Fibrosing Cholehepatitis in Broiler Chickens Induced by Bile Duct Ligations or Inoculation of *Clostridium perfringens*

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ABSTRACT

The pathogenesis of fibrosing hepatitis causing condemnations in broiler chickens was investigated. Three to four week old broilers were inoculated via the hepatoenteric bile duct with saline washed suspensions of *Clostridium perfringens* (10^7 and 10^8 organisms). In another group of broilers, both bile ducts were ligated. The sequential development of liver and gall bladder lesions was studied at intervals ranging from 1-28 days postsurgery. The lesions were similar in both experiments in that the liver became mottled and swollen by five to seven days. Fibrinoid necrosis, heterophil and lymphocyte infiltration, bile duct hyperplasia and fibrosis with reticulin fiber proliferation occurred. By 14-17 days, the liver was enlarged, tan colored and firm with red and white foci. By 28 days, bile duct proliferation and fibrosis were massive with only a few hepatocytes remaining. The liver capsule was not involved. Jaundice was not present but the birds with ligated bile ducts excreted intensely yellow stained droppings after six to seven days. The gall bladder in inoculated birds was edematous and distended with flocculent or inspissated material. *Clostridium perfringens* was reisolated from gall bladder and/or liver of inoculated birds up to 28 days postsurgery. It is suggested that this organism plays a role in the pathogenesis of fibrosing cholehepatitis by inducing septic intrahepatic cholestasis.

RÉSUMÉ

Les auteurs étudient la pathogénèse de l'hépatite fibrosante, cause de condamnations chez le poulet à griller. À un premier groupe d'oiseaux âgés de trois à quatre semaines on a inoculé une suspension de *Clostridium perfringens* dans le canal biliaire hépatoentérique (*ductus hepatoentericus*). Sur le second groupe on a ligaturé les deux canaux biliaires (*ductus hepatoentericus* et *ductus cystoentericus*). À intervalles déterminés, du jour 1 au jour 28 après la chirurgie, on a étudié le développement des lésions au foie et à la vésicule biliaire. Les sujets des deux groupes, aux jours 5-7 post-chirurgie, ont présenté les mêmes lésions hépatiques: tâches et enflure. On a noté aussi nécrose fibrinoïde, infiltration d'hétérophiles et de lymphocytes, hyperplasie du canal biliaire, fibrose avec prolifération des fibres de réticuline. Pour la période de 14-17 jours après l'opération, le foie avait pris du volume, une couleur brunâtre, une consistance ferme avec foyers rouges et foyers blancs. Au 28e jour, la prolifération était marquée, la fibrose était massive et il ne restait plus que quelques hépatocytes. La capsule du foie ne portait pas d'atteinte. Les oiseaux ne présentaient aucun ictère mais les sujets soumis à la ligature des canaux biliaires excrétaient des selles fortement teintées de jaune à partir des 6-7e jours. Les sujets inoculés présentaient une vésicule biliaire oedémateuse et distendue par du matériel floconneux et épaissi. Jusqu'au vingt-huitième jour après l'opération il fut possible d'isoler *Clostridium perfringens* de la vésicule biliaire et/ou du foie. On croit que cet organisme joue un rôle dans la pathogénèse de la choléhepatite fibrosante en provoquant un choléstase septique intrahépatique.

INTRODUCTION

The veterinary inspection directorate of Agriculture Canada defines hepatitis as an enlarged liver with or without necrotic foci (1). During the last five years, broiler flocks in western Canada experienced considerable slaughterhouse condemnations due to hepatitis characterized by an enlarged firm liver sometimes with a slightly knobby surface and a medium tan color. A number of these livers were submitted to the Edmonton Diagnostic Laboratory. Texture and color were consistent throughout the parenchyma. In most cases, small discrete, pale, or red foci were scattered throughout the liver (Fig. 1). In over half of the cases cholecystitis was present and *Clostridium perfringens* was isolated from affected gall bladders but less often from the liver. Microscopically, the lesion consisted of massive bile duct proliferation with variable amounts of heterophil infiltrations. There was extensive fibrosis replacing all but a few islets of hepatocytes. The liver capsule was not thickened. Ascites was not present but many of these birds had smaller body

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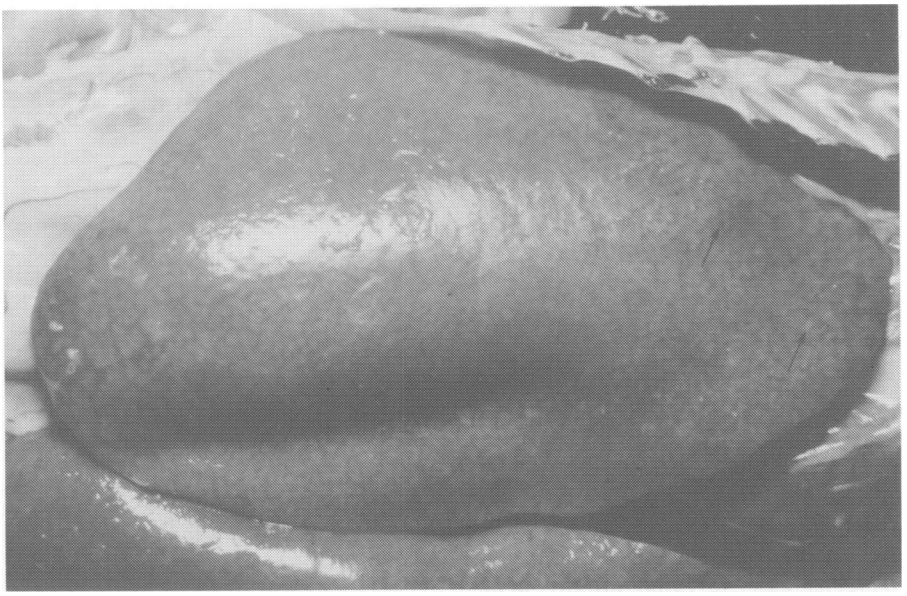

Fig. 1. Liver from a 6 wk old broiler chicken condemned at slaughter for hepatitis. The liver is tan colored and firm with a slightly irregular surface. Numerous pale and red foci are visible (arrows).

size, were poorly fleshed and some were jaundiced. Flock owners did not report any clinical signs except that some birds were smaller than normal.

The purpose of this study was to describe liver lesions due to extrahepatic obstructive bile stasis and to evaluate the role of *C. perfringens* in the pathogenesis of hepatitis.

MATERIALS AND METHODS

BIRDS

Forty one-day-old Hubbard broiler chicks were reared in a commercial multitier electrically heated brooder (Petersime Incubator Co., Gettysburg, Ohio) for two weeks and then transferred to 76 x 55 x 74 cm wire floored cages where they were housed at room temperature in groups of three or four until surgery at 21-28 days of age. The birds were maintained on a commercial 22% protein broiler starter ration (Masterfeeds, Edmonton, Alberta) and tap water. Throughout the experiment the guidelines of the Canadian Council on Animal Care were observed.

SURGERY

Birds were preanesthetized with Ketamine (Ketaset, rogar/STB, London, Ontario) at a dose of 30 mg/kg injected intramuscularly. After 5 min, methoxyfluorane (Metofane, Pitman Moore Ltd., Mississauga, Ontario) was delivered in an open system consisting of a cardboard cone plugged by cotton onto which methoxyfluorane was applied. This was placed more or less over the bird's head thus regulating the desired depth of anesthesia. Aseptic surgical procedures were used to expose the bile ducts through a 3-4 cm long right side incision parallel to the xyphoid process. Ligations of the hepatic and cystic bile ducts were made with 3-0 absorbable surgical sutures. Inocula were delivered only into the hepatoenteric duct with a 26 G hypodermic needle proximal to a ligature which prevented the inoculum from flowing into the duodenum. The cystic duct remained patent. To reduce leakage of inoculum at the injection site, the procedure was later changed utilizing an 8 cm long polyethylene cannula (Intramedic, O.D. 0.050″, Clay Adams, Parsippany, New Jersey) inserted proximal to the duct ligation, secured by a loop of suture applied around the duct and cannula and the inoculum was delivered through a 20 G needle fitted into the tube. As the cannula was withdrawn, the suture loop was tightened and knotted. After surgery, birds were allowed to recover on a heated cloth pad.

INOCULA

Clostridium perfringens bacteria were obtained from a pool of cultures from four submissions of birds with necrotizing enteritis. The isolates were stored in sheep blood at -70°C. They were recultured on fresh blood agar plates (BAP, Difco, Detroit, Michigan), checked for purity and verified as *C. perfringens* by the Rapid ANA Identification System (Innovative Diagnostic Systems, Inc., Atlanta, Georgia) and the Vitek Anaerobe ANI Identification System (Vitek Systems, Hazelwood, Missouri). A fresh 24 h *C. perfringens* culture was heavily inoculated onto BAP for overnight anaerobic incubation. The resulting 24 h growth was aseptically scraped into sterile normal saline. This suspension was centrifuged for 30 min at 5000 x *g* and washed twice with saline. The pellicle was resuspended in sterile saline to approximate the number 4 McFarland turbidity standard. This preparation was then stored in 1 mL aliquots at -70°C. Viability counts were performed using consecutive tenfold dilutions plated on BAP and incubated anaerobically overnight. To determine the loss of viability during handling of the sample for inoculation, the count was repeated with an aliquot that was thawed for 2 h at 4°C.

EXPERIMENTS

In the first series, both bile ducts were ligated in ten birds. They were necropsied 3, 5, 8, 10, 14 and 28 days postligation according to the schedule in Table I. In the second series, a group of three birds was injected with 2.5 x 10^7 viable *C. perfringens* (0.25 mL, batch 1), into the hepatoenteric bile duct and a second group of 14 birds was injected with 5 x 10^8 viable organisms (0.5 mL, batch 2). A third group of 11 birds was inoculated via a cannula in the bile duct with 5 x 10^8 viable *C. perfringens*. Birds were examined 1, 3, 5, 6, 8, 12, 17 and 28 days postinoculation (Table I). Two birds were subjected to surgery but injected only with 0.5 mL saline into one bile duct and necropsied after 13 days.

Liver tissue was collected consistently from the posterior third of both primary lobes, fixed in 10% buffered formalin and routinely processed for microscopic paraffin sections. They

TABLE I. Number of birds necropsied and cultured from four experimental groups

Days postinoculation	Dual ligation	0.25 mL[a] via needle	0.5 mL via needle	0.5 mL via canula
1			1	1
3	2	1 (c)		
5	2		4 (3c)	
6		1 (c)	1 (c)	2
8	2	1 (c)	3 (c)	
10	1			
12			4 (2c)	1 (c)
14	1			
17			1	3
28	2			4 (c)

[a]Volume of inoculum and method of inoculation with *C. perfringens*
(c) = Culture of liver and gall bladder for *C. perfringens*

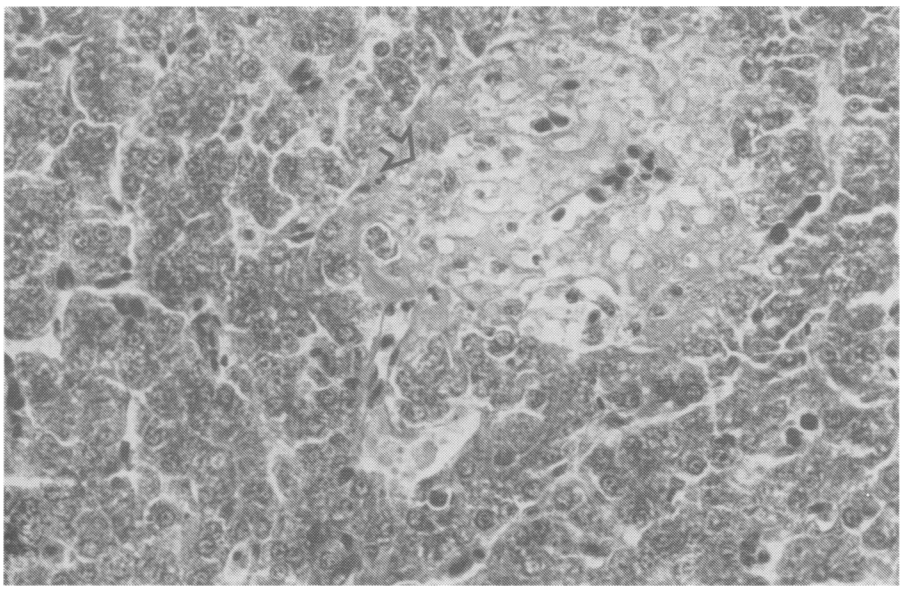

Fig. 2. Liver from broiler three days after ligation of both bile ducts. Coagulative and lytic necrosis (arrow) in portal area. H&E. X452.

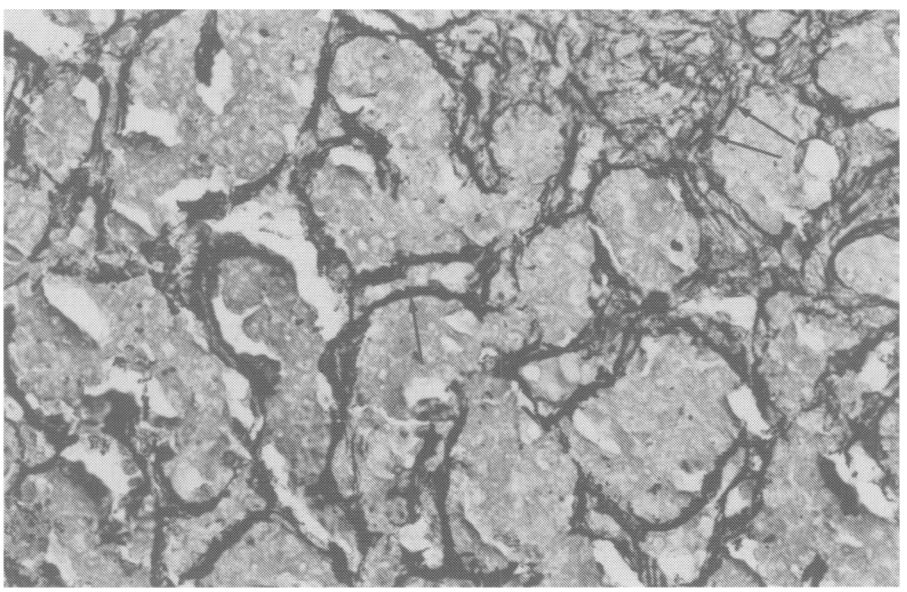

Fig. 3. Liver from broiler five days after ligation of both bile ducts. Marked increase of reticulin particularly in areas of early bile duct proliferation (arrows). Gomori's reticulin stain. X452.

were stained with hematoxylin/eosin, Gomori's reticulin and Masson's trichrome stain. Slides were examined by one pathologist who recorded and rated the lesions subjectively without knowing from which group the tissues came. Liver tissue and gall bladder swabs were cultured as indicated in Table I.

RESULTS

LIGATION OF BOTH BILE DUCTS

Broilers with both bile ducts ligated maintained good appetite but their fecal droppings became intensely yellow after six to seven days. Microscopic lesions three days after ligation consisted of multifocal areas of fibrinoid necrosis (Fig. 2). By day 5, there was periportal bile duct proliferation associated with marked increase in membrane reticulin (Fig. 3). After eight days, fibrinoid necrosis was replaced by cell debris, heterophil and lymphocyte infiltrations and there was more extensive bile duct proliferation and fibrosis. By days 10 and 14 the liver became olive-brown in color and microscopic lesions were more extensive (Fig. 4). Twenty-eight days after ligation, the liver was enlarged, firm and tan colored with multiple red and white foci. The capsule was not thickened. Bile duct proliferation and fibrosis were massive with few hepatocytes remaining. At this time, mature collagen was demonstrated with Masson's trichrome stain. Jaundice was not present.

BILE DUCT INOCULATION WITH *C. PERFRINGENS*

Birds inoculated with either batch 1 or batch 2 of *C. perfringens* through a hypodermic needle often had an abscess at the injection site or even perihepatitis involving one liver lobe. In two cases, bile and inoculum leaked from the injection site causing peritonitis which was evident when the birds were examined five and six days later. In one case, the injected duct was necrotic and severe cholecystitis was present. Gall bladders had edematous walls or were distended with flocculent or inspissated material in 5 of 17 birds injected (Fig. 5). *Clostridium perfringens* was recovered as late as 12 days postinoculation primarily from gall bladders.

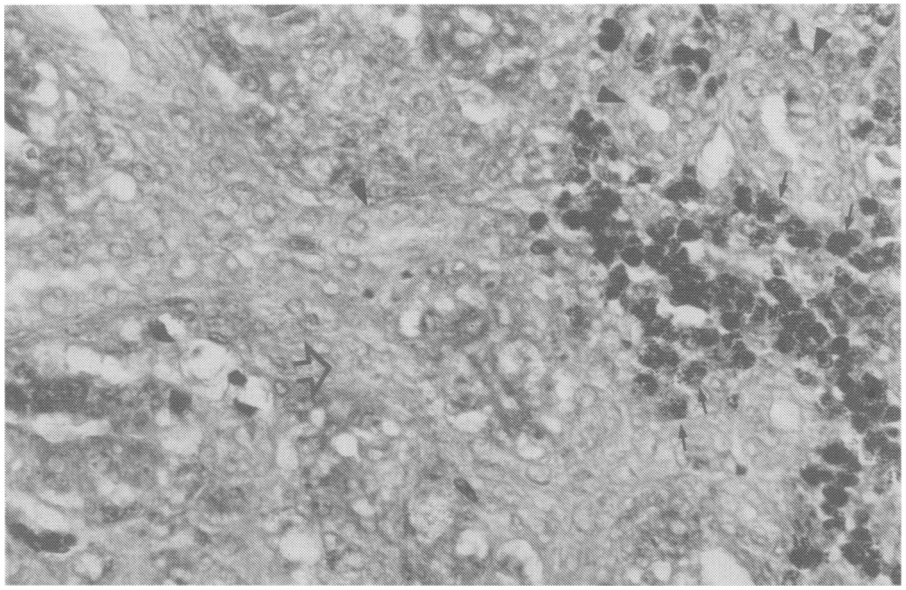

Fig. 4. Liver from broiler 14 days after ligation of both bile ducts. There is extensive bile duct proliferation (arrowheads) and fibrosis (open arrow) with scattered foci of heterophils (arrows). H&E. X452.

There was less leakage of inoculum when a canula was used. Some of these birds were kept for 28 days postinoculation at which time *C. perfringens* was still reisolated from liver and gall bladder.

The gross and microscopic lesion development was similar in both *C. perfringens* inoculated groups. Mottling and slight swelling of the liver were seen five to seven days postinoculation. By 12 days, the liver became pale and firm with multiple pale foci. By 17 days the liver was much enlarged, tan colored and firm with red and white foci. There was bile stasis. At 28 days the lesions were similar to those at 17 days (Fig. 6). None of the birds had yellow droppings. Microscopic lesions were relatively extensive by five days postinoculation and consisted of fibrinoid necrosis, heterophil and lymphocyte infiltration, bile duct hyperplasia and fibrosis with reticulin fiber proliferation (Fig. 7). By 17 days the lesions were massive (Fig. 8). Very scant Masson's trichrome positive collagen was present but became more extensive by day 28.

Examination of infected gall bladders showed low cuboidal epithelium and submucosal edema, submucosal lymphocyte infiltration and in one case severe necrosis. The nonligated bile ducts had no lesions and were patent. Other organ systems had no gross lesions and were not further examined. The saline injected birds showed no lesions 13 days after surgery.

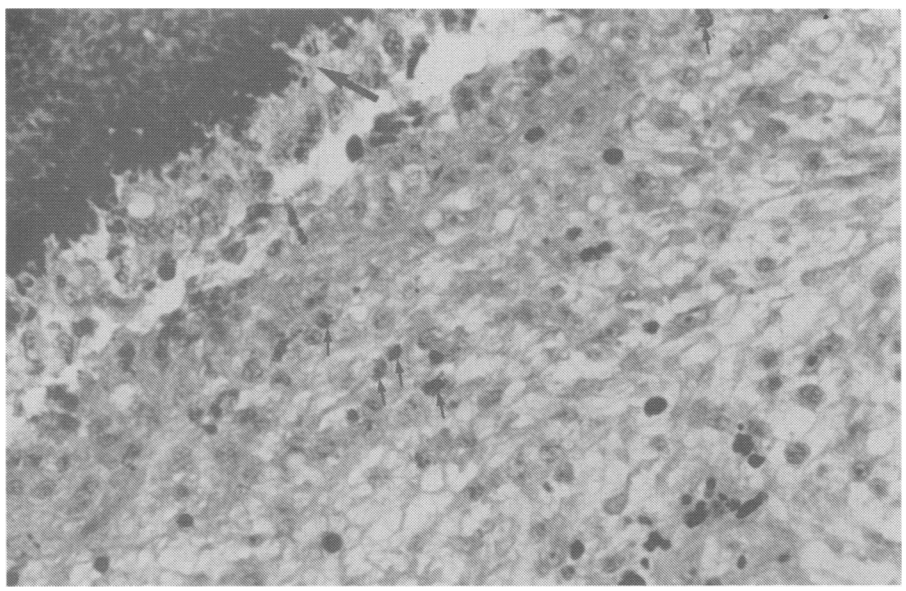

Fig. 5. Gall bladder from broiler five days after inoculation of 2.5×10^7 *C. perfringens* organisms via the bile duct. The wall is thickened and edematous with scant heterophil infiltration (arrows) and the lumen (large arrow) is filled with inspissated necrotic debris. H&E. X452.

DISCUSSION

Hepatitis characterized by an enlarged, firm, tan colored liver often with cholecystitis is of concern to Alberta broiler growers. A similar condition has been described in Scotland (2). Birds with this condition are usually not recognized prior to slaughter and are fed to market weight only to be condemned at slaughter. The microscopic appearance at that time is one of a chronic, possibly toxic insult to the liver resulting in degeneration and death of hepatocytes with replacement by proliferating ductal epithelium, pseudoduct formation and fibrosis. This is accompanied by variable degrees of inflammatory cell infiltrations. The progressive and finally massive bile duct hyperplasia suggests a relatively slow process with the initial insult occurring in the portal region. Mycotoxins have been considered as a possible cause but extensive feed analyses from affected flocks failed to demonstrate mycotoxins such as fusarium trichothescenes, aflatoxin and ochratoxin. Furthermore, fatty vacuolation, increased hepatocellular glycogen and nodular hyperplasia typical of mycotoxicosis (3,4) have not been observed.

The presence of *C. perfringens* in many of the gall bladders of affected livers suggested some involvement of either the bacterium or its toxin. In a pilot study, unpurified *C. perfringens* toxin contained in the culture medium

Fig. 6. Liver from broiler 28 days after inoculation of 5 x 10^8 *C. perifringens* organisms via the bile duct. The liver is swollen and hard with multiple pale areas of fibrosis visible on cut section.

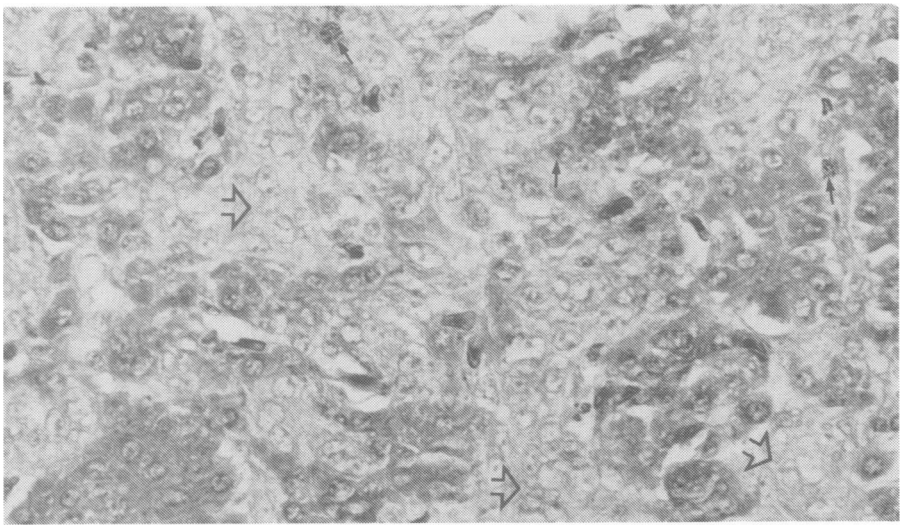

Fig. 7. Liver from broiler five days after inoculation of 5 x 10^8 *C. perfrigens* organisms via the bile duct. There is fairly extensive bile duct proliferation and fibrosis (open arrows) with scattered heterophil infiltration (arrows) in remaining parenchyma. H&E. X452.

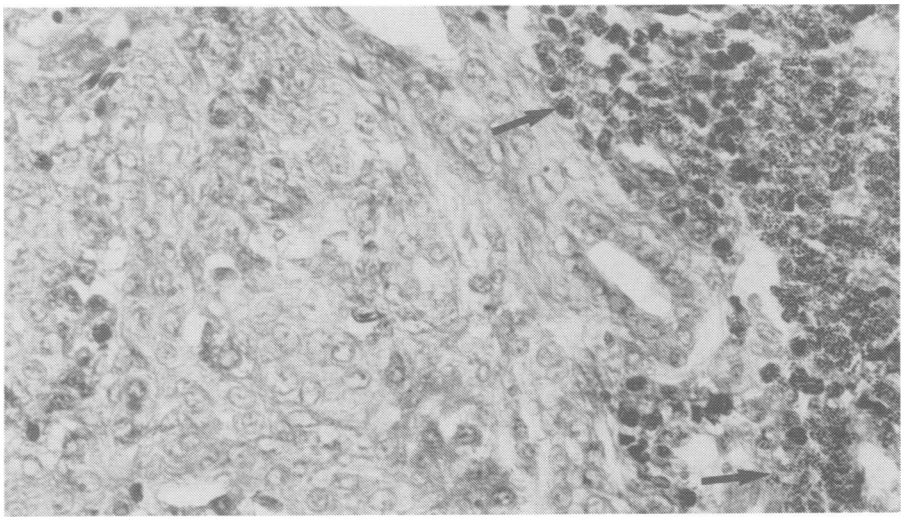

Fig. 8. Liver from broiler 17 days after inoculation of 5 x 10^8 *C. perfringens* organisms via the bile duct. Massive bile duct proliferation and fibrosis has replaced most hepatocytes. Extensive heterophil infiltration (arrows) is present. H&E. X452.

and titrated by intraperitoneal inoculation of mice, was injected into broiler chickens at 20 min intervals for 12 h via the cannulated pancreatoduodenal vein. No lesions were observed. The experiment was repeated using the hepatoenteric bile duct. This resulted in granulomas in the liver and necrosis of the bile duct. However, the same lesions were produced when the culture medium alone was used, indicating that the granulomas may well have been a reaction to the protein contained in the meat broth growth medium.

In the present study, liver lesions identical to those seen at slaughter were produced by ligation of both bile ducts or the inoculation of saline washed *C. perfringens* organisms into one bile duct. The lesions observed with total extrahepatic bile duct obstruction concur with those described by Lind *et al* (5). Their study showed a sharp rise in plasma bilirubin about ten days after obstruction. Bilirubinemia was likely present in the birds in our study when renal excretion of the pigment started to give a yellow stain to fecal droppings six to seven days after ligation of both bile ducts. Single bile duct ligation on the other hand apparently has no influence on plasma bilirubin levels due to the intrahepatic anastomosing network of bile ducts between the two duct systems (5).

Hepatic lesions caused by extra or intrahepatic cholestasis are well documented (6-8) and are attributed to the toxic effect of bile acids. Obstruction of bile ducts leads to retrograde passage of bile through the hepatocytes and through intercellular junctions.

Intrahepatic cholestasis was demonstrated with toxic drug reactions, bacterial sepsis and endotoxins (9-11). The toxic effect may alter the sodium dependent carrier mechanism for bile acids thus inhibiting bile acid dependent bile flow (12) or influence canaliculi membrane integrity and intercellular tight junctions (6,7,13). The end result is again escape of bile acids into pericanalicular passages causing necrosis and inflammation. It is, therefore, not surprising to see similar lesions produced by the two experimental procedures used in this study.

The results suggest that *C. perfringens* can play an important role in fibrosing cholehepatitis of broilers. On premises where a high incidence of this disease occurs repeatedly, treatment as suggested for *C. perfringens* induced necrotic enteritis (14,15) might be beneficial.

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REFERENCES

1. **BISAILLON JR, MEEK AH, FELTMATE TE.** An assessment of condemnations of broiler chicken carcasses. Can J Vet Res 1988; 52: 269-276.
2. **RANDALL CJ, KIRKPATRICK KS, PEARSON DB.** Liver abnormality in broilers. Vet Rec 1986; 119: 576.
3. **DWIVEDI P, BURNS RB.** Pathology of ochratoxicosis A in young broiler chicks. Res Vet Sci 1984; 36: 92-103.
4. **HUFF WE, KUBENA LF, HARVEY RB, CORRIER DE, MOLLENHAUER HH.** Progression of aflatoxicosis in broiler chickens. Poultry Sci 1986; 65: 1891-1899.
5. **LIND GW, GRONWALL RR, CORNELIUS CE.** Bile pigments in the chicken. Res Vet Sci 1967; 8: 280-282.
6. **SCHAFFNER F, POPPER H.** Morphologic studies of cholestasis. Gastroenterology 1959; 37: 565-573.
7. **MIYAI K, RICHARDSON AL, MAYR W, JAVITT NB.** Subcellular pathology of rat liver in cholestasis and choleresis induced by bile salts. Lab Invest 1977; 36: 249-258.
8. **PHILLIPS MJ, POUCELL S, ODA M.** Biology of disease. Mechanisms of cholestasis. Lab Invest 1986; 54: 593-608.
9. **SCHAFFNER F, JAVITT NB.** Morphologic changes in hamster liver during intrahepatic cholestasis induced by taurolithocholate. Lab Invest 1966; 15: 1783-1792.
10. **MANGINELLO FP, JAVITT NB.** Parenteral nutrition and neonatal cholestasis. J Pediatr 1979; 94: 296-298.
11. **UTILI R, ABERNATHY CO, ZIMMERMAN HJ.** Cholestatic effects of *Escherichia coli* endotoxin on the isolated perfused liver. Gastroenterology 1976; 70: 248-253.
12. **BLITZER BL, RATOOSH SL, DONOVAN CB.** Effects of inibitors of Na^+ — coupled ion transport on bile acid update by isolated rat hepatocytes. Am J Physiol 1982; 243: G48-53.
13. **DE VOS R, DE WOLFE-PEETERS C, DESMET V, BIANCHI L, ROHR HP.** Significance of liver canalicular changes after experimental bile duct ligation. Exp Mol Pathol 1975; 23: 12-34.
14. **LONG JR, TRUSCOTT RB.** Necrotic enteritis in broiler chickens III. Reproduction of the disease. Can J Comp Med 1976; 40: 53-59.
15. **PRESCOTT JF, SIVENDRA R, BARNUM DA.** The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can Vet J 1978; 19: 181-183.