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Communication

[*Comunicação*]

Effects of the food contaminant semicarbazide on testicular morphology of juvenile Wistar rats

[*Efeitos do contaminante alimentar semicarbazida na morfologia testicular de ratos Wistar jovens*]

A. *Ramos*[1], A. *Pereira*[2,3], A.S. *Cabrita*[4], F. *Capela e Silva*[3,5*]

[1]Departamento de Medicina Veterinária - Universidade de Évora e Universidade Lusófona de Humanidades e Tecnologias, Portugal
[2]Departamento de Zootecnia - Universidade de Évora, Portugal
[3]Instituto de Ciências Agrárias e Ambientais Mediterrânicas-ICAAM - Universidade de Évora, Portugal
[4]Instituto de Patologia Experimental - Faculdade de Medicina - Universidade de Coimbra, Portugal
[5]Departamento de Biologia - Universidade de Évora, Portugal

Semicarbazide is a hydrazine derivative by-product of azodicarbonamide, that can occur naturally, e.g. in algae, shrimps and eggs, and is formed from natural substances, e.g. arginine and creatine (Hoenicke *et al*., 2004). Formation of semicarbazide in processed food samples, such as carrageenan-containing food, treated with hypochlorite for disinfection and bleaching have been reported (Hoenicke *et al*., 2004). Semicarbazide is also present in glass jars and bottles closed with metal lids sealed with plastic gaskets that are foamed using the azodicarbonamide as blowing agent (Opinion..., 2005). Azodicarbonamide is not listed as a permitted food additive in Council Directive 95/2/EC [European Parliament and Council Directive No. 95/2/EC of 20 February 1995 on food additives other than colors and sweeteners]; however, it is used as a flour additive in some countries such as Canada, USA and Brazil owing to its dough-improving properties (de la Calle and Anklam, 2005). Recent studies proved that semicarbazide can also be formed in processed food such as coated poultry products and in bread prepared with azodicarbonamide-containing flour (de la Calle and Anklam, 2005). The toxicity of semicarbazide is not well understood (Opinion..., 2005 and Nestmann *et al*., 2005), but experimental data showed semicarbazide acting as osteolathyrogen agent (Ramamurti and Taylor, 1959; Kundel, 1964; Maranghi *et al*., 2009), and exerting pleiotropic effects on several organs/tissues (Maranghi *et al*., 2009) and marked alterations of spontaneous motor and exploratory behaviours (Maranghi *et al*., 2009). This study aimed to evaluate the effects of semicarbazide on testicular morphology of juvenile Wistar rats.

After ten days of acclimatizing, thirty weaning male Wistar rats, four-week-old, were individually weighed, identified, divided into three groups (ten animals/group) and kept in polypropylene cages with wood chip bedding under a 12h light/dark cycle and room temperature of 22-24ºC, with water and food available *ad libitum*. The animals of control group (G_0) received the standard diet; the animals of the G_3 and G_6 groups received the semicarbazide hydrochloride (Sigma-Aldrich, Refª S2201) in the diet at a concentration of 3g/kg and 6g/kg, respectively. Body weight changes were recorded weekly on an electronic balance (0.1g). At the end of the thirty days of experimental period, the animals were weighed and sacrificed by anesthesia overdose. After sacrifice, the testes were excised and fixed in 10% neutral phosphate-buffered (pH 7.4) formalin, dehydrated, embedded in paraffin, cut into 5µm sections and stained with hematoxylin and eosin, for the examination under light microscopy (Nikon Eclipse 600 microscope). All

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*Autor para correspondência (*corresponding author*)
E-mail: fcs@uevora.pt

the ten animals from each group were used and for each animal, 15 pictures from random seminiferous tubules were collected with a digital camera (Nikon DN100) at 200X magnification. For each picture, the area and the perimeter of the semineferous tubules, the area and the perimeter of the lumen of the semineferous tubules and the height of the germinative epithelium were measured using SigmaScan Pro5.0 software (SPSS, Chicago, IL, USA). For each seminiferous tubules, the height of the germinative epithelium was the mean of ten measures. From the results obtained, the average values for each animal and group were evaluated. All procedures involving the animals were approved by the scientific committee, supervised by a Federation of European Laboratory Animal Science Associations (FELASA) trained scientist and conforming to the regulations of the Portuguese law (Portaria 1005/92), following the European Union Laboratory Animal Experimentation Regulations. Statistical procedures were done as follows. The dependent variable "weight" was tested for the assessment of normality according to Kolmogorov Smirnoff test and homogeneity of variance according to Levene's test (Underwood, 1997). Following the findings of normality and homocedasticity, the variable weight was analyzed according to general linear model analysis of variance with two fixed factors: diet (three levels) and dates of weighing (with five levels). In the analysis of variance, it was considered as value significantly different, one whose probability of occurrence was greater than 95% ($P<0.05$). When significant differences were found, means were compared by Tukey Kramer test (Underwood, 1997). All statistical analysis were performed by SPSS 16.0 software package.

The animals which were administered the semicarbazide showed significant reductions in body weight in a dose-dependent manner (Table 1).

Table 1. Body weight (BW) of rats (n=10) during the experimental period (g; mean ± standard deviation)

Groups	BW_1	BW_2	BW_3	BW_4	BW_5
G_0	80.70±10.1aA	115.30±9.5bA	154.40±13.3cA	188.10±16.8dA	214.70±24.5eA
G_3	78.10±14.4aA	84.2±14.7aB	92.40±15.7bB	96.10±17.0bB	97.40±25.3bB
G_6	74.90±14.8aA	75.45±13.3aB	79.18±16.0aC	79.54±18.4aC	76.18±20.6aC

Means followed by distinct lower letters in the same row and capital letters in the same column are different ($P<0.05$).

The results showed that animals in G_0 exhibited a behavior quite distinct from other groups ($P<0.01$). From the second weighing, there were higher weights ($P<0.01$) in the animals of G_0 for the remaining. This trend continued and was accentuated with the progress of time, resulting in greater differences in weight achieved at the last weighing. For the G_0, there were differences in the G_3 and G_6 greatly mitigated in increments of weight compared to initial weight. In G_3, only after weighing three significant differences were observed in the results, at which point they began to show significant differences regarding the G_6. Note that in G_6, the highest dose of semicarbazide prevented significant increases in animal weight ($P>0.05$) over a number of weighings.

Untreated rats showed mostly normal testicular architecture with an orderly arrangement of germinal cells and Sertoli cells and normal successive stages of the spermatogenesis. Semicarbazide treatment induced testicular atrophy accompanied by the degeneration of germ cells within the seminiferous tubules, and the tubules were shrunken and greatly depleted of germ cells (Table 2 and Figure 1).

Table 2. Histomorphometric changes in the seminiferous tubules of rats (n=10) according to treatments (pixels; mean ± standard deviation)

Groups	Epithelia area	Epithelia height	Tubular area	Tubular diameter	Lumen area	Lumen diameter
G_0	60,947.01± 7,416.36a	82.21± 3.52a	77,083.09± 10,587,40a	1,083.70± 73.97a	16,136.08± 3,503.32a	558.49± 52.06a
G_3	46,186.84± 9,524.99b	61.53± 7.82b	57,227.41± 12,280.02b	971.48± 102.29b	11,040,57± 3,121.04b	464.70± 66.08b
G_6	39,384,20± 13,800.95b	64.57± 16.05b	47,494.95± 16,816.06b	853.68± 158.03c	8,110.75± 3,775.71b	437.58± 86.39b

Means followed by distinct letters in the same column are different (P<0.05).

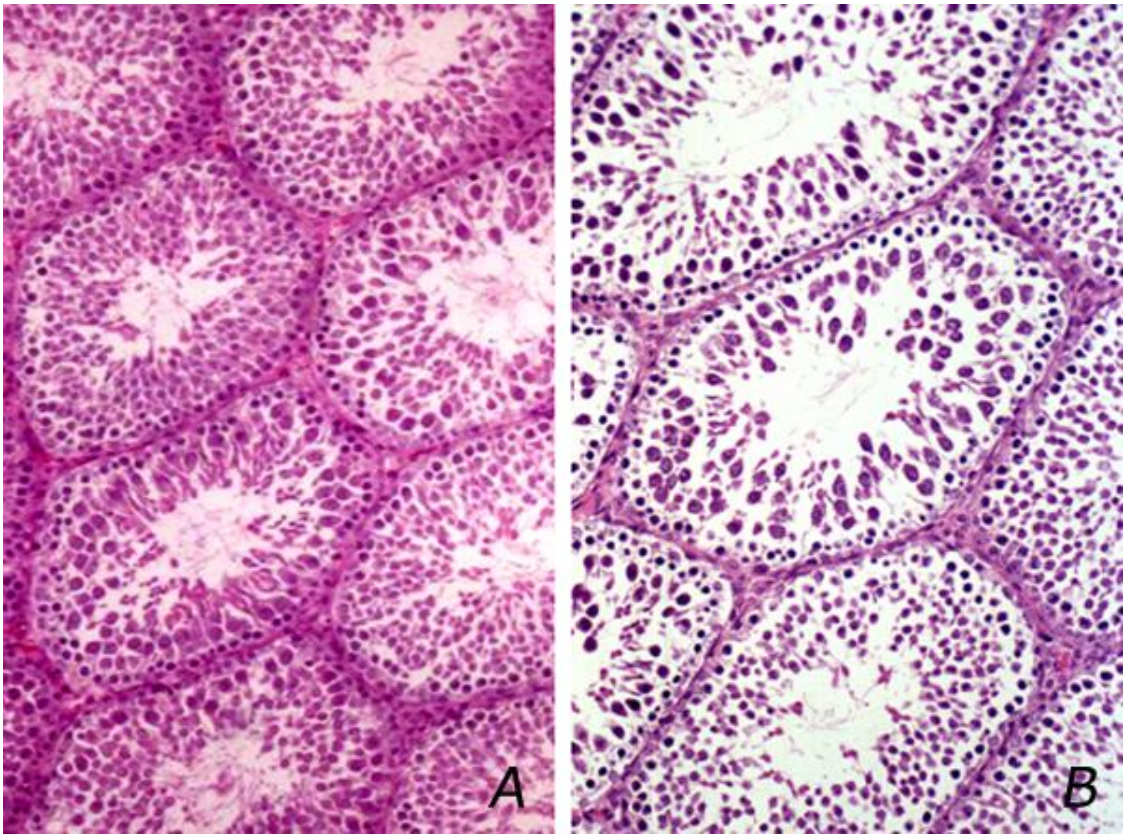

Figure 1. Photomicrograph of the seminiferous tubules of control group (A) showing the normal arrangement of germinal cells (H&E, 100X). Testis of semicarbazide-treated rats (B) showing tubular atrophy with extensive degeneration of the germinal epithelium. The atrophic tubules contained degenerated Sertoli cells with few germ cells (H&E, 100X).

The administration of semicarbazide to juvenile rats showed significantly decrease of body weight gain as reported by other authors (Maranghi *et al*., 2009) and exerted adverse effects by altering the normal testicular architecture and normal successive stages of the spermatogenesis. Similar effects were reported in rats treated with phthalates (Kondo *et al*., 2006) and dye blend (tomato red) (Sharma *et al*., 2008). However, Maranghi *et al*. (2009) observed only subtle adverse effects of semicarbazide in testis of juvenile Sprague-Dawley rats by altering the percentage of testicular tissue programmed for spermatogenesis without affecting spermatogenesis itself. The mechanism of semicarbazide-induced toxic effects in the cardiovascular (i.e. aorta) and skeletal systems results from its binding to at least two

enzymes, lysyl oxidase (Dawson *et al*., 2002) and semicarbazide-sensitive amine oxidase (Langford *et al*., 1999), both involved in the proper production and cross-linking of extracellular matrix (ECM) proteins, especially collagen and elastin. At high concentrations *in vitro*, semicarbazide may be weakly mutagenic as a result of the production of reactive oxygen species (ROS) (Hirakawa *et al*. 2003) which are involved in a variety of pathophysiological conditions of testes (Agarwal *et al*., 2006). Superoxide dismutase and glutathione peroxidase are major enzymes that scavenge harmful ROS in male reproductive organs (Fujii *et al*., 2003). Semicarbazide is a potent enzyme inhibitor, and the toxic effects in the reproductive system may result from inhibition of activities of those ROS-scavenging enzymes. The present results showed that semicarbazide induce important changes during juvenile period in rat testicular morphology which probably may affect reproductive functions and further detailed studies are necessary to elucidate the mechanisms of action. This can be considered relevant for food safety in particular for children who represent a group of major exposure and susceptibility to semicarbazide

Keyword: semicarbazide, rat, Wistar, testes

RESUMO

O objectivo do presente trabalho foi avaliar os efeitos da semicarbazida na morfologia testicular de ratos Wistar jovens. Os animais foram tratados durante 30 dias com hidrocloreto de semicarbazida incorporado na dieta, nas concentrações de 0, 3 e 6g/kg. Os resultados obtidos revelaram uma diminuição estatisticamente significativa no diâmetro dos túbulos seminíferos, no diâmetro do lúmen dos túbulos seminíferos e na área ocupada pelo epitélio seminífero nos animais dos grupos experimentais em comparação com os animais do grupo controlo. Estes resultados evidenciam que a semicarbazida induz alterações importantes no desenvolvimento testicular e sugerem um estudo mais aprofundado sobre os mecanismos de acção, dos efeitos a longo prazo, da reversibilidade das lesões e nas capacidades reprodutivas. Estes aspectos são relevantes numa perspectiva de segurança alimentar, em particular para as crianças, as quais representam um grupo de maior exposição e susceptibilidade à semicarbazida.

Palavras-chave: semicarbazida, rato, Wistar, testículos

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