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Studies on the Transmissibility of Pathogenic-Organisms to Liver by Larvae of Liver Fluke and Hookworm

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ABSTRACT

In order to confirm whether the migrating larvae of parasites could carry pathogenic organisms into liver and cause hepatitis, a series of experiments has been carried out. The summary of the results is as follows:

1. *Clonorchis sinensis*

A few of the excysted larvae of *Clonorchis sinensis* penetrated into the peritoneal cavity, but they could not penetrate the liver tissues. The artificially introduced *Clonorchis sinensis* in the tissues were all destroyed within 3-5 days. There was no manifestation of diffuse inflammatory changes due to the inoculation of the parasites, though the sampled micro-organisms, *Staphylococcus aureus*, were confirmed from the surrounding area.

2. Hookworm

The larvae carried pathogenic organisms to liver tissues either by cutaneous or oral infection, but there was no manifestation of hepatitis due to the micro-organisms.

In conclusion, it is indicated that liverfluke and hookworm may transmit pathogenic organisms to the liver during their migration.

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INTRODUCTION AND OBJECT

Larson (1964) reported on biological aspects of enterohepatitis in fowl relative to *Histomonas meleagridis* and *Heterakis gallinarum*. Groups of fowl were given embryonated eggs of *Heterakis gallinae* orally or intracecally, or hatched larvae orally, intracecally or intra-duodenally, and found that Enterohepatitis developed in some groups irrespective of the infection technique. However, none of the fowls which received crushed larvae developed enterohepatitis. Bradley and Reid (1964, 1966) reported production of typical infectious enterohepatitis in bacteria-free turkeys inoculated with *H. meleagridis* plus *Escherichia coli* or *Clostridium perfringens*. The use of *H. meleagridis* plus *Bacillus subtilis* yielded only lesions of cecal ulceration. But the combination of *H. meleagridis* plus *Aerobacter cloacae* and *Streptococcus fecalis* did not produce the infectious enterohepatitis. As a result, they emphasized that a certain combination of protozoan and bacterium was required for the development of infectious enterohepatitis.

Hutchison (1967) fed the cystic forms of *Toxoplasma gondii* to cats which were infected or non-infected with *Toxocara cati*, and found that the eggs of *Toxocara cati* were capable of infecting mice with *Toxoplasma*, whereas the latter one did not. These experiments suggest that *Toxoplasma gondii* may be transmitted by *Toxocara cati* ova.

Previous to the above reports, Wykoff et al.

(1657) found that excysted larvae of *Clonorchis sinensis* in duodenum were capable of making their way through portal vein instead of common bile ducts. He obtained the results by ligaturing the ductus choledochus, and in some cases introducing the tube into the gall-bladder. Excysted metacercariae or adolescariae which were injected directly into the mesenteric vein reached the hepatic biliary passages in one of five animals. Higashi (1960) observed that *Fasciola hepatica* was capable of penetrating the intestinal wall and capsule of the liver, and reached the bild-ducts through the parenchymatous tissue of liver. It indicates that *Clonorchis sinensis* larva may utilize another route than through the common bile duct.

It would be intesting to know whether the excysted or hatched larvae of parasites in the intestine could carry bacterial flora from the intestine to the liver during their migration, and thus causing hepatitis. It is known fact that the ingested hookworm and ascaris larvae migrate into the liver before lung circulation and cause temporary hepatic inflammation.

The present study has been designed to confirm whether the liver fluke and hookworm, which are the common parasites in Korea, could carry the pathogenic bacteria from intestine to liver.

MATERIALS AND METHODS

A. *Clonorchis sinensis*

Male rabbits weighing about 0.8–2.0 kg body weight were used as experimental animals. The metacercariae of *Clonorchis sinensis* were collected from raw fish by a digestion method with artificial gastric juice, and 900–1,200 of them were introduced directly into the stomach by a 5 mm diameter polyethylene tube. On the planned data (1–7th day), animals were sacrificed. The abdominal cavity was washed with saline solution. The solution was centrifuged to examine whether the sediment contained the adolescaria. The liver, small intestine, mesenterium and other organs in the abdominal cavity were separated surgically and washed gently with saline solution, and the solutions were also subjected to examination for the larvae. The liver tissues and the common bile ducts were examined for larvae.

In another experiment, 15–90 days old *Clonorchis sinensis* from the bile ducts of rabbits were collected and introduced directly into the abdominal cavity of rabbits. After a designated date, the abdominal cavity and organs were carefully examined to find the introduced worms. The contents of the ducts were also examined. The area of the liver tissues where the worm was attached was sectioned for pathological and bacteriological examination.

In the 3rd experiment, the young worms were collected from the bile ducts of rabbits which were infected with *Clonorchis sinensis* 11, 16 and 21 days before. The surface of the liver of rabbits was incised and the collected worms were introduced into the wound, then covered gently with the outer lobe of the wounded liver and the abdominal wall was closed by routine surgical procedure. Three, six and ten days later, the animals were sacrificed, and the inoculated area was examined as above.

In the 4th experiment, adult worms of *Clonorchis sinensis* were incubated in saline containing *Staphylococcus aureus* for 30 hours at 25°–26°C. The surface of the rabbit liver was incised and the living adult worms, which had been incubated in the saline solution containing *Staphylococcus aureus*, were introduced in the wound. Three days later, the inoculated area of liver was examined pathologically and bacteriologically. Intestinal contents of the *Clonorchis sinensis* from liver tissues were cultured in nutrient-agar plate and examined bacteriologically.

In the 5th experiment, the collected metacercariae of *Clonorchis sinensis* were excysted in artificial intestinal juice (trypsin 0.5 gm, sodium bicarbonate 0.2 gm, saline 50.0 ml) which was contaminated by coagulase and catalase positive-staphylococcus. One ml of the juice, which contained

about 200 metacercariae, were introduced into portal vein of rabbit by syringe needle. The rabbits were sacrificed 5, 9, 69 and 75 days later. The bile ducts were examined carefully in order to detect *Clonorchis sinensis*, and even the peribiliary tissues.

On one side, pieces of liver tissue were smeared on blood agar plate, and cultured for 24–48 hours in 37°C incubator. In another series of experiment, the excysted larvae were washed with saline.

One ml of saline which contained the larvae was introduced into portal vein. And bile ducts were examined at 28 and 49 days after the inoculation.

B. Hookworm

Cutaneous infection: Laboratory cultured rhabditoid larvae of canine hookworm were incubated in normal tap water containing abundant *Diplococcus pneumoniae*. Mice were narcotized with ether and fixed on the board. The abdominal surface of the mice was shaved following soap washing and 75% alcohol disinfection. A 500–1,000 filariform larvae suspension was smeared on the shaved area and left until it dried. On the 4–8th day after the infection, the mice were sacrificed and the liver was examined pathologically and bacteriologically.

Oral infection: Rhabditoid larvae of canine hookworm were incubated in normal tap water containing *Staphylococcus albus* or *Staphylococcus aureus*. The larvae which developed to filariform stage in the media were fed to mice orally without washing. One to five days later, the liver was examined pathologically and bacteriologically. Methylene blue and Gram's stain were applied.

RESULTS

A. *Clonorchis sinensis*

Recovery rate of larvae in the abdominal cavity of rabbits

One to seven days after the administrated adolescariae were recovered from the abdominal cavity in less than 1% of the total number of metacercariae given. Generally, 1–6 larvae were found from each animal, though many larvae were already found in the common bile ducts or remained still in intestine (Table 1).

Table 1. Number of larvae of *Clonorchis sinensis* in abdominal cavity after feeding its metacercariae to healthy rabbits

No.	Wt. (Kg)	No. of metacercariae given	Period to sacrifice (day)	No. of worms found	
				Abdominal cavity (%)	Common bild duct
1.	0.8	1200	3	4	+
2.	1.1	1200	3	5(0.4)	+
3.	0.9	1200	5	1	+
4.	1.0	1200	5	6	+
5.	1.0	900	1	0	×
6.	1.0	1000	1	5(0.5)	×
7.	1.9	1200	5	0	—
8.	1.8	1200	7	4	—
9.	2.0	1200	5	2	—

Remarks: —···not found, +···found, ×···not examined.

Fate of *Clonorchis sinensis* in abdominal cavity

The young or mature worms which were introduced directly into the abdominal cavity were examined 15, 32, 40 and 42 days after the inoculaion (Table 2.).

Table 2. The fate of the liver fluke introduced into the abdominal cavity of rabbits

No.	Age of the given worm (day)	No. of the worms introduced	Period after the introduction	No. of worms		Pus nodule
				Surface of liver	Abdominal cavity	
1.	15	100	40	7	2	—
2.	15	82	42	2	—	—
3.	60	75	32	13	—	—*
4.	90	88	15	—	30	—

* In No. 3, one nodule which contained eggs was found on the wall of the intestine.

Several larvae were found on the surface of the liver in four animals. All the worms on the surface of the liver were dead and the biopsied liver tissues on the area where the worms were attached showed no pathological changes. Two of them were between the bile duct and liver tissue but pus cell infiltration surrounding them was observed. In every case, pus cell infiltration was found in the peripheral portion of the liver and pus nodules on the surface of the intestine and mesentery. The

nodule in the intestinal wall contained the eggs of *Clonorchis sinensis* (Fig. 1). Two worms in the abdominal cavity were still alive. A few of the larvae of *Clonorchis sinensis* penetrated the intestinal wall and reached the organs in the abdominal cavity surviving for 15–42 days, but they were unable to penetrate the organs. No bacterial flora appeared from the lesions by culture method.

Fate of *Clonorchis sinensis* which was inoculated into the peripheral region of liver

A small abscess was observed at the area of inoculation. Microscopically, the area became edematous and the vessels in the peripheral region were dilated. The parasites became necrotic and amorphous. Pathologically the lesions appeared as eosinophilic masses, and neutrophile leukocytes were infiltrated surrounding the masses (Fig. 2). In some cases, the dead worms were found apart from the original place of inoculation, but no leukocyte infiltration was found. There was linear infiltration between the original site and the portion where the dead worm was found (Fig. 3). The distance from the capsule varied from 0 to 4 mm (Fig. 4). The eggs of *Clonorchis sinensis* were also found, although no living worms were seen in the liver tissues and hepatic ducts. In all cases, the bacteriological examination was negative.

Transmission of micro-organism by *Clonorchis sinensis*

Five adult worms of *Clonorchis sinensis* were incubated in the saline solution containing *Staphylococcus aureus*. The intestinal contents of these worms were isolated aseptically under dissecting microscope. The materials isolated were cultured on the nutrient-agar plate. The colonies on the plate were smeared on the slide and examined by methylene blue and Gram's stain (Table 3).

Table 3. Bacteriological examination of the intestinal contents of *Clonorchis sinensis* which were incubated in the saline containing *staphylococcus aureus*

No.	Incubation time	*Staphylococcus aureus* in the intestinal content
1.	30 hours	—
2.	〃	—
3.	〃	—
4.	〃	—
5.	〃	—

The area of liver tissue where the *Clonorchis sinensis* were inoculated showed no inflammatory changes after 3 days of inoculation, and no living *Staphylococcus aureus* was found in the culture media with which the pieces of liver tissues were smeared.

Fate of the larvae of *Clonorchis sinensis* which were introduced into portal vein.

No *Clonorchis sinensis* was found in the bile ducts in any of the cases, and the Staphylococcus cultures on the blood-agar plate, by smearing the liver tissues, were negative.

The gross section of 75 days-liver showed no pathological changes, though a slight hyperplasia of biliary ducts were found in several liver pieces (Table 4).

B. Hookworm

Cutaneous infection: The mice were sacrificed on four to eight days after the cutaneous infection

Table 4. Fate of the larvae of *Clonorchis sinensis* which were introduced into portal vein of rabbits

Rabbit No.	Staphylococcus in the artificial intestinal juice	No. of larvae introduced	Duration hatch-introduction	Period after introduction	No. of worm in bile duct	*S. aureus* in the liver
1.	no	200	24 hours	28	—	×
2.	no	200	24 hours	49	—	×
3.	yes	150	1 hour	5	—	×
4.	yes	150	〃	9	—	×
5.	yes	150	〃	69	—	×
6.	yes	150	〃	75	—	×
7.	no	150	〃	75	—	×

Remarks: ×…not examined —…negative

of *Ancylostoma caninum*. Grossly, there was no abnormal finding in the liver. The pieces of liver tissues were smeared on the nutrient-agar plate, and cocci were found in four out of six examined. The micro-organisms were confirmed as the same species of *Diplococcus pneumoniae* which were grown in the hookworm culture media (Table 5).

Table 5. Bacteriological examination of the liver tissues of mice, after the infection of Ancylostoma caninum which were grown in the media containing *Diploccus pneumoniae*

Mouse No.	Duration (day) Infection-sacrifice	*D. pneumoniae* in the liver
1	4	—
2	4	—
3	4	—
4	8	—
5	8	—
6	8	—

Oral infection: 1,000 filari-form larvae of *Ancylostoma caninum* were given orally. 24 hours later, the mice were sacrificed and the pieces of liver tissue were smeared on the nutrient-agar plate. After 50 hours at 36°C, the bacterial colonies were examined. *Staphylococcus albus* was found from two out of four samples. Grossly there was no abnormality on the surface of liver, but microscopically there were spot-like micro-abscesses which were infiltrated by leukocytes. The larvae were also found from other portions of liver tissues and they were surrounded by yellow colored material (Table 6, Fig. 5).

Table 6. Bacteriological examination of the liver tissues of mice, after the infection of *Ancylostoma caninum* which were grown in the media containing *Staphylococcus albus*

Mouse No.	Duration (hrs.) Infection-sacrifice	*Staphylococcus albus* in the liver
1	24	—
2	24	—
3	24	—
4	Death	

In another experiment, a combination of *Ancylostoma duodenale* and *Staphlococcus aureus* was fed to mice. The mice were sacrificed five days after the oral administration of *Ancylostoma duodenale* which had been cultivated in the media containing *Staphylococcus aureus*.

The liver pieces were examined routinely. The larvae cultivated in normal tap water, which contained no *Staphylococcus aureus*, was used as control. In the experimental mouse, the cocci appeared in the liver (Table 7).

Table 7. Bacteriological examination of the liver tissues of mice, after the infection of *Ancylostoma duodenale* which were grown in the media containing *Staphylococcus aureus*

Mouse No.	Culture media	Duration (day) Infection-sacrifice	*Staphylococcus aureus* in the liver
1	tap water with *S. aureus*	5	—
2	tap water	5	—

Pathologically, microabscesses infiltrated with neutrophile leukocytes were found, but there was no manifestation of inflammatory change due to *Staphylococcus aureus*. There was only mechanical trauma due to the larvae penetration (Fig. 6). Haemorrhage appeared only where the larvae were found.

DISCUSSION

There have been several opinions about the migration route of the hatched larvae of *Clonorchis sinensis* from the upper portion of intestine to the bile ducts. Mukoyama (1921) could not recover the adults after ligaturing the common bile duct in dogs, cats and guinea pigs in which the metacercariae of *Clonorchis sinensis* were fed orally. Hsü (1938) gave support to the bile duct theory, which states that the adolescariae migrate from the duodenum to the bile ducts. Sutn et al. (1968) also confirmed that the common bile duct is the only natural route taken by *Clonorchis sinensis* from the duodenum to the liver. But the haematogenic theory has been raised on one hand i.e. hatched larvae penetrating the intestine and blood vessel transfer them to the bile ducts through the parenchymatous tissue of the liver. Wykoff and Lepes

(1957) injected, directly, 1,000 adolescariae into the mesenteric vein of rabbits, and after 30 days adult worms were found in the hepatic biliary passages. In the present study the authors obtained no *Clonorchis sinensis* from the bile ducts until 7-75 days after their introduction into portal vein.

There has been no report whether the excysted larvae of *Clonorchis sinensis* reach the liver via intestital wall and abdominal cavity. However, Higashi (1960) found a few larvae of *Fasciola hepatica* in the abdominal cavity, surface and parenchyma of liver of guinea pig on 55 days after the infection. In the present study, five larvae were found in the abdominal cavity of two rabbits out of nine. which 900~1,200 metacercoriae given orally each. These data mean that larvae of *Clonorchis sinensis* are able to penetrate the intestinal wall into the abdominal cavity. It is noteworthy to examine whe ther these larvae are able to penetrate into liver tissues through the capsule. But the experiment showed that larvae were found only on the surface of liver even though they were inoculated directly into the abdominal cavity. The worms on the liver surface were dead. Although a small number of worms in the abdominalc avity were still alive for 15-40 days, they were too feeble to survive. Some pus nodules were found on the surface of the intestine. All the worms in the nodules were dead, and no evidence that the larvae had entered directly into parenchymatous tissue of liver was observed.

Some young worms were introduced artificially beneath the liver capsule But no surviving worm was found 3-10 days after the operation, except some linear infiltration with length of about 4 mm from the original site.

The findings suggest that the larvae which penetrated out from the intestine can survive for a certain period, but are not able to invade into the liver tissues.

Wykoff and Lepes (1957) found the excysted larvae of rabbits stayed in the duodenum from 22 through 72 hours after inoculation. During this period, the worm may ingest bacteria in the intestine. The results in the present study confirm the hypothesis. When worms, which were cultured in the bacteria containing media, were introduced beneath the capsule of liver, the bacteria were present in the inoculated area. It was proven by culture method. However, none of the bacteria was found from the case in which the contaminated hatched larvae were introduced into the portal vein. The controversy is not still clear. It is presumed that the larvae may regurgitate their intestinal contents, including the bacteria, during their migration. The ascites and Kupffer cells may kill bacteria before an inflammatory reaction occur. It will need further axenical experiments to confirm the correlation between liver disease and the introduced bacteria.

Bradley et al. (1964), Bradley and Reid (1966) observed that typical infectious enterohepatitis was produced in bacteria-free turkeys by oral inoculation with a combination of *Histomonas meleagridis* and *Escherichia coli* or *Clostridium perfringens*, but the combination of *Histomonas meleagridis* and *Bacillus subtilis* produced ulcers on the cecal mucosa. The use of *Aerobacter cloacae* or *Streptococcus faecalis* plus *Histomonas meleagridis* did not produce characteristic lesions. It indicates that parasites sometimes act as synergist with bacteria to produce pathogenecity. *Escherichia coli* was considered to supply one or more heat-labile or filtrable factors for disease production by *Histomonas meleagridis* (Bradley et al., 1964). It is known fact that infectious hepatitis is caused by micro-organisms such as protozoa, fungi, bacteria, rickettsia, virus and by *Fasciola hepatica* (Sapuna and Donckaster, 1967). *Fasciola hepatica* will damage the liver tissues and act as a causative agents by carrying pathogenic agents. Higashi (1960) found pathologic findings were more severe in a mixed infection with *Fasciola hepatica* and *Bacillus subtilis* than in a single infection with *Fasciola hepatica.*

Lewert and Lee (1954) reported the mechanism of penetration of parasites is related to the

production of a collagenase-like enzyme. This enzyme may be necessary for larvae of *Clonorchis sinensis* to penetrate the capsule of the liver, but it is yet to be determined whether *Clonorchis sinensis* produces a collagenase-like enzyme.

The majority of hookworm pass through the pulmonary cycle via the liver in case of an oral infection. This has been proven by Soh (1958) and other investigators. McCoy (1929-a, 1929-b) demonstrated that certain bacteria alone may furnish adequate food for the growth of hookworm larvae to the filariform larvae, but the larvae did not grow on dead bacteria. The bacteria ingested by the rhabditoid larvae may remain in the intestinal lumen of the larvae as nutrients. After maturation to filariform larva, the bacteria in the gut and even on the body surface of the worm may be introduced passively with the larvae into the host. Such circumstances may cause corresponding inflammatory changes when the bacteria propagate in the tissue of the host.

In the present experiments, filariform larvae of *Ancylostoma caninum* which were grown in the media containing the *Diplococcus pneumoniae*, *Staphylococcus albus* and *Saphylococcus aureus* respectively, were infected to mice, the same bacteria were found in the liver tissues of mice. The data seem to indicate that pathogenic bacteria can be transmitted to the liver by migrating larvae of hookworm. However, the pathologic findings showed that there were only hemorrhages due to the mechanical trauma of the larvae penetration in the liver tissues and no linear infiltration, granulomatous infiltration and eosinophilic granuloma which are the characteristic findings of larva migrans. The etiology of small white spots which were infiltrated by neutrophilic leukocytes appeared is not yet clear.

Beaver (1956) described the typical lesion seen in liver tissues of an abnormal host was an eosinophilic granuloma, and Esslinger (1962) observed the same findings in the mice infected with immature larvae of *Porocephalus crotali*.

In this study, it was investigated whether hookworm and liver fluke, which are related to the liver in their life cycle, could carry micro-organisms from the outside to the liver, and thus cause inflammatory changes. However, no evidence of pathologic findings was seen.

The results suggest only that parasites migrating through the parenchymatous tissue of liver in their larval stage may transmit some pathogenic organisms and induce hepatitis.

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—Legend for Figures—

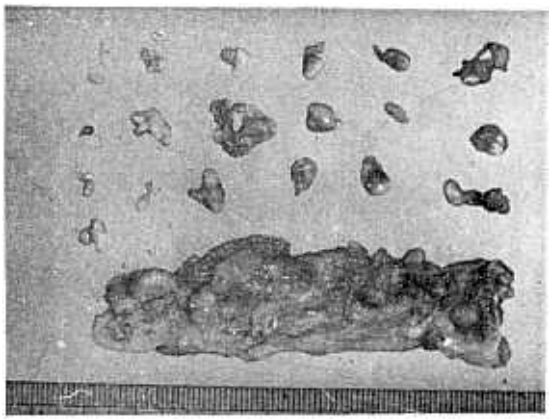

Fig. 1. Pus nodule on the surface of intestine and mesentery. The nodule contained the eggs of *Clonorchis sinensis.*

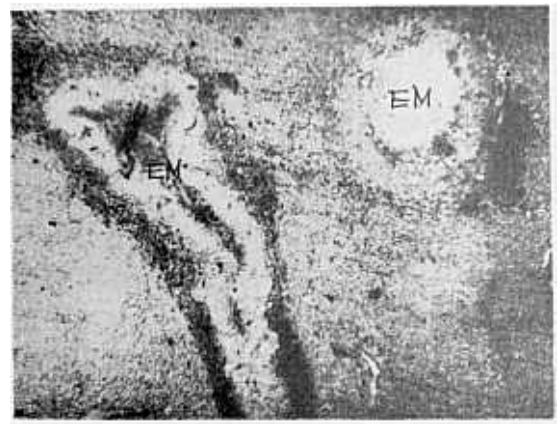

Fig. 2. Amorphous eosinophilic masses (EM) formed by necrosis of worm of *Clonorchis sinenis* in the liver tissue of the rabbit (H-E stain, ×40).

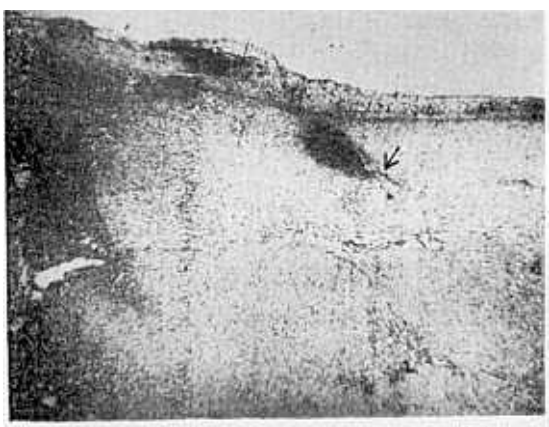

Fig. 3. Linear infiltration due to migration of *Clonorchis sinensis* in the liver tissue of the rabbit (H-E stain, ×40).

Fig. 4. *Clonorchis sinensis* in the liver capsule of the rabbit (H-E stain, ×40).

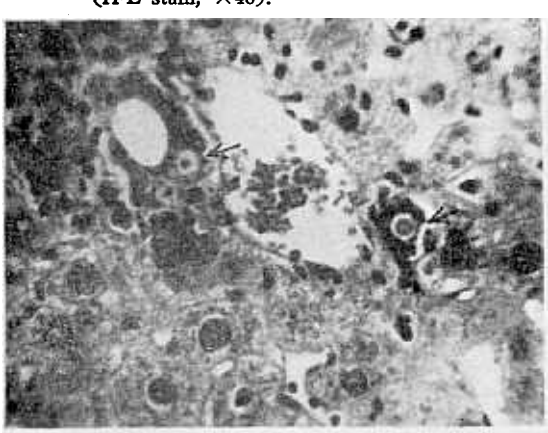

Fig. 5. Histopathology of the liver tissue of mice after the infection of *Ancylostoma canium* which were grown in the media containing *Staphlococcus albus.* Larvae were surrounded by yellow colored materials (H-E stain, ×400).

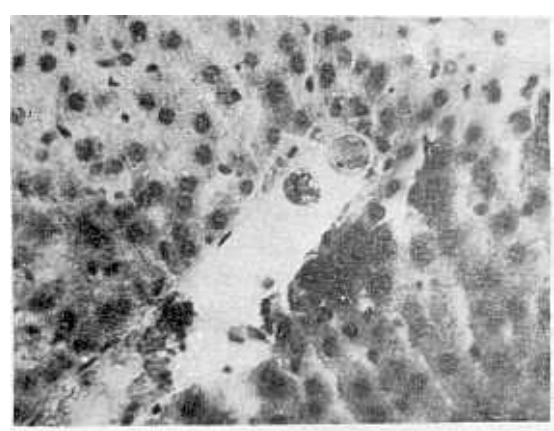

Fig. 6. Liver tissue of mice after the infectian of *Ancylostoma duodenale* which were grown in the media containing *Staphylococcus aureus.* Mechanical trauma due to the larva penetartion is seen (H-E stain, ×400).