Effects of the Mixture of Cashew Nut Shell Liquid (CNSL) on the Testicular Function of Sexually Mature Mice

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Abstract

Cashew nut shell liquid (CNSL) is a byproduct of cashew nut processing that has an efficient larvicidal potential against Aedes aegypti. Recent study published by our group indicated that pregnant Swiss females exposed to CNSL showed no changes in embryo-fetal development, reproductive performance and genetic stability. However, there are no studies in the literature that demonstrate CNSL safety in the reproductive performance of males. Then, the aim of this study is investigating the toxicity of CNSL on male reproductive function. Sexually mature Swiss male mice (n = 36) were used. The animals were divided into 5 groups: a) control (n = 6): received only water; b) CNSL 5 mg / kg (n = 6); c) CNSL 50 mg / kg (n = 6); d) CNSL 100 mg / kg (n = 6); e) CNSL 1000 mg / kg (n = 6). The results showed that testicular weight and the testicular parenchyma were similar in all groups evaluated. The nuclear volume and the total number of Sertoli cells did not change in the groups treated with CNSL. The same trend occurred with the nuclear and cytoplasmic volume of Leydig cells, which were similar between those animals exposed to CNSL and the control. Taken together, our results provide strong evidence that CNSL is not toxic for testicular function and spermatogenesis.

Keywords: Cashew nut shell liquid; Testis function; Spermatogenesis.

Abbreviations

CNSL: Cashew nut shell liquid; COBEA: Brazilian Society of Science in Laboratory Animals; GI: gonadosomatic index; LC: Leydig cells; SC: Sertoli cell

Introduction

The cashew tree, Anacardium occidentale, belongs to the Anacardiaceae family and it is a very common plant in some Brazilian states with great economic importance in the country [1-3]. From the chestnut shell, a caustic and flammable liquid, dark almost black in color is obtained: the cashew nut shell liquid [1-3]. The use of this compound is reinforced by the fact that this compound is not toxic to the environment, which makes it an agent in favor of sustainability [2,9,10].

CNLS is a byproduct of cashew nut processing, renewable and of low added value, becoming the interest of several researchers due to its potential applications as analgesic, diuretic, for the treatment of asthenia, respiratory problems, infant scurvy, eczema, genital infections, skin diseases, besides being rich in fibers that can help in intestinal problems [1].

In addition, anacardic acid, the main component of natural CNLS, has an efficient larvicidal potential [2,7]. Its use has been an alternative to the control of Aedes aegypti, a mosquito that transmits diseases such as Dengue, Chikungunya Fever and the Zika virus [2,7,8]. Thus, the use of CNSL is reinforced by the fact that this compound is not toxic to the environment, which makes it an agent in favor of sustainability [2,9,10].

However, it is known that the use of larvicides is usually due to fumigation and/or deposition in water tanks with drinking water or not [11]. The use of the larvicides are directly associated with genetic instability, which can lead to increased predisposition to cancer, as well as alter reproductive performance, causing hormonal disorders and male infertility [12,13].

Recent study published by our research group indicated that pregnant Swiss females exposed to CNSL showed no changes in embryo-fetal development, reproductive performance and genetic stability [9]. However, there are no studies in the...
literature that demonstrate CNSL safety in the reproductive performance of males. It is known that the testicles fulfill two essential functions: spermatogenesis in the seminiferous tubules, which produces gametes and the synthesis and secretion of sex hormones in the interstitial. Nevertheless, for spermatogenesis to occur without damage, it is necessary the proper function of the endocrine regulatory system and the two testicular compartments, the tubular and interstitial compartments. The occurrence of damage to germ cells as well as the destruction and/or functional alteration of Sertoli cells and Leydig cells could lead to irreversible impairment of spermatogenesis [12].

Given the above, it is important to study the impacts of exposure, in male sexually mature mice, to different doses of CNLS, in order to assess testicular function and the spermatogenic process, thus investigating the toxicity of CNLS on male reproductive performance.

**Materials and Methods**

**Ethics statement**

All experimental procedures utilized in this study were previously approved by the Animal Experimentation Ethics Committee of the Federal University of Mato Grosso do Sul (protocol number 401/2012), following to the standards required by the Brazilian Society of Science in Laboratory Animals (COBEA), according to international ethical principles for animal experimentation.

**Obtaining Cashew Nut Shell Liquid (CNLS)**

The present study used five hundred grams of cashew nut shells (*Anacardium occidentale*) donated by Kardol Indústria Química in 2014. The plant material was verified by Msc. Juliana Miron Vani, and a voucher specimen was deposited (No. 51838) in the herbarium of the Federal University of Mato Grosso do Sul (UFMS). The cashew nut shells were cut into small fragments, which were exhaustively extracted with ethanol for 6 hours in a Söhdet system. After evaporation of the solvent, a dark liquid was obtained (compared in ccd with technical CNSL, hexane: ethyl acetate 4:1; 157g; 31%) [14]. Vani and colleagues [7], described the method of preparing CNSL and its constituents.

**Experimental Design**

Sexually mature Swiss male mice (n = 36) were used. The animals were divided into 5 groups: a) control (n = 6); received only water; b) CNSL 5 mg/kg (n = 6); c) CNSL 50 mg / kg (n = 6); d) CNSL 100 mg / kg (n = 6); e) CNSL 1000 mg / kg (n = 6). The CNSL was administered orally, by gavage. After 30 days of treatment, the animals were weighed and euthanized. The dose of 5 mg/kg (body/weight, via orally) was based on the larvicide dose [7], and the security dose was defined as 10× greater than the indicated for guidelines, i.e., 50 mg/kg. [15,16]. The remaining doses (CNSL 100 and 1000 mg/kg) were used for safety testing. Moreover, considering that the time required for a complete spermatogenetic cycle in the mouse is about 34.5 days [17], the present study established a treatment period of 30 days.

The testicles were fixed by immersion, dehydrated and embedded in parafin. The 4 μm histological sections were obtained on the rotating microtome, stained with hematoxylin-eosin, mounted with Entellan and analyzed using a trinocular microscope. For each animal investigated, the GI gonadosomatic index (percentage ratio between testicular mass and body) was calculated.

**Histomorphometric studies**

Histomorphometric studies were used to assess testicular function in mice submitted to different treatments. The tubular diameter was estimated by measuring 30 cross sections of the tubules for each animal, and the mean between the largest and the smallest diameter of each section was considered. For this measurement, we used 400x magnification. The volume densities of testicular components were determined by a reticulum that contained 494 points of intersection obtained by the Image J software [18]. Atotal of 7410 points were scored for each animal. Each point was classified into one of the following divisions: tunica propria, epithelium, lumen, Leydig cells, connective tissue, blood and lymph vessels [19]. The volume of each testicular component was determined by the product of the testicular volume and the volume density. The specific gravity of the testicular tissue was considered 1.0 [20]. The albuginea tunica was not taken into account in the testicular weight.

The mean nuclear volume of Sertoli cells (SC) was established by measuring the diameter of 40 nuclei in each animal and the volume (μm³) could be calculated using the sphere formula. The total number of SC per testis was determined as the total nucleus volume of SC in the testicular parenchyma / nuclear volume of SC (μm³). In addition, the volume of Leydig cells (LC) was established from the evaluation of nuclear LC and cytoplasmic volume. Because the LC nucleus in mice, under light microscopy, is practically round or almost round, its volume is easily defined from its average nuclear diameter. Thus, 30 nuclei were measured that showed a nucleus quite evident for each animal. The nuclear volume, in turn, was estimated based on the sphere formula. To calculate the proportion between the nucleus and the cytoplasm, a 494-point intersection grid was placed on the sectioned material with a magnification of 1000x. Approximately 1000 points above the LC per testis were counted per animal. The number of LC per testis was estimated using the individual LC volume and the LC volumetric density in the testicular parenchyma [21].

**Statistical Analysis**

The results obtained were analyzed with the aid of the program "Excel for Windows", being estimated the means, standard deviations and standard errors of the mean (SEM). The values obtained were expressed as mean ± SEM and the data were analyzed using the ANOVA test / Student-Newman-Keuls test using the GraphPad Prism program (version 5; GraphPad Software Inc., San Diego, CA). The level of significance considered was p <0.05.
Results

Biometric data

The biometric parameters of the animals exposed to the CNSL are shown in Figure 1. The body weight was similar (p>0.05) between control and animals treated with CNSL, with the exception of the animal exposed to a dosage of 5 mg/kg of CNSL, who had a higher body weight compared to the control. This is due to the random distribution of animals in this experimental group and the fact that the group is not homogeneous. However, testicular weight was similar between all groups (p>0.05). In addition, the gonadosomatic index (GI), expressed by the testicular mass divided by body weight, indicates that animals treated with 5 mg/kg of CNSL did not present impaired testicular function because the GI did not show significant difference (p>0.05) between the groups in the present study.

Histology and volumetric proportion of testicular components

Histologically, the testicular parenchyma is normal in all groups evaluated (Figure 2). In testicular microscopy, it is possible to analyze the germ and somatic elements and the similarity in the testicular parenchyma of control and CNSL exposed animals, in relation to the tubular structures: tunica propria, seminiferous epithelium, lumen, and to the intertubular compartment: connective tissue, LC, blood and lymphatic vessels. More specifically, important results were found when analyzing the LC and SC in a quantitative way. LC is arranged in cellular nests, in the interstitial compartment, between connective tissue, blood and lymph vessels. These showed classic morphology with intense cytoplasmatic vacuolations. On the other hand, in the seminiferous epithelium, it is possible to observe the SC with a very evident nucleus and central nucleolus. According to the section plane, it is also possible to identify the two heterochromatin spots, bordering the nucleolus, characteristic of this cell type in its mature phase. It should be noted that it is not possible to observe, under light microscopy, the cytoplasm of SC, since they emit crypts to support the other spermatogenic cells (spermatogonia, spermatocytes and spermatids). Furthermore, the diameter of the seminiferous tubules was similar to the control in all investigated groups (Table 1).

Regarding the volumetric proportion of seminiferous tubules there was a significant decrease in the experimental groups that received the doses of 5 and 50 mg/kg of CNSL compared to the control group. In the proportion of volume of the tunica propria there is a reduction (p<0.5) in all experimental groups compared to the control. However, with regard to the seminiferous epithelium and lumen, there were no changes compared to the control. On the other hand, the volume density of the interstitial compartment increased significantly in the groups of 5 and 50 mg/kg of CNSL. In this compartment, it can also be observed that the volume density of blood vessel, connective tissue and Leydig cells treated at different doses of CNSL did not change (p>0.05) in relation to the control group. In addition, a significant increase was noted for the volumetric density of lymphatic space in the 5 and 50 mg/kg groups.
Number of Sertoli and Leydig cells

All quantitative parameters related to the Sertoli cell are shown in Figure 3. It is possible to observe that both the nuclear volume and the total number of Sertoli cells did not change (p > 0.05) in the groups treated with CNSL. The same trend occurred with the nuclear and cytoplasmic volume of Leydig cells, which were similar (p > 0.05) between those animals exposed to CNSL and the control. In addition, the individual volume of Leydig cells and the total number per testis did not change in CNSL treated animals (Figure 4).

Discussion

Dengue is an epidemic in Brazil and worldwide. Associated with Chikungunya fever and Zika virus, these diseases cause extensive public health problems transmitted by the vector *Aedes aegypti* (Brazil, 2019). It is known that, generally, to combat the vector synthetic pesticides are used and these can act as endocrine disruptors, which can generate genetic instability, leading to an increased predisposition to cancer, as well as changes in the reproductive performance of individuals to whom exposes[22]. Thus, the discovery of products capable of inhibiting the development of the vector and at the same time being less harmful to the environment and human health would have a huge impact. Recent studies have shown that cashew nut liquid is a potent larvicide for *Aedes aegypti* that does not cause embryotoxic teratogenic effects in females [7]. Therefore, the main objective of the present study was to evaluate whether sexually mature male animals exposed to different dosages of CNSL had effects on testicular function and on the spermatogenic process.

The biometric data revealed that exposure to CNSL did not change the body weight of the experimental groups compared to the control except for the animal exposed to the dosage of 5 mg / kg, which showed an increase in weight. Changes in body weight are sufficient evidence to initially classify a compound as systemic toxicity and its reduction may be related to the effects of exposure

| Table 1: Histomorphometric parameters of control animals exposed to CNSL (Mean ± SEM). |
| Parameter             | Group         |
| Tubular Diameter (μm) | Control       | CNSL 5 mg/kg | CNSL 50 mg/kg | CNSL 500 mg/kg | CNSL 1000 mg/kg |
| Tubular Diameter (μm) | 203.8 ± 1.80  | 206.7 ± 2.66  | 206.9 ± 1.35  | 203.5 ± 1.87  | 198 ± 2.08     |
| Volume density (%)    |              |              |              |              |               |
| Seminiferous tubules  | 95.5 ± 0.19   | 94.0 ± 2.03   | 94.4 ± 1.59   | 94.9 ± 0.72   | 94.7 ± 1.69    |
| Tunica propria        | 2.5 ± 0.06    | 2.2 ± 0.07    | 2.0 ± 0.05    | 2.1 ± 0.08    | 2.2 ± 0.12     |
| Seminiferous epithelium | 82.9 ± 0.33  | 80.2 ± 1.14   | 82.0 ± 0.69   | 82.3 ± 0.32   | 82.0 ± 0.77    |
| Lume                  | 10.2 ± 0.69   | 11.6 ± 0.82   | 10.4 ± 0.9     | 10.5 ± 0.31   | 10.6 ± 0.80    |
| Intertubular compartment | 4.4 ± 0.17  | 6.0 ± 0.50    | 5.6 ± 0.51    | 5.2 ± 0.30    | 5.3 ± 0.28     |
| Leydig cell           | 3.3 ± 0.12    | 4.1 ± 0.23    | 3.9 ± 0.33    | 3.6 ± 0.12    | 4.0 ± 0.08     |
| Blood vessel          | 0.4 ± 0.07    | 0.6 ± 0.06    | 0.5 ± 0.07    | 0.5 ± 0.05    | 0.4 ± 0.08     |
| Lymphatic space       | 0.6 ± 0.08    | 1.3 ± 0.18    | 1.1 ± 0.10    | 1.0 ± 0.12    | 0.8 ± 0.07     |
| Connective tissue     | 0.2 ± 0.06    | 0.1 ± 0.03    | 0.1 ± 0.02    | 0.1 ± 0.02    | 0.1 ± 0.01     |

Figure 3 Sertoli cell nuclear volume and total number per testis. Results expressed in (Mean ± SEM). There is no significant difference between control and treated groups. (Test:ANOVA/Student-Newman-Keuls; p > 0.05)
to toxic substances [23]. However, the gonadosomatic index (GI) and testicular weight were similar between all groups studied. It is well established in the literature that the GI, corresponding to the relative size of the gonads as a proportion of the total body size, is widely used as an indicator of the reproductive stage, as it measures sexual maturity in relation to testicular development [24]. It is also known that changes in the absolute and relative weights of the reproductive organs are indicative signs of toxicity risk in the male reproductive system [23], and that sperm production is highly correlated with testicular weight [25-27]. In this way, testicular weight can be used as an indicator of sperm production since SC support the germ cells. The Sertoli cell is the primary determinant of testicular weight and sperm production and conditions in which the proliferation/maturation of these cells are affected result in changes in testicular weight and sperm production [28].

However, in this study, there were no variations in the GI and testicular weights of the treated groups, which suggests the absence of CNSL interference in the testicular function of the mice, thus corroborating that this increase in body weight of the group exposed to 5mg / kg of CNSL comes from the groups not being homogeneous with each other.

According to Lirdi and colleagues [29], apoptosis is a programmed cell death that occurs in tissues in order to guarantee the maintenance of physiological homeostasis. However, toxic chemical agents can cause interruption of testicular homeostasis, causing apoptotic cell death [30]. In histology, the usual appearance of the testis after the administration of any testicular toxicant consists of varying degrees of germ cell degeneration, depletion and disorganization of the germ cell layers in the seminiferous epithelium. This reflects the fact that germ cells are sensitive to any disruption to their support, whether mechanical, biochemical, nutritious or regulatory. Therefore, the action of a toxic agent, regardless of the mechanism of action - acting on SC, vasculature or LC - will be the most affected germ cells, presenting evidence of cell degeneration, death and depletion [31]. However, in our study, the CNSL testicular parenchyma remained similar to the control, with no damage to the germinal epithelium. Corroborating this, the weight of the organ in question was maintained.

In addition, when massive loss of seminiferous epithelial cells occurs, a marked decline in testicular morphometric parameters is observed. The weight of the testicles is also related to the total length of the seminiferous tubules, which consequently depends on the tubular volume, the tubular diameter and the volumetric proportion of the seminiferous tubules [25]. When analyzing the average tubular diameter, it did not vary between the control and experimental groups. According to Auharek and França [32], the diameter of the seminiferous tubules is an excellent indicator to evaluate the spermatogenic activity during the postnatal development of the testis and the degree of SC maturation and fluid secretion that result in the formation of the lumen. Thus, our results, although obtained in sexually mature animals, indicate a correspondence between the results obtained for the lumen of the seminiferous tubules and those found for the nuclear volume of SC, thus following the pattern previously established in the literature.

According to Blanco and collaborators [33], for heavy metals such as cadmium, the intertubular compartment is the region...
most sensitive to changes in the testicles. The animals exposed to the dosages of 5 and 50 mg /kg of CNSL showed a percentage increase in the intertubular compartment due to the increase in the lymphatic space. This could indicate, in a first analysis, the occurrence of lymphatic and/or interstitial edema, which consists of a testicular lesion and a direct consequence of the rupture of the endothelial layer, releasing fluids from the blood flow to the interstitial [29]. However, this hypothesis is not valid in the present study because it only identifies this percentage increase at lower dosages. This result, therefore, can be analyzed as a variant of normality, since important parameters in relation to toxicity, such as GI, testicular weight, tubular diameter and SC and LC histomorphometry did not change in CNSL exposed animals.

Regarding the quantitative parameters related to Sertoli cells, it is possible to notice that the nuclear volume and the number of SC per testicle remained unchanged between the groups. These findings are particularly relevant, because SC is considered the primary determinant of testicular weight and sperm production [28,34]. This is because this cell is the first somatic element to differentiate in the testis in fetal life and is responsible for playing a central role in coordinating the differentiation of other types of testicular cells and in testicular development in general [21,35,36]. In addition, SC start to proliferate in fetal life and this process extends postnatally up to about 2 weeks (in mice) to 3 weeks (rats) after birth [37]. After this period, the number of SC per testicle is considered stable throughout the animal’s life [32,38].

Although the mechanism by which it occurs is not well understood, it is well established in the literature that androgens are involved in the regulation of Sertoli cell proliferation in perinatal life [37,39] being Leydig cells, responsible for the production of testosterone in the testes (Russell et al. 1990). In this sense, the investigation of Leydig cell morphology is relevant. Although hormonal testosterone measurement has not been performed, it is known that the total number of Leydig cells per testis and the nuclear volume of these cells is highly related to testicular and serum testosterone levels [40]. Thus, these results corroborate the hypothesis that the CNSL does not alter the production of endogenous androgens in treated mice.

**Conclusion**

In conclusion, our results provide strong evidence that CNSL is not toxic for testicular function and spermatogenesis. Therefore, the use of CNSL is notoriously attractive, due to the possibility of using different industrial by-products of the nut, in addition to being a renewable resource, of low cost and that does not show signs of testicular toxicity within the parameters analyzed in this work. In view of the above, the absence of toxicity of CNSL, at least in the doses analyzed in this study, supports the view that this compound could be used in humans without causing adverse effects to spermatogenesis.

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