time after *in-vivo* exposure, and (3 to 6 or 24 h) recommended for *in-vitro* exposure, starting from 40 and 44 h from cultures initiation time [36]. To the moment, experiments for chemicals or drug genotoxicity evaluation, which are developed according to the present requirements for *in-vitro* exposure time, are rare [42].

This study reported mildly increased levels of binucleated cells with micronuclei, as well total micronuclei after 4 h exposure to omeprazole. These results are in support of the mild genotoxic effect. Moreover, the data presented here establish a dose-dependent relationship for BNMN, MN, arrested metaphases, nuclear buds, nuclear plasmic bridges, and necrotic cells, which directly support a genotoxic effect for omeprazole. Using the same cells, peripheral blood lymphocytes [7] showed weak, inconclusive *in-vitro* genotoxic effect after 20 hours exposure to omeprazole, starting from 24 to 44 h after cultures initiation. Similar, inconclusive results on lymphocytes through the CBMN test were also reported after *in-vivo* exposure of patients to low doses of omeprazole [12].

Our results are in support of and showed dose-dependence for arrested metaphases (AM) in binucleated cells, which revealed omeprazole as a possible aneugenic agent, similar to the colchicineaneugenic effect [43]. In the literature, the aneugenic effect of omeprazole was also reported after *in-vitro* exposure [5]. Taking into account the mode of cell suspension-hypotonisation before fixation used in the above-cited articles [7, 12], serious loss of binucleated cells cannot be excluded, which logically will lead to negative genotoxicity conclusion of the evaluated drug.

Therefore, omeprazole genotoxicity continues to be a matter of debate, primarily on the method or protocol used for assessment. Ourexperience elaborating with CBMN assay showed that a cold 4 ºC hypotonisation combined with subsequent treatment within first fixation with formaldehyde for 1 h, effectively preserves the cell cytoplasm. This is a critical finding as such an approach avoids the loss of binucleated cells with well-preserved cytoplasm and may lead to a more accurate assessment of cells with/or without micronuclei, to save binucleated cells with well-preserved cytoplasm with/or without micronuclei, presented in cell suspension. Moreover, this treatment of cell suspension facilitates successful recognition of all CBMN assay endpoints [37-39]. The results regarding bleomycin aneugenicity is in support of the recently reported aneugenic effect of bleomycin on human lymphocytes [33-35]. The baseline of DNA damage, assessed by the Comet and Cytokinesis-block micronucleus assay were dose-dependent and these results were statistically significant, which confirm genotoxicity of omeprazole.

CONCLUSION: In conclusion, the results of *in-vitro* mutagenicity test showed the negative mutagenic effect of omeprazole. Nevertheless, the results from the Comet assay suggest positive support for genotoxicity, as well as genotoxicity and aneugenicity of omeprazole proven by the cytokinesis-block (CBMN) assay in human peripheral blood lymphocytes. Additionally, the results indicate a strong genotoxic, as well as a weak aneugenic effect of bleomycin. Further comprehensive investigations are needed to verify the genotoxic and/or aneugenic potential of omeprazole and other clinically relevant proton pump inhibitors for precise evaluation of its genotoxic/carcinogenic risk to humans.

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