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Toxicological evaluation of the João da Costa e Associações® on Beagle dogs

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Abstract: A toxicological study was performed in Beagle dogs treated for 180 days with the product João da Costa e Associações. Were used six males and six females distributed in control and treated groups (n=3). We used a dose of 566 mg/kg of the product according to preclinical study in rodents. The animals were weighed and evaluated by clinical and laboratory aspects. The product did not cause mortality or alter the normal behavior of animals, but interfered with the weight gain on males in the middle phase of the treatment. The group treated had a lower incidence of clinical abnormalities compared to control, checked by veterinary consultations. Laboratory data showed elevated blood glucose levels perhaps due to the high amount of sucrose present in the product; about the histopathological data no significant change was found. We conclude that the product Joao da Costa and Associações, at the dose tested, has low toxicity in Beagle dogs treated chronically.

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Introduction

The need for studies before the marketing of drugs is determined by Brazilian Federal Law nº 6360 (Brasil, 1976), which requires the development of all the necessary information to acquire knowledge of the efficacy, safety and quality of products. Specific complementary rules for drugs of phytomedicine origin are provided in the Anvisa Resolution RDC 14 (Anvisa, 2010) and basic safety guidelines are set forth in the Anvisa Resolution RE nº 90 (Anvisa, 2004). As a result, pharmaceutical companies in Brazil, upon seeking to make their products compliant, have implemented the development of security, efficacy and quality studies within the aforementioned statutory provisions, particularly in the areas of pharmacology and toxicology (Zaupa et al., 2002; Etges, 2007; Tagliati et al., 2008), with the addition of traditional aspects in order to obtain the product's final validation.

João da Costa e Associações® (JCA) is a phytomedicine product, which has been marketed in Brazil for at least 35 years, composed of decoctions of five species: agoniada (barks of *Himatanthus lancifolius* (Muell. Arg.) Woodson, Apocynaceae), abútua (roots of *Chondodendron platyphyllum* Miers, Menispermaceae), cotton (root peels of *Gossypium herbaceum* L., Malvaceae), rosemary (flowering tops of *Rosmarinus officinalis* L., Lamiaceae) and joao-da-costa (barks of *Echites peltata* Vell. Apocynaceae), in solutions with sucrose. It is recommended for intermittent ovarian pains and menstruation anomalies, chronic uterus and ovary inflammations and to relieve menopause symptoms (Laboratório Belém Jardim, 2011).

Three of these five species are traditionally indicated for inflammation and problems related to women's health: abútua is considered an emmenagogue, agoniada is used in amenorrhea, complicated and painful menstruations, and cotton is also recommended as an emmenagogue; joao-da-costa is recommended for inflammations in general and European rosemary is also mentioned as being abortive and emmenagogue (Hoehne, 1920; Coimbra, 1942). The product is, therefore, composed of the association of five species traditionally related to the improvement of menstrual activity, with probable anti-inflammatory, analgesic and antispasmodic properties.

Chemically, literature mentions the presence of benzyl tetrahydroisoquinoline alkaloids in abutua (Gottlieb&Mors, 1980), uleine in agoniada (França et al., 2000), gossypol in cotton (Etges, 2007), essential oils, flavonoids and phenolic acids in rosemary (Al-Sereiti et al., 1999) and pentacyclic triterpenes in joao-da-costa barks (Humberto et al., 2004).

The JCA product was evaluated for its antinociceptive activity, as well as its chemical composition by means of high-performance liquid chromatography. The results showed a weak inhibition in the second phase of the formalin test and an inadequate chromatogram profile when compared to what was expected from the five species used, thereby suggesting that problems had arisen due to the preparation of the product (Brandão et al., 2010) .

In relation to toxicity in rodents, acute (oral and intraperitoneal routes) and 180 day (oral route) treatments study in male and female Wistar rats did not cause mortality, or physiological and behavioral alterations, even with a 1120 mg/kg dosage. The biochemical results presented a significant hypoglycemic effect at a 560 mg/kg dose in males (control 173±64; treated 108±22; $p<0.05$) and females rats (control 133±17; treated 77±42; $p<0.05$) and a significant increase in total proteins in males rats treated with 1120 mg/kg (control 7.57±0.48; treated 9.10±0.88; $p<0.05$). The histopathological evaluation showed no significant alteration, except in the lungs, which presented chronic pneumonia and bronchitis, and the liver which revealed hepatic steatosis with sinusoidal capillaries dilatation, conditions also presented in the control group animals. The alterations mentioned above are common in animals treated over long periods and problably should not be related with the treatment (Brandão et al., 2003).

This study was carried out in order to complete the pre-clinical toxicological evaluation of JCA, in compliance with the Brazilian legislation which also requires assessment in non-rodent species.

Material and Methods

Phytomedicine product

The evaluated product JCA was produced by the Belém Jardim Ltda. Laboratory, which prepared batch nº 0330 in 9 Sep 2002, packed it in 500 mL amber glass packing, which were forwarded to the research institution. The Belém Jardim pharmaceutical company has stored parts of batches produced used in this study and details of the preparation are given by Brandão et al. (2010). Samples of this batch were concentrated in a rotary-evaporator, lyophilized, stored in a freezer, and weighed daily shortly before administration.

Animals

Twelve tri-color Beagle dogs were used, six males and six females, aged 3 to 4 months, in accordance with the literature (Mesa et al., 1994; Guzman et al., 2000). The animals were kept in animal facilities, separated by gender and fed with all the standard pet food and water *ad libitum*. Initially they remained thirty days without any treatment so they could adapt to the new environment; during this period fecal coprologic tests were performed, the dogs were dewormed and their vaccination was completed (octuple and rabies). For the purpose of the experiment, the animals were divided in two groups, experimental and control (n=3).

Ethical aspects

The project was forwarded for assessment by the Maringá State University Ethical Committee under registration nº 068/2002, an opinion was issued (nº 077/2002) and the protocol was approved.

Chronic test protocol

JCA was administered during 180 days (24 weeks), from September 2002 to March 2003, in the Maringá State University animal facilities; the experimental groups received the product orally at a dose of 566 mg/kg (six times higher than the recommended product therapy), as per instructions for rodent tests, and the controlled animals received only water. Administration was done daily, always in the morning (from 9 to 10 am), with animals that had not eaten since the previous afternoon and were fed only 1 h after receiving the product.

The animals were supervised daily for general and behavioral evaluation (food and water ingestion, consistency of feces, urine color, aggressivity, sedation, tremors, convulsions and other unusual behavior) and were weighed weekly. Biweekly veterinary consultations were conducted, with the same professional, the findings were registered in individual clinical records.

Blood sampling were performed for laboratorial evaluation every two months during the treatment (November 2002, January 2003). At the end of the treatment, the animals were euthanized, according to a pre-established protocol (Prado Filho et al., 2000), with the performance of a final collection of blood and urine, fluids evaluation in the abdominal cavity, and organs macroscopic observation followed by removal in blocks (thoracic and abdominal) for histopathology.

Clinical evaluation

Clinical sessions were held with the checking of body temperature, skin irritation, corporal itching, pustules, hair loss (ocorrence and extension of possible alopecia), auricular pavillion edema, buccal and ocular mucosa, as well as sub-mandibular glands palpation.

Scores were defined based on to the clinical profile, with a protocol adapted from CADESI - *Canine atopic dermatitis* extents and severity index and the clinical veterinary practice (McCurnin & Poffenbarger, 1991; Olivry et al., 2002), according to the following classification:
Score 5 = no clinical signs;
Score 4 = mild alopecia or any other light clinical signs;
Score 3 = localized alopecia, redness of the oral and/or ocular mucosa, ganglia infarction;
Score 2 = generalized alopecia, hyperemia of the oral and/or ocular mucus, corporal body pustules, ganglia infarction, pruritus;
Score 1 = anterior or posterior limbs with erythematosus aspect, inflammatory lesions, increased occurrence of pustules on the body, alopecia intensification, oral and/or ocular mucosa hyperemia, ganglia infarction, severe pruritus.

Histopathological evaluation

Procedures were conducted according to routine techniques (Montenegro&Franco, 1992; Mikel, 1994). After total viewing, target organs were removed, dissected, and weighed, and a subsequent analysis of their macroscopic aspects were performed; after that, the organs were getting ready to obtain permanent slides that were analyzed in a blind test, by optical light microscopy, for the detection of possible histopathological alterations.

Laboratorial evaluation

The following laboratory tests were also performed on the dogs, also blindly:

Blood: complete blood count, glucose, total cholesterol, triacylglycerides, urea, creatinine, glutamic oxalacetic transaminase, glutamic pyruvic transaminase, total protein, albumin, uric acid, amylase, bilirubin (direct, indirect an total) and alkaline phosphatase.

Urine I (only in the last sampling): physical examination (volume, color, aspect, deposit, pH and density), chemical examination (protein, ketones, urobilinogen, glucose, hemoglobin and nitrite), sedimentoscopy (epithelial cells, leukocytes, erythrocytes, mucus crystals, cylinders and filaments) and bacterioscopy.

Statistical analysis

A one-way analysis of variance was conducted (Anova) for parametric tests with variable intervals, followed by a Duncan a posteriori test. The frequency of clinical and hystopathological alterations were evaluated by Fisher's exact test. The accepted level of significance was of $p \leq 0,05$ for all the experiments. These analyses were performed in static software (Statistica®).

Results

General observations

Over the 24-week treatment, none of the twelve animals involved in the study died or presented behavioral alterations, according to the daily observation of the animals technical attendant, under the supervision of the veterinary responsible for the dog facility.

Ponderal evolution

The dogs went through several weightings during the six months of treatment, as described in Table 1. The date showed that the JCA provoked a progressive and significant loss of body weight in the males, which occurred as early as the third week of treatment and continued until at least the fifteenth weighing, with subsequent recovery. Differently, the females showed no significant weight variation during any of the evaluation periods.

Table 1. Ponderal evolution (kg) of male and female dogs chronically treated with water (control) or 566 mg/kg of JCA product.

Weighing week	Male		Female	
	Control	JCA	Control	JCA
1 th	5.7±0.6	5.3±0.6	3.7±0,6	3.3±0.6
5 th	9.9±0.5	8.1±0.6**	7.7±0.3	7.3±0.8
10 th	11.7±0.3	10.3±0.3*	9.3±0.4	9.1±1.0
15 th	13.1±0.1	11.7±0.2*	10.3±0.3	10.1±0.9
20 th	12.5±0.5	11.3±0.1	10.3±0.6	9.9±1.0
25 th	12.3±0.1	12.0±0.6	9.9±0.1	9.5±1.1

*$p<0.05$; **$p<0.01$

Clinical evaluation

Ten clinical evaluation sessions were conducted during the treatment, with the verifiation on the parameters established in the clinical protocol, and the determination of a final score (5 to 1) for the animal's general condition. No animal presented significant body temperature alteration during the sessions (data not submitted). Regarding other alterations, both groups presented various clinical manifestations, in different degrees and treatment periods, according to the established protocol.

The sum of the scores in the ten sessions

showed higher values for the groups treated with the JCA product, for both the males (control 37.97±5.8; JCA 44.0±1.0; n.s.) and the females (control 32.0±4,4; JCA 43.0±2,0; $p<0.05$). The clinical score assessment during the whole treatment shows that there was favorable significance in the females treated with JCA detected in three evaluation periods, the 6th , 7th and 8th sessions (Figure 1).

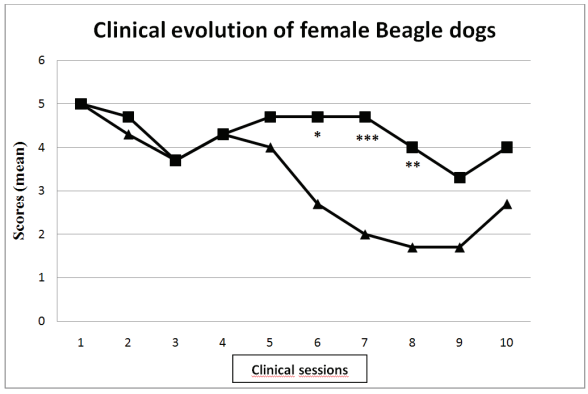

Figure 1. Clinical evolution, expressed in clinical scores (mean), of female Beagle dogs chronically treated with water (▲) or 566 mg/kg of JCA product (■) over ten sessions (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Laboratorial data

Laboratory test results were obtained during days 60, 120 and 180 of the treatment (hematological and biochemical values of the males and females), with the urine data obtained only in the third collection.

In the hematological data, the only significant alterations occurred during the second collection (on the 120th day of the treatment). A statistically significant increase in the hematimetric indices of Average Globular Volume was found in the males (control 67.96±0.25; treated 70.65±1.36; $p<0.05$); in females occurred an increase in the segmented cells (control 7984±929; treated 9826±468; $p<0.05$) and in the neutrophils cells (control 8027±967; treated 9874±550; $p<0.05$) numbers.

With regard to biochemical tests, there were few significant changes, like a glucose value increase in the animals treated and a decrease in the total proteins levels in the second collection in females (Table 2).

The urinalysis data results highlighted three cases of hematuria (probable infection or inflammation of the urinary tract), one in a female control and two in the treated animals (one male, one female). All other values were normal for this kind of test.

Histopathological data

Macroscopic evaluations were performed on all the thoracic and abdominal cavities, with no occurrence of cavitary effusions, inflammatory alterations or external abnormalities in the organs; measurements and weight were also within normal parameters, as well as consistency and color characteristics.

Table 2. Biochemical data of total proteins, albumin and glicemia of three blood sampling of male and female Beagle dogs chronically treated with water (control) or 566 mg/kg of JCA product.

Parameter	Groups	Treatment	1th blood sampling	2th blood sampling	3th blood sampling
Total proteins (g/dL)	male	control	5.8±0.5	5.8±0.5	6.8±0.4
		JCA	6.1±1.0	6.1±0.2	7.0±0.5
	female	control	5.7±0.6	7.2±0.3	7.9±0.6
		JCA	5.6±0.5	6.1±0.3*	7.1±0.7
Albumin (g/dL)	male	control	3.9±0.2	3.4±0.3	4.0±0.6
		JCA	3.7±0.2	3.5±0.2	3.8±0.1
	female	control	3.8±0.1	3.8±0.4	3.8±0.3
		JCA	3.8±0.1	3.8±0.1	3.8±0.2
Glicemia (mg/dL)	male	control	34.7±5.1	32.7±10.7	44.7±15.3
		JCA	54.7±9.6*	52.3±8.7	47.0±10.1
	female	control	45.3±10.0	25.0±15.1	38.7±9.5
		JCA	53.7±10.5	48.3±10.1	60.0±13.5

*$p< 0.05$.

In microscopic terms, the animals organs in both groups (control and treated) presented histological integrity, with a few occasional findings. Two control animals, one male and the other female, presented linfoplasmocytic portal hepatitis, with no parenchymal necrosis or steatosis, which can sometimes be related to a parasitic process or intestinal infection.

In a second case, two dogs, one treated male and one control female, revealed a chronic pyelonephritis focus, which can be related to urethra or bladder infections. Urine exams confirmed these data, since the male dog nº 1 (treated group) presented 66000 erythrocytes/ml and the female nº 2 (control group) presented 84000 erythrocytes per ml. The treated female nº 1 also presented high values of erythrocytes in his urine (23000/mL), but the microscopic evaluation did not indicate pyelonephritis foci .

Other detected microscopic alterations were an acute pneumonia focus in a control female and a pulmonary septal fibrosis focus in a treated male animal. In statistical terms, there are no differences between the frequency of occurrence of these alterations in both animal groups.

Discussion

In vivo toxicological studies are the most common method for evaluating the toxic properties of a test substance, because they supply information on

the risks resulting from a short or long-term exposure. In this sense, despite guidelines that long term studies would not be a priority for traditionally used products that have proved to be harmless (WHO, 2000), data obtained through the study that examined JCA product prolonged administration effect in Beagle dogs helped gain a better understanding of the possible risks involved when using this medicine, improving the safety level of its use and contributing to its validation in modern regulatory terms.

Study results show that the medicine does not cause mortality, modify normal behavior, promote changes in the food and water intake, nor in the aspect of feces and urine. On the other hand, they show interference in weight gain and ponderal evolution, particularly in males in the intermediate stage of the treatment. In a related aspect, both groups organs showed no difference in weight, showing that interference with weight gain did not affect dogs tested vital organs.

In clinical terms, the product was well tolerated, revealing no higher clinical parameter alterations than those of the control animals. Moreover, the JCA product seems to have promoted some protection degree against general environmental aggressions in the treated animals, evidenced by a lower incidence of clinical alterations, with higher scores in the evaluation produced over the ten sessions. Therefore, the males, and mainly the female animals treated with JCA, had clinical scores that revealed that their clinical condition was much better than the control animals. This finding can be related to the pharmacological effects of the species used in the medicinal formula, such as the antioxidant and antimicrobial properties of rosemary - *Rosmarinus officinalis* (Al-Sereiti et al., 1999), or even the anti-inflammatory actions attributed to both abutua and Joao da Costa (Coimbra, 1942), which certainly must have contributed to the decrease in skin and mucosa irritations, and other clinical parameters evaluated.

As for laboratorial and histopathological results, the changes found were minimal and unlikely related to the JCA treatment. The glicemia parameter, which had higher levels in the treated animals than in the control ones, has an obvious relation with the sucrose present in the product, which must have been responsible for the increase observed in all the treated groups. However, the product presented opposite responses, with a decrease in glicemia levels when tested on rodents, which must have metabolic reactions to the daily intake of a carbohydrate-rich solution different to those of the dogs.

In relation to the values of total proteins, the second collection revealed a decrease in its level in females treated (control 7.2±0.3; JCA 6.1±0.3; $p<0.05$) although no changes occurred in the first collection (control 5.7±0.6; JCA 5.6±0.5, n.s,) nor in the end of treatment (control 7.9±0.6; JCA 7.1±0.7; n.s.). However in males such change did not occur in any phase of treatment, there were no changes in other parameters related to the liver such as albumin, transaminases and alkaline phosphatase. The histopathological data also do not showed any liver ou renal parenchymal process after treatment. Thus, this alteration in the amount of total protein should represent an isolated finding with no clinical relation.

We can therefore conclude that the João da Costa e Associações product, in a dosage six times higher than the prescribed therapy, shows low toxicity in chronically treated Beagle dogs, demonstrating the safe use in the dose studied. However, the use of the product by diabetic patients must be controlled, due to its high sucrose concentration.

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