

## Grape juice concentrate protects reproductive parameters of male rats against cadmium-induced damage: a chronic assay

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### Abstract

The aim of the present study was to investigate the effects of long-term grape juice concentrate (GJC) consumption, in two dosages, on the reproductive parameters of cadmium-exposed male rats. The effects of the concentrate on body mass gain, plasma testosterone levels, reproductive organ weights, daily sperm production, sperm morphology, testis histopathological and histomorphometrical parameters, and testicular antioxidant markers were investigated. Wistar rats ( $n$  54) were distributed into six groups: CdCl<sub>2</sub>; cadmium and grape juice I (1.18 g/kg per d); cadmium and grape juice II (2.36 g/kg per d); grape juice I (1.18 g/kg per d); grape juice II (2.36 g/kg per d); control. A single dose of CdCl<sub>2</sub> (1.2 mg/kg body weight (BW)) was injected intraperitoneally and the grape juice was administered orally for 56 d. The results indicated that cadmium changed all reproductive and antioxidant parameters. At dosage I (1.18 g/kg BW), GJC consumption did not show the effects against cadmium-induced damages. In contrast, at dosage II (2.36 g/kg BW), the GJC improved the gonadosomatic index ( $P=0.003$ ), serum testosterone levels ( $P=0.001$ ), the relative weight of epididymis ( $P=0.013$ ) and ventral prostate ( $P=0.052$ ), the percentage of normal sperm ( $P=0.001$ ), and histopathological and histomorphometrical parameters. In addition, at this dosage, normalisation of the enzymatic activity of superoxide dismutase ( $P=0.001$ ) and of testicular levels of glutathione ( $P=0.03$ ) were observed. The parameters of the non-exposed rats did not depict significant alterations. In conclusion, the product was able to act as a protector of reproductive function against cadmium-induced damage. Such a property was expressed in a dose-dependent manner as the more effective dose was dosage II. The GJC acted possibly by antioxidant mechanisms.

**Key words:** Functional foods: Reproduction: Cadmium

Cadmium is a metal found in nature in low concentrations<sup>(1)</sup>. However, high levels of the metal can be found in the environment due to the burning of fossil fuels, the manufacture of batteries and the production of pigments and stabilisers<sup>(2)</sup>. The consequent contamination of soil and water results in the exposure of plants and animals to the metal and its bioaccumulation<sup>(3,4)</sup>. Moreover, tobacco smoke is another major source of human exposure to cadmium<sup>(5)</sup>.

In the male reproductive system, cadmium is known to impair reproductive physiology and decrease sperm quality<sup>(6,7)</sup>. In addition, *in vitro* studies have shown that cadmium can stimulate Sertoli cell apoptosis, leading to the disruption

of the blood–testis barrier<sup>(8,9)</sup>. Endocrine disruption is another consequence of cadmium exposure, caused by Leydig cell apoptosis and decreased serum levels of testosterone, as demonstrated in animal studies<sup>(10,11)</sup>. Clinical trials have shown an inverse correlation between high serum levels of cadmium and semen quality, including sperm DNA damage, sperm count, motility and morphology<sup>(12–15)</sup>.

Oxidative stress is related to cadmium-induced damage, increasing the formation of free radicals and decreasing the activity of antioxidant enzymes<sup>(16)</sup>. This event promotes the oxidation of cell structures, consisting mainly of PUFA which are present in high amounts in mammalian sperm,

**Abbreviations:** BW, body weight; CAT, catalase; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; CTRL, control; Dosage I, 1.18 g/kg BW of grape juice concentrate; Dosage II, 2.36 g/kg BW of grape juice concentrate; DSP, daily sperm production; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate; GSH, glutathione; SOD, superoxide dismutase.

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and facilitates DNA fragmentation and mitochondrial and plasma membrane peroxidation damage<sup>(17,18)</sup>. This results in a decrease in the motility and viability of the gametes<sup>(17,18)</sup>.

Grape and its derivatives are rich in polyphenols, including flavonoids (flavanols, flavonols and anthocyanins) and non-flavonoids (hydroxycinnamic acid derivatives, benzoic acid, hydrolysable tannins and stilbenes)<sup>(19,20)</sup>. The antioxidant activities of polyphenols include the modulation of endogenous antioxidant system, the scavenging of free radicals, the inhibition of lipid peroxidation and the formation of hydroperoxides<sup>(21)</sup>. In addition, flavonoids have demonstrated anti-inflammation and anti-carcinogenic activities, as well as metal-chelating properties<sup>(21–23)</sup>.

Grape juice concentrate (GJC) is an alternative natural food colourant obtained by the nanofiltration of the juice from red grapes (*Vitis labrusca*, mostly of the Concord variety), with subsequent concentration to 68° Brix, by evaporation. This concentrated product provides five times more polyphenols than grape juice and, for this reason, might exert physiological effects. Aguiar *et al.*<sup>(24)</sup> have previously studied the phenolic amount and composition of this grape product and reported its capacity to decrease oxidative DNA damage induced by H<sub>2</sub>O<sub>2</sub> in peripheral blood cells. The authors have suggested that further investigations should be conducted *in vivo* to verify the possible physiological effects. Thus, the objective of the present study was to examine the effects of GJC consumption, in two dosages, on long-term cadmium-induced reproductive damage in male rats.

## Materials and methods

### Animals

All experimental protocols involving animals conformed to the procedures described in the Guiding Principles for the Use of Laboratory Animals. The study was approved by the Animal Committee of the Federal University of Sao Paulo, UNIFESP, SP, Brazil.

Male adult Wistar rats (90 d old) weighing approximately 350 g were obtained from Centro de Desenvolvimento de Modelos Experimentais, Federal University of Sao Paulo, SP, Brazil. They were maintained under controlled conditions of temperature (24 ± 2°C) and light–dark periods of 12 h, with free access to water and a commercial diet (Nuvital®). All the rats were acclimatised for 2 weeks before the experiment.

### Experimental design

A total of fifty-four rats were randomly divided into six groups. CdCl<sub>2</sub> (Nuclear®) was administered in a single dose of 1.2 mg/kg body weight (BW) intraperitoneally, according to the method described by Predes *et al.*<sup>(25)</sup>, and GJC was given orally daily. The non-treated rats received a single dose of saline solution intraperitoneally and/or daily water by oral administration. The first group (Cd) was treated only with cadmium (CdCl<sub>2</sub>) injection (*n* 10). The second group (CdGJ1) received the CdCl<sub>2</sub> injection and 1.18 g/kg BW of GJC (*n* 10). The third group (CdGJ2) received the CdCl<sub>2</sub> injection and

2.36 g/kg BW of GJC (*n* 10). The fourth group (GJ1) was treated only with 1.18 g/kg BW of GJC (*n* 7). The fifth group (GJ2) was treated only with 2.36 g/kg BW of GJC (*n* 7). The sixth group (CTRL) did not receive any treatment (*n* 10). The rats were euthanised by decapitation after 56 d, chosen considering the period necessary to complete a spermatogenic cycle<sup>(26)</sup>. BW was recorded four times per week.

### Grape juice concentrate intake

The rats were given 1.18 or 2.36 g/kg BW per d of GJC (G8000™; Golden Sucos) by oral administration. The samples were tested for their polyphenol content, and the doses were calculated to be equivalent to four or eight glasses (200 ml each) of natural grape juice and adjusted to the faster metabolism of the rats, according to the method proposed by Aguiar *et al.*<sup>(24)</sup>. The dosage was adjusted every day according to the weight of the rats.

### Plasma testosterone levels

Immediately after decapitation, blood samples were collected in EDTA tubes and centrifuged at 5000 rpm for 15 min at 4°C. Plasma samples were kept at –80°C before analysis. Plasma testosterone levels were determined by chemiluminescence immunoassay, using acridine ester as the chemiluminescent marker (ADVIA Centaur® XP Immunoassay System; Bayer Corporation). The CV for testosterone was 7.7%.

### Tissue collection and preparation

After decapitation, the left testis and epididymis, ventral and dorsolateral prostate and seminal vesicle (with the coagulating gland) were immediately removed and fixed in ALFAC solution (80% ethanol, formaldehyde and glacial acetic acid, 8.5:1.0:0.5, by vol.) for 24 h and then weighed. Relative weight was used for a comparative analysis (absolute weight/total BW × 100). Paraplast-embedded testes were sectioned at 3–5 μm thickness and stained with haematoxylin and eosin.

### Histopathological analysis

Inflammatory characteristics of the testis were analysed by the semi-quantitative method adapted from Zhang *et al.*<sup>(27)</sup>, establishing the following scores: 0, no inflammatory characteristic; 1, vascular congestion/necrosis; 2, mononuclear cell infiltrate; 3, tissue degeneration. For the evaluation of spermatogenesis, fifty horizontally sectioned seminiferous tubules per animal were scored from 10 (complete spermatogenesis and organised seminiferous epithelium) to 1 (no cells in a tubular section/tubular sclerosis) using Johnsen's score as described by Lee *et al.*<sup>(28)</sup>.

### Histomorphometrical analysis

The percentage of interstitial and tubular areas of the testis was determined by measuring the area occupied by

the seminiferous tubules and interstitium in fifteen fields per animal<sup>(25)</sup>. For each animal, thirty round tubules were selected randomly to measure the tubular diameter<sup>(25)</sup>. This analysis was performed using AxioVision 4.8 software (Carl Zeiss) linked to a microscope (Carl Zeiss) at 200X magnification.

### Sperm parameters

**Daily sperm production.** A major portion of the right testis was stored at  $-20^{\circ}\text{C}$  and used to determine the daily sperm production (DSP). After tissue homogenisation in a standard solution (0.9% NaCl<sub>2</sub>, 0.05% Triton X-100 and 0.01% Thimerosal) and dilution in the same solution in 1:10 proportion, the spermatids were counted in four Neubauer haemocytometer chambers to obtain the average count. The calculation was performed according to the method described by Robb *et al.*<sup>(29)</sup>, corrected by testis mass used, and the data are expressed as the number of sperm ( $\times 10^6$ ) per testis per d.

**Sperm morphology.** To analyse the morphology of sperm stored in the epididymis, a small incision was made on the right epididymal cauda (previously frozen at  $-20^{\circ}\text{C}$ ) that was immersed in a PBS solution for diffusion for 15 min. Drops of the sperm suspension were put on microscope slides and exactly 200 spermatozoa per animal were analysed under a light microscope at 400X magnification, and classified as normal or abnormal, according to the method described by Seed *et al.*<sup>(30)</sup>. The abnormalities included head alterations (isolated, amorphous, absent or reduced hook) and tail alterations (isolated, coiled, broken or bent). The results are expressed as a percentage of normal sperm.

### Testicular antioxidants

A small piece of the right testis, stored at  $-80^{\circ}\text{C}$ , was used for the evaluation of antioxidant markers. The enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) and the tissue levels of glutathione (GSH) were analysed.

**Superoxide dismutase.** The enzymatic activity of testicular SOD was measured through the microplate method proposed by Ewing & Janero<sup>(31)</sup>, in a spectrophotometer at 560 nm, for 3 min at  $25^{\circ}\text{C}$ , using SoftMax software (Bioanalytical Company). Mitochondrial SOD (Mn-SOD) was quantified by the same method, adding potassium cyanide to block cytoplasmic SOD (Cu,Zn-SOD). The activity of cytoplasmic SOD was established by the difference between total SOD and mitochondrial SOD. The obtained values were corrected by tissue protein and are expressed in U SOD/mg protein.

**Catalase.** The enzymatic activity of testicular CAT was obtained according to the method described by Adamo *et al.*<sup>(32)</sup> by the kinetic of H<sub>2</sub>O<sub>2</sub> degradation in a spectrophotometer (Hitachi-200; Hitachi) at 230 nm, for 3 min at  $30^{\circ}\text{C}$ , using UV Solutions software (Hitachi High Technologies America). The obtained values were corrected by tissue protein and are expressed in U CAT/mg protein.

**Glutathione.** The tissue levels of GSH were measured based on the method proposed by Tietze<sup>(33)</sup>. An acid extract of the testicular tissue was obtained using perchloric acid and analysed in a spectrophotometer (Hitachi U-2010; Hitachi)

at 412 nm and  $25^{\circ}\text{C}$ , using UV Solutions software (Hitachi High Technologies America). Data are expressed as  $\mu\text{mol}$  GSH/g tissue.

### Statistical analysis

Considering that the present study is a part of a larger experimental design, including an additional (sub-chronic) period of treatment, statistical procedures were performed using the ANOVA model for three fixed factors (cadmium, grape juice and time) and multiple-comparisons method of Bonferroni. For semi-quantitative histopathological analyses, simultaneous non-parametric comparisons were established. The significance level was set at  $P \leq 0.05$ .

The DSP was chosen for planning the sample size. The minimal differences were established according to the researchers' expectation about cadmium and GJC effects. The DSP was expected to be normal (approximately  $30 \times 10^6$  sperm/d per testis) in the CTRL group, being reduced to approximately  $8-10 \times 10^6$  in the Cd group, possibly being elevated by the first GJC dose (to approximately  $14 \times 10^6$ ) and further elevated by the second dose (approximately  $20-22 \times 10^6$ ) without returning to the normal values. By the presence of resveratrol, the GJC was expected to increase the normal DSP in healthy rats (to approximately  $37-40 \times 10^6$ ). On the basis of these assumptions and fixing the significance level at 5%, the power of the tests using different sample sizes was calculated, and this resulted in the following value: 0.906 (90.6%). Such a value was only 1% lower when compared with the value obtained with all groups containing ten rats (91.6%).

## Results

### Body mass gain

The Cd group showed less body mass gain than the CTRL group ( $P = 0.009$ ). No statistically significant differences were observed after GJC consumption. These results are given in Table 1.

**Table 1.** Values of body mass gain and plasma levels of testosterone (Mean values and standard deviations)

Groups	Body mass gain (g)		Testosterone (nmol/l)	
	Mean	SD	Mean	SD
CTRL	89.7	11.3	22.11	2.40
Cd	69.0**	6.5	10.49**	2.52
CdGJ1	73.3	20.8	18.51	1.93
CdGJ2	83.0	22.4	15.62††	6.33
GJ1	53.0	21.4	10.89	5.80
GJ2	62.8	17.0	16.80	5.19

CTRL, control; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

\*\*Mean value was significantly different from that of the CTRL group ( $P \leq 0.01$ ).

††Mean value was significantly different from that of the Cd group ( $P \leq 0.01$ ).

**Plasma testosterone levels**

Plasma testosterone decreased in the Cd group ( $P = 0.001$ ), as described in Table 1. Whereas dosage I of GJC (1.18 g/kg BW) showed no effect, the intake at dosage II (2.36 g/kg BW) was able to recuperate the hormonal levels after cadmium exposure ( $P = 0.002$ ). GJC consumption did not significantly change this parameter in the non-exposed rats.

**Reproductive organ weight**

Cadmium exposure decreased the relative weight of the testis ( $P = 0.001$ ), epididymis ( $P = 0.001$ ), ventral prostate ( $P = 0.008$ ) and seminal vesicle ( $P = 0.002$ ) in the Cd group (Table 2). Dosage I of the GJC showed no effect on the weight of any organ, while dosage II successfully recovered the relative weight of the testis ( $P = 0.003$ ), epididymis ( $P = 0.013$ ) and ventral prostate ( $P = 0.052$ ). In the GJ1 group (non-exposed), decreased seminal vesicle weight ( $P = 0.002$ ) was observed. Interestingly, the effect was not observed in rats of the dosage II group.

**Histopathological analysis**

In the analysis of the inflammatory process, the Cd and CdGJ1 groups showed statistically significant histopathological changes when compared with the CTRL group ( $P = 0.001$  and  $P = 0.048$ , respectively). Nevertheless, there were significant differences between the Cd *v.* CdGJ1 ( $P = 0.002$ ) and the Cd *v.* CdGJ2 ( $P = 0.001$ ) groups. GJC consumption did not change testicular histopathology in the healthy rats (Table 3). The spermatogenic process was disturbed by cadmium exposure, according to Johnsen's score, in the Cd ( $P = 0.001$ ), CdGJ1 ( $P = 0.001$ ) and CdGJ2 ( $P = 0.001$ ) groups. The main findings were the presence of inflammatory processes, tubular atrophy and tissue necrosis, with variable degrees among the groups. In spite of less pronounced damage in the CdGJ2 group, a statistically based analysis showed that the GJC was not able to attenuate cadmium-induced changes or modify spermatogenesis in the

non-exposed groups (Table 4). The results of the histopathological analysis are illustrated in Fig. 1.

**Histomorphometrical analysis**

In the histomorphometrical analysis, the Cd, CdGJ1 and CdGJ2 groups showed a decrease in the tubular area ( $P = 0.001$ ,  $P = 0.001$  and  $P = 0.009$ , respectively) and an increase in the interstitial area ( $P = 0.001$ ,  $P = 0.001$  and  $P = 0.008$ , respectively). Dosage II of the GJC attenuated tubular area ( $P = 0.016$ ) and interstitial area ( $P = 0.015$ ) alterations. Tubular diameter was decreased only in the Cd group ( $P = 0.001$ ). Although the GJC at dosage I did not promote histomorphometrical improvements, at dosage II, the values of tubular diameter were normalised ( $P = 0.001$ ). No changes were observed in the histomorphometrical parameters of the non-exposed groups (CTRL, GJ1 and GJ2; Table 4).

**Sperm parameters**

DSP was decreased by cadmium exposure in the Cd ( $P = 0.001$ ), CdGJ1 ( $P = 0.001$ ) and CdGJ2 ( $P = 0.001$ ) groups. The GJC was not able to attenuate the cadmium-induced effect. The parameters were not changed in the non-exposed groups at any dosage (Fig. 2). Moreover, CdCl<sub>2</sub> was able to decrease the percentage of normal sperm in the Cd ( $P = 0.001$ ), CdGJ1 ( $P = 0.001$ ) and CdGJ2 ( $P = 0.006$ ) groups. In this case, GJC intake at dosage II improved the sperm morphology of the exposed rats ( $P = 0.003$ ). GJC consumption did not change sperm morphology in the non-exposed groups (Fig. 3).

**Testicular antioxidants**

The enzymatic activity of total SOD in the Cd group was increased ( $P = 0.001$ ), as well as that of Mn-SOD ( $P = 0.001$ ) and Cu,Zn-SOD ( $P = 0.006$ ). A recovery was not observed in the group treated with the GJC at dosage I. At dosage II, however, a decrease in total SOD ( $P = 0.001$ ) and Mn-SOD ( $P = 0.007$ ) activities was observed. Additionally, CAT activity

**Table 2.** Relative weight (%) of the testis, epididymis, prostate (ventral and dorsolateral) and seminal vesicle (Mean values and standard deviations)

Groups	Testis		Epididymis		Ventral prostate		Dorsolateral prostate		Seminal vesicle	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CTRL	0.429	0.060	0.145	0.009	0.088	0.020	0.059	0.021	0.273	0.036
Cd	0.141†††	0.026	0.073†††	0.006	0.053**	0.017	0.054	0.015	0.161**	0.036
CdGJ1	0.180	0.117	0.092	0.027	0.077	0.015	0.064	0.014	0.205	0.047
CdGJ2	0.323‡	0.139	0.122‡	0.027	0.084§	0.022	0.041	0.010	0.211	0.047
GJ1	0.422	0.041	0.152	0.016	0.095	0.034	0.059	0.027	0.141**	0.053
GJ2	0.424	0.048	0.151	0.016	0.105	0.018	0.071	0.026	0.218	0.063

CTRL, control; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

\*\*Mean value was significantly different from that of the CTRL group ( $P \leq 0.01$ ).  
 †††Mean value was significantly different from that of the CTRL group ( $P \leq 0.001$ ).  
 ‡Mean value was significantly different from that of the Cd group ( $P \leq 0.01$ ).  
 §Mean value was significantly different from that of the Cd group ( $P \leq 0.05$ ).

**Table 3.** Total number of rats in all groups according to the degree of histopathological changes in the testicular tissue

Groups	Score			
	0	1	2	3
CTRL	7	0	0	0
Cd†††	0	0	1	4
CdGJ1*‡	3	0	3	1
CdGJ2§	5	0	2	1
GJ1	5	0	0	0
GJ2	5	0	0	0

CTRL, control; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

\*Values were significantly different from those of the CTRL group ( $P \leq 0.05$ ).  
 †††Values were significantly different from those of the CTRL group ( $P \leq 0.001$ ).  
 ‡Values were significantly different from those of the Cd group ( $P \leq 0.01$ ).  
 §Values were significantly different from those of the Cd group ( $P \leq 0.001$ ).

was not changed in any group, in spite of the significant differences between the CdGJ1 and GJ1 groups ( $P = 0.001$ ). The tissue levels of GSH decreased in the Cd ( $P = 0.004$ ) and CdGJ1 ( $P = 0.01$ ) groups. Dosage II of the GJC effectively recovered the GSH levels after cadmium exposure ( $P = 0.03$ ). The GJ1 and GJ2 groups did not show statistically significant differences when compared with the CTRL group. Detailed results are given in Table 5.

**Discussion**

It is well established that cadmium accounts for several biological changes, mainly leading to oxidative stress and inflammation<sup>(34)</sup>. Conversely, GJC has demonstrated anti-inflammatory, pro-apoptotic and cell-cycle regulation properties<sup>(35,36)</sup>. The major phenolic compounds in GJC have been assessed earlier by Aguiar *et al.*<sup>(24)</sup> by a qualitative analysis. The authors have observed the relevant presence of flavonoids (quercetin, kaempferol, peonidin-glucoside, malvidin-glucoside, petunidin-3-*O*-acetylglucoside, peonidin-3-*p*-coumaroylglucoside, malvidin-3-*O*-*p*-coumaroylglucoside and dimethoxyflavylum) and non-flavonoids (ferric acid, caffeic acid derivatives and resveratrol).

From a general perspective, the metal induced an overall toxicity evidenced by reduced body mass gain, in agreement with previous studies<sup>(37–39)</sup>. Although the mechanism is not clear, Rencuzogullari & Erdogan<sup>(40)</sup> suggested that free radical production may be involved in cadmium-induced weight changes. GJC consumption did not change body mass gain in any condition, in agreement with previous clinical studies<sup>(41,42)</sup>.

Severe cadmium-induced damage was observed in the male reproductive system, including testicular and epididymal atrophy as well as prostate and seminal vesicle weight reduction. The effects of cadmium on testis and epididymis weight are well known<sup>(37–39)</sup>. However, data on other organs are controversial. Although some authors have observed no change in seminal vesicle and prostate weight<sup>(25,43)</sup>, others have found a decrease<sup>(44,45)</sup>. Harmful effects of cadmium on reproductive organs may be related to low testosterone levels after cadmium exposure, as evidenced in the present study. This result was probably caused by DNA damage-induced Leydig cell apoptosis as a consequence of oxidative stress, also observed in the present study<sup>(11,44,46)</sup>. The present results suggest that the protective effects of GJC consumption on reproductive organs are related to the maintenance of plasma levels of testosterone, observed after the administration of dosage II in cadmium-exposed rats. It has been described that resveratrol and other polyphenols are able to increase testosterone levels and avoid Leydig cell apoptosis<sup>(47,48)</sup>. In the non-exposed rats, changes in seminal vesicle after the consumption of dosage I were observed. Given that similar results were not found in the literature, these findings require further investigations.

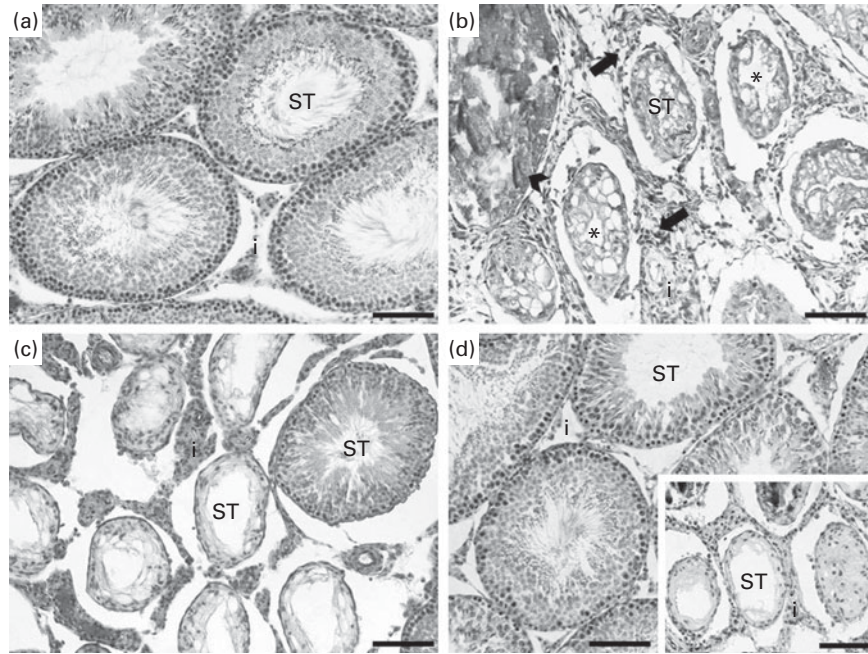
Spermatogenesis disruption may be attributed to the high prevalence of Sertoli cell-only seminiferous tubules, as a consequence of cadmium exposure, in agreement with previous studies<sup>(49,50)</sup>. Furthermore, CdCl<sub>2</sub> caused histomorphometrical changes, similar to the findings reported by Predes *et al.*<sup>(25)</sup>, and intense inflammatory processes, including interstitial mononuclear cell infiltrate, vascular congestion, necrosis and tissue degeneration. These effects were generally due to the stimulation of pro-inflammatory cytokines<sup>(50)</sup>. Although GJC

**Table 4.** Johnsen's score and histomorphometrical analysis (Mean values and standard deviations)

Groups	Johnsen's score		Tubular area (%)		Interstitial area (%)		Tubular diameter (µm)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CTRL	9.95	0.03	71.90	3.40	26.84	3.54	307.09	18.70
Cd	1.26†††	0.50	48.19†††	8.50	51.80†††	8.50	150.08†††	19.32
CdGJ1	3.72†††	3.03	49.89†††	9.58	50.10†††	9.58	196.08	50.60
CdGJ2	7.08†††	4.11	65.11**‡	14.73	34.88**‡	14.73	250.93‡	65.60
GJ1	9.90	0.05	76.60	2.12	23.39	2.12	288.05	13.49
GJ2	9.94	0.04	75.52	3.40	24.47	3.40	293.91	10.96

CTRL, control; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

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 ‡Mean value was significantly different from that of the Cd group ( $P \leq 0.01$ ).

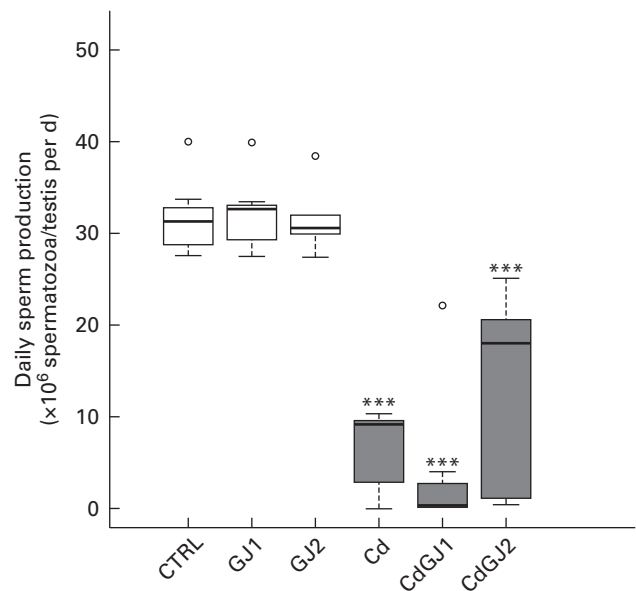


**Fig. 1.** Histopathological analysis of the testis. Control rats presented normal testis tissue organisation with intact seminiferous tubules (ST) (a), while in the cadmium-treated group, tissue disorganisation with inflammatory infiltrate (arrows) in the interstitium (i), tissue necrosis (arrowhead) and general tubular atrophy (\*) were found (b). In spite of the presence of few intact ST, the CdGJ1 group presented the same degree of damage as that observed in the group exposed only to cadmium (c). Qualitatively, less pronounced damage was observed in the group receiving the second grape juice concentrate dosage (CdGJ2) (d), in spite of extensive areas of some rats showing intense damage (d, onset) (scale bar 100  $\mu$ m). The GJ1 and GJ2 groups (not shown) presented the same histological architecture as that observed in the control group. CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

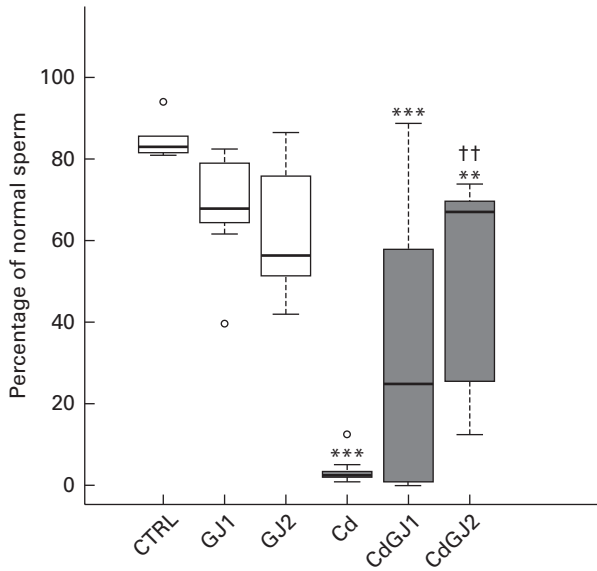
consumption did not improve spermatogenesis changes, both dosages I and II of the GJC attenuated the cadmium-induced inflammatory profile and dosage II attenuated the histomorphometrical changes. It is known that grape polyphenols modulate endothelial function and cytokine expression, which may be related to the beneficial effects observed<sup>(51–56)</sup>.

Morphological changes are directly related to physiological dysfunction<sup>(57)</sup>, and spermatogenesis disruption may have led to reduced DSP, as has been also demonstrated in earlier studies<sup>(7,44,58)</sup>. In a recent review, Wong & Cheng<sup>(59)</sup> have reported that cadmium-induced oxidative stress seems to be the main cause for the decline observed in sperm count. Oxidative stress has been stated to occur after cadmium exposure and may cause DNA damage and lipid peroxidation of sperm, contributing to sperm morphology changes as found in the present investigation<sup>(60,61)</sup>. In spite of the inability of the GJC to completely restore the testicular physiology, a dose-dependent attenuation of cadmium-induced changes in sperm morphology was observed.

An imbalance between oxidant and antioxidant agents, triggered by cadmium, was shown in the present study, characterised by decreasing GSH levels, increasing SOD activity and the maintenance of CAT activity. Low levels of GSH in the testicular tissue, after cadmium exposure, have been well described<sup>(62–64)</sup> and mainly occur because the thiol group binds covalently with metals<sup>(65,66)</sup>. After GJC consumption, the tissue levels of GSH were normalised by dosage II. In general, flavonoids are able to modulate both



**Fig. 2.** Daily sperm production. CTRL, control; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate. \*\*\* Values were significantly different compared with that of the CTRL group ( $P \leq 0.001$ ). □, No cadmium; ■, cadmium; ○, outliers.



**Fig. 3.** Percentage of normal sperm. CTRL, control; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate. Values were significantly different compared with that of the CTRL group: \*\**P* ≤ 0.01, \*\*\**P* ≤ 0.001. †† Values was significantly different compared with that of the Cd group (*P* ≤ 0.01). □, No cadmium; ■, cadmium, ○, outliers.

γ-glutamylcysteine synthetase and glutamate cysteine ligase involved in the synthesis of GSH<sup>(67–69)</sup>.

SOD is especially abundant in the mitochondria due to the release of reactive molecules by the respiratory chain<sup>(66)</sup>. Cadmium targets the mitochondria, changing its membrane permeability and accumulating semi-ubiquinone molecules, which can transfer electrons to molecular oxygen<sup>(66,70)</sup>. This mechanism can be involved in the overproduction of superoxide anions in the testicular tissue, which explains the increase in SOD activity, especially in the mitochondrial fraction, found in the present study. A decrease in SOD levels was found after GJC consumption at dosage II, mainly in the mitochondrial fraction. This event may be explained by the property of

grape bioactive compounds to reduce reactive oxygen species production in the mitochondria and to scavenge superoxide anions, preserving enzymatic activity<sup>(71)</sup>.

In contrast, CAT activity was not altered, which was unexpected, as CAT activity is directly related to SOD activity<sup>(72)</sup>. These findings suggest that a possible increment in CAT activity was inhibited by cadmium, probably due to the metal's capacity to bind sites for other metals, such as Fe, which catalyses the reactions of CAT<sup>(73,74)</sup>. In this sense, a statistically significant difference was observed between the CdGJ1 and GJ1 groups, showing not only the potentiality of cadmium to increase CAT activity but also the capacity of GJC to revert cadmium-induced inhibition, in agreement with the Eybl *et al.*<sup>(75)</sup> data. No changes were found in the CdGJ2 group as expected, since SOD activity maintained the levels that were similar to those observed in the CTRL group.

Although alterations in enzymatic antioxidant activities indicate oxidative stress, some studies have demonstrated a reduction in testicular SOD and/or CAT activity after cadmium exposure<sup>(46,58,64,76,77)</sup>. Disparity between the present results and those reported in the literature may be related to different administration routes and CdCl<sub>2</sub> concentrations, generally higher in a single dose or in a high-frequency exposure. In agreement with our hypothesis, Wang *et al.*<sup>(78)</sup> demonstrated that while low cadmium concentrations can maintain or increase CAT, SOD and GSH peroxidase activities, high dosages inhibit these enzymes in the testicular tissue. Similarly, Kojima *et al.*<sup>(79)</sup> reported an increase in testicular CAT activity and the maintenance of SOD activity 30 min after a 5 mg/kg BW CdCl<sub>2</sub> injection. This may indicate that the increase/maintenance of antioxidant enzymatic activity is an event that take place before its inhibition, as has been widely described in the literature.

In conclusion, the present results show that regular dietary doses of GJC were able to attenuate long-term cadmium-induced reproductive damage in a dose-dependent manner, through various possible mechanisms, including modulation of the antioxidant system.

**Table 5.** Enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) and testicular levels of glutathione (GSH) (Mean values and standard deviations)

Groups	Total SOD (U/mg protein)		Mn-SOD (U/mg protein)		Cu,Zn-SOD (U/mg protein)		CAT (U/mg protein)		GSH (μmol/g tissue)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CTRL	10.76	2.68	6.87	3.19	3.89	0.72	5.38	1.38	251.58	83.32
Cd	20.72†††	4.74	13.69†††	4.05	7.02**	0.83	6.34	1.73	60.79**	20.11
CdGJ1	17.07	4.24	11.29	4.01	5.78	2.05	9.72‡	6.42	80.91**	34.13
CdGJ2	12.95‡	4.40	6.91§	3.19	6.04	2.60	4.66	3.10	213.41	96.98
GJ1	8.20	1.69	4.46	2.07	3.75	2.49	2.14	1.30	202.18	36.57
GJ2	10.26	1.47	5.69	2.92	4.57	2.89	2.18	0.55	118.55	62.29

CTRL, control; Cd, cadmium injection; CdGJ1, CdCl<sub>2</sub> injection + 1.18 g/kg BW of grape juice concentrate; CdGJ2, CdCl<sub>2</sub> injection + 2.36 g/kg BW of grape juice concentrate; GJ1, 1.18 g/kg BW of grape juice concentrate; GJ2, 2.36 g/kg BW of grape juice concentrate.

\*\*Mean value was significantly different from that of the CTRL group (*P* ≤ 0.01).

†††Mean value was significantly different from that of the CTRL group (*P* ≤ 0.001).

‡Mean value was significantly different from that of the Cd group (*P* ≤ 0.001).

§Mean value was significantly different from that of the Cd group (*P* ≤ 0.01).

||Mean value was significantly different from that of the Cd group (*P* ≤ 0.05).

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