Histopathological study of encephalomalacia in neonatal calves and application of neuronal and axonal degeneration marker

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ABSTRACT. Five calves that had shown neurological symptoms within 9 days after birth were histopathologically diagnosed as encephalomalacia. Two calves showed bilateral laminar cerebrocortical necrosis and neuronal necrosis in the corpus striatum and hippocampus. Since the distributional pattern of the lesions was consistent with that of global ischemia in other species, the lesions were probably hypoxic/ischemic encephalopathy consistent with the history of dystocia and perinatal asphyxia. One calf also showed bilateral laminar cerebrocortical necrosis. However, the lesions were chronic ones, because the calf had survived for long time and necropsied at postnatal day 118. Additionally, the lesions did not involve the corpus striatum and hippocampus. The other two calves showed multifocal necrosis with vascular lesions characterized by fibrin thrombi, perivascular edema and perivascular hyaline droplets in the cerebral cortex, corpus striatum, thalamus, brain stem and cerebellum. Considering the age of onsets and histopathological appearance, it was possible that latter three calves were also hypoxic/ischemic encephalopathy, however, exact cause of them was not revealed. In all calves, degenerated/ necrotic neurons showed positive reactions for Fluoro-Jade C and degenerated axons showed immunoreactivity for Alzheimer precursor protein A4. Therefore, these markers were applicable to examination of brain injury in neonatal calves.

KEY WORDS: Alzheimer precursor protein A4, encephalomalacia, Fluoro-Jade C, ischemic/ hypoxic encephalopathy, neonatal calf

In clinical practice of cattle, dystocia and perinatal asphyxia remains as an important problem [22]. However, detailed pathological study on brain injury in these cases has not been conducted. In human, neonatal ischemic/hypoxic encephalopathy is well known as perinatal brain injury [23]. The most widely used definition of perinatal period is the period from 20 weeks fetal gestation to 28 days postnatal [16]. Perinatal brain injury shows different pattern of lesion owing to several factors such as maturity of brain and strength and duration of insults [43]. In term infant, which is defined as babies born after 37 completed weeks fetal gestation [16], major variation of lesions include selective neuronal necrosis that means necrotic lesions of gray matter areas vulnerable for hypoxic/ischemic stress such as the cerebral cortex, basal nuclei, hippocampus and cerebellum (Purkinje's cell) and parasagittal cerebral injury [23, 43, 44]. Meanwhile, in preterm infant, which is defined as babies born on or before completion of 37 weeks fetal gestation [16], white matter injury such as periventricular leukomalacia (PVL) is well known [23, 43, 44]. These perinatal brain injuries are often observed in bilateral and symmetrical both in term and preterm infants [11, 16, 23]. Especially, deep nuclear pattern of selective neuronal necrosis, parasagittal cerebral injury and PVL are predominantly symmetrical [11, 16, 23]. In neonatal foals, perinatal brain injury is well known as neonatal maladjustment syndrome (NMS) and characterized by laminar cerebrocortical necrosis and ischemic neuronal degeneration irregularly in the hippocampus, basal nuclei, thalamus,

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The Journal of
**Veterinary
Medical
Science**

Table 1. Clinical and pathological features of the calves

Calf No.	Bleed[a)	Sex[b)	Age at onsets (days)	Age at necropsy (days)	Status of delivery	Clinical symptoms	Histological findings of the brain	Histological findings other than the brain
1	JB	F	0	3	Placental abruption, dystocia, perinatal asphyxia	Consciousness disturbance, astasia	Laminar cerebrocortical necrosis, neuronal necrosis in the corpus striatum and hippocampus	-
2	JB	F	0	1	Dystocia, perinatal asphyxia	Consciousness disturbance, astasia, opisthotonus	Laminar cerebrocortical necrosis, neuronal necrosis in the corpus striatum and hippocampus	Traumatic changes in the tongue
3	H	F	0	118	Normal	Convulsion, extension of four limb, robot-like walking, blindness	Laminar cerebrocortical necrosis	-
4	JB	M	7	9	Normal	Astasia, extension of four limbs	Multifocal necrosis with vascular lesions	Fatty degeneration of hepatocytes, mild interstitial edema in the heart
5	H	M	9	13	Normal	Astasia, opisthotonus	Multifocal necrosis with vascular lesions	Focal necrosis and hemorrhage in the retina, mild hydropic degeneration of hepatocytes, mild interstitial edema in the heart, mild mononuclear cell infiltration in the jejunum

a) JB, Japanese black; H, Holstein. b) M, male; F, female.

amygdala, medial and lateral geniculate nuclei and colliculi [7, 39, 47]. Also in sheep, dogs and pigs, perinatal brain injury is reported [7, 35]. We have recently reported spontaneous PVL in a neonatal calf [25]. As mentioned above, various pattern of necrotic brain lesions, in other words encephalomalacia, has been reported in human and other animals, it has remained unclear whether patterns other than PVL occur also in calves. Therefore, to elucidate this matter, we selected five neonatal calves with encephalomalacia from calves subjected to pathological examinations in our laboratory from April 1, 2005 to September 30, 2017 and evaluated their histopathological features and causes.

We have experienced calves of which responsible lesions were not detected by routine histological examination despite of their clinical neurological symptoms. The fact that there are calves with no morphological changes in the brain despite clinical neurological symptom is also described in the literature [38]. Therefore, alternative method has been required to detect lesions in these cases. Ruby *et al.* have recently reported utility of Fluoro-Jade C (FJC) as a neuronal degeneration marker in foals with NMS, some of which show no histologic abnormities with standard hematoxylin and eosin (HE) staining [36]. Alzheimer precursor protein A4 (APP) has been applied as an axonal degeneration marker in studies on *Clostridium perfringens* type D enterotoxemia of sheep and cattle, neuroaxonal dystrophy of sheep and experimentally induced PVL of sheep [17, 24, 28, 29]. In the present study, we also investigated utility of these neuronal and axonal degeneration markers in neonatal calves with encephalomalacia.

MATERIALS AND METHODS

Animals

Five calves were examined in this study (Table 1). The calves had shown neurological symptoms within 9 days after birth, and they were necropsied at 1–118 days old. Calf No. 1 showed respiratory arrest immediately after delivery with human assistance. The calf was treated with artificial respiration and an injection of respiratory stimulant, and started to breath on her own after 10 min of birth. Calf No. 2 was also delivered with human assistance, and was hypothermic and severely debilitated. Other calves were delivered normally. Calf No. 1–3 had shown neurological symptoms (summarized in Table 1) since just after birth. Calf No. 4 had shown no clinical abnormality until postnatal day (PD) 7, but suddenly had shown neurological symptoms. Calf No. 5 had also seemed normal until PD 9, but had presented diarrhea and neurological symptom. Calf No. 1, 4 and 5 died spontaneously and the others were euthanized under deep barbiturate anesthesia due to poor prognosis. The clinical and pathological examination procedures were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine.

Pathological examination

At necropsy, central nervous system (CNS), major organs including the liver, spleen, kidneys, heart and lungs, and other organs were examined. Sera were collected at necropsy or a day before necropsy and then frozen at −30°C until use. For histological examination, CNS and other organs were fixed in 15% neutral-buffered formalin. Fixed samples were trimmed, embedded in paraffin-wax and cut into 5-μm thick sections. Paraffin sections were stained with HE. Selected sections were subjected to periodic acid-Schiff (PAS) reaction.

Fluoro-Jade C and APP immunohistochemistry

Selected sections were subjected to Fluoro-Jade C (Biosensis, Thebarton, Australia) and immunohistochemical staining using monoclonal anti-APP (dilution 1 in 10; Boehringer Mannheim, Mannheim, Germany). For immunohistochemistry, microwave antigen retrieval (15 min in 0.01 M citrate buffer, pH 6.0) was performed. As negative controls for FJC and immunohistochemistry, three calves (7-day-old, Holstein x Japanese black crossbreed calf, 13-day-old, Holstein calf and 22-day-old, Holstein calf) without neurological symptoms were used. As a semiquantitative assessment, the number of axons positive for APP was evaluated. The number of axons was graded by the three authors using the following scale according to a previous report [18]: 0, none; 1, slight; 2, scattered; 3, extensive.

Measurement of serum thiamine concentrations

The serum total thiamine concentrations of calf No. 1–3 were measured using HPLC with precolumn method [27] with modifications as follows. To 500 μl of serum, an equal volume of 10% trichloroacetic acid was added and centrifuged. The supernatant was hydrolyzed with takadiastase for converting thiamine esters to free thiamine. After derivatization reaction and adjustment pH to 7–8, chromatographic analysis was performed using a component system (Automated-LC System, Shimadzu Co., Ltd., Kyoto, Japan) with Purospher® STAR RP-18 endcapped column (5 μm) (Merck KGaA, Darmstadt, Germany). Mobile phase was dibasic sodium phosphate (25 mmol/l, pH 7.0): methanol (70:30, vol/vol) and pumped at flow rate of 1.0 ml/min.

Examination of serum Clostridium perfringens epsilon toxin

The serum epsilon toxin concentrations of calf 4 and 5 were examined using *Clostridium perfringens* epsilon toxin serological ELISA kit (Bio-X Diagnostics, Rochefort, Belgium).

Measurement of serum endotoxin concentrations

The serum endotoxin concentrations of calf 4 and 5 were examined with ES (Endotoxin Specific) method by Daiichi Kishimoto at their clinical examination center (Obihiro, Japan).

Detection of the BVDV gene

RT-PCR targeting the highly conserved region of BVDV was performed according to a previous report with the following primer set: forward primer 103 (5′TAGCCATGCCCTTAGTAGGAC 3′) and reverse primer 372 (5′ACTCC ATGTGCCATGTACAGC 3′) [46]. Sample RNA was extracted from the sera using a QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Each sample was processed by the single-tube RT-PCR method using a Gene-Amp® EZ rTth RNA PCR Kit (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS

Gross findings

In calf No. 1, a focal hemorrhage in the cortex of the right occipital lobe was found. No other gross change was detected (Fig. 1A). Calf No. 2 showed no obvious lesion in the brain. In calf No. 3, the dorsal surface of the cerebral cortex was discolored yellowish, especially in the parietal-occipital areas. At the cut surface of these areas, discoloration and thinning of the cerebral cortex was noted. In calf No. 4, at the cut surface of the brain, multifocal pale reddish lesions were observed bilaterally in the entire thalamus and caudal colliculus and cerebral peduncles of the mesencephalon (Fig. 2A). In calf No. 5, the brain was totally edematous. At the cut surface, multifocal reddish lesions were observed in the cerebral cortex of the parietal lobe and occipital lobe, thalamus and the dentate nuclei of the cerebellum.

Histological findings

All calves were diagnosed as encephalomalacia. Briefly, calves No.1–3 had laminar cerebrocortical necrosis, and calves No. 4 and 5 had multifocal necrosis with vascular lesions. Histological findings are summarized in Table 1. Detailed histological findings were described below.

In calf No. 1, there was bilateral laminar necrosis in the deep layer of whole cerebral cortex except the ventral regions (Fig. 1B) and multifocal necrosis in the corpus striatum. Eosinophilic and condensed degenerated neurons were noted diffusely in the cerebral cortex and corpus striatum, and partially in the hippocampus (in CA1–CA3 region, hilar region and dentate gyrus) (Fig. 1C and 1D), cerebellum (Purkinje cells), mesencephalon, pons and medulla oblongata. Vascularization with swelling of vascular endothelial cells was also observed in and around the necrotic layers and foci. In calf No. 2, there was bilateral laminar loosening of neuropil in the deep layer of whole cerebral cortex except the ventral regions. Degenerated neurons were scattered in the corpus striatum and identified sparsely in the cerebral cortex and hippocampus. In calf No. 3, bilateral laminar cerebrocortical necrosis was observed severely in the parietal lobes, temporal lobes and occipital lobes, and mildly in the frontal lobes. The affected areas of the cerebral cortex were severely cavitated. There were infiltrations of gitter cells and vascular endotheliosis. Degenerated/necrotic neurons were found around the necrotic foci. Astrogliosis was observed in superficial layers of the cerebral cortex, dorsal areas of the mesencephalon, and the olivary nucleus and vestibular nucleus of the medulla oblongata.

Calf No. 4 had bilateral multifocal symmetrical encephalomalacia (necrosis) in the basal ganglia, thalamus (Fig. 2B), nuclei of the brainstem including the caudal colliculi, pontine nuclei, vestibular nuclei and cuneate nuclei, and cerebellar nucleus. Necrotic
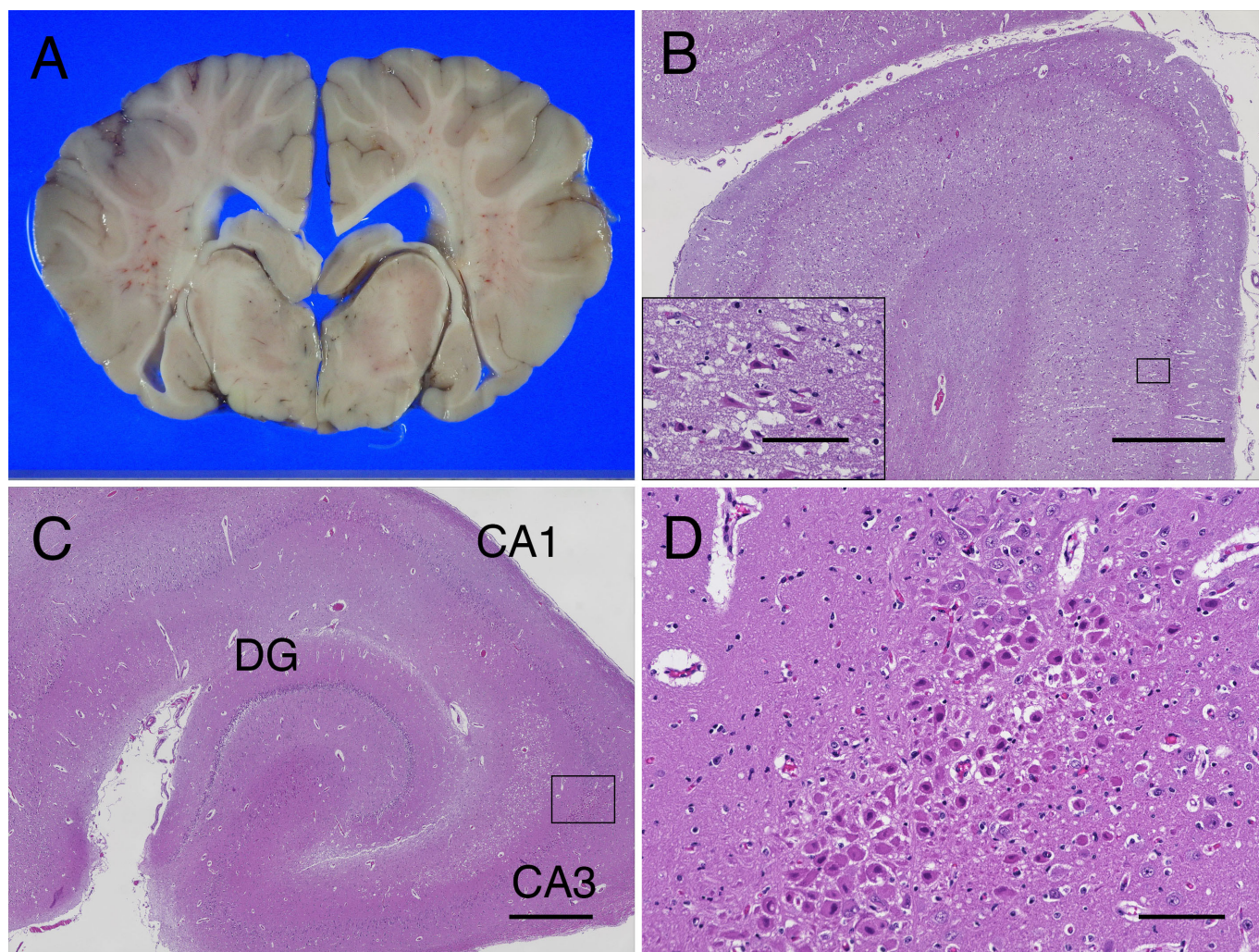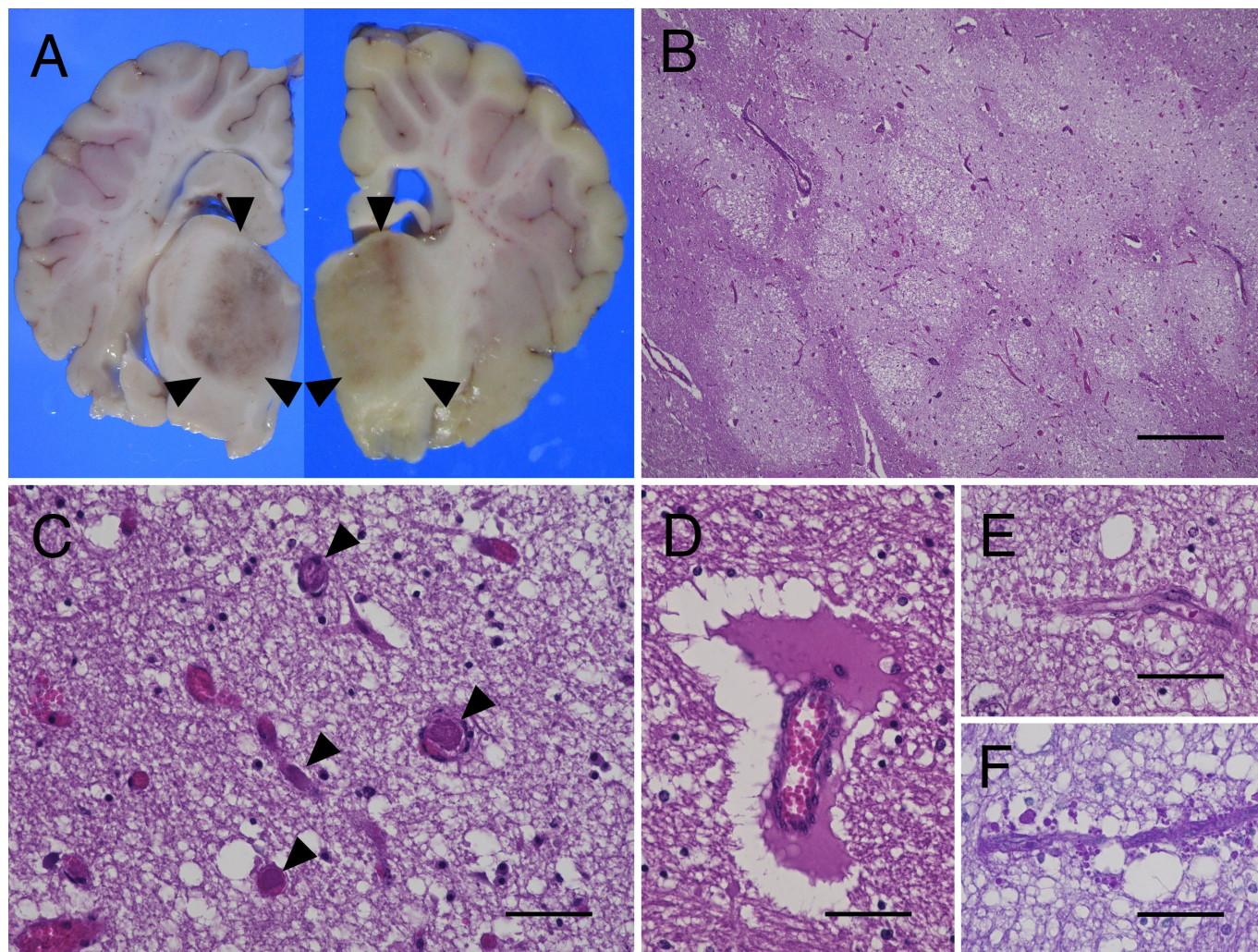
Fig. 1. Calf No. 1. A. Cerebrum, level of diencephalon. No obvious change is observed. B. Frontal lobe. Laminar cortical necrosis in the deep layer. HE. Bar=1,000 μm. Inset. Higher magnification (indicated by rectangle). Eosinophilic, condensed degenerated neurons. Bar=100 μm. C. Hippocampus. HE. Bar=1,000 μm. D. Higher magnification of C (indicated by rectangle). Eosinophilic, condensed degenerated neurons. HE. Bar=100 μm.

lesions and loosening of neuropil were sparsely observed in the cerebral cortex. Degenerated neurons and numerous fibrin thrombi were scattered in and around the necrotic foci (Fig. 2C). There was perivascular exudation of pale eosinophilic leakage mainly in and around the necrotic foci (Fig. 2D). Perivascular eosinophilic hyaline droplets were observed in the cerebral cortex of the frontal and parietal lobe and mesencephalon, prominently in the areas of cerebral sulci (Fig. 2E). These hyaline droplets were stained positive with PAS reaction (Fig. 2F). Calf No. 5 had similar necrotic lesions and vascular lesions mainly in the basal ganglia, thalamus, nuclei of the brainstem including the caudal colliculi, pontine nuclei, vestibular nuclei and cuneate nuclei, and cerebellar nucleus. Multifocal necrotic lesions and loosening of the neuropil were seen in the cerebral cortex and cerebellar cortex. Perivascular hyaline droplets were extensively observed in almost all the cerebral cortex.

Fluoro-Jade C and APP immunohistochemistry

In all calves, FJC positive reactions were mainly observed in the same area in which degenerated/necrotic neurons were recognized in HE sections (Fig. 3A and 3B). In calf No. 2, positive reactions were observed not only in the parietal lobe, corpus striatum and hippocampus in which degenerated/necrotic neurons were observed with HE staining but also in the thalamus and mesencephalon in which morphological changes were not recognized with HE staining. In all control calves, no positive reaction was detected. In calf No. 1–3, APP immunostaining revealed slight to extensive positive axons in the cerebral and cerebellar white matter (Table 2) (Fig. 3C and 3D). In these calves, neurons in the affected area were also showed intense positive reactions (Fig 3F). In calf No. 4 and 5, positively stained axons were extensively distributed in and around the necrotic foci (Fig. 2E). In all calves, immunoreactivity was also found in axons without obvious swelling. Although control calves also showed neuronal and axonal immunoreactivity for APP, the number of axonal immunoreactivity was small. In addition, control calves showed higher

Fig. 2. Calf No. 4. A. Cerebrum, the level of diencephalon. Bilateral pale reddish lesions in the thalamus (surrounded by arrowheads). B. Thalamus. Multifocal necrosis. HE. Bar=500 μm. C. Thalamus. Fibrin thrombi in the necrotic focus (arrowheads). HE. Bar=50 μm. D. Thalamus. Eosinophilic perivascular edema around the necrotic focus. HE. Bar=50 μm. E. Mesencephalon. Eosinophilic perivascular hyaline droplet. HE. Bar=50 μm. F. Mesencephalon. Perivascular hyaline droplet. PAS. Bar=50 μm.

background staining of the neuropil than affected calves. Therefore, contrast between the neurons and neuropil was weak in these control calves.

Serum thiamine concentrations of calf No. 1–3

Serum thiamine concentrations of calves were as below, calf No. 1; 774.5 ng/ml, calf No. 2; 772.5 ng/ml, calf No. 3; 15.1 ng/ml (standard value: 10–20 ng/ml).

Serum Clostridium perfringens epsilon toxin of calf No. 4 and 5

Both calf No. 4 and 5 showed negative against epsilon toxin.

Serum endotoxin concentrations of calf No. 4 and 5

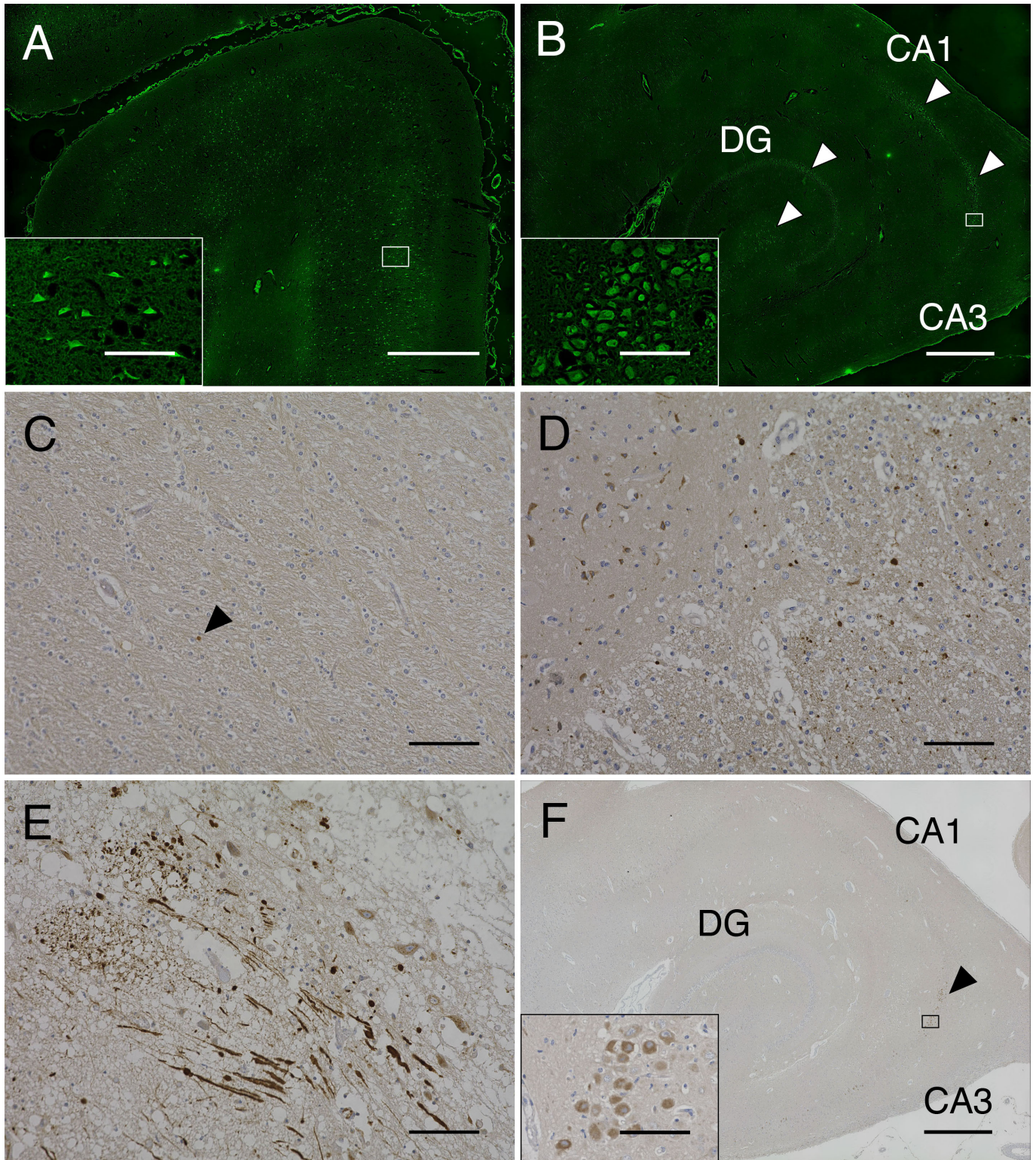In both calves, serum endotoxin level was under 5.0 pg/ml, within standard value.

Detection of the BVDV gene

The BVDV gene was not detected in the sera of all calves.

DISCUSSION

Human perinatal brain injury shows different pattern of lesions owing to several factors such as maturity of brain and strength

Fig. 3. A. Calf No. 1. Frontal lobe. Positive neurons are diffusely observed. FJC. Bar=1,000 μm. Inset. Higher magnification (indicated by rectangle). Bar=100 μm. B. Calf No. 1. Hippocampus. Positive neurons are observed in the CA1–CA3 region, hilar region and dentate gyrus (DG) (arrowheads). FJC. Bar=1,000 μm. Inset. Higher magnification (indicated by rectangle). Bar=100 μm. C. Calf No. 3. Corpus striatum. Slight immunoreactivity (grade 1). Immunohistochemistry for APP. Bar=100 μm. D. Calf No. 1. Corpus striatum. Scattered immunoreactivity (grade 2). Immunohistochemistry for APP. Bar=100 μm. E. Calf No. 4. Thalamus. Extensive immunoreactivity (grade 3). Immunohistochemistry for APP. Bar=100 μm. F. Calf No. 1. Hippocampus. Limited number but intense immunoreactivity was observed consistent with degenerated neurons in CA1–CA3 (arrowhead). Immunohistochemistry for APP. Bar=1,000 μm. Inset. Higher magnification (indicated by rectangle). Bar=100 μm.

Table 2. Distributions and degrees of APP positive axons

Calf No.	Parietal lobe	Corpus striatum	Thalamus	Mesencephalon	Cerebellum
1	2	2	3	3	1
2	1	2	1	1	2
3	1	1	1	1	1
4	3	3	3	3	3
5	3	2	1	3	3
Cont. 1	1	1	1	1	1
Cont. 2	1	1	1	2	1
Cont. 3	1	1	1	1	1

0, none; 1, slight; 2, scattered; 3, extensive.

and duration of insults [43]. In term infants, major patterns include selective neuronal necrosis and parasagittal cerebral injury [23, 43, 44]. In preterm infants, meanwhile, lesions are formed mainly in the cerebral white matter such as PVL [23, 43, 44]. In experimental studies with term fetal or neonatal monkeys, total asphyxia (complete cessation of respiratory gas exchange) induced lesions principally in the thalamus and brainstem. In contrast, partial asphyxia induced lesions in the cerebral cortex, white matter and basal ganglia [4, 32, 33]. In cattle, it is possible that different patterns appear owing to the strength and time of injury in similar to humans and experimental models.

Two out of the five present calves had laminar cerebrocortical necrosis with degenerated/necrotic neurons in the corpus striatum and hippocampus. Causes of bovine laminar cerebrocortical necrosis include thiamine deficiency, ischemia, sulfur poisoning, lead poisoning and sodium toxicosis [7, 9]. Thiamine deficiency is most common, but occurs in calves older than 2 months of age [7]. In addition, it has been reported that some cases of thiamine deficiency showed mesencephalic and cerebellar lesions, however, striatum and hippocampal lesions are not common [7]. Since distributional pattern of the lesions was consistent with that of global ischemia in other species, such as selective neuronal necrosis in human neonates and NMS in foals, the lesions observed in these calves were probably caused by hypoxic/ischemic insult during dystocia and perinatal asphyxia [7, 43, 47]. Because whole blood is more suitable for measurement of thiamine concentration rather than serum [27], it is difficult to discuss about thiamine deficiency involvement based on serum thiamine concentrations. However, thiamine concentrations in the two calves were far higher than standard value of serum thiamine concentrations (10–20 *ng*/m*l*) and also whole blood concentrations (26.8 ± 4.8 *ng*/m*l*) [21]. High thiamine concentrations in these calves might indicate the possibility that thiamine administration was done.

One out of the five calves had laminar cerebrocortical necrosis. The calf had shown clinical symptoms since just after birth, however, had survived for long time and necropsied at postnatal day 118. The histological lesions were chronic ones involving severe cavitation and gliosis in the cerebral cortex. Although distributional pattern of the lesions was consistent of that of thiamine deficiency, it has not been reported in neonatal calves. In addition, serum thiamine concentration was not low, though there were long span between onset and collecting serum. Although hypoxic/ischemic insult was also suspected to be cause as with aforementioned two calves, it was conflicting that there was no hippocampal lesion, characteristically observed in perinatal brain injury in human and other animals [7, 43].

The remaining two calves had multifocal necrosis with vascular lesions. These lesions were similar to microangiopathy and focal symmetrical encephalomalacia (FSE) associated with *C. perfringens* type D enterotoxemia in sheep [7, 29]. Although FSE-like lesions have also been reported sporadically in cattle, examinations for epsilon toxin were not conducted in any of these cases [2, 6, 14, 30, 31]. Apparently, cattle have low sensitivity for *C. perfringens* infections compared to small ruminants [42]. Only three calves of microangiopathy have ever showed evidence of epsilon toxin in intestinal contents [29, 45]. In experimental study of type D enterotoxemia in calves, in addition to brain lesions, severe pulmonary edema was observed [15, 41]. However, the present calves did not show pulmonary edema and significant intestinal lesion. In addition, considering the negative result of immunoserological test against epsilon toxin, the lesions were probably not due to type D enterotoxemia. Fibrin thrombi are also formed systemically associated with DIC and shock conditions [26]. In cattle, these lesions are induced by endotoxin shock due to *Escherichia coli* [20]. However, in our calves, thrombi were observed only in the brain. Additionally, serum endotoxin levels were within standard value. Brain hypoperfusion by ischemia activates vascular endothelial cells and coagulation system, and then induces microthrombi formation and vascular hyperpermeability [10]. These changes tend to occur in the corpus striatum in which blood flow is slow [10]. In the present calves, fibrin thrombi formation and vascular lesions in deep gray matter might be indicate that they had fallen into brain ischemic status for some reason in the past. Applying to human perinatal brain injury, the distributional pattern of these calves resembled deep nuclear-brain stem pattern or cerebral cortex-deep nuclear pattern of selective neuronal necrosis [43].

There are differences in blood supply between cattle and other animals including sheep, horses and dogs in which perinatal brain injury have been reported [7, 35, 39]. In cattle, vertebral artery supplies blood to all regions of the brain via rete mirable [12, 13, 19]. In non-rete mirable animals such as horse and dog, meanwhile, vertebral artery supplies blood to caudal regions of the brain, and branches of common carotid artery supply rostral regions [12, 19]. The basilar artery is a blood supply route to the arterial circle in cattle [13]. Conversely, it leads blood from the arterial circle in sheep [13]. These anatomical differences between cattle and other animals might be underlie the fact that bovine perinatal brain injury has not been reported except our previous report

about PVL [25].

FJC staining is specific for degenerated neurons regardless of the mechanism of degeneration [37]. In a study with middle cerebral artery occlusion (MCAO) model of rats, degenerated neurons were detected in the corpus striatum at shortest 6 hr and in the hippocampus at 12 hr after insult [5]. The result showed that FJC could detect lesions at earlier period than the methods in previous study, such as silver impregnation for neuronal degeneration and immunohistochemistry against glial fibrillary acidic protein and vimentin for reactive astrocytes [5, 8, 34]. In a study with rat MCAO models, positive reactions in the striatum had remained until 21 days (not examined further period) after insult [5]. They suspected ongoing reactivity might reflect not only individual degenerated neurons remaining for long periods but also degenerated neurons in penumbra or newly de-innervated neurons [5]. In our study, calf No. 3 showed positive reaction around necrotic and cavitated lesions. Although long time had passed from putative insult, ongoing degeneration might have continued around necrotic and cavitated lesions. Application of FJC in veterinary natural disease has only been reported in neonatal foal [36]. The present study clarified high sensitivity and specificity of FJC in neonatal calves.

The present study also confirmed high sensitivity of APP immunohistochemistry for axonal degeneration in neonatal calves. APP is an indicator of anterograde axonal transport, and highly accumulates in axons injured by various mechanism including trauma, hypoxia, hypoglycemia, infarct and abscess [3]. In human traumatic brain injury cases, positive immunoreactivity appear 1.75–3 hr after axonal injury, and the number and size of swelling increase over the next 24 hr [3]. Moreover, this immunoreactivity is detectable for about a month or much longer [3] In our calves, immunoreactivity was also found in axons without swelling. Some of these reactions might indicate axonal injury in its early stages although APP immunoreactivity does not necessarily represent irreversible axonal damage [3]. Positive reactions in calf No. 3, necropsied at 118 days PD, probably reflected long-term accumulation or ongoing axonal degeneration. Hypoxic condition also induces increase of neuronal APP expressions [1, 40]. Intense neuronal immunoreactivities in calf No. 1–3 might indicate elevation of APP expressions. Although immunoreactivities were also found in control calves, the number of axonal immunoreactivity was small. Therefore, APP immunostaining will be helpful for detailed examination as an axonal degeneration marker. Additionally, control calves showed low contrast between the neurons and neuropil. Hence, to clarify significance of such neuronal immunoreactivity alteration, further investigation is required.

In the present study, at least two calves out of the five calves was probably hypoxic/ischemic encephalopathy because of the distributional pattern of the lesions and the history of dystocia and perinatal asphyxia. This means that the pattern of perinatal brain injury other than PVL occur also in calves. To the best of our knowledge, this is a first report that describes encephalomalacia related to perinatal accidents in calves. Additionally, we confirmed usefulness of FJC and APP for detailed examination of neonatal calves. In food-producing animals such as cattle, time between insult and necropsy is often short. Therefore, neuronal and axonal markers that can detect early-stage of degeneration will be helpful to examination of brain injury in neonatal animals.

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