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Communication

[*Comunicação*]

Effect of dithiocarbamate thiram on Wistar rat growth plate and articular cartilage

[*Efeito do ditiocarbamato tirame sobre a placa de crescimento e cartilagem articular de ratos Wistar*]

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Dithiocarbamates, as disulfiram, ziram, and thiram, are extensively used as agricultural fungicides for both foliage application and seed treatment. Thiram is also used as fruit and mushrooms disinfectant, as well as a repellent for rodents and certain large animals that cause damage to field crops, and as ingredient of medicated soaps, suntan, and antiseptic sprays. In addition, it is used as a rubber accelerator in tire industry and as a lubricating oil additive. Thiram is available as dust, wettable powder, water suspension formulations, and in mixtures with other fungicides (Dalvi *et al.*, 1988; see Extonet, 2011). Toxic and some tumorigenic effects have been observed in different animal species exposed to thiram (Dalvi, 1988; Maita *et al*., 1991; Extonet, 2011), numerous tests indicate that it is genotoxic (Crebelli *et al*., 1992; Hemavathi and Rahiman, 1996) and effects on cartilaginous tissues *in vitro* (Rath *et al.*, 1995) and *in vivo*, in different animal species exposed to dithiocarbamtes, including thiram, were reported (Enomoto *et al.*, 1989; Suzuki *et al.*, 2000, 2001; Rath *et al.*, 2004; 2007; Simsa *et al.*, 2007). The purpose of this study was to evaluate the effects of the dithiocarbamate thiram on the growth plate and articular cartilage of mammals, using Wistar rats as experimental model.

Twenty-four male Wistar rats, seven-day-old were individually marked, weighed, and allocated to three experimental groups, each with eight rats, having no significant differences in body weight (13.31±1.53g). They were housed in rat cages according to European Union (EU) recommendations and revision of Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) and maintained on a 12:12h, light/dark cycle at a constant temperature of 22°C with *ad libitum* access to water and to a standard diet. 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 (1005/92), following European Union Laboratory Animal Experimentation Regulations.

The first group was used as control and received the standard diet without any manipulation. The second group, the corn oil group, received the standard diet and 0.1mL of corn oil twice a week. The third group, the thiram group, received the standard diet and thiram (CAS n.º37-26-8, SIGMA, n.º T5638), administered by gavage at 100mg/kg-body weight suspended in 0.1mL of corn oil twice a week. The animals of all groups were weekly individually weighted, using a digital balance (0.01g).

The animals were sacrificed 35 days after the onset of the experiment, by an excess of

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anesthetic and long bones were removed by careful dissection. The left tibias were removed, the soft tissues excised and a sagital section from the proximal epiphysis was fixed in 10% neutral-buffered formalin, and subsequently decalcified in 5% formic acid during three days. The tissues were dehydrated in a graded ethanol series, cleared in xylene, and paraffin embedded. Sections with 5μm thickness were made using a rotary microtome and stained with Hematoxylin and Eosin for general structure observation and histomorphometric analyses. Growth plates were divided into resting, proliferating, and hypertrophic zones according to morphologic criteria. The height of the growth plate and its different zones and the height of the articular cartilage were calculated as the mean of three different measurements performed at three locations randomly chosen on each section with an eyepiece micrometer.

The slides were examined by light microscopy with a Nikon Eclipse 600 microscope (Kanagawa, Japan) and pictures were collected with a Nikon DN100 camera (Kanagawa, Japan). Statistical analysis was performed with NCSS97 program. Normality and homocedasticity were evaluated by Kolmogorov- Smirnov and Levene test respectively. Data analyses were performed by two factors ANOVA, using two fixed factors groups and period. For evaluation of treatment effects and interactions and whenever were found significant effects the Tukey test was used to determine the statistical significance of differences among averages. Differences in statistical analysis of data were considered significant at $P<0.05$. Data were expressed as means ± standard deviation (SD).

Body weight evolution is shown in Table 1. Statistically significant differences were found in the body weight evolution among the thiram group and the other two groups. Significant differences were not found between control and corn oil groups.

There were no differences in the height of articular cartilage (Table 2 and Figure 1).

A reduction in the height of the tibial growth plate was observed in the animals submitted to the administration of thiram (Table 2 and Figure 2). There were no differences in the relative height of the different zones of the tibial growth plate (Table 3).

Table 1. Weekly body weight evolution in grams (mean ± SD) of male Wistar rats

Group	P_0	P_1	P_2	P_3	P_4
Control	13.80±1.43a	27.96±2.07a	39.94±3.43a	61.83±7.01a	89.13±9.07a
Corn oil	12.93±1.42a	26.33±2.59a	38.01±3.48 a	56.19±8.00a	84.66±16.59a
Thiram	12.90±1.87a	20.98±3.67b	28.07±5.11b	37.00±9.38b	53.92±17.36b

Means in the same column followed by distinct letters are different ($P<0.05$).
P_0... P_4: 7.... 35-day-old.

Table 2. Articular cartilage and growth plate height (μm, mean ± SD) of male Wistar rats

Group	Articular cartilage	Resting zone	Proliferation zone	Hypertropic zone	Growth plate
Control	266.4±28.0a	32.9±4.2a	255.7±34.1a	234.6±26.0a	523.2±57.7a
Corn oil	268.7±18.9a	32.6±2.1a	252.0±23.9a	225.5±25.5a	510.1±44.5a
Thiram	289.3±22.2a	30.7±1.8a	208.6±39.1b	177.3±45.3b	416.6±79.0b

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

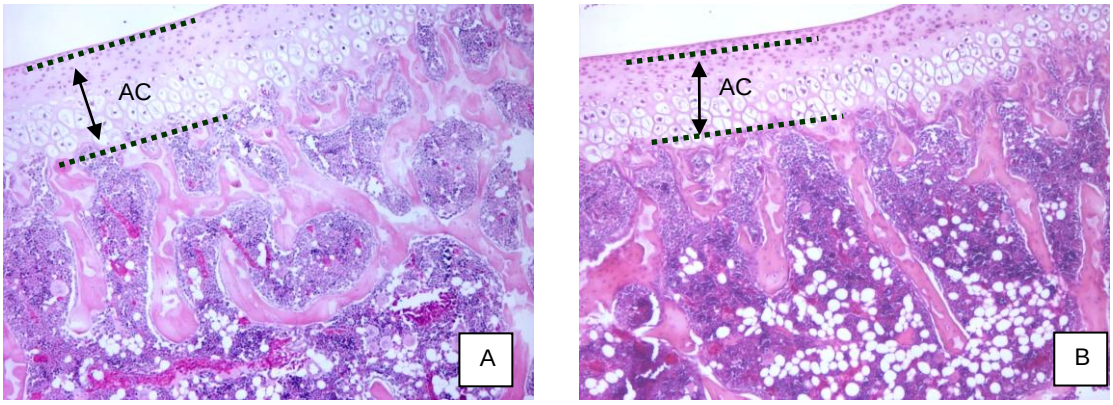

Figure 1. Articular cartilage (AC) of control group (A) and thiram group (B) (40x, H&E).

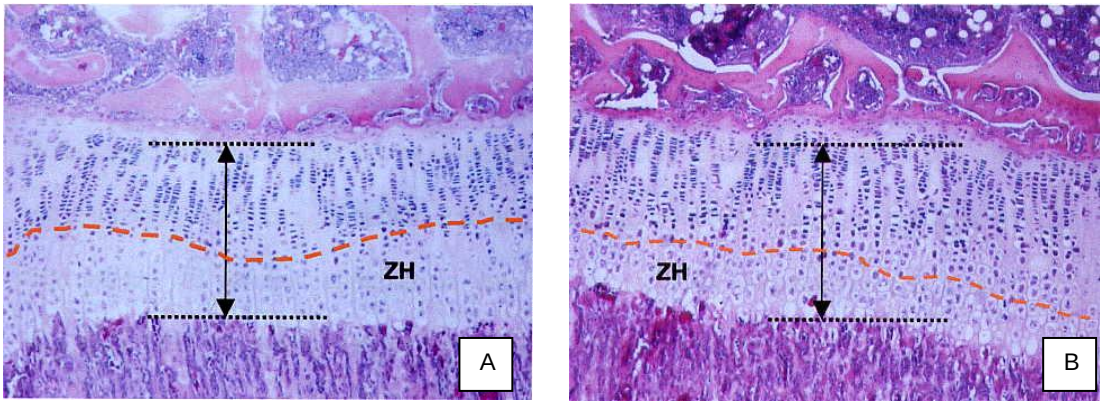

Figure 2. Growth plate of control group (A) and thiram group (B) (40x, H&E). Growth plate of control group is larger, with a greater hypertropic zone (ZH).

Table 3. Relative height (%) of the zones of the tibial growth plate (mean ± SD) in male Wistar rats

Group	Growth plate (μm)	Resting	Proliferating	Hypertropic
Control	523.2±57.7a	6.3±0.8a	48.8±2.3a	44.9±2.3a
Corn oil	510.1±44.5a	6.4± 0.5a	49.4±2.3a	44.2±2.2a
Thiram	416.6±79.0b	7.6±1.7a	50.3±4.0a	42.0±4.8a

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

In this study, the body weight decrease is in agreement with the results of other studies in which it was also observed a reduction in the growth of the animal fed diets containing different levels of thiram (Lee *et al.*, 1978 appud Dalvi, 1988; Gultart *et al.*, 1996) and ziram (Enomoto *et al.*, 1989). Several authors (Rath *et al.*, 2004; 2007; Simsa *et al.*, 2007), observed the development of tibial dyschondroplasia in poultry chicks fed a diet containing dithiocarbamates. This pathology is characterized by the development of an atypical accumulation of matrix in the proximal growth plates of tibia and tibiotarsal bones, accompanied by the failure of cartilage remodeling and bone formation.

Rath *et al.* (1995) observed a cytotoxic *in vitro* effect of thiram to chondrocytes, at high concentrations, probably due to its damaging effect on the cell membrane, which may be responsible for chondrocyte death. In the present study, histopathological examination showed no such injuries in thiram treated rats. In fact, no histological lesions were observed neither in growth plate chondrocytes nor in articular chondrocytes as well as differences in articular cartilage height. On the other hand, the growth plates exhibited well demarcated and

proportionated zones of resting, proliferation, and hypertrophy of chondrocytes and were acompained by bone trabecula formation in the metaphysic showing a normal endochondral ossification process. However, a reduction in the epiphyseal growth plate height in tibia was observed in the animals submitted to the administration of thiram, but it was an absolute decrease since when considered the proportion of the different zones of the growth plate, no differences were found among the groups. These results may be related to the dose and duration of the assay.

Enomoto *et al*. (1989) observed epiphyseal lesions of the femur and tibia in rats following oral chronic administration of ziram, but did not find significant changes in other cartilaginous tissues. Mechanisms of biological effects of dithiocarbamates include the chelation of certain important elements as copper and zinc, which are essential as cofactors for many enzymes (Dalvi, 1998), disruption in proteoglicans synthesis, collagen maturation, secondary effects in prolil-hydroxilase activity (Suzuki *et al*., 2000; 2001), and anti-angiogenic activity (Marikovsky, 2002).

In tibial dyschondroplasia, it appears that some of the early effects of thiram on the growth plate may be the failure of genes encoding VEGF receptors and Bcl-2 resulting from endothelial cell death, which compromise vascularization, cartilage remodeling, and the removal of dead chondrocytes leading to cartilage lesions (Rath *et al.*, 2005, 2007). However, the results of Gay *et al.* (2007), using the dithiocarbamate disulfiram, suggest that disruption of VEGF expression probably is not a key factor in the development of tibial dyschondroplasia.

Although dithiocarbamates might have a direct or indirect effect in cartilaginous tissues and on the chondrogenesis of the epiphyseal plate, probably affecting growth plate vascularization, there was no clear evidence in the present study suggesting disturbed endochondral ossification. The results suggest that thiram might have a chronic toxicity to mammals that can be shown affecting their growth. In future studies it, would be interesting to evaluate the molecular effects of chronic exposure to thiram in the growth, cartilage, and bone.

Keywords: rat, dithiocarbamate, thiram, growth plate, articular cartilage

RESUMO

Avaliou-se o efeito do tirame, ditiocarbamato largamente utilizado na agricultura como antifúngico e repelente de roedores, na ossificação endocondral de mamíferos, usando, como modelo, ratos Wistar. Não foram observadas lesões na cartilagem articular, nem nas placas de crescimento, o que pode ser atribuído à dose utilizada e à duração do ensaio. A diminuição da altura da placa de crescimento nos animais aos quais foi administrado o tirame parece traduzir o atraso verificado no crescimento em geral, e não um efeito específico na cartilagem, uma vez que as diferentes zonas da placa epifisária mantiveram as proporções dos animais do grupo-controle. Embora não tenham sido verificados, no presente trabalho, os efeitos registrados para outras espécies nos tecidos cartilaginosos, sugere-se a avaliação dos efeitos crónicos do tirame no crescimento e no desenvolvimento dos ossos longos em mamíferos.

Palavras-chave: rato, ditiocarbamatos, tirame, placa de crescimento, cartilagem articular

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