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PRELIMINARY RESULTS IN THE EVALUATION OF THE CHEMOTHERAPY ACTIVITY OF BETA-SITOSTEROL IN A MODEL OF OSTEOSARCOMA IN RATS

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Abstract: Osteosarcoma (OS) is a malignant bone tumor, constitutes 75% in young patients, mainly affects bones of the lower extremities. There is no effective method to prevent this type of cancer and it can induce amputation. Beta-sitosterol (BS) is a plant compound. Studies have shown immunomodulatory, antimutagenic, anti-inflammatory, and antioxidant biomedical properties. Benzopyrene (BZP) is a hydrocarbon, capable of inducing OS. Considering this background, this research project was raised. Therefore, the objective was to determine, in a model of bone carcinogenesis in rats, the chemotherapeutic activity of beta-sitosterol in the development of osteosarcoma. Neoplastic lesions were induced by perifemoral administration of BZP. From the 7th to the 9th week BS was administered orally. The weight of the rats was recorded, blood samples were taken for genotoxicity studies; biochemical tests were performed to determine sedimentation rate (ESR), alkaline phosphatase (FA) and lactic dehydrogenase (DHL). At the ninth week, euthanasia was performed, femurs, tibiae, liver and lungs were removed for histopathological study.
Keywords: Osteosarcoma, Benzopyrene, beta-sitosterol, chemotherapeutic, Murine model.

INTRODUCTION

Osteosarcoma is a primary malignant neoplasm, derived from bone mesenchyme and typically forms osteoid tissue or immature bone. It usually occurs during adolescence, during the growth spurt. It is the most common primary solid bone tumor [1] and constitutes approximately 20% of primary bone sarcomas (American Cancer Society, 2018). Between 400 and 1000 new cases are diagnosed each year in the United States [2,3], which is an incidence of 8/1,000,000 in the general population. It is considered a

juvenile disease as 7.5 out of 10 cases occur in patients younger than 25 years [4]. Most cases in older patients are secondary sarcomas, i.e. sarcomas arising as a complication of pre-existing bone disease (Paget's disease, chronic osteomyelitis, chronic bone infarcts) or in previously irradiated tissues. It is one of the few tumors that begin in bone and metastasize to other parts of the body [5]. Osteosarcoma is considered a complex of resistance to conventional treatments. There is currently no effective method to prevent this type of cancer. Treatment of osteosarcoma includes chemotherapy, followed by surgery (amputation or salvage surgery) and post-surgical chemotherapy. Despite significant advances in the treatment of osteosarcoma, the prognosis of patients with metastases remains poor, with an overall survival of 55% after surgery and intensive chemotherapy [6,7].

ANIMAL MODELS

The animal models used in bone tissue research are as diverse as the materials and repair strategies. Generally, and as in other areas of science, animal studies begin in models such as the mouse or rat, followed by rabbits, pigs and sheep. Most studies focus on medium and large animals, because the surgery is complex and requires a large area to be performed and the results obtained in these cases are more easily extrapolated to a possible clinical use. As for musculoskeletal tissue injury models, both critical injury models (injuries that do not repair spontaneously) and non-critical models (injuries that heal spontaneously) are used [8]. The anatomical location of these defects depends on the objective of the study. In this regard, the most common are lesions and osteotomies in long bones (femur, tibia, humerus and radius), skull, lumbar lesions and lesions in the maxillofacial region. There is some controversy when comparing the results obtained in different animal models. This is because, in some cases, compounds that seem to work in small animals do not work in larger animals. Therefore, the correct choice of the study material, the type of lesion to be performed and the animal model chosen is considered of great importance [9].

MURINE CANCER INDUCTION MODELS

From the beginning of the last century to the present day, murine models have contributed to the understanding of the pathogenesis of many diseases and to the development of new therapies. The current trend in biomedical research suggests that, in the near future, the availability of murine models will be greatly improved by the large number of existing genetic manipulation techniques and chemical mutagenesis projects. Therefore, the use of these models will be essential for the functional study of sequences obtained from human and murine genome sequencing projects [10,11]. The use of murine models is because these models help in the understanding of the pathogenesis of many diseases and the development of therapies to replace the defective function of a given gene. In experimental medicine, the rodent is a model organism that offers many advantages over other genetic models such as the Drosophila fly, Caenorhabditis elegans benatode fly, among others [12,13].

These advantages are:

- To be a mammal, much of their biochemical processes are similar although not identical to humans.
- They have a very short gestation time, are very prolific and adapt easily to life in a biotherium, which allows control of environmental variables in experiments.
- After humans, they are the most studied species from a genetic point of view.
- There are a large number of genetically

defined lines, such as inbred and congenic lines, as well as hundreds of mutations and a large number of chromosomal rearrangements available.

- It is the only animal with efficient systems for culturing pluripotent embryonic cells (ES cells), which allows targeted mutations (constitutive and conditional KO mice).

Some models reported in the literature for OS development are as follows: An animal model for human OS was established in newborn Syrian golden hamsters by injecting cultured human OS cells adjacent to the femur, all animals developed OS and lung metastases with a survival of 36 days. median [8,14]. Another model was developed with tibial intramedullary injections of Moloney murine sarcoma virus in three strains of neonatal inbred rats, 10 days later they presented highly malignant tumors, producing mortality in all treated rats [10]. Another group of investigators induced OS with virus by lymphocyte microtoxicity assay, with intratibial injection of Moloney murine sarcoma virus. Seventy-three percent of the injected animals progressed to OS and caused pulmonary metastases with a survival of no more than 2 months [15]. However, these models have disadvantages; mortality is high in each of the benzopyrene models used.

BENZOPYRENE

Benzopyrene (BZP) is a potentially carcinogenic polycyclic aromatic hydrocarbon (PAH). The high content of BZP in some foods (nuts, sausages, chorizo, spices, pizzas, wood-fired bread, grilled meats, roasted coffee) is due to its manufacturing process that involves incomplete combustion processes, it can also be found in tobacco. BZP is produced by condensation of five benzene rings during combustion processes at temperatures of 300 to 600 °C. Since 1775, the British doctors Pott and Hill observed a high incidence of scrotal cancer in the personnel in charge of cleaning chimneys (chimney sweeps) [16]. About a hundred years later, similar finds were reported in Germany and Scotland, among workers in the coal tar and paraffin industry. These observations led to the conclusion that oil and coal derivatives contained substances capable of inducing tumors. confirmed in 1918, when the Japanese researchers Yamagiwa and Ichikawa demonstrated that tar applied to the skin of rabbits induced cancer [17]. Similar findings in rats were documented in 1920, in England by Kennaway reported by Hugues. However, since tar and soot are mixtures of various substances and the isolation of the possible chemical compounds responsible for such results was complex, it was not until 1920 that PAHs isolated from tar were identified as possibly responsible for producing tumors in the mice skin [18]. confirmed in 1918, when the Japanese researchers Yamagiwa and Ichikawa demonstrated that tar applied to the skin of rabbits induced cancer [17]. Similar findings in rats were documented in 1920, in England by Kennaway reported by Hugues. However, since tar and soot are mixtures of various substances and the isolation of the possible chemical compounds responsible for such results was complex, it was not until 1920 that PAHs isolated from tar were identified as possibly responsible for producing tumors in the mice skin [18]. confirmed in 1918, when the Japanese researchers Yamagiwa and Ichikawa demonstrated that tar applied to the skin of rabbits induced cancer [17]. Similar findings in rats were documented in 1920, in England by Kennaway reported by Hugues. However, since tar and soot are mixtures of various substances and the isolation of the possible chemical compounds responsible for such results was complex, it was not until 1920 that PAHs isolated from tar were identified as possibly responsible for

producing tumors in the mice skin [18]. In 1931 one of the PAHs, benzopyrene (BZP), was isolated from coal, in the same year it was synthesized and it was shown that it was responsible for the production of cancerous tumors in experimental animals. It was also found in coal tar, tar and soot [19], one of the first clearly identified carcinogens.

In epidemiological studies in workers exposed to coke ovens, during the industrial coal coking process, it was found that asphalt plants, foundries and aluminum facilities have a higher rate of lung cancer than those who did not carry out these work activities, which was attributed to PAH exposure [20]. Subsequent investigations revealed that PAHs were also found in foods consumed by humans [21]. BZP apparently acts on the K-Ras gene, causing a mutation in a specific area of the gene that can be matched in the DNA of lung cancer patients. This finding not only serves to determine that tobacco is carcinogenic, but may also be useful for developing new therapeutic strategies based on the specific information available for DNA damaged by cancer [22].

BETA-SITOSTEROL

It is a phytosterol with a structure similar to that of cholesterol from animal fat, it differs by the presence of an ethyl group in the side chain (figure 1). All plants, including fruits, vegetables, grains, spices, and seeds, have this compound. Plant oils are a particularly rich source of beta-sitosterol, which is used in the treatment of hypercholesterolemia, as well as to modulate immune function, inflammation, and is involved in the control of cytokine production. Research has also shown that beta-sitosterol helps normalize the function of T-helper cells and suppressor cells. [23].

MATERIAL AND METHODS

ANIMALS

Ten male Sprague Dawley rats and 10 Wistar rats were used, donated by the IMSS-Siglo XXI and the National Institute of Rehabilitation LGII National School of Biological Sciences, with an average weight of 180 g, divided into 4 groups and kept in polycarbonate boxes. The environmental conditions were: Light/dark period of 12/12 hours; temperature of 22 ± 2 °C and relative humidity of 60-70%, with free access to food (Rodent Lab Chow 5001, Purina) and water. The experimental procedure was approved by the Ethics and Biosafety Committee of the National Institute of Rehabilitation LGII.

REAGENTS

Beta-sitosterol, benzopyrene, mineral oil, dimethylsulfoxide, ketamine, xylazine, isoflurane, Giemsa stain, and NaCl were purchased from Sigma Chemicals (St. Louis, MO, USA).

OS INDUCTION WITH ADMINISTRATION OF BZP AND BS

Lesions were induced by perifemoral administration every 24 hours for 30 days at a dose of 20 mg/kg of BZP (Sigma-Aldrich), dissolved in dimethylsulfoxide, the administration volume was 0.3 mL. Negative control groups were given a daily administration of 0.3 ml dimethyl sulfoxide (BZP vehicle) to the distal right femur in a perifemoral three-point injection for 30 days. 2 experimental groups (one of Sprague Dawley rats and the other of Wistar rats) to which a daily administration of 20 mg/kg of BZP diluted in dimethyl sulfoxide was administered in the distal portion of the right femur in a perifemoral injection and at three points, for 30 days, Subsequently, the negative

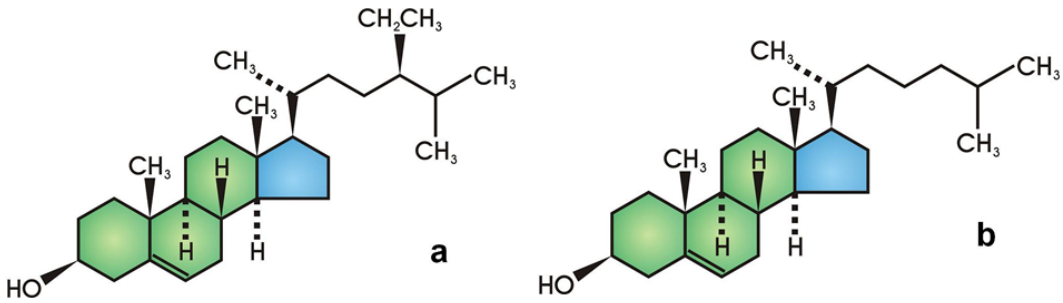

Figure 1. Chemical structure of cholesterol (a) and beta-sitosterol (b).

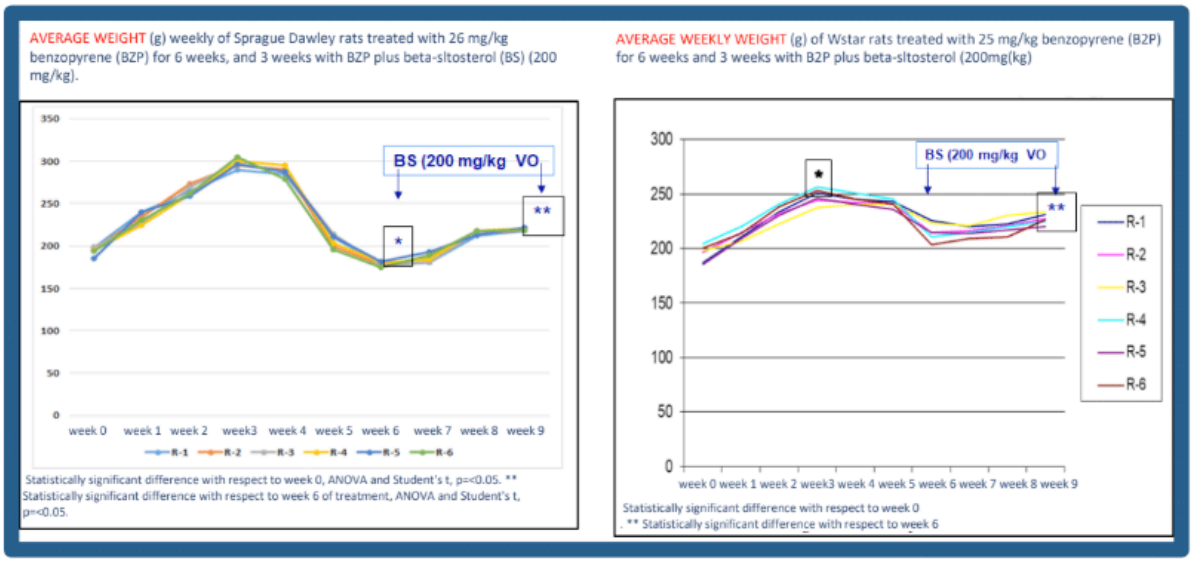

Figure 2. Average weights obtained from Sprague Dawley rats and Wistar rats treated with benzopyrene (BZP) and beta-sitosterol (BS).

controls were administered VO mineral oil and the treatment groups were administered 200 mg/kg beta-sitosterol for 3 weeks, all animals were euthanized at the completion of 9 weeks for study, morphological evaluation was performed and blood samples were obtained.

DESCRIPTION OF PROCEDURES

1. From day 0 the weight of the rats was recorded daily.

2. On day 0 and weekly until the end of the study, blood samples (two drops) were obtained from the tip of the tail by making a small cut (1 mm), to perform a blood smear for genotoxicity and cytotoxicity studies, using the micronucleus test.

3. From the end of the first week after the pharmacological manipulation and until the sacrifice of the rats, the condition of the treated femurs was evaluated.

4. At week 0 and every three weeks, blood samples (1 mL) were taken from the mandibular and/or retroorbital vein to determine sedimentation rate (Westergreen method), C-reactive protein (ELISA), alkaline phosphatase, and lactic dehydrogenase (colorimetric analysis).

5. For the procedures in which sedation of the animals was required, isoflurane placed in a wide-mouthed bottle was used, in which the animal under study was introduced for the time necessary for it to reach a sedated state.

After euthanasia and at the time of surgical opening of the abdomen, a macroscopic evaluation of the organic status of the rat was performed, with special attention to the lung, liver, kidney, spleen, and intestine. Femora, tibiae, lungs, and liver were removed and washed with buffer. Then they were placed in 10% formalin buffer and the samples were fixed and kept at 4°C until their histological study.

HISTOPATHOLOGICAL STUDY OF RAT FEMUR AND TIBIA

Once the rat femurs and tibias were obtained, each sample was washed with a buffer, then fixed in 10% buffered formalin, keeping them overnight at a temperature of 4 °C for 12 hours. The femurs and tibias were placed in a decalcifying solution (5% nitric acid for 96 hours). The bone was neutralized by placing it in a 10% carbonate solution. solution and washed with running water for 15 minutes. Samples were embedded in paraffin. Histological slides were immersed in xylene to remove excess paraffin. Subsequently, histological sections were made on a Minot-type rotary microtome. Tissue was cut to intact tissue in 5 micron slices, and then brushed into a flotation bath. The slides were marked with the number corresponding to the sample. The selection and placement of the cut in the slider was carried out. Excess water was drained from the histological slides and heat-fixed (thermostat plate at 56-58 °C). The deparaffinization of the histological sections was carried out in an oven or oven at 60°C for 30 min. Then they went through a series of alcohols in decreasing concentration to rehydrate the sample (100°, 95° and 70°). They were washed with water to remove excess alcohol. They were immersed in hematoxylin for 10 minutes, washed with water to remove excess, and rapidly passed through acidic alcohol. A wash was performed again, they were submerged for 30 seconds in eosin. They went through another series of alcohols, this time in increasing order (70°, 95° and 100°). dehydrate the sample, achieve the mount with a non-water soluble glue. Finally, they were left to soak for 10 minutes in xylol, before carrying out the final assembly and observation under a microscope [15]. They went through another

series of alcohols, this time in increasing order (70°, 95° and 100°). dehydrate the sample, achieve the mount with a non-water soluble glue. Finally, they were left to soak for 10 minutes in xylol, before carrying out the final assembly and observation under a microscope [15]. They went through another series of alcohols, this time in increasing order (70°, 95° and 100°). dehydrate the sample, achieve the mount with a non-water soluble glue. Finally, they were left to soak for 10 minutes in xylol, before carrying out the final assembly and observation under a microscope [15].

HISTOPATHOLOGICAL STUDY OF LUNG AND LIVER

Organs were placed in 10% formalin for 24 hours. After embedding in paraffin, the tissues were sliced 2 to 4 mm thick. They were immersed in xylol to remove excess paraffin. Then they were sequentially immersed in alcohol of decreasing concentration to rehydrate them (100°, 95° and 75°). They were washed with tap water to remove excess alcohol, then immersed in hematoxylin for 10 minutes, washed with tap water, rapidly passed through acidic alcohol, and finally immersed in eosin for 30 seconds. They were then dehydrated by dipping them sequentially in increasingly concentrated alcohol (75°, 95° and 100°). to be mounted on the slides with non-hydrophilic glue. Finally they were soaked with xylol for 10 min before finalizing the mounting, and observed under the microscope [18].

EVALUATION OF GENOTOXICITY AND CYTOTOXICITY IN PERIPHERAL BLOOD BY GIEMSA STAINING

Once the blood samples were obtained from the rat's tail, they were placed on perfectly clean slides and blood smears were made. For the staining of the samples, as a first step, they were fixed with methanol by immersing them for 5 minutes, then they were washed with running water and stained for 18 minutes in 4% Giemsa stain in phosphate buffer pH 6.8, the plates were observed at immersion microscope. Polychromatic erythrocytes (EPC) stained violet and normochromic erythrocytes (ENC) stained blue. The micronuclei were observed to have an intense violet color. To assess cytotoxicity, the relationship between the number of EPN and ENC was determined. The count was made in 2000 cells per group at the established times and the number of micronucleated normochromic erythrocytes was determined to assess genotoxicity [24,25].

RESULTS

The partial results during the administration of BZP and after the administration of BS were the following: The weight of the animals in both strains presented a statistically significant decrease from the third to the 7th week, however, the weight began to stabilize at from the 8th week (Figure 2). The biochemical tests presented statistically significant differences from the 3rd week of treatment with BZP in the values of ESR, FA and DHL, in all the treated rats, however, from the 8th week a decrease in FA was observed. and DHL in some animals administered with BS (Figure 3). Histopathological study demonstrated OS formation in the treated femur. BZP was highly genotoxic in both strains of rats, a statistically significant difference being observed with the micronucleus (MN) test from the first week of BZP treatment with a frequency of up to 27.36±0.32 MN in 1000 polychromatic erythrocytes, being slightly less evident in W rats (Figure 4).

DISCUSSION

Experimental animal studies on benzopyrene have classified it as a toxicant that affects fertility in rats and other animal

species, because the resulting metabolites interfere with normal ovarian function [26]. Tsai-Turton, et al [27], in 2007, evaluated the accumulation of BZP metabolites in liver and testicular microsomes of rats, hamsters and pigs, finding that several of these interfere with gamete formation and function, thus contributing to infertility. In liver and ovarian cell tissues of various species (rats, mice, goats, sheep, pigs and cows) it was observed that upon exposure to 5 μg/g of BZP the resulting metabolites bind to the estrogen receptor, reducing estrogen receptor activity; Prolonged exposure to BZP causes a sequestration of these in high-density lipoproteins that are essential for the biosynthesis of steroid hormones in the ovary, leading to reduced secretion of gonadotropins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), with negative results. results in the late stages of follicular development, decreasing fertility [28,29]. BZP has been reported to be a potent inducer of OS, with features similar to the pathophysiology of OS in humans and survival is up to 8 months in treated animals [14]. Given the carcinogenic and mutagenic effects of Benzopyrene, we propose this compound as a chemical to induce osteosarcoma in Sprague Dawlwy rats. Wester and colleagues [30], demonstrated the carcinogenic potential of oral administration of BZP, these researchers exposed 104 Wistar rats for five days a week to amounts of 0, 3, 10 or 30 mg of BZP per kg body weight, for two years and subsequently histopathological studies were performed. The results showed the development of liver tumors in 99 of the 104 rats, with an estimated dose of 3-5 mg/kg, with a confidence interval of 90%, as well as sarcomas in soft tissues, such as skin and breasts with a dose of 10 mg/kg. Both skin and liver tumors were considered relevant to humans at high doses.

Regarding beta-sitosterol in a review published by Ovesna et al [31], they recorded the experimental inhibition of colon and breast cancer development by taraxasterol and β-sitosterol. They indicated that these compounds can affect different levels of tumor development, such as their inhibitory effects on cancer cell creation, promotion and induction, as well as inhibition of tumor cell invasion and metastasis. Dietary supplementation with BS decreases the levels of 17β-estradiol (E2) which suggested that high levels of phytosterols may have beneficial effect in women with breast cancer [32].

The genotoxic assay is used to determine how much DNA damage is exerted by xenobiotics, which can therefore affect humans exposed to them. Paniagua-Pérez [33] reported the genotoxicity of β-sitosterol, including the acute toxicity test, which demonstrated low lethal potential (38%) of this compound. The results indicated that no SCE (sister chromatid exchanges) increase was induced by the doses tested (200, 400, 600 and 1000 mg/kg), as well as no changes in the kinetics of cell proliferation, or in the mitotic index. In said report, the highest dose applied showed 80% of the LD50. For this reason, β-sitosterol is not considered to be genotoxic and cytotoxic. The safety of this compound stimulates scientists to carry out more pharmacological investigations of this sterol [33].

The results of a study demonstrated that beta-sitosterol in 1,2-dimethylhydrazine-induced colon carcinogenesis in rats caused elevation in enzymatic and non-enzymatic antioxidants, recommending the compound as an effective chemopreventive drug for colon carcinogenesis [3. 4]. Beta-sitosterol stimulates antioxidant enzymes by activating the estrogen receptor/PI3-kinase-dependent pathway. The ratio of glutathione GSH and total GSH recovered after β-sitosterol treatment, suggesting that this phytosterol

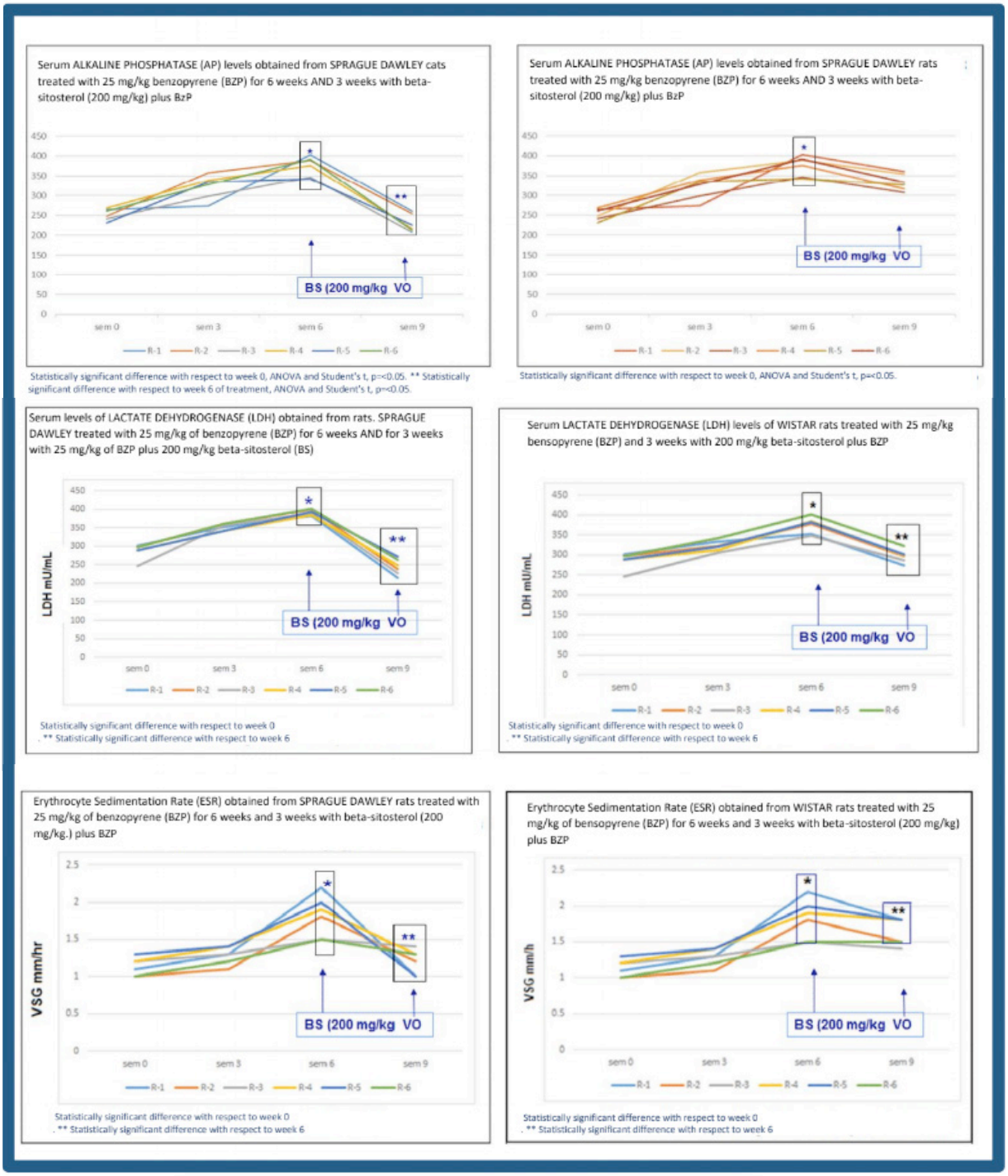

Figure 3. Biochemical values obtained from Sprague Dawley rats and Wistar rats treated with benzopyrene (BZP) and beta-sitosterol (BS).

Columna1	R-1 SD	R-1 W	R2-SD	R-2 W	R-3 SD	R-3 W	R-4 SD	R-4 W	R-5 SD	R-5 W	R-6 SD	R-6 W
Sem 0	1.13±0.16	1 ± 0.5	1.5 ± .08	2.1±0.4	2.0±0.23	0.93±0.3	1±0.12	1.3±0.4	1.74±0.21	1.98±0.2	1.14±0.32	1.3±0.6
Sem 1	*18.26±0.26	4.97±0.2	*19.8±0.36	5.63±0.2	*19.3±0.36	*7.10±0.1	*20±0.93	*6.73±0.1	*22±0.62	*6.1±0.23	*19.1±0.38	*8.73±0.71
Sem 2	*19.3±0.29	*8.1±0.14	*26.8±0.17	*7.9±0.14	*17.7±0.52	*9.5±0.08	*23.5±0.44	*7.1±0.14	*18.6±0.19	*11.2±0.13	*17.33±0.61	*9.1±0.35
Sem 3	*26.4±0.31	*7.5±0.08	*25±0.12	*11.5±0.8	*29.76±0.11	*13.1±0.1	*26.93±o.61	*9.5±0.12	*29.5±0.24	*13.3±0.32	*25.38±0.33	*11.3±0.36
Sem 4	*20.5±0.22	*14.5±0.4	*24.4±0.38	*18.2±0.1	*28.16±0.21	*19.4±0.5	*23.83±0.27	*16.3±0.23	*27.5±0.34	*14.14±0.21	*27.36±0.14	*16.6±0.17
Sem 5	*27.4±0.51	*19.1±0.6	*28±0.19	*19.8±0.6	*29.76±0.11	*18±0.24	*31.43±0.41	*15.45±0.35	*28.9±0.29	*16.4±0.45	*30.38±0.30	*17.8±0.31
Sem 6	*30.5±0.41	*18.2±0.7	*29.5±0.28	*18.0±0.26	*30.16±0.34	*18.2±0.43	*33.5±0.58	*20.4±0.71	*32.5±0.13	*19.3±0.22	*31.33±0.32	20.6±0.23
Sem 7	*20.5±05	**11.2±0.7	*22.2±0.3	*15.8±0.11	*20.5±0.42	*16.4±0.52	*29.1±0.31	*16.4±0.33	*23.4±0.45	*17.4±0.62	*26.4±0.12	**13.8±0.6
Sem 8	*21.2±0.1	**10.6±0.6	*17.5±0.34	*16.8±0.34	*18.24±0.22	*15.4±0.67	**14.3±0.22	**12.3±0.11	**17.4±0.35	*14.2±0.91	**14.5±0.32	**10.1±0.6
Sem 9	**12.6±0.43	**10.3±0.5	**13.2±0.11	*15.2±0.23	**11.16±0.21	*14.8±0.45	**12.5±0.11	**13.6±0.36	**14.2±0.11	**13.7±0.11	**12.5±0.56	**8.3±0.5

Figure 4. Frequency of micronuclei in Sprague Dawley (SD) rats and Wistar (W) rats treated with benzopyrene (BZP) 25 mg/kg for 6 weeks and 3 weeks with beta-sitosterol (BS) 200 mg/kg plus BZP.

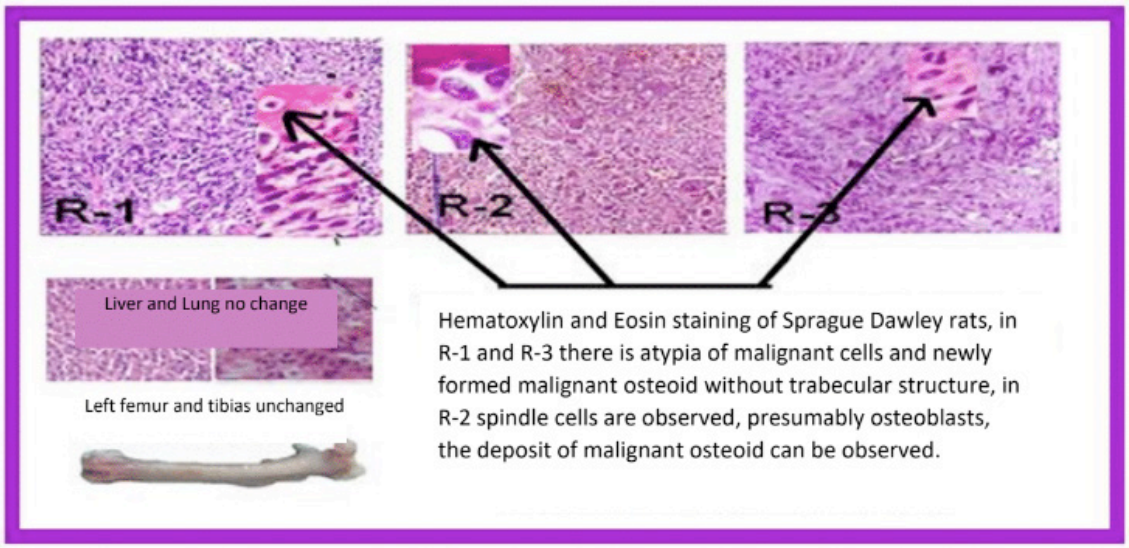

Figure 5. Histological results of Sprague Dawley rats treated with benzopyrene and beta-sitosterol.

may be a good ROS treatment alternative [35].

CONCLUSIONS

The results suggest that the administration of BZP contributes to the induction of OS in Sprague Dawley and Wistar rats. Preliminary data suggest that the administration of BS can help reduce the carcinogenic activity of BZP, which gives us the guideline to continue with other studies. that allow considering the use of BS in people with OS.

AUTHOR CONTRIBUTIONS

Paniagua-Pérez Rogelio, Franco and Bourland Rebecca E, Madrigal-Bujaidar Eduardo, Álvarez-González Isela, designed this research topic and wrote the manuscript; Martínez-Canseco Carlos J, Araujo Monsalvo Victor M, Domínguez Hernández Victor M, Martínez Coria Elisa, They designed and executed part of the methodology; Cruz Hernández Lidia, Ruiz Rosano Lidia, García Campillo Hiram, Quintana Armenta Alejandra, carried out the experiments and collected and analyzed the data.

GRATITUDE

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CONFLICT OF INTEREST DECLARATION

The authors of this manuscript we have no conflict of interest to declare.

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