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Spirulina (*Arthrospira maxima*) protects from cyclophosphamide teratogenicity in mice

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Abstract

We evaluated whether *Arthrospira maxima*, known as spirulina (Sp), counteracts the teratogenic effects induced by cyclophosphamide (Cp) in mice. Ninety pregnant CD-1 mice were divided into 6 groups: control, Cp 20 mg/kg, Sp 400 mg/kg, and three with Sp at 100, 200, and 400 mg/kg with Cp. Sp was administered intragastrically from the day of gestation (DG) 6 to 16 and Cp intraperitoneally to DG 10. Females did not differ in weight, except for DG 10. In gravid parameters, Cp and Sp alone or in association did not show significant effects, except for umbilical cord length, placental diameter, weight, and size of fetuses. At DG 17, the females were sacrificed to obtain pregnancy parameters. In the fetuses, macroscopic malformations such as anasarca, exencephaly, hydrocephalus, open eye, cleft palate, absence and deformations of upper and lower extremities and tail were evaluated, in skeletal anomalies absences, deformations, supernumerary bones and delay in mineralization were observed, antioxidant enzymes were determined in the livers as well as markers of damage due to oxidative stress. Sp 400 along with Cp counteracted the malformations significantly. Sp protects against Cp teratogenicity in mice by decreasing the reactive oxygen species and increasing the concentrations of superoxide dismutase and glutathione peroxidase, but not catalase.

Keywords: Cyclophosphamide; Spirulina; *Arthrospira maxima*; Antitheratogenesis; Mouse.

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Study contribution

Congenital malformations in humans and animals can be caused by the consumption of some drugs, environmental causes, genetic factors, and other circumstances of a multifactorial order, administered or present during the critical periods of gestation. In the case of drugs, cyclophosphamide is known to be used as an antitumor and immunosuppressant, which produces by the generation of reactive species, among other mechanisms, serious external and skeletal malformations in the progeny of mothers who consume it freely or by medical prescription. Preclinical experiments in experimental animals such as rodents using antioxidant agents such as Sp, cyanobacterium administered in the present work to pregnant mice during organogenesis, by their property of extrapolation to humans, can alert and avoid such risks that occur during the embryonic period thus contributing to the scientific development and understanding of antitheratogenesis, current research area, through the use of natural products.

Introduction

Cyclophosphamide (Cp) is an alkylating pro-drug used as an antitumor and immunosuppressant, which through liver microsomal enzymes is biotransformed mainly into phosphoramide mustard and acrolein,[1] causing some toxic effects. Teratogenicity, one such effect, is of great concern worldwide, so to mitigate its severity antioxidant agents such as vanadium,[2] quercetin,[3] N-acetyl-L-cysteine,[4] and green tea extract[5] have been investigated, and have resulted in good protective effects. In a review of cases of women exposed to Cp, it has been pointed out that one of the reasons for the production of teratogenic effects is the prooxidant-antioxidant imbalance, which leads to intrauterine growth restriction and craniofacial malformations including ocular abnormalities, cleft palate, hydrocephalus, micrognathia, microtia, craniosynostosis, and facial asymmetry, in addition to limb defects such as radial, ulnar, and tibial hypoplasia, clubfoot, digital hand, and foot defects, as well as vertebral fusion.[6]

Arthrospira maxima, better known by tradition and commercially as "spirulina", belongs to the phyllo Cyanobacteria, also formerly called green-blue microalgae, has a high nutritional value, so it has been recommended to fight hunger and malnutrition,[7] in addition to attributing different pharmacological activities in humans and laboratory animals, many of them based on its high antioxidant capacity. Such is the case of its antitoxic,[8] anti-inflammatory,[9] anticancer, chemopreventive and DNA repair,[10] antibacterial,[11] antifungal,[12] immunoregulatory,[13] antiallergic and antiviral,[14] antianemic,[15] antihypertensive and lipid-lowering,[16] hypoglycemic,[17] and hepatoprotective effect,[18] in addition to body weight reduction,[19] among others. Therefore, this study aimed to determine whether Sp as an antioxidant agent also protects against congenital malformations induced by Cp in pregnant mice administered during organogenesis.

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Materials and methods

Ethical statement

All animal handling was carried out according to the ethical principles approved by the CEI-ENCB with folio ENCB/CEI/076/2020 CONBIOÉTICA 09-CEI-002-20190327 and by NOM-062-ZOO-1999, "Technical specifications for the production, care, and use of laboratory animals".

Animals

For this study, female and male CD-1 mice weighing 30-35 grams, 7 to 8 weeks of age, acquired in the vivarium of the Autonomous University of the State of Hidalgo, Mexico, were used and kept in individual cages in a vivarium under controlled conditions of temperature (23 ± 2ºC), humidity (60 ± 10 %), and light-dark cycles of 12 hours each. The animals had free access to standard food (Rodent Lab Chow 5001, Purina, St. Louis, MO, USA) and purified water for an acclimatization period of two weeks before the experiment.

Mating

Mating was carried out during the last three hours of darkness (7 am to 10 am), randomly exposing three females to a male, and when the vaginal plug was evidenced it was considered as the day of gestation 0 (DG 0), placing the mated females in individual cages carrying a harmless marking on the fur.

Treatments

The mated females were randomly distributed into 6 groups of 15 animals each, which were intended for the following treatments: control, Cp 20 mg/kg, Sp 400 mg/kg, and the remaining three were concomitantly administered Cp 20 mg/kg and 100, 200, and 400 mg/kg Sp doses, respectively. Sp was acquired in Essential Foods for Humanity (Mexico City) and was administered intragastrically using purified water as a vehicle at a constant volume of 10 mL/kg. The Cp (Sigma-Aldrich CAS Number: 6055-19-2, product C3250000), was injected intraperitoneally at doses of 20 mg/kg using the vehicle at a constant volume of 5 mL/kg; the administrations were carried out between 11:30 am and 12:30 pm. Pregnant females were weighed on DG 0, DG 6, DG 10, and DG 17.

Euthanasia

The females were euthanized on DG 17 by cervical dislocation, and the uterus was obtained through an abdominal incision and weighed with its contents. The number of implantations and early and late embryonic resorption were counted in the uterus in addition to obtaining the weight of the females after the removal of the uterus.

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Macroscopic evaluation of the fetuses

When removing the fetuses, it was recorded whether they were alive or dead, the length of the umbilical cord, and the length and diameter of the placenta. The weight, size, and sex were determined in each of the fetuses, together with a visual examination to detect macroscopic malformations with the help of a stereoscopic microscope (American Optical 30×) following the cephalocaudal and proximal-distal planes.

Skeletal analysis of fetuses

For the skeletal analysis of the fetuses, after death, the skin was removed and, through an abdominal incision, the internal organs were removed, placing the livers in PBS (Phosphate-Buffered Saline) at -70 °C. They were then set to 70 % ethanol to proceed 48 h later to the modified bichromatic staining.[20] Skeletal alterations were observed in a stereomicroscope, as described by Menegola et al.,[21] evaluating the number, presence/absence/supernumeraries, shape (fusion, division, increase or decrease in size, curvatures, amorphous, lack of development), as well as the degree of ossification.

Determination of oxidative stress

Fetal livers placed in PBS were thawed to homogenize the tissue (Omni Tissue Homogenizer) per litter. The homogenized tissue was centrifuged at 13 000 rpm for 15 min at a temperature of 4 °C; using the resulting supernatant to determine the enzymatic activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and as markers of oxidative stress, malondialdehyde (MDA) and carbonyl groups. For determination of SOD and GPx activity, RANSOD and RANSEL kits (Randox Laboratories Ltd., Crumlin, Co. Antrim, UK) were used and the manufacturer's instructions were followed, reading the absorbances at 480 and 340 nm, respectively. For the CAT analysis, the Aebi method was used,[22] reading the absorbance at 570 nm.

The determination of carbonyl groups was performed by the method of Parvez and Raisuddin,[23] reading the absorbance at 360 nm, while the MDA analysis was performed according to the method of Buege and Aust,[24] to read the absorbances at 535 nm.

Statistical analysis

Statistical inferences were made with the SigmaPlot program version 14. The study of the quantitative data was carried out using one-way variance analysis and *post hoc* Student-Newman-Keuls. The Kruskal-Wallis test was used to compare the number and type of implantations. In turn, to analyze the data of the type of percentages, the chi^2 test was used followed by the exact Fisher test. In each analysis, statistically significant differences were considered when obtaining a value of $P < 0.050$.

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Results

Maternal weights

In DG 0, before starting the treatments, the weight of the mated females did not vary among the groups; only in DG 10, they were significantly reduced $P < 0.001$ in all Sp-treated compared to the control and Cp groups, except the control and the Cp + Sp 200 group with a $P = 0.001$. In the remaining days, the weights showed growth very similar to that of the control and Cp groups. Once the uterus was removed after euthanization, the weights of the females showed no significant differences.

Gravid parameters

Regarding the gravid parameters, there were no significant differences among the groups, although a tendency was observed to decrease early and late resorption in the Cp + Sp groups compared to the Cp group (Table 1).

In Table 1, the different groups correspond to: Control (vehicles: water and serum, depending on whether Sp or Cp); Cf 20 mg/kg (single administration on day 10 of gestation); Sp 400mg/kg; Cf + Sp 100 = Cf, 20 mg/kg + Sp, 100 mg/kg; Cf + Sp 200 = Cf 20 mg/kg + Sp, 200 mg/kg, and Cf + Sp 400 = Cf, 20 mg/kg + Sp, 400 mg/kg. Sp was administered from day 6 to 16 of gestation.

The length of the umbilical cord was lower in the Cp and Cp + Sp 200 groups, with no difference between them, but with the rest of the groups, that is, with the Control group ($P \leq 0.001$), with the Sp, Cp + Sp 400 ($P \leq 0.001$) and with group Cp + Sp 100 ($P = 0.022$) (Table 1). The same effects were observed in the diameter of the placenta, compared with the control group, showing a significant difference ($P \leq 0.001$) with the Cp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 groups, although the Cp group, only had a difference with the Sp group ($P \leq 0.001$), without observing changes between the groups concerning the weight of the placenta (Table 1).

Concerning fetal weight and size, the group of mothers treated with Sp significantly outperformed all groups, even with the control group ($P = 0.001$); this control group did not show a difference from the Cp + Sp 400 group. (Table 1). The fetuses presented a lower weight in the Cp, Cp + Sp 100, and Cp + Sp 200 groups than the Control group with a significant difference ($P \leq 0.001$). The fetuses with the lowest weight were those of the Cp group, with a difference with all the groups, Sp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 ($P \leq 0.001$) (Table 1).

Regarding the size of the fetuses, the Control group did not show a difference between Cp + Sp 400, but it did with the groups' Cp ($P \leq 0.001$), Cp + Sp 100 ($P = 0.026$), and Cp + Sp 200 ($P = 0.027$), indisputably. the smallest fetuses are located in the Cp group with a significant difference from the rest, Sp, Cp + Sp 100 and Cp + Sp 200, Cp + Sp 400 ($P \leq 0.001$) (Table 1).

Macroscopic analysis of fetuses

Table 2 and Figure 1 show the results of the external analysis performed on the fetuses: no malformations were found in the negative control or the animals treated with only Sp. When evaluating macroscopic malformations, significant differences of $P \leq 0.001$ between the groups were found.

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Table 1. Gravid parameters of mice treated with cyclophosphamide and spirulina

	Control	Cp	Sp 400	Cp + Sp 100	Cp + Sp 200	Cp + Sp 400
Mating females	18	18	17	18	20	18
Pregnant females	15	15	15	15	15	15
Fertility rate[1] (%)	83.33 %	83.3 3%	88.23 %	83.33 %	75.00 %	83.33 %
Females with live fetuses	15	15	15	15	15	15
Pregnancy rate[2] (%)	83.33 %	83.33 %	88.23 %	83.33 %	75.00 %	83.33 %
Implantations	15.4 ± 0.59	14.6 ± 0.61	14 ± 0.76	16.3 ± 0.50	13.86 ± 0.52	15.6 ± 0.57
Early resorption	1.2 ± 0.22	1.66 ± 0.56	0.93 ± 0.23	1.60 ± 0.56	1.53 ± 0.43	1.13 ± 0.36
Late resorption	0.6 ± 0.25	0.8 ± 0.26	0.53 ± 0.19	0.86 ± 0.40	0.66 ± 0.30	0.4 ± 0.23
Dead fetuses	0.00 ± 0.00	0.66 ± 0.66	0.00 ± 0.00	0.66 ± 0.66	0.00 ± 0.00	0.00 ± 0.00
Post-implantation losses[3] (%)	12.55 %	17.59 %	10.48 %	15.57 %	15.86 %	9.70 %
Live fetuses	87.44 ± 0.7	82.42 ± 0.8	89.52 ± 0.7	84.43 ± 0.8	84.13 ± 0.6	90.29 ± 0.8
Female/male fetuses (%)	50.99 / 49.01 %	50.00 / 49.00 %	50.53 / 49.47 %	50.48 / 49.51 %	50.86 / 49.14 %	50.46 / 49.53 %
Uterine weight (g)	1.23 ± 0.08	1.20 ± 0.07	1.14 ± 0.06	1.23 ± 0.05	1.08 ± 0.06	1.24 ± 0.04
Umbilical cord length (cm)	1.0 1± 0.41	0.72 ± 0.01 [a]	0.94 ± 0.00 [b]	0.80 ± 0.01 [b]	0.71 ± 0.02 [a]	0.87 ± 0.01 [b]
Placental weight (g)	0.13 ± 0.07	0.10 ± 0.00	0.12 ± 0.00	0.10 ± 0.01	0.12 ± 0.02	0.12 ± 0.00
Diameter of the placenta (cm)	0.82 ± 0.12	0.72 ± 0.01 [a]	0.83 ± 0.01 [b]	0.75 ± 0.00 [a]	0.76 ± 0.01 [a]	0.79± 0.01 [a]
Fetal weight (g)	0.94 ± 0.01	0.67 ± 0.01 [a]	1.02 ± 0.01 [a, b]	0.75 ± 0.01 [a, b]	0.75 ± 0.02 [a, b]	0.93 ± 0.01 [b]
Fetal size (cm)	1.99 ± 0.01	1.66 ± 0.01 [a]	2.06 ± 0.01 [a, b]	1.78 ± 0.01 [a, b]	1.79 ± 0.02 [a, b]	1.96 ± 0.01 [b]

Results are expressed as the mean ± SE. [a] Significant difference from Control; [b] Significant difference with Cf, $P < 0.05$.
[1] Fertility rate (%) = (N° of pregnant females / # of mated females) × 100.
[2] Pregnancy rate = (N° of females with live fetuses / N° of mated females) × 100.
[3] Post-implantation loss = (N° of implantation sites – N° of live fetuses / N° of implantation sites) × 100.

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Table 2. Macroscopic malformations in fetuses (n = 1163) due to treatments with cyclophosphamide (Cp) and spirulina (Sp)

Groups	Control	Cp	Sp 400	Cp + Sp 100	Cp + Sp 200	Cp + Sp 400
Number of fetuses examined (n)	202	178	188	206	175	214
(%) Macroscopic malformations	0 (0.00 %)	168 [a] (94.38 %)	0 [b] (0.00 %)	138 [a, b] (66.99 %)	116 [a, b] (66.28 %)	48 [a, b] (25.53 %)
Anasarca	0 (0.00 %)	27 [a] (15.17 %)	0 [b] (0.00 %)	55 a (26.69 %)	33 [a] (18.85 %)	0 [b] (0.00 %)
Exencephaly	0 (0.00 %)	16 [a] (8.98 %)	0 [b] (0.00 %)	13 [a] (6.31 %)	4 (2.28 %)	0 [b] (0.00 %)
Hydrocephalus	0 (0.00 %)	4 (2.25 %)	0 (0.00 %)	19 (9.22 %)	24 [a, b] (13.71 %)	0 (0.00 %)
Open eye	0 (0.00 %)	34 [a] (19.10 %)	0 [b] (0.00 %)	42 [a] (20.38 %)	45 [a] (25.71 %)	0 [b] (0.00 %)
Microtia	0 (0.00 %)	17 [a] (9.55 %)	0 [b] (0.00 %)	4 [b] (1.94 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
Cleft palate	0 (0.00 %)	114 [a] (64.04 %)	0 [b] (0.00 %)	67 [a, b] (32.52 %)	42 [a, b] (24.00 %)	9 [b] (4 %)
Iniencephaly	0 (0.00 %)	67 [a] (37.64 %)	0 [b] (0.00 %)	80 [a] (38.83 %)	53 (30.28 %)	0 [b] (0.00 %)
Spina bifida	0 (0.00 %)	45 [a] (25.28 %)	0 [b] (0.00 %)	27 [a, b] (13.10 %)	14 [a, b] (8.00 %)	0 [b] (0.00 %)
Short tail	0 (0.00 %)	53 [a] (29.77 %)	0 [b] (0.00 %)	51 [a] (24.75 %)	51 [a] (29.14 %)	0 [b] (0.00 %)
Coiled tail	0 (0.00 %)	16 [a] (8.9 8%)	0 [b] (0.00 %)	12 [a] (5.82 %)	10 [a] (5.71 %)	0 [b] (0.00 %)
Front limbs	0 (0.00 %)	124 [a] (69.66 %)	0 [b] (0.00 %)	72 [a, b] (34.95 %)	82 [a, b] (46.86 %)	33 [a, b] (15.42 %)
Hind limbs	0 (0.00 %)	126 [a] (70.79 %)	0 [b] (0.00 %)	81 [a, b] (39.32 %)	44 [a, b] (25.14 %)	8 [b] (3.74 %)

Sp was administered to the females from the 6th to the 16th day of gestation. The results indicate the number of fetuses with the malformation; in parentheses, the percentage that corresponds to each group. [a] Significant difference from Control; [b] Significant difference with Cf; $P < 0.05$.

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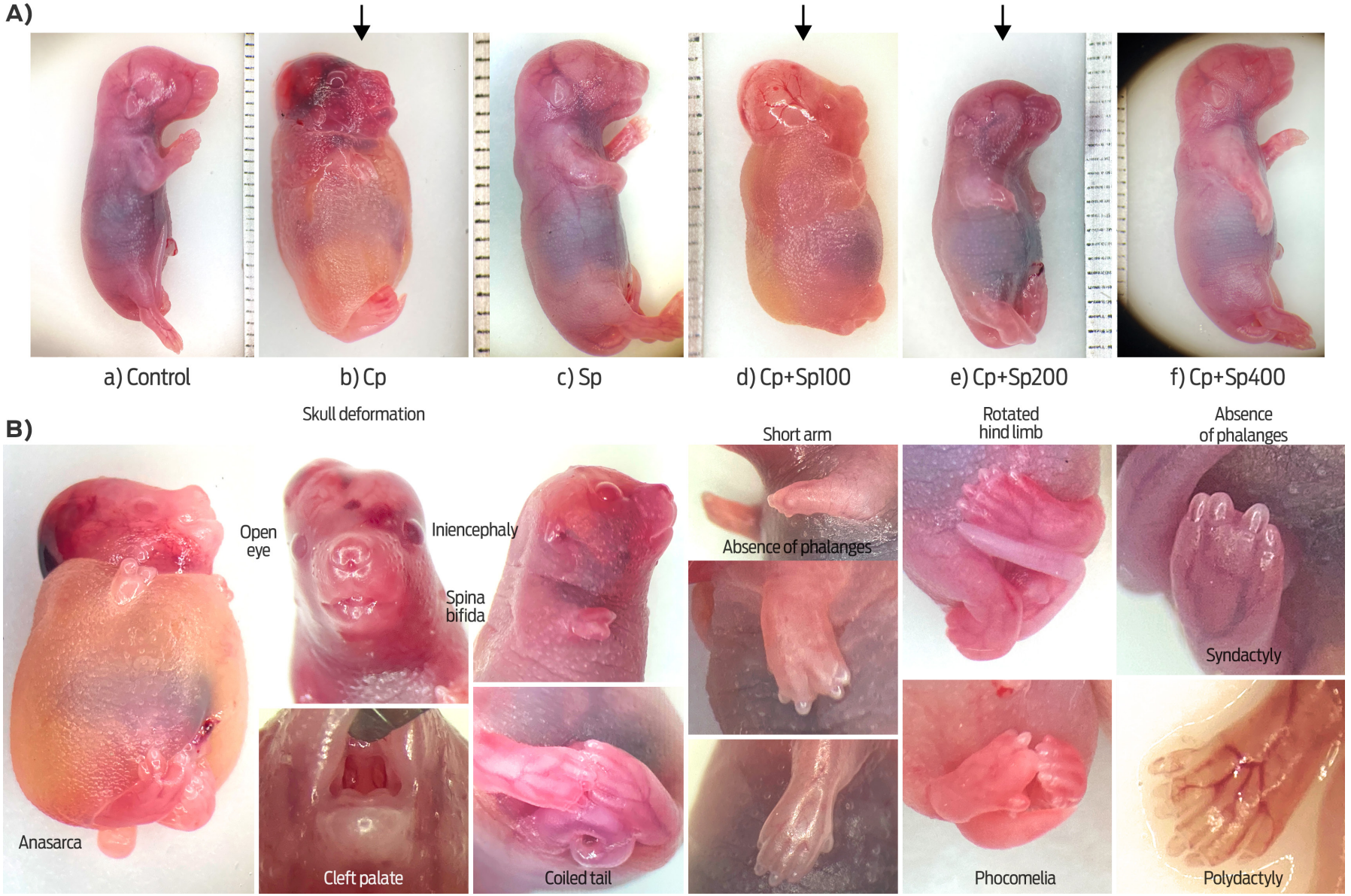

Figure 1. Malformations induced in fetuses of pregnant mice treated with cyclophosphamide (Cp), administered in one dose on day 10 of gestation, and different doses of spirulina (Sp), administered from day 6 to 16 of gestation.

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Control with Cp, Cp + Sp 100, Cp + Sp 200 an,d Cp + Sp 400, as well as the Cp group with the groups' Sp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400. The most affected animals were those of the group treated only with Cp, with 70% forelimbs and 71% hindlimbs. Cleft palate was a malformation with a high percentage (64 %).

1163 fetuses were obtained from 15 females distributed in the following groups: 202, which corresponds to 100 % of the control group (vehicles: water and serum, depending on whether Sp or Cp); 178 fetuses (100 %) in the (Cp) cyclophosphamide 20 mg/kg (single administration day of gestation 10); 188 fetuses (100 %) in the (Sp) spirulina 400 mg/kg; 206 fetuses (100 %) in the (Cp + Sp 100) cyclophosphamide 20 mg/kg plus spirulina 100 mg/kg; 175 fetuses (100 %) in the (Cp + Sp 200) cyclophosphamide 20 mg/kg plus spirulina 200 mg/kg and 214 fetuses (100 %) in the (Cp + Sp 400) cyclophosphamide 20 mg/kg plus spirulina 400 mg/kg.

Figure 1A) shows one fetus for each group corresponding to: a) control (vehicles: water and serum, depending on whether Sp or Cp); b) Cp, 20 mg/kg (single administration day of gestation 10); c) Sp, 400 mg/kg; d) Cp + Sp 100 = Cp 20 mg/kg Sp 100 mg/kg; e) Cp + Sp 200 = Cp 20 mg/kg + Sp, 200 mg/kg, and f) Cp + Sp 400 = Cp 20 mg/kg Sp 400 mg/kg; where a), c) and f) no malformations are observed, while, the black arrow (↑) indicates evident alterations in fetuses with treatments b, d, and e). B) shows Cp-induced malformations in fetuses in different areas of the body.

Although concomitant administration of Sp with Cp decreases the percentages of malformations, there are still significant differences in the number and/or severity of those abnormalities in the fetuses, having in most cases, a dose-response relationship, although this relationship is slightly lost in fetuses with hydrocephalus and open eyes. In the treatment with Cp + Sp 400, except for the percentage of damage in the palate, front, and hind limbs, all reached the percentage value of 0, as in the control group.

Specifically, the anasarca presented significant differences between the control group with the Cp, Cp + Sp 100, and Cp + Sp 200 groups ($P \leq 0.001$), the same difference between the Cp group with Sp and Cp + Sp 400. About the malformations present in the skull, exencephaly showed a significant difference between the Control, Sp, and Cp + Sp 400 groups with the Cp group ($P = 0.006$); in addition, the Control group also had a difference with the Cp + Sp 100 group. ($P = 0.038$). Unlike hydrocephalus, only the Cp + Sp 200 group has a difference with the control group ($P \leq 0.001$) and with the Cp group ($P = 0.004$).

Regarding the presence of an open eye, the control group has differences with the Cp, Cp + Sp 100, and Cp + Sp 200 groups ($P \leq 0.001$), while the Cp group presents a $P \leq 0.001$ with the Sp and Cp + Sp 400. Microtia was one of the less frequent malformations, with a significant difference between the Cp group and all the others ($P = 0.004$), even though the difference is smaller but significant with the Cp + Sp 100 group ($P = 0.037$). Cleft palate is one of the three most frequent malformations, and its presence showed a significant difference in the Cp group when compared with all the other groups ($P \leq 0.001$), at the same time in the Control group as well as in the Sp group. This malformation was reported, with a significant difference with the Cp, Cp + Sp 100, and Cp + Sp 200 groups ($P \leq 0.001$).

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Within the defects in the vertebral column, it was observed that iniencephaly in the control group presented a difference with the Cp and Cp + Sp 100 groups ($P \leq 0.001$), the same difference is shown between the Cp group when compared with the Sp and Cp + Sp 400 groups. In addition, the presence of spina bifida showed a difference between the control group and the Cp + Sp 200 group ($P = 0.012$), as well as between the Cp and Cp + Sp 100 groups ($P \leq 0.001$), while the Cp group presented a significant difference with the Sp group, Cp + Sp 100 and Cp + Sp 200 and Cp + Sp 400 ($P \leq 0.001$).

In particular, the tail presents alterations, considering short or coiled. The short tail in the Control group presented differences with the Cp, Cp + Sp 100, and Cp + Sp 200 ($P \leq 0.001$) groups, as well as the Cp group showed a difference with the Sp and Cp + Sp 400 groups ($P \leq 0.001$ While the Control group, when determining the presence of coiled tail, differed from the Cp groups ($P = 0.006$), Cp + Sp 100 and Cp + Sp 200 ($P = 0.038$), as well as the Cp group differed from the Cp groups. Sp and Cp + Sp 400 groups ($P = 0.006$).

The most recurrent malformations occur in the extremities, presenting more anteriorly, which vary in severity and are related to Cp, observing dose-dependent protection with Sp. Regarding the forelimbs, the control group presented differences with the Cp groups, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 ($P \leq 0.001$); at the same time, the Cp group showed a difference with Sp ($P \leq 0.001$), Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 ($P = 0.001$). When comparing the malformations of the hind limbs, the control group presents differences with the Cp, Cp + Sp 100, and Cp + Sp 200 groups ($P \leq 0.001$), when the Cp group differs with the Sp, Cp + Sp 100 groups, Cp + Sp 200, and Cp + Sp 400 ($P \leq 0.001$).

Skeletal disorders

Table 3 and Figure 2 show that the skeletal alterations induced by Cp are evident and each of them included a decrease in ossification, absence or deformation of bones, lack of bone development or differentiation, fusions, or divisions (results not presented). None of the skeletal abnormalities found in the Cp group were observed in the control or Sp groups.

In Table 3, the results represent the total number of fetuses with alteration per group and in parentheses the percentage of alterations per bone. The different treatments correspond to control (vehicles: water and serum, depending on whether Sp or Cp (Cp) 20 mg/kg (single administration day 10 of gestation); Sp 400 mg/kg; Cp + Sp 100 = Cp 20 mg/kg + Sp 100 mg/kg; Cp + Sp 200 = Cp 20 mg/kg + Sp 200 mg/kg and Cp + Sp 400 = Cp 20 mg/kg + Sp 400 mg/kg. Sp was administered from the 6th to the 16th day of gestation.

Control (vehicles: water and serum, depending on whether Sp or Cp); (Cp) cyclophosphamide 20 mg/kg (single administration day 10 of gestation); Sp 400 mg/kg; Cp + Sp 100 = Cp 20 mg/kg + 100 mg/kg; Cp + Sp 200 = Cp 20 mg/kg + 200 mg/kg; and Cp + Sp 400 = Cp 20 mg/kg + 400 mg/kg.

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Table 3. Fetal skeletal alterations present at cyclophosphamide treatments and spirulina

Area	Bone	Control	Cp	Sp	Cp + Sp 100	Cp + Sp 200	Cp + Sp 400
Skull	Occipital	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	68 [a] (38.86 %)	0 [b] (0.00 %)
	Interparietal	0 (0.00 %)	162 [a] (91.01 %)	0 [b] (0.00 %)	193 [a] (93.69 %)	48 [a, b] (27.43 %)	17 [a, b] (7.94 %)
	Parietal	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	91 [a, b] (52.00 %)	17 [a, b] (7.94 %)
	Temporal	0 (0.00 %)	172 [a] (96.63 %)	0 [b] (0.00 %)	206 [a] (100 %)	76 [a, b] (43.43 %)	17 [a, b] (7.94 %)
	Ethmoidal	0 (0.00 %)	162 [a] (91.01 %)	0 [b] (0.00 %)	193 [a] (93.69 %)	32 [a, b] (18.28 %)	0 [b] (0.00 %)
	Vomer	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Basisphenoid	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	39 [a, b] (22.28 %)	0 [b] (0.00 %)
	Frontal	0 (0.00 %)	176 [a] (98.88 %)	0 [b] (0.00 %)	206 [a] (100 %)	52 [a, b] (29.71 %)	0 [b] (0.00 %)
Facial	Nasal	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	26 [a, b] (12.62 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Incisor	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	51 [a, b] (24.76 %)	20 [a, b] (11.43 %)	0 [b] (0.00 %)
	Palatine	0 (0.00 %)	114 [a] (64.04 %)	0 [b] (0.00 %)	67 [a, b] (32.52 %)	43 [a, b] (24.57 %)	9 [b] (4.20 %)
	Maxilla	0 (0.00 %)	114 [a] (64.04 %)	0 [b] (0.00 %)	67 [a, b] (32.52 %)	43 [a, b] (24.57 %)	9 [b] (4.20 %)
	Mandible	0 (0.00 %)	14 [a] (7.86 %)	0 [b] (0.00 %)	3 (1.46 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Zygomatic	0 (0.00 %)	94 [a] (52.80 %)	0 [b] (0.00 %)	94 [a] (45.63 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
Spine	Cervical	0 (0.00 %)	156 [a] (87.64 %)	0 [b] (0.00 %)	154 [a, b] (74.76 %)	25 [a, b] (14.28 %)	0 [b] (0.00 %)
	Thoracic	0 (0.00 %)	115 [a] (64.61 %)	0 [b] (0.00 %)	165 [a, b] (80.10 %)	43 [a, b] (24.57 %)	0 [b] (0.00 %)
	Lumbar	0 (0.00 %)	39 [a] (21.91 %)	0 [b] (0.00 %)	57 [a] (27.67 %)	16 [a, b] (9.14 %)	0 [b] (0.00 %)
	Sacral	0 (0.00 %)	63 [a] (35.39 %)	0 [b] (0.00 %)	72 [a] (34.95 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Caudal	0 (0.00 %)	81 [a] (45.50 %)	0 [b] (0.00 %)	83 [a] (40.29 %)	61 [a] (34.86 %)	0 [b] (0.00 %)
Sternum	Sternebra I	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	204 [a] (99.03 %)	60 [a, b] (34.28 %)	0 [b] (0.00 %)
	Sternebra II	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	64 [a, b] (36.57 %)	0 [b] (0.00 %)
	Sternebra III	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	59 [a, b] (33.71 %)	0 [b] (0.00 %)
	Sternebra IV	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	41 [a, b] (23.43 %)	0 [b] (0.00 %)
	Sternebra V	0 (0 %)	156 [a] (87.64 %)	0 [b] (0.00 %)	153 [a] (74.27 %)	48 [a, b] (27.43 %)	0 [b] (0.00 %)
	Sternebra VI	0 (0.00 %)	156 [a] (87.64 %)	0 [b] (0.00 %)	127 [a] (61.65 %)	38 [a, b] (21.71 %)	0 [b] (0.00 %)

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Area	Bone	Control	Cp	Sp	Cp + Sp 100	Cp + Sp 200	Cp + Sp 400
Ribs	True	0 (0.00 %)	153 [a] (85.95 %)	0 [b] (0.00 %)	92 [a, b] (44.66 %)	45 [a, b] (25.71 %)	0 [b] (0.00 %)
	False	0 (0.00 %)	156 [a] (87.64 %)	0 [b] (0.00 %)	52 [a, b] (25.24 %)	51 [a, b] (29.14 %)	0 [b] (0.00 %)
	Floating	0 (0.00 %)	149 [a] (83.71 %)	0 [b] (0.00 %)	46 [a, b] (22.33 %)	20 [a, b] (11.43 %)	0 [b] (0.00 %)
Hip	Ilium	0 (0.00 %)	62 [a] (34.83 %)	0 [b] (0.00 %)	16 [a, b] (7.77 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Ischium	0 (0.00 %)	157 [a] (88.20 %)	0 [b] (0.00 %)	123 [a, b] (59.71 %)	91 [a, b] (52.00 %)	0 [b] (0.00 %)
	Pubis	0 (0.00 %)	163 [a] (91.57 %)	0 [b] (0.00 %)	189 [a] (91.75 %)	112 [a, b] (64.00 %)	0 [b] (0.00 %)
Scapular waist	Clavicle	0 (0.00 %)	133 [a] (74.72 %)	0 [b] (0.00 %)	52 [a, b] (25.24 %)	0 [b] (0.00 %)	0 [b] (0.00 %)
	Scapula	0 (0.00 %)	157 [a] (88.20 %)	0 [b] (0.00 %)	63 [b] (30.58 %)	3 [b] (1.71 %)	0 [b] (0.00 %)
Front limb	Humerus	0 (0.00 %)	109 [a] (61.23 %)	0 [b] (0.00 %)	49 [a, b] (23.79 %)	44 [a, b] (25.14 %)	7 [b] (3.27 %)
	Radius	0 (0.00 %)	92 [a] (51.68 %)	0 [b] (0.00 %)	54 [a, b] (26.21 %)	56 [a, b] (32.00 %)	38 [a, b] (17.76 %)
	Ulna	0 (0.00 %)	92 [a] (51.68 %)	0 [b] (0.00 %)	54 [a, b] (26.21 %)	56 [a, b] (32.00 %)	38 [a, b] (17.76 %)
	Carpus	0 (0.00 %)	165 [a] (92.69 %)	0 [b] (0.00 %)	73 [a, b] (35.43 %)	29 [a, b] (16.57 %)	7 [b] (3.27 %)
	Metacarpus	0 (0.00 %)	165 [a] (92.69 %)	0 [b] (0.00 %)	36 [a, b] (17.47 %)	87 [a, b] (49.71 %)	0 [b] (0.00 %)
	Phalanges	0 (0.00 %)	178 [a] (100 %)	0 [b] (0.00 %)	206 [a] (100 %)	175 [a] (100 %)	72 [a, b] (33.64 %)
Hind limb	Femur	0 (0.00 %)	133 [a] (74.72 %)	0 [b] (0.00 %)	124 [a, b] (60.19 %)	69 [a, b] (39.43 %)	8 [b] (3.74 %)
	Tibia	0 (0.00 %)	162 [a] (91.01 %)	0 [b] (0.00 %)	114 [a, b] (55.34 %)	32 [a, b] (18.29 %)	0 [b] (0.00 %)
	Fibula	0 (0.00 %)	162 [a] (91.01 %)	0 [b] (0.00 %)	114 [a, b] (55.34 %)	32 [a, b] (18.29 %)	0 [b] (0.00 %)
	Metatarsus	0 (0.00 %)	9 (5.06 %)	0 (0.00 %)	5 (2.43 %)	7 (4.00 %)	0 (0.00 %)
	Phalanges	0 (0.00 %)	162 [a] (91.01 %)	0 [b] (0.00 %)	68 [a, b] (33.01 %)	39 [a, b] (22.28 %)	0 [b] (0.00 %)

The results are expressed as the mean ± SE. [a]Significant difference from control; [b]Significant difference with Cf, $P < 0.05$.

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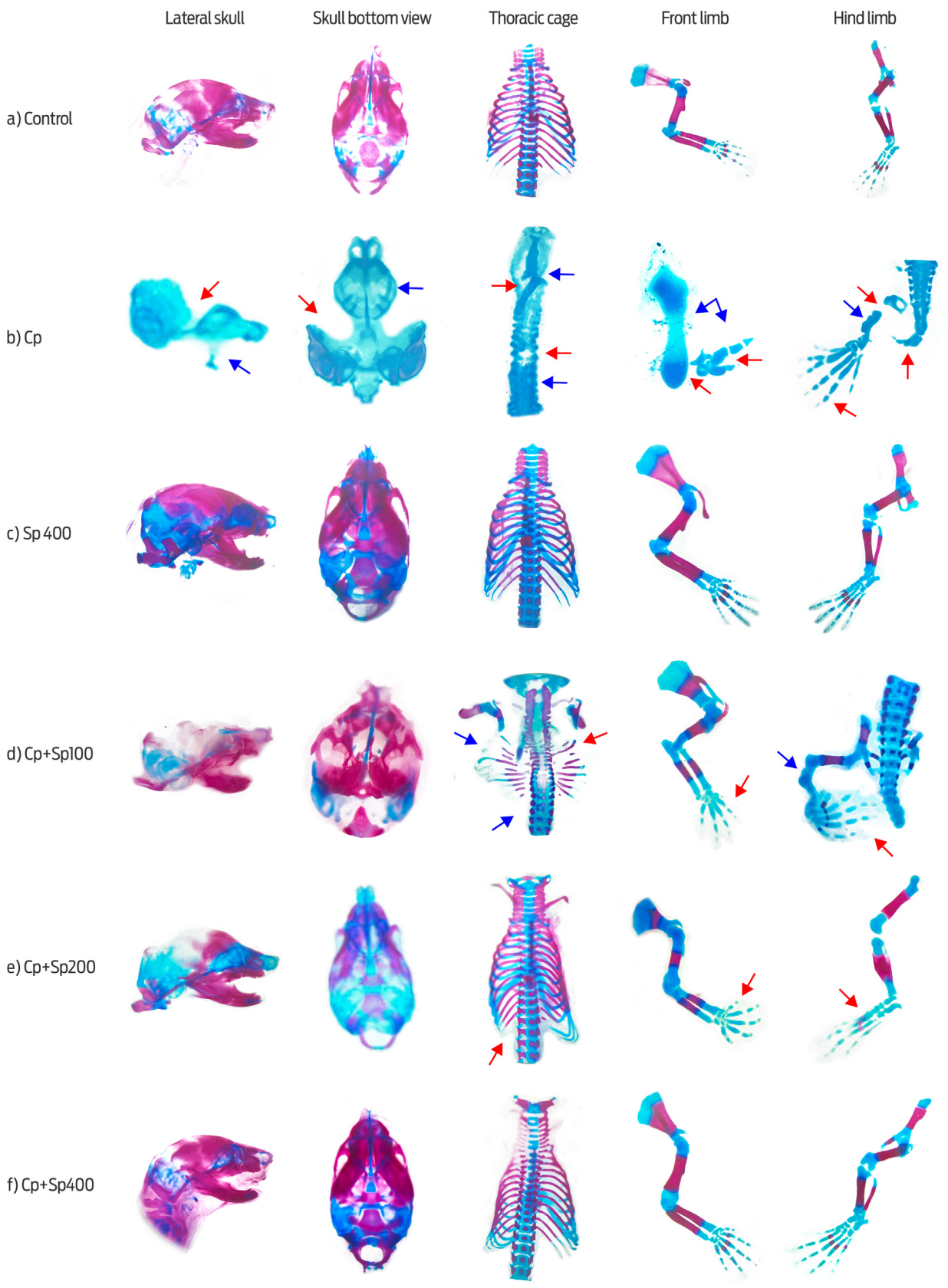

Figure 2. Skeletal disorders in fetuses treated with the bichromatic technique, a representative specimen of each group.

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In Figure 2. a), c), and f) without obvious alterations; b) Cp absence of bones (red arrow ↑) in the skull (frontal, parietal, intraparietal), in the thoracic cage (ribs, vertebrae), front limbs (radius, ulna, carpus, phalanges) and hind limbs (tibia, fibula, phalanges), alterations in the shape (blue arrow ↑) facial (maxillary, palatine, mandible), thoracic cage (sternebra, vertebrae) and limbs, as well as lack of generalized ossification of the skeleton, conditions that decrease according to the administered dose of Sp d) Cp + Sp 100, e) Cp + Sp 200 and f) Cp + Sp 400.

When evaluating the skull, skeletal alterations related to Cp were observed, exceeding 90%. The occipital bone without involvement in the group's Control, Sp, and Cp + Sp 400 differed from the groups' Cp, Cp + Sp 100, and Cp + Sp 200 ($P \leq 0.001$). The interparietal bone presented a difference between the control group with the groups' Cp, Cp + Sp 100, Cp + Sp 200 ($P \leq 0.001$), and Cp + Sp 400 ($P = 0.012$), while Cp showed a difference with the groups' Sp, Cp + Sp 200 and Cp + Sp 400 ($P \leq 0.001$). In the parietal bones, the alterations showed differences between the control group versus Cp, Cp + Sp 100, Cp + Sp 200 ($P = 0.001$), and the Cp + Sp 400 group ($P = 0.012$), while the Cp group differed from the groups' Sp and Cp + Sp 400 ($P = 0.001$). The temporal presented a difference between the control group with all the Cp groups, that is, Cp, Cp + Sp 100, Cp + Sp 200 ($P = 0.001$) and with Cp + Sp 400 ($P = 0.012$), regarding the group Cp with the groups' Sp, Cp + Sp 400, and Cp + Sp 200 ($P = 0.001$), showing the dose-dependent protective effect of Sp. A similar effect occurs when evaluating the ethmoid bone, in which the difference with the control group is given with Cp, Cp + Sp 100, Cp + Sp 200 ($P < 0.001$) and with the Cp group with Sp, Cp + Sp 400, and Cp + Sp 200 ($P < 0.001$). When evaluating the vomer, the control group presented a difference with the groups' Cp and Cp + Sp 100 ($P \leq 0.001$), a difference that the Cp group also has with the groups' Sp, Cp + Sp 400, and Cp + Sp 200. Meanwhile, the basisphenoid and frontal difference between the control group occurs with the Cp and Cp + Sp 100 ($P \leq 0.001$) groups, while the same difference occurs between the Cp with the groups' Sp, Cp + Sp 400, and Cp + Sp 200.

Within the bony alterations of the head, it stands out that the nasal bone presented a difference between the control group with the Cp and Cp + Sp 100 groups ($P \leq 0.001$), while the same difference between the Cp group with the groups' Sp, Cp + Sp 400, and Cp + Sp 200. While the incisor showed a difference between the control group and the Cp, Cp + Sp 100 ($P \leq 0.001$), Cp + Sp 200 ($P = 0.002$) groups, when comparing the Cp group with the groups' Sp, Cp + Sp 400, Cp + Sp 200 and Cp + Sp 100 ($P \leq 0.001$). The palatines and maxilla have the same relationship as the control group with the groups' Cp, Cp + Sp 100 and Cp + Sp 200 ($P \leq 0.001$), as well as the Cf group with Sp, Cp + Sp 400, Cp + Sp 200 and Cp + Sp 100 ($P \leq 0.001$). Among the bones less affected by Cp is the mandible (8%), even in this the Control group presented a difference with the group Cp ($P = 0.012$), Cp + Sp 100, and Cp + Sp 200 ($P \leq 0.001$), the difference between the group Cp remains the Cp group when compared with the Sp, Cp + Sp 400 and Cp + Sp 200 groups ($P \leq 0.001$). On the other hand, the zygomatic bone without reported alterations in the control group presented differences with the Cf and Cp + Sp 400 groups ($P \leq 0.001$) and the Cf group with the groups' Sp, Cp + Sp 400, and Cp + Sp 200.

The damage produced by Cp affects the vertebrae. The Control group without alterations in the cervical vertebrae with a significant difference from the

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groups with alterations, these are Cp, Cp + Sp 100 (P ≤ 0.001), and Cp + Sp 200 (P = 0.001), instead when comparing the Cf group, the group with the greatest alterations in said vertebrae had differences with the Sp, Cp + Sp 400, Cp + Sp 200 (P ≤ 0.001) and Cp + Sp 100 (P = 0.004) groups. The differences occurred in other vertebrae; in the thoracic vertebrae, the control group presented a difference with the Cp, Cp + Sp 100, and Cp + Sp 200 groups (P ≤ 0.001), while the Cp group presented the same difference with Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 (P ≤ 0.001). The least affected vertebrae were the lumbar and sacral (22 and 35 %, respectively), even so, in the lumbar the Control group presented a difference with Cp, Cp + Sp 100 (P ≤ 0.001) and Cp + Sp 200 (P =0.006), while the Cp group with the groups' Sp, Cp + Sp 400 (P ≤ 0.001) and Cp + Sp 200 (P=0.002), on the other hand, when analyzing the sacral vertebrae, the Control group showed a difference with the Cp and Cp + Sp 100 groups (P ≤ 0.001) and the Cp group with the groups' Sp, Cp + Sp 400 and Cp + Sp 200 (P ≤ 0.001). In the caudal vertebrae, the control group differed from the groups' Cp, Cp + Sp 100, and Cp + Sp 200 (P ≤ 0.001); on the other hand, the Cp group had the same difference with Sp and Cp + Sp 400 (P ≤ 0.001).

The sternebra, like the rest of the skeleton, was affected by the administration of Cp as well as a protective effect of Sp, which is related to its dose. The sternebra I, without alterations in the Control, Sp, and Cp + Sp 400 groups, differs from the groups' Cp, Cp + Sp 100, and Cp + Sp 200 (P ≤ 0.001). The sternebra II, III, IV, and VI the Control group with differences with Cp, Cp + Sp 100 and Cp + Sp 200 (P ≤ 0.001), while the Cp group with Sp, Cp + Sp 400 and Cp + Sp 200 (P ≤ 0.001). In the case of sternebra V, the control group maintained a difference with the Cp, Cp + Sp 100 (P ≤ 0.001), and Cp + Sp 200 (P = 0.038) groups, while the Cp group showed a difference with the groups' Sp, Cp + Sp 400 and Cp + Sp 200 (P ≤ 0.001).

In ribs, both true, false, and floating in the Control group, there are no alterations, I keep a difference with Cp, Cp + Sp 100, and Cp + Sp 200 (P ≤ 0.001), on the other hand, the Cp group with the groups' Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 (P ≤ 0.001).

The hip bones are affected by exposure to Cp, an effect that is counteracted as the dose of Sp increases. Specifically, when evaluating the ilium of the Control group, there was a difference with Cp (P ≤ 0.001), Cp + Sp 100 (P = 0.012), while the Cp group presented a significant difference with the Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 groups (P ≤ 0.001). In the ischium and pubis, the control group differed from the Cp, Cp + Sp 100, and Cp + Sp 200 (P ≤ 0.001) groups, while Cp presented a difference with Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 (P ≤ 0.001). The differences between the groups are also manifested in the clavicle and scapula, where the control group presented a difference with Cp and Cp + Sp 100 (P ≤ 0.001), on the other hand, the Cp group presented it with the groups' Sp, Cp + Sp 400, Cp + Sp 200 and Cp + Sp 100 (P ≤ 0.001).

The bones of the forelimbs are the most affected by Cp, which is why the humerus, carpus, and metacarpus showed differences between the control group and the Cp, Cp + Sp 100, and Cp + Sp 200 groups (P ≤ 0.001); the group with the most alterations was the Cp group that presented a difference with Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 (P ≤ 0.001).

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Table 4. Antioxidant enzymes and oxidative damage in fetal livers from females treated with cyclophosphamide and spirulina

Treatment	SOD (U/mL)	GPx (U/L)	CAT (U)	nmol MDA/g	nmol C = O / mg/g
Control	299.3 ± 59.34	105.5 ± 13	4578 ± 83	55.4 ± 6.74	0.73 ± 0.12
Cp	275.6 ± 84.51	143.8 ± 10 [a]	3964 ± 11	48.0 ± 5.11	0.56 ± 0.05 [a]
Sp 400	991.3 ± 96.65 [a, b]	181.2 ± 25 [a, b]	6090 ± 99	43.7 ± 3.32	0.71 ± 0.16 [b]
Cp + Sp 100	302.9 ± 64.6	139.1 ± 55 [a, b]	4862 ± 10	46.3 ± 4.70	0.75 ± 0.06 [b]
Cp + Sp 200	361.9 ± 66.13	224.8 ± 46 [a, b]	4600 ± 16	41.2 ± 5.22	0.70 ± 0.08 [b]
Cp + Sp 400	531.5 ± 91.20	276.8 ± 48 [a, b]	6635 ± 17	47.2 ± 4.44	1.28 ± 0.34 [b]

The results are expressed as the mean ± SE. The different treatments correspond to: control (vehicles: water and serum, depending on whether Sp or Cp); Cp, 20 mg/kg (single administration day 10 of gestation); Sp 400 mg/kg; Cp + Sp 100 = Cf, 20 mg/kg + Sp, 100 mg/kg; Cp + Sp 200 = Cf, 20 mg/kg + Sp, 200 mg/kg; Cp + Sp 400 = Cf, 20 mg/kg + Sp, 400 mg/kg. Sp was administered from day 6 to 16 of gestation. The activity of superoxide dismutase (SOD) glutathione peroxidase (Gpx), and catalase (CAT) antioxidant enzymes is shown, while oxidative damage was determined by the concentration of malondialdehyde (MDA) and carbonyl groups (C = O).

In the radius and ulna, the control group had a difference with Cp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 ($P \leq 0.001$), and the Cp group presented a difference with Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100 ($P \leq 0.001$).

The parts of the extremities most affected by Cf are the phalanges, showing a significant difference between the control group with the Cp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 groups ($P \leq 0.001$), presenting the same difference between the Cp group with the groups' Sp, Cp + Sp 400, Cp + Sp 200, and Cp + Sp 100.

Of the posterior extremity, the femur, tibia, fibula, and phalanges, presented differences between the Control group that maintained a significant difference with the groups' Cp, Cp + Sp 100, Cp + Sp 200, and Cp + Sp 400 ($P \leq 0.001$), while the Cp group had it with the groups' Sp, Cp + Sp 400, Cp + Sp 200 and Cp + Sp 100 ($P \leq 0.001$), except in the femur with Cp + Sp 100 ($P = 0.002$).

Biochemical determination

The results of the biochemical study are presented in Table 4. In the case of antioxidant enzymes, SOD is higher in Sp 400 mg/kg group with a significant difference compared to the control group. On the other hand, GPx activity increased significantly $P = 0.047$, compared to the control and Cp groups. However, CAT activity does not differ significantly, although a tendency to decrease in the Cp group and to increase in the Sp 400 group is observed.

Regarding oxidative damage measured by the MDA concentration, the groups showed no significant differences $P < 0.050$ with the control group or with the Cp group, while, in protein oxidation, all those treated with Sp differ significantly from the Cp group (Table 4).

Discussion

It has been widely demonstrated that Sp has different pharmacological and antitoxic activities that have been proven in humans and experimental animals.[13, 25] In

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animals, it has been found that some of them are mainly attributed to their antioxidant activity, due to the abundant content of beta-carotene, catalase, superoxide dismutase, and glutathione.[7, 26]

Although there is some research on the effectiveness of Sp, mainly associated with its antioxidant effects to protect against the damage that some agents can cause in embryonic development in animals,[27, 28] no studies have been carried out on the action it can have against the teratogenic effects caused by the Cp, a prodrug with different and particular chemical and pharmacological characteristics.

In addition to the above, Sp has proven to be a safe cyanobacterium that does not produce toxicity when administered to rodents in acute, subchronic, chronic, and genotoxic studies.[25] At the same time, in research carried out on reproductive toxicity at high doses, it is free from producing harmful effects.[29]

Regarding the effect of Cp on maternal weight, authors such as Miller et al.,[4] do not report significant differences among the groups, while others find a significant reduction, even when administered concomitantly with oxidants such as vanadium[2] or green tea extract.[6]

In this study, after starting the administration of Sp, pregnant mice began to decrease their weight compared with the control and Cp groups. However, at the end of pregnancy, there was recovery, with no difference among the groups. This is associated with the effects of *Arthrospira maxima*, which like Heo and Choung,[30] is attributable to a decrease in white adipose tissue in the CD-1 mouse strain, due to the decrease of lipogenesis and adipogenesis combined with the activation of thermogenesis, which promotes the metabolism of brown adipose tissue.[31]

Also, Sp contains a large amount of protein, which provides nutritional deficiencies and modulates food intake (reduces leptin and phenylalanine that promotes the release of cholecystokinin).[31] On the other hand, long-chain polyunsaturated fatty acids (PUFAs) of Sp are converted into docosahexaenoic acid (DHA), which is essential during pregnancy because the body does not produce them efficiently to cover those required in placental and fetal development.[32]

The fatty acids supplied by the Sp promote changes in the metabolism of lipids by favoring thermogenesis, as noted, which causes the offspring to have a higher weight, although it does not influence that of the mothers, as reported by Carpio et al.[31] in pregnant-lactating rats.

Rezaei et al.,[32] in rats administered Cp on DG 13, reported a decrease in the weight and diameter of the placenta, due to a reduction in the thickness of the labyrinth, a situation that favors the passage of this agent to the fetus.[33] In our study, a significant reduction in the diameter of the placenta and the length of the umbilical cord was found in the Cp and Cp + Sp 200 groups, although without a difference in weight. The poorly developed spiral arteries cause hypoxia and therefore reperfusion, which exacerbates oxidative stress, contributing to damage to the placental tissue itself and causing retardation of fetal growth.[34]

Several authors have confirmed that the Cp administered by DG 10 produces malformations in mice without being embryo-lethal or causing apparent toxicity in pregnant females.[2, 4, 6] What was found in our study agrees with the above, in addition to not observing alterations in reproductive parameters, although there is an increasing trend in resorption and fetal deaths in the group treated with Cp and low doses of Sp.

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Cyclophosphamide, on the other hand, has been the subject of several toxicity studies reporting damage to different organs and systems, both in humans and animals.[2-4, 6, 35] On the other hand, Sp or some of its components such as β-carotenes promote growth, differentiation of epithelial tissues and embryonic development.

Polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are essential for the development of the neural system,[10] counteracting the toxic effects of Cp. This is corroborated by the results of this study, which show that the fetuses of the mothers treated with Sp did not present macroscopic or skeletal malformations and a significant increase in weight, size, and degree of ossification was observed compared to the fetuses in the control group.

A high percentage (94.38 %) of fetuses in the Cp group had at least one malformation, while the protective effect provided by Sp is significant, even in low doses, Cp + Sp 100 (66.99 %) and Cp + Sp 200 (66.28 %), although the most important results are found in the Cp + Sp 400 group.

To protect against the teratogenic effects of Cp, antioxidants have been used, such as Miller et al.,[4] that when administering N-acetyl-L-cysteine they found a decrease in some defects in ribs, vertebrae, sternum, and ossification. In our study, using Sp, damage to the total skeleton is significantly reduced, especially at the dose of 400 mg/kg, combined with better ossification.

The incidence of cleft palate induced by Cp in our study (64 %) is very similar to that found by some authors.[30] In this regard, it should be mentioned that its closure is embryologically influenced by other local structures such as the tongue and mandible. The latter, which can manifest as micrognathia, has been observed as an effect of Cp.[4, 6] Its smaller size corresponds to that of the maxilla, which is a physiological characteristic of mammals for nursing as indicated by Leite et al.,[36] what is important is to consider the decrease in ossification due to the effect of Cp,[37] which affects the intrauterine mobility of the jaw, therefore of the tongue, which in turn favors the presence of the cleft palate.[38]

Suárez et al.,[39] have pointed out that the increase in SOD activity is a reflection of a high generation of superoxide anion for a long time, which ends up inactivating this antioxidant enzyme. In this case, the exogenous contribution of SOD through Sp favors its presence and activity, avoiding damage by oxidative stress, which leads to fetuses in Sp 400 mg/kg group having a larger size, heavier weight, and mineralization as well as no malformations. Also, the presence of SOD in Sp counteracts the oxidative stress produced by Cp and/or its metabolites, achieving protective effects, especially at 400 mg/kg, a dose at which the fetuses have a size and weight similar to those of the control, significantly reducing the teratogenic damage induced by Cp.

In turn, CAT is considered an antioxidant enzyme dispensable during gestation,[40] since genetically modified mice, in which the synthesis of this enzyme in the liver is inhibited, continue to convert hydrogen peroxide into water, which does not compromise fetal development.[41] On the other hand, it has been observed that in the case of Cp, glutathione (GSH) is an antioxidant enzyme that plays an important role, so that when it binds to the drug or its metabolites, it can become saturated in its activity.[4] Actually, mouse embryos have a very low capacity for GSH synthesis, the lack of which can lead to abnormal embryonic development.[40]

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It is important to consider that Sp contains carotenoids that are a source of vitamin A, which may influence the synthesis and/or activity of GPx. GPx activity in groups administered Sp alone or concomitantly with Cp shows a significant difference, both with the control group and with the Cp group, suggesting protective activity against oxidative stress. De Haan et al.[42] pointed out that the liver is the organ with the highest levels of SOD production in the cytosol and GPx in the embryonic stage and mouse neonates, independent of oxygen consumption or exposure, these antioxidant enzymes being the ones that most counteract the damage produced by reactive oxygen species (ROS).

Ufer and Wang,[40] consider an alternative to determine the effects of ROS, the damage they cause through malformations, as well as the oxidation of lipids and proteins, although the half-life of these macromolecules should be considered as the repair mechanisms, which may indicate short-term or cumulative damage by ROS.

In our study, there are no differences among the groups concerning the determination of malondialdehyde (MDA), a biomarker of the final product of lipoperoxidation, damage that could be repaired if we consider the time elapsed between the administration of Cp (DG 10) and obtaining the liver (DG 17), together with the effect of its components such as phycocyanin and β-carotenes that inhibit lipoperoxidation.[19] On the other hand, proteins have significantly greater oxidation in the Cp group compared to all the others, so it must be considered as indicated by Jové et al.[43] that proteins damaged by ROS or by the lipoxidation compounds themselves are preserved for longer, that is, they are more stable damages.

The relationship of low concentrations of CAT and GSH as well as the elevation of MDA, with the increase of oxidants in amniotic fluid and peripheral blood in women whose fetuses have congenital abnormalities of the nervous system, has been reported.[44] For this reason, the use of different antioxidants has been proposed in experimental mice treated with Cp that, by increasing the ROS, lead to ectodermal and mesenchymal defects during organogenesis.[2-4, 6]

SOD is essential during the development of mouse embryos; other enzymes such as GPx and CAT, despite their poor synthesis capacity, can compensate for their antioxidant functions between them or by other mechanisms.[40] In addition, SOD even after birth, can lead to alterations or susceptibility to different diseases. Sp promotes an adequate antioxidant effect, with extensive studies in humans and animals, which constitutes a new proposal for its use to avoid malformations associated with the damage produced by ROS caused by Cp.

Conclusions

Sp as an antioxidant, administered to CD1 mice during organogenesis, protects against malformations caused by Cp, by decreasing the reactive oxygen species (ROS) induced by this agent, preventing oxidation, especially of proteins, being 400 mg/kg the most effective dose, which increases the concentration of SOD and GPx.

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Data availability

All data can be found in the manuscript and supporting written material.

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Conflicts of interest

The authors state that they do not have conflicts of interest, personal financial relationships, or relationships with organizations that could inappropriately influence or harm the content of this article.

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