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Effect of dietary fluorine from Araxá rock phosphate on the hepatic production of cyclic-adenosine monophosphate in broilers

(Efeito do flúor presente no fosfato de rocha de Araxá sobre a produção hepática de AMP-cíclico em frangos de corte)

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ABSTRACT

The cyclic adenosine 3', 5'-monophosphate (cAMP) production was evaluated in liver thin sections of broiler chicks fed on a experimental diet containing bicalcium phosphate or Araxá rock phosphate (ARP) as source of P, with a high content of fluorine, at different ages: from the first to the 42nd and from the 21st to the 42nd day of age. The intake of the ARP formulated diet starting from birth elicited an increase of cAMP production in broiler liver. However, when this diet was offered after the 21st day of age, the hepatic cAMP production in broilers was not significantly (P>0.05) affected, suggesting that the effect of high fluorine present in Araxá rock phosphate, on hepatic cAMP of broiler chicks depends on the age in which the experimental diet is started.

Key words: Fluorine, cyclic-adenosine monophosphate, broiler

RESUMO

A produção de adenosina 3', 5'- monofosfato cíclico (AMPc) foi avaliada em fatias de fígado de frangos de corte que receberam dieta experimental contendo fosfato bicálcico ou fosfato de rocha de Araxá, com alto teor de flúor, como fonte de fósforo, em dois períodos distintos: do 1º ao 42º e do 21º ao 42º dias de idade. A ingestão da dieta formulada com fosfato de rocha de Araxá desde o 1º dia de vida, provocou elevação da produção de AMPc no fígado dos frangos de corte. Entretanto, a utilização da mesma dieta a partir do 21º de idade não alterou significativamente (P>0,05) a produção hepática de AMPc nas aves, sugerindo que a influência da alta ingestão do flúor presente no fosfato de rocha de Araxá sobre o AMPc hepático dos frangos de corte é dependente da idade em que a administração da dieta é iniciada.

Palavras chave: Flúor, adenosina monofosfato cíclico, frango de corte

INTRODUCTION

Phosphorus (P) is probably the mineral that is involved in most functions in the animal body. In the recent years, for economical reasons, many investigations were done to evaluate the utilization of natural phosphates as feeding source of P for poultry and pigs. The main advantage of Araxá rock phosphate (ARP) is low cost. ARP also has several disadvantages, such as low content in P and high levels of fluorine (F). However, fluorine relative bioavailability in the ARP was low as compared to sodium fluoride (NaF) used as an available 100% standard (Veloso et al., 1998 a).

It is known that high ingestion of F reduces weight gain of chicks (Phillips et al., 1935) and chickens (Hauck et al., 1933). In rats, NaF as source of F, caused a reduction in feed intake and a consequent lower weight gain than that observed in control rats (Carlson & Suttie, 1966). The intake of high levels of F from NaF added to experimental diets increased glycemia in broiler chicks (Abdelhamid & Dorra, 1992).

The regulatory effects of cAMP, the nucleotide synthesized within the cell from ATP by action of adenylate cyclase, on enzymes involved in energetic metabolism are well known. The elevation of intracellular levels of cAMP enhances the conversion of glycogen to glucose by inhibiting glycogen synthesis and increasing glycogen breakdown in liver and skeletal muscle. In addition, there is an increase in lipolysis.

Gilman (1987), showed that fluoride activates adenylate cyclase, independently of the complex hormone-receptor formation, as a result of the interaction of the anion with the G protein. Studies using rat liver tissue (Katz, 1988) showed that the activation of adenylate cyclase by fluoride is dependent on animal age.

The aim of this work was to study the effects of F present in Araxá rock phosphate on hepatic cAMP production of broilers at different ages.

MATERIAL AND METHODS

Materials were obtained from the following sources: cAMP protein kinase, cAMP, fiberglass filters 24mm (retention> 2.3µm), MF–Millipore nitro-cellulose membranes 25mm (retention > 0.22µm), theophylline, trichloracetic acid (TCA), bovine serum albumin (BSA) and Trizma base (Sigma Chemical Company, St Louis, MO, USA) ^{3}H-cAMP (specific activity 34 Ci/mmol) (Amersham Laboratories, Buckinghamshire, England), Dowex AG 50-X4 (200-400 mesh) (BioRad, Richmond, CA, USA) Folin-Ciocateau's phenol reagent and toluene (Merck KGaA, Darmstadt, Germany) and bicalcium phosphate (Fertilizantes Mitsui S.A., São Paulo, Brazil) The other chemicals used were analytical grade.

The basal diets (Tab. 1) were formulated to supply the broilers nutritional requirements according to their different growing phases (Rostagno et al., 1987), except in phosphorus.

Table 1. Composition and analytical composition of the basal diets

	Growing phase		
	1-21days	21-35 days	35-45 days
Ingredient		(%)	
Ground yellow corn	58.00	63.23	65.50
Soybean meal	33.40	28.50	25.94
Soybean oil	1.90	2.00	2.70
Iodized salt	0.30	0.30	0.30
Vitamin premix [1]	0.40	0.40	0.40
Mineral premix [1]	0.10	0.10	0.10
Methionine-HCl	0.14	0.14	0.14
Variable composition [2]	5.76	5.35	4.96
Total (%)	100.00	100.00	100.00
Analytical composition			
Crude protein (%)	21.00	18.90	17.60
Metabolizable energy [3] (kcal/kg)	2922	3000	3099
Total calcium (%)	0.13	0.11	0.107
Available calcium [4] (%)	0.13	0.11	0.107
Total phosphorus (%)	0.34	0.33	0.32
Available phosphorus [5] (%)	0.11	0.11	0.11

[1]See Veloso et al. (1998 a)
[2]Fulfilled with variable amounts of phosphate source, limestone and sand.
[3]Calculated value.
[4]Considered calcium availability of 100%.
[5]Considered 1/3 of total phosphorus available.

The test diets (Tab. 2) were supplemented using appropriate levels of each phosphorus source. The control diets were fortified with commercial bicalcium phosphate (0.2% F) and the experimental diets with Araxá rock phosphate (10.48% P and 1.16% F). The diets were analyzed and the protein, calcium and phosphorus contents were determined by the AOAC methods (AOAC, 1984). Fluoride was determined by the specific electrode method (Singer & Amstrong, 1968).

Table 2. Composition of test diets at different phases.

	Control diets (days)			Experimental diets (days)		
	1-21	21-35	35-45	1-21	21-35	35-45
Ingredient	(%)					
Basal diet	94.24	94.65	95.04	94.24	94.65	95.04
Bicalcium phosphate	2.20	2.00	1.80	-	-	-
Araxá rock phosphate	-	-	-	5.12	4.72	4.20
Limestone	0.90	0.86	0.90	0.64	0.63	0.76
Sand	2.66	2.49	2.26	-	-	-
Total (%)	100.00	100.00	100.00	100.00	100.00	100.00
Analytical composition						
Crude protein (%)	20.09	19.07	18.44	20.87	19.50	18.37
Total calcium (%)	1.10	1.04	0.95	1.61	1.48	1.34
Available calcium [1](%)	0.97	0.90	0.88	0.97	0.90	0.88
Total phosphorus (%)	0.51	0.42	0.45	0.90	0.78	0.72
Available phosphorus[2](%)	0.48	0.45	0.41	0.48	0.45	0.41
Total fluorine (ppm)	71	60	56	946	933	893
Available fluorine[3] (ppm)	71	60	56	458	447	427

[1]Considered calcium bioavailability obtained by Veloso et al. (1998 b).
[2]Considered phosphorus bioavailability obtained by Dell'Isola (1996).
[3]Considered fluorine bioavailability obtained by Veloso et al. (1998 a) for experimental diets.
In control diets fluorine was considered 100% available.

Hubbard male broiler chicks were used in the experiments. Control chicks were fed from hatch on control diets containing bicalcium phosphate as source of P. Experimental chicks of the same age and source were divided in two groups. The first group received, the experimental diets from hatching onwards. The second group received a control diet from hatching to the 20^{th} day of age and the experimental diets thereafter. Feed and water were provided *ad libitum* to control and experimental groups.

Analysis of cAMP was performed in liver tissue of control and experimental groups. Five chicks of each treatment were killed by decapitation at 0, 7, 14, 21, 28, 35 and 42 days of age. Their livers were dissected, placed immediately on ice and used for the measurement of the individual cAMP production. Each liver was assayed in duplicate.

For cAMP determination, 50mg of liver slices 0.5mm thick were incubated in a medium containing: NaCl, 20mM; KCl, 20mM; $MgSO_4$, 1mM; Trizma base (Tris[hydroxymethyl] aminomethane), 0.2mM; theophylline, 2mM; ascorbic acid, 0.1mM, pH 7.4, in a shaker bath at 37^oC and about 120 oscillations/min, for 20min. The incubation was stopped using 0.2ml of trichloroacetic acid (TCA) 3M. The contents were transferred to centrifuge tubes and the flasks were washed with 0.5ml of TCA 0.3M. After the carrier addition (20nCi of ^{3}H-cAMP) to the tubes and mixing, the samples were centrifuged for 40min at 11,200g at 4ºC. The supernatants obtained were used in cAMP separation.

The camp was separated from trichloroacetic acid and endogenous interfering compounds using ion exchange chromatographic columns.

The supernatant solutions of the deproteinized samples were applied to Dowex 50W-X4 columns (0.8×6.0cm), previously washed and equilibrated. The columns were eluted with fractions of 3.0ml of deionized water. The cAMP was isolated using a fractionation procedure similar to that described by Matsuzawa & Niremberg (1975) and confirmed by ^{3}H-cAMP recovery after elution. The recuperation was determined in fractions of

0.15ml of samples applied to fiberglass filters, dried for 30min at 100°C, and counted in 5.0ml of an anhydrous scintillation solution made with 1,000ml of toluene, 4g of 2,5-diphenyloxazol (PPO) and 100mg of 1,4 bis[5-phenyl-2-oxazol]benzene,2,2'-p-phenylene-bis[5-phenyloxazole] (POPOP) in a Packard Liquid Scintillation Spectrophotometer. The average recovery of ^{3}H-cAMP was 75%. The pellet obtained after centrifugation was digested with NaOH 1N and then assayed for protein determination (Lowry et al., 1951). Bovine serum albumin was used as standard. Cyclic AMP concentrations in the chromatographically purified samples were determined by the method of Gilman (1970). After the end of the reaction, the samples were filtered on Millipore nitro-cellulose membranes, that were dried for 10min at 40°C, put to vials with 5.0ml of toluene scintillation solution and counted in the liquid scintillation spectrophotometer to determine their contents in ^{3}H-cAMP. A standard curve for cAMP assay obtained in the presence of saturating concentrations of ^{3}H-cAMP was used in these determinations. Each cAMP sample was assayed at two concentrations. Each value shown corresponds to 100% recovery of cyclic nucleotide and is the mean of duplicate determinations. The values were expressed in ρmoles of cAMP/mg of tissue protein.

The characterization of the *in vitro* F effect over hepatic cAMP production was studied in liver slices of 7 and 21 day-old chicks fed on control diet. The slices were incubated during 20min in presence or absence (controls) of 5mM of fluoride added as NaF. The cAMP produced after the stimulation was determined in the supernatants.

Statistical analysis were performed by analysis of variance of the data, using SAS software (SAS, 1985). The coefficient of variation was determined. The means were compared by the Student's t test, and the statistical meaning of the differences between treatments was established (Snedecor & Cochran, 1967). Statistical significance was accepted at $P<0.05$. A linear regression was calculated to illustrate the pattern of hepatic cAMP in broilers at different ages and dietary treatments.

Broiler chicks used in the experiments were weighed once a week, from hatching to the 45th day of age, to determine the body weight gain during the experimental period, in each dietary treatment. The food intake was also measured weekly and the available fluorine intake was calculated. Statistical analysis of these values was not performed. The results were only used as references. Data of productive performance obtained in similar conditions were published by Rezende et al. (1998).

RESULTS

The cAMP produced when 5mM of fluorine was added as NaF to the liver slices of 7 and 21 day-old broilers fed on control diet were 48.36±4.07 and 65.45±6.35 ρmoles cAMP/mg of tissue protein, respectively (mean±SEM, n=5). The values obtained in controls groups were 30.42±3.41 in 7 days old and 39.20±5.56 in 21 days old chicks. The results were significantly different at the 7th ($P< 0.001$) and 21st ($P< 0.01$) days of age. The rise in cAMP levels produced by the addition of NaF was 59 and 69%, respectively. The increase in the hepatic production of cAMP could be related to an *in vitro* activation of adenylate cyclase by fluoride anion (Sterweis & Gilman, 1982).

Significative differences were observed in hepatic cAMP production of broiler chicks fed on diet containing Araxá rock phosphate at the 28th ($P< 0.001$) and 42nd ($P< 0.05$) days of age, when the diet was provided from hatching (Tab. 3). Despite the numeric difference between the camp values at the 35th day of age, they were not significant by Student's t test. The coefficient of variation (CV) was 28.40%, in contrast with 16.64% and 21.70% at the 28th and 42nd days of age, respectively. The rise in CV at the 35th day indicates that there was an interfering factor at that time.

Table 3. Hepatic cAMP production (ρmoles/mg of protein) of broiler chicks that were fed *ad libitum* with control diet and experimental diet prepared using Araxá rock phosphate as source of phosphorus.*

Age (days)	Control diet	Experimental diet starting at the birth date	Experimental diet starting at the 21st day of age
1	39.43 ± 2.48 ac	-	-
7	30.42 ± 4.94 Aab	33.41 ± 3.47 Aa	-
14	34.12 ± 1.39 Aab	30.52 ± 1.59 Aa	-
21	39.19 ± 5.56 Aabc	34.88 ± 2.96 Aa	-
28	32.17 ± 1.64 Ab	55.03 ± 3.69 Bb	31.26 ± 3.10 Aa
35	48.44 ± 3.71 Ac	65.66 ± 9.66 Ab	52.16 ± 6.41 Ab
42	49.99 ± 3.31 Ac	72.38 ± 6.10 Bb	58.95 ± 4.33 ABb

Mean ± SEM (n =5). Statistical difference ($P<0.05$) in Student's test was indicated by different capital letters in the same row, and by different minuscule letters in the same column.
*Araxá rock phosphate with 10.48% P and 1.16%F.

The elevation of the cAMP hepatic production of broilers that received the diet with a high fluorine content since the 1st day of age suggested that the fluoride ion present in the Araxá rock phosphate could be probably identified as an *in vivo* stimulator of adenylate cyclase. When the use of the experimental diet started at the 21st day of age, the cAMP levels in the broiler's livers were similar in control and experimental groups from the 28th to the 42nd days of age. The intake of fluorine from Araxá rock phosphate at that age did not significantly affect the hepatic adenylate cyclase. These findings suggested that the nonreceptor-mediated activation of adenylate cyclase by F^- was affected by the age of the broilers when the exposure occurred and by the high levels of this element in natural phosphate.

In order to determine the behaviour of cAMP hepatic production in broilers at different ages and treatments, a regression analysis was performed. The best equation that represents the evaluated parameter was a quadratic regression (Fig. 1). In the equation, y expressed the cAMP production in liver as ρmoles of cAMP/mg of protein, and x the age of the broilers in days. The regression equations obtained were $Y= 37.53 – 0.629X+0.022989X^2$ ($R^2=0.3739$; ep=0.009519) for the broilers treated with control diet since their birth date and $Y = 36.76 – 0.653X + 0.038314X^2$ ($R^2=0.6258$; ep=0.013375) for those that received the diet prepared with Araxá rock phosphate with high levels of fluorine from the 1st to the 42nd day of age. The curves were similar during the developmental period, but at the 21st day of age slope differences could be observed. The same aspect was observed in the regression curve of the treatment in which the broilers started on the diet containing natural phosphate at the 21st day of age (data not shown).

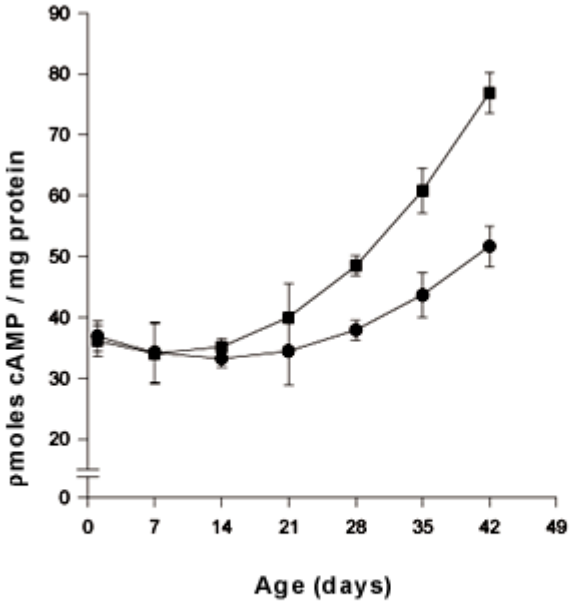

Figure 1. Age related increase in hepatic cAMP production of broilers fed on a diet using Araxá rock phosphate with high content of fluorine as source of phosphorus (■) and its corresponding control (●).

The productive performance of broiler chicks fed at different stages of their development on the experimental diet that uses the Araxá rock phosphate with high fluorine content as source of P is shown in Tab. 4. The results suggest the occurrence of a reduction in food intake and body weight gain in the group that received the experimental diet from hatching onwards when compared with the control group, however, the feed conversion was unaffected. The data obtained here are similar to those described by Rezende et al. (1998). These authors showed that there was no significant difference in productive performance when broilers started to receive different diets using natural phosphates after the 21st day of life, if the values of phosphorus and calcium bioavailability were previously determined and considered in the diet formulation.

Table 4. Performance of broiler's feeding at different stages of their development with a diet using Araxá rock phosphate with high fluorine content as phosphorus source, and its respective control.

Treatment	Food intake (g)	Body weight gain (g)	Feed Conversion (g/g)	Available fluorine intake (ppm)
Control diet (1st - 45th day)	4003	1988	2.01	298
Experimental diet (1st - 45th day)	3472	1698	2.04	1282
Experimental diet (21st - 45th day)	3849	1907	2.02	1044

DISCUSSION

Alterations in the responsive adenylate cyclase activity appear to be mediated by changes in receptor and nonreceptor components. The activation of adenylate cyclase by binding of a hormone or neurotransmitter to its specific receptor is mediated by the stimulatory G protein (G_s), that promotes the inter-conversion between inactive GDP and active GTP forms. The stimulation of the catalytic activity of adenylate cyclase by an action in nonreceptor components requires the guanine nucleotide (GTP) interaction with a stimulatory guanine nucleotide-binding regulatory component of the enzyme (Gilman, 1984).

Fluoride activates adenylate cyclase, independently of the complex hormone-receptor formation, as a result of the interaction of the anion with G_s (Gilman, 1987). This is a characteristic effect of F^- on all G proteins. Sternweis & Gilman (1982) showed that Al^{3+} at micromolar concentrations was required for activation of G_s by F^-, and suggested that the activating ligand was AlF^-_4. The Al^{3+} source on the *in vitro* experiments, was attributed by the authors to a contamination that occurs in preparations of commercial ATP or in the disposable glass test tubes. The effect of Al^{3+} on the *in vivo* activation of adenylate cyclase by F^- can be attributed to a formation of a physiological equivalent to the aluminum-fluoride complex. Bigay et al. (1985) suggested that this compound persistently activates G proteins because it has the same shape as PO^{3-}_4 and binding next to GDP where it mimics the g -phosphate group of GTP.

The use the experimental diet made with Araxá rock phosphate with high content of fluorine from hatching elicited a significant increase of the hepatic cAMP production in broiler chicks that can be attributed to the activation of adenylate cyclase by the ion fluoride present in the Araxá rock phosphate. The activation causes a rise in cAMP production in liver and in consequence an increase in energetic metabolism. The reduction food intake and weigh gain in broilers that received the diet with high content of fluorine since the first day of age can be correlated with the activation of adenylate cyclase. From the 21st to the 42nd days of age, dietary supplementation of F did not significantly affect food intake and growth. The absence of differences in productive parameters, when the broiler chicks were started to receive the experimental diet at 21st day of life can be associated with the fact that their hepatic adenylate cyclase levels were not significantly affected. The 21st day of age is coincident with the period in which the nutritional requirements of broiler chicks modify. At this age, the requirements for dietary protein decreases, and for metabolizable energy increases.

Katz et al. (1985) demonstrated changes of nonreceptor-mediated adenylate cyclase activities of rat liver during development using homogenates of rat livers with ages between 6 and 60 days. The maximum value obtained after F^- stimulation of adenylate cyclase was 450 ρmoles of cAMP/10min/mg of protein at the 6th day of age. In non-stimulated (control) group, they found a maximum of 100 ρmoles of cAMP/10min/mg of protein at the same age. Their data showed that the fluoride stimulated adenylate cyclase activity decreased in liver homogenates between the 6th and 20th days of age, but increased again after 20 days. These authors suggested developmental alterations of the nucleotide regulatory component of the adenylate cyclase and/or its interaction with the catalytic component. Afterwards, Katz (1988) showed the F^- stimulation of the enzyme

in rat liver persists during ageing. In liver homogenates from 6 to 30 months, of *ad libitum* fed and food restricted rats, the fluoride raised the levels of adenylate cyclase. The maximum stimulation measured as ρmoles of cAMP/10min/mg of protein was reached by 18 months of age, in both groups, but the values obtained at that time were smaller than the values observed by Katz et al. (1985) in liver homogenates of 6^{th} day old rats. The results obtained *in vivo* with the broilers in this study were in agreement with their findings. Michel et al. (1984) reported that fluorine deleterious effect on food intake and, in consequence, in weight gain was more effective during the broiler's first weeks of age. Significant reductions in 21-day live weights of broilers were observed by Huyghebaert et al. (1988), when fluoride was added at 200 or 400ppm to the diet. The reductions were accompanied by a significant lower food intake.

The present study concludes that the experimental diet made with Araxá rock phosphate, which contains a high content of fluorine, given from hatching elicited a significant increase of the hepatic cAMP production in broiler chicks and reduced their productive parameters. The broilers that received the same diet from the 21^{st} day of age did not show significant differences in the cAMP levels of liver, food intake and body weight gain as compared to those fed with control diet, suggesting that the effect of a high fluorine intake on adenylate cyclase activation and consequently in the energetic metabolism of broilers was more effective during the early weeks of age. The significant increase in cAMP production by the addition of NaF to incubation medium of liver slices of broilers fed with control diet from hatching confirms the occurrence of nonreceptor mediated activation of the hepatic adenylate cyclase of broilers by the ion fluoride.

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