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Chlorophyllin in the intra-uterine development of mice exposed or not to cyclophosphamide

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ABSTRACT. Chlorophyllin, a sodium-copper salt synthesized from chlorophyll, has already proved to have anticlastogenic, antimutagenic and anticarcinogenic activity, however few are the studies in the teratogenicity area. The present study evaluated the effects of chlorophyllin in intra-uterine development of mice exposed or not to cyclophosphamide. Pregnant females were divided into 8 groups of 15 animals each, G01 - PBS (0.1 mL 10.0^{-1} g) orally; G02 – cyclophosphamide (20.0 mg kg^{-1}) i.p.; G03, G04 and G05 - chlorophyllin at concentrations of (5.0, 10.0 and 15.0 mg kg^{-1}) orally; G06, G07 and G08 (5.0, 10.0 and 15.0 mg kg^{-1}) orally, of chlorophyllin, respectively, and (20.0 mg kg^{-1}) i.p. of cyclophosphamide. In the 18th day the females were submitted to laparotomy and females and fetuses analyzed. The results showed that the chlorophyllin was not effective in protecting the reproductive parameters as well as teratogenicity. Finally, it was observed that the presence of chlorophyllin increased the frequency of some malformations when combined with cyclophosphamide. However, it was not teratogenic and not embryo lethal in this experimental design.

Keywords: antimutagenic, anticarcinogenic, food additives, pregnancy, teratogenesis.

Clorofilina no desenvolvimento intra-uterino de camundongos expostos ou não à ciclofosfamida

RESUMO. Clorofilina é um sal de cobre e sódio sintetizado a partir da clorofila. Provou-se ter atividade anticlastogênica, antimutagênica e anticarcinogênica. No entanto, poucos são os estudos sobre esta substância na área de teratologia. Dessa forma, o presente trabalho avaliou os efeitos da clorofilina no desenvolvimento intrauterino de camundongos expostos ou não à ciclofosfamida. Para tal, fêmeas prenhez foram divididas em oito grupos experimentais contendo 15 animais cada: G01 - PBS (0,1 mL 10.0^{-1} g) via oral; G02 - ciclofosfamida (20,0 mg kg^{-1}), intraperitoneal; G03, G04 e G05 - clorofilina em concentrações de (5,0; 10,0 e 15,0 mg kg^{-1}) via oral; G06, G07 e G08 (5,0, 10,0 e 15,0 mg kg^{-1}) via oral, de clorofilina, respectivamente, e 20,0 mg kg^{-1}, via intraperitoneal, de ciclofosfamida. No 18º dia de gestação, os animais foram submetidos à laparotomia e os fetos, analisados para parâmetros teratogênicos. Os resultados mostraram que a clorofilina não foi eficaz para proteger os parâmetros reprodutivos, bem como a teratogenicidade. Finalmente, foi observado que a clorofilina quando combinada com a ciclofosfamida aumentou a frequência de algumas malformações. No entanto, a clorofilina não se apresentou teratogênica e nem letal para este desenho experimental.

Palavras-chave: antimutagênico, anticarcinogênico, aditivos alimentares, gestação, teratogenese.

Introduction

With the growth and development of modern society, there has been an increase in the number of substances that pollute the environment, resulting in the development of chronic diseases and also in germinative alterations that determine the birth of adverse offsprings. However, this last fact seems to be continuously worsened.

It is therefore important to develop and to use test-systems that allow evaluation of substances with teratogenic potential, as well as natural and synthetic products able to prevent the adverse development of the offspring of mammals due to ability to prevent damage to the DNA.

Chlorophyllin is a sodium-copper salt derivative of chlorophyll where the central nucleus of magnesium is substituted by another metal such as copper, iron or cobalt and where the phytyl and methyl ester groups are substituted by sodium or potassium (SARKAR et al., 1994),

converting it into water soluble. Despite the artificial chemical changes caused in chlorophyllin, this continues to present the same functional properties of chlorophyll, a product found in various sources such as fruits, vegetables and greens.

Due to produce more stability than the chlorophylls (SARKAR et al., 1996), chlorophyllin has been extensively used as part of food additives, in the acceleration of healing, in the treatment of kidney stones by the calcium oxalate and in the control of body, urinary and fecal odors of geriatric patients.

This substance has been investigated because of its antigenotoxic, anticlastogenic, antimutagenic and anticarcinogenic potential (RENNER, 1990; GHOSH et al., 1991; SEN et al., 1991; ABRAHAM et al., 1994; SARKAR et al., 1994; EDENHARDER et al., 1995; SURH et al., 1995; NEGISHI et al., 1997; PIMENTEL et al., 1999; BEZ et al., 2001a; RAMPAZO et al., 2002), as well as by several other effects, but few are the studies in the area of teratogenicity, which is a process closely linked to mutagenesis, since that results from mutations in DNA.

In the study of García-Rodríguez et al. (2001) it was evaluated the protective effects of chlorophyllin on the clastogenic damage caused by chromium trioxide (CrO_3). It was verified the frequency of micronuclei in polychromatic erythrocytes from blood of rats, it was showed that the CrO_3 induced the formation of micronuclei 12 and 48 hours after treatment, that the administration of chlorophyllin (20 μg g^{-1} body weight) did not increase the frequency of micronuclei and that when the chlorophyllin was administered prior to treatment with CrO_3 there was a significant decrease in the frequency of micronuclei observed 12 hours after treatment. Still considering the chemopreventive effects of chlorophyllin, Ibrahim et al. (2007) evaluated the potential of this substance as a natural antioxidant to reduce oxidative stress markers induced by cyclophosphamide and benzo[a]pyrene in male albino rats, finding that chlorophyllin was effective in the prevention of oxidative damage caused by these oxidants in rats.

However, other studies show that both the chlorophylls and chlorophyllin may have genotoxic, clastogenic and carcinogenic effects. Romert et al. (1992), working with several test-systems, observed that chlorophyllin can act as a positive or negative mutagenicity-modifier agent, but without dose-response effect.

Woo et al. (2007) evaluated the effects of dietary supplementation with chlorophyllin in male Sprague-Dawley rats on the toxicity induced by acrylamide. The animals received 1% of chlorophyllin in the diet and a week after 0.02% of acrylamide in the drinking water for four weeks. It was considered the testicular toxicity and neurotoxicity, which were evident in the treatment of acrylamide and were not suppressed by dietary supplementation with chlorophyllin.

Thus, the field of investigation of chlorophylls and related compounds in the process of protecting the DNA or as agents that induce or modulate the DNA damage, still needs more data to a better understanding of the mechanisms of action of these molecules, which have been ambiguous, in the prevention and in the induction of damage to genetic material. Thus, this study aimed to investigate the chlorophyllin, as well as its effects against the morphophysiological damage caused by exposure to cyclophosphamide during embryonic development, through the evaluation of congenital malformations and reproductive parameters of females.

Material and methods

Teratogenic agent

For the induction of teratogenesis it was used the indirect-acting alkylating agent cyclophosphamide (Fosfaseron-Ítaca Laboratórios) in a final dose of 20.0 mg kg^{-1} of body weight (intraperitoneally, i.p.), diluted in phosphate buffer solution (PBS), Ca^{+2}- and Mg^{+2}-free, pH 7.4. This chemotherapeutic and immunosuppressive suffers mainly hepatic bioactivation and its metabolites have potent teratogenic action.

Chlorophyllin

For the evaluation of the teratogenesis protection the chlorophyllin was administered in doses of 5.0, 10.0 and 15.0 mg kg^{-1} of body weight (orally, with probe aid), diluted in phosphate buffer solution (PBS), Ca^{+2}- and Mg^{+2}-free, pH 7.4.

Animals and experimental design

It was used Swiss mice (*Mus musculus*) of both sexes (120 females and 60 males) in reproductive age, with a mean weight of 30 g, from the Biotério Central da Universidade Estadual de Londrina. The experiment was conducted at Biotério Setorial do Departamento de Biologia Geral. The animals were kept in polypropylene cages, in double in the case of females and isolated in the case of males. A minimum period of 7 days was allowed for acclimatization. Lighting and temperature were controlled, using a photoperiod of 12h light: 12h

dark and a temperature of 22 ± 2ºC. The animals were fed with filtered water and commercial ration. To evaluate the teratogenicity, females were submitted to crossing, at a proportion of 1 male to 2 females. Pregnancy was determined by the 'vaginal plug's' detection, in which day was considered gestation zero day. The experiment was conducted acording to the Ethics committee rules on animal experimentation from Universidade Estadual de Londrina.

Teratogenicity assay

Pregnant females (n = 120) were divided into 8 experimental groups. The animals of the control group (Group 1) received PBS in a 0.1 mL 10^{-1} g body weight's volume, orally, on the 9th, 10th and 11th gestation day and intraperitoneal (i.p.) on the 10th day of pregnancy. The animals in the cyclophosphamide group (Group 2) received this chemotherapeutic agent at a dose of 20 mg kg^{-1} of body weight (i.p.) on the 10th gestation day and PBS in a volume of 0.1 mL 10^{-1} g of body weight (orally) on the 9th, 10th and 11th gestation day. The animals in the groups chlorophyllin (Group 3, 4 and 5) received on the 9th, 10th and 11th gestation day chlorophyllin at concentrations of 5.0, 10.0, and 15.0 mg kg^{-1} of body weight (orally), respectively, and PBS in the volume of 0.1 mL 10^{-1} g of body weight (i.p.) on the 10th gestation day. The animals in the associated groups (Group 6, 7 and 8) received the doses of chlorophyllin (orally) above mentioned, respectively, in the 9th, 10th and 11th gestation day and 20.0 mg kg^{-1}(orally) of body weight (i.p.) of cyclophosphamide on the 10th day of pregnancy.

In the 18th day of pregnancy, females were submitted to euthanasia by cervical dislocation, followed by laparatomy, since newborns that are malformed or have low viability are often cannibalized by their genitors.

To determine possible maternal toxic effects, the visceral organs were inspected for macroscopic abnormalities. Next, the lungs, heart, liver and kidneys were collected and weighed.

Then, a hysterectomy and omphalectomy were performed, recording the number of implantation sites, presence of resorptions, number of live and dead fetuses, and fetal and placental weights. A systematic analysis was also performed in the fetuses to detect external structural malformations and to determine sex. Based on these data, the following fertility parameters were determined: resorption's level (no. of resorptions x 100 / no. of implantations); post-implantation losses' level (no. of implantations – no. of live fetuses x 100 / no. of implantations); fetal viability's level (no. of live fetuses x 100 / no. of implantations); placental index (placental weight / fetal weight); external malformations' level (no. of malformed fetuses x 100 / no. of fetuses examined).

Adequate weight for gestational age was determined based on the Calderon's method. According to this author, fetuses can be classified as: fetuses of adequate weight for gestational age (AWGA) – weighing between the mean weight of fetuses in the control group more or less the standard deviation; fetuses of low weight for gestational age (LWGA) – body weight less than the mean weight of fetuses in the control group minus the standard deviation of the same group; and high weight's fetuses for gestational age (HWGA) – body weight above the mean weight of fetuses in the control group plus the standard deviation of that group.

The offspring of the groups was divided randomly into two subgroups, each consisting of half the litter. The first was fixed in Bodian´s solution for visceral examination, which was performed using the incisions/microdissection proposed by Barrow and Taylor (1969) for the thorax and abdomen's study, and using the strategic incisions proposed by Wilson (1965) for the head's study. The classification of the visceral alterations was based mainly on the works of Taylor (1986) and Manson and Kang (1994). The second subgroup was reserved for skeletal examination according to the 'Alizarin red' technique described by Staples and Schnell (1964). The examination of visceral and skeletal from fetuses was performed using a dissecting stereomicroscope.

The comparison of quantitative results was carried out using parametric and non-parametric tests (ANOVA, Kruskal-Wallis and Chi-Square), depending on the nature of the data's distribution. For the qualitative data and frequencies, the litter was utilized as the unit basis, as recommended in the relevant literature (HANSEMAN; HOGAN, 1995). In all cases, differences with $p < 0.05$ were considered statistically significant.

Results

The ponderal development, absolute and relative weight of organs, weight gain and net weight gain are presented in Table 1.

The initial weights showed that there is no difference between the groups. The values for the initial weights ranged from 29.61 ± 0.97 to 33.60 ± 1.18.

Table 1. Parameters related to ponderal development, absolute and relative weight of organs, weight gain and net weight gain.

Parameters	Experimental Groups							
	Control	Cyclophosphamide	Chlorophyllin			Associated Groups		
	G01	G02	G03	G04	G05	G06	G07	G08
	Ponderal Development							
Total of Female	15	15	15	15	15	15	15	15
Initial weight(g)	33.60 ± 1.18^{a}	30.72 ± 0.87^{a}	32.36 ± 1.09^{a}	30.55 ± 0.59^{a}	33.24 ± 1.04^{a}	29.68 ± 0.71^{a}	29.61 ± 0.97^{a}	31.13 ± 0.79^{a}
Final weight(g)	56.48 ± 1.55^{a}	47.60 ± 1.61^{b,c}	54.70 ± 2.10^{a,b}	53.08 ± 1.85^{a,b,c}	57.51 ± 1.56^{a}	48.13 ± 1.28^{b,c}	45.84 ± 1.52^{c}	48.94 ± 1.81^{b,c}
Úterus(g)	17.41 ± 0.85^{a,b}	9.74 ± 1.27^{c}	15.88 ± 1.77^{a,b,d}	16.17 ± 1.42^{a,d}	17.48 ± 1.32^{a}	13.04 ± 0.90^{b,c,d}	10.86 ± 1.06^{c,d}	12.15 ± 1.19^{b,c}
WG (g)	22.88 ± 1.24^{a,b}	16.88 ± 1.55^{b}	22.34 ± 2.00^{a,b}	22.52 ± 1.60^{a,b}	24.27 ± 1.42^{a}	18.45 ± 1.43^{a,b}	16.23 ± 1.47^{b}	17.81 ± 1.42^{a,b}
NWG(g)	5.39 ± 0.68^{a}	7.14 ± 0.52^{a}	6.46 ± 0.70^{a}	6.36 ± 0.54^{a}	6.79 ± 0.73^{a}	5.40 ± 0.80^{a}	5.38 ± 0.91^{a}	5.56 ± 0.97^{a}
	Absolute and relative weight of organs							
Liver (g)	2.26 ± 0.05^{a,b}	2.08 ± 0.09^{a,b}	2.21 ± 0.08^{a,b}	2.12 ± 0.10^{a,b}	2.34 ± 0.09^{a}	2.10 ± 0.07^{a,b}	1.91 ± 0.10^{b}	2.04 ± 0.08^{a,b}
RW	0.0403 ± 0.0010^{a}	0.0436 ± 0.0009^{a}	0.0406 ± 0.0006^{a}	0.0398 ± 0.0009^{a}	0.0407 ± 0.0009^{a}	0.0436 ± 0.0009^{a}	0.0418 ± 0.0018^{a}	0.0418 ± 0.0011^{a}
Kidneys (g)	0.37 ± 0.01^{a}	0.33 ± 0.02^{a,b}	0.35 ± 0.01^{a,b}	0.32 ± 0.01^{a,b}	0.35 ± 0.01^{a,b}	0.31 ± 0.01^{b}	0.33 ± 0.01^{a,b}	0.35 ± 0.01^{a,b}
RW	0.0065 ± 0.0002^{a,b}	0.0070 ± 0.0004^{a,b}	0.0065 ± 0.0002^{a,b}	0.0060 ± 0.0002^{a}	0.0060 ± 0.0002^{a}	0.0064 ± 0.0002^{a,b}	0.0072 ± 0.0002^{b}	0.0072 ± 0.0004^{b}
Lung (g)	0.20 ± 0.01^{a}	0.19 ± 0.01^{a}	0.19 ± 0.01^{a}	0.18 ± 0.01^{a}	0.21 ± 0.01^{a}	0.17 ± 0.01^{a}	0.20 ± 0.01^{a}	0.20 ± 0.1^{a}
RW	0.0036 ± 0.0002^{a}	0.0041 ± 0.0002^{a}	0.0036 ± 0.0002^{a}	0.0035 ± 0.0002^{a}	0.0036 ± 0.0002^{a}	0.0037 ± 0.0002^{a}	0.0043 ± 0.0002^{a}	0.0042 ± 0.0002^{a}
Heart (g)	0.17 ± 0.01^{a}	0.16 ± 0.00^{a}	0.17 ± 0.01^{a}	0.17 ± 0.02^{a}	0.18 ± 0.01^{a}	0.15 ± 0.00^{a}	0.16 ± 0.01^{a}	0.16 ± 0.01^{a}
RW	0.0030 ± 0.0001^{a}	0.0035 ± 0.0001^{a}	0.0032 ± 0.0002^{a}	0.0032 ± 0.0003^{a}	0.0031 ± 0.0002^{a}	0.0032 ± 0.0001^{a}	0.0036 ± 0.0002^{a}	0.0032 ± 0.0002^{a}

Legend: G01 – phosphate buffer solution (PBS) – 0.1 mL 10.0^{-1} g b.w., orally; G02 – cyclophosphamide – 20.0 mg kg^{-1} b.w., i.p.; G03, 04 e 05 – chlorophyllin at concentrations of 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally; G06, 07 e 08 – 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally of chlorophyllin, respectively, and 20.0 mg kg^{-1} b.w., i.p. of cyclophosphamide. WG – weight gain; NWG – net weight gain; RW – relative weight. Data expressed as mean ± standard deviation. Different letters means statistically significant difference. (Test: Analysis of Variance / Tukey, $p < 0.05$).

The final weights (45.84 ± 1.52 - 57.51 ± 1.56) showed that animals that received cyclophosphamide had lower body weight. The gravid uterus weight (9.74 ± 1.27 - 17.48 ± 1.32) and weight gain (16.23 ± 1.47 - 24.27 ± 1.42) reflect the same events. However, the net weight gain (5.39 ± 0.68 - 7.14 ± 0.52) shows no statistically significant differences.

The analysis of absolute and relative weights showed that there are no significant differences in relation to control group for the absolute and relative weight of liver, lung and heart and relative weight of kidneys. To the absolute weight of kidneys was found that there is a statistically significant difference between the control group and the group that received cyclophosphamide and the lower dose of chlorophyllin; to the relative weight of that organ there is only difference between the groups that received chlorophyllin in intermediate and higher doses and in these associated groups.

Table 2 presents data related to the reproductive performance and intrauterine development. The results demonstrate that there is no statistically significant difference between groups for resorption's number, fetal viability, post-implantation losses' level, resorption's level and placental index. However, statistically significant differences were noted for the number of implants, number of live fetuses, fetal weight, fetal length, placental weight, external, visceral and skeletal malformations' level.

The lower number of implants was observed in the group that received cyclophosphamide alone and combined with chlorophyllin in the intermediate dose. For this parameter values ranged from 9.20 ± 0.64 to 12.67 ± 0.51.

The number of live fetuses showed the same behavior, in other words, the significant differences are between groups that received cyclophosphamide alone and in association with the intermediate dose; values for this item ranged from 5.87 ± 0.56 to 10.00 ± 0.55.

The fetal weight has been reduced in those groups receiving cyclophosphamide associated to chlorophyllin and the values ranged from 1.14 ± 0.15 to 1.46 ± 0.11. When evaluated the adequacy of fetal weight to gestational age, it was found that exactly the three groups that received cyclophosphamide and chlorophyllin had low birth weight. The fetal length also corroborates the data previously reported and the values relating to it ranged from 2.34 ± 0.08 to 2.60 ± 0.05.

The placental weight was decreased in those groups that received cyclophosphamide. But only the group receiving cyclophosphamide and chlorophyllin in the lowest dose showed a statistically significant difference. The values of this parameter ranged from 0.0728 ± 0.0038 to 0.1108 ± 0.0066.

The external malformations' level ranged from 9.59 ± 1.76 to 59.69 ± 8.55. It is verified that the chlorophyllin did not cause malformations in a statistically significant manner, but there was an increase of occurrence. The groups receiving cyclophosphamide with chlorophyllin did not demonstrate prevention and values were similar to that group that received cyclophosphamide alone.

Table 2. Parameters related to reproductive performance and intrauterine development.

Parameters	Experimental Groups							
	Control	Cyclophosphamide	Chlorophyllin			Associated Groups		
	G01	G02	G03	G04	G05	G06	G07	G08
Total of Female	15	15	15	15	15	15	15	15
Implants	12.67 ± 0.51^{a,b}	9.20 ± 0.64^{c}	12.07 ± 0.57^{d}	11.07 ± 0.44^{a,b,c}	12.07 ± 0.51^{a,d}	11.13 ± 0.31^{a,b,c}	10.33 ± 0.46^{c,d}	11.20 ± 0.62^{a,b}
Resorptions	2.47 ± 0.52^{a}	3.27 ± 0.44^{a}	2.53 ± 0.48^{a}	2.27 ± 0.47^{a}	1.93 ± 0.47^{a}	2.60 ± 0.56^{a}	3.33 ± 0.57^{a}	3.07 ± 0.56^{a}
Live Fetuses	9.93 ± 0.37^{a}	5.87 ± 0.56^{b}	9.47 ± 0.71^{a}	9.07 ± 0.59^{a,c}	10.00 ± 0.55^{a}	8.53 ± 0.43^{a}	7.00 ± 0.51^{b,c}	8.13 ± 0.59^{a,b}
Fetal weight (g)	1.40 ± 0.14^{a}	1.29 ± 0.22^{a,b}	1.38 ± 0.15^{a}	1.46 ± 0.11^{a}	1.38 ± 0.10^{a}	1.14 ± 0.15^{b}	1.15 ± 0.20^{b}	1.17 ± 0.23^{b}
AW		AWGA	AWGA	AWGA	AWGA	LWGA	LWGA	LWGA
Fetal length (cm)	2.53 ± 0.04^{a,b}	2.40 ± 0.05^{a,b}	2.47 ± 0.06^{a,b}	2.60 ± 0.05^{a}	2.49 ± 0.04^{a,b}	2.35 ± 0.05^{b}	2.34 ± 0.08^{b}	2.35 ± 0.07^{b}
Placental weight (g)	0.1078 ± 0.0083^{a}	0.0913 ± 0.0056^{a,b}	0.0985 ± 0.0046^{a,b}	0.1055 ± 0.0072^{a}	0.1108 ± 0.0066^{a}	0.0728 ± 0.0038^{b}	0.0903 ± 0.0040^{a,b}	0.0904 ± 0.0118^{a,b}
Fetal Viability (%)	97.94 ± 1.15^{a}	98.33 ± 1.67^{a}	99.44 ± 0.56^{a}	100.00 ± 0.00^{a}	98.97 ± 0.71^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
PILL (%)	18.84 ± 5.06^{a}	33.70 ± 6.39^{a}	22.13 ± 5.71^{a}	18.33 ± 6.09^{a}	16.37 ± 5.23^{a}	21.82 ± 5.74^{a}	30.60 ± 7.14^{a}	30.60 ± 7.14^{a}
Resorption's level (%)	17.17 ± 4.94^{a}	32.96 ± 6.16^{a}	21.62 ± 5.78^{a}	18.33 ± 6.09^{a}	15.39 ± 5.34^{a}	21.83 ± 5.74^{a}	30.60 ± 7.14^{a}	24.34 ± 6.05^{a}
Placental index	0.0777 ± 0.0062^{a}	0.0723 ± 0.0044^{a}	0.0722 ± 0.0039^{a}	0.0720 ± 0.0042^{a}	0.0811 ± 0.0055^{a}	0.0643 ± 0.0034^{a}	0.0798 ± 0.0034^{a}	0.0769 ± 0.0070^{a}
EML	9.59 ± 1.76^{a}	43.79 ± 7.97^{b,c,d}	12.42 ± 4.00^{a,b}	25.61 ± 8.77^{a,b,c,d}	19.95 ± 6.44^{a,b,c}	40.44 ± 7.57^{a,b,c,d}	59.69 ± 8.55^{d}	49.00 ± 7.39^{c,d}
VML	3.00 ± 2.06^{a}	43.89 ± 11.74^{a,b}	15.91 ± 5.34^{a,b}	13.44 ± 5.60^{a,b}	22.56 ± 7.47^{a,b}	52.78 ± 10.78^{b}	45.89 ± 11.62^{a,b}	35.19 ± 11.12^{a,b}
SML	41.97 ± 8.62^{a}	100.00 ± 0.00^{b}	70.89 ± 5.58^{a}	60.83 ± 7.85^{a}	67.98 ± 8.15^{a}	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}	98.33 ± 1.67^{b}

Legend: G01 – phosphate buffer solution (PBS) – 0.1 mL 10.0^{-1} g b.w., orally; G02 – cyclophosphamide – 20.0 mg kg^{-1} b.w., i.p.; G03, 04 e 05 – chlorophyllin at concentrations of 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally; G06, 07 e 08 – 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally of chlorophyllin, respectively, and 20.0 mg kg^{-1} b.w., i.p. of cyclophosphamide. AW – adequacy of weight; AWGA – adequate weight for gestational age; LWGA – low weight for gestational age; PILL – post-implantation losses' level; EML – external malformations' level; VML – visceral malformations' level; SML – skeletal malformations' level. Data expressed as mean ± standard deviation. Different letters means statistically significant difference. (Test: Analysis of Variance / Tukey, $p < 0.05$).

The visceral malformations' level, which ranged from 3.00 ± 2.06 to 52.78 ± 10.78, was similar among different groups. However, the group that received chlorophyllin in the lowest dose associated to cyclophosphamide showed higher rates of birth defects, which were statistically significant.

The skeletal malformations' level were the ones that showed the highest values (41.97 ± 8.62 to 100.00 ± 0.00) and the groups that received cyclophosphamide associated or not to chlorophyllin presented increased statistically significant values.

The external malformations are shown in Table 3. The changes in brain (ectodermic dysplasia, ear's low implantation and exencephaly), limbs (retroversion and phocomelia) and trunk (scoliosis and lordosis) were not significant. The changes of eyes (opened eye and exophtalmus) had values that ranged from 2.42 ± 2.42 to 32.09 ± 12.86 and the groups that had higher incidence were those treated with cyclophosphamide associated to chlorophyllin in the highest doses tested.

The changes of mouth (retrognathism, micrognathia, tongue protrusion, labial-palatal fissure), which had averages between 0.61 ± 0.61 and 54.34 ± 14.36, presented significant changes and the most affected groups were the two highest doses of chlorophyllin associated to cyclophosphamide. Changes of paws (oligodactyly, polydactyly and sindactyly) were shown to be increased in groups that received cyclophosphamide, independently of the association with chlorophyllin.

Table 4 shows the data of visceral malformations. The analysis indicated that there is greater frequency among the groups that received cyclophosphamide, independently of receiving chlorophyllin, for the parameter changes in brain (hydrocephalus), however this was only significant in the group of cyclophosphamide associated with the lower dose of chlorophyllin. The control group did not show this change. But the animals treated with chlorophyllin presented rates of this visceral malformation that ranged from 6.67 ± 4.54 to 17.28 ± 7.19.

Table 3. External malformations observed in mice's fetuses.

Parameters	Experimental Groups							
	Control	Cyclophosphamide	Chlorophyllin			Associated Groups		
	G01	G02	G03	G04	G05	G06	G07	G08
Total of Fetuses	149	88	142	136	150	128	105	122
Changes of brain	0.00 ± 0.00^{a}	3.17 ± 2.35^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.67 ± 0.67^{a}	11.65 ± 7.49^{a}	2.31 ± 1.65^{a}	1.12 ± 1.15^{a}
Changes of eyes	8.74 ± 2.50^{a,b}	14.08 ± 7.41^{a,b}	2.42 ± 2.42^{a}	6.50 ± 3.97^{a,b}	2.37 ± 1.54^{a}	14.51 ± 5.72^{a,b}	32.09 ± 12.86^{b}	20.46 ± 11.55^{a,b}
Changes of mouth	0.61 ± 0.61^{a}	21.33 ± 9.40^{a,b}	9.66 ± 5.06^{a,b}	25.62 ± 13.74^{a,b}	26.70 ± 13.39^{a,b}	41.99 ± 14.77^{a,b}	54.34 ± 14.36^{b}	37.49 ± 7.85^{b}
Changes of limbs	3.23 ± 1.24^{a}	14.53 ± 7.01^{a}	4.72 ± 2.34^{a}	7.32 ±3.35^{a}	3.94 ± 1.83^{a}	14.77 ± 4.58^{a}	19.76 ± 7.33^{a}	12.06 ± 3.83^{a}
Changes of paws	0.00 ± 0.00^{a}	30.49 ± 8.48^{b}	0.44 ± 0.44^{a,c}	5.60 ± 3.21^{a,b}	1.41 ± 0.96^{a,b}	29.38 ± 9.68^{b}	29.58 ± 9.68^{b,c}	22.10 ± 6.52^{b,c}
Changes of trunk	0.00 ± 0.00^{a}	2.22 ± 2.22^{a}	0.00 ± 0.00^{a}	0.48 ± 0.48^{a}	0.44 ± 0.44^{a}	5.94 ± 3.49^{a}	3.17 ± 1.87^{a}	5.66 ± 3.90^{a}

Legend: G01 – phosphate buffer solution (PBS) – 0.1 mL 10.0^{-1} g b.w., orally; G02 – cyclophosphamide – 20.0 mg kg^{-1} b.w., i.p.; G03, 04 e 05 – chlorophyllin at concentrations of 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally; G06, 07 e 08 – 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally of chlorophyllin, respectively, and 20.0 mg kg^{-1} b.w., i.p. of cyclophosphamide. Changes of brain – ectodermic dysplasia, ear's low implantation and exencephaly; Changes of eyes – opened eye and exophtalmus; Changes of mouth – retrognathism, micrognathia, tongue protrusion, labial-palatal fissure; Changes of limbs – retroversion and phocomelia; Changes of paws – oligodactyly, polydactyly and sindactyly; Changes of trunk – scoliosis and lordosis. Data expressed as mean ± standard deviation. Different letters means statistically significant difference. (Test: Kruskal Wallis/Dunn, $p < 0.05$).

Table 4. Visceral malformations observed in mice's fetuses.

Parameters	Experimental Groups							
	Control	Cyclophosphamide	Chlorophyllin			Associated Groups		
	G01	G02	G03	G04	G05	G06	G07	G08
Total of Fetuses	72	41	68	64	72	64	49	61
Changes of brain	0.00 ± 0.00^{a}	37.22 ± 11.35^{b}	6.67 ± 4.54^{a,b}	10.11 ± 5.51^{a,b}	17.28±7.19^{a,b}	35.89 ± 10.19^{b}	30.44 ± 10.37^{a,b}	22.86 ± 9.97^{a,b}
Changes of head and neck	0.00 ± 0.00^{a}	6.67 ± 6.67^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.29±1.58^{a}	5.22 ± 2.87^{a}	5.55 ± 3.87^{a}	3.57 ± 3.57^{a}
Cardiac changes	0.00 ± 0.00^{a}	2.22 ± 2.22^{a}	1.79 ± 1.22^{a}	0.00 ± 0.00^{a}	0.83±0.83^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	3.40 ± 2.52^{a}
Urogenital changes	3.00 ± 2.06^{a}	28.89 ± 11.67^{a}	10.79 ± 4.97^{a}	3.33 ± 2.41^{a}	3.00±2.06^{a}	29.22 ± 10.27^{a}	19.33 ± 9.18^{a}	16.67 ± 7.36^{a}

Legend: G01 – phosphate buffer solution (PBS) – 0.1 mL 10.0^{-1} g b.w., orally; G02 – cyclophosphamide – 20.0 mg kg^{-1} b.w., i.p.; G03, 04 e 05 – chlorophyllin at concentrations of 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally; G06, 07 e 08 – 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally of chlorophyllin, respectively, and 20.0 mg kg^{-1} b.w., i.p. of cyclophosphamide. Changes of brain – hydrocephalus; Changes of head and neck – esophageal atresia, absence of nasal septum, and choana's hypoplasia; Cardiac changes – expansion of heart ventricle; Urogenital changes – hydronephrosis, renal agenesis and hermaphroditism. Data expressed as mean ± standard deviation. Different letters means statistically significant difference. (Test: Kruskal Wallis/Dunn, $p < 0.05$).

And the animals of the associated groups had rates of 22.86 ± 9.97 to 35.89 ± 10.19. These values were close to those observed in the group treated only with cyclophosphamide (37.22 ± 11.35), demonstrating that there was no prevention of this malformation. To the head and neck's malformations (esophageal atresia, absence of nasal septum, and choana's hypoplasia), urogenital changes (hydronephrosis, renal agenesis and hermaphroditism) and expansion of heart ventricle there was no statistically significant differences between the different experimental groups.

The skeletal changes are shown in Table 5. Changes of the brain (parietal with incomplete ossification), changes in the mouth (palatal fissure and short mandible and maxilla), changes of fore and hind members and waist (asymmetric, decresead and away) did not differ from control. However, there were statistically significant differences in changes of the brain (increased fontanelle), changes in the brain (asymmetrical, decreased, divided and absent supraoccipital), fore and hind paws (oligodactyly, polydactyly and sindactyly), sternum (supernumerary, asymmetrical, fused, change in the shape and incomplete ossification) and trunk (supernumerary rib, number of ribs less than 13, rib morphologically changed, fused vertebrae and vertebral center morphologically changed).

The fontanelle, average ranging from 0.00 ± 0.00 to 95.55 ± 4.44, only occurred in groups that received cyclophosphamide associated or not to chlorophyllin. In the groups with association an increase in the incidence of malformations has occurred. Changes of supraoccipital have occurred more frequently in the same groups that the changes of fontanelle and showed average between 0.95 ± 0.95 and 56.56 ± 9.96. Changes of fore paws (average of 0.00 ± 0.00 to 57.22 ± 15.95) and hind paws were also evident in the group that received cyclophosphamide. However, in spite of not presenting statistically significant prevention, the administration of chlorophyllin demonstrates reduction of hind paws' changes (average of 0.00 ± 0.00 to 83.67 ± 28.65).

Changes of sternum (41.97 ± 8.62 to 100.00) and trunk (0.00 ± 0.00 to 38.33 ± 14.92) were also more evident in groups that received cyclophosphamide. In the last case, occurrence has increased when there was administration of chlorophyllin in the two highest doses.

Discussion

In the pertinent literature it was noted that the chlorophyllin causes embryolethality and changes in intra-uterine development, as well as presents a potential clastogenic effect; however some studies

Table 5. Skeletal malformations observed in mice's fetuses.

Parameters	Experimental Groups							
	Control	Cyclophosphamide	Chlorophyllin			Associated Groups		
	G01	G02	G03	G04	G05	G06	G07	G08
Total of Fetuses	77	47	74	72	78	64	56	61
Changes of brain - fontanelle	0.00 ± 0.00^{a}	88.33 ± 8.04^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	95.55 ± 4.44^{b}	94.44 ± 3.01^{b}	90.00 ± 6.81^{b}
Changes of brain - parietal	0.00 ± 0.00^{a}	11.67 ± 8.04^{a}	3.33 ± 3.33^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	4.44 ± 4,44^{a}	8.89 ± 6.88^{a}	3.89 ± 2.68^{a}
Changes of brain - supraoccipital	1.67 ± 1.67^{a}	32.33 ± 11.33^{a,b,c}	14.17 ± 6.53^{a,b,c}	0.95 ± 0.95^{a}	6.56 ± 3.00^{a,b}	44.00 ± 12.83^{a}	56.56 ± 9.96^{c}	50.56 ± 10.90^{b,c}
Changes of mouth	0.00 ± 0.00^{a}	14.67 ± 7.61^{a}	7.33 ± 5.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	27.78 ± 14.70^{a}	18.56 ± 9.00^{a}	25.78 ± 10.28^{a}
Changes of fore limbs and waist	0.00 ± 0.00^{a}	11.67 ± 7.26^{a}	1.11 ± 1.11^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	20.00 ± 10.81^{a}	19.00 ± 13.59^{a}	9.11 ± 8.00^{a}
Changes of hind limbs and waist	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	1.33 ± 1.33^{a}	6.67 ± 6.67^{a}	5.33 ± 5.33^{a}
Changes of fore paws	0.00 ± 0.00^{a}	37.56 ± 15.83^{a,b}	6.67 ± 6.67^{a}	6.67 ± 6.67^{a}	4.44 ± 4.44^{a}	57.22 ± 15.95^{b}	28.56 ± 8.28^{a,b}	27.66 ± 13.00^{a,b}
Changes of hind paws	0.00 ± 0.00^{a}	83.67 ± 28.65^{b}	9.33 ± 7.00^{a,b}	0.00 ± 0.00^{a}	6.67 ± 6.67^{a,b}	62.11 ± 18.74^{b}	47.11 ± 14.82^{a,b}	45.44 ± 17.49^{a,b}
Sternum changes	41.97 ± 8.62^{a}	100.00 ± 0.00^{b}	67.56 ± 7.23^{a}	60.83 ± 7.85^{a}	67.98 ± 8.15^{a}	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}
Changes of trunk	0.00 ± 0.00^{a}	26.33 ± 10.49^{a,b}	6.67 ± 6.67^{a,b}	5.33 ± 5.33^{a,b}	1.33 ± 1.33^{a,b}	26.89 ± 9.85^{a,b}	38.33 ± 14.92^{b}	31.67 ± 14.69^{a,b}

Legend: G01 – phosphate buffer solution (PBS) – 0.1 mL 10.0^{-1} g b.w., orally; G02 – cyclophosphamide – 20.0 mg kg^{-1} b.w., i.p.; G03, 04 e 05 – chlorophyllin at concentrations of 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally; G06, 07 e 08 – 5.0, 10.0 e 15.0 mg kg^{-1} b.w., orally of chlorophyllin, respectively, and 20.0 mg kg^{-1} b.w., i.p. of cyclophosphamide. Changes of brain - fontanelle – increased fontanelle; Changes of brain – parietal – parietal with incomplete ossification; Changes of brain - supraoccipital – asymmetrical, decreased, divided and absent supraoccipital; Changes of mouth – palatal fissure and short mandible and maxilla; Changes of fore limbs and waist – asymmetric, decresead and away; Changes of hind limbs and waist – asymmetric, decresead and away; Changes of fore paws – oligodactyly, polydactyly and sindactyly; Changes of hind paws – oligodactyly, polydactyly and sindactyly; Sternum changes – supernumerary, asymmetrical, fused, change in the shape and incomplete ossification; Changes of trunk – supernumerary rib, number of ribs less than 13, rib morphologically changed, fused vertebrae and vertebral center morphologically changed. Data expressed as mean ± standard deviation. Different letters means statistically significant difference. (Test: Kruskal Wallis/Dunn, $p < 0.05$).

show the antigenotoxic potential both *in vitro* and *in vivo*, and these processes are intimately linked, as they come from damage to the DNA. In front of this ambiguous character, this work studied the chlorophyllin and its effects against the damage caused by the chemotherapeutic agent cyclophosphamide.

It is important to note that several protocols were developed for the effects of substances associated to cyclophosphamide and in some of these were found preventive effects on teratogenicity induced by the chemotherapeutic in question. According Shubert (1982), teratogenic and embryolethal effects in mice can be prevented by the combined administration of cyclophosphamide and a bovine blood proteins free extract, known as Solcoseryl. In another study, Bailey et al. (2005) present two protocols of treatment with indole-3-carbinol administered 24 and 48 hours before the exposure to cyclophosphamide. It was demonstrated that indole-3-carbinol prevents changes of limbs and tails in the pre-treatment protocol of 48 hours.

Thus, the current study used a protocol in which the females of the associated groups were exposed to chlorophyllin for a period of 3 days and the exposure to the chemotherapeutic agent occurred only on the second day. Thus, it was intended, in this experimental protocol, to combine a pre-treatment, simultaneous treatment and post-treatment of chlorophyllin in relation to cyclophosphamide. It was chosen this manner of administration due to studies indicate that chlorophyllin has antimutagenic activity, being the mechanism of action characterized by both desmutagenesis and bioantimutagenesis (BRONZETTI et al., 1990; NEGISHI et al., 1990; DASHWOOD; LIEW, 1992; ARIMOTO et al., 1993; DASHWOOD; GUO, 1993; TACHINO et al., 1994; EDENHARDER et al., 1995; DASHWOOD et al., 1996; BEZ et al., 2001b; RAMPAZO et al., 2002). Therefore, this protocol promoted the exposure of chlorophyllin at three moments, in an attempt to achieve a better effectiveness in preventing teratogenesis. The females of chlorophyllin groups received the same experimental design, however, without association, so that the effects of chlorophyllin were evaluated.

García-Rodrígues et al. (2002) proposed doses of chlorophyllin known as protective of genotoxic damage on embryonic development. So the pregnant females received doses (20, 30, 40, 50 and 100 $\mu g\ g^{-1}$) of the chlorophyllin intraperitoneal in the eighth day of gestation. The study showed that chlorophyllin induced total loss of litters in a dose-dependent response. Furthermore, it was demonstrated that the embryolethality in the groups treated with chlorophyllin are more associated to the toxic effects instead of the teratogenic effects. Thus, all the three proposed doses in this study (5.0, 10.0 and 15.0 mg kg^{-1} of body weight) are located below the first dose suggested by that author.

The values related to the initial weight show no statistical differences between the groups. In relation to the values of final weight, these indicated that females treated with cyclophosphamide, associated or not to chlorophyllin, had lower body weight; the uterus' weight and weight gain has also followed the same pattern, however, there is no difference in the net weight gain. It probably happened due to the animals treated with cyclophosphamide have fewer fetuses than the other. So, when it is considered the net weight gain, the weight of the uterus is discarded, showing no differences in this parameter.

Considering the absolute and relative weight of females' organs it was observed that for the absolute weight of the kidneys there was only difference in the associated group with the lowest chlorophyllin dose when compared to the control group, however these organs did not show significant difference in their relative weight, indicating that the difference is related to the animal's weight.

Considering the number of implants and live fetuses the lowest values are in the groups cyclophosphamide alone and associated to chlorophyllin in the intermediate dose. There was also reduction in placental weight in the groups receiving cyclophosphamide, associated or not to chlorophyllin, however, the placental index showed no such difference. This observation suggests fetal malnutrition unrelated to the increased placental index, facts that are corroborated by fetal weight and length of these experimental groups. Although these data have been statistically significant in the groups in which cyclophosphamide had been associated with chlorophyllin. The study of the adequacy of weight to gestational age also strengthens this finding, which demonstrated that the administration of cyclophosphamide, associated to chlorophyllin, was the responsible for fetuses who presented low weight for gestation age. Thus, these data suggest that chlorophyllin alone did not change these parameters, however in combination with cyclophosphamide there is a trend to increase the damage already caused by cyclophosphamide in some of these parameters. In concordance with the results of this study Mauthe et al. (1998), when treating pregnant rats with 2-Amino-1-methyl-6-phenilimidazo[4,5-b]pyridine (PhIP), a mammary carcinogen, and associating it to chlorophyllin, observed that the animals of the associated groups had in breast milk and in the stomach contents of newborns a greater amount of PhIP than the not

associated groups, showing that the chlorophyllin can enhance the effect of certain substances.

In an overview, it was observed that the chlorophyllin was not effective at preventing external malformations, or in induce them, although chlorophyllin groups present a greater frequency of external malformations when compared to control group. Also it was found that for some changes as those in eyes, mouths and limbs, caused by cyclophosphamide, the association with chlorophyllin increased the frequencies, especially in the groups of intermediate and higher doses. To paws' changes (oligodactyly, polydactyly and sindactyly), there was an increase in the groups treated with cyclophosphamide independent of association. Chaube et al. (1967) observed that rats treated with cyclophosphamide showed paws' changes like these. In humans Paskulin et al. (2005) reported changes in the fingers of a newborn whose mother went through sections of chemotherapy during pregnancy.

Among the visceral malformations found, the brain one, more specifically hydrocephalus, was the only one that showed statistically significant differences for groups cyclophosphamide alone and the association with the lower dose of chlorophyllin. Therefore, it is important to stress that when the control group is evaluated this change is not found, while in the groups treated only with chlorophyllin the frequency is increased, and is even higher in the groups receiving cyclophosphamide, associated or not.

The evaluation of skeletal malformations showed that in all the treatments in which there is the presence of cyclophosphamide the parameter increased fontanelle was statistically significant when compared to other groups, which did not show this alteration. It can be suggested that the association with chlorophyllin or even it alone does not change this parameter. Supraoccipital and fore and hind paws changes demonstrated that the groups who received cyclophosphamide, independent of receiving chlorophyllin, showed the increased frequency, however, the groups treated only with chlorophyllin also presented increased frequency of these changes when compared with the control group, but not being statistically significant. Moreover, it should be emphasized that on fore and hind paws changes the intermediate and higher doses of chlorophyllin associated to cyclophosphamide showed a decrease in the frequency of changes compared with the cyclophosphamide alone group; however, these data do not suggest a possible prevention of damage by chlorophyllin since they are isolated data that are not in agreement with the other parameters evaluated in this study.

The changes in sternebrae showed a statistically significant diference in the groups treated with cyclophosphamide and increased frequencies in the groups with chlorophyllin when compared to the control group. It should be mentioned that some skeletal changes occur spontaneously in fetuses and newborns, and it is known that the incidence of these anatomical variants increases after the treatment of pregnant females with teratogenic agents. These skeletal variations can be seen as effects of drugs at higher doses (14^{th} extra rib), or just normal variations (14^{th} rudimentary rib and variations of sternebrae), but they may not be classified as abnormalities when they are the only signs of embryotoxicity. Sternum variations, for example, seem to have a dubious value in predicting the teratogenic potential, since they only increase significantly when the doses are already seen as teratogenic. It should be emphasized that some of these variations are species-specific and therefore may show different magnitudes of responses in other strains or species (KIMMEL; WILSON, 1973).

To stem variations it was observed that the groups receiving cyclophosphamide in their treatment had increased frequencies and that in the association with the intermediate and higher doses of chlorophyllin these values were statistically significant, suggesting that these doses of chlorophyllin may increase the adverse effects of cyclophosphamide.

This research suggests that the chlorophyllin was not effective in protecting the reproductive parameters and the teratogenicity, despite the anticlastogenic effects of chlorophyllin shown in other studies (GHOSH et al., 1991; SEN et al., 1991; SARKAR et al., 1996; GARCÍA-RODRÍGUEZ et al., 2001) and its inhibitory capacity on the P450 enzymes (YUN et al., 1995) that are involved in citotoxic processes and in teratogenic properties. Finally, it was observed that the presence of chlorophyllin increased the frequency of certain birth defects when combined with cyclophosphamide. However, it was neither teratogenic nor embryolethal in this experimental design.

Conclusion

The evaluation of chlorophyllin in intra-uterine development of mice exposed or not to cyclophosphamide, showed that the chlorophyllin was not effective in protecting the reproductive parameters as well as teratogenicity.

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