≪Research Note≫

Comb Atrophy after Bile Duct Ligation in Chickens

Kazuki Yoshioka[1], Shiho Imai[1], Hiromi Ikadai[2], Jun Nagasao[3] and Masashi Tsujio[4]

[1] Laboratory of Veterinary Anatomy, School of Veterinary Medicine, Kitasato University,
35-1 Higashi 23, Towada, Aomori 034-8628, Japan
[2] Laboratory of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University,
35-1 Higashi 23, Towada, Aomori 034-8628, Japan
[3] Honma Pet Clinic, 1315-1 Ninomiya, Iwata, Shizuoka 438-0074, Japan
[4] Department of Veterinary Anatomy, Joint Faculty of Veterinary Medicine, Kagoshima University,
1-21-24 Korimoto, Kagoshima 890-0065, Japan

Gross, histological, and immunohistochemical changes in the combs of chickens after bile duct ligation (BDL) are described. Gross reductions in comb size and volume and lower serum testosterone levels were evident in chickens after BDL. Histologically, atrophic combs were characterized by reduced blood capillary diameter, decreased acid mucopolysaccharides, thinning of the stratum germinativum of the epidermis and dermis, and reduced immuno-staining intensity of androgen receptors. These results suggest that the affected cells in atrophic combs are androgen targets. BDL caused testicular atrophy in chickens, a primary complication of liver disease, and the resultant low serum testosterone levels subsequently caused atrophy of the comb. In other words, the atrophy of the comb observed in BDL chickens was a secondary complication of liver dysfunction that simulated the effects of liver disease.

Key words: androgen receptor, atrophy, bile duct ligation, comb

Introduction

Bile duct ligation (BDL) has been used in animal models to produce liver fibrosis and cirrhosis. Histologically, BDL can induce chronic cholestatic liver dysfunction, jaundice, proliferation of the intrahepatic bile ducts (ductular reaction), and liver fibrosis (Slott *et al*., 1990; Yoshioka *et al*., 2005). In humans, liver cirrhosis and chronic cholestatic liver disease frequently result in hypogonadism, metabolic bone loss, loss of muscle bulk, and abnormal thyroid hormone regulation and metabolism (Van Thiel *et al*., 1980; McIntyre, 1999; Hay, 1995; Gemma *et al*., 2001). The pathogenesis of these changes is not yet completely understood. To date, there has been only one report on the effects of BDL in chickens. Previous research conducted by our laboratory has demonstrated that chickens with BDL-induced liver fibrosis develop testicular atrophy and hypogonadism and that Leydig cell involution causes low serum testosterone (Yoshioka *et al*., 2004). Further research found that comb atrophy was common in BDL chickens. However, there has

been no investigation of the morphological changes in the comb after BDL in chickens. In this paper, we describe gross, histological, and immunohistochemical changes in the combs of chickens after BDL.

Materials and Methods

Nine 1-year-old male Australorp chickens were randomly assigned to either one control or one of two treatment groups. Both the common hepatoenteric duct and hepatocystic duct in six of these birds were ligated (Handharyani *et al*., 2001). Birds were bled and killed under ether anesthesia at either 5 ($n=3$) or 10 ($n=3$) weeks after BDL. The bile ducts of the remaining three chickens were left intact as controls; these birds were killed under ether anesthesia at the same time as the last surviving group of BDL birds (which were killed 10 weeks after BDL). Control and BDL chickens were housed in individual cages at a temperature of $18\pm1℃$ under a 12 h light–dark cycle and a light intensity of 10 lux. All birds had access to standard chicken chow and water *ad libitum*. Sera samples were collected from the heart under ether anesthesia just before birds were killed, and tissue samples were collected immediately after death. Serum was stored at $-80℃$ until assayed for testosterone levels. Serum testosterone levels were determined using a competitive solid-phase radioimmunoassay (SRL, Inc., Tokyo). Histologically, BDL chickens showed liver fibrosis with proliferation of the

intrahepatic bile ducts and obstructive cholestasis. Experimental procedures and the care of animals were conducted in accordance with the requirements of the Institutional Animal Care Committee at Kitasato University.

Comb specimens were fixed with 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Tissue sections, 4−5-μm thick, were then cut from each sample, stained using routine methods with hematoxylin and eosin, alcian blue (pH 2.5), which stains acid mucopolysaccharides blue, and Azan to detect collagen. For immunohistochemistry, peroxidase-labeled antibody was used with anti-androgen receptor (AR) polyclonal rabbit serum (NCL-ARp; Novocastra Laboratories, Ltd., Newcastle, UK; cross-reacts with rat, mouse, guinea pig, and chicken) as the primary antibody. Deparaffinized sections were rinsed in 0.1 M PBS, and the antigen was retrieved by autoclaving in 0.1 M citrate buffer for 20 min at 120℃. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide diluted in methanol for 20 min. The sections were incubated with the primary antibody for 12 h at 4℃, then incubated with the secondary antibody biotinylated anti-rabbit IgG (Nichirei Biosciences Inc., Tokyo, Japan) for 1 h at room temperature; this was followed by incubation with peroxidase-conjugated streptavidin (Nichirei Biosciences Inc.) for 30 min at room temperature; 3,3′-diaminobenzidine tetrahydrochloride (Nichirei Biosciences Inc.) was used as a chromogen. For morphometric analyses, the major axis of blood capillaries in the peripheral layer, and the thickness of the stratum germinativum of the epidermis and dermis was calculated using imaging software (Motic Images 2000, Shimadzu Rika Co., Tokyo, Japan). Twenty-five sections for each experimental and control group were prepared for morphometric analyses. Two-tailed Student's t-tests were used to assess the statistical significance of differences in mean serum testosterone levels and morphometric values between the experimental groups. Differences were considered significant at P values less than 0.05.

Results and Discussion

Compared to the controls, BDL chickens had reduced comb size and volume. Ten weeks after BDL, atrophic combs were pale red, sclerotic, and dry (Fig. 1). Serum testosterone levels were significantly lower ($P<0.05$) in BDL chickens than in the control chickens (Table 1). Histologically, the comb consists of epidermis, dermis, which comprises peripheral dermis layer and intermediate dermis layer, and a central core layer. Many large and small capillaries comprising vascular endothelial cells were observed in the peripheral dermis layer of control chicken combs (Fig. 2A). The average length of the major axis of blood capillaries in BDL chickens was significantly smaller than that of control chickens (Table 2), and there were fewer red blood cells in the capillaries (Fig. 2B and C). The intermediate dermis layer in control chickens consisted of a loose network of collagen fibers and spindle-shaped fibroblasts, which were stained blue by Azan (Fig. 2D), as well as a deposit of intercellular acid mucopolysaccharides that were heavily stained with alcian blue (Fig. 2G). BDL chickens had thinning of the stratum germinativum of the epidermis and dermis and decreased acid mucopolysaccharides (Fig. 2H and I) (Table 2). The fibroblasts were rounded, but the borders of the peripheral, intermediate, and central core layers were unclear because the fibroblasts and collagen fibers were too dense (Fig. 2E and F). In the control chickens, AR were localized in the nuclei of the stratum germinativum of the epidermis, the capillary endothelial cells in the peripheral dermis layer, and in the fibroblasts of the intermediate dermis layer (Fig. 3A and D). In contrast, all positive cells in the BDL chickens had reduced AR immu-

Table 1. **Serum testosterone levels (ng/dl) in chickens that were or were not (controls) subject to bile duct ligation (BDL).** Serum testosterone levels were significantly lower ($P<0.05$) in BDL chickens than in control chickens.

	Serum testosterone (mean±SD)
Controls ($n=3$)	154±113
5 weeks after BDL ($n=3$)	≦5※
10 weeks after BDL ($n=3$)	≦5※

※ Statistically significant ($P<0.05$) compared with the control group.

Table 2. **The major axis of blood capillaries in the peripheral dermis of the comb, thickness of the stratum germinativum of the epidermis and dermis, and thickness of dermis in chickens that were or were not (controls) subject to bile duct ligation (BDL) (μm).** The average length of the major axis of blood capillaries in BDL chickens was significantly smaller than that in control chickens, and had thinning of the stratum germinativum of the epidermis and dermis.

	Major axis of blood capillaries (mean±SD)	Thickness of stratum germinativum (mean±SD)	Thickness of dermis (mean±SD)
Controls ($n=3$)	49±5	37±6	4693±1787
5 weeks after BDL ($n=3$)	38±7※	14±4※	2570±939※
10 weeks after BDL ($n=3$)	28±4※	25±9※	1629±865※

※ Statistically significant ($P<0.05$) compared with the control group.

Fig. 1. **Gross appearance of the combs of chickens that were or were not (controls) subject to bile duct ligation (BDL).** (A) Control, (B) 5 weeks after bile duct ligation (BDL), (C) 10 weeks after BDL. Note the reduction in comb size and volume in BDL chickens compared to those in the control chickens. Atrophic combs were pale red, sclerotic, and dry.
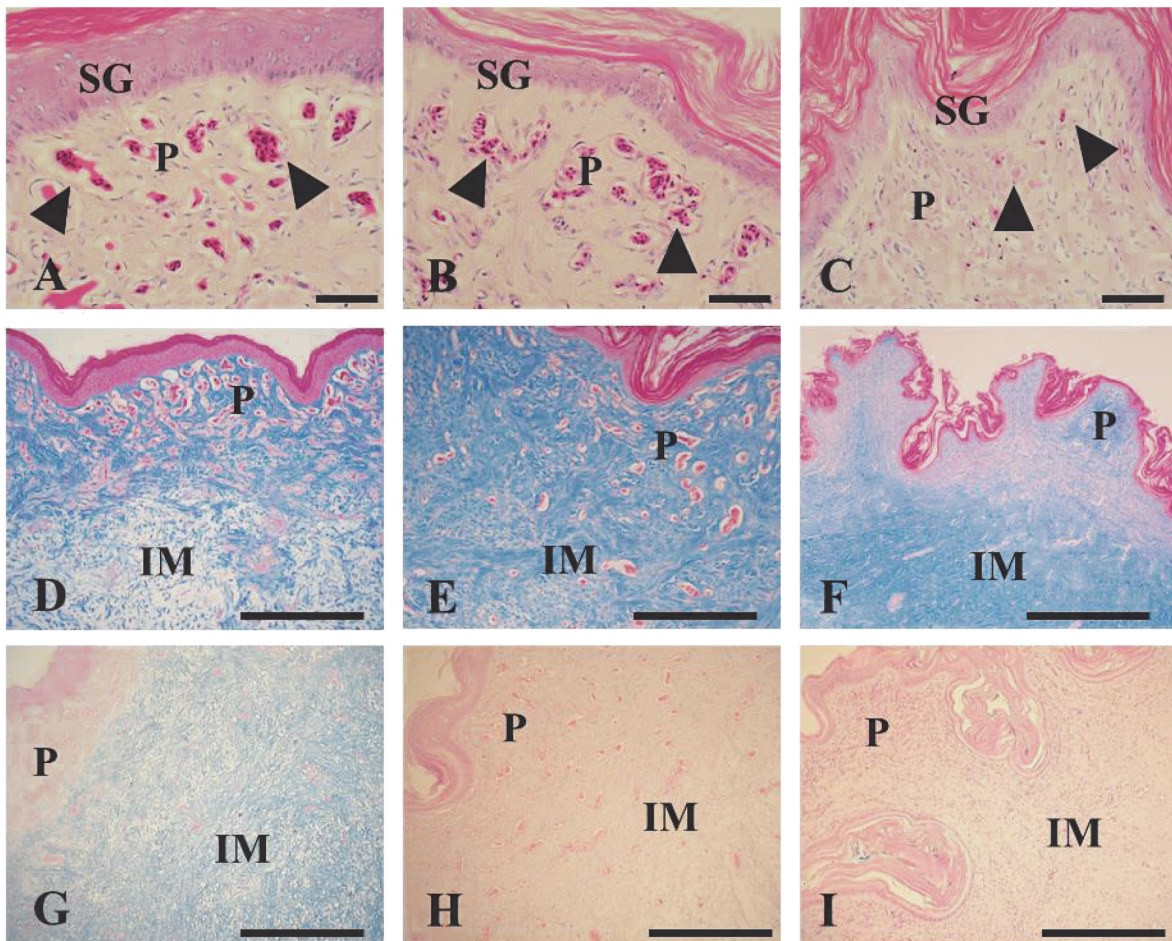

Fig. 2. **Histological photomicrographs of the combs of chickens that were or were not (controls) subject to bile duct ligation (BDL).** (A, D, G) Control, (B, E, H) 5 weeks after BDL, (C, F, I) 10 weeks after BDL. Atrophic combs in BDL chickens had blood capillaries (arrowheads) with smaller diameters in the peripheral dermis layer (P); BDL chickens had thinning of the stratum germinativum of the epidermis (SG), lower acid mucopolysaccharides, and dense collagen fibers in the intermediate dermis layer (IM). (A, B, C) Hematoxylin and eosin stain. (D, E, F) Azan stain. (G, H, I) Alcian blue stain (pH 2.5). Scale bar＝50 μm (A−C), 200 μm (D−I).
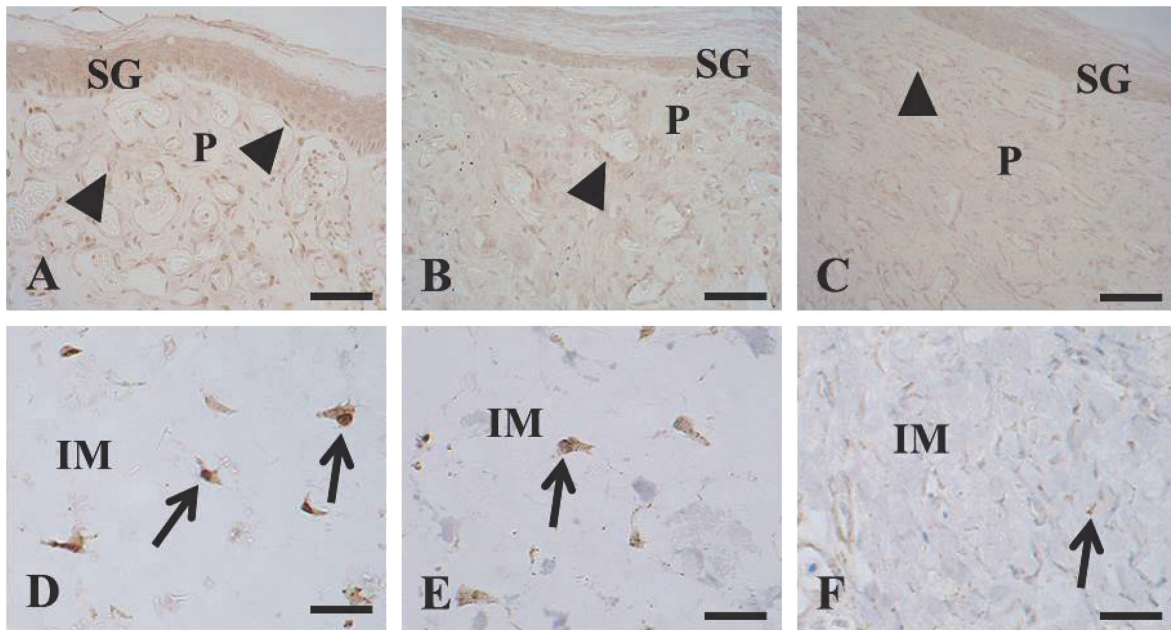
Fig. 3.   **Results of immunostaining of androgen receptors (AR) in the combs of chickens that were or were not (controls) subject to bile duct ligation (BDL).**   (A, D) Control, (B, E) 5 weeks after BDL, (C, F) 10 weeks after BDL.   AR immunoreactivity was observed in the nuclei of the stratum germinativum (SG), in the capillary endothelial cells (arrowheads) in the peripheral dermis layer (P), and in the fibroblasts (arrows) of the intermediate dermis layer (IM) in the control chickens.   All positive cells in BDL chickens showed reduced AR immunostaining intensity.   Scale bar＝50 μm (A–C), 20 μm (D–F).

nostaining intensity (Fig. 3B, C, E, and F).

These results demonstrate that AR immunoreactivity in the control chickens occurred in the capillary endothelial cells of the peripheral dermis layer, in addition to the stratum germinativum of the epidermis and the fibroblasts of the intermediate dermis layer. However, the atrophic combs of BDL chickens were characterized by blood capillaries with smaller diameters, reduced acid mucopolysaccharides, thinning of the stratum germinativum of the epidermis and dermis, and reduced AR immunostaining intensity. These results suggest that affected cells in atrophic combs are androgen targets (Shanbhag and Sharp, 1996; Yoshioka *et al*., 2010). The histological characteristics of atrophic combs were similar to those observed in androgen antagonist flutamide-treated chickens (Yoshioka *et al*., 2010). That study found AR in the nuclei of the stratum germinativum, the capillary endothelial cells in the peripheral dermis layer as well as fibroblasts in the comb. The intensity of staining in these cells increased in the testosterone-treated group but was reduced in the androgen antagonist flutamide-treated group. Thus, we suggest that the capillary endothelial cells in the peripheral dermis layer of the comb, in addition to the stratum germinativum and fibroblasts, are also androgen targets (Yoshioka *et al*., 2010).

Serum testosterone levels were significantly reduced ($P<$ 0.05) in BDL chickens compared to control chickens. This was likely the result of hypogonadism caused by liver dysfunction. We have previously reported that chickens with BDL-induced liver fibrosis show testicular atrophy and hypogonadism and that Leydig cell involution causes low serum testosterone (Yoshioka *et al*., 2004). Testicular atrophy and hypogonadism from advanced cirrhosis is well documented in humans. Conversely, experimental models of hypogonadism associated with liver injury are rare. Prepubertal male rats that underwent BDL showed a reduction in testis size (Van Thiel *et al*., 1985), and portocaval shunts in rats reportedly caused testicular atrophy. This atrophy, manifested histologically by the loss of germinal epithelium, was due to decreased mitosis and increased apoptosis, loss of spermatogonia and spermatozoa in the lumen of the seminiferous tubules, and eventual atrophy of the seminiferous tubules, leaving them lined only with Sertoli cells (Zaitoun *et al*., 1998). Administration of insulin-like growth factor-I (IGF-I) reverses testicular atrophy and improves testicular function in a carbon tetrachloride model of liver cirrhosis in the rat (Castilla-Cortazar *et al*., 2000).

It is interesting that the macroscopic findings of this study show the coincidence of liver damage, which is caused by BDL, with comb atrophy. It is well known that testosterone is the growth factor for combs. Since no other factors have been reported for comb growth, this study demonstrates the possibility that a growth factor originating from the liver also

affects comb atrophy.

In conclusion, BDL causes testicular atrophy as a primary complication of liver dysfunction, which then causes atrophy of the comb as a result of low serum testosterone. In other words, atrophy of combs observed in BDL chickens is a secondary complication of a liver injury that simulates the effects of liver disease.

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