

# Compounds used to produce cloned animals are genotoxic and mutagenic in mammalian assays *in vitro* and *in vivo*

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## Abstract

The compounds 6-dimethylaminopurine and cycloheximide promote the successful production of cloned mammals and have been used in the development of embryos produced by somatic cell nuclear transfer. This study investigated the effects of 6-dimethylaminopurine and cycloheximide *in vitro*, using the thiazolyl blue tetrazolium bromide colorimetric assay to assess cytotoxicity, the trypan blue exclusion assay to assess cell viability, the comet assay to assess genotoxicity, and the micronucleus test with cytokinesis block to test mutagenicity. In addition, the comet assay and the micronucleus test were also performed on peripheral blood cells of 54 male Swiss mice, 35 g each, to assess the effects of the compounds *in vivo*. The results indicated that both 6-dimethylaminopurine and cycloheximide, at the concentrations and doses tested, were cytotoxic *in vitro* and genotoxic and mutagenic *in vitro* and *in vivo*, altered the nuclear division index *in vitro*, but did not diminish cell viability *in vitro*. Considering that alterations in DNA play important roles in mutagenesis, carcinogenesis, and morphofunctional teratogenesis and reduce embryonic viability, this study indicated that 6-dimethylaminopurine and cycloheximide utilized in the process of mammalian cloning may be responsible for the low embryo viability commonly seen in nuclear transfer after implantation *in utero*.

Key words: Somatic cell nuclear transfer; 6-Dimethylaminopurine; Cycloheximide; Genotoxic; Mutagenic

## Introduction

The somatic cell nuclear transfer (SCNT) technique is being used to clone a variety of animal species, including cattle, sheep, mice, rats, goats, pigs, cats, rabbits, and horses. As the efficiency of SCNT improves, the technique offers more applications in agriculture, biomedical science, and endangered species preservation.

Animal cloning by SCNT has the potential to improve the productivity of enormously valuable livestock and may

eventually prove to be a more economical venture than current breeding methods (1,2). Additionally, SCNT may also provide the ability to produce both embryonic stem cells for therapeutic use and transgenic animals that synthesize large quantities of human proteins for use in medicine (3,4). Furthermore, SCNT can help in understanding gene expression and nuclear-cytoplasmic contributions to cellular function and their respective roles in

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growth, development, and aging (1,5). However, the efficiency of the SCNT procedure and the percentage of live offspring obtained by transfer of the resulting embryos into surrogate recipients remain low in most situations (6).

The success of SCNT techniques depends on a wide range of known factors, including the reconstruction process, culture conditions, and cell cycle stages of both the donor and recipient cells (6,7). Oocyte activation may also be an important component of the precise reprogramming that involves chromatin configuration, histone composition, methylation and acetylation patterns, and genomic imprinting for further development of the embryos (8). Therefore, oocyte maturation is considered an important step in achieving *in vitro* embryo production, since it is one of the essential elements that determine the success of nuclear transfer and the subsequent development of cloned embryos (9-11). To circumvent this problem, additional treatments with 6-dimethylaminopurine (6-DMAP), a phosphorylation inhibitor, or cycloheximide (CHX), a protein synthesis inhibitor (12), have been used to induce the inactivation of mitosis promoting factor. 6-DMAP has been used to improve *in vitro* maturation and developmental competence of human and mouse oocytes (13) as well as the successful development of cloned monkey (10) and rabbit (14) embryos. Furthermore, 6-DMAP and CHX, independently or together, have been used in the successful production of cloned mammals, including cattle, rabbits, goats, and horses (15).

These treatments induce the inactivation of mitosis promoting factor (12) and/or cytostatic factor, leading to the resumption of meiosis and entry into the first embryonic cell cycle. Both 6-DMAP and CHX act nonspecifically as broad-spectrum inhibitors in several metabolic pathways in oocytes and may consequently impair further embryonic development (12). Bovine embryos activated by these chemicals are characterized by abnormal chromosomal complements (16). Retrospective studies of the chromosomal makeup of the embryos of food-producing domestic animals have shown that the incidence of chromosomal alterations appears to be influenced by the methods of embryo handling and production (17). In embryos, chromosomal change is usually associated with reduced developmental potential or impaired viability.

The literature has shown that, regardless of which protocol or animal is used, intrauterine development is rare in all species cloned to date. Although several mammalian species have been cloned successfully using SCNT, the efficiency of neonate development has been low. Cloned embryos produced by SCNT frequently die, are aborted, or develop fetal abnormalities. An investigation is necessary to determine whether these phenomena are due to gene mutations induced by the chemical mutagens used to produce the reconstructed cloned embryos.

The purpose of the present study was to investigate whether 6-DMAP or CHX are genotoxic or mutagenic, using comet and micronucleus assays in mammals *in vitro* and *in vivo*.

## Material and Methods

### Experiments *in vitro*

**Chemical agents.** In the cell line HepG2, the direct-acting alkylating agent methyl methane sulfonate (MMS; CAS No. 66-27-3, Merck, Germany) was used as the positive control at concentrations of 4.23, 4.23, 12.53, and 37.60  $\mu\text{g}/\text{mL}$  in the comet assay, trypan blue exclusion assay, micronucleus test, and cytotoxicity assay, respectively. The different concentrations were defined in prior pilot experiments for each assay with the aim of determining the lowest effective concentration.

The compounds tested were 6-DMAP (CAS No. 938-55-6, Sigma, USA) and CHX (CAS No. 66-81-9, Sigma). The concentrations utilized in the genotoxicity and mutagenicity assays were determined by the colorimetric thiazolyl blue tetrazolium bromide (MTT) assay (CAS No. 298-93-1, Sigma), which was used to determine cytotoxicity. 6-DMAP was tested at 13 concentrations between 0.014 and 60.8  $\text{mg}/\text{mL}$ , and CHX was tested at 13 concentrations of 1.25 to  $5120 \times 10^{-3} \text{ mg}/\text{mL}$  in culture medium.

In the comet, cell viability, and micronucleus assays, the following concentrations were tested: 0.01, 0.10, 0.50, and 1.00  $\text{mg}/\text{mL}$  6-DMAP and 0.16, 1.60, 16.00, and  $160.00 \times 10^{-3} \text{ mg}/\text{mL}$  CHX in culture medium. These concentrations were determined based on the MTT assay results. Also, an extensive literature review revealed that the concentration of the doses of 6-DMAP and CHX ranged from 0.0003263 to 0.8159  $\text{mg}/\text{mL}$  (18,19) and from 2 to  $10 \times 10^{-3} \text{ mg}/\text{mL}$  (20,21), respectively.

**Cell line.** The human hepatic cell line HepG2 utilized in this study was provided by Prof. Dr. Sigfried Knasmüller of the Research Institute of Cancer of the University of Vienna, Austria.

The cells were grown in 25-cm<sup>2</sup> culture flasks in 10.0 mL minimum essential medium (Lot 1262854, Cat. 41500-018, Gibco, USA) supplemented with 20% fetal bovine serum (Gibco) and 1% sodium pyruvate (Lot 1271397, Cat. 11360-070, Gibco), in a 5% CO<sub>2</sub> incubator (37°C) until reaching confluence. Under these conditions, the cell cycle time was approximately 24 h.

**MTT assay.** For the MTT assay,  $2.5 \times 10^4$  cells/well were seeded on 96-well culture plates. However, the cells were seeded only in columns 2-6 and 8-12, and columns 1 and 7 were filled with only reagents without the presence of cells (designated in the text as blanks). On the plate, the rows were filled with the following treatments: control, positive control (MMS,  $37.6 \times 10^{-3} \text{ mg}/\text{mL}$ ), 6-DMAP (0.014 to 60.8  $\text{mg}/\text{mL}$ ), and CHX (1.25 to  $5120 \times 10^{-3} \text{ mg}/\text{mL}$ ). The experiments were carried out in duplicate, with five

replicate plates used for each experiment.

The cells were seeded in a final volume of 100  $\mu$ L culture medium and incubated for a period of 24 h. Medium was added with and without the drugs, for a total volume of 200  $\mu$ L per well. The cells were exposed to the drugs for a period of 30 h. The medium was then discarded, and the plate was incubated at 37°C for 3 h with 150  $\mu$ L MTT diluted in phosphate-buffered saline (PBS) at a concentration of  $1.0 \times 10^{-6}$  mg/mL. Afterward, the MTT solution was discarded, and 100  $\mu$ L dimethylsulfoxide (Lot 16148, Biotec, Brazil) was added. Plates were then read in a plate spectrophotometer (Universal Microplate Reader, ELx 800, Bio-Tek Instruments, USA) using filters for 490 and 630 nm. Values are reported as means  $\pm$  standard deviation. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer test ( $P < 0.05$ ).

**Comet assay.** The comet assay was performed according to Tice et al. (22), using HepG2 cells that were grown for one complete cycle (24 h) before the drug treatments for 30 h. The cells ( $4 \times 10^6$ ) were trypsinized (trypsin-EDTA, 0.1%) and harvested. A 20- $\mu$ L aliquot of cell suspension along with 120  $\mu$ L 1.5% low melting point agarose at 37°C was deposited on a slide precoated with normal agarose (5%). The slide was covered with a glass coverslip and cooled at 4°C for 20 min. Slides were immersed in lysis solution for 1 h. Next, the slides were transferred to electrophoresis buffer for 20 min for denaturation and then electrophoresed with a pH >13.0 buffer at 4°C for 20 min. Afterward, the slides were neutralized, air-dried, and fixed in absolute ethanol for 10 min.

Cells on the slides were stained with 100  $\mu$ L ethidium bromide ( $20 \times 10^{-3}$  mg/mL) and evaluated using a fluorescence microscope (Nikon, Labophot-2, Japan) at 40 $\times$  magnification, using an excitation filter of 420-490 nm and a barrier filter of 520 nm.

Three independent repetitions were performed. Cells (100) were scored per treatment, and the comets were classified as follows: cells without a comet tail (Class 0), cells with a tail less than the diameter of the nucleus (Class 1), cells with a tail one to two times the diameter of the nucleus (Class 2), and cells with a tail greater than the diameter of the nucleus (Class 3). Apoptotic cells, which had a totally fragmented nucleus, were not counted (23).

The total score was calculated by summing the resultant values after multiplication of the total cells observed in each class of lesion by the number of the class. Statistical analysis was performed using ANOVA followed by the Tukey-Kramer test ( $P < 0.05$ ).

**Cell viability assay.** After the cells were harvested for the comet assay, a 20- $\mu$ L aliquot of the cell suspension was mixed with 20  $\mu$ L trypan blue (0.2%; Lot 1275150, Cat. No. 15250, Gibco, USA). Next, 20  $\mu$ L of this mixture was placed in a Neubauer chamber, and the numbers of viable and nonviable cells were counted in the four outer

squares of the chamber using a light microscope. Values are reported as percentages and statistical analysis was performed using the Kruskal-Wallis test ( $P < 0.05$ ).

**Micronucleus test with cytokinesis block.** Mutagenicity was determined by the micronucleus test using HepG2 cells that had been grown for one complete cell cycle (24 h) and treated for a period of 30 h with test agents simultaneously with cytochalasin-B.

The procedures utilized for the assay, cell harvest, preparation of slides, and analysis of cells were carried out according to those described by Phelps et al. (24).

Cell cultures were washed with PBS and trypsinized (trypsin-EDTA, 0.1%), and cells were harvested. After centrifugation for 5 min at 26,226.56 g, the supernatant was discarded, and cells were placed in hypotonic 1.5 mL sodium citrate (1%) and fixed with a drop of 40% formaldehyde. Cells were then fixed with 5.0 mL methanol-acetic acid (3:1) and dropped on slides that had been cleaned and kept in distilled water at 4°C. After mounting on a film of water, the slides were air-dried and later stained with 5% Giemsa for 10 min.

Three independent repetitions were performed for each treatment. The criteria for selection of binucleated cells were as follows: i) distinguishable nuclear boundaries by light microscopy; ii) nuclei with similar sizes and the same staining pattern and intensity; iii) main nuclei being well separated or close, but well outlined and not superimposed; and iv) cytoplasmic boundary distinguishable from adjacent cells. The micronucleus, present in binucleated cells, should not be more than one-third the size of the main nuclei and, furthermore, should have the same staining pattern and not be refractive.

A total of 3000 binucleated cells were examined per treatment (1000 cells/treatment/repetition), except for cytotoxic treatments, and the chi-square test ( $P < 0.05$ ) was used for statistical analysis.

The same slides used for the micronucleus test were used to determine the nuclear division index to compare the mitogenic responses and cytostatic effects of the agents tested. According to Salvadori et al. (25), this parameter is calculated using the following formula:  $MI = [M1 + 2(M2) + 3(M3) + 4(M4)]/N$ , where M1-M4 are the numbers of cells with 1, 2, 3, and 4 nuclei, respectively, and N is the total number of viable cells. Statistical analysis was carried out using ANOVA followed by the Tukey-Kramer test for comparison of means ( $P < 0.05$ ).

**Statistical analysis.** The comparison of quantitative results was carried out using parametric (ANOVA/Tukey-Kramer) and nonparametric tests (ANOVA/Kruskal-Wallis; chi-square test), depending on the nature of the data distribution (26).

## Experiments *in vivo*

**Animals.** Male Swiss mice of reproductive age and a mean weight of 35 g were obtained from the Central

Animal Facility, Universidade Estadual de Londrina, PR, Brazil. The mice were randomly distributed into groups of three animals per polypropylene cage with hardwood chips as bedding and were maintained under controlled conditions of temperature ( $22 \pm 2^\circ\text{C}$ ), humidity ( $55 \pm 10\%$ ), and a 12:12-h light-dark cycle, with at least 10 changes per hour of filtered air. Food (Nuvital, Brazil) and filtered water were available *ad libitum*. A minimum period of 7 days was allowed for acclimatization. In this phase, the animals were inspected daily. At the end of the experiment, all animals were killed by cervical dislocation because the use of any anesthetic could interfere with the effects of the chemical compounds under investigation. The euthanasia procedure was selected to minimize pain and stress, in agreement with the guidelines for the ethical treatment of animals, according to the Ethics Committee in Animal Experimentation of the Universidade Estadual de Londrina (Protocol #72/2005).

**Chemicals.** Cyclophosphamide (Fosfaseron), an indirect-acting alkylating agent, was obtained from Itaca Laboratórios (REG. M.S. No. 1.2603.0056.002-1; Batch 063020, Brazil) and used to induce DNA damage. Cyclophosphamide was prepared in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS, pH 7.4, and administered at a final dose of 50 mg/kg body weight (BW) by the intraperitoneal (*ip*) route.

6-DMAP and CHX were also diluted in PBS, and the tested doses (0.33, 3.30, 16.50, and 33.00 mg/kg BW, *ip*, for 6-DMAP and 0.053, 0.53, and 5.30 mg/kg BW, *ip*, for CHX) were established in pilot experiments.

**Experimental design.** Animals were distributed into nine groups (6 animals in each) as follows: group 1 (control) received PBS in a volume of 0.1 mL/10 g BW, *ip*; group 2 received cyclophosphamide (a DNA damage-inducing agent) at a dose of 50.0 mg/kg BW, *ip*; groups 3 to 6 received 6-DMAP at doses of 0.33, 3.30, 16.50, and 33.0 mg/kg BW, *ip*, respectively; groups 7 to 9 received CHX at doses of 0.053, 0.53, and 5.30 mg/kg BW, *ip*, respectively.

Four hours after the administration of 6-DMAP or CHX, peripheral blood was collected by caudal venipuncture from all experimental groups to evaluate genotoxicity using the comet assay.

Peripheral blood was collected by caudal venipuncture from all of the experimental groups 24, 48, and 72 h after the administration of 6-DMAP or CHX, to evaluate mutagenicity using the micronucleus assay. These collection times were designated T1 (24 h), T2 (48 h), and T3 (72 h).

**Comet assay.** The alkaline comet assay was performed under indirect light (22). Briefly, 20  $\mu\text{L}$  blood cell suspension was embedded into 120  $\mu\text{L}$  0.5% low melting point agarose, which was then layered on a precoated slide with a thin layer of normal melting point agarose. The remaining procedures were performed according to the method already described for the

comet assay *in vitro* (22,23).

Comet assay data were analyzed using ANOVA followed by the Tukey-Kramer test. Differences were considered to be statistically significant when  $P < 0.05$ .

**Micronucleus test in peripheral blood cells.** Mutagenicity was determined using the micronucleus technique in peripheral blood as described by Hayashi et al. (27). Briefly, a drop of peripheral blood was deposited on a slide previously prepared with a layer of acridine orange (1.0 mg/mL). The slide was then coverslipped and placed in a freezer ( $-20^\circ\text{C}$ ) for a minimum of 7 days. Slides were analyzed by fluorescence microscopy, using blue light (488 nm) and an orange filter, with a  $100\times$  objective. A total of 2000 randomly selected cells from each animal were examined for the frequency of micronuclei.

The micronucleus assay data were analyzed using ANOVA followed by the Tukey-Kramer test. Differences were considered to be statistically significant when  $P < 0.05$ .

## Results

### *In vitro*

Table 1 presents the MTT assay results. Concentrations of 60.8 and 30.4 mg/mL 6-DMAP precipitated in the wells of the plate, impeding the test reaction at these concentrations. The two highest concentrations of 6-DMAP (15.2 and 7.6 mg/mL) were shown to cause the death of all of the cells in culture because the absorbance values were statistically equal to that of the blank. The concentration of 3.8 mg/mL was cytotoxic but did not cause death of all of the cells in culture. However, the concentrations between 1.9 and 0.475 mg/mL showed absorbances similar to those found for the controls, indicating that this compound did not exert a cytotoxic effect at the lower concentrations. The lowest concentrations, 0.237-0.014 mg/mL, showed significantly higher absorbances than the control, indicating the presence of a higher number of cells per well.

In the evaluation of CHX, the highest concentration tested ( $5120 \times 10^{-3}$  mg/mL) was found to kill all of the cells; the absorbance reading was similar to that of the blank. Concentrations between  $2560$  and  $320 \times 10^{-3}$  mg/mL were cytotoxic, whereas lower concentrations ( $160$ - $1.25 \times 10^{-3}$  mg/mL) did not affect cell viability and cell proliferation.

Table 2 presents the data for the comet assay and cell viability. All of the concentrations of 6-DMAP and CHX tested were found to be genotoxic, except the lowest concentration of CHX ( $0.16 \times 10^{-3}$  mg/mL), and the data did not indicate a dose-response relationship. Genotoxicity, determined by the number of cells with damaged DNA, was essentially the same (99-100) after treatment with all concentrations of 6-DMAP. After CHX treatment, the number of damaged cells varied from 79.00 to 97.00. Thus, the increase in frequency of damaged

**Table 1.** Mean absorbance values  $\pm$  SD in the thiazolyl blue tetrazolium bromide colorimetric assay.

Treatment	Mean $\pm$ SD	Treatment	Mean $\pm$ SD
Blank	0.0314 $\pm$ 0.0005 <sup>a</sup>	Blank	0.0295 $\pm$ 0.0007 <sup>a</sup>
Control	0.1859 $\pm$ 0.0016 <sup>c,d</sup>	Control	0.1799 $\pm$ 0.0451 <sup>c</sup>
MMS	0.1516 $\pm$ 0.0327 <sup>d</sup>	MMS	0.1669 $\pm$ 0.0097 <sup>c</sup>
6-DMAP (mg/mL)		CHX (10 <sup>-3</sup> mg/mL)	
0.014	0.2220 $\pm$ 0.1803 <sup>e</sup>	1.25	0.1580 $\pm$ 0.0145 <sup>c</sup>
0.028	0.2384 $\pm$ 0.0097 <sup>e</sup>	2.50	0.1691 $\pm$ 0.224 <sup>c</sup>
0.059	0.2253 $\pm$ 0.0226 <sup>e</sup>	5.00	0.1708 $\pm$ 0.0095 <sup>c</sup>
0.118	0.2164 $\pm$ 0.0129 <sup>e</sup>	10.00	0.1544 $\pm$ 0.0097 <sup>c</sup>
0.237	0.1974 $\pm$ 0.0249 <sup>d,e</sup>	20.00	0.1620 $\pm$ 0.0173 <sup>c</sup>
0.475	0.1555 $\pm$ 0.0123 <sup>c,d</sup>	40.00	0.1623 $\pm$ 0.0290 <sup>c</sup>
0.950	0.1288 $\pm$ 0.0226 <sup>c,d</sup>	80.00	0.1565 $\pm$ 0.0180 <sup>c</sup>
1.900	0.1231 $\pm$ 0.0027 <sup>c</sup>	160.00	0.1458 $\pm$ 0.0156 <sup>b,c</sup>
3.800	0.0946 $\pm$ 0.0175 <sup>b,c</sup>	320.00	0.1073 $\pm$ 0.0438 <sup>b</sup>
7.600	0.0386 $\pm$ 0.0019 <sup>a</sup>	640.00	0.1500 $\pm$ 0.0297 <sup>b</sup>
15.200	0.0352 $\pm$ 0.0028 <sup>a</sup>	1280.00	0.1124 $\pm$ 0.0220 <sup>b</sup>
30.400	-	2560.00	0.1036 $\pm$ 0.0228 <sup>b</sup>
60.800	-	5120.00	0.0374 $\pm$ 0.0044 <sup>a</sup>

SD: standard deviation; MMS: methyl methane sulfonate; 6-DMAP: 6-dimethylaminopurine; CHX: cycloheximide. Different letters indicate a statistically significant difference ( $P < 0.05$ , ANOVA/Tukey-Kramer).

cells after 6-DMAP exposure was approximately 37.45 times that of the control cultures. The increase in DNA damage after treatment with CHX was 29.59 times that of the control. The indices of cell viability were statistically similar for all the treatments and varied from 81.33 to 87.67%.

The data for the micronucleus test with cytokinesis block and nuclear division index are reported in Table 3. Statistical analysis demonstrated that the lowest concentration of 6-DMAP tested (0.01 mg/mL) was not mutagenic and did not reduce the nuclear division index. However, the other three concentrations significantly reduced the nuclear division index and the lowest concentration showed a mutagenic effect.

The two lowest concentrations of CHX (0.16 and  $1.60 \times 10^{-3}$  mg/mL) did not cause a reduction in the nuclear division index and had no mutagenic effect. However, the concentration of  $1.60 \times 10^{-3}$  mg/mL was effective in causing a 2.92-fold increase in mutagenicity. The two highest concentrations of CHX studied (16.00 and  $160.00 \times 10^{-3}$  mg/mL) reduced the nuclear division index and showed mutagenic activity, causing increases in the frequency of micronuclei of 4.22 and 1.80 times that of the control, respectively.

### *In vivo*

The mean number of damaged cells, the distribution of classes of DNA damage, and the score in the comet assay in peripheral blood cells of mice treated with 6-DMAP and CHX are reported in Table 4. Statistically significant differences were observed in the total number

of damaged cells at the three highest 6-DMAP doses (3.30, 16.50, and 33.00 mg/kg BW) when compared to the negative control group. At these three doses, 6-DMAP induced a positive genotoxic response, with 15.3 times the number of damaged cells compared to the negative control. The data in Table 4 also show the effect of 6-DMAP-induced DNA damage on the score of the damaged cells. At the three highest doses, the scores were higher than that of the positive control. For CHX-induced DNA damage, the total number of damaged cells was significantly higher than the negative control only after treatment with the highest dose (5.30 mg/kg BW), and the frequency of damaged cells was 2.5 times higher than that of the negative control.

Table 5 summarizes the data obtained from the micronucleus test in peripheral blood cells of mice treated with 6-DMAP and CHX, at the three different times (T1, T2, and T3) of analysis. After 6-DMAP treatment, a statistically significant difference was observed at T1 (analysis at 24 h after treatment) only for the highest dose (33.00 mg/kg BW). 6-DMAP induced a positive mutagenic response 2.5 times greater than that of the negative control. At T2 (analysis at 48 h after treatment), the mutagenic responses for the two highest doses (15.50 and 33.00 mg/kg BW) were 2.3 and 2.8 times the negative control value, respectively. At T3 (analysis at 72 h after treatment), all three doses of 6-DMAP induced a positive mutagenic response that ranged from 1.2 to 1.9 times the negative control value.

After treatment with CHX, a positive mutagenic response was produced only at the highest dose

**Table 2.** Frequency of cells with damaged DNA (DNA migration in the comet assay), distribution among the different classes of damage, score, and cell viability index in the comet assay in HepG2 cells.

Treatment	Concentration	Total number of cells with damaged DNA <sup>1,2</sup>	Class of damage			Score	Cell viability index <sup>3</sup>
			0	1	2		
<b>6-DMAP</b>							
Control	—	2.67 ± 3.05 <sup>a</sup>	97.33 ± 3.05	2.00 ± 2.00	0.33 ± 0.58	3.67 ± 4.73	87.33 ± 4.16 <sup>a</sup>
MMS	4.23 µg/mL	99.33 ± 1.15 <sup>b</sup>	0.67 ± 1.15	71.33 ± 6.66	23.67 ± 7.09	131.67 ± 5.13	86.67 ± 4.93 <sup>a</sup>
6-DMAP	0.01 mg/mL	99.33 ± 0.58 <sup>b</sup>	0.67 ± 0.58	92.67 ± 1.53	4.33 ± 1.53	108.33 ± 2.08	87.67 ± 2.31 <sup>a</sup>
	0.10 mg/mL	100.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00	95.67 ± 3.51	3.00 ± 3.60	105.67 ± 3.51	86.33 ± 3.21 <sup>a</sup>
	0.50 mg/mL	100.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00	94.67 ± 2.08	3.67 ± 2.08	107.00 ± 2.65	84.00 ± 2.65 <sup>a</sup>
	1.00 mg/mL	100.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00	95.33 ± 3.79	3.33 ± 3.21	106.00 ± 4.36	81.33 ± 3.20 <sup>a</sup>
<b>CHX</b>							
Control	—	2.67 ± 3.05 <sup>a</sup>	97.33 ± 3.05	2.00 ± 2.00	0.33 ± 0.58	3.67 ± 4.73	87.33 ± 4.16 <sup>a</sup>
MMS	4.23 × 10 <sup>-3</sup> mg/mL	99.33 ± 1.15 <sup>c</sup>	0.67 ± 1.15	71.33 ± 6.66	23.67 ± 7.09	131.67 ± 5.13	86.67 ± 4.93 <sup>a</sup>
CHX	0.16 × 10 <sup>-3</sup> mg/mL	8.67 ± 1.53 <sup>a</sup>	91.33 ± 1.53	8.67 ± 1.53	0.00 ± 0.00	8.67 ± 1.53	85.67 ± 5.03 <sup>a</sup>
	1.60 × 10 <sup>-3</sup> mg/mL	79.00 ± 5.57 <sup>b</sup>	21.00 ± 5.57	73.33 ± 5.13	5.00 ± 2.00	85.33 ± 6.35	87.33 ± 3.21 <sup>a</sup>
	16.00 × 10 <sup>-3</sup> mg/mL	96.67 ± 3.05 <sup>c</sup>	3.33 ± 3.05	89.00 ± 8.18	5.00 ± 2.65	107.00 ± 6.24	85.33 ± 5.51 <sup>a</sup>
	160.00 × 10 <sup>-3</sup> mg/mL	97.00 ± 1.00 <sup>c</sup>	3.00 ± 1.00	93.00 ± 2.00	3.00 ± 1.00	102.00 ± 2.65	85.00 ± 4.04 <sup>a</sup>

MMS: methyl methane sulfonate; 6-DMAP: 6-dimethylaminopurine; CHX: cycloheximide. <sup>1</sup>Mean ± SD of number of total cells per 300 cells (100 cells/treatment/repetitions). Different letters indicate a statistically significant difference (<sup>2</sup>P < 0.05, ANOVA/Tukey-Kramer; <sup>3</sup>P < 0.05, Kruskal-Wallis).

**Table 3.** Total number of cells analyzed, total number of micronuclei, frequency of micronuclei, and cell division index in the micronucleus test with cytokinesis block in the HepG2 cells.

Treatment	Concentration	Total number of cells analyzed	Total number of micronuclei <sup>1</sup>	Frequency of micronuclei (%)	Nuclear division index <sup>2</sup>
6-DMAP					
Control	–	3000	49	1.63	1.9227 ± 0.0323 <sup>b</sup>
MMS	12.53 µg/mL	3000	89*	2.97	1.8927 ± 0.0050 <sup>b</sup>
6-DMAP	0.01 mg/mL	3000	38	1.27	1.9820 ± 0.0362 <sup>a</sup>
	0.10 mg/mL	1200	42*	3.50	1.0620 ± 0.0080 <sup>c</sup>
	0.50 mg/mL	551	12	2.18	1.0273 ± 0.0061 <sup>c</sup>
	1.00 mg/mL	715	18	2.52	1.0233 ± 0.0031 <sup>c</sup>
CHX					
Control	–	3000	49	1.63	1.9227 ± 0.0323 <sup>a</sup>
MMS	12.53 × 10 <sup>-3</sup> mg/mL	3000	89*	2.97	1.8927 ± 0.0050 <sup>a</sup>
CHX	0.16 × 10 <sup>-3</sup> mg/mL	3000	56	1.87	1.8733 ± 0.0151 <sup>a</sup>
	1.60 × 10 <sup>-3</sup> mg/mL	3000	143*	4.77	1.8733 ± 0.0284 <sup>a</sup>
	16.00 × 10 <sup>-3</sup> mg/mL	3000	207*	7.00	1.1053 ± 0.0284 <sup>b</sup>
	160.00 × 10 <sup>-3</sup> mg/mL	889	88*	9.90	1.0473 ± 0.0061 <sup>c</sup>

MMS: methyl methane sulfonate; 6-DMAP: 6-dimethylaminopurine; CHX: cycloheximide. <sup>1</sup>Chi-square test: \*P<0.05 statistically different compared with control; <sup>2</sup>ANOVA/Tukey-Kramer test: P<0.05, different letters indicate a statistically significant difference.

(5.30 mg/kg BW) at T1, T2, and T3, with values ranging from 1.8 to 1.9 times that of the negative control.

The distribution of the frequency of micronuclei in peripheral blood cells of mice treated with 6-DMAP and CHX at three different analysis times (T1, T2, and T3) is shown in Figure 1. In groups 1 (control), 3 (0.33 mg/kg 6-DMAP), 8 (0.53 mg/kg CHX), and 9 (5.30 mg/kg CHX), the frequency of micronuclei gradually increased over the three time periods of evaluation. In contrast, in groups 2

(DNA damage-inducing agent), 4 (3.30 mg/kg 6-DMAP), 5 (16.50 mg/kg 6-DMAP), 6 (33.00 mg/kg 6-DMAP), and 7 (0.053 mg/kg CHX), the increase was more substantial at T2 (48 h after treatment).

Table 6 summarizes the effects found for the test compounds and demonstrates that, considering the different concentrations and doses tested, 6-DMAP and CHX were cytotoxic *in vitro* and genotoxic and mutagenic *in vitro* and *in vivo*, and altered the nuclear division index *in vitro*.

**Table 4.** Means ± SD of cells with damaged DNA, distribution between classes of DNA damage, and score from blood of mice in the comet assay.

Treatment	Concentration	No. of cells with damaged DNA	Class of damage				Score
			0	1	2	3	
PBS (G1)	0.1 mL/10 g	6.50 ± 3.27 <sup>a</sup>	93.50 ± 3.27	6.33 ± 3.27	0.17 ± 0.41	0.00 ± 0.00	6.67 ± 3.33
CPA (G2)	50.00 mg/kg	97.33 ± 3.50 <sup>b</sup>	2.67 ± 3.50	93.83 ± 1.47	3.17 ± 2.48	0.33 ± 0.52	101.17 ± 6.62
6-DMAP							
G3	0.33 mg/kg	9.33 ± 1.75 <sup>a</sup>	89.00 ± 3.46	9.17 ± 1.57	0.17 ± 0.37	0.00 ± 0.00	9.50 ± 1.71
G4	3.30 mg/kg	99.67 ± 0.52 <sup>b</sup>	0.33 ± 0.52	96.50 ± 1.64	3.17 ± 1.33	0.00 ± 0.00	102.83 ± 1.17
G5	16.50 mg/kg	99.67 ± 0.52 <sup>b</sup>	0.33 ± 0.52	93.17 ± 4.17	5.50 ± 4.32	1.00 ± 0.89	107.17 ± 5.12
G6	33.00 mg/kg	100.00 ± 0.00 <sup>b</sup>	0.00 ± 0.00	64.00 ± 18.42	34.67 ± 18.02	1.33 ± 0.94	137.33 ± 18.86
CHX							
G7	0.053 mg/kg	8.00 ± 2.45 <sup>a</sup>	92.00 ± 2.45	7.33 ± 1.86	0.67 ± 0.82	0.00 ± 0.00	8.67 ± 3.14
G8	0.53 mg/kg	8.00 ± 2.61 <sup>a</sup>	92.00 ± 2.61	7.67 ± 2.25	0.33 ± 0.82	0.00 ± 0.00	8.33 ± 3.14
G9	5.30 mg/kg	16.50 ± 3.62 <sup>c</sup>	83.50 ± 3.65	14.67 ± 3.93	1.83 ± 1.17	0.00 ± 0.00	18.33 ± 3.70

G1: phosphate-buffered saline (PBS; negative control); G2: cyclophosphamide (CPA; positive control); G3 to G6: 6-dimethylaminopurine (6-DMAP); G7 to G9: cycloheximide (CHX). Different letters indicate a statistically significant difference (P<0.05, ANOVA/Tukey-Kramer).

**Table 5.** Frequency of micronuclei in the peripheral blood of mice.

Treatment	Concentration	Total frequency of MN <sup>1</sup>			Mean ± SD		
		T1	T2	T3	T1	T2	T3
PBS (G1)	0.1 mL/10 g	79	91	102	13.17 ± 2.86 <sup>a</sup>	15.17 ± 4.11 <sup>a</sup>	17.00 ± 4.24 <sup>a</sup>
CPA (G2)	50.00 mg/kg	248	325	187	41.33 ± 7.26 <sup>b</sup>	54.17 ± 8.03 <sup>b</sup>	31.17 ± 12.45 <sup>b</sup>
6-DMAP							
G3	0.33 mg/kg	78	117	125	13.00 ± 4.52 <sup>a</sup>	19.50 ± 2.59 <sup>a</sup>	20.83 ± 4.66 <sup>a,b</sup>
G4	3.30 mg/kg	62	129	121	10.33 ± 3.88 <sup>a</sup>	21.50 ± 4.09 <sup>a</sup>	20.17 ± 6.76 <sup>a,b</sup>
G5	16.50 mg/kg	122	174	79	20.33 ± 7.74 <sup>a</sup>	34.8 ± 7.76 <sup>c</sup>	23.2 ± 5.54 <sup>a,b</sup>
G6	33.00 mg/kg	204	172	131	34.00 ± 8.05 <sup>b</sup>	43.00 ± 5.48 <sup>c</sup>	32.75 ± 1.26 <sup>b</sup>
CHX							
G7	0.053 mg/kg	92	134	129	15.33 ± 6.25 <sup>a</sup>	22.33 ± 2.50 <sup>a</sup>	21.50 ± 8.50 <sup>a</sup>
G8	0.53 mg/kg	80	126	162	13.33 ± 3.78 <sup>a</sup>	21.00 ± 6.32 <sup>a</sup>	27.00 ± 6.90 <sup>a</sup>
G9	5.30 mg/kg	150	161	197	25.00 ± 5.21 <sup>c</sup>	26.83 ± 7.25 <sup>a,c</sup>	32.83 ± 7.88 <sup>a,b</sup>

MN: micronuclei; SD: standard deviation; G1: phosphate-buffered saline (PBS; negative control); G2: cyclophosphamide (CPA; positive control); G3 to G6: 6-dimethylaminopurine (6-DMAP); G7 to G9: cycloheximide (CHX); T1, T2, and T3: peripheral blood collected at 24, 48, and 72 h after treatment, respectively. <sup>1</sup>Total number of MN in 12,000 cells analyzed (2000 cells/animal). Different letters indicate a statistically significant difference ( $P < 0.05$ , ANOVA/Tukey-Kramer).

## Discussion

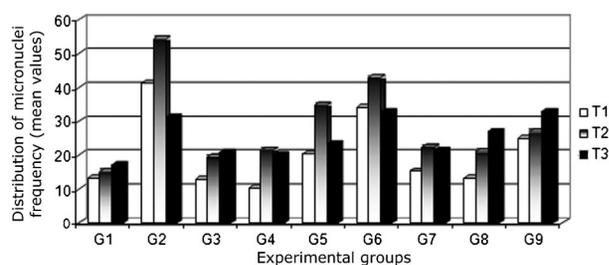
The results of this study demonstrate that oocyte-activation agents 6-DMAP, a protein serine/threonine kinase (or phosphorylation) inhibitor, and CHX, a protein synthesis inhibitor, have different effects on the induction of genotoxic and mutagenic damage in HepG2 cells and mice.

The *in vitro* results indicate that both compounds can be cytotoxic at high concentrations. The data indicate that the higher concentrations are capable of killing all of the cells in culture. Lower concentrations also showed cytotoxicity, but did not kill all of the cells. The concentrations of 1.90-0.475 mg/mL CHX and 160.0-1.25 × 10<sup>-3</sup> mg/mL 6-DMAP did not affect cell viability, and the concentrations of 0.237-0.014 mg/mL of 6-DMAP caused an increase in the number of cells. These observations

indicate that high concentrations cause damage to cells, leading to death, whereas lower concentrations allow progression of the cell cycle, leading to an increase in the number of dividing cells and thus in the number of cells present on the culture plate.

According to de la Fuente and King (28), treatment of bovine oocytes with Ca<sup>2+</sup>, CHX, and 6-DMAP stimulates the reinitiation of the cell cycle in embryonic cells and thereby produces a high rate of blastocyst formation. Therefore, based on studies by Presicce and Yang (29) and Susko-Parrish et al. (12), treatment with 6-DMAP has been suggested to be directly related to the initiation of mitosis for cleavage of the zygote and consequent formation of the blastocyst.

The effects of 6-DMAP on chromatin and microtubule arrangement, which have been the most extensively studied effects, are mediated by inhibition of protein



**Figure 1.** Distribution of the micronuclei frequency (means) in peripheral blood of mice during the 72 h of experimentation. G1: phosphate-buffered saline (PBS; negative control); G2: cyclophosphamide (CPA; positive control); G3 to G6: 6-dimethylaminopurine (6-DMAP); G7 to G9: cycloheximide (CHX). T1, T2, and T3: collection of peripheral blood at 24, 48, and 72 h after treatment, respectively.

**Table 6.** Summary of 6-DMAP and CHX activities.

Activity	6-DMAP	CHX
<i>In vitro</i>		
Cytotoxicity	+	+
Genotoxicity	+	+
RCV	-	-
Mutagenicity	+	+
RNDI	+	+
<i>In vivo</i>		
Genotoxicity	+	+
Mutagenicity	+	+

6-DMAP: 6-dimethylaminopurine; CHX: cycloheximide; RCV: reduction in cell viability; RNDI: reduction in nuclear division index; +: presence of activity; -: absence of activity.

kinases and, consequently, proteins involved in the processes of phosphorylation (28,30,31). The dephosphorylation induced by treatment with 6-DMAP is capable of inactivating the proto-oncogene *c-mos* and initiating mitosis by activating mitogen-activated protein kinases (28,32,33). This cascade could be the mechanism by which 6-DMAP acts as a mitogen in HepG2 cells in the MTT assay. In the micronucleus test *in vitro*, the lowest concentration of 6-DMAP (0.01 mg/mL) showed a significant increase in the nuclear division index, which could have been due to the same inhibitory activity of 6-DMAP in the production of kinases that leads to mitosis.

The comet assay, which showed a good index of cell viability, demonstrated that all of the concentrations of 6-DMAP and CHX, except the lowest concentration of CHX, induced DNA damage. Thus, the use of these agents can produce genetic lesions and compromise the development of the embryo.

The micronucleus test *in vitro* showed that the three highest concentrations of 6-DMAP reduced the nuclear division index and that only the lowest of these was mutagenic. The results demonstrated that the frequency of micronuclei observed with treatment with the two highest concentrations was less than the frequency observed for the negative control. However, it cannot be inferred that these two concentrations were not mutagenic, because a reduction in the nuclear division index (which was much larger for these two concentrations) can lead to a substantial underestimation of the frequency of micronuclei. This underestimation could occur because the DNA damage could be quantified only in cells that passed through a cycle of cell division and had an interruption in cytokinesis due to the use of cytochalasin-B. With respect to CHX, the two lowest concentrations did not reduce the nuclear division index, and only the concentration of  $1.60 \times 10^{-3}$  mg/mL showed mutagenic activity. The two highest concentrations tested significantly decreased the nuclear division index, and both concentrations were found to be mutagenic. However, it should be noted that the higher concentration was shown to be less mutagenic than  $16.00 \times 10^{-3}$  mg/mL.

Mutagenesis studies of 6-DMAP and CHX are scarce. However, some studies have indicated that the utilization of these two compounds is associated with alterations in mitosis and meiosis (10,28,34).

A study by de la Fuente and King (28), which was performed using bovine embryos developed by the process of parthenogenesis, demonstrated that an elevated frequency of tetraploid metaphases (which could have been induced by the utilization of 6-DMAP and CHX) was related to embryonic mortality. Moreover, Mitalipov et al. (10) have reported, in a study conducted with oocytes of Rhesus monkeys, that one-third of the embryos produced by parthenogenesis extruded one of the two polar bodies containing chromatin, but the ploidy of these embryos was not determined. Thus, it is believed that most

pregnancy failures could, in part, be attributed to the high incidence of polyploidy observed in cloned embryos exposed to 6-DMAP (1,35,36). However, alternative treatments with dehydroleucodine combined with cytochalasin B could have better results as oocyte activators than activation by an often-used protocol using ionomycin combined with 6-DMAP (36). Another study (37) showed that DNA damage caused by cell cycle inhibitors could also be evaluated by the presence of phosphorylated histone H2AX foci (markers of DNA double-stranded breaks). They also showed that treatment with 6-DMAP in transgenic embryos is a promising alternative for transgenic animal production.

The extrusion of genetic material involved the loss of parts of chromosomes or whole chromosomes. Thus, alterations in the cell cycle can result from the use of compounds that interfere with the activation of the oocyte cytoplasm, in parthenogenesis and in the transfer of somatic nuclei to the cytoplasm.

Previous studies have reported that treatment with CHX at a concentration of 1 mM for 6, 10, or 16 h causes an absence of or alterations in chromosome condensation during cell division and the absence of nuclear envelope fragmentation during prophase (34). Other reports have indicated that, at a concentration of 0.1 mM, chromosomes that do not localize adequately in the equatorial plate are later found dispersed, suggesting alterations in the formation of the mitotic spindle (34,38). These events can be related to those that occur in the formation of micronuclei, since these can arise from clastogenic and/or aneugenic damage.

According to Sentein (34,38), treatment with CHX can produce a reduction in astral expansion, condensation of the centrosphere, and a separation of poles and asters from the nuclei. These three symptoms indicate a weakened polar activity. Cytokinesis is blocked irreversibly after 6 h of treatment. After 10 h of treatment, the recovery of cytokinetic activity is possible, but it is not efficient. Treatment for 30 h, as utilized in this study, may favor the appearance of damage, as demonstrated by the micronucleus test *in vitro*.

When analyzing the percentages of DNA damage, measured by the quantification of cells with micronuclei *in vitro* in relation to the number of cells analyzed, a dose-response relationship can be seen for CHX, and the frequency of DNA damage increases with the concentration of the compound. However, this type of relationship could not be demonstrated upon treatment with 6-DMAP because of the drastic reduction in binucleated cells resulting from the interruption of the cell cycle.

*In vivo* studies were carried out in mice, testing four doses of 6-DMAP and three doses of CHX. In the comet assay, which was performed 4 h after the administration of the agents, the lowest dose of 6-DMAP did not show genotoxic activity. However, the other three doses displayed genotoxic effects with scores higher than that

for the positive control, indicating high genotoxic activity. Only the highest dose of CHX had a genotoxic effect. However, the score for this dose was 5.52 times lower than that of the positive control, indicating a lower genotoxic activity when compared to 6-DMAP.

The assays to determine mutagenicity *in vivo* were performed at three different times: 24, 48, and 72 h after the administration of the compounds. At 24 h, both compounds were found to be mutagenic only at the highest dose. At 48 h, the two highest doses of 6-DMAP and the highest dose of CHX increased the frequency of micronuclei. However, at 72 h after treatment, mutagenic activity could be observed with all of the doses of 6-DMAP tested and with only the highest dose of CHX.

A comparison of the different times demonstrates that the peak of micronucleus frequency upon treatment with 6-DMAP occurred at 48 h. However, even the lowest dose caused DNA damage at 72 h, suggesting that this compound can exert a mutagenic effect even after being metabolized and later excreted.

The only mutagenic dose of CHX caused a gradual increase in the frequency of micronuclei over the 72 h of the experiment, suggesting that the metabolites of CHX can also exert mutagenic effects. Thus, the peak of micronuclei after treatment with this compound occurred at 72 h. In addition, the three highest doses of 6-DMAP induced a positive genotoxic response, with 15.3 times the number of damaged cells compared with the negative control. 6-DMAP treatment also induced a positive mutagenic response (chromosomal breaks, revealed by the presence of micronuclei) that ranged from 1.2 to 2.8 times that of the negative control, depending on the time of the analysis. The genotoxic activity of CHX was 2.5 times that of the negative control. The positive mutagenic response was induced only at the highest concentration of CHX and ranged from 1.8 to 1.9 times that of the negative control, also depending on the time of analysis. These results suggest that 6-DMAP and CHX enhance the incidence of DNA damage and chromosomal abnormalities in mammals.

Katoh et al. (39) conducted studies to determine whether 6-DMAP and CHX are mutagenic agents in bacterial mutagenicity tests. *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 and *Escherichia coli* WP2 *uvrA* were used as test strains. The authors observed that, although CHX was not mutagenic in any of the strains, 6-DMAP induced a positive mutagenic response that ranged from 2 to 5 times above background in TA1535.

According to de la Fuente and King (28), the utilization of 6-DMAP and CHX is related to the successful production of mammalian clones, including those of cattle, rabbits, goats, and horses. However, despite providing satisfactory activation rates, the utilization of 6-DMAP has

resulted in aneuploidy in embryos produced using this protocol. This result was corroborated by Katoh et al. (39), who reported that cloned embryos produced by nuclear transfer frequently die, are aborted, or have fetal abnormalities. The authors noted the need for new studies to determine if these adverse events are truly the result of using chemical agents in the process of embryo reconstruction. In addition, Basic-Zaninovic et al. (40) have performed a comprehensive study of the mutagenic effects of CHX using the Ames test (*S. typhimurium* TA98 and TA100; *Saccharomyces cerevisiae* D7), *Allium cepa* root tip assay, and a micronucleus test in mouse bone marrow. The results demonstrated that there was no increase in frequency or reversion in *S. typhimurium*, suggesting that CHX does not induce frameshift or base pair substitution mutations in *S. typhimurium*, regardless of metabolic activation. In *S. cerevisiae*, no mutations were observed that could be determined by gene conversion in this yeast. However, treatment with CHX significantly diminished viability, indicating toxic effects. In the *A. cepa* root tip test and micronucleus test in bone marrow, treatment with CHX causes different types of DNA damage that could be determined by chromosomal breaks, mitotic perturbations, and nuclear abnormalities.

A general analysis of the data demonstrates that the two compounds are cytotoxic *in vitro*, mutagenic *in vitro* and *in vivo*, alter the nuclear division index *in vitro*, and do not affect cell viability *in vitro*. It should be noted that numeric or structural chromosomal aberrations, caused by clastogenic and/or aneugenic agents, are of major importance in the study of mutagenesis, carcinogenesis, spontaneous abortion, prenatal death, and stillbirth and in the development of offspring with congenital malformations and physiological and/or behavioral alterations. Considering the latter and based on our findings, the present investigation can be viewed as the beginning of these aforementioned studies.

Although other related studies have been reported, our data contraindicate the process of cloning of mammals using 6-DMAP and CHX and present a hypothesis that improves our understanding of the low viability of embryos produced by nuclear transfer, after their implantation *in utero*. Studies must still be performed to correlate reproductive performance and teratogenic, morphological, and functional analyses, including the study of learning and physical and reflexologic development, in the progeny of animals produced or treated with 6-DMAP and CHX. These types of studies will lead to greater insight into the effects of these agents.

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