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Effects of Dietary Calcium Levels on Testicular Function in the White Leghorn Cocks

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Sixty White Leghorn cocks fed 3 levels (basal, moderate and high) of dietary Ca were used in this experiment. High dietary Ca caused significant decrease in body, testes, comb, wattle, pituitary and adrenals weights, and caused increase in thyroids weight. Ca and cholesterol levels in the blood plasma were increased, but P, Zn and glucose levels were decreased by high dietary Ca administration. Significant increase of Ca and decrease of P and Zn levels in the testicular tissue were also detected. Histological examination of the testes in the high dietary Ca group revealed impaired spermatogenesis, and reactivities for several oxidative enzymes were retarded in these tissues. Semen volume, sperm concentration and the motility did not differ significantly. Zn administration at the recovery period returned to normal Ca, P and Zn levels in the blood plasma and testicular tissue, and oxidative enzyme activities in the testes were also returned to normal level. These results indicate that the excess dietary Ca (Ca/P imbalanced diet) produce a physiological antagonistic function to that of P, Zn and various enzymes.

INTRODUCTION

It is well known that Ca is essential for normal reproductive function in mature female fowl (Roland et al., 1973), but limited research has been reported dealing with Ca requirement of the adult male and effects of diteary Ca levels on the fertility and reproduction.

It was believed that parathyroid hormone was solely responsible for the regulation of blood Ca from the vast skeletal reserve in response to hypocalcemia. Conversely, hypercalcemia was indicated as suppressing the production of parathyroid hormone, thus constituting a simple negative feed-back mechanism in the maintenance of a constant physiological level of circulating Ca (Mclean and Urist, 1955). Additional complicating factors must also be recognized as affecting the endocrine balance controlling the Ca homestasis. These include peptide hormones of pituitary gland, steroid hormones of gonads and adrenal cortex, minerals. pariticularly with reference to Ca/P ratio in nutrients and vitamin D.

It is impossible to state the requirement for a particular mineral element

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without considering the dietary levels of the other elements. For example, Zn status of an animal is influenced by a number of dietary factors other than the level of Zn in the diet. Some factors which appeared to affect Zn requirement are the dietary levels of Ca, P and Ca/P ratio. Deficiency syndrome of Zn is often precipitated or aggravated by additional Ca (Forbes, 1960).

The present study was conducted with cocks to study Ca requirement and to determine whether high dietary Ca is detrimental to reproduction and affects the nature of Zn deficiency syndrome. Effects of high dietary Ca on chemiical constituents of blood plasma were also ascertained. Problems of evaluating alteration that occur in the testes of cocks receiving different levels of dietary Ca were investigated from a histological and histochemical viewpoint.

MATERIALS AND METHODS

A total of 60 White Leghorn cocks of 12 weeks old were randomly divided into 3 experimental groups of *20* cocks each. One group was fed a basal diet (Eltohamy ***et al.,*** 1979) containing 0.0032 % Zn, 0.65 % Ca, 0.43 % P, 0.05 % Mg, 0.0125 % Fe and 8500 IU/kg vitamin D (basal group). Supplements of tricalcium phosphate were added to the basal diet to provide added levels of 1.1% Ca and 0.62 % P (moderate group), and 5.6 % Ca and 0.9 % P (high group). Ca/P ratio of the basal, moderate and high groups were 1.5 : 1, 1.8 : 1 and 6.2 : 1, respectively. Food intake was limited throughout the experimental period to prevent the excess growth. That is effort to eliminate the differences of diet requirement due to varying growth rates.

After 12 weeks experimental period, semen was collected and 30 cocks (10 cocks from each experimental group) were sacrificed by decapitation. Semen was examined for sperm concentration and the motility. The remainder 25 cocks (10 cocks of basal group, 10 cocks of moderate group and 5 cocks of high group) were placed on the basal diets without addition of excess Ca for 21 days (recovery period), and the others (5 cocks of high group) were received 50 ppm Zn into the basal diet for 21 days, and then sacrificed. This was done in order to find out whether or not cocks could recover from the high levels of Ca, or if Zn administration will correct the apparent deficiency.

Pituitary gland, testes, adrenals and thyroids for the experimental and recovery periods were removed and weighed. Testes were used for histological and histochemical examinations. They were fixed in Bouin's solution and stained with hematoxylin-eosin (HE). Frozen sections of the testes were stained with Sudan black for lipid and Schultz method for cholesterol.

Fresh specimens of the testes were cut into 18 μm thick sections in a -20°C cryostat with a sliding microtome. The sections were incubated in a substrate solution with nitro-BT for a histochemical detection of several oxidative enzymes. After incubation, they were fixed in 10 % neutral formalin solution for over 10 minutes, rinsed in distilled water, and mounted with glycerol.

Following enzymes were histochemically demonstrated; lactic dehydro-

genases (LDH), malic dehydrogenases (MDH), β-hydroxybutyric dehydrogenases (β-HDH), steroid 3β-ol dehydrogenases (Ster-D), glucose-6-phosphate dehydrogenases (G6PDH), nicotinamide-adenine dinucleotide dehydrogenases (NADH), nicotinamide-adenine dinucleotide phosphate dehydrogenases (NADPH) and alkaline phosphatases (AP). Details of incubating solution and the time employed were described elsewhere (Barka and Anderson, 1963).

Blood samples were collected and immediately placed in ice. Blood plasma was separated off by centrifugation within 10 min. of collection and the samples were stored at -10°C until used. Ca, Zn and Mg levels in the blood plasma were determined by atomic absorption spectrophotometry and P level was determined using a method of Fiske and Subbarow (1925). Enzymatic determination of glucose was carried out using Biomérieux glucose PAP kit. Total cholesterol was estimated by the modification of an enzymatic method using cholesterol oxidase (Varley, 1969). Plasma testosterone was extracted, isolated and quantitated via gas-liquid chromatography with electron capture detection.

RESULTS

Values of body and organ weights of the experimental period are tabulated in Table 1. Data of the body weight showed that the basal and moderate dietary Ca levels did not affect the growth, but the high dietary Ca level caused significant decrease in the body weight below those of the basal group. Decreases in comb, wattle, pituitary gland, adrenals weights and increase in thyroids weight were observed in the high group. Significant reduction in testes weight was detected in the high group.

Table 1. Effects of dietary Ca levels on body and organ weights at the end of the experimental period.

	Basal group (10)	Moderate group (10)	High group (10)
Body weight (g)	1790.00 ± 192.00	1742.00± 110.00	1494.00±123.40[1)]
Organ weights			
Comb (g)*	2.90±0.80	3.00±0.70	2.50±0.30
Wattle (g)*	0.77±0.06	0.68±0.09	0.66±0.26
Pituitary gland (mg)*	0.65±0.15	0.68±0.08	0.62±0.17
Adrenals (mg)*	9.60±1.70	8.80±1.10	9.20±0.60
Thyroids (mg)*	8.60±2.80	8.50±2.30	9.60f1.70
Testes (g)*	1.60±0.37	1.60±0.14	1.00±0.58[1)]

Values are means (±SEM) per group.
Parenthesized number shows a number of cocks.
* g or mg/100 g body weight.
1) Significantly different (P<O. 05) from the basal group by analysis of variance.

Date illustrating the effects of dietary Zn in the high group at the end of the recovery period and the controls data are tabulated in Table 2. Body

weight of Zn free group in the high dietary Ca was still lower than that in the controls or Zn added group. No differences were found between 4 groups in organ weights.

Table 2. Effects of dietary Zn on body and organ weights of the high group cocks at the end of the recovery period.

	Controls		High group	
	Basal group (10)	Moderate group (10)	Zn addedgroup (5)	Zn free group (5)
Body weight (g)	1896.00-t 29.07	*1900.00 ± 30.00*	1860. 00 ± *35.83*	*1750.00 ± 96.00"*
Organ weights				
Comb (g)*	3. 63 ± 0. 30	3. 99 ± 0. 40	3. 74 ± 0. *50*	3. 20 ± 0. 70
Wattle (g)*	0. 81 ± 0. 09	0. 91t0. 09	0. 84 ± 0. 09	0. 79 ± 0. 06
Pituitary gland (mg)*	0. 6910. 07	*0. 71 ± 0. 08*	*0. 67f0. 06*	0. 60 ± 0. 15
Adrenals (mg)*	9. 99 ± 1. 79	10. 99 ± 2. 00	9. 89 ± 1. 10	8. 84 ± 1. 43
Thyroids (mg)*	9. 46 ± 1. 64	10. 44 ± 1. 55	9. 63tl. 69	8. 99 ± 1. 74
Testes (g)*	1. 90 ± 0. 14	2. 14 ± 0. 20	1. 89 ± 0. 16	1. 69 ± 0. 38

Values are means (±SEM) per group.
Parenthesized number shows a number of cocks.
* g or mg/100 g body weight.
1) Significantly different ($P < 0.05$) from the basal group by analysis of variance.

Data showing chemical constituents of blood plasma and testicular tissue in the experimental period are tabulated in Table 3. Ca levels in the plasma and testes were significantly higher in the high group than those in the basal

Table 3. Effects of dietary Ca levels on chemical constituents of blood plasma and testicular tissue for the experimental period.

	Basal group (10)	Moderate group (10)	High group (10)
Plasma			
Ca (%)	7. 70 ± *0. 47*	8. 39 ± *0. 37*	*12. 51* Il. *81"*
P (%)	5. 11 ± *0. 42*	5. 47 ± *0. 62*	3. 39 ± 0. 14[1)]
Zn (μg/ml)	3. 44 ± *0. 06*	2. 94 ± *0. 07*	0. 90 ± 0. 13[1)]
Mg (%)	2. 06 ± *0. 33*	2. 04 ± *0. 28*	1. 94 ± 0. *11*
Glucose (mg/100 ml)	168.004 10.00	160.00 ± 12.00	137. 00 ± 6. 00[1)]
Cholesterol (mg/100 ml)	*70.00 ± 12.00*	*74.00 ± 14.00*	134. 00 ± 5. 00[1)]
Testosterone (μg/100 ml)	1. 85 ± 0. 09	1. 99 ± 0. 06	1. 40 ± 0. 09
Testes			
Ca (%)	14. 23 ± 1. 13	15. 50 ± 1. 90	*17. 92 ± 2. 57"*
P (%)	1. 59 ± *0. 07*	1. 66 ± *0. 07*	1. 38 ± 0. *18"*
Zn (μg/mg)	11. 23 ± *0. 81*	11. 28 ± *0. 90*	*8. 00* ± 2. 23[1)]
Mg (μg/g)	1. 49 ± *0. 14*	1. 49 ± *0. 17*	1. 64 ± 0. 13

Values are means (±SEM) per group.
Parenthesized number shows a number of cocks.
1) Significantly different ($P < 0.05$) from the basal group by analysis of variance.

group. On the other hand, values of P and Zn in the plasma and testes were significantly lower in the high group than those in the basal group. No significant differences were detected between groups for Mg concentration in the plasma and testes. After the recovery period, Zn administration returned Ca, P and Zn levels in the plasma and testes to the levels near to the controls (Table 4). The same trend occurred after removal of excess Ca from the diet without addition of Zn, while Zn concentration in the plasma was significantly lower than that in the controls.

Table 4. Effects of dietary Zn on chemical constituents in blood plasma and testicular tissue of the high group cocks at the end of the ***recovery*** period.

	Controls		High group	
	Basal group (10)	Moderate group (10)	***Zn*** added group (5)	Zn free group (5)
Plasma				
Ca (%)	9.78± 0.23	10.40± 0.25	9.99± 0.52	9.81± 0.20
P (%)	7.33± 0.39	6.88± 0.11	7.77± 0.42	6.76± 0.23
Zn (μg/ml)	3.70± 0.16	3.45± 0.21	3.90± 0.19	2.48± 0.14[1)]
Mg (%)	2.34± 0.51	2.44± 0.60	2.39± 0.63	2.41± 0.54
Glucose (mg/100 ml)	170.00± 25.00	169.60 ± 25.00	169.00 ± 32.00	157. ooi 4.00
Cholesterol (mg/100 ml)	90.00±18.00	79.90± 16.87	87.00±21.00	129.00 ±26.00
Testes				
Ca (%)				
P (%)	15.1.86±90± 0.07 3.00	16.00±1.78± 0.06 3.11	17.1.69 00±± 0.09[1)] 1.00	16.90±1.60± 0.08[1)] 2.91
Zn (μg/mg)	12.47 ± 3.00	12.55± 3.11	15.08± 5.80	12.90± 6.39
Mg (μg/g)	2.00± 0.11	1.89± 0.21	2.40± 0.17	1.90± 0.14

Values are means (±SEM) per group.
Parenthesized number shows a number of cocks.
1) Significantly different ($P<0.05$) from the basal group by analysis of variance.

High dietary Ca caused significant increase in plasma cholesterol, while resulted in a significant decrease in plasma glucose level as compared with the basal group (Table 3). No significant differences were found between groups in plasma glucose at the recovery period, while cholesterol level was still higher in the Zn free group than those in controls and in Zn added group (Table 4). No measurable changes were noted in the concentration of plasma testosterone between experimental groups.

The results shown in Table 5 indicate that semen volume, sperm concentration and the motility were nearly identical between the three experimental groups.

According to the histological examination of testes, no apparent differences were observed between basal and moderate groups. They revealed normal maturation which presented complete spermatogenesis (Pl. III, Fig. 1). Cessation of spermatogenesis was observed in the cocks received the high level of dietary Ca (Pl. III, Fig. 2). Secondary spermatocyte and early stage spermatid were comparatively few, and maturing spermatozoa were not observed

Table 5. Effects of dietary Ca levels on semen quality for the experimental period.

	Basal group (10)	Moderate group (10)	High group (10)
Semen Volume (ml)	0.33±0.04	0.35f0.07	0.31±0.06
Sperm concentration (billion/ml)	6.78±0.25	6.98±0.25	6.00±2.40
Sperm motility*	1.96±0.32	1.99±0.20	1.87±0.24

Values are means (±SEM) per group.
Parenthesized number shows a number of cocks.
* Motility was graded between 0 to 6.

in these testes. No conspicuous effects were seen in the non-germinal sustentacular cells of all experimental groups. Lipid droplets within the seminiferous tubules were relatively few in basal group cocks (Pl. III, Fig. 3). They gave negative Schultz reaction. Histological changes in the interstitial tissues were accompanied by parallel changes in the seminiferous tubules. Interstitial cells were atrophic in the high group cocks. They had small nuclei and large amount of lipid droplets which were positive to Schultz test (PI. III, Fig. 4). On the basis of these observations, functional activity of these testicular cells were probably low.

Marked alterations in the activity of the enzymes studied are summarized in Table 6. Enzyme activities of the seminiferous tubules and interstitial cells were similar in the testes of the basal and moderate groups. However, there were striking differences in several histochemical reactions in the testes of the high group. In the seminiferous tubules of the high group, moderate activities of LDH and MDH were observed, but considerably less activities were noted with β-HDH, Ster-D, GGPDH, NADH, NADPH and AP (Pl. III, Figs. 5 and 6). The activities of β-HDH and Ster-D were markedly decreased in the inter-

Table 6. Histochemical distribution of oxidative enzyme activity in the testicular tissues evaluated arbitrarily on a zero to four plus scale for the experimental period.

Enzyme	Basal group		Moderate group		High group	
	ST	IC	ST	IC	ST	IC
LDH	++++	+	++++	++	+	+
MDH	++++	++	++++	++	+	+
β-HDH	+	++++	+	++++	−	±
Ster-D	+	++++	+	++++	−	−
G6PDH	++++	++++	++	++++	+	++
NADH	−	++++	+	++++	±	++
NADPH	++++	++++	++	++++	±	++
AP	++++	+	++++	+	−	+

Abbreviations: ST; seminiferous tubules, IC: interstitial cells, LDH; lactic dehydrogenase, MDH; malic dehydrogenase, β-HDH; β-hydroxybutyric dehydrogenase, Ster-D; steroid 3β-ol dehydrogenase, GGPDH; glucose-6-phosphate dehydrogenase, NADH; nicotinamide-adenine dinucleotide dephydrogenase, NADPH; nicotinamide-adenine dinucleotide phoshate dehydrogenase, AP; alkaline phosphatase.

stitial cells and ranged from very weak to absent.

At the recovery period, removal of high dietary Ca or Zn supplement returned LDH, MDH and AP activities in spermatogenic cells of the tubules to a normal level. Recovery of β-HDH and Ster-D activities was observed after Zn supplementation while the recovery tendency was still subnormal after removal of Ca from the diet.

DISCUSSION

The present study showed that excess dietary Ca (Ca/P imbalanced diet) produced an antagonism to Zn, P, Mg and various enzymes. The result clearly suggested that a plasma Zn level (0.9 μg/ml) would certainly reflect an inadequacy of Zn intake. An inadequate intake of Zn causes growth retardation, testicular atrophy, spermatogenic arrest and abnormalities of reproductive system in cocks (Eltohamy, 1981). Specificity of Zn deficienty was established by showing that plasma Zn level could be reversed by oral administration of Zn and could not be reversed by removal of Ca from the diet.

Moderate changes in the testes weights, semen volume, sperm concentration, sperm motility and impairment of normal spermatogenesis in the cocks receiving the high level of Ca clearly demonstrated the important role of Ca in the reproductive process in the male fowl. Histological examination of the testes revealed loss of spermatozoa, regression of spermatids and secondary spermatocytes from the seminiferous tubules of the high group. This observation indicated that the transitional stages between the primary and secondary spermatocytes seemed to be critical stage and were dependent on the level of dietary Ca. This finding seemed to be at variance with results obtained by Wilson *et al.* (1969). They reported that the level of 3.0 % dietary Ca were not detrimental to the reproduction.

Lipid and cholesterol contents of the seminiferous tubules were depleted by the level of high dietary Ca. This observation was interpreted on the basis of Lacy Hypothesis (Lacy, 1960). He reported that large quantities of lipid and cholesterol in the seminiferous tubules were due to temporary cessation of gonadotrophin output from pituitary gland. Histochemical reaction for LDH, MDH and AP in the spermatogenic cells of the seminiferous tubules of the group receiving high dietary Ca was lowered. Histochemical reactivity of these enzymes represents different metabolic pathways, LDH is involved in glycolysis and MDH in tricarboxylic cycle. Decline in glycolytic activities of the testicular cells of the cocks receiving high dietary Ca suggested that anaerobic pathways for glucose metabolism were altered concomitantly. The observed decline in plasma glucose may indicate a marked impairment in utilization of glucose in the cocks receiving high dietary Ca.

Interstitial cells of the cocks receiving high dietary Ca were also affected. There were reduction in the cell size, high accumulation of lipid and cholesterol contents and decrease in both β-HDH and Ster-D activites. Reaction of these, enzymes is useful indicator of the level of testicular hormone production. G6PDH showed little reactivity in the interstitial cells of the high group.

This enzyme is involved in the first step of the hexose monophosphate shunt, which is linked with the production of NADPH, required in the testes for steroid hormone synthesis. Therefore, the present observations were supported by significant reduction in comb and testes weights which are known to be depended upon the androgen level of the testes. These collective observations can be taken to suggest that interstitial tissues of the testes of the cocks receiving high dietary Ca were not capable of steroid synthesis.

Enzymes selected for this study are Zn-containing enzymes. These activities were found to parallel to tissue Zn contents in several instances (Prasad *et al.*, 1971). It may be true Zn is a regulator of these enzymes. Effects of dietary Ca levels on the synthesis of testicular enzymes might be via alteration in tissue Zn contents. Thus, the lowered activities of the testicular enzymes detected in the present study were account for by lack of sufficient Zn to support the lack of synthesis of these enzymes. While, McCuaig *et al.* (1972) reported that the dietary Ca/P ratio was the important factor regulating the activity of these enzymes. From the results of the present study, it is evident that the changes in dietary Zn level may be important factor exceed those of Ca/P ratio. The results of the recovery period would support this suggestion, in which Zn administration returned blood plasma Zn and P levels, and the enzyme activities of the testicular tissues of the high group to a normal level.

Lehninger (1970) reported that excess Ca tended to slow down cellular metabolism by reducing testicular membrane permeability and interfering with AP production. These effects of excess Ca, together with the effect of Zn deficiency, may account for the lowered enzyme activities *as* detected in the present study.

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Explanation of Plate III

Fig. 1. Testis from a cock fed the basal diet. Seminiferous tubules show various stages of spermatogenesis which is supposed in functionally active phase. HE stain. ×320.

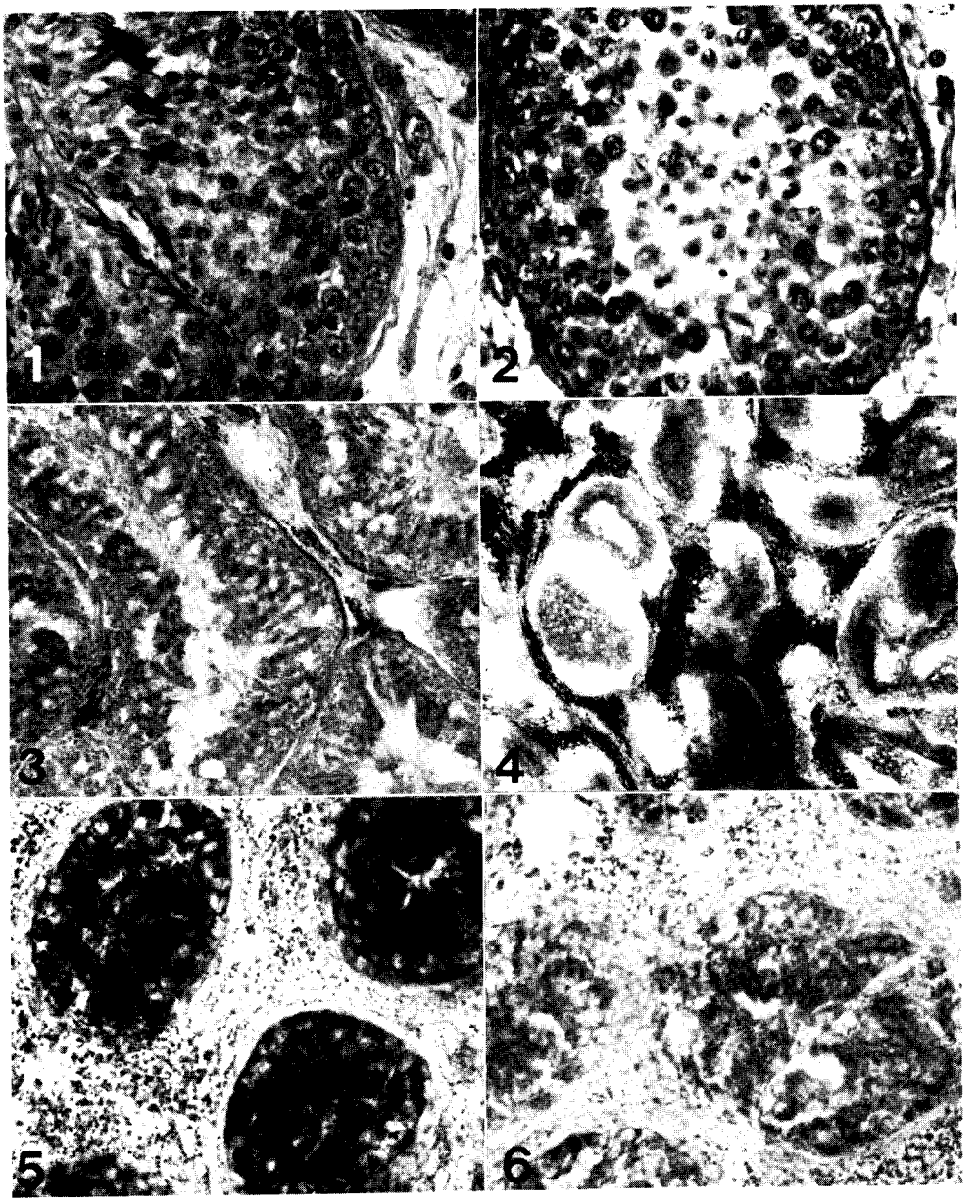
Fig. 2. Testis from a cock fed diet supplemented with high dietary Ca. Spermatogenesis is ceased. Seminiferous tubules contain spermatogonia. primary spermatocytes and sustentacular cells. Maturing spermatozoa are rarely seen. Interstitial cells are regressed and their nuclei are shrunk. HE stain. ×320.

Fig. 3. Histochemical demonstration of lipids in the testis from a cock fed the basal diet. Relative amount of lipid droplets seems to be contained in the interstitial cells. Sudan black stain. ×120.

Fig. 4. Histochemical demonstration of lipids in the testis from a cock fed diet supplemented with high dietary Ca. A large amount of lipid seems to be be contained not only in the interstitial tissue but also in the seminiferous tubules. Sudan black stain. x80.

Fig. 5. Histochemical reaction for G6PDH activity in the testis from a cock fed the basal diet. The tubules and interstitial cells showed clearly high activity for this enzyme. Acetone fixation. Nitro-BT. ×120.

Fig. 6. Histochemical reaction for G6PDH activity in the testis from a cock fed diet supplemented with high dietary Ca. The activities in the tubules and interstitial cells decreased but some cells still exhibit positive activities. Acetone fixation. Nitro-BT. ×120.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