JSM Urology and Research

Research Article

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

Rodrigo Juliano de Oliveira[1], Fernando Oliveira Figueiredo Andrade[2], Ana Carolina Damasceno Cavalcanti[2], Silvia Cordeiro das Neves[1], Dênis Pires de Lima[3], Adilson Beatriz[3], and Sarah Alves Auharek[2]*

[1]Department of Cellular Therapy and Toxicological Genetics (CeTroGen), Maria Aparecida Pedrossian University Hospital (HUMAP), Brazil

[2]Department of Medicine of Mucuri (FAMMUC), Federal University of Vales do Jequitinhonha and Mucuri (UFVJM), Brazil

[3]Department of Chemistry (INQUI), Federal University of Mato Grosso do Sul (UFMS), Brazil

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 CNLS 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 CNLS 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 (CNSL), which constitutes approximately 25% of the total weight of the chestnut and approximately 30% to 35% of the nut peel weight [1,4-6]

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 CNLS 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 CNLS showed no changes in embryo-fetal development, reproductive performance and genetic stability [9]. However, there are no studies in the

Submitted: 29 September 2023 | **Accepted:** 06 November 2023 | **Published:** 08 November 2023

***Corresponding author:** Dr. Sarah Alves Auharek, Federal University of Vales do Jequitinhonha and Mucuri, Faculty of Medicine of Mucuri-FAMMUC, Rua do Cruzeiro, 01, Jardim São Paulo-Teófilo Otoni/MG, Brazil

Citation: de Oliveira RJ, Figueiredo Andrade FO, Damasceno Cavalcanti AC, das Neves SC, de Lima DP, et al. (2023) Effects of the Mixture of Cashew Nut Shell Liquid (CNSL) on the Testicular Function of Sexually Mature Mice. JSM Urol Res 6: 7.

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 properly 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 cut into small fragments, which were exhaustively extracted with ethanol for 6 hours in a Söhxlet 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 paraffin. 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]. A total 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 (um^3) 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 (um^3). 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.

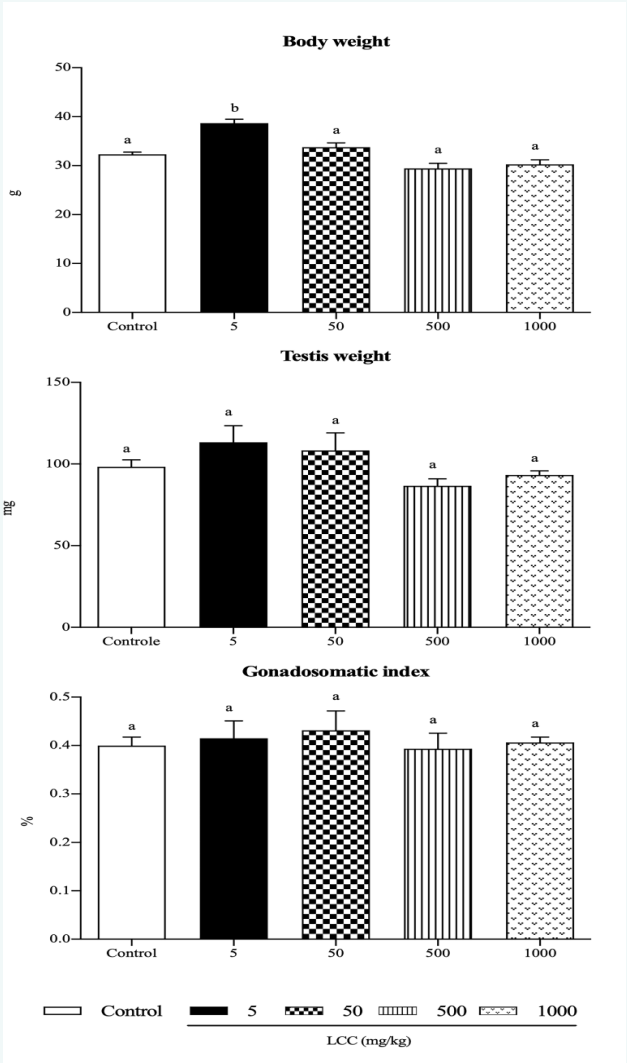

Figure 1 Biometric parameters of control animals and exposed to LCC (Mean ± SEM).

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 cytoplasmic 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.

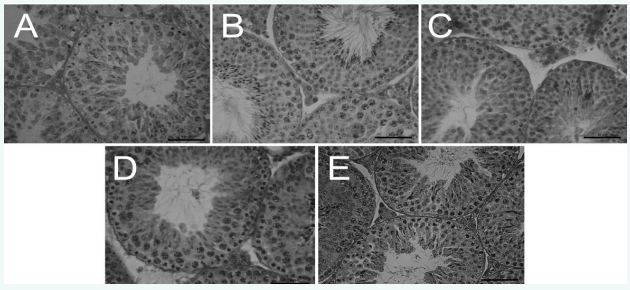

Figure 2 Seminiferous tubule cross sections of mice that received water (a), CNSL 5 mg/kg (b), CNSL 50 mg/kg (c), CNSL 100 mg/ kg (d), CNSL 1000 mg/kg (e). Scale bars represent 50 µm.

Table 1: Histomorphometric parameters of control animals exposed to CNSL (Mean ± SEM).

Parameter	Group				
	Control	CNSL 5 mg/kg	CNSL 50 mg/kg	CNSL 500 mg/kg	CNSL 1000 mg/kg
Tubular Diameter (µm)	203.8 ± 1.80[a]	206.7 ± 2.66[a]	206.9 ± 1.35[a]	203.5 ±1.87 [a]	198 ± 2.08[a]
Volume density (%)					
Seminiferous tubules	95.5 ± 0.19[a]	94.0 ± 2.03[b]	94.4 ± 1.59[b]	94.9 ± 0.72[ab]	94.7 ± 1.69[ab]
Tunica propria	2.5 ± 0.06[a]	2.2 ± 0.07[b]	2.0 ± 0.05[b]	2.1 ± 0.08[b]	2.2 ± 0.12[b]
Seminiferous epithelium	82.9 ± 0.83[a]	80.2 ± 1.14[a]	82.0 ± 0.69 [a]	82.3 ± 0.32 [a]	82.0 ± 0.77 [a]
Lume	10.2 ± 0.69[a]	11.6 ± 0.82[a]	10.4 ± 0.9 [a]	10.5 ± 0.31 [a]	10.6 ± 0.80 [a]
Intertubular compartment	4.4 ± 0.17[a]	6.0 ± 0.50[b]	5.6 ± 0.51[b]	5.2 ± 0.30[ab]	5.3 ± 0.28[ab]
Leydig cell	3.3 ± 0.12[a]	4.1 ± 0.23[a]	3.9 ± 0.33[a]	3.6 ± 0.12[a]	4.0 ± 0.08[a]
Blood vessel	0.4 ± 0.07[a]	0.6 ± 0.06[a]	0.5 ± 0.07 [a]	0.5 ± 0.05 [a]	0.4 ± 0.08 [a]
Lymphatic space	0.6 ± 0.08[a]	1.3 ± 0.18[b]	1.1 ± 0.10[b]	1.0 ± 0.12[a]	0.8 ± 0.07[a]
Connective tissue	0.2 ± 0.06[a]	0.1 ± 0.03[a]	0.1 ± 0.02[a]	0.1 ± 0.02[a]	0.1 ± 0.01[a]

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 aegypyti* (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 them 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 larvicity 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

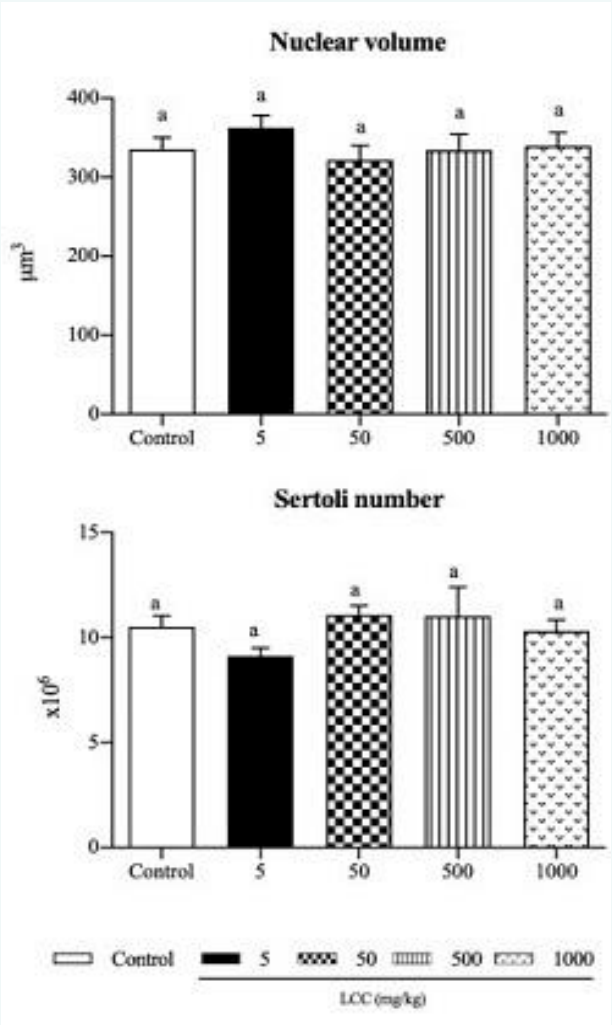

Figure 3 Sertoli cell nuclear volume and total number per testis. Results expressed in (Mean ± *SEM*). There is no significant difference between control and treated groups. (Test:ANOVA/Student–Newman–Keuls.; $p > 0.05$)

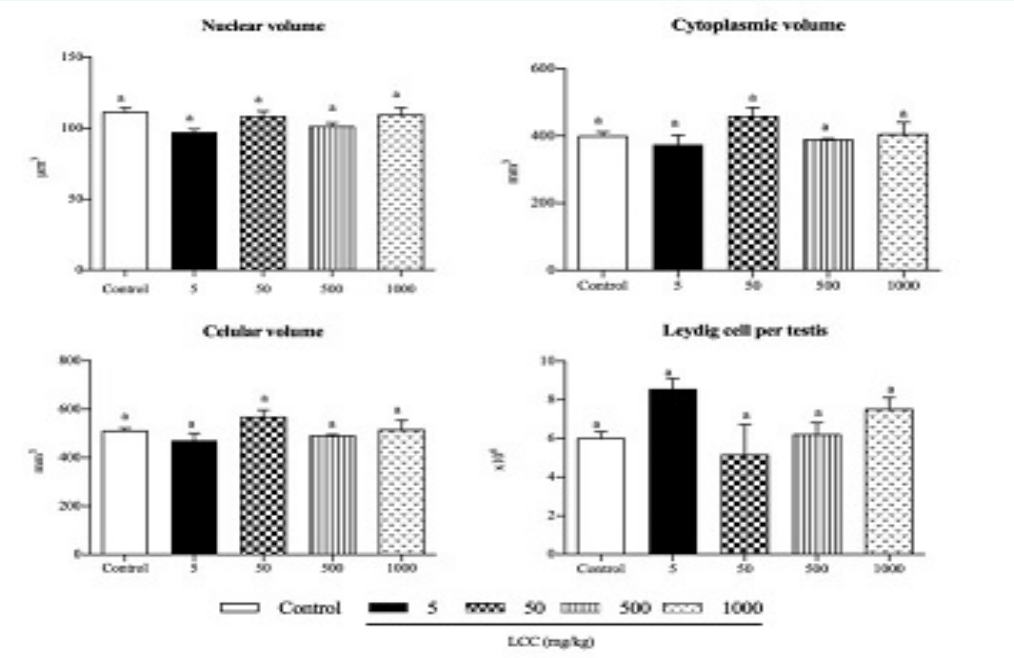

Figure 4 Leydig cells nuclear, cytoplasmic and individual volume and total number per testis. Results expressed in (Mean ± SEM). (Test:ANOVA/ Student–Newman–Keuls.; $p > 0.05$).

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.

References

1. Mazzetto SE, Lomonaco D. Óleo da castanha de caju: oportunidades e desafios no contexto e sustentabilidade industrial. Química Nova. 2009; 32: 732-741.
2. Guissoni ACP, Silva IG, Geris R, Cunha LC, Silva HHG. Atividade larvicida de Anacardium occidentale como alternativa ao controle de Aedes aegypti e sua toxicidade em Rattus norvegicus**.** Revista Brasileira de Plantas Medicinais. 2013; 15: 363-367.
3. Carvalho ALN, Annoni R, Torres LHL, Durão ACCS, Shimada ALB, Almeida FM, et al. Anacardic acids from cashew nuts ameliorate lung damage induced by exposure to diesel exhaust particles in mice. Evid Based Complement Alternat Med. 2013; 1-13.
4. Amorati R, Pedulli GF, Valgimigli L, Attanasi OA, Filippone P, Fiorucci C, Saladino R. Absolute rate constants for the reaction of peroxyl radicals with cardanol derivatives. J Chem Society. Perkin Transactions. 2001; 2: 2142-2146
5. Hamad FB, Mubofu EB. Potential biological applications of bio-based anacardic acids and their derivatives. Int J Molecular Sci. 2015; 16: 8569-8590.
6. Osmari MP, de Matos LF, Salab BL, Diaz TG, Giotto FM. Líquido da casca da castanha de caju: características e aplicabilidades na produção animal. Pubvet. 2015; 9: 143-149.
7. Vani JM, Monreal MTFD, Auharek SA, Cunha-Laura AL, Arruda EJ, Lima AR, et al. The mixture of cashew nut shell liquid and castor oil results in an efficient larvicide against Aedes aegypti that does not alter embryo-fetal development, reproductive performance or DNA integrity. PLoS One. 2018; 13: 1-21.
8. Brasil—Secretaria de Vigilância em Saúde/ Ministério da Saúde. Boletim Epidemiológico. Monitoramento dos casos de dengue, chikungunya e zika até a semana epidemiológica. 2019; 50.
9. Silva ECR. Atividade larvicida de mistura de surfactantes aniônicos do líquido da casca da castanha do caju frente ao mosquito Aedes aegypti. 2014. (Dissertação apresentada ao programa de Pós- Graduação em Farmácia – Nível de Mestrado). Universidade Federal de Mato Grosso do Sul, Campo Grande, Brasil.
10. Torres RC, Garbo AG, Walde RZML. Characterization and bioassay for larvicidal activity of Anacardium occidentale (cashew) shell waste fractions against dengue vector *Aedes aegypti.* Parasitol Res. 2015; 114: 3699-3702.
11. Jerônimo CE, Nascimento LP, Balbino CP. Impacto ambiental derivado das ações de controle e combate a Dengue no Rio Grande do Norte. Revista Monografias Ambientais. 2012; 9: 2021-2030.
12. Bretveld R, Brouwers M, Ebisch I, Roeleveld N. Influence of pesticides on male fertility. Scandinavian J work environment health. 2007; 33: 13-28.
13. Barros AL, Cavalheiro GF, de Souza AVM, Traesel GK, Franci JAA, Kassuya CAL, et al. Subacute Toxicity Assessment of Diflubenzuron, an insect growth regulator, in adult male rats. Wiley Periodicals. Inc. Environ Toxicol. 2014; 31: 407-414.

14. Matos JEX, Silva FJA, Vieira PB. Solventes para extração do líquido da casca da castanha de caju (LCC) e compatibilidade ambiental. Rev Tecnol Fortaleza. 2008; 29: 101-109.

15. OECD. Guidelines for Testing of Chemicals Section 4: Health Effects. Test No. 414: Prenatal Development Toxicity Study. 2001.

16. OECD. Guidelines for Testing of Chemicals. Draft Proposal for an Extended One-Generation Reproductive Toxicity Study. 2009.

17. Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat. 1956; 99:507-516

18. Rasband W, Image J. US, National Institute of Health. Maryland, USA: Bethesda. 2005.

19. Costa DS, Faria FJC, Fernandes CAC, Silva JCB, Auharek SA. Testis morphometry and kinetics of spermatogenesis in the feral pig (Sus scrofa). Animal Reproduction Sci. 2013; 142: 63-70.

20. França LR, Godinho CL. Testis morphometry, seminifer- ous epithelium cycle length, and daily sperm production in domestic cats (Felis catus). Biol Reproduction. 2003; 68: 1554-1561.

21. Auharek SA, Lara NLM, Avelar G F, Sharpe RM, França LR. Effects of inducible nitric oxide synthase (iNOS) deficiency in mice on Sertoli cell proliferation and perinatal testis development. Int J Androl. 2012; 35: 741-751.

22. Mnif W, Hassine AIH, Bouaziz A, Bartegi A, Thomas O, Roig B. Effect of endocrine disruptor pesticides: a review. Int J Environ Res Public Health. 2011; 8: 2265-2303.

23. Clegg ED, Perreault SD, Klinefelter GR, Assessment of male reproductive toxicity. In A. W. Hayes (Ed.), Principles and Methods Toxicol. 2001; 1263-1300.

24. Tamilselvan S, Dhote BS, Singh I, Mrigesh SM, Sathapathy Mahanta, D. Gross morphology of testes and gonadosomatic index (GSI) of guinea fowl (*Numida meleagris*). J Entomology Zoology Studies. 2018; 6: 156-159.

25. Amann RL. The male rabbit. IV. Quantitative testicular histology and comparisons between daily sperm production as determined histologically and daily sperm output. Fertility Sterility. 1970; 21: 662-672.

26. Russell LD; Ettlin RA, Sinha HAP, Clegg ED. Histological and histopathological evaluation of the testis. 1990. Clearwater. FL: Cache River Press.

27. França LR, Russell LD. The testis of domestic animals. In F. Martinez, & J. Regadera (Eds.), Male reproduction: A multidisciplinary overview. 1998;197-219.

28. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction. 2003; 125: 769-784.

29. Lirdi LC, Stumpp T, Sasso-Cerri E, Miraglia SM. Amifostine protective effect on cisplatin - treated rats testis. The Anatomical Record. 2008; 291: 797-808.

30. Stumpp T, Sasso-Cerri E, Freymüller E, Miraglia SM. Apoptosis and testicular alterations in albino rats treated with etoposide during the prepubertal phase. The Anatomical Record Part A. 2004; 279A: 611-622.

31. Crease DM, Chapin RE. Male reproductive system. In: WM Haschek, CG Rousseaux and MA Wallig, eds. Haschek and Rousseaux's handbook of toxicologic pathology. 2003; 2493-2598.

32. Auharek SA, França L. Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL / 6J mice made transiently hypo- and hyperthyroidic during the neonatal period. J Anatomy. 2010; 216: 577–588.

33. Blanco A, Moyano R, Vivo J, Flores-Acuña R, Molina A, Blanco C, et al. Quantitative changes in the testicular structure in mice exposed to low doses of cadmium. Environ Toxicol Pharmacol. 2007; 23: 96-101.

34. Auharek SA. Avelar GF, Lara NLM, Sharpe RM, França LR. Sertoli cell numbers and spermatogenic efficiency are increased in inducible nitric oxide synthase mutant mice. Int J Andrology. 2011; 34: e621-e629.

35. DiNapoli L, Capel B. SRY and the standoff in sex determination. Mol Endocrinol. 2008; 22: 1-9.

36. Rebourcet D, Darbey A, Monteiro A, Soffientini U, Tsai YT, Handel I, et al. Sertoli cell number defines and predicts germ and Leydig cell population sizes in the adult mouse testis. Endocrinology. 2017; 158: 2955-2969.

37. Auharek SA, Franca LR, McKinnell C, Jobling MS, Scott HM, Sharpe RM. Prenatal plus postnatal exposure to Di(n-butyl) phthalate and/ or flutamide markedly reduces final sertoli cell number in the rat. Endocrinology. 2010; 151: 2868-2875.

38. França LR, Avelar GF, Almeida FFL. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology. 2005; 63: 300-318.

39. Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, et al. Role of androgens in fetal testis development and dysgenesis. Endocrinol. 2007; 148: 2027-2036.

40. Castro ACS, Berndtson WE, Cardoso FM. Plasma and testicular testosterone levels, volume density and number of Leydig cells and spermatogenic efficiency of rabbits. Braz J Med Biol Res. 2002; 35: 493-498.