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Blood Parameters and Toxicity of Chromium Picolinate Oral Supplementation in Lambs

Bruno Stéfano Lima Dallago[1,7] · **ShélidaVasconcelos Braz**[2] · **Tatiana Guerrero Marçola**[3] · **Concepta McManus**[1,7] · **Denise Ferreira Caldeira**[4] · **Aline Campeche**[5] · **Edgard Franco Gomes**[5] · **Tiago Prado Paim**[6] · **Bárbara Oliveira Borges**[1] · **Helder Louvandini**[5,7]

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Abstract The effects of oral supplementation of chromium picolinate (CrPic) on various blood parameters and their possible toxicity on the liver, kidneys, lungs, heart, and testis were investigated. Twenty-four Santa Inês (SI) lambs were treated with four different concentrations of CrPic (six animals/treatment): placebo, 0.250, 0.375, and 0.500 mg CrPic/animal/day for 84 days. The basal diet consisted of hay *Panicum maximum* cv Massai and concentrate. Blood and serum were collected fortnightly for analysis. On day 84, the animals were euthanized, and histopathological analysis in the liver, kidney, heart, lung, and testis was made. The liver and kidney were also submitted to electronic microscopy analysis. Differences between treatments ($P<0.05$) were observed for packed cell volume (day 84), hemoglobin (day 84), total plasm protein (day 56 and day 84), and triglycerides (day 70). There was no statistically significant relationship between Cr supplementation and histopathology findings, although some animals treated with supplementary Cr showed morphological changes in the liver, kidney, and testis. Thus, the effectiveness of supplementation with Cr remains in doubt as to its physiological action and toxicity in sheep.

Keywords Hemogram · Leukogram · Serum biochemistry · Mineral supplement

Introduction

Chromium picolinate (CrPic) is a chelated compound used as food supplement for humans and animals. The picolinic acid

✉ Bruno Stéfano Lima Dallago
dallago@unb.br

ShélidaVasconcelos Braz
shelidabraz@gmail.com

Tatiana Guerrero Marçola
tatigmarcola@hotmail.com

Concepta McManus
concepta@unb.br

Denise Ferreira Caldeira
denise_caldeira@hotmail.com

Aline Campeche
aline.campeche@gmail.com

Edgard Franco Gomes
icedtoad@gmail.com

Tiago Prado Paim
pradopaim@hotmail.com

Bárbara Oliveira Borges
barbaraoliveirab@hotmail.com

Helder Louvandini
louvandini@cena.usp.br

[1] Faculdade de Agronomia e Medicina Veterinária/UnB, Campus Darcy Ribeiro, ICC-Sul, Brasília, DF, Brazil 70910-900

[2] Laboratório de Microscopia Eletrônica, Insitituto de Ciências Biológicas/UnB, Campus Darcy Ribeiro, Brasília, DF, Brazil 70910-900

[3] Laboratório de Patologia Clínica Veterinária/UnB, Campus Darcy Ribeiro, ICC-Sul, Brasília, DF, Brazil 70910-900

[4] Diretoria de Defesa e Vigilância Agropecuária, Secretaria de Agricultura e Desenvolvimento Rural—SEAGRI/DF, Parque Estação Biológica—Asa Norte, Brasília, DF, Brazil 70770-914

[5] Centro de Energia Nuclear na Agricultura/USP, Av. Centenário, no 303, Caixa Postal 96, Piracicaba, SP, Brazil 13400-970

[6] Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Iporá, Av. Oeste s/n, Iporá, GO, Brazil 76200-000

[7] Instituto Nacional de Ciência e Tecnologia-Informação Genético-Sanitária da Pecuária Brasileira (CNPq/INCT/IGSPB), Belo Horizonte, MG, Brazil

(PIC), the organic part of CrPic, presents excellent chelation properties being used widely as a mean of introducing bioactive metals into biological systems. In these formulations, PIC is generally considered the non-active ingredient, and its biological function as well as its toxicological activity are not well established [1]. The chromium (Cr), in turn, have been demonstrated that should no longer be considered an essential element [2], but the addition of supranutritional amounts of Cr to the diet showed a pharmacological effect increasing insulin sensitivity. Thus, supplemental Cr can increase insulin sensitivity and interfere with general metabolism and other body processes [3]. However, sheep have a rumen, a fermentation chamber which is a primary compartment of the digestion process, and thus, these interferences may be different from those observed in monograstric animals.

Therefore, the use of Cr as a nutritional supplement presents some information gaps, especially about its safety: Cr is a heavy metal, it may be toxic and cause genetic damage, impairment to gametes, and general metabolic interference on essential function or can generate free radicals [4–7] increasing the oxidative stress in the animal body. Some researchers have shown that the study of Cr as dietetic supplement is important as some toxicological effects (DNA cleavage, increase of oxidative stress, and reproductive impairments) are associated to its use [8–10]. In addition, Dallago et al. [11] highlighted the negative aspects of the use of CrPic supplements to lambs in ruminal protozoa and, moreover, Cr concentration increased in the heart, lungs, and testis after CrPic oral supplementation (Dallago et al., submitted). As these tissues could be used for human consumption or are essential to the reproduction of the species, it is imperative to evaluate the consequences of Cr on these organs.

Despite attempts to warn farmers about the risks of Cr use without scientific support [11, 12], indiscriminate use of mineral formulas with Cr as a dietetic supplement persists. Thus, the evaluation of possible toxicological effects of oral CrPic supplementation in animal production is crucial, as this product may affect the health of livestock, and also enter the food chain. Therefore, this study aims to investigate the effects of CrPic oral supplementation in lambs on blood parameters and possible toxicity on target organs previously identified as prone to Cr accumulation.

Material and Methods

This study was approved by the Ethics Committee for Animal Use (CEUA/UnB no. 033/2009) and was carried out at the Sheep Management Center of the University of Brasília, in the Federal District, Brazil. Twenty-four, 14-week-old Santa Inês (hair) male lambs were used. The initial mean body mass was 22.89±2.23 kg. The lambs were assigned to four treatments (six animals/treatment) with different dosages of CrPic offered in capsules: placebo, 0.250, 0.375, and 0.500 mg of CrPic/animal/day giving, respectively, a total amount of 0.025, 0.296, 0.399, and 0.702 mg of Cr/kg of dry matter. Thus, daily CrPic was about 0.8, 9.0, 12.0, and 21.0 μg per kilogram of body mass. For Cr measurements in food, water, and capsules, an inductively coupled plasma atomic emission spectrometer (model Thermo Jarrel Ash IRIS/AP®) was used. Details on Cr measurements, total amount of Cr intake, and Cr content in the basal diet are available in Dallago et al. [11]. These doses were established according to formulas used by the companies that produce mineral supplements in Brazil, where mineral supplementation formulation has 12 mg of Cr/kg of mineral salt with a daily mean intake of 20 g of salt/animal/day. This supplementation gives a total of 0.240 mg of Cr/animal/day.

Lambs were kept in individual pens for a 2-week adaptation period and 84 days of CrPic supplementation. They were fed with *Panicum maximum* cv Massai hay and concentrate (85 % cassava flour, 11.5 % mineral salt, and 3.5 % urea) maintaining 10 % orts (ad libitum). Water was also offered ad libitum. Before the start of the trial, all animals were treated with Levamisol P.O. (Fort Dodge Ripercol-L®—1 mL/10 kg of body weight). Clinical exams were carried out by a veterinarian three times during the trial (before—on the first day of adaptation period, in the middle—day 35, at the end of the study—day 83, 1 day before the euthanasia). This included the pulmonary system, superior airways, cardiac auscultation and temperature measurements, mucous color evaluation, and coprology exam.

Blood samples were collected by jugular venipuncture in tubes with and without EDTA (both Vacutainer®) fortnightly. Blood parameters analyzed included packed cell volume—PCV (%)—obtained using a micro centrifuge, total plasma proteins—TPP (g/100 mL)—using refractometer, and number of erythrocytes—HEM ($\times 10^6$/mm^3)—and concentration of hemoglobin—Hb (g/100 mL)—that were carried out using an automatic counter (ABCVet-ABX®, Montpellier, Héraut, France). Hematimetric parameters were determined by calculation (mean corpuscular volume (fL)—MCV, mean corpuscular hemoglobin (pg)—MCH, and mean corpuscular hemoglobin concentration (%)—MCHC). For biochemical analysis, glucose (GLU), cholesterol (COL), high-density lipoproteins (HDL), triacylglycerol (TRI), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), serum urea (BUN), and creatinine (CREAT) serums were obtained by centrifugation of tubes without EDTA at 2000 rpm for 5 min. Then, serum was frozen at −20 °C until analysis to measure the concentration of these metabolites by spectrophotometry using specific kits (LABTEST®, Lagoa Santa, MG, Brazil).

At the end of trial, all animals were euthanized, and samples from the liver, kidney, lungs, testis, and heart were fixed in 10 % formol buffered until dehydration in series of ascending ethanol concentrations (70–100 %), clarified in xylene,

Table 1 Interaction between supplementary CrPic and time on blood parameters of control and CrPic-supplemented SI lambs

Day	Treatment (mg of CrPic/day)				SD_m	Significance	Reference value[a] (mean)
	Placebo	0.250	0.375	0.500			
Packed cell volume (%)							
0	32.5	33.8	32.7	32.8	2.72	ns	27–45 (35)
14	33.0	34.2	37.7	34.7	2.76	ns	
28	33.2	33.3	36.8	34.5	4.01	ns	
42	31.5	31.7	33.8	30.7	2.44	ns	
56	31.1ab	30.3b	35.1a	31.3a	2.64	0.0249	
70	31.8	30.8	33.0	32.3	2.65	ns	
84	28.0a	29.0ab	31.6b	31.5ab	2.10	0.0210	
Erythrocytes ($\times 10^6/\mu L$)							
0	11.3	11.8	13.3	12.3	1.50	ns	9–15 (12)
14	13.2	12.9	11.6	12.2	2.28	ns	
28	13.0	11.1	14.2	11.5	2.78	ns	
42	11.9	11.9	12.3	11.8	1.50	ns	
56	12.7	12.0	14.5	12.9	1.48	ns	
70	12.9	12.7	12.3	11.5	1.90	ns	
84	10.8	11.9	11.8	12.3	1.73	ns	
Hemoglobin (g/100 mL)							
0	11.05	11.50	11.11	11.16	0.92	ns	9–15 (11.5)
14	11.22	11.62	12.81	11.79	0.94	ns	
28	10.7a	10.9ab	12.3b	11.2ab	0.95	0.0322	
42	10.71	10.77	11.50	10.43	0.83	ns	
56	11.65ab	11.45a	13.1b	11.5a	0.93	0.0161	
70	11.93	11.82	12.50	12.33	1.04	ns	
84	8.3a	8.8ab	9.9b	10.1b	0.93	0.0114	
MCV (fL)							
0	29.22	29.35	24.71	27.04	3.96	ns	28–40 (34)
14	25.31	27.19	33.42	30.03	5.70	ns	
28	26.56	30.23	27.36	30.44	4.43	ns	
42	26.65	26.71	27.57	26.45	2.29	ns	
56	24.53	25.41	24.57	24.87	3.26	ns	
70	24.76	25.37	27.03	28.14	3.34	ns	
84	26.52	24.71	26.89	26.91	4.49	ns	
MCHC (g/100 mL)							
0	34.0	34.0	34.0	34.0	0.00	ns	31–34 (32.5)
14	34.0	34.0	34.0	34.0	0.00	ns	
28	32.6	32.8	34.2	33.0	4.10	ns	
42	34.0	34.0	34.0	34.0	0.00	ns	
56	37.3	38.1	37.6	36.8	2.68	ns	
70	37.5	38.4	38.0	38.5	3.08	ns	
84	29.8	30.4	31.4	32.2	2.68	ns	
MCH (pg)							
0	9.9	10.0	8.4	9.2	1.35	ns	8–12 (10)
14	8.6	9.2	11.4	10.2	0.62	ns	
28	8.7	9.9	9.2	10.1	1.80	ns	
42	9.1	9.1	9.4	9.0	0.78	ns	
56	9.2	9.7	9.2	9.2	1.41	ns	
70	9.3	9.7	10.3	10.8	1.53	ns	
84	7.9	7.5	8.4	8.7	1.75	ns	

Table 1 (continued)

Day	Treatment (mg of CrPic/day)				SD_m	Significance	Reference value[a] (mean)
	Placebo	0.250	0.375	0.500			
TPP (g/100 mL)							
0	5.9	5.9	5.8	6.1	0.34	ns	6–7.5
14	6.5	6.4	6.1	6.6	0.30	ns	
28	6.5	6.5	6.3	6.7	0.29	ns	
42	6.1	5.9	6.0	6.2	0.32	ns	
56	6.0	6.1	6.1	6.5	0.38	ns	
70	6.4	6.3	6.2	6.5	0.33	ns	
84	6.0a	5.8a	6.0a	6.8b	0.28	<0.0001	

Different letters (a, b) in the same row means significantly different using *F* test ($P<0.05$)

SD_m medium standard deviation, *ns* not significant

[a] According to Jain [17]

and embedded in Histosec® (ref. 115161, Merck Millipore/Brazil). Semi-serial sections were cut (5 μm each) and stained with hematoxylin and eosin (H&E). Sections were mounted on glass slides and covered with cover slips. Slides were visualized and analyzed using a Leica microscope model DM1000 (Leica Microsystems, Switzerland) and digitally photographed using a Leica DFC280 camera and Leica Application Suite Version 2.7.0 2003–2007 software (Leica Microsystems, Switzerland). For each tissue, three slides were evaluated for the presence/absence and intensity (+, ++, or +++) of pathological lesions typically involved in heavy metal intoxication or damage that could be inferred as Cr-induced lesions.

Immediately after euthanasia, liver and kidney fragments were rinsed with phosphate-buffered saline (PBS) (pH 7.2) and then cut into small sections of about 1 mm^3. Tissues were fixed in a solution containing 2.5 % glutaraldehyde, 5 mM CaCl, and 5 % sucrose in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C overnight. Samples were then post-fixed for 1 h in osmium tetroxide. Material was dehydrated in a series of ascending acetone concentrations and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Finally, material was analyzed using a JEOL® 1011C Transmission Electron Microscope (Jeol®, Japan) and digitally photographed using Gatan Digital Micrograph®.

Data were analyzed in a completely randomized design with total Cr levels as the independent source of variation. PROC GLM and PROC REG (to the pertinent regressions—linear or quadratic) were carried out using Statistical Analysis System (SAS® v.9.3, Cary, NC, USA) at 5 % of significance level. For histopathology analysis, the frequency of tissue lesion was used to evaluate statistical difference by the Fischer's exact test with 5 % of significance level. For data measured more than once during the trial (blood parameters), PROC MIXED and a repeated-measure analysis were carried out and means were compared by multiple comparison adjustment for Tukey test ($P<0.05$). Parameters with high coefficient of variance were previously transformed using log or radical transformations.

Results

Clinical exams showed no alterations in clinical aspects, all remaining within the normal standard ranges for this species. PCV, TPP, and Hb showed a significant interaction between time and treatment (Table 1). When the effect of time was excluded, on day 84, a positive linear relationship was observed between PCV and CrPic treatment ($P=0.0082$) and between Hb and CrPic treatment ($P=0.0024$). Depending on the treatment, a quadratic positive effect was observed on TPP ($P<0.0001$) on days 56 and 84. Time on experiment influenced PCV, Hb, MCHC ($P<0.0001$), and MCV ($P=0.0242$), but without a specific profile throughout time. HEM and MCH did not show differences between treatments and time effect.

Supplementary CrPic did not present a significant effect on serum glucose, cholesterol, and HDL concentration. An interaction between treatment and time was observed on days 70 and 84 for TRI ($P=0.0029$ and $P=0.0347$, respectively) (Table 2). Serum concentrations of AST, GGT, BUN, and CREAT were not modified by CrPic addition to the diet (Table 3). Time on experiment influenced ($P<0.0001$) all biochemical traits (AST, GGT, BUN, and CREAT), but no specific profile throughout time was observed.

Under light microscopy, the analysis of tissue from control animals (placebo group) showed normal morphological architecture except for the lungs. For this tissue, independent of supplementary CrPic added to diet, extended bronchoalveolar

Table 2 Interaction between supplementary CrPic and time on serum glucose, cholesterol, high-density lipoproteins (HDL), and triacylglycerol of control and CrPic-supplemented SI lambs

Day	Treatment (mg of CrPic/day)				SD_m	Significance	Reference value[a] (mean±SD)
	Placebo	0.250	0.375	0.500			
Glucose (mg/100 mL)							
0	66.3	65.0	64.2	64.0	10.4	ns	50–80 (68±6)
14	59.0	59.2	61.7	61.7	5.0	ns	
28	60.8	61.2	67.7	61.8	9.5	ns	
42	42.0	46.8	48.5	49.8	13.7	ns	
56	67.3	58.3	63.7	56.7	9.0	ns	
70	62.0	63.2	72.0	75.8	13.8	ns	
84	51.3	47.7	52.7	50.7	5.0	ns	
Cholesterol (mg/100 mL)							
0	29.8	39.3	40.0	36.5	8.9	ns	52–76 (64±12)
14	29.3	29.0	30.7	33.7	8.0	ns	
28	32.8	37.5	41.2	37.7	8.3	ns	
42	33.3	34.8	38.8	35.2	5.7	ns	
56	33.5	33.0	38.5	32.2	8.5	ns	
70	37.7	34.2	41.5	34.8	7.0	ns	
84	57.2	50.3	59.2	52.7	10.3	ns	
HDL (UI/L)							
0	19.7	21.9	23.8	25.1	5.6	ns	–
14	158.8	147.2	160.6	148.1	45.0	ns	
28	123.4	123.4	140.8	141.7	25.1	ns	
42	117.6	110.7	127.7	114.8	20.6	ns	
56	18.8	19.7	19.5	16.4	3.9	ns	
70	133.7	117.6	138.8	132.4	32.3	ns	
84	12.3	12.4	14.9	14.7	5.1	ns	
Triacylglycerol (mg/100 mL)							
0	75.5	107.8	319.7	80.5	216.6	ns	–
14	10.2	9.3	12.2	12.2	4.7	ns	
28	16.7	82.7	166.2	34.2	112.7	ns	
42	11.5	15.3	15.0	17.0	5.5	ns	
56	22.2	20.7	26.3	20.2	15.8	ns	
70	21.0a	14.0b	19.7ab	23.5a	3.7	0.0029	
84	21.3a	11.0b	19.0a	11.2b	6.3	0.0347	

Different letters (a, b) in the same row means significantly different using *F* test ($P<0.05$)

SD_m medium standard deviation, *ns* not significant, *SD* standard deviation

[a] According to Kaneko et al. [16]

lymphoid tissue (BALT) and increased thickness of alveolar septum were observed (Table 4) (Fig. 1).

Heart muscle did not show any histopathologic lesions in any experimental group. Liver of animals from control and lower CrPic dose did not present tissue damage (Fig. 2a, b). However, one animal (16 %) supplemented with 0.500 mg of CrPic/day presented liver damage with marked vacuolization of parenchyma in the periportal region (Table 4, Fig. 2d). In addition, lymphocytic infiltrate cells could be seen in this region (Fig. 2d). Also, in lambs supplemented with 0.375 mg of CrPic/day, one (16 %) showed a liver with diffuse regions containing some inflammatory lymphocytic infiltrate (Table 4) but normal liver architecture, without signs of hepatobiliary damage (Fig. 2c).

Under transmission electron microscopy, no ultrastructural changes in the liver of control animals or those supplemented with 0.250 mg of CrPic/day were observed (Fig. 3a). The liver of animals supplemented with 0.375 mg of CrPic/day (Fig. 3b) showed a vacuolated cytoplasm with many electron-dense materials inside fat vesicles which could suggest chromium accumulation in the liver. On the other hand, no nucleus and other cytoplasm changes were seen. In the

Table 3 Interaction between supplementary CrPic and time on AST, GGT, BUN, and CREAT of control and CrPic-supplemented SI lambs

Day	Treatment (mg of CrPic/day)				SD_m	Significance	Reference value[a] (mean±SD)
	Placebo	0.250	0.375	0.500			
AST (UI/L)							
0	188.5	156.9	273.0	180.1	88.9	ns	60–280 (307±43)
14	78.9	83.6	86.3	97.5	19.8	ns	
28	94.7	89.1	108.6	121.6	20.3	ns	
42	131.8	134.6	167.1	163.4	42.4	ns	
56	82.6	77.0	89.1	92.8	22.9	ns	
70	98.4	103.9	106.8	107.7	26.8	ns	
84	86.3	83.6	91.0	66.8	27.4	ns	
GGT (UI/L)							
0	29.2	31.3	29.3	40.8	9.8	ns	20–52 (33.5±4.3)
14	32.3	27.2	25.2	37.7	11.2	ns	
28	41.8	47.0	45.0	51.5	13.3	ns	
42	33.5	36.7	37.7	39.8	12.3	ns	
56	41.0	42.8	38.5	55.8	18.5	ns	
70	30.2	39.7	27.0	32.3	9.5	ns	
84	20.8	24.2	27.2	25.0	8.8	ns	
Serum urea (mg/100 mL)							
0	50.0	45.0	47.8	40.0	13.5	ns	8–20
14	26.3	15.2	19.7	16.8	8.7	ns	
28	27.5	18.7	19.7	22.5	8.4	ns	
42	35.2	30.2	33.8	36.7	6.5	ns	
56	28.7	23.5	23.5	22.8	5.5	ns	
70	29.7	31.2	26.0	27.2	4.8	ns	
84	26.3	21.3	23.5	21.7	4.8	ns	
Creatinine (mg/100 mL)							
0	0.6	0.6	0.6	0.6	0.1	ns	1.2–1.9
14	0.5	0.5	0.5	0.5	0.1	ns	
28	0.6	0.6	0.6	0.5	0.1	ns	
42	0.7	0.7	0.7	0.6	0.1	ns	
56	0.7	1.1	0.8	0.9	0.3	ns	
70	0.6	0.6	0.7	0.7	0.1	ns	
84	0.5	0.6	0.6	0.5	0.2	ns	

SD_m medium standard deviation, *ns* not significant, *SD* standard deviation

[a] According to Kaneko et al. [16]

same manner, the liver of animals supplemented with 0.500 mg of Cr/day (Fig. 3c) showed vacuolated cytoplasm with electron-dense structures. However, no ultrastructural changes in the nucleus and plasmatic membrane were observed (Fig. 3c).

The kidneys were, in general, healthy (Fig. 4a). The exception was some glomeruli showing increased thickness of Bowman capsule from two (33 %) animals supplemented with 0.500 mg of CrPic/day (Fig. 4b, Table 4). Under electron microscopy, no structural modification was detected in any sample.

In the testis, the extensive retraction of Leydig cells was observed in all samples which could be an artifact, as all testes were fixed in 10 % buffered formol. Nevertheless, morphology and normal architecture of seminiferous tubules were preserved (Fig. 5a, b). Different levels of testicular maturity were observed between animals, and one animal (16 %) supplemented with 500 mg of CrPic/day did not show spermatogenesis, and only one or two layers of primordial cells in the seminiferous epithelium (Fig. 5c, d, Table 4) could be seen. As animals were 28 weeks old and puberty in Santa Inês lambs is seen 28.2±0.8 weeks [13], and the seminiferous tubules of this animal were pervious and had a large lumen, therefore, the possibility of this animal not reaching puberty can be put away [14]. Thus, this animal should present some degree of spermatogenesis, and actually, this was not observed.

Table 4 Histopathology summary of tissue evaluation from control and CrPic-supplemented SI lambs

Tissue	Lesion observed		Treatment (mg of CrPic/day)				*P* value
			Placebo	0.250	0.375	0.500	
Lung	BALT	Presence Intensity	6/6 (100 %) +++	6/6 (100 %) +++	6/6 (100 %) +++	6/6 (100 %) +++	ns
	Alveolar septum swollen	Presence Intensity	6/6 (100 %) +++	6/6 (100 %) +++	6/6 (100 %) +++	6/6 (100 %) +++	ns
Liver	Lymphocytic infiltrate cells	Presence Intensity	0/6 (0 %) −	0/6 (0 %) −	2/6 (33 %) +	1/6 (16 %) +	ns
	Periportal vacuolization	Presence Intensity	0/6 (0 %) −	0/6 (0 %) −	0/6 (0 %) −	1/6 (16 %) ++	ns
Kidney	Increased thickness of Bowman's capsule	Presence Intensity	0/6 (0 %) −	0/6 (0 %) −	0/6 (0 %) −	2/6 (33 %) +	ns
Testis	Absence of spermatogenesis	Presence Intensity	0/6 (0 %) −	0/6 (0 %) −	0/6 (0 %) −	1/6 (16 %) +++	ns

+, ++, and +++—lesion intensity

P value significance, *BALT* bronchoalveolar lymphoid tissue, *ns* not significant

Discussion

Blood is responsible for essential functions of the life of complex organisms and has a high potential for cell proliferation and high susceptibility to intoxication. Thus, hematopoietic tissue and blood per se are target organs for potential toxicants. According to Klaassen [15], the blood, liver, and kidneys are the major organs used to evaluate toxicants for populations exposed to potentially toxic elements, such as Cr. The capability of proliferation and regeneration makes the blood particularly sensitive to antimitotic agents and to secondary effects of substances which can impair the nutritional supply to the animal, the excretion of toxins and metabolites, and the production of vital factors for homeostasis. However, at least with the doses and time used here, no signs of toxicity of CrPic on hematopoietic tissue could be seen: with exception to marginally reduced TPP and Hb, no noteworthy change was observed. This is not an exception: although using rats and other Cr formulations, studies aiming to evaluate Cr toxicity, generally, report no consistent dosage-dependent results on blood parameters [16–18].

The differences registered between treatments for red blood cell traits are not consistent as they did not present specific profile throughout time or they were only observed on the last day of supplementation. Thus, trends in increase or decrease in values for any blood parameter did not consolidate over the time. In the same way, parameters whose differences were detected only in the last experimental day do not support extrapolation merely speculative. Therefore, inferences on maintenance or consolidation of these differences could not be extrapolated beyond 84 days of CrPic supplementation. Complementarily, Dallago et al. [12] report no direct effect of CrPic supplementation on white cell production. Therefore, our results corroborate with the need, pointed by Hepburn and Vincent [19], that studies evaluating the chronic effects of CrPic supplementation are important. On the other hand, our results suggest subtle advantages for animals supplemented with Cr; for example, raised PCV (Table 1) or TPP (Table 1) represent, at least in principle, lower propensity to anemia and dysproteinemia, respectively [20].

Hb values were all within the reference values for sheep (9–15 g/100 mL, according to Jain [21]). Nevertheless, increased Hb concentration represents higher capacity to carry oxygen to tissues, and at least on day 84, animals supplemented with CrPic had an advantage when compared with control animals. Maybe this could influence some carcass quality traits such as flavor or tenderness, as oxygen plays an important role in meat pH [22]. However, meat quality was not evaluated here. Regardless, the increased MCHC on days 56 and 70 may be due to in vitro hemolysis, as true hyperchromia (raised Hb quantity in erythrocytes) does not exist [23] and values for this parameter were uniformly increased in all treatments.

The link between Cr supplementation, carbohydrate metabolism, and glycemia is undeniable [24, 25]. Uyanik [26] observed slight reductions in glycemia of lambs supplemented with 200 μg of Cr/kg of diet fed as $CrCl_3$, while Zanetti et al. [24] showed a tendency to excrete glucose faster in supplemented animals. Additionally, Zhou et al. [27] observed a significant decrease of serum insulin in lambs supplemented with yeast Cr, leading to an increased ratio of glucose to insulin in supplemented animals. On the other hand, Kitchalong et al. [28] reported similar results as seen here when growing lambs were supplemented with 0.250 mg of CrPic/kg. It is noteworthy that in these last three experiments, serum glucose

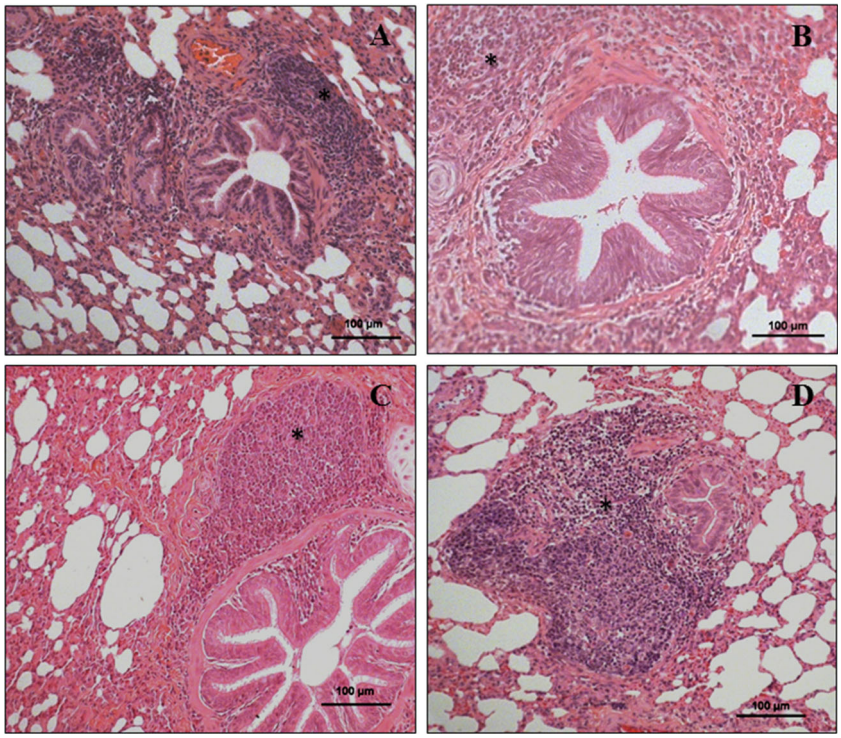

Fig. 1 Lungs of control animal (**a**), supplemented with 0.250 mg of CrPic/day (**b**), 0.375 mg of CrPic/day (**c**), and with 0.500 mg of CrPic/day (**d**). *Asterisk* means bronchus-associated lymphoid tissue (BALT). Observe the increased thickness of septum in all treatments. ×20 magnified, H&E

concentration did not change during supplementation, although Zhou et al. [27] reported a decreased glucose trend in Cr-supplemented lambs.

As energetic metabolism is a complex issue, especially in ruminants, and considering the differences in methodology between experiments and also the blurred line between what can considered stress or not, controversial conclusions between experiments may occur. Thus, while Zhou et al. [27] observed high insulin sensitivity (by decreased serum insulin) in supplemented animals, here, there was no glucose effect

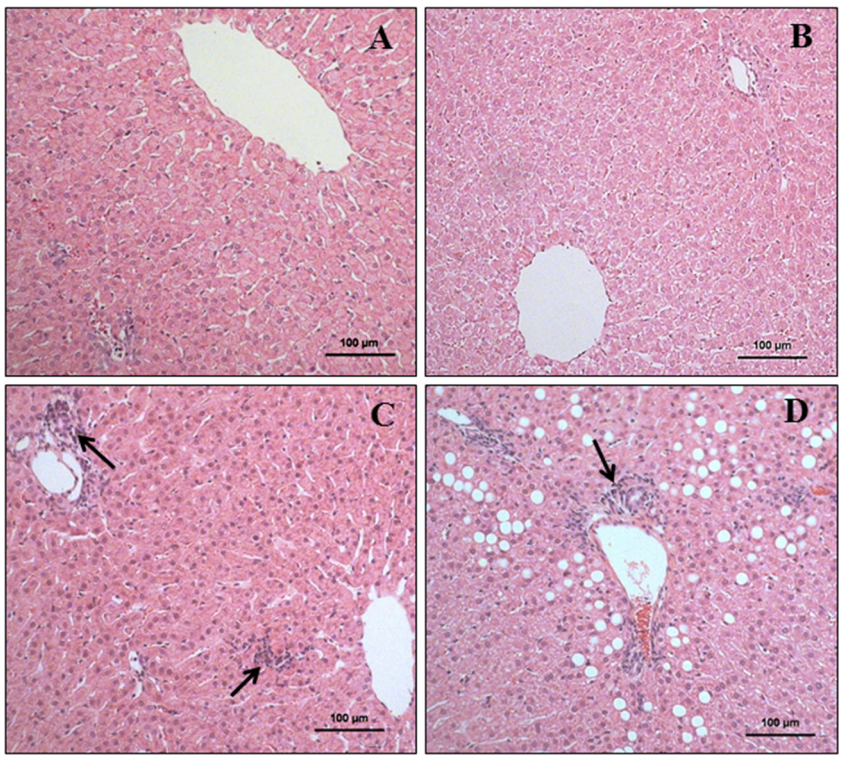

Fig. 2 Liver from control (**a**), supplemented with 0.250 mg of CrPic/day (**b**), 0.375 mg of CrPic/day (**c**), and 0.500 mg of CrPic/day (**d**). *Arrows* indicate lymphocytic inflammatory infiltrate cells. In **d**, marked vacuolization in periportal region from one (16 %) animal supplemented with 0.500 mg of CrPic/day. H&E, ×20 magnified

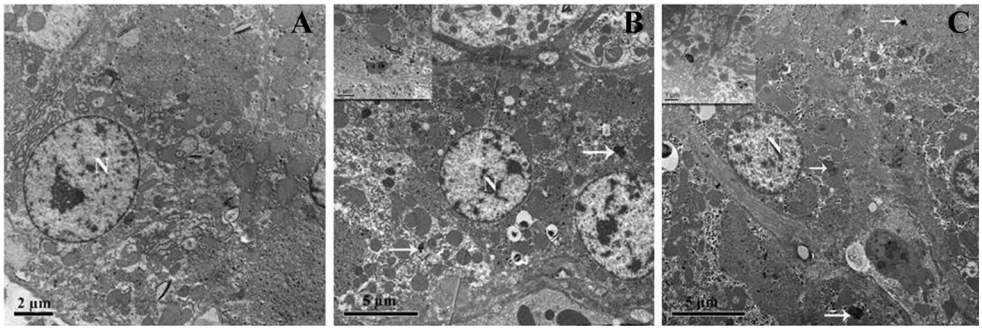

Fig. 3 Liver of control animal (**a**), supplemented with 0.375 mg of CrPic/day (**b**), and supplemented with 0.500 mg of CrPic/day (**c**). In **b** and **c**, note the vacuolated cytoplasm with abundant electron-dense particles inside fat vesicles (*arrow*). *Inset* fat vesicles with electron-dense material inside. *N* nucleus

after supplementation, but serum insulin was not measured. Therefore, our results corroborate with the hypothesis of Dallago et al. [11] that the effects on glucose of Cr supplementary are only apparent when a stress factor is involved. When there is no stress, supplementary Cr does not significantly affect glucose metabolism [11, 29]. According to Dallago et al. [11], this may be associated with its possible role in facilitating insulin action. When serum cortisol concentration is higher, more glucose is needed by the cells. This causes an increase in the use of Cr by insulin-sensitive cells, and in consequence, Cr excretion via urine increases, depleting body Cr. This lost Cr may be replaced by supplemental Cr, or if the animal is not supplemented, the entrance of glucose into the cell may be impaired by the lack of Cr. On the other hand, when a stress factor is not associated, the demand for Cr decreases and supplemental mineral is not necessary.

This hypothesis can be used to explain the lack of differences between treatments on serum cholesterol and HDL concentrations. However, it cannot explain the effects observed on TRI. Kitchalong et al. [28] suggest the requirement of Cr for normal fatty acid metabolism. According to Lefavi et al. [30], Anderson [31], and Zhou et al. [27], Cr supplementation reduces total cholesterol concentration and increases the proportion of HDL, with consequent reduction of LDL and TRI. In addition, Uyanik [26] observed reduction in TRI concentration in lambs treated with Cr. On the other hand, Sano et al. [32] found no changes in serum glucose and non-esterified fatty acids in supplemented sheep, and Zhou et al. [27] reported higher concentration of serum free fatty acids and lower serum triglycerides in supplemented animals. Hence, effects of Cr on glucose and fatty acid modulation are inconsistent between experiments. Lefavi et al. [30] disagree with other authors and postulate that Cr effects on fatty acid metabolism are independent of the effects of Cr on glucose metabolism. This conclusion can explain the results observed here for TRI. However, this concept is, at least, weak and open to criticism, as lipid metabolism depends on carbohydrate metabolism.

Hepatic enzyme (AST and GGT) values remained within the reference values, except on day 56 for animals supplemented with 0.500 mg of CrPic/day, removing the hypothesis of liver damage caused by Cr supplementation. Even in those animals with increased values on day 56, the quick return to normality of GGT values (day 70) reflects the possibility of exogenous reasons that caused this increase. Moreover, ultrastructural analysis of the liver could lead one to misplaced conclusions thinking that the electron-dense material visualized in the liver is Cr deposits, especially due to the association between supplementary Cr dose and the quantity of electron-dense material visualized (higher doses presented more electron-dense particles). This association was not supported by quantification of Cr in the liver by ICP-MS (Dallago et al., submitted), light microscopy analysis, and leakage enzyme quantification. However, the deposits of electron-dense material observed were thought to be Fe^{3+} (iron), as there exists a relationship between hemochromatosis, overload of

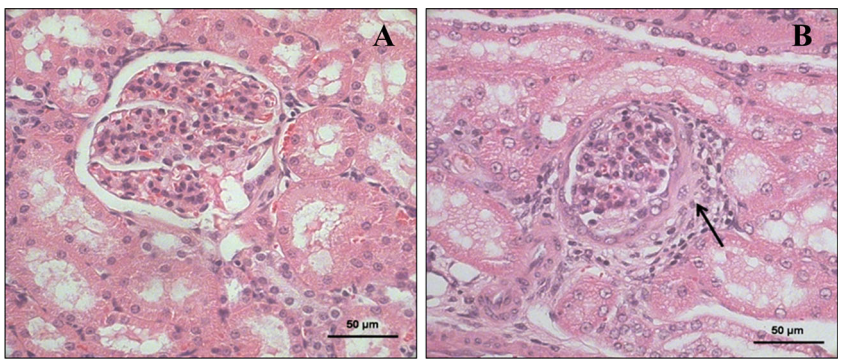

Fig. 4 Glomeruli from control animal (**a**) and supplemented with 0.500 mg of CrPic/day (**b**). *Arrow* indicates increased thickness of Bowman capsule. ×40 magnified, H&E

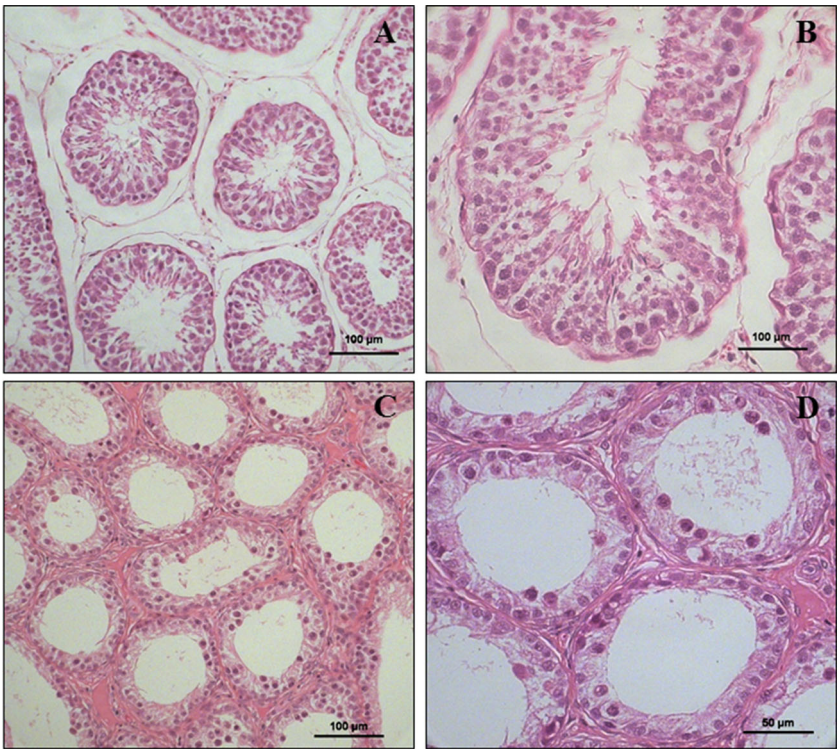

Fig. 5 Testis from control animal (**a**—×20 magnified and **b**—×40 magnified) and testis from one animal (16 %) supplemented with 0.500 mg of CrPic/day (**c**—×20 magnified and **d**—×40 magnified). Observe the absence of spermatogenesis in previous seminiferous tubules (**c**, **d**). H&E

liver iron, insulin, transferrin, and Cr. This relationship is well reported by Vincent [33].

In addition, histopathology analysis reinforces the biochemistry analysis of serum for both liver and kidney. Thus, although many studies point to Cr as a toxic element [10, 19, 34], we found no statistical proof of Cr toxicity here. However, even though the damage observed is minimal and in few animals, it still exists. In this sense, we strongly recommend caution and, at least for the moment, to avoid the use of CrPic as dietetic supplement for animal production. Thus, as some supplemented animals showed liver damage, kidney lesions, and, especially, testes alterations, this indicates the need for further studies on Cr toxicity, with longer experiments (>84 days).

Lung lesions were possibly due to food presentation: concentrate was offered in the form of flour, with low granulometry, being easily inspired. Thus, the small particles of concentrate could elicit a local immunogenic response, causing high proliferation of BALT. This explains why control animals also showed extensive BALT.

High values of BUN observed in all treatments are in line with a high level of urea added to the diet (3.5 %). In this way, the uptake of urea nitrogen is also increased. CREAT is a secondary product of protein metabolism. A low quantity of serum CREAT is observed when there is muscle catabolism, insufficient dietetic intake of proteins, or concurrent hepatic disease [35]. Nevertheless, experiments conducted by Forbes et al. [36] and Kegley and Spears [37] indicate no muscle loss due to Cr supplementation, and Mostafa-Tehrani et al. [38] reported improvements in carcass composition using chromium nicotinate (CrNic) and chromium chloride ($CrCl_3$) supplement in sheep. Here, the possibilities mooted in the bibliography to explain low values of CREAT concentration (muscle catabolism, insufficient dietetic proteins, or hepatic disease) do not make sense. Actually, carcasses of these animals were evaluated—results are described in Dallago et al. [11]—and did not show any weight loss due to Cr level. In addition, animals did not show any signs of muscle loss, dietetic protein offered was adequate in quality and quantity during the trial, and there was no evidence of concomitant hepatic disease. Thus, as reference values for CREAT in Santa Inês (SI) have not been established, especially in SI lambs, low values are probably normal for this breed.

Conclusion

Although dietetic CrPic supplementation did not lead to significant toxicity or consistently change blood parameters, the use of CrPic as a food supplement is suspect due to histopathology findings in the liver, kidneys, and testis in animals supplemented with the mineral. In this sense, we strongly recommend caution and, for the moment, to avoid the use of CrPic as dietetic supplement for animal production.

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