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Hypophosphatemia Induced by Dietary Aluminium Hydroxide Supplementation in Growing Pigs: Effects on Erythrocytes, Myocardium, Skeletal Muscle and Liver

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Håglin, L., B. Essén-Gustavsson and A. Lindholm: Hypophosphatemia induced by dietary aluminium hydroxide supplementation in growing pigs: Effects on erythrocytes, myocardium, skeletal muscle and liver. Acta vet. scand. 1994, 35, 263-271. – Three groups of pigs were studied during and after 10 weeks of treatment with either $Al(OH)_3$ ($Al[OH]_3$-group, n=8) to induce hypophosphatemia, $AlPO_4$ ($AlPO_4$-group, n=8, aluminium control without hypophosphatemia) or no addition to the feed (control group, n=8). Blood samples were taken at the start of the experiment and after 3, 6 and 10 weeks and were analyzed for phosphate, calcium and 2,3-diphosphoglycerate (2,3-DPG). Samples from myocardium, skeletal muscle and liver were obtained in connection with exsanguination and analyzed for glycogen, adenosine-tri-phosphate (ATP), creatine phosphate (CP), glucose-6-phosphate (G-6-P) and lactate. The $Al(OH)_3$-group became hypophosphatemic and hypercalcemic with low levels of 2,3-DPG in erythrocytes within 3 weeks and showed a retarded growth rate. After 10 weeks the $Al(OH)_3$-group had low levels of ATP in myocardium as compared with the control-group and low levels of G-6-P as compared with the $AlPO_4$-group. No disturbances on electro-cardiograms registered at rest could be documented. G-6-P concentration was low in the biceps muscle in the $Al(OH)_3$-group as compared with the $AlPO_4$-group and in the liver low G-6-P concentration was seen in addition to high lactate concentration. The fibre type composition in M. Longissimus did not differ between groups, but the $Al(OH)_3$-group had, due to retardation in growth, smaller mean fibre-areas than pigs in the $AlPO_4$-group. Hypophosphatemia gave rise to high serum calcium levels, low concentration of 2,3-DPG in erythrocytes and influenced G-6-P concentration in skeletal muscle, G-6-P and ATP in myocardium, G-6-P and lactate in liver. Retarded growth was one serious consequence of hypophosphatemia and the disturbed energy metabolism.

ATP; G-6-P; 2,3-DPG; lactate; glycogen; ECG; hypercalcemia; growth; muscle fibres.

Introduction

Hypophosphatemia with symptoms of muscle weakness is documented in humans treated with moderate doses of aluminium-containing antacids (*Lotz et al.* 1968). Clinical manifestations on the myocardium in connection with hypophosphatemia include cardiomyopathy (*Darsee & Nutter* 1978) and decreased mean left ventricular stroke work (*O'Connor et al.* 1977). Hypophosphatemia is also associated with low levels of 2,3-DPG in erythrocytes (*Lichtman et al.* 1971).

Energy metabolism in skeletal muscle and myocardium is affected by hypophosphatemia (*Fuller et al.* 1978, *Brautbar et al.* 1982 and 1983, *Hörl et al.* 1983). In skeletal muscle of rats given a phosphate-deficient diet, a decrease is seen in mitochondrial oxygen consumption together with reduced intracellular inorganic phosphate (*Brautbar et al.* 1983), glycogen and G-6-P (*Brautbar et al.* 1984 a), whereas ATP and CP levels are unaltered. In the constantly active myocardium, however, the levels of ATP and CP are decreased (*Brautbar et al.* 1982) in addition to decreases in inorganic phosphate, glycogen and G-6-P (*Brautbar et al.* 1984 b). Studies on dogs fed low-phosphate diet show depressed myocardial performance with a strong correlation to low total skeletal muscle phosphorus and low serum phosphate levels (*Fuller et al.* 1978).

The question of whether decreased growth, described in hypophosphathemic animals (*Aubel et al.* 1936, *Lee et al.* 1977, *Håglin et al.*1988) has adverse effects on muscle fibre types (type I, type IIA and IIB) has not been raised or studied earlier. Type IIB fibres may be less well preserved than type I and IIA fibres in connection with starvation, anorexia or malnourishment (cf. *Henriksson* 1990).

The aim of this study was to induce hypophosphatemia by antacid treatment in a fast growing animal like the pig and to study the influence on both substrates and metabolites involved in energy metabolism in erythrocytes, skeletal muscle, myocardium and liver. In addition, the influence on muscle fibre type composition and fibre areas was studied.

Materials and methods

Animals and experimental design

Twenty-four Swedish landrace pigs (13-14 weeks old at the start of the experiment with a live weight of 20-42 kg) were used and randomly divided into 3 groups with 8 pigs in each, treated with a suspension of aluminium hydroxide ($Al[OH]_3$-group), aluminium phosphate ($AlPO_4$-group, treated control-group) or left untreated (untreated control-group). They were given 500 ml/day, 250 ml/meal well mixed with the feed, corresponding to 17.5 and 16.2 g aluminium, respectively. The animals were killed after 10 weeks of the experimental period by exsanguination through the carotid arteries while under intravenous anesthesia with Sodium Pentothal (Abbot Laboratories, Chicago, Ill., USA) and a complete necropsy was performed. A more detailed description of the experimental procedure is presented in an earlier paper (*Håglin et al.* 1988).

Electrocardiograms

ECG were recorded with a mingograf (Mingograf minor 3, Siemens-Elema, Solna, Sweden) at week 10 using leads 1, 2, 3, AVR, AVL, AVF, and 3 chest leads placed in the 5-6; th intercostal space on the left chest wall. The QT-interval was calculated from an average of 6 complexes from lead AVF which showed accurate amplitudes. All recordings were made during anaesthesia.

Blood samples

Samples were drawn from the cranial vena cava under anaesthesia with Sodium Pentothal on 4 occasions (0,3,6 and 10 weeks) and analyzed for serum-phosphate, serum-calcium and erythrocyte 2,3-DPG. Analytic methods have been described earlier (*Håglin et al.* 1988).

Creatin kinase (CK) and serum glucose were analyzed with strips in a seralyzer (AMES-seralyzer, Miles Inc. Indiana, USA).

Tissue samples

Organ specimens were collected following exsanguination. The samples were taken from

longissimus dorsi and biceps muscles, liver and heart. All samples were frozen immediately in liquid nitrogen and stored in -80°C until analyzed.

Biochemical analyses

The tissue from liver, heart and skeletal muscle was freeze-dried and dissected free from connective tissue, fat and blood. One-two mg of tissue was extracted in perchloric acid and the supernatant was neutralized with $KHCO_3$ and centrifuged. This supernatant was used for measurement of ATP, CP, G-6-P, and lactate according to *Lowry & Passaneau* (1973). For analysis of glycogen 2-5 mg of tissue was boiled in 1 ml of 1M HCl in a water bath for 2h. The glycogen was analyzed as glucose-residues by the method of *Lowry & Passaneau* (1973).

Histochemical analyses

Serial sections of the longissimus muscle were stained for myofibrillar ATPase after both acid (pH 4.3 and 4.6) and alkaline (pH 10.3) preincubation in order to identify fibre types I, IIA and IIB (*Brocke & Kaiser* 1970). A computerized image analysis system designed for muscle fibre analysis (Bio-Rad, Scan Beam, Hadsund, Denmark) was used to calculate relative distribution of fibre types and fibre areas from the stained sections (pH 4.6).

Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations (SD) and results are presented as mean±SD. Unpaired and paired Student's t-test were used to assess group differences. Correlation coefficients (Pearson correlations) were computed according to *Armitage* (1974). Statistical significance was declared at a probability of $p<0.05$.

Table 1. Phosphate, calcium and 2,3-DPG levels after 0, 3, 6 and 10 weeks treatment with Al(OH)3, or AlPO4 and from untreated controls (n=8 in each group). Data are given as mean ±SD.

Group treatment	Serum P mmol/L				Serum Ca mmol/L				2,3-DPG mmol/L		
	0 wk	3 wk	6 wk	10 wk	0 wk	3 wk	6 wk	10 wk	3 wk	6 wk	10 wk
$Al(OH)_3$	2.33±0.21	1.12±0.12[a,b]	0.93±0.28[a,b]	1.01±0.44[a,b]	2.67±0.16	3.84±0.53[a,b]	4.21±0.79[a,b]	3.71±0.45[a,b]	2.71±0.27[a,b]	2.70 ±0.39[a,b]	2.60±0.44[a,b]
$AlPO_4$	2.13±0.21	2.53±0.14	2.40±0.18	2.26±0.20	2.71±0.09	2.61±0.15	2.52±0.07	2.57±0.14	3.71 ±0.27	3.60±0.27	3.70±0.22
Control	2.36±0.15	2.48±0.10	2.40±0.17	2.36±0.18	2.63±0.14	2.58 ±0.14	2.58±0.19	2.61±0.13	3.66±0.29	3.61±0.31	3.90±0.44

a: Significant difference between $Al(OH)_3$ and $AlPO_4$
b: -«- -«- and control

Table 2. Glucose-6-Phosphate (G-6-P), creatine-phosphate (CP), adenosine-triphosphate (ATP), lactate and glycogen concentrations in myocardium, skeletal muscles and liver from pigs treated with $Al(OH)_3$ or $AlPO_4$ or untreated controls. Data are given as mean±SD (n=8 in each group).

	G-6-P mmol/kg	CP mmol/kg	ATP mmol/kg	Lactate mmol/kg	Glycogen mmol/kg
Myocardium:					
$Al(OH)_3$	3.4 ± 1.0[a]	26 ± 5	11.5 ± 1.9[b]	87 ± 10	155 ± 50
$AlPO_4$	4.9 ± 0.8	28 ± 6	13.5 ± 1.7	95 ± 9	142 ± 19
Control	4.6 ± 1.3	28 ± 9	14.6 ± 2.9	86 ± 11	134 ± 31
M. Biceps:					
$Al(OH)_3$	6.7 ± 3.0b	42 ± 17	22 ± 1	15 ± 12	456 ± 52
$AlPO_4$	9.8 ± 6.0	51 ± 6	21 ± 2	11 ± 5	433 ± 28
Control	13.0 ± 4.0	47 ± 18	21 ± 2	24 ± 21	440 ± 49
M. Longissimus dorsi:					
$Al(OH)_3$	9.8 ± 11.6	16 ± 8	21 ± 1	29 ± 25	480 ± 51
$AlPO_4$	11.2 ± 9.3	19 ± 6	21 ± 1	57 ± 64	484 ± 48
Control	7.4 ± 5.4	16 ± 7	20 ± 1	27 ± 18	523 ± 85
Liver:					
$Al(OH)_3$	42.5 ± 18.4[a]	– – –	15 ± 8	37 ± 8[a,b]	1298 ± 525
$AlPO_4$	58.2 ± 3.7	– – –	21 ± 7	25 ± 4	1657 ± 154
Control	47.6 ± 7.7	– – –	21 ± 11	29 ± 5	1545 ± 355

a: Significant difference between $Al(OH)_3$ and $AlPO_4$
b: Significant difference between $Al(OH)_3$ and control

Results

Blood

The results on serum phosphate, serum calcium and erythrocyte 2,3-DPG, shown in Table 1 has been presented earlier (*Håglin et al.* 1988, *Håglin et al.* 1993). Three weeks after introducing $Al(OH)_3$-treatment, the $Al(OH)_3$-group had lowered S-P and erythrocyte 2,3-DPG levels and elevated calcium levels as compared with the $AlPO_4$- and control-groups. After 6 and 10 weeks the differences were still significant (Table 1). The concentrations of CK after 10 weeks were 349±353, 342±136 and 347±125 IU/l in the $Al(OH)_3$-, $AlPO_4$- and control-groups, respectively. One hypophosphatemic pig developed high CK levels in its blood. The glucose level in the $Al(OH)_3$-group was not different from the control-group (6.9±1.4 and 6.9±0.9 mmol/L, respectively). Glucose was not analyzed in the $AlPO_4$-group.

ECG recordings

The heart rates during anaesthesia in the $Al(OH)_3$-, $AlPO_4$- and control-groups were 162±24, 146±21 and 162±26(beats/min), respectively. No arrhythmias or other ECG abnormalities were observed. No differences in QT-times were seen between groups.

There was a negative correlation between QT-time and heart rate which was calculated from the recorded electrocardiograms at 10 weeks, (r=-0.83;n=23).

Table 3. Fibre type composition, fibre area and relative fibre area and mean fibre area in M. longissimus dorsi from pigs treated with $Al(OH)_3$ (n=8), $AlPO_4$ (n=8) and from an untreated control group (n=7). Data are given as mean ± SD.

Group treatment	Fibre type composition (%)			Fibre area ($\mu m^2 \times 10^2$)			Relative fibre area (%)			Mean fibre area ($\mu m^2 \times 10^2$)
	I	IIA	IIB	I	IIA	IIB	I	IIA	IIB	
$A1(OH)_3$	10±4	7±5	83±5	23±6[a]	14±5	29±5[a]	9±5	4±4	87±6	27±5[a]
$A1PO_4$	12±5	8±5	80±7	33±9	17±9	48±9	9±3	4±4	88±5	44±8
Control	10±3	8±5	83±4	28±11	13±5	35±13	8±1	3±2	89±3	33±11

a: significant difference between $A1(OH)_3$ and $A1PO_4$

Myocardium

The ATP content in the myocardium from the $Al(OH)_3$-group was lower than in the control-group and almost significantly lower than in the $AlPO_4$-group ($p<0.07$, Table 2). The G-6-P content in myocardium was lower in the $Al(OH)_3$-group as compared with the $AlPO_4$-group and was almost significantly lower than the control-group ($p<0.07$).

Skeletal muscle

Glycogen, ATP, CP, G-6-P, and lactate concentrations in the longissimus dorsi and biceps muscles were not different among the 3 groups exept for a lower G-6-P level in biceps of the $Al(OH)_3$-group compared with the control group (Table 2).

Liver

The content of glycogen and the concentrations of G-6-P, ATP, and lactate in liver are presented in Table 2. G-6-P concentration was lower in the $Al(OH)_3$-group as compared with the $Al(PO)_4$-group. Lactate concentration was higher in the $Al(OH)_3$-group as compared with the $AlPO_4$-group and the control-group. The glycogen concentration varied markedly among the pigs in the $Al(OH)_3$-group with a tendency toward a lower mean level as compared with the $AlPO_4$-group ($p<0.08$). A negative correlation was seen between liver glycogen content and serum calcium ($r=-0.55$; $n=23$).

Body weight and fibre areas

The body weight was lower after 10 weeks in the $Al(OH)_3$-group (57.1±11.3 kg) as compared with the $AlPO_4$-group (81.5±8.5 kg) and the control group (83.3±16.3 kg), (*Håglin et al.* 1988). No differences between groups were found in the percentage fibre types or the relative area of each fibre type (Table 3). A correlation was found between mean fibre area and body weight (Fig. 1). The $Al(OH)_3$-group had smaller mean fibre areas as compared with the $AlPO_4$-group but not with the control-group.

Discussion

This study showed that treatment with aluminium hydroxide for 10 weeks made pigs hypophosphatemic, hypercalcemic and induced retarded growth (*Håglin et al.* 1988). Hypophosphatemia and low energy intake due to anorexia during the last weeks contributed to the retardation in growth. Smaller mean mus-

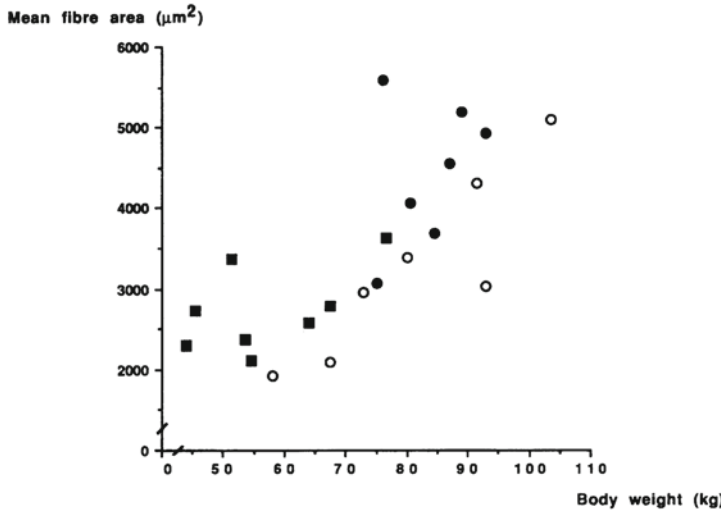

Figure 1. Mean fibre area in M. Longissimus in relation to body weight of pigs treated with $Al(OH)_3$ (■), $AlPO_4$ (●) and in control pigs (○).

cle fibre areas were therefore seen in hypophosphatemic pigs compared with pigs in the $AlPO_4$-group. From the correlation between body weight and mean fibre area (Fig. 1) it can be seen that the final body weight of 3 control pigs were within the range seen for the $Al(OH)_3$-group. These control pigs had a low body weight at the start of the experiment and explains why mean fibre area did not show a significant difference between the $Al(OH)_3$-group and the control-group. The retarded growth in the hypophosphatemic pigs was not accompanied by any change in percentage of fibre types. The percentage relative area of fibre types was thus similar in all groups of pigs. There is evidence both in man and in rats that in a state of energy deficiency, the size of type I-fibres is better preserved than the type-II fibres (*Henriksson* 1990). In the present study the hypophosphatemic pigs had with time a lower energy intake due to anorexia. The energy deficiency due to this did not seem to be severe enough to influence the size of type II fibres.

A large variation in metabolic response was observed among the pigs in the 3 groups as shown by the large standard deviations. This is likely related to different stress tolerance among the pigs even if all pigs were handled and treated in a similar manner before exsanguination.

The low concentration of G-6-P and ATP in the myocardium and lower G-6-P concentration in the biceps muscle and liver of the hypophosphatemic pigs indicate that energy metabolism was affected to some extent. These findings agree with previous data on phosphate deficient rats, where ATP levels were low in myocardium (*Brautbar et al.* 1982) but not in skeletal muscle (*Brautbar et al.* 1983), whereas G-6-P was low in both skeletal muscle (*Brautbar et al.* 1984a) and myocardium (*Brautbar et al.* 1984b).

If S-P is limiting for the phosphorylation

within the cells, the lower G-6-P might have been caused by a diminished capacity to phosphorylate glucose.

The strongest indication for a connection between S-P and glucose phosphorylation is shown by *Davis et al.* (1979) in an in vitro study; they found decreased glucose uptake in hind limb muscle from phosphate-depleted rats. Further, an epidemiological study reveals that low S-P is associated with higher basal levels of insulin and glucose in humans (*Ljunghall et al.* 1979); this indicates a connection between glucose uptake and S-P levels. The hypophosphatemic effect on glucose transport is also illustrated by a decreased glucose metabolism in patients with hypophosphatemia of different origins (*DeFronzo & Lang* 1980).

In an earlier study on pigs it was shown that an exercise test with a high energy demand on muscle induced lower muscular G-6-P concentrations in hypophosphatemic pigs as compared with normophosphatemic pigs (*Håglin & Essén-Gustavsson* 1992). A diminished capacity to resynthesize high energy substrates like ATP has been observed after repeated muscular contractions in phosphate-depleted mice (*Hettleman* 1983); the decreased ATP synthesis might in turn also cause a decreased capacity to phosphorylate glucose.

In addition it has been shown, in humans, that the continously working respiratory muscles are affected by hypophosphatemia (*Agusti* 1984, *Newman* 1977, *Planas et al.* 1982). With the higher energy demand in respiratory muscle (diaphragm), it is hypothezised that depletion of both G-6-P and ATP might predispose for decreased contractility (*Newman et al.* 1977).

In the present study on hypophosphatemic pigs, the levels of G-6-P in biceps but not in longissimus, were reduced. Biceps is a more active and oxidative muscle than longissimus, which might contribute to the differences in G-6-P response. Aerobic metabolism is also shown to be disturbed in phosphate depletion by a reduced 0_2-consumption within the mitochondria of rats fed low-phosphate diet (*Brautbar et al.* 1983). This might be due to low levels of 2,3-DPG in erythrocytes which give rise to a reduced oxygen release (*Benesch & Benesch* 1967). The decrease in 2,3-DPG seen in the hypophosphatemic pigs in the present study indicates that a disturbance of oxygen release also occurred. This may cause an impairment in ATP regeneration, especially in the myocardium which depends on its high oxidative capacity for energy production. Electrocardiograms did not reveal a disturbed myocardial function in the hypophosphatemic pigs. The ECG's, however, were only analyzed under resting (anaesthetized) conditions and it is not known how the myocardium would react if the energy turn-over would increase.

The liver is a major organ in the regulation of the blood glucose level. That energy metabolism was disturbed in the liver in association with hypophosphatemia was indicated by low G-6-P and high lactate concentrations. This could also be due to an impaired aerobic metabolism caused by low levels of 2,3-DPG. Three of the pigs in the hypophosphatemic group had very low glycogen concentrations (<1000 mmol/kg) and these were also those with the lowest serum phosphate, highest calcium concentrations and lowest G-6-P concentrations. Besides this, the serum calcium was correlated with glycogen content in the liver. A study concerning glycogenolysis confirms our findings that either the hypophosphatemia, and possibly also the hypercalcemia, affect the enzyme activity in liver glycogen metabolism (*Hörl et al.* 1982). Hypophosphatemia may be associated with impaired glucose phosphorylation, glycosysis and/or glycogen synthesis in the liver.

The results from this study emphasize that hypophosphatemia in pigs during 10 weeks of $Al(OH)_3$-treatment influenced energy metabolism and especially the concentrations of 2,3-DPG in erythrocytes, ATP and G-6-P in myocardium, and G-6-P in liver. Whether these effects on energy metabolism resulted from hypophosphatemia or from hypercalcemia or both could not be determined, but the hypothesis is that hypophosphatemia is the critical factor. In addition, growth was retarded, partly due to reduced feed intake and partly due to the disturbed energy metabolism.

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Sammanfattning

Hypofosfatemi hos gris framkallad genom supplementering av fodret med aluminium hydroxid: effekter på myokardium, skelettmuskel, lever och erytrocyter.

Tre grupper med grisar (13-14 veckor gamla) studerades under och efter 10 veckors behandling med antingen aluminium hydroxid ($Al[OH]_3$-gruppen, n=8) för att inducera hypofosfatemi, eller aluminium fosfat ($AlPO_4$-gruppen, n=8). Den tredje gruppen utgjorde obehandlad kontrollgrupp. Blodprov togs vid inledningen av experimentet och efter 3, 6 och 10 veckor för analys av fosfat, kalcium och 2,3-difosfoglycerat (2,3-DPG). Vävnadsprov från myokardium, skelettmuskel och lever insamlades i samband med obduktionen och analyserades på glykogen, adenosintrifosfat (ATP), kreatinfosfat (CP), glukos-6-fosfat (G-6-P) och laktat. Grisarna i $Al(OH)_3$-gruppen blev hypofosfatemiska och hyperkalcemiska med låga nivåer av 2,3-DPG i erytrocyterna inom 3 veckor och hade en sämre tillväxt. Efter 10 veckor noterades även låga halter ATP i myokardium jämfört med kontrollgruppen och låga nivåer av G-6-P jämfört med $AlPO_4$-gruppen. Inga störningar i EKG registrerades i vila under anestesi. G-6-P koncentrationen var också låg i bicepsmuskeln och levern från $Al(OH)_3$-gruppen jämfört med $AlPO_4$-gruppen. Muskelfibersammansättningen (M.Longissimus) var den samma i grupperna, medan fiberytan pga tillväxthämning var mindre i $Al(OH)_3$-gruppen än i $AlPO_4$-gruppen. Hypofosfatemin medförde höga serumkalcium nivåer och låga koncentrationer av 2,3-DPG i erytrocyterna, ATP i myocardium och påverkade G-6-P i skelettmuskel, myokardium och lever. En hämmad tillväxt blir konsekvensen av hypofosfatemi och en rubbad energimetabolism.

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