Clinical Application of Milbemycin D as a Prophylactic Agent against *Dirofilaria immitis* Infection in Dogs: Pathological Findings following Administration

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ABSTRACT. Pathological findings following milbemycin D (Milbe) administration were examined in 54 dogs. No significant changes were seen in the uninfected group (9 dogs). In microfilaria (mf)-positive group (22 dogs), yellowish-brown spots in the liver, splenomegaly, and reddish-brown spots in the lungs were seen on macroscopic examination, and inflammatory reactions phagocytizing the microfilariae by macrophage were noted in the liver, lungs, spleen and heart on the microscopic examination. These lesions were observed with almost the same frequency and severity in the shock-like (7 dogs) and caval syndrome (22 dogs) groups. However, they showed a lower rate and slighter degree in the mf-negative group (11 dogs). To examine the correlation of hepatic lesions and microfilariae, hepatic biopsies and calculations of circulating microfilariae were performed with time in 3 dogs. The number of circulating microfilariae was markedly reduced by 6 hr after administration. Microfilariae in the sinusoid increased in number at 3 hr after administration, neutrophils adhered to microfilariae at 6 hr, macrophages infiltrated around microfilariae and focal necroses containing microfilariae were observed in parenchyma at 9 hr, and macrophage masses were formed at 12 hr.—**KEY WORDS:** canine heartworm, dog, microfilaricide, milbemycin D.

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Milbemycin D (Milbe) is a clinically safe drug when administered to heartworm (*Dirofilaria immitis*)-uninfected or microfilaria-negative dogs at the recommended dose as a prophylactic for heartworm infection. However, some adverse reactions including shock-like reaction and caval syndrome (CS) have occurred in some cases in microfilaria-positive dogs [5, 9, 10]. These adverse reactions may be associated with microfilariae [9]. Number of circulating microfilariae reduced markedly after Milbe administration [9, 10], however, pathological changes were not known yet. The present study describes the pathological findings in heartworm-uninfected and -infected dogs after Milbe administration.

MATERIALS AND METHODS

Fifty-four dogs were selected at random from our previous experiments [5, 9, 10] for this study; 9 dogs were heartworm-uninfected (uninfected group), 11 dogs heartworm-infected without circulating microfilaria (mf-negative group), 22 dogs heartworm-infected with circulating microfilaria (mf-positive group), 7 dogs showed shock-like reaction after Milbe administration (shock-like group) and 5 dogs showed CS after Milbe administration (CS group). Circulating microfilariae were detected in all dogs of the shock-like and CS group, except one case in the former group which did not detect circulating microfilaria before administration. Their symptoms and clinical findings were described in the previous

reports [5, 9, 10].

The dogs of each group were administered a dose of 1 mg of 5 mg of Milbe per kg body weight. But there were no differences in the pathological findings between two doses of Milbe, so their findings were shown together. The dogs were autopsied at 24 hr after Milbe administration except 1 case of CS group which done after 3 days. Euthanasia was carried out by exsanguination after anesthesia. After macroscopic examination and calculation of adult heartworms, the specimens for histopathological examination were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned by routine procedure. Sections were stained with hematoxylin and eosin. Each lobe of the lungs and liver, heart, gallbladder, spleen, kidneys, regional lymph nodes, urinary bladder, and gastrointestinal were examined by light microscopy. The pathological findings induced by heartworm infection described in previous reports [4, 11, 16] were observed in the lungs, liver and kidneys in almost all heartworm-infected dogs, but almost all of these findings were excluded, because they had no direct relation to Milbe administration.

To examine the correlation of the hepatic lesions and microfilariae, hepatic biopsies and circulating microfilaria counts were made at 3, 6, 9, 12, and 24 hr after Milbe administration in 3 dogs which were not included in the above mf-positive group. The hepatic biopsies were made by cutting off a piece of the liver (5×5×2 mm) with laparotomy under general anesthesia. The dogs were autopsied 24 hr after administration and the lungs, spleen, and kidneys were also examined.

RESULTS

The mean numbers of adult heartworms were 20, 25, 20 and 28 in the mf-negative, mf-positive, shock-like and CS groups, re-

spectively. Adult heartworms of both sexes were found in 8 of 11 cases of the mf-negative group and in 1 case of the shock-like group in which circulating microfilariae were not detectable before Milbe administration. The mean number of circulating microfilariae markedly decreased 24 hr after administration in the mf-positive (from 2037 to 277/200 μ l), shock-like (2184 to 262/200 μ l) and CS (3016 to 360/200 μ l) groups. The decrease number of circulating microfilariae also showed no significant differences among the each group.

The incidence of macroscopic lesions is shown in Table 1. In some cases in the mf-positive, shock-like and CS groups and a few cases in the uninfected and mf-negative groups, there were a few to numerous yellowish-brown spots in the liver, edematous thickening with ecchymosis in the serous membrane of gallbladder, splenomegaly with dark-red mottle, numerous spots in the lungs which were reddish-brown in color and miliary to rice-grain in size, and edematous enlargement in the hepatic and pulmonary lymph nodes. The severity of these lesions tended to parallel with the circulating microfilaria counts before Milbe administration. In other lesions, petechiae in the mucous membrane of urinary bladder were seen in a few cases in all groups.

The incidence of the main microscopic lesions is shown in Table 2. Microgranulomas and necroses were seen in the liver. Microgranulomas (Fig. 1) were spherical or ovoidal in shape, measuring about 50 μ m in diameter. They usually consisted of epithelioid cells, but occasionally contained microfilarial fragments and showed infiltration of lymphocyte or neutrophils. The yellowish-brown spots on macroscopic examination corresponded to two types of necrosis on microscopic examination. Both types of lesions were irregular in shape, measuring 100 to 500 μ m in long diameter. Necroses of one type contained some microfilariae with

Table 1. Incidence of macroscopic lesions in dogs administered milbemycin D

Lesion	Group				
	Uninfected (n=9)	Mf-negative (n=11)	Mf-positive (n=22)	Shock-like ^{a)} (n=7)	CS ^{b)} (n=5)
Liver					
Yellowish-brown spot	0	2 (18%)	9 (41%)	3 (43%)	3 (60%)
Gallbladder					
Edematous thickening with ecchymosis	0	0	5 (23)	2 (29)	1 (20)
Spleen					
Splenomegaly	1 (11%)	1 (9)	13 (59)	4 (57)	2 (40)
Lung					
Reddish-brown spot	1 (11)	4 (36)	20 (91)	7 (100)	3 (60)
Hepatic Lymph node					
Edematous enlargement	1 (11)	3 (27)	8 (36)	4 (57)	3 (60)
Pulmonary lymph node					
Edematous enlargement	0	1 (9)	6 (27)	3 (43)	2 (40)
Urinary bladder					
Petechia	2 (22)	4 (36)	7 (32)	4 (57)	1 (20)

a) Shock-like reaction appeared after milbemycin D administration.

b) Caval syndrome appeared after milbemycin D administration.

Table 2. Incidence of microscopic lesions in dogs administered milbemycin D

Lesion	Group				
	Uninfected (n=9)	Mf-negative (n=11)	Mf-positive (n=22)	Shock-like ^{a)} (n=7)	CS ^{b)} (n=5)
Liver					
Microglanuroma	0	2 (18%)	21 (95%)	7 (100%)	5 (100%)
Focal necrosis with microfilariae	0	0	13 (59)	5 (71)	3 (60)
Spleen					
Necrosis	0	0	17 (100)	5 (71)	1 (20)
Lung					
Thickening of alveolar septa with microfilariae	0	3 (27)	22 (100)	7 (100)	5 (100)
Heart					
Mass of macrophages around microfilariae	0	1 (9)	6 (27)	1 (14)	3 (60)

a) Shock-like reaction appeared after milbemycin D administration.

b) Caval syndrome appeared after milbemycin D administration.

infiltration of macrophages, lymphocytes and neutrophils (Fig. 2). Necroses of another type were accompanied by infiltration macrophages, eosinophils, neutrophils, lymphocytes and/or plasma cells, but not

any microfilariae. The lesions with microfilariae were found in cases in which a large number of circulating microfilariae were detected before Milbe administration, but lesions without microfilaria had no relation

Fig. 1. Microgranuloma in the liver. $\times 650$.

Fig. 2. Necrosis containing microfilarial fragments (small arrows) in the liver. Macrophages, neutrophils and lymphocytes infiltrated in the lesion. Some macrophages (large arrows) closed on the body surface of microfilariae. $\times 650$.

Fig. 3. Necrosis in the spleen. Macrophages infiltrated around microfilariae (arrows). $\times 200$.

Fig. 4. Thickening in the alveolar septa owing to macrophages infiltration around microfilarial fragments (small arrows). Some macrophages (large arrows) closed on the body surface of microfilariae. $\times 650$.

Table 3. Correlation between development of liver lesions and number of circulating microfilariae in 3 dogs after milbemycin D administration

Item	Before administration	Hours after administration				
		3	6	9	12	24
No. of circulating microfilariae (per 200 μ l of blood)	18396 $\pm$ 1651 ^{c)}	2480 $\pm$ 538	490 $\pm$ 320	370 $\pm$ 248	350 $\pm$ 260	338 $\pm$ 275
No. of microfilariae in the sinusoid in the liver (per visual field) ^{a)}	0.85 $\pm$ 0.65	3.61 $\pm$ 1.91	4.07 $\pm$ 4.42	4.2 $\pm$ 4.2	4.3 $\pm$ 3.5	1.2 $\pm$ 0.8
No. of macrophage masses (per visual field) ^{b)}	0	0	0	2.0 $\pm$ 1.0	4.5 $\pm$ 1.5	12.2 $\pm$ 4.3
No. of necroses with microfilariae (per visual field) ^{b)}	0	0	0	0.6 $\pm$ 0.6	0.8 $\pm$ 1.1	0.6 $\pm$ 0.6

a) $\times 200$.b) $\times 100$.c) Mean $\pm$ standard deviation. Fig. 5. The liver at 6 hr after milbemycin D administration. Neutrophils adhered to microfilariae (arrows) in the liver sinusoid. $\times 750$.Fig. 6. The liver at 9 hr after milbemycin D administration. Macrophages (large arrows) proliferated around the microfilaria (small arrow). $\times 750$.Fig. 7. The liver at 12 hr after administration. Mass of macrophages contained microfilaria (arrow). $\times 400$.

with the circulating microfilaria count.

Necroses containing a few microfilarial fragments and accompanied with macrophage infiltration and hyalin substance were mainly seen in the peri-white pulp areas of the spleen and a few of them also in

red pulp (Fig. 3). The almost cases with splenomegaly in the mf-positive, shock-like and CS groups had necrosis of the spleen, but the cases in the uninfected and mf-negative groups had no significant changes.

Alveolar septa were thickened by mac-

rophages infiltrated around the microfilariae (Fig. 4). Hyalin substance and focal hemorrhages were occasionally observed around or near the microfilariae. Severe lesions were seen throughout the lungs in the cases which revealed a large number of circulating microfilariae before Milbe administration. A few focal lesions were noted in the cases which were detected a small number of circulating microfilariae. The reddish-brown spots on macroscopic findings corresponded to the focal hemorrhages or focal hyalin substances.

There were focal infiltrations of macrophages surrounding microfilariae in the myocardium in a few cases. Edema, infiltration of the inflammatory cells and a few microfilariae without the cell reaction in the hepatic and pulmonary lymph nodes were observed in some cases which had severe lesions in the liver or lungs.

Upon the examination for correlation of the hepatic lesions and microfilariae after Milbe administration, circulating microfilariae were markedly reduced by 6 hr after Milbe administration, and lesions began to be formed after 9 hr (Table 3). There were only a few microfilariae in the sinusoid throughout the specimens before administration. The number of microfilariae increased in the sinusoid, but their morphological appearances were not changed at 3 hr after Milbe administration. Neutrophils and lymphocytes adhered to some but not all microfilariae at 6 hr (Fig. 5). Macrophages infiltrated around microfilariae in the sinusoid (Fig. 6), and the focal necroses with infiltration of macrophages, neutrophils and hemorrhages occurred in the areas in which a large number of microfilariae aggregated at 9 hr. Masses of macrophages containing microfilarial fragments were formed at 12 hr (Fig 7). Macrophage masses increased still more, but microfilariae in the almost lesions were disappeared and microglanulomas were formed at 24 hr. The lesions in the

lungs and spleen of the dogs done hepatic biopsy were as the same types as those in the mf-positive group.

DISCUSSION

No significant lesions were evident in the uninfected group, but some lesions were noted in the mf-negative, mf-positive, shock-like and CS groups after Milbe administration. The incidences and severity of macroscopic and microscopic lesions paralleled with the decrease of circulating microfilaria count, but not with clinical signs and number of adult heartworms. Microgranuloma and necrosis containing microfilariae in the liver, necrosis in the spleen, thickening of alveolar septa and masses of macrophages in the myocardium, were observed with a high incidence in the mf-positive, shock-like and CS groups, and at a lower incidence in the mf-negative group. These lesions were considered to result from the trapping of Milbe killing microfilariae by macrophages. The formation of microgranuloma and necrosis in the liver were confirmed by the examination of hepatic biopsies with time after Milbe administration. Microgranulomas were formed at the place where a small number of microfilariae were seen, and necroses formed the place at which a large number of microfilariae aggregated. The necroses with microfilariae was assumed to occur owing to some cytotoxic substances which might be released from dead microfilariae. On the other hand, it would be appeared that the necroses without microfilariae were formed before Milbe administration, because the lesions were somewhat chronic in nature, but not known the true cause. Petechiae in the lungs and urinary bladder might have occurred by anoxia at agony.

It seemed from the number of trapped microfilariae in a specimen that the largest number of microfilariae were treated in the

lungs, followed by the liver and spleen after Milbe administration. Other microfilaricidal drugs such as ivermectin [7, 14] and levamisole [12, 13], also induced the lesions which resulted from trapping of microfilariae in some organs in dogs with circulating microfilariae. The trapping of microfilariae in the glomerulus of the kidney was reported in levamisole and ivermectin, but not in Milbe. Infiltrations of eosinophils around dead microfilariae were seen in levamisole and ivermectin, but scarcely seen in Milbe. The causes of these differences were not clear.

It seemed that the time from drug administration until liver lesions developed was different according to the kinds of microfilaricide. In ivermectin, microfilariae with normal appearance increased in the sinusoid in the liver at 18 hr after subcutaneous injection of a dose of 0.5 mg/kg in dogs with microfilaremia of heartworm, and granulomas observed at 2 or 3 days after [7]. In diethylcarbamazine (DEC), microfilariae increased in the sinusoid in the liver at 20 to 60 min after intraperitoneal injection of a dose of 250 mg/kg in rats with microfilaremia of *Litomosoides carinii*. Also phagocytes around the microfilariae formed an extensive cluster and displaced the liver cells at 4 to 6 hr after [3]. Thus formation time of the liver lesions in Milbe was later than DEC, but earlier than ivermectin. Since the formation of microgranulomas were related to killing microfilariae, above evidence might be depended on the differences of time from drug administration to killing microfilariae. The same tendencies were observed in the onset time of the adverse reaction in dogs with microfilaremia after drug administration. In DEC reactions occurred at 30 to 60 min after the drug administration [1], in Milbe at 1.5 to 4 hr after [9], and in ivermectin at 3.5 hr to 3 days after [unpublished data]. From these findings, it was suspected that the pharmacokinetics in animal bodies or in the drug

mechanisms for killing microfilariae were different.

The characteristic lesions, which were reported previously in shock-like reaction by DEC [2, 15] and spontaneous CS [6, 8], were not observed in shock-like and CS groups in this study. These facts may be explain by the following evidence. The severity of shock-like reaction by Milbe was slighter than that by DEC [9], and the time from onset of the disease to autopsy in CS induced by Milbe was shorter than that of spontaneous CS [5]. The cause of the shock-like reaction and CS by Milbe was not suggested from the pathological findings, but they were probably associated with functional disorders of the circulation system.

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要 約

犬糸状虫予防薬ミルベマイシンの臨床応用：投薬後の病理学的所見：佐々木栄英・北川 均・石原勝也・森みどり（岐阜大学農学部家畜内科学講座）——54例の実験犬を用いてミルベマイシン D 投薬に伴う病理学的所見を検索した。犬糸状虫寄生犬群（9例）では、特記すべき病変はみられなかった。犬糸状虫寄生犬のうち末梢ミクロフィラリア（mf）陽性群（22例）では、肉眼的に肝臓の黄褐色斑、脾腫、肺の赤褐色斑が認められた。病理組織学的検査では、肝臓、肺、脾臓および心臓にマクロファージによる mf 貪食に関連した炎症性病変が認められた。ショック様症状発症群（7例）および caval syndrome 発症群（5例）にも、同様の病変がほぼ同頻度に認められたが、これらの群に特有な病変は認められなかった。また、mf 陰性群（11例）でも、軽度ではあるが同質の病変が少数例に認められた。mf と肝臓病変との関連を観察するため、投薬後経時的に肝臓の生検を3例で実施した。投薬3時間後には類洞内に mf 数が増加し、6時間後には好中球の mf への付着、9時間後には mf 周囲におけるマクロファージの浸潤および壊死巣の形成、12時間後には微小肉芽腫の形成が認められた。