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Studies on Chemotherapy of Parasitic Helminths (XIII) Efficacy of Ivermectin on the Circulating Microfilaria and Embryonic Development in the Female Worm of *Dirofilaria immitis*

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Key words: ivermectin, *Dirofilaria immitis*, microfilaricide, embryonic development

Introduction

A few chemotherapeutic agents have been known to be effective on the filariasis in man and animals. Among these drugs, diethylcarbamazine has been the drug of the first choice for clinical use in filariasis including that caused by *Dirofilaria immitis* in dogs (Rollo, 1975). However, avermectins, a series of macrocyclic lactone derivatives, were reported to be effective against various nematodes and ectoparasites in animal experiments (Egerton *et al.*, 1979; James *et al.*, 1980; Sano *et al.*, 1981a). For filarial worms, it has been demonstrated that avermectins are effective against the migrating larvae of *Dirofilaria immitis* in ferrets (Blair and Campbell, 1978), the migrating larvae and circulating microfilariae but not adult of *D. immitis* in dogs (Campbell and Blair, 1978; Blair and Campbell, 1979), and also microfilariae of *Litomosoides carinii* in jirds (McCall and Campbell, 1979).

Ivermectin (22,23-dihydroavermectin B_1), a synthetic derivative of avermectins, has also been reported to be effective against larvae and adults of many filarial worms, such as; migrating larvae of *D. immitis* before reaching the heart of ferrets and dogs (Egerton *et al.*, 1980; Blair and Campbell, 1980a, b), microfilariae of *Onchocerca cervicalis* in horses (Egerton *et al.*, 1981), and skin microfilariae of *O. cervicalis* and adult *Setaria equina* in ponies (Klei *et al.*, 1980).

It was also reported that avermectin B_{1a} elicits paralysing effect through its action on γ-aminobutyric acid (GABA) mechanism in various animals (Kass *et al.*, 1980; Sano *et al.*, 1981b). Besides the effect on GABA mechanism, it has been suggested that ivermectin has a direct effect on the reproductive system (Sano *et al.*, 1982).

The present study aimed to clarify the effects of ivermectin on *D. immitis* in dogs, and to determine the mechanism of its action.

Materials and Methods

The experiment was carried out on four dogs naturally infected with *D. immitis*. Microfilarial counts were carried out on blood films made from 0.02 ml of blood obtained from the saphenous vein at 3–5 P.M. Samples were prepared by the Giemsa's stain-

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ing method, and an average count was obtained from three samples for each collection. Blood sampling of dogs was carried out in three consecutive days before treatment. Ivermectin (L-640, 471-00W), which was kindly offered from Merck Sharp & Dohme Research Laboratories, was mixed with the 100 times of glucose (w/w), filled in a gelatin capsule, and then administered orally in a single dose of 0.2 mg/kg B.W. to three dogs. The dose was sufficiently effective for *D. immitis* in literatures (Blair and Campbell, 1980a, b; Egerton *et al.*, 1980). One dog was given only vehicle as an untreated control. The microfilarial counts were carried out at 6 hr and 1, 2, 4, 6, and 9 days after treatment, and then every week and later every two weeks. At the 26th week after treatment, the second dose of ivermectin was administered orally in the same dose. The microfilarial counts were carried out on the 1, 2, 4, 7, and 10 days after treatment and every week, later every two weeks. Two dogs No. 3 and 2 were sacrificed 4 and 14 weeks after the second treatment, respectively. The worms in the right ventricle and the pulmonary artery were transfered into Tyrode's solution and then examined as follows.

For the histological observation, the worms were cut into five equal pieces; the first to fifth parts from the anterior to posterior ends. In females, the 1st part corresponded to the location of the Y-shaped vagina and two uteri. The 5th part was further divided into two; the anterior and posterior corresponded to the seminal receptacle and the ovaries, respectively. For light microscopy, fixed specimen with 70% alcohol were embedded in paraffin, sectioned, and stained with haematoxylin and eosin. For electron microscopy, the whole worms were fixed with 2% glutaraldehyde for 4 hr and cut into five pieces as mentioned above. The specimens again fixed overnight were washed with 0.12 M phosphate buffer (pH 7.4), further fixed with 2% osmium tetroxide for 2 hr, and dehydrated in the ethanol series. The dehydrated specimens were transferred to propylene oxide and embedded in epoxy resin. The ultrathin sections were double-stained with uranyl acetate and lead citrate.

For fresh observation, the removed uteri in Tyrode's solution were placed on a glass slide and cut into small pieces. Eggs and embryos that flowed from the cut end of the uteri were examined under a differential interference microscope.

To examine the *in vitro* effect of the drug, female worms were obtained from the heart of naturally infected dogs, washed with Tyrode's solution, and transferred into the fresh medium with ivermectin (2.6 mg/l) containing 100 units/ml penicillin G potassium salt and 100 μg/ml streptomycin. After exposure for 24 hr to ivermectin at 37 C, the worms were washed and incubated in the medium without ivermectin. The embryos in the uteri and free microfilariae were examined 1, 3, 5, and 7 days after exposure.

Results

The microfilarial count decreased remarkably from the peripheral circulation of all three dogs one day after treatment, as shown in Fig. 1. The low circulating microfilarial density was stable from the 1st week to the 8th week after treatment. In one dog (No. 3), microfilariae could not be detected in the circulation from the 6th day, and reappeared in the 13th week, but the microfilarial count was still low until the 19th week after treatment. The microfilarial density gradually increased from the 8th week after treatment in two dogs (No. 1 and 2) and from the 19th week in dog No. 3.

The second dose, given at the time when the microfilarial levels had recovered to as high as that of control dog, resulted in a similar pattern to the first treatment. The number of circulating microfilariae decreased also one day after the treatment. The duration of the low density was prolonged. In dog No. 3, microfilariae in the peripheral circulation

could not be detected from the 10th day up to the sacrifice 4 weeks after the second treatment. In dog No. 2, the number of microfilariae was stable at a low level up to the sacrifice 14 weeks after the second treatment. In dog No. 1, the microfilariae were not demonstrable in the circulation on the 4th week, then reappeared on the 9th week, but were stable at a low level at least until the 48th week.

The number of living worms recovered from dog No. 3 was 104, 52 males and 52 females. Dog No. 2 had 13 living worms, 7 males and 6 females, and additionally one dead worm was found in the lungs.

The adult female worms, recovered both 4 and 14 weeks after the treatment, showed normal motility, but grossly abnormal appearance due to the uteri being alternately empty (Fig. 2).

The H&E stained sections of males showed no morphological change in testis and sperm compared with normal worms. In the female worms, as shown in Fig. 3, also no morphological change was observed in ovaries and embryos in the uteri posterior to the mid body, whereas the sections of the mid body and anterior to mid body showed marked differences. In the two uteri of the normal females, there were the developing microfilariae in egg shells in the second part, and free microfilariae in the anterior part (Fig. 3a). The sections of the treated worms showed multi-cell eggs in posterior part, but few eggs, which caused the empty uteri, in some parts of the anterior end (Fig. 3b).

The embryos of the treated worms observed with a light microscope were indistinguishable from normal in the regions of seminal receptacle and posterior uteri. Cell division and development had occurred; that is, two-cell eggs and multi-cell eggs were found in the seminal receptacle and adjacent region. The developmental process might

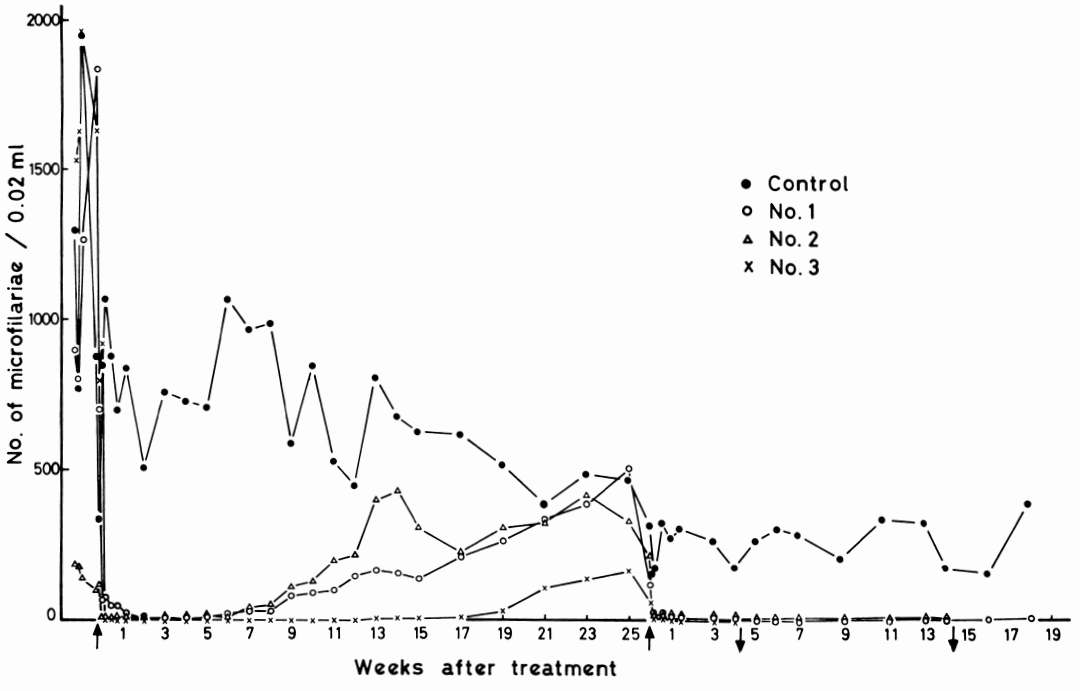
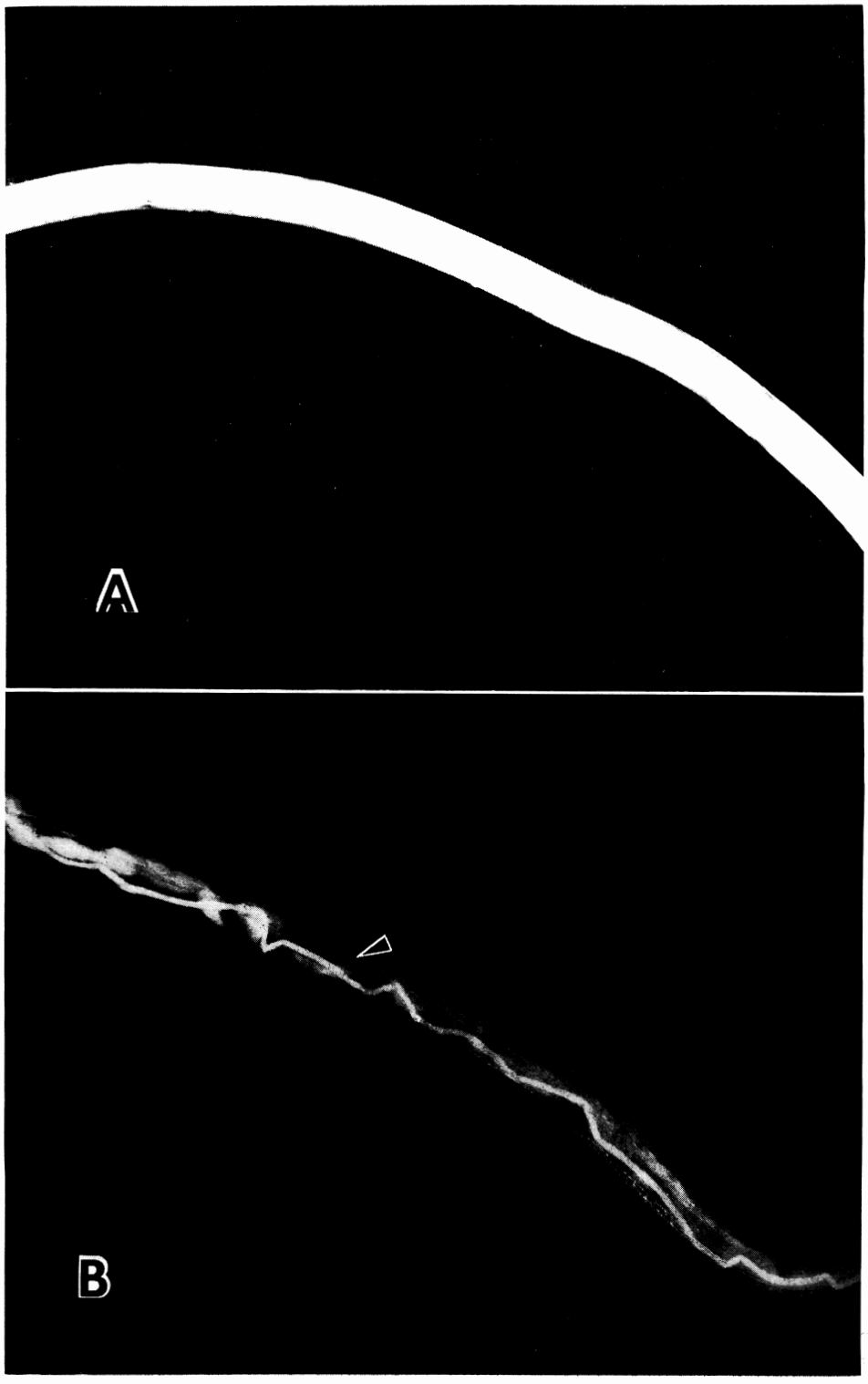


Fig. 1 Effect of ivermectin (0.2 mg/kg) on circulating microfilariae of *Dirofilaria immitis* in dogs. Arrows indicate the administration (up) and the sacrifice (down), respectively.


A
B

progress to gastrulation. The deformed eggs were observed in the uteri posterior to the mid body, prior to the tadpole stage (Fig. 4). Electron microscopy of the parts of female worms corresponding to those in H&E stained sections showed clear details. In the seminal receptacle, there were matured oocytes, sperm and fertilized eggs with fertilization membrane in the normal worms (Fig. 5a) and with engulfed sperm in the egg in the treated worms (Fig. 5b). In both cases, cleavage was also observed in the seminal receptacle (Fig. 6a, b), and advanced multi-cell eggs fully occupied the uteri in the 4th part of the body. In the normal worms, the anterior half of the body was occupied with various stages of microfilariae (Fig. 7a). In the 3rd part (mid body) of the treated worms, most of the embryos showed degeneration. In the 2nd part, all of the embryos showed complete degeneration; the cells were shrunken, the cytoplasm was granular and contained many vacuoles, and the nuclei were largely disintegrated (Fig. 7b). However, the morphological structure of the uterine wall of the treated worms was indistinguishable from that of the normal worms.

The compound was also tested *in vitro* in order to supplement the *in vivo* observations. The *in vitro* experiments demonstrated that ivermectin has no direct effect on free microfilariae and early embryos in the uteri.

Discussion

Although diethylcarbamazine has been shown to be highly effective in reducing microfilariae of filarial worms, long term administration is required for effective results. Avermectin B_{1a} (0.1 mg/kg) suppressed microfilariae of *D. immitis* for 9 weeks, and B_{2a} (0.05 mg/kg) suppressed for 10 weeks (Campbell and Blair, 1978). In the case of ivermectin in this study, the suppressive effect lasted for 18–19 weeks in the first treatment. Though the suppressive effect was prolonged in the second treatment, the mode of action of ivermectin seems to be similar to that of other avermectins. The suppressive effect consisted of two processes; the reduction in circulating microfilariae and the inhibition of the embryonic development in female worms.

In the ivermectin treated dogs, the circulating microfilariae were probably destroyed by the host's defence mechanism in the liver and/or in the reticulo-endothelial system. The mechanism may be activated by the drug associated with relating immunity in the host tissue, as the reported action of diethylcarbamazine (Baqui and Ansari, 1978; Hawking, 1979; Kobayashi *et al.*, 1969). This is supported by the absence of direct microfilaricidal effect *in vitro*. The survival of a few microfilariae in the peripheral circulation probably resulted either from escaping from host's defence mechanism or from small percentage of successfully developed embryos in the female worms.

The female worms from treated dogs showed a partially empty body appearance. This was due to fewer or no eggs or microfilariae in the uteri from mid to anterior body. With light microscopy, though the embryonic development progressed to multi-cell stage at about mid body, no further embryonic stage was found in the anterior half of the body. With electron microscopy, the embryos in the anterior half of the body showed a regressive degeneration. These evidences mean that the long lasting low level of microfilariae was caused by the inhibition of the embryonic development which led to no production of new microfilariae in the female worms.

The occurrence of the fertilization was demonstrated. Spermatozoon engulfed in

Fig. 2 Gross appearance of female *Dirofilaria immitis* at mid body. A). Normal worm. B). Treated worm from dog sacrificed 4 weeks after treatment, showing partially transparent empty uteri (arrow).

oocyte was observed at the seminal receptacle close to the oviduct of the treated worm, and the fertilization membrane was observed at the anterior seminal receptacle of the normal worm. Harada *et al.* (1970) stated that the fertilization mechanism of *D. immitis* in the seminal receptacle was accompanied with the fertilization membrane which was formed by sperm and ovum. On the other hand, Foor (1970) reported that the spermatozoon entered into the oocyte by a phagocytosis-like mechanism. It is probable that fertilization occurs immediately after oocyte has passed the oviduct to the seminal receptacle by an engulfing mechanism, thereafter the sperm membrane dissolves, and the fertilization membrane is formed when the fertilization is completed.

There may be many factors which inhibit the development of the embryos and these factors may work together or function independently. These factors inhibited the further development of embryos at the stage of early gastrulation, which was observed in the worms from both dogs sacrificed 4 and 14 weeks after the second treatment, respectively. However, the second treatment seems to cause a stronger effect on the worms than that of the first treatment, for the low level of circulating microfilariae lasted longer after the second treatment.

Taylor and Terry (1960) reported the effect of several drugs on the developing embryos of *Litomosoides carinii* both *in vivo* and *in vitro*. They showed that different drugs affected the embryos at different stages, such as, arsenic and antimony compounds destroyed all the developmental stages, and the bis-isoquinolinium compounds acted mainly on the early embryo. Ashburn *et al.* (1945) observed the worms of *D. immitis* recovered from dogs treated with various antimonial and mercurous compounds, and stated that the unicellular eggs and the ovaries were affected, and resulted in the absence of microfilariae in the genital tubes of adult worms. In the case of ivermectin, the drug affected the development of multi-cell eggs to further satges, which resulted in the absence of microfilariae. Harada *et al.* (1970) stated that the embryonic eggs depend upon the external nutritional sources. Although the morphological change in the uterine wall of the treated worms was not observed, its function may be abnormal or incomplete, which may cause the depletion of metabolic substances or energy sources that are necessary for the normal embryonic development. Lee (1975) indicated that the first germ cells were derived from a terminal cap cell of the ovary, which was morphologically indistinguishable from other oogonia. Ivermectin possibly acts on this stem cell; if so, chromosomes or genes become abnormal in their function which is followed by disorganization of embryos during the developmental process. Abnormal meiosis of male and/or female reproductive cells, which may result the haploid or triploid chromosomes, may also influence the normal embryonic development. Another possible factor which may be considered is polyspermy which was sometimes seen even in the normal process of fertilization of *D. immitis* (Taylor, 1960). In this study, although it was not observed in either treated and normal worms, ivermectin may induce this phenomenon which inhibits further embryonic development.

In conclusion, the present study showed that ivermectin is an effective antifilarial agent for *D. immitis,* since it reduces the number of microfilariae and inhibits the production of the microfilariae for a long time.

Summary

Ivermectin 0.2 mg/kg body weight is a good microfilaricide for *Dirofilaria immitis* in dogs. The suppressive effect on the circulating microfilariae lasts from the 1st week of the treatment up to the 48th week or more after the second treatment. No lethal effect on the adult worms was observed in either of the dogs sacrificed 4 and 14 weeks

after the second treatment, but the normal embryonic development of eggs in the uteri of female worms was inhibited. Light microscopy of adult females from treated dogs showed developing eggs of early stage in the uteri of the posterior half of the body. The later stages of eggs in the uteri of the anterior half of the body were affected by this compound. With electron microscopy, the degeneration of the embryonated eggs in the uteri was observed from the mid body up to the anterior end of the worms, therefore no microfilariae were released to the peripheral circulation.

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寄生蠕虫症の化学療法に関する研究 (XIII)
犬糸状虫ミクロフィラリアおよび子宮内虫卵の胚発生に及ぼす Ivermectin の影響

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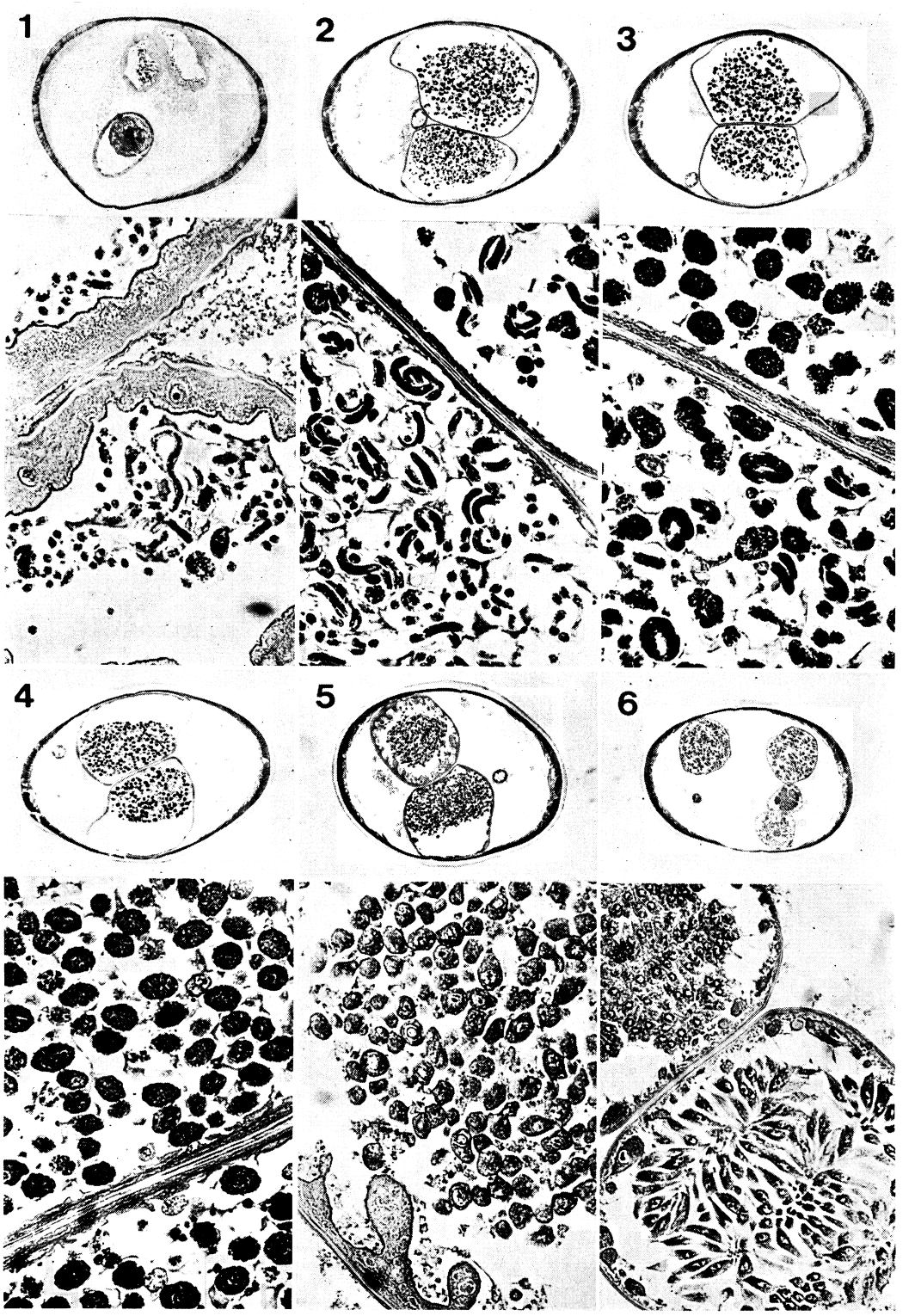
Ivermectin は，犬に感染した犬糸状虫に対し，すぐれた抗ミクロフィラリア作用を示した．すなわち，犬の末梢血中のミクロフィラリア数は，Ivermectin 0.2 mg/kg 投与後，1日で著しく減少し，1頭の犬では6日間で消失した．この低レベルは，投与後8週ないし19週で回復した．2回目の投与を行なうと同様に減少し，1頭の犬では少なくとも，48週低レベルが持続した．

2回投与後，4週および14週経過した雌虫の子宮内虫卵は，光顕的には胚発生中期で発育が停止し，膣および体前半部の子宮内には，正常卵はみられなかった．電顕的には，体中部以降の子宮内虫卵はいずれも変性していることが観察された．従って，Ivermectin による抗ミクロフィラリア作用は，肝および網内系を介すると思われる直接的な殺虫作用に加えて，胚発生に対する阻害効果に由来することが明らかとなつた．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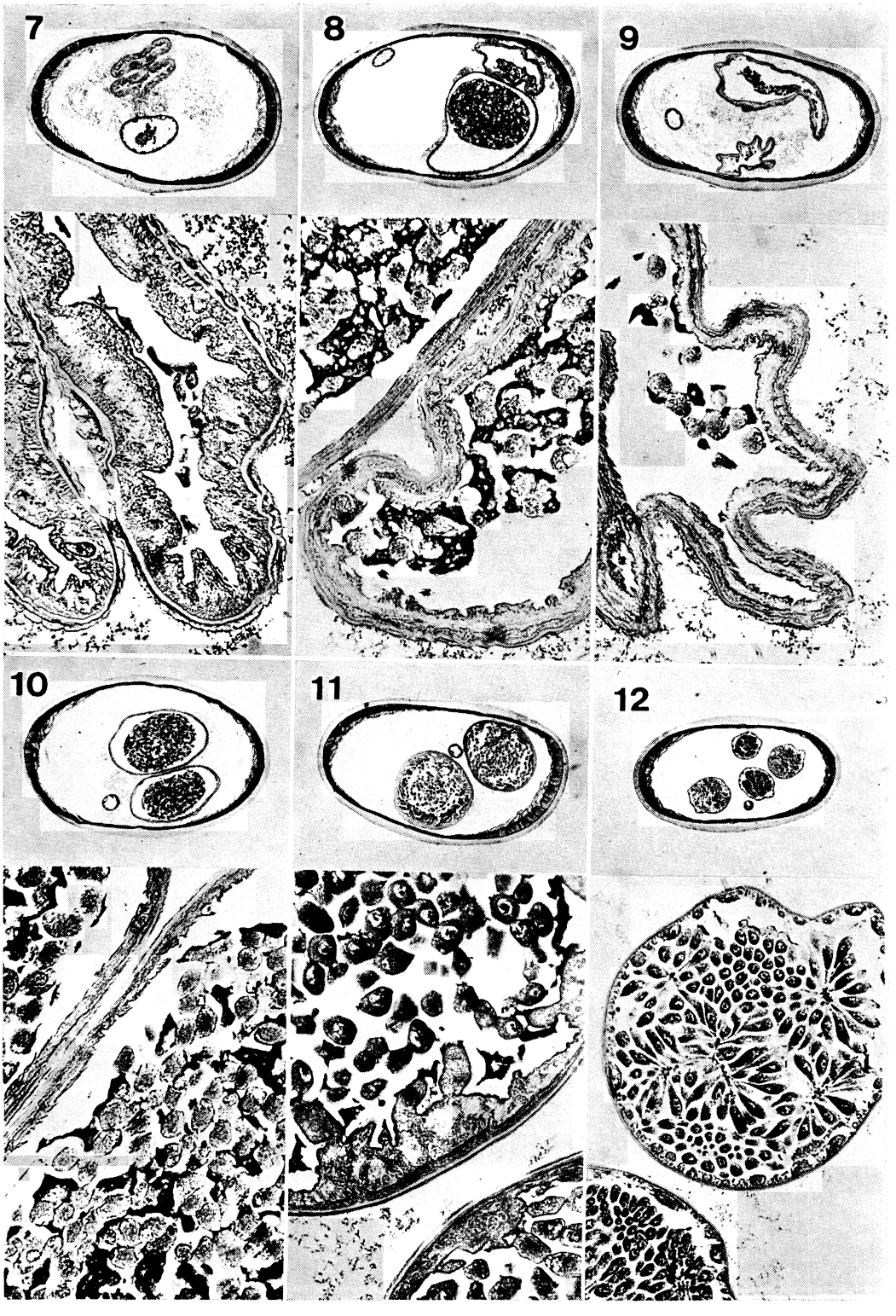
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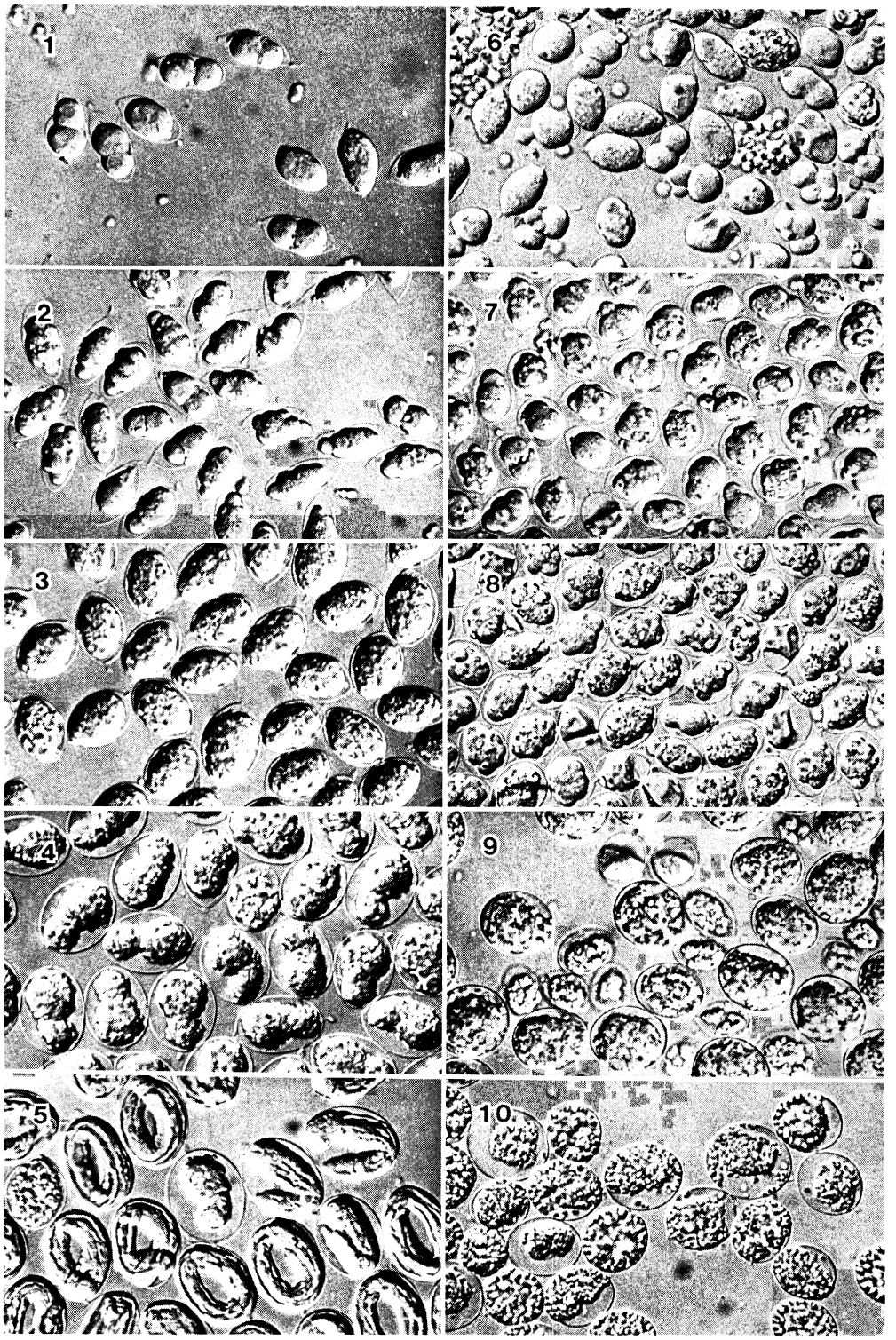
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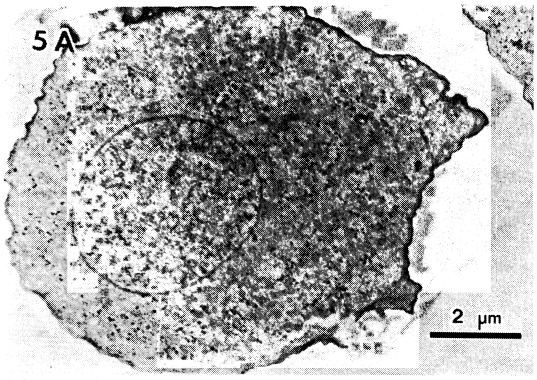
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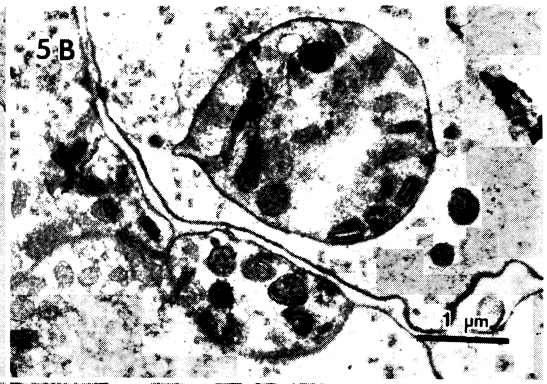
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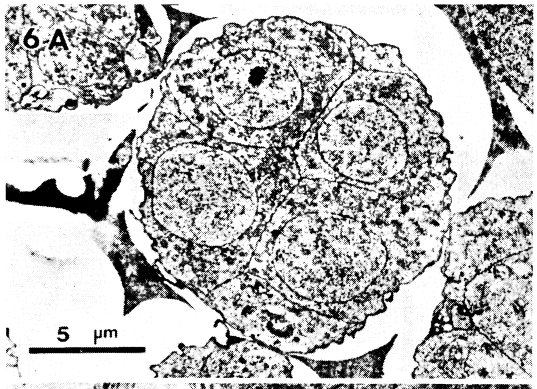
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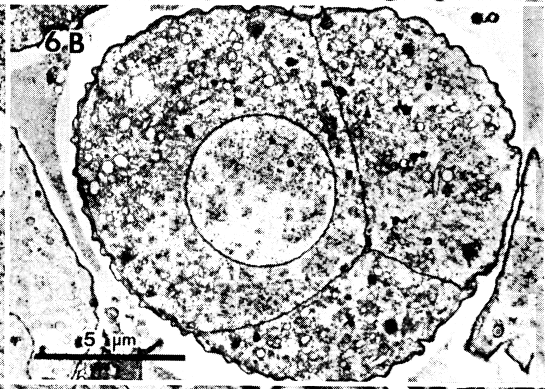
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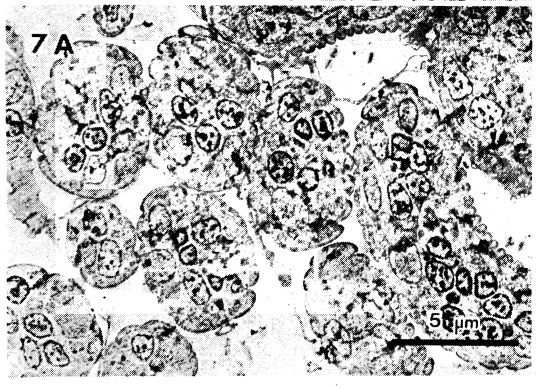
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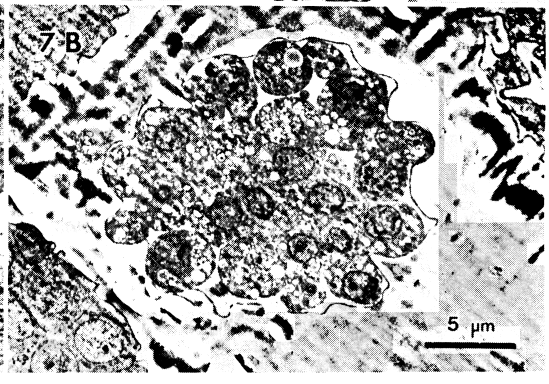
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Explanation of Figures

Fig. 3 H&E stained sections of female *Dirofilaria immitis*. 1–6: Normal worm. 7–12: Treated worm from dog sacrified 4 weeks after treatment. Upper and lower ones of each figure are at low and high magnifications, respectively.

1. Anterior portion showing microfilariae in the uteri.
2. 2nd part showing microfilariae in egg shells in the uteri.
3. Mid body showing developing microfilariae in egg shells.
4. 4th part showing developing embryos in egg shells.
5. Seminal receptacle showing oocytes and sperm.
6. Posterior portion showing ovaries.
7. Anterior portion showing empty uteri.
8. 2nd part showing unfully occupied eggs in the uteri.
9. Mid body showing few eggs in both uteri.
10. 4th part showing eggs in the uteri.
11. Seminal receptacle showing oocytes and sperm.
12. Posterior portion showing ovaries.

Fig. 4 Embryonic development of *Dirofilaria immitis* in the uteri of female worms. 1–5: Normal worm. 6–10: Treated worm.

1. Oocytes, sperm, and two-cell eggs in seminal receptacle.
2. Early multi-cell eggs.
3. Advanced multi-cell eggs.
4. Early gastrulation eggs.
5. Young microfilariae in egg shells in the mid body.
6. Oocytes, sperm, and two- to four-cell eggs in seminal receptacle.
7. Early multi-cell eggs.
8. Advanced multi-cell eggs.
9. Degenerated eggs.
10. Fully degenerated eggs in the mid body.

Fig. 5 Electron micrograph of fertilized eggs in seminal receptacle. A). Fertilization membrane of egg of normal worm. B). Engulfed sperm in the cytoplasm of egg of treated worm.

Fig. 6 Electron micrograph of eggs at cleavage. A). Normal worm. B). Treated worm.

Fig. 7 A). Electron micrograph of microfilariae in the 2nd part of female worm.
B). Electron micrograph of degenerated egg. Note that the cells are shrunken, the cytoplasm is granular and contained many vacuoles, and nuclei are largely disintegrated.