Biochemical and Pathological Findings on Sheep and Calves Dying of Experimental Cerebrocortical Necrosis

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ABSTRACT. For observing biochemical and morphopathological changes in experimental cerebrocortical necrosis, 6 sheep and 6 calves were given amprolium (600 mg/kg/day). All of the amprolium-dosed animals showed neurological signs and died on days 35 to 57 after the onset of daily administration. They were pathologically confirmed as cerebrocortical necrosis. Total thiamine levels in blood and tissues were markedly reduced at death. Blood total thiamine level decreased significantly on day 7 of amprolium administration, and reached to the level as low as that seen at death about 2 weeks before the onset of abnormal electroencephalograms. Significant decrease in a thiamine-dependent enzyme, erythrocyte transketolase activity, and increase in thiamine pyrophosphate effect were detected about 2 weeks before the onset of abnormal electroencephalograms. Pathological changes and cerebral autofluorescence observed under ultraviolet light were also examined at death.—KEY WORDS: calf, cerebrocortical necrosis, sheep, thiamine, transketolase.

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Cerebrocortical necrosis (CCN) seen in domestic ruminants has been known as a disease due to thiamine deficiency because affected animals have low thiamine levels in tissues [1, 13] and respond to thiamine therapy [13]. Induction of experimental CCN has been tried by administration of amprolium, a thiamine antagonist, and clinical, morphological and biochemical changes were examined [7, 9, 11, 15]. However, results obtained in these studies on experimental CCN and spontaneous cases were not in agreement in some aspects including blood thiamine levels, transketolase activities and its thiamine pyrophosphate (TPP) effect in blood or erythrocyte [10, 14, 16].

In this series of study [3, 5] including the present one, we describe the pathogenesis of CCN on clinical, biochemical and pathological aspects. No systematic report has been available on these points, although some papers [6, 7, 9, 11, 16] had described some of them separately.

In the present study, thiamine-related biochemical changes in blood which were induced by amprolium administration in sheep and calves were observed chronologically from the beginning of administration to death. Pathological and thiamine-related biochemical changes in tissues were examined at death. The study on electroencephalographic (EEG) aspect and clinical signs has been already reported [5]. Chronological alterations in tissues are reported elsewhere [3].

MATERIALS AND METHODS

Nine Suffolk sheep, 7 to 17 months of age, and 6 Holstein calves, 4 to 7 months of age, were employed (Table 1). All sheep and calves Nos. 3 to 6 were fitted with rumen cannulae and EEG electrodes as noted in the previous report [5]. Calves Nos. 1 and 2 were equipped with neither rumen cannulae nor electrodes. Sheep Nos. 1 to 9 and calves Nos. 3 to 6 were given amprolium [1-(4-amino-2-*n*-propyl-5-pyrimidinyl-methyl)-2-picolinium chloride hydrochloride, MSD Japan Co., Ltd., Tokyo] and/or thiamine propyldisulfide (TPD, Takeda Industries Co., Ltd., Osaka) via the cannulae as noted in the previous report [5]. The dose of the drugs and the allotment design are summarized in Table 1. Calves Nos. 1

Table 1. Experimental animals and starting days of abnormal EEG and behaviors

Animal[a]				Days after administration			
No.	Sex	Month after birth	Body weight (kg)	Abnormal EEG	Anorexia	Convulsion	Death (Euthanasia)
SHEEP							
Amprolium 600 mg/kg/day							
1	♂	14	52	48	51	52	55
2	♂	14	50	36	38	39	41
3	♂	17	45	50	51	52	57
4	♂	17	54	36	36	37	40
5	♀	7	29	42	43	45	47
6	♀	7	27	37	38	39	41
Amprolium 600 mg/kg/day and thiamine propyldisulfide 200 mg/day							
7	♂	9	25	—[b]	—	—	(58)
8	♀	14	43	—	—	—	(58)
Thiamine propyldisulfide 50 mg/day							
9	♂	14	33	—	—	—	(58)
CALVES							
Amprolium 600 mg/kg/day in amprolium-containing feed supplement							
1	♂	4	138	ND[c]	35	—	(37)
2	♂	6	179	ND	40	44	(45)
Amprolium 600 mg/kg/day							
3	♂	7	250	31	31	34	35
4	♀	4	145	39	41	42	44
5	♂	5	175	40	40	41	49
6	♂	4	138	40	45	47	56

a) Suffolk breed and Holstein breed.
b) Normal.
c) Not determined. Calf Nos. 1 and 2 were not fitted with electrodes for EEG recording.

and 2 were given 600 mg/kg/day of amprolium orally in amprolium-containing feed supplement (ACFS; Amprol Plus; amprolium 25%, ethopabate 1.6%; Dainippon Pharmaceutical Co., Ltd., Osaka) suspended in tap water.

Blood was collected from the jugular vein at weekly intervals during the course of experiment. Blood was also sampled at the onset of clinical signs and immediately before death. For determination of transketolase activity, hemolysate was prepared according to the method of Takeuchi *et al.* [17]. Immediately after death, the liver and heart were taken for thiamine determination. The brain was soaked in cold physiological saline and observed under ultraviolet (UV) light at 365 nm in the dark room. After this observation, the cerebrum was partly taken for thiamine determination. Whole blood, hemolysate and tissues collected as noted above were stored at −75°C until determination.

Total thiamine concentration was determined by the thiochrome method using high-performance liquid chromatographic (HPLC) system [8] with modifications as follows. To 1 m*l* of blood, an equal volume of 20% trichloroacetic acid was added and centrifuged. The cerebral cortex was divided from the medulla. Approximately 1 g of tissues were homogenized with 4 m*l* of 10% trichloroacetic acid, filled up to 25 m*l* with 5% trichloroacetic acid and centrifuged. The supernatant solution was filtrated through a 0.45 μm of disposable syringe filter unit, and hydrolysis of thiamine esters with takadiastase. Chromatographic analysis was performed using a component system (Automated-LC System, Shimadzu Co., Ltd., Kyoto). A column (shim-pack CLC-ODS(M) 4.6 mmϕ × 15 cm, Shimadzu) and a guard column (shim-pack G-ODS(4) 4.0 mmϕ × 1 cm, Shimadzu) were also used in this system.

Erythrocyte transketolase activity (ETKA) was determined by the method of Takeuchi *et al.* [17]. The ETKA was expressed as nanomoles of sedoheptulose-7-phosphate formed per m*l* of erythrocyte per min (nmol-S7P/m*l*-ery/min). TPP effect on ETKA, which is the per cent difference in ETKA with and without excess TPP, was also determined.

For histopathological examination, organs and tissues were fixed in 10% buffered formalin solution. Cerebral brocks with or without autofluorescence were dissected from formalin-fixed brains under UV light at 365 nm. All specimens were stained with hematoxylin and eosin.

RESULTS

Clinical manifestations observed in animals employed in the present study are summarized in Table 1. No significant difference was seen in the clinical course between amprolium-dosed and ACFS-dosed calves. Calves Nos. 1 and 2 were killed on day 37 and 45 of administration, respectively, when they were showing ataxia and lateral recumbency. Sheep Nos. 7 and 8 given both amprolium and TPD and sheep No. 9 given TPD alone remained normal and killed on day 58 of administration (Table 1).

Biochemical findings: In the sheep dosed with amprolium, the blood total thiamine concentrations decreased to about half of the control value at the first sampling on day 7 of administration (Table 2). The concentrations reached to the level as low as that observed at death 14 to 16 days

Table 2. Blood total thiamine concentration, ETKA and TPP effect in sheep given amprolium and/or TPD.

	Blood total thiamine concentration (*ng*/m*l*)	ETKA (nmol-S7P/m*l*-ery/min)	TPP effect (%)
All sheep (controls)			
Before administration	22.2±4.5(9)	120.4±36.0(9)	88.6±20.7(9)
Amprolium-dosed sheep			
A week after amprolium administration	11.9±3.5(6)***	110.3±52.9(6)	121.5±36.0(6)
2 weeks after amprolium administration	8.3±3.3(4)***	84.2±37.1(6)	150.4±49.4(6)**
14–16 days before the onset of abnormal EEG	5.7±2.9(4)***	63.2±25.5(6)**	200.4±45.4(6)***
7–9 days before the onset of abnormal EEG	5.2±2.2(5)***	49.1±20.0(6)**	273.5±71.5(6)***
Onset of abnormal EEG	4.9±1.8(5)***	40.6±18.8(5)**	267.3±67.9(5)***
Convulsion	4.3±1.2(5)***	41.3±12.0(5)***	267.2±64.0(5)***
Death	6.3±1.9(5)***	34.2±15.0(5)***	345.5±95.4(5)***
Amprolium+TPD-dosed sheep			
At euthanasia	29.5±6.1(2)	144.6±16.0(2)	62.4±5.5(2)
TPD-dosed sheep			
At euthanasia	30.5(1)	103.2(1)	77.5(1)

Values are expressed as mean±standard deviation.
Numbers in parentheses are numbers of animals tested.
**: Significantly different from the value of controls at $p<0.01$.
***: Significantly different from the value of controls at $p<0.001$.

Table 3. Blood total thiamine concentration, ETKA and TPP effect in calves given ACFS or amprolium

	Blood total thiamine concentration (*ng*/m*l*)	ETKA (nmol-S7P/m*l*-ery/min)	TPP effect (%)
All calves			
Before administration (controls)(6)	26.8±4.8	131.4±54.2	55.7±32.6
A week after administration(4)	14.3±1.7**	100.6±28.1	87.1±26.9
2 weeks after administration(2)	6.6±0.1	110.4±35.8	119.9±18.7
ACFS-dosed calves			
14 days before the onset of anorexia(2)	5.2±0.9	51.4±4.2	325.0±99.3
7 days before the onset of anorexia (2)	4.5±0.5	59.2±20.4	417.3±148.6
Onset of anorexia (2)	5.0±2.4	35.8±13.4	450.3±99.1
At euthanasia (2)	4.7±0.4	27.6±13.4	593.9±125.7
Amprolium-dosed calves			
10 days before the onset of abnormal EEG (2)	6.0±0.2	39.4±1.0	440.2±23.0
5 days before the onset of abnormal EEG (2)	5.5±0.1	35.7±0.9	450.3±4.0
Onset of abnormal EEG (2)	3.3±1.3	24.7±1.9	549.0±16.9
Convulsion (2)	4.7±2.5	22.8±1.2	558.0±5.5
Death (4)	3.3±1.1***	25.5±11.9*	690.1±183.7**

Values are expressed as mean±standard deviation.
Numbers in parentheses are numbers of animals tested.
*: Significantly different from the value of controls at $p<0.05$.
**: Significantly different from the value of controls at $p<0.01$.
***: Significantly different from the value of controls at $p<0.001$.

Table 4. Total thiamine concentration in tissues (μg/g wet weight)

	Liver	Heart	Cerebral	
			Cortex	Medulla
SHEEP				
Amprolium-dosed (6)	0.304±0.133[a)]	0.259±0.077[a)]	0.075±0.022[a)]	0.123±0.035[a)]
Amprolium+TPD-dosed (2)	3.833±0.315	3.553±0.005	1.039±0.140	1.031±0.120
TPD-dosed (1)	4.186	3.369	1.216	1.046
CALVES				
Amprolium-dosed (4)	0.526±0.182[b)]	0.253±0.120[b)]	0.075±0.022[b)]	0.085±0.020[b)]
ACFS-dosed (2)	0.634±0.138[b)]	0.430±0.066[b)]	0.063±0.027[b)]	0.125±0.025[b)]
Intact normal calves (5)	1.357±0.080	2.679±0.505	0.908±0.177	0.499±0.076

Values are expressed as mean±standard deviation.
Numbers in parentheses are numbers of animals employed.
a) Significantly different from the value of amprolium+TPD-dosed sheep at $p<0.001$.
b) Significantly different from the value of intact normal calves at $p<0.001$.

before the onset of abnormal EEG, and remained low until death. The results obtained in the calves dosed with amprolium or ACFS are shown in Table 3. Although the number of animals was not sufficiently large to permit statistical analysis of the data, the results were similar to those of sheep dosed with amprolium (Table 2). In sheep Nos. 7 to 9 dosed with amprolium + TPD or TPD alone, blood thiamine levels remained over 27 *ng*/m*l* until euthanasia.

ETKA decreased significantly 14 to 16 days before the onset of abnormal EEG and remained low until death in the amprolium-dosed sheep (Table 2). TPP effect increased significantly 2 weeks after the beginning of daily administration and remained high until death in the amprolium-dosed sheep (Table 2). After the onset of abnormal EEG, values of TPP effect ranged from 260.7 to 452.5% in the amprolium-dosed sheep excluding sheep No. 4, in which it ranged from 148.1 to 181.7%. In sheep Nos. 7 to 9 dosed with amprolium + TPD or TPD alone, neither ETKA decrease nor TPP effect increase was observed throughout the experiment.

The changes in ETKA and TPP effect observed in the

calves dosed with amprolium or ACFS were similar to those in the amprolium-dosed sheep (Tables 2 and 3). TPP effects determined after the onset of abnormal EEG in calves were much higher than those observed in sheep.

Total thiamine concentrations of the liver, heart and cerebrum in sheep and calves given amprolium or ACFS were markedly lower than those in sheep dosed with amprolium + TPD or TPD alone, and intact normal calves (Table 4). These intact calves are not listed in Table 1. In the cerebrum of intact calves, thiamine level in the cortex was twice as high as that in the medulla. There was no significant difference between the levels in the cortex and medulla in sheep dosed with amprolium + TPD or TPD alone. In the amprolium-dosed sheep and calves, however, the level in the cerebral cortex tended to be lower than that in the medulla.

Histopathological findings: Pathological changes found in the lesions were classified into 4 categories (see the footnote of Table 5). Most lesions in the cerebral cortex of affected animals were classified into ++ or +++ (Table 5). In the cerebral medulla adjacent to severe cortical lesions, edematous and vascular changes presented. Necrotic changes similarly to those in the cerebral cortex were present in the midbrain and the cerebellum from all the sheep and calves given amprolium or ACFS. In some cases, necrotic lesions were found in the thalamus and pons.

No significant lesions were detected in the liver, heart, kidney, lung, spleen, small and large intestines examined.

Under UV light, many autofluorescent spots were observed over the brain surface of both hemispheres in the affected animals. In transverse section, the autofluorescent areas were confined to the cerebral cortex. Neither histological lesions noted above nor autofluorescence were found in brains from sheep given amprolium + TPD or TPD alone.

DISCUSSION

No differences were observed in all the aspects examined between the amprolium-dosed and the ACFS-dosed calves. Total thiamine levels in the liver and heart of sheep and calves dosed with amprolium or ACFS decreased similarly to those found in naturally occurring CCN [1], but the level in the brain of ours was lower than Edwin's [1]. A decrease in ETKA and an increase in TPP effect were also observed in affected sheep and calves employed in the present study. This result confirmed the observation found in sheep and cattle affected with naturally occurring CCN [1, 13].

Blood total thiamine concentration was reported not to be lowered significantly in spite of decreases in tissue thiamine level in amprolium-dosed calves [7]. However, in the present study, not only tissue thiamine levels but also blood thiamine level decreased significantly at death. Loew *et al.* [10] reported that blood thiamine levels in normal cattle did not differ from those in animals with spontaneous CCN, but Rammell and Hill [14, 15] found decreased blood thiamine levels in suspected cases of CCN although tissue thiamine levels and pathological changes were not examined in these reports. We have also experienced that some cattle confirmed histologically as CCN had low thiamine levels in blood and tissues. Thus it could be confirmed that the decrease in blood thiamine level was an important aspect of not only experimental CCN but also spontaneous one.

Table 5. Histopathological findings in brains of amprolium-dosed animals.

	Cerebral cortex				Thalamus	Midbrain	Pons	Cerebellum
	Frontal lobe	Temporal lobe	Parietal lobe	Occipital lobe				
SHEEP[a)]								
No. 1	++	+++	+++	+++	−	+++	+	+++
2	++	NT[b)]	++	+++	−	+++	++	+++
3	+++	+++	+++	+++	+	+++	−	++
5	+	+++	+++	+++	−	+++	+	+++
6	++	++	++	++	−	++	−	++
CALVES								
No. 1	++	+++	+++	+++	−	++	−	+
2	+	+	+	+	−	++	−	+
3	+++	++	+++	++	−	+	+	+
4	++	+++	+++	++	+	++	++	+
5	++	+++	+++	+++	+	++	−	+
6	+++	+++	+++	+++	++	+++	++	+++

−: Normal.
+: Necrotic change of nerve cells and general looseness of neuropils.
++: Necrotic change with increasing of vascular endothelial cells in size and number.
+++: Malacic change with increase of grial cells and migration of macrophages and neutrophils.
a) The brain of sheep No. 4 was damaged before examination.
b) Not tested.

In amprolium-dosed sheep, Loew and Dunlop [9] reported that a change in blood thiamine level was inconsistent with alterations in ETKA and TPP effect which were regarded as indices of thiamine status in ruminants [1]. In the present study, however, decreases in blood thiamine level and ETKA, and an increase in TPP effect were observed 2 weeks before the onset of abnormal EEG until death in amprolium-dosed animals. The significant change in blood thiamine level before the onset of abnormal EEG would indicate subclinical thiamine deficiency, and assessment of blood thiamine level may be useful to diagnose not only clinical but also subclinical thiamine deficiency.

The concentrations of blood total thiamine determined in this study were different from those reported in some studies [7, 9, 10], in which manual thiochrome method was employed. In the present study, thiochrome corresponding to the thiamine was determined separately from other fluorescent compounds by HPLC. In addition, HPLC profiles for blood of amprolium-dosed animals indicated many fluorescent compounds which were not seen in blood collected before amprolium administration. All these fluorophors may be measured as thiochrome by manual thiochrome method. In tissue samples, these contaminating fluorescent compounds were much less than those in blood. This may be the reason why blood thiamine level but tissue levels determined in the present study differed from that found by early investigators.

Significant changes in ETKA and TPP effect were observed before the onset of nervous signs in the present study. This result confirmed the observations of Spicer and Horton [16] in amprolium-dosed sheep, but the TPP effect measured in the control animals and amprolium-dosed ones of ours were much higher than Spicer's [16]. Significant changes in ETKA and TPP effect were observed later than the decrease in blood total thiamine level in the present study. This result suggests that blood thiamine level may be more sensitive than ETKA and TPP effect to detect thiamine deficiency. TPP effect measured in the amprolium-dosed sheep No. 4 was lower than those found in the other amprolium-dosed sheep. Normal ETKA and TPP effect were reported in some field cases of CCN [10, 16]. TPP effect was unsuitable for detecting thiamine deficiency in humans with liver disorders [2] or alcoholism [4]. Therefore measurement of ETKA and TPP effect might not be the best indication of thiamine deficiency in the diagnosis of ruminants.

Histopathological changes and cerebral fluorescence observed in the brains of affected animals examined in the present study were similar to those observed in naturally occurring CCN [6, 12, 18]. It could be agreed that these changes including biochemical and behavioral ones and observed at death were confirmed as CCN induced experimentally.

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REFERENCES

1. Edwin, E. E., Markson, L. M., Shreeve, J., Jackman, R., and Carroll, P. J. 1979. Diagnostic aspects of cerebrocortical necrosis. *Vet. Rec.* 104: 4–8.
2. Fennelly, J., Frank, O., Baker, H., and Leevy, C. M. 1967. Red blood cell-transketolase activity in malnourished alcoholics with cirrhosis. *Am. J. Clin. Nutr.* 20: 946–949.
3. Horino, R., Itabisashi, T., and Hirano, K. 1994. Biochemical and pathological observations on sheep showing various clinical manifestations of experimental cerebrocortical necrosis. *J. Vet. Med. Sci.* 56: 573–576.
4. Inokuchi, T. 1979. Thiamin metabolism in chronic alcoholics. *Vitamins (Kyoto)* 53: 493–498 (in Japanese).
5. Itabisashi, T., Horino, R., Hirano, K., and Maeda, M. 1990. Electroencephalographic observation on sheep and cattle with experimental cerebrocortical necrosis. *Jpn. J. Vet. Sci.* 52: 551–558.
6. Jackman, R. and Edwin, E. E. 1983. Cerebral autofluorescence and thiamine deficiency in cerebrocortical necrosis. *Vet. Rec.* 112: 548–550.
7. Kasahara, T., Ichijo, S., Osame, S., and Sarashina, T. 1988. Clinical and biochemical findings in bovine cerebrocortical necrosis produced by oral administration of amprolium. *Jpn. J. Vet. Sci.* 51: 79–85.
8. Kimura, M., Fujita, T., and Itokawa, Y. 1982. Liquid-chromatographic determination of the total thiamin content of blood. *Clin. Chem.* 28: 29–31.
9. Loew, F. M. and Dunlop, R. H. 1972. Induction of thiamine inadequacy and polioencephalomalacia in adult sheep with amprolium. *Am. J. Vet. Res.* 33: 2195–2205.
10. Loew, F. M., Bettany, J. M., and Halifax, C. E. 1975. Apparent thiamin status of cattle and its relationship to polioencephalomalacia. *Can. J. Comp. Med.* 39: 291–295.
11. Markson, L. M., Edwin, E. E., Lewis, G., and Richardson, C. 1974. The production of cerebrocortical necrosis in ruminant calves by the intraruminal administration of amprolium. *Br. Vet. J.* 130: 9–16.
12. Markson, L. M. and Wells, G. A. H. 1982. Evaluation of autofluorescence as an aid to diagnosis of cerebrocortical necrosis. *Vet. Rec.* 111: 338–340.
13. Pill, A. H. 1967. Evidence of thiamine deficiency in calves affected with cerebrocortical necrosis. *Vet. Rec.* 81: 178–181.
14. Rammell, C. G. and Hill, J. H. 1986. A review of thiamine deficiency and its diagnosis, especially in ruminants. *N. Z. Vet. J.* 34: 202–204.
15. Rammell, C. G. and Hill, J. H. 1988. Blood thiamine levels in clinically normal goats and goats with suspected polioencephalomalacia. *N. Z. Vet. J.* 36: 99–100.
16. Spicer, E. M. and Horton, B. J. 1981. Biochemistry of natural and amprolium-induced polioencephalomalacia in sheep. *Aust. Vet. J.* 57: 230–235.
17. Takeuchi, T., Nishino, K., and Itokawa, Y. 1984. Improved determination of transketolase activity in erythrocytes. *Clin. Chem.* 30: 658–661.
18. Terlecki, S. and Markson, L. M. 1961. Cerebrocortical necrosis in cattle and sheep. *Vet. Rec.* 73: 23–27.