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Cytoprotective effects of L-carnitine on testicular biometry and stereology of albino rats treated with vincristine sulfate in the prepubertal phase

Efeitos citoprotetores de L-carnitine sobre a biometria e estereologia testicular de ratos albinos, tratados com sulfato de vincristine na fase pré-púbere

Efectos citoprotectores de la L-carnitina en la biometría y estereología testicular de ratas albinas tratadas con sulfato de vincristina en la fase prepuberal

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Abstract
This research evaluated the cytoprotective effects of L-carnitine on the testis of albino rats treated with vincristine sulfate in the prepubertal stage. Ninety animals were used, 30 of which were controls, 30 treated with vincristine sulfate, and 30 treated with L-carnitine and vincristine sulfate. Drug applications occurred at 15 days of life, and euthanasia at 40, 64, and 127 days of life, allowing the analysis of the gonads at different stages of development. Biometric measurements (body weight, absolute testicular weight, relative testicular weight, testicular volume, major and minor testicular axes) and stereological measurements (length and volume densities of the seminiferous tubules and interstitial tissue) were performed. The results showed that the preventive use of L-carnitine can reduce the testicular deleterious effects caused by vincristine sulfate, since variables such as absolute testicular weight, relative testicular weight, and testicular minor axis indicated the mitigation of damages resulting from the action of the antineoplastic, mainly in acute and subacute phases.
Keywords: L-carnitine; Vincristine sulfate; Testis.

Resumo

Esta pesquisa avaliou os efeitos citoprotetores de L-carnitine sobre os testículos de ratos albinos tratados com sulfato de vincristine na fase pré-púbere. Foram utilizados 90 animais, sendo 30 animais controles, 30 tratados com sulfato de vincristine e 30 animais tratados com L-carnitine e sulfato de vincristine. As aplicações das drogas ocorreram aos 15 dias de vida, e a eutanásia aos 40, 64 e 127 dias de vida, possibilitando a análise da gônada em diferentes fases de desenvolvimento. Foram realizadas medidas biométricas (peso corpóreo, peso testicular absoluto, peso testicular relativo, volume testicular, eixos testiculares maior e menor) e medidas estereológicas (densidade de comprimento, densidades de volume dos túbulos seminíferos e do tecido intersticial). Os resultados demonstraram que o uso preventivo da L-carnitine pode reduzir os efeitos deletérios testiculares provocados pelo sulfato de vincristine, uma vez que variáveis como peso testicular absoluto, peso testicular relativo e eixo menor testicular apontaram mitigação dos danos decorrentes da ação do antineoplásico, principalmente em fase aguda e subaguda.
Palavras-chave: L-carnitine; Sulfato de vincristina; Testículo.

Resumen

Esta investigación evaluó los efectos citoprotectores de la L-carnitina en los testículos de ratas albinas tratadas con sulfato de vincristina en la fase prepuberal. Se utilizaron 90 animales, 30 de los cuales eran controles, 30 tratados con sulfato de vincristina y 30 animales tratados con L-carnitina y sulfato de vincristina. Las aplicaciones de fármacos ocurrieron a los 15 días de vida, y la eutanasia a los 40, 64 y 127 días de vida, lo que permitió el análisis de las gónadas en diferentes etapas de desarrollo. Se realizaron mediciones biométricas (peso corporal, peso testicular absoluto, peso testicular relativo, volumen testicular, ejes testiculares mayor y menor) y estereológicas (densidad de longitud, densidades de volumen de los túbulos seminíferos y tejido intersticial). Los resultados mostraron que el uso preventivo de L-carnitina puede reducir los efectos deletéreos testiculares causados por el sulfato de vincristina, ya que variables como peso testicular absoluto, peso testicular relativo y eje menor testicular indicaron la mitigación de los daños resultantes de la acción del antineoplásico, principalmente en las fases aguda y subaguda.
Palabras clave: L-carnitina; Sulfato de vincristina; Testículo.

1. Introduction

Vincristine sulfate is a chemotherapy drug widely used in human medicine, effective against various types of neoplasms in pediatric, young, and adult patients. In Veterinary Medicine, it is the drug of choice in the treatment of Transmissible Venereal Tumor in dogs and integrates several chemotherapy protocols against leukemias and lymphomas, however, the non-selectivity by neoplastic cells causes normal tissues and cells in the replication process to be affected by the drug, which is a limiting factor to the therapy (Borges et al., 2021; Groth et al., 2021; Jark et al., 2020; Lima et al., 2021; Morais et al., 2021; Miranda et al., 2021; Oliveira et al., 2021). Several studies have described the deleterious effects of vincristine sulfate on the testes (Daleck et al., 1995; Delbès et al., 2010; Diniz et al., 1999; Hodel et al., 1984; Martins et al., 2011; Vaisheva et al., 2007).

L-carnitine is a derivative of the amino acid lysine, found in mitochondria, contributing to the lipid oxidation process (Gomes et al., 2020). L-carnitine has been identified as a substance with cytoprotective potential for tissues exposed to chemotherapy drugs. Minimizing or neutralizing the harmful effects of chemotherapy on these tissues is crucial for young patients who intend to reproduce and cannot yet conserve gametes due to sexual immaturity. (Okada, Stumpp & Miraglia, 2009; Salehinezhad et al., 2019; Zarbakhsh et al., 2019; Zhu et al., 2015).

This research aims to evaluate the cytoprotective capacity of L-carnitine on the testes of albino rats treated with vincristine sulfate in the prepubertal stage, and euthanized in the prepubertal, pubertal, and adult stages.

2. Methodology

The research was conducted at the Academic Unit of Veterinary Medicine (UAMV) of the Rural Health and Technology Center (CSTR) of the Federal University of Campina Grande (UFCG). The research project was submitted and approved by the Research Ethics Committee from the same Institution, under protocol CEUA/CSTR No. 099/2018.

2.1 Obtaining the animals

Ninety (90) male Wistar rats (Rattus norvegicus albinus) born from matrices from the vivarium of CSTR/UFCG, and treated with vincristine sulfate, were used to evaluate the cytoprotective effects of L-carnitine. Mating took place at night and the following day, vaginal smears were performed to check for the presence of sperm, thus proving the pregnancy. This was considered day 0 of pregnancy.

The animals were weaned at 21 days and kept in cages with 06 (six) ones each, under shavings, and monitored for hygiene and health. The nurturing conditions were standardized and maintained throughout the experiment, with a daylight regime of 12 hours (circadian), under controlled temperature. The animals were fed with commercial ration and water ad libitum.

2.2 Formation of experimental and control groups

After weaning, the animals were divided into three main groups: Group C (GC) (control), with 30 animals; Group V (GV) (treated with vincristine sulfate), with 30 animals; and Group CV (GCV) (treated with L- carnitine and vincristine sulfate) with 30 animals.

The GC group received saline solution intraperitoneally at the same dose used in the GV and GCV groups at 15 days of age. The objective was to simulate the handling conditions of the treated groups by performing the same procedures in all groups. The animals in the GV group received 0.05 mg/kg body weight of vincristine sulfate in a single dose intraperitoneally at 15 days of age (Hodel et al., 1984). The GCV group received 250mg/kg body weight of L-carnitine "inner salt" SIGMA® in a single dose intraperitoneally at 15 days of age, in a process similar to that described by other authors (Okada, Stumpp & Miraglia, 2009). After one hour of observation, these animals received the same dose of vincristine sulfate used in the GV group. The day of drug administration was chosen based on the development of Sertoli cells and the blood-testis barrier since up to the 18th day of life the first is not yet fully mature and the second is still forming (Clegg, 1960; Zarbakhsh et al., 2019).

The GC, GV, and GCV groups were divided into three subgroups of ten animals each, according to the ages at which they were euthanized: 40 days of life (subgroups GC40, GV40, and GCV40); 64 days of life (subgroups GC64, GV64, and GCV64) and 127 days of life (subgroups GC127, GV127, and GCV127). The age-matched experimental groups were compared. The ages were chosen based on the developmental stages of the male reproductive system of the species (Clegg, 1960), since animals at 40 days of life are considered prepubertal, at 64 days they are pubertal but not sexually mature, and at 127 days they are sexually mature. At the specified ages, they were anesthetized with Sodium Thiopental (89 mg/kg of body weight) (Brilhante et al., 2012) and euthanized followed by removal of both testes for further stereological evaluation.

2.3 Sample collection and processing

At the specified ages, the rats were weighed and euthanized. The testes were removed and prefixed by immersion in Bouin's solution. After 30 minutes, they were sectioned and the sections were returned to Bouin's solution, for 24 hours. After fixation, the samples were washed and preserved in 70% alcohol, then cut, dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in paraffin (Michalany, 1960). 5µm sections of each testicle (right/left) were obtained using the manual microtome LEICA RM2125 RT and disposable razors Easy Path DURAEDGE. The samples were stained with Hematoxylin and Eosin (HE), enabling the performance of stereological and testicular parenchyma evaluation.

2.4 Biometrics and Stereology

Biometrics included body weight at the time of euthanasia. Testicle weight was also measured, after removal, on a BG 1000 GEHAKA® semi-analytical precision balance. The relative testicular weight was calculated according to the formula

(testicular weight/100g of live weight) (Brilhante, Stumpp & Miraglia, 2011). The major and minor axes were measured using a MITUTOYO caliper. Testicular volume was measured using Scherle's method (Mandarim-de-Lacerda, 1995; Scherle, 1970). The total testicular volume was obtained from the following formula:

VOLUME=Density X Mass

The average of the total testicular volume of each group was obtained by calculating the weighted averages of the volumes of the two testes of each animal in their respective control and experimental groups.

Stereological analysis was performed on sections of the left and right testes, the sections were subjected to analysis of tubular volume density (Vv) and the testicular interstitial tissue. The stereological variables of volume density (Vv) were obtained by counting the points by systematic and random allocation of the images obtained through the IMAGE-PRO EXPRESS 6.0 software in a computer coupled to a binocular light microscope OLYMPUS BX40 at 20x magnification and later used in the IMAGEJ software to count the points, totaling 840 points per testis for the tissue volume density, and 3190 points for the volume density of Sertoli cells and spermatogonia (Mandarim-de-Lacerda, 1995; Gundersen et al., 1988).

The volume density of interstitial tissues and seminiferous tubules in mm^3 was calculated from the percentage obtained in the volume density (Vv) of each tissue, the volume density of Sertoli cells and spermatogonia was also measured using the same methodology (Miraglia & Hayashi, 1993). The mean tubular and interstitial volumes per animal were also obtained by calculating the data obtained for the total volume of the right and left testes.

The length density of the seminiferous tubules (Lv) was obtained using the formula Lv=2Qa, with Qa being the sum of the existing tubular sections in the test area recorded on a 1mm scale and metric system intervals of 10µm. This way, Qa=∑perfis/At (Gundersen et al., 1988).

2.5 Statistical analysis

Data were evaluated by the nonparametric Kruskal-Wallis ANOVA test, which is applied to paired comparisons between treated groups and their respective controls. Results were considered significant when $p \leq 0.05$. Statistical analysis was performed using the Bioestat 5.0 software.

The results of the evaluated variables were obtained by calculating the weighted average of the individual data of each testis (right and left), of each animal, and, consequently, of each group (40, 64, and 127 days). The results were arranged in tables containing three groups treated with vincristine sulfate (GV), three groups treated with L-carnitine and vincristine sulfate (GCV), and their respective controls (GC). The research covered biometric and stereological assessments.

3. Results

The body weights (Table 1) of the animals in the GCV127 group did not differ statistically ($p>0.05$) when compared to the control group GC127 and the treated group GV127. In groups GC64, GV64 and GCV64, no differences were observed ($p>0.05$). However, the GC40 group differed from the GV40 and GCV40 groups. When comparing the GC40 and GV40 groups, $p=0.0023$; when comparing the GC40 and GCV40 groups, $p=0.0096$.

Table 1: Bodyweight (BW), absolute testicular weight (ATW), relative testicular weight (RTW), total testicular volume (TTV), testicular major axis (TMAA), and testicular minor axis (TMIA) in groups of albino control rats (GC), treated with vincristine sulfate (GV) and treated with L-carnitine and vincristine sulfate (GCV) at 40, 64 and 127 days of age (GC40, GV40, GCV40, GC64, GV64, GCV64, GC127, GV127, and GCV127).

	GROUPS					
	BW (g)	**ATW (g)**	**RTW (%)**	**TTV (mm^3)**	**TMAA (mm)**	**TMIA (mm)**
GC127	308,7a±27,2	1,8a*±0,06	0,59a*±0,04	1657,16a*±88,6	17,02a*±0,3	7,73a*±0,4
GV127	283,0a±39,8	0,9b*±0,18	0,34b*±0,05	1081,11b*±323	12,55b*±1,1	5,46b*±0,6
GCV127	277,4a±40,3	1,2b*±0,2	0,44b*±0,08	1172,17b*±197	13,17b*±2,5	6,52b±0,9
GC64	228,2a±17	1,6a*±0,04	0,71a*±0,05	1450,12a±131	16,88a*±0,4	6,79a*±0,3
GV64	225,0a±23,2	1,2b*±0,06	0,56b*±0,08	1293,01b±109	13,43b*±0,8	5,55b*±0,5
GCV64	226,7a±45,6	1,3b*±0,1	0,60b±0,11	1297,09a±211	13,56b*±2,4	6,07b±0,7
GC40	136,4a*±9,1	0,6a*±0,03	0,51a*±0,03	600,16a±69	16,88a*±0,4	4,41a*±0,3
GV40	116,6b*±6,2	0,4b*±0,02	0,42b*±0,04	517,11b±58	6,43b*±0,7	2,28b*±0,4
GCV40	114,2b*±25,6	0,5a±0,3	0,45a±0,02	527,17a±288	9,49b*±4,6	3,27c±1,1

Different letters: Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0,05$). Different letters with "*": Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0,01$). Equal letters: Means did not differ from each other by the Kruskal-Wallis ANOVA test ($p > 0,05$). Source: Elaborated by the authors.

Absolute and relative testicular weights tended to progressively decrease. It is worth noting that in all experimental groups (GV40, GV64 and GV127) treated only with vincristine sulfate there were significant reductions ($p \leq 0.01$) in absolute testicular weight. Relative testicular weight also decreased ($p \leq 0.05$) in all chemotherapy-treated groups.

The total testicular volumes followed the trend of reduction observed in the other biometric variables, however, such reduction was only consolidated in the group treated at 127 days (G127). The animals in the GV127 and GCV127 groups showed significant reductions ($p \leq 0.01$) compared to those in the GC127 group, showing that the cytotoxic effects of the chemotherapy affect the testis volume in a delayed manner.

Animals in groups GC40, GV40, GCV40, GC64, GV64, and GCV64 did not show significant reductions ($p > 0.05$) in gonad volume. At the same time, there was no difference ($p > 0.05$) between the GV127 and GCV127 groups, proving that the use of L-carnitine was not effective in preserving the total testicular volume of rats treated with vincristine sulfate.

The major and minor testicular axes decreased in the groups treated with vincristine sulfate (major axis $p=0.0001$, minor axis $p=0.0001$) and in those treated with vincristine sulfate and L-carnitine (major axis $p= 0.0004$, minor axis $p=0.0448$) when compared to their respective controls.

The smaller testicular axis showed a reduction trend in all treated groups, but in the GCV40 there was mitigation ($p=0.0308$) in this trend concerning the GV40, this result is a strong indication of the cytoprotective effect of L-carnitine in the first days after application.

It was found that the volume densities of the seminiferous tubules (Table 2) of G64 and G40 had slight oscillations when comparing the experimental groups with their relative controls, however, such variations did not differ significantly ($p > 0,05$).

Table 2: Seminiferous tubule volume density (DVT), interstitial tissue volume density (IVD), seminiferous tubule volume (VT), interstitial tissue volume (VI) in groups of control albino rats (GC), treated with vincristine sulfate (GV) and treated with L-carnitine and vincristine sulfate (GCV) at 40, 64 and 127 days of age (GC40, GV40, GCV40, GC64, GV64, GCV64, GC127, GV127, and GCV127).

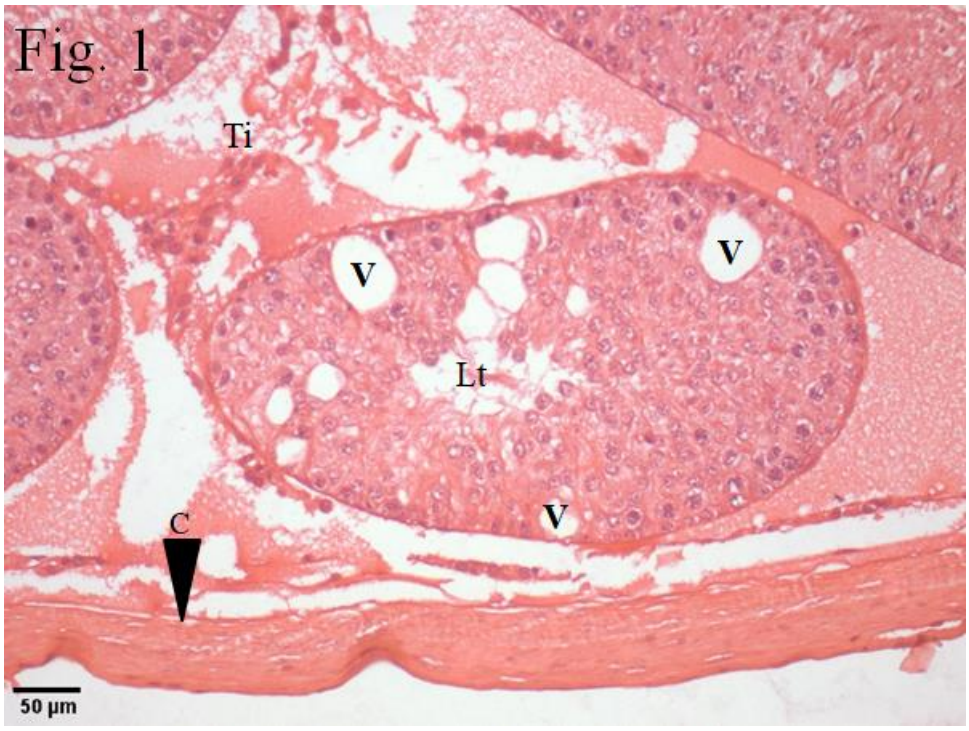
	GROUPS			
	TVD (%)	**IVD (%)**	**TV (mm^3)**	**IV (mm^3)**
GC127	71,42a*±11,7	28,57a*±11,7	1185,55a*±219,3	471,6a±191
GV127	37,42b*±5,6	62,57b*±5,6	407,13b*±141,1	673,97b±206
GCV-127	43,42b*±3,7	59,57b*±3,7	521,16b*±70,3	687,85b±142
GC64	73,42a±9,4	26,57a±9,4	1058,81a*±119,7	391,31a±148
GV64	69,14a±8	30,85a±8	891,43b*±105,7	401,57a±119
GCV-64	69,71a±4,7	30,28a±4,7	907,23b±176,6	389,85a±68
GC40	77,42a±12,5	22,57a±12,5	462,72a*±77,2	137,44a±75
GV40	72,57a±7,9	27,42a±7,9	375,1b*±58,7	142a±44
GCV-40	72,28a±5	27,71a±5	385,75a±217,3	141,4a±81

Different letters: Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0,05$). Different letters with "*": Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0,01$). Equal letters: Means did not differ from each other by the Kruskal-Wallis ANOVA test ($p > 0,05$). Source: Elaborated by the author.

The comparison between the GC127 and GV127 (p=0.0001), and between the GC127 and GCV127 (p=0.0022), demonstrates the deleterious effects of vincristine sulfate on the volume density of the seminiferous tubules were delayed and significantly affected adult animals (127 days). In contrast, there were no differences between GV127 and GCV127.

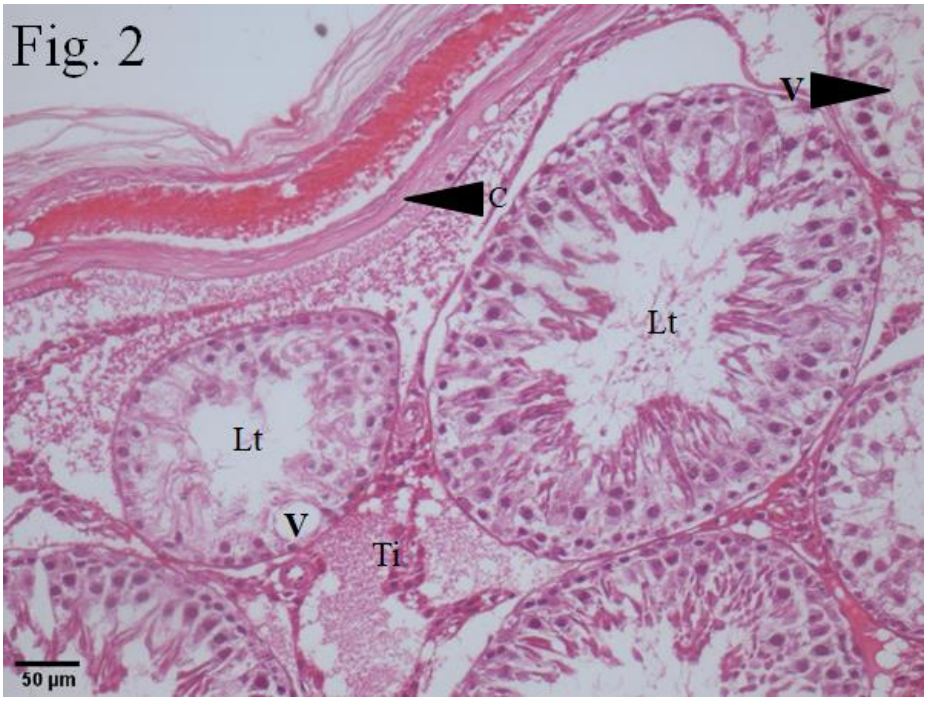
However, the GCV127 (Figure 1) presented statistically different means of volume density of the seminiferous tubules concerning the GV127 (Figure 2) and the GC127 (Figure 3). Likely, the cytoprotective effect of L-carnitine does not extend into the adult life of the animals.

Figure 1: Photomicrograph of tubular sections of testes from albino rats treated with L-carnitine and vincristine sulfate and sacrificed at 127 days of life (GCV_{127}). HE staining, 20x magnification. An increase in vacuolization of the seminiferous epithelium is observed. Testicular capsule (C); interstitial tissue (Ti); tubular lumen (Lt); vacuolization (V).



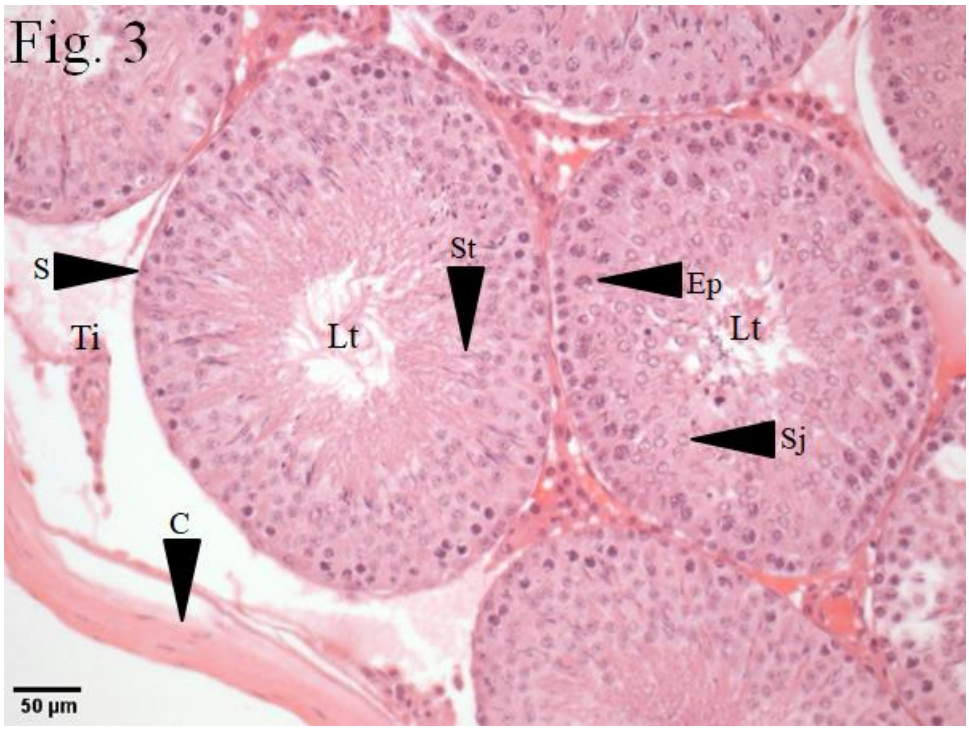
Source: Elaborated by the authors.

Figure 2: Photomicrograph of tubular sections of testes from albino rats treated with vincristine sulfate and sacrificed at 127 days of life (GV127). HE staining, 20x magnification. Severe degeneration of the seminiferous tubules, disorganization, and vacuolization of the seminiferous epithelium are evidenced. Testicular capsule (C); interstitial tissue (Ti); tubular lumen (Lt); vacuolization (V).



Source: Elaborated by the authors.

Figure 3: Photomicrograph of tubular sections of the testes of albino rats from the control group and sacrificed at 127 days of life (GC127). HE staining, 20x magnification. Testicular capsule (C); interstitial tissue (Ti); Sertoli cell nucleus (S); tubular lumen (Lt); primary spermatocyte (Ep); young spermatid (Sj); late spermatid (St).

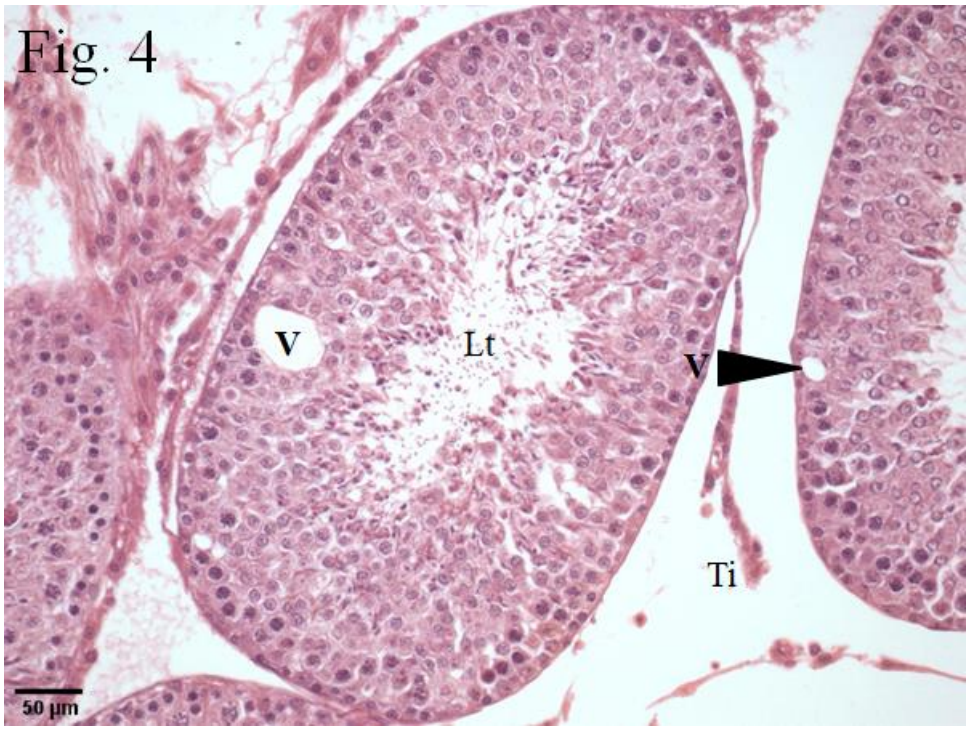


Source: Elaborated by the authors.

The volume densities of the interstitial tissue followed the trends observed in the volume density of the seminiferous tubules, however, in an inverse way, given the proportionality relationship between the two variables. Thus, the GV127 and GCV127 demonstrated a significant increase (p=0.0001) in the volume density of the testicular interstitial tissue when compared to their respective control (GC127).

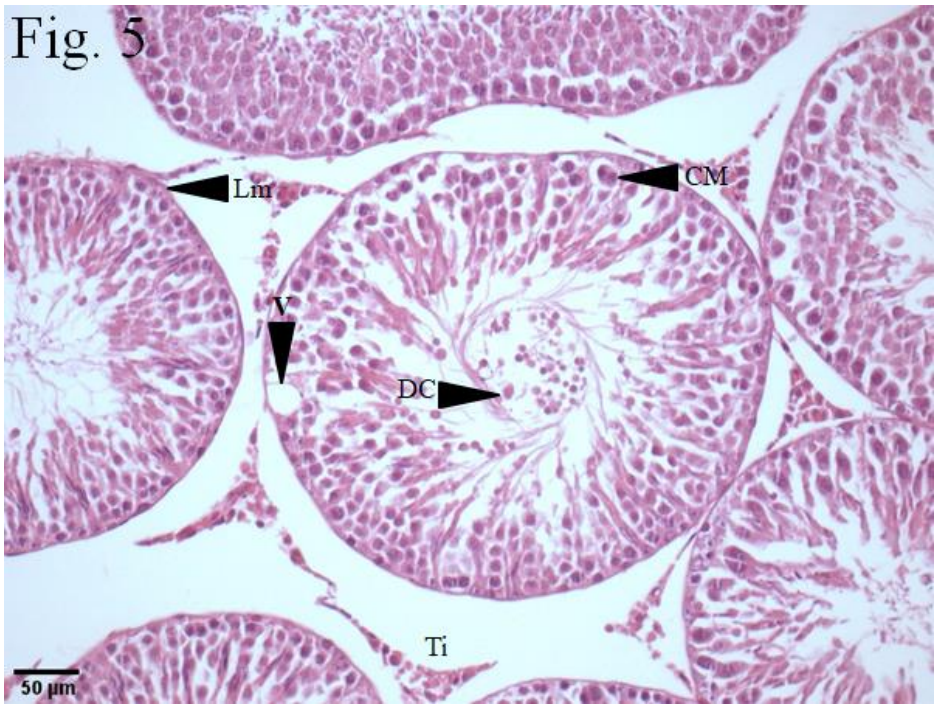
The volume of the seminiferous tubules showed a tendency to reduce the averages, similar to that observed in the total testicular volume, showing significant differences ($p \leq 0.05$) in the experimental groups GCV64 (Figure 4), GV64 (Figure 5), GC64 (Figure 6), GCV127, GV127, and GC127 when compared the treated ones with their respective controls.

Figure 4: Photomicrograph of tubular sections of testes from albino rats treated with L-carnitine and vincristine sulfate and sacrificed at 64 days of age (GCV64). HE staining, 20x magnification. Vacuolization is observed, however, to a lesser extent. Interstitial tissue (Ti); tubular lumen (Lt); vacuolization (V).



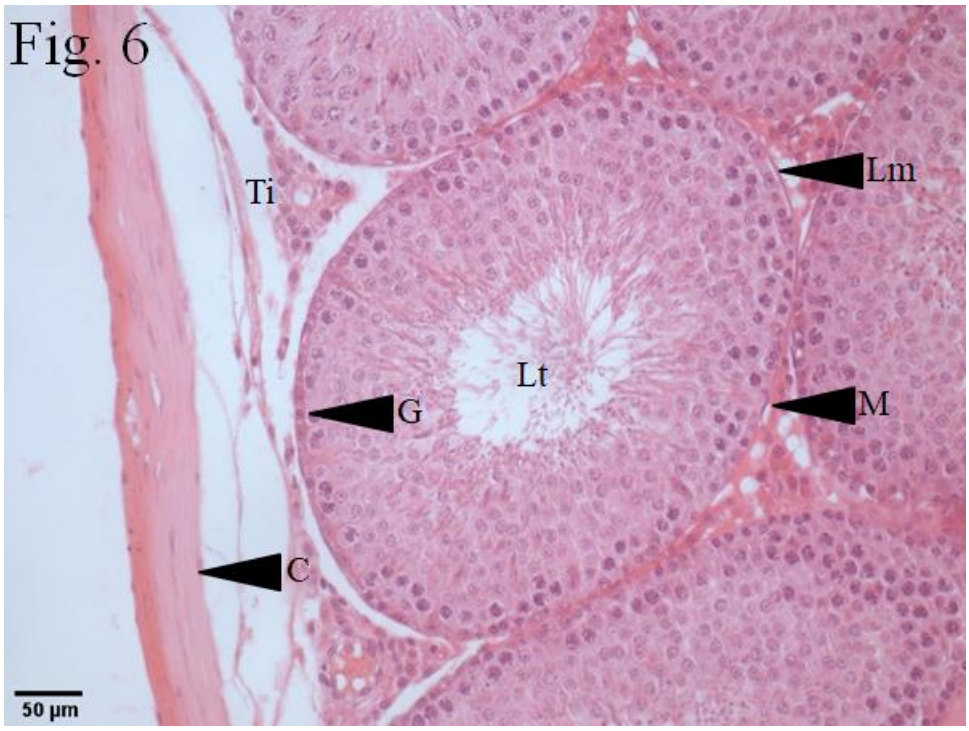
Source: Elaborated by the authors.

Figure 5: Photomicrograph of tubular sections of testes from albino rats treated with vincristine sulfate and sacrificed at 64 days of age (GV64). HE staining, 20x magnification. Cell debris from the desquamation of the seminiferous epithelium is observed. Interstitial tissue (Ti); limiting lamina (Lm); multinucleated cell (CM); vacuolization (V); cell debris from the desquamation of the seminiferous epithelium (DC).



Source: Elaborated by the authors.

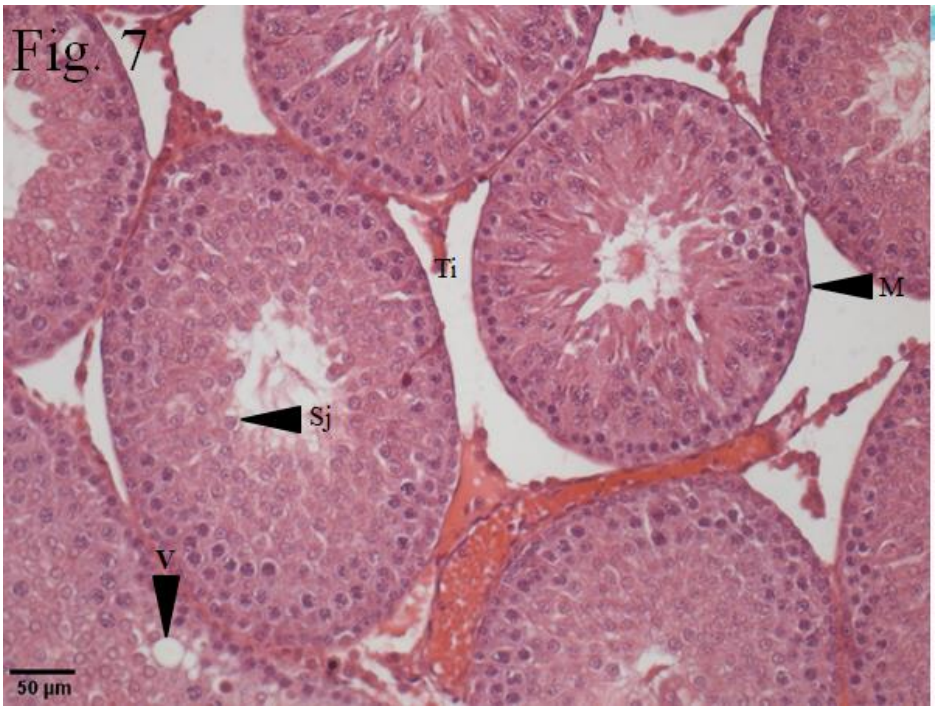
Figure 6: Photomicrograph of tubular sections of the testes of albino rats from the control group and sacrificed at 64 days of life (GC64). HE staining, 20x magnification. Testicular capsule (C); interstitial tissue (Ti); spermatogonia (G); tubular lumen (Lt); myoid cell (M); limiting lamina (Lm).



Source: Elaborated by the authors.

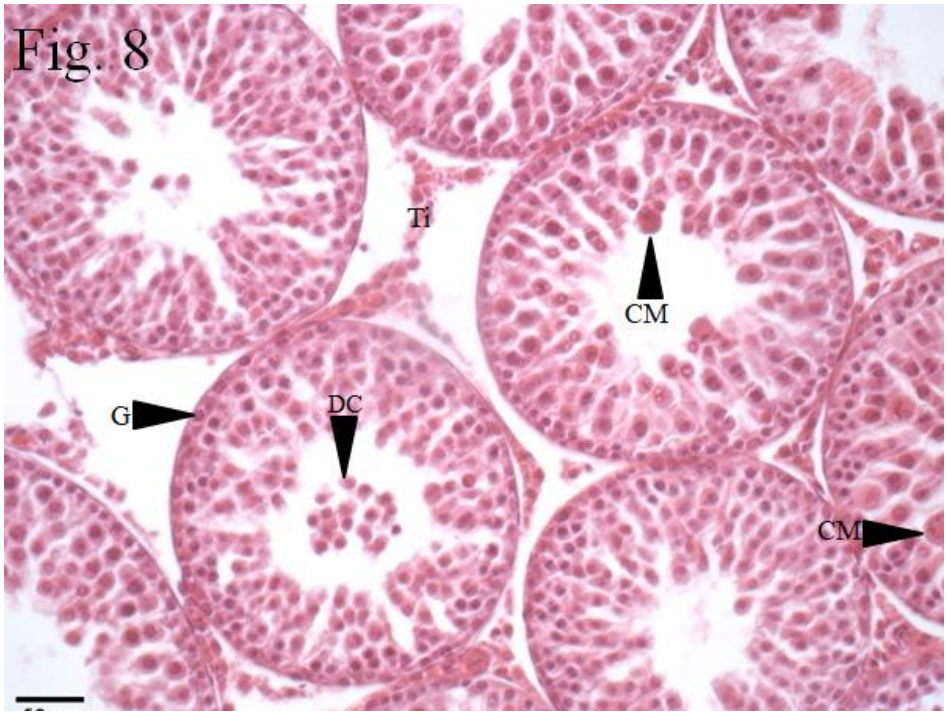
Testicular interstitial tissue volume showed non-significant variations ($p>0.05$) in groups GCV64, GV64, GC64, GCV40 (Figure 7), GV40 (Figure 8), and GC40 (Figure 9); when compared the treated groups and respective controls. However, the GV127 and GCV127 showed a significant increase ($p\leq0.05$) in the volume of testicular interstitial tissue when compared to the control group of the same age (GC127).

Figure 7: Photomicrograph of tubular sections of testes from albino rats treated with L-carnitine and vincristine sulfate and sacrificed at 40 days of age (GCV40). HE staining, 20x magnification. There is a reduction in the deleterious effects on the seminiferous epithelium. Interstitial tissue (Ti); myoid cell (M); young spermatid (Sj); vacuolization (V).



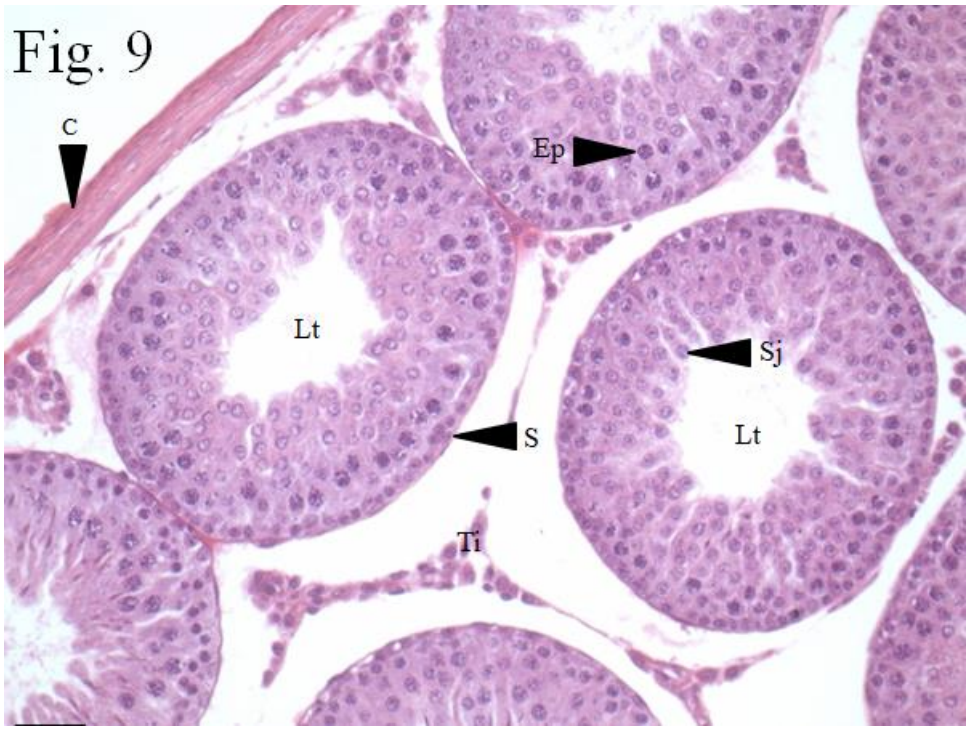
Source: Elaborated by the authors.

Figure 8: Photomicrograph of tubular sections of testes from albino rats treated with vincristine sulfate and sacrificed at 40 days of life (GV40). HE staining, 20x magnification. Deleterious effects are observed on the seminiferous epithelium. Interstitial tissue (Ti); spermatogonia (G); multinucleated cell (CM); cell debris from the desquamation of the seminiferous epithelium (DC).



Source: Elaborated by the authors.

Figure 9: Photomicrograph of tubular sections of the testes of albino rats from the control group and sacrificed at 40 days of life (GC40). HE staining, 20x magnification. Testicular capsule (C); tubular lumen (Lt); interstitial tissue (Ti); primary spermatocyte (Ep); Sertoli cell nucleus (S); young spermatid (Sj).



Source: Elaborated by the authors.

The volume densities of Sertoli cells (Table 3) and spermatogonia showed a reduction when comparing the control and treated groups with 127 days of life.

Table 3: Sertoli cell volume density (SDV), Sertoli cell volume (SCV), spermatogonia volume density (SVD), spermatogonia volume (SV), and length density (LD) in groups of albino control rats (GC), treated with vincristine sulfate (GV) and treated with L-carnitine and vincristine sulfate (GCV) at 40, 64 and 127 days of age (GC40, GV40, GCV40, GC64, GV64, GCV64, GC127, GV127, and GCV127).

	GROUPS				
	SDV (%)	**SCV (mm^3)**	**SVD (%)**	**SV (mm^3)**	**LD (μm^2)**
GC127	1,77a*±0,2	29,41a*±5,2	1,37a*±0,1	22,93a*±3,8	28,34a*±2,2
GV127	1,03b*±0,1	11,33b*±4	0,86b*±0,1	9,25b*±3,1	34,31b*±1,7
GCV-127	1,55a*±0,1	18,79c*±3,9	0,76b*±0,1	9,26b*±2,1	32,7b*±2,6
GC64	0,92a±0,1	13,37a±1,8	0,94a±0,1	13,70a±2,5	20,74a±2,2
GV64	0,95a±0,1	12,34a±1,9	0,92a±0,1	11,93a±1,6	23,67b±2,7
GCV-64	0,90a±0,1	11,85a±3,3	0,95a±0,1	12,39a±2,4	23,04b±1,7
GC40	1,64a±0,1	9,90a±1,7	0,75a±0,1	4,55a±1,1	37,88a*±2,9
GV40	1,58a±0,1	8,19a±1,2	0,73a±0,1	3,82a±0,9	43,69b*±3
GCV-40	1,75a±0,1	9,36a±5,2	0,72a±0,1	3,77a±2,2	43,73b*±2,3

Different letters: Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0.05$). Different letters with "*": Means differed from each other by the Kruskal-Wallis ANOVA test ($p \leq 0.01$). Equal letters: Means did not differ from each other by the Kruskal-Wallis ANOVA test ($p > 0.05$). Source: Elaborated by the authors.

The volume density of Sertoli cells was different when comparing GC127 and GV127 (p=0.0001) and GCV127 and GV127 (p=0.0016), however, there was no difference when comparing GC127 and GCV127. The volume density of spermatogonia showed a reduction when comparing GC127 and GV127 (p=0.0007), and GC127 and GCV127 (p=0.0001), but there was no difference when comparing GV127 and GCV127 (p=0.44). The experimental groups of 64 and 40 days did not show differences in the volume density of Sertoli cells and spermatogonia.

The volumes of Sertoli cells and spermatogonia followed the trend of reduction observed in the volume density of these cell types. The volume of Sertoli cells showed differences between the three experimental groups of 127 days of life when compared to each other, including when GC127 and GCV127 were compared (p=0.0119). There were also differences when comparing the spermatogonia volume of GC127 and GV127 (p=0.0002), and GC127 and GCV127 (p=0.0001), respectively. However, there were no differences between GV127 and GCV127 in this variable (p=0.91).

As for length density, there were no differences between the experimental groups of 40 (G40) and 64 (G64) days of life, respectively. Groups GV127 and GCV127 differed from the age-matched control group, and this difference was significant (*p*=0,0001).

4. Discussion

The body weight oscillations of the treated animals in relation to their respective controls, in this experiment, are similar to the data presented in other studies (Daleck et al., 1995; Delbès et al., 2010; Diniz et al., 1999; Hodel et al., 1984; Martins et al., 2011; Vaisheva et al., 2007).

It is known that vincristine sulfate, as well as other chemotherapeutic agents, produce deleterious effects on the intestinal epithelium and glands, indirectly interfering with the digestive process and reducing the ability to absorb nutrients, and consequently weight gain. Similar data were reported by other authors (Moura et al., 2006; Peixoto Júnior et al., 2009; Russell & França, 1995; Vendramini et al., 2010).

The body weights of treated and euthanized animals at 40 days of life (GV40 and GCV40) showed significant differences ($p \leq 0.01$) when compared to the age-matched control group (GC40). However, these same groups (GV40 and GCV40), when compared to each other, did not differ in relation to body weights. These results confirm that the previous use of L-Carnitine does not prevent the occurrence of adverse effects of vincristine on the bodyweight variable, that is, the cytoprotective drug was not able, in the short term, to prevent or mitigate the deleterious action of the chemotherapeutic on the digestive mucosa, a fact expressed by the maintenance of body weight reduction in the animals of the GCV40. This fact has been reported in studies conducted with other chemotherapeutic agents in association with L-carnitine, which showed similar characteristics regarding the bodyweight variable (Cabral et al., 2014; Cao et al., 2017; Coşkun et al., 2013; Dehghani et al., 2013; Okada et al., 2009; Yaman & Topcu-Tarladacalisir, 2018).

On the other hand, the non-significant oscillations observed in the groups of 64 and 127 days suggest that the increase in the time between the application of chemotherapy and the age of euthanasia allowed the recovery of the digestive tract. The regenerative capacity of intestinal gland cells and intestinal epithelium has already been described (Dekaney et al., 2009), and this characteristic makes it possible to recover the body weight lost in the acute phase of treatment with vincristine sulfate. Reductions in the bodyweight of animals treated with chemotherapy have been reported as common changes not only with the use of vincristine sulfate but with other anticancer drugs (Brilhante et al., 2011; Coşkun et al., 2013; Dehghani et al., 2013; Hodel et al., 1984; Okada et al., 2009; Vasiliausha et al., 2016; Vendramini et al., 2010).

Testicular weight is considered directly proportional to sperm production, however, there is no proportionality between testicular weight and body weight, for this reason, in this study, there are no correlations between body weight and testicular weight, evidenced by the difference in the relative weight of the organ. The testicular size directly interferes with the

reproductive behavior of the animal, individuals with larger testicular size have greater sperm production and, consequently, tend to have greater reproductive activity, a correlation based on the gonadosomatic index (Amann & Schanbacher, 1983; França & Russell, 1998; Kenagy & Trombulak, 1986; Olar et al., 1983).

The reduction in testicular weight, as well as the other biometric variables studied, has a dose-dependent relationship in most chemotherapy drugs, the reductions observed in this research are in agreement with other studies (Okada et al.,2009; Vendramini et al., 2010). Furthermore, the decrease in testicular weight is considered an important indication of loss of seminiferous epithelial cells (Bordallo et al., 2001; Hacker-Klom et al., 1986; Howell & Shalet, 2001), although changes in the interstitium should also be considered.

In studies where a reduction in total testicular volume is observed, the need to discriminate the volumes of the seminiferous tubules and interstitial tissue becomes evident so that it is possible to determine which of the members of the testicular tissue was altered, or if both were affected, determine the severity and extent of changes.

Animals treated with L-carnitine and vincristine sulfate, and euthanized at 40 days of age, showed a slowing of the reduction in absolute and relative testicular weights, demonstrating that there were cytoprotective effects that reduced the harmful effects of chemotherapy on the male gonad. These results corroborate other studies conducted with the cytoprotective agent associated with other chemotherapy drugs, which suggests that the cytoprotective activity of L-carnitine is independent of the pharmacological characteristics of the chemotherapy agents used. However, due to the diversity of chemical interactions that can occur between drugs of different classes, more research is needed regarding the mechanism of action of L-carnitine (Okada et al., 2009; Zhu et al., 2015; Dehghani et al., 2013).

Research that compared the cytoprotective effects of L-carnitine with the protection provided by Homogenized Testis Tissue (THT), in the testes of busulfan-treated rats, found that THT is more effective in conserving variables such as testicular volume and weight, tubular and interstitial tissue volume, and sperm count than L-carnitine-treated ones (Dehghani et al., 2013).

The reduction in testicular weight of animals treated with the drug suggests the occurrence of persistent harmful effects on the gonads, which are expressed cumulatively and are evidenced by the inability to recompose testicular weight in the long term.

Rats treated with x-rays may demonstrate significant decreases in seminiferous tubule and interstitial tissue volumes with consequent gonad atrophy, depending on the ages at which exposure occurred (Cabral et al., 1997). However, in this study, a reduction in total testicular volume was observed without necessarily reducing interstitial tissue components.

Reductions in total testicular volume and major and minor axes of testes in animals treated with antineoplastic agents, such as vincristine sulfate, corroborate the findings by other authors and reinforce that their gonads were severely damaged by the cytotoxic action of the chemotherapeutic agent (Brilhante et al., 2011).

Decreased testicular volume is a strong indication of damage to the seminiferous epithelium since much of the testicular tissue is formed by the seminiferous tubules, which accommodate the germinal epithelium. However, the absence of variations in testicular volume does not mean that there are no harmful effects on the germ cells, since the total testicular volume is composed of the sum of the volume of the seminiferous tubules and the volume of the testicular interstitial tissue, and these two tissue components are subject to variations for more and less (Amann & Schanbacher, 1983).

The volume densities of the seminiferous tubules and interstitial tissue are percentage variables of the measurements of the seminiferous tubules, structures, cells and interstitial matrix blood vessels, lymphatics, innervation, and interstitial spaces of the male gonads. In this way, they are measures that represent, in a proportional way, the amount of seminiferous tubules and interstitial tissue existing in the organ.

L-carnitine was ineffective in preserving the volume density of the seminiferous tubules of rats treated with

vincristine sulfate and euthanized in the adult phase. Research on the effects of L-carnitine on the testes of rats treated with etoposide reported a significant increase in the number of germ cells undergoing apoptosis (Okada et al., 2009). The loss of cells from the seminiferous tubules of testes treated with vincristine sulfate still needs to be further investigated. Although the reduction in the volume density of the seminiferous tubules is a strong indication of a reduction in the population of testicular parenchyma cells, especially germline cells, as they are in greater quantity.

In this study, the reduction in the volume densities of the seminiferous tubules in rats treated with L-carnitine and vincristine sulfate, as well as in the one treated with vincristine sulfate alone, may have occurred due to the death of germ cells, leading to the belief that the L-carnitine was not efficient in protecting these cells, and that this fact had an indirect impact on the volume density of the seminiferous tubules. Damage to Sertoli cells can also indirectly result in a reduction in the population of germline cells due to disruption of the seminiferous epithelium. The effects of vincristine sulfate on Sertoli cells have already been described (Salehinezhad et al., 2019).

Studies focused on the dynamics of the seminiferous epithelium cycle reveal that spermatogonia surviving the acute effects of chemotherapy would need 56 days to produce the first generation of spermatids after exposure to the drug (Cabral et al., 1997; Clermont & Bustos-Obregon, 1968). Thus, it becomes evident that some of the harmful effects of the antineoplastic on the seminiferous tubules (including volume density reduction) could not be observed in the 40-day experimental group (GV40). Regarding GV64, it is possible that the action of the chemotherapeutic agent results in a delay in the spermatogenic process, making the deleterious effects late (Clermont & Hermo, 1975; Hodel et al., 1984).

The stereological results revealed an increase in the volume and volume density of the interstitial tissue, which may have occurred due to the increase in the dispersion of the seminiferous tubules of the treated animals (GV127 and GCV127). The enlargement of testicular lymphatic structures is an effect commonly described in animals undergoing chemotherapy (Madhu et al., 2016; Nikpour et al., 2018; Rosental, 1981; Sherif et al., 2018).

The reduction in the volume densities of Sertoli and spermatogonia cells associated with reductions in their volume are strong indicators of a decrease in the cell population within the seminiferous tubules.

There was the preservation of Sertoli cell volume density in the animals of the GCV127, indicating a cytoprotective effect of L-carnitine on this cell type, however, the same effect was not observed in the volume density of spermatogonia. The reduction in the volume of Sertoli cells is a sign of probable irreversible structural damage to the seminiferous tubules, since there is no replacement of the lost population of this cell type, making its preservation crucial (Salehinezhad et al., 2019).

Length density is considered by some authors as the ideal parameter in the evaluation of tubular structures with rectilinear distribution (Mandarim-de-Lacerda, 1995). It was observed that the animals treated with vincristine sulfate presented in adulthood (127 days), an increase in the number of profiles in a given test area as a result of the decrease in the major (diameter) and minor axes of the seminiferous tubules.

Substances with deleterious effects on spermatogenesis can lead to an increase in the number of profiles in a given test area, because, among the changes caused by the substances, the decrease in the diameter of the seminiferous tubules stands out, thus, the structures occupy smaller physical spaces, increasing the number of computed profiles (Bustos-Obregon & Lopez, 1973; Eboetse et al., 2011).

5. Conclusion

The administration of vincristine sulfate causes a reduction of important parameters that demonstrate a severe degree of testicular damage. The previous administration of L-carnitine at the dosage used, in association with treatment with vincristine sulfate, is a viable alternative that provides a reduction in the damage observed in testicular tissues.

More complete evaluations of testicular morphophysiology are needed to determine the cytoprotective mechanisms of L-carnitine on the treated testis in the prepubertal phase. Morphometric, molecular, immunohistochemical assessments and analysis of the seminiferous epithelium cycle are desired, which may provide important clues in understanding the mechanisms by which the deleterious effects of vincristine sulfate on germ cells and immature Sertoli cells are established, in addition to the mechanisms L-carnitine cytoprotectors on these cell types.

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