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Fibrosis and Collagen-I Accumulation in Bali Cattle Liver Tissue Infected with *Fasciola gigantica*

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ABSTRACT

This study aims to determine the histopathological changes of fibrosis and accumulation of collagen I in liver tissue of Bali cattle infected with *Fasciola gigantica* in the Badung district, Bali Indonesia. On examination of 100 Bali cattle livers at the traditional abattoir of Badung, 37 of them were found to contain *F. gigantica* worms in both the bladder and bile ducts. The liver tissue was then fixed with 10% formalin for processing and stained with hematoxylin-eosin dyes. The liver sample preparation in the coating slide was also reacted with rabbit anti-collagen-I antibody. On histopathological examination, it was found that the distribution of fibrosis varied in the portal, interlobular, and bridging fibrosis areas. Immunohistochemical examination showed an immunoreactive reaction using rabbit anti-collagen I in the portal area with strong and weak intensity in the interlobular area and in bridging fibrosis. Fibrosis and accumulation of collagen I in Bali cattle infected with *F. gigantica* at the traditional abattoir of Badung district, Bali Indonesia was found in the portal, interlobular, and bridging fibrosis areas.

Key words: Bali Cattle, Collagen I, *Fasciola gigantica*, Fibrosis, Liver.

INTRODUCTION

Fasciolosis is a parasitic disease caused by flatworms (trematodes) and generally attacks ruminants, including cattle, buffalo, sheep, and other livestock. This disease is provoked by the trematodes *F. hepatica* and *F. gigantica*. These two types of worms have different habitats and hosts. The life cycle of *F. hepatica* requires an intermediate host, namely the snail *Lymnaea truncatula* found in Europe and Asia, while *F. gigantica* is generally found in subtropical and tropical countries such as India, Indonesia, Japan, Philippines, Malaysia, and Cambodia. The incidence of Fasciolosis in Indonesia is caused by the species *F. gigantica,* which is associated with the intermediate host of the snail *L. rubiginosa* (Gandahusada et al. 2004). This disease is also zoonotic, with a centralized incidence in South America, Africa, and Asia (Hotez et al. 2008). Furthermore, human fasciolosis has been reported in other areas such as Turkey (Boşnak et al. 2016), Serbia (Pavlović et al. 2014) and Denmark (Stensvold et al. 2018) and Germany (Salzer and Schmiedel 2015). In beef liver tissue, migration of young worms of *Fasciola spp.* can cause necrosis, inflammation and fibrosis (Lalor et al. 2021).

Several things have been known to trigger liver fibrosis, such as alcohol, drugs, genetic disorders, metabolic disorders, cholestasis, parasitic infections, viral hepatitis, cytokines, chemokines, and cryptogenic (Acharya et al. 2021). Fasciola infection has been linked to liver fibrosis, cirrhosis, and hepatocellular carcinoma, according to a meta-analysis study (Machicado et al. 2016). The fibrosis mechanism is derived from the activation of HSC by cathepsin, which is secreted from the tegument *Fasciola spp.* (Marcos et al. 2011). Worm load appears to be a crucial determinant of fibrosis incidence, as it is in schistosomiasis. Liver fibrosis develops as a result of repeated injury, and parenchymal cells regenerate after acute injury to replace necrotic or apoptotic cells. Inflammatory response and restricted extracellular matrix (ECM) deposition are related to this process. If the liver injury persists, followed by failure of the regeneration process, the hepatocytes are replaced by an abundant extracellular matrix, including collagen fibrils. The extent to which this fibrous material is distributed is determined by the cause of the liver injury (precipitating fibrosis). Prolonged liver cell injury is followed by chronic inflammatory stimulation accompanied by deposition of

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the extracellular matrix and gradual replacement of normal liver cells by fibrous tissue (Higashi et al. 2017; Zhangdi et al. 2019). Fibrotic tissue is initially located around the portal tract in chronic viral hepatitis and cholestatic disorders. It is distributed in the pericentral and perisinusoidal regions in alcohol-induced liver disease (Pinzani 1999). Liver fibrosis is a pathophysiological process caused by several pathogens and if the cause cannot be eliminated, fibrosis develops into cirrhosis (Hernandez-Gea and Friedman 2011; Parola dan Pinzani 2019). Rats who were given alcohol orally for 16 weeks showed changes in liver tissue such as steatosis, necrosis, inflammation, and fibrosis (Zhou et al. 2013). Changes such as steatosis, inflammation and fibrosis were found in mice after 7 weeks given the combination of CCl4 and ethanol. For this reason, mice can be used as animal models for drug development (Brol et al. 2019). Liver fibrosis is not a unique disease because whatever the trigger, the process of fibrosis is the same (Sebastiani et al. 2014; Kamdem et al. 2018).

As liver fibrosis progresses, the disease progresses from collagen bands to bridging fibrosis to the early stages of cirrhosis. The quantity and composition of the extracellular matrix are changed dramatically in liver fibrosis (Benyon and Iredale 2000). Collagen (I, III, and IV), undulin, fibronectin, elastin, hyaluronan, proteoglycans, and laminin are among the extracellular matrix (ECM) found in advanced stages of the disease. ECM accumulation occurs due to increased synthesis and decreased degradation (Arthur 2000). The decrease in matrix metalloproteinase (MMP) elimination activity against ECM was mostly due to overexpression of tissue-specific inhibitors of metalloproteinases (TIMPs). The main ECM-producing cells in the injured liver are HSCs (Gabele et al. 2003), residing in Disse's space and acting as a storage site for vitamin A in the normal liver. HSCs activate or differentiate into contractile, proinflammatory, and fibrogenic myofibroblast-like cells after a chronic injury (Milani et al. 1990; Marra 1999). Activated HSCs migrate and accumulate at tissue repair sites, secrete large amounts of ECM and regulate ECM degradation. PDGF (Platelet-Derived Growth Factor), primarily produced by Kupffer cells, is a major mitogen for HSC activation. The transcriptional and posttranscriptional control of collagen synthesis in HSCs are both important (Lindquist et al. 2000), and other liver cell types may also have fibrogenic potential. In cholestasis-induced hepatic fibrosis, myofibroblasts from small portal vessels proliferate around the bile duct to start collagen deposition (Kinnman and Housset 2002; Magness et al. 2004). Specific cell markers and responses to apoptotic stimuli vary between HSCs and portal myofibroblasts (Knittel et al. 1999). HSCs and myofibroblasts have been found infiltrating human livers undergoing tissue remodeling after CD34+CD38– hematopoietic stem cells were cultured with various growth factors (Forbes et al. 2004; Suskind and Muench 2004). These results suggest that fibrogenic cells in the injured liver may come from bone marrow-derived cells. In the liver, other potential sources of fibrogenic cells, such as the transitional mesenchymal epithelium and circulating fibrocytes have not been discovered (Kalluri and Neilson 2003; Phillips et al. 2004). Animal models suitable for liver fibrosis and organoids similar to the physiology of the human body can be used as a basis for studies of the pathogenesis of liver fibrosis and the development of therapeutic drugs Bao et al. 2021). Chusilp et al, (2020) stated that biliary atresia due to fibrosis can be made on culture medium of an apoptotic animal model of intrahepatic bile ducts. In addition, this model can also be used to identify new compounds to treat patients with non-alcoholic steatohepatitis (NASH) using the 3D spheroids method (Pingitore et al. 2019). This study aims to determine the histopathological changes of fibrosis and accumulation of collagen I in liver tissue of Bali cattle infected with *F. gigantica* in the Badung district, Bali Indonesia.

MATERIALS AND METHODS

The sample used in this study was the liver of Bali cattle infected with *F. gigantica* obtained from a traditional slaughterhouse in the Badung district, Bali Indonesia. The organ samples were put into a container filled with 10% neutral formalin buffer, then further processed to be prepared for examination under a microscope using routine hematoxylin-eosin staining. The variety of lesions found was documented and analyzed descriptively. Collagen expression in liver tissue was detected immunohistochemically using a standard peroxidase technique, with Meyer's hematoxylin as a counterstain. Preparations of 4-5micron thick paraffin were coated onto glass slides, deparaffinized with xylene, and rehydrated using graded ethanol solution. After three times of washing with phosphate-buffered saline (PBS), the preparation was immersed in a buffered citrate solution and heated in a microwave. Blocking endogenous peroxidase, it was again dripped with 3% H_2O_2 for 30 minutes. After being washed with PBS, the preparations were dripped with primary rabbit anti-collagen I antibody (1:100) and incubated in a refrigerator at 4°C for 12 hours. After incubation, the preparations were washed with PBS, dripped again with histofine simple stain (MULTI), and left for 1 hour. Collagen was visualized by adding a diamino benzidine substrate. The preparations were again stained with Meyer's hematoxylin counterstain and were ready to be observed under a microscope.

RESULTS

This research was initiated by surveying traditional slaughterhouses in Badung Regency. The survey was conducted to find Bali cattle infected by *F. gigantica*. Bali cattle with Fasciolosis indicated the presence of several *F. gigantica* liver worms in the bladder and bile ducts. Field observations were carried out from January to February 2021. On examination of 100 liver organs, 37 of them were found to contain several *F. gigantica* worms both in the bladder and bile ducts. Based on the sex, 30 Bali Fasciolosis cattle were female and 7 were male. Bali cattle infected with *F. gigantica* worms at the traditional Badung slaughterhouse is shown in Table 1.

Examination of liver tissue of Bali cattle infected with *F. gigantica* worms showed an accumulation of fibrous connective tissue with dense intensity in the portal area and mild intensity in the interlobular area. In addition, fibrous tissue deposits were also found in bridging fibrosis. The accumulation of fibrosis in the liver tissue of Bali cattle can be seen in Fig. 1.

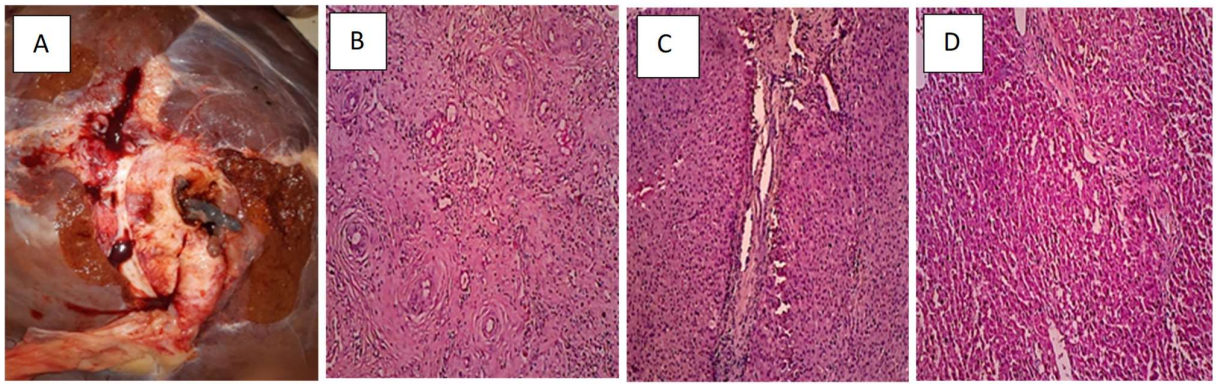

Fig. 1: Photomicrograph of liver tissue fibrosis in Bali cattle with fasciolosis using hematoxylin-eosin staining; Several *Fasciola gigantica* worms were found in the bile duct (A), accumulation of fibrous connective tissue with dense intensity was found in the portal area (B), and mild intensity in the interlobular area (C) and fibrous tissue deposits were also found in the form of bridging fibrosis (D). H and E. X200

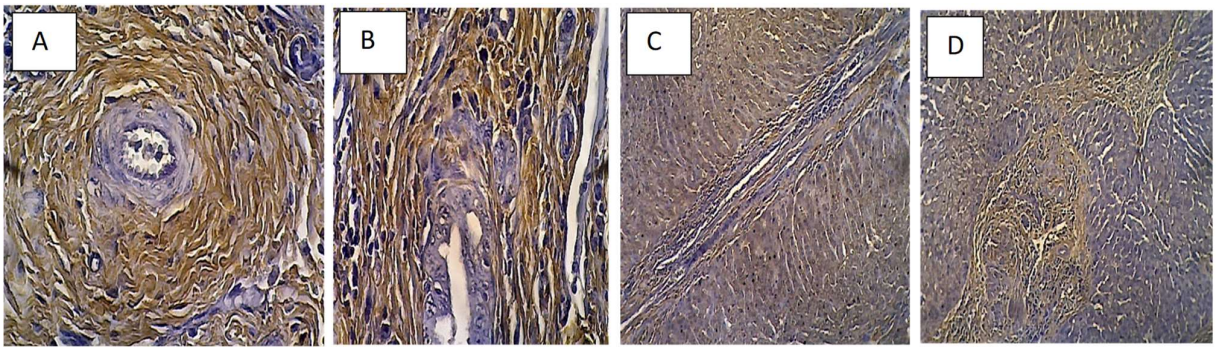

Fig. 2: Photomicrograph of Bali cattle liver tissue infected with *F. gigantica* showing an immunoreactive reaction (brown color) around the bile ducts in the portal area with strong intensity (A and B) and weak intensity in the interlobular area and bridging fibrosis (C and D). Staining: Immunohistochemically using rabbit anti-collagen I antibody. X400 (A and B) and X200 (C and D).

Table 1: Bali cattle infected with *Fasciola gigantica* at the traditional Badung slaughterhouse

Sex	Total	*Fasciola gigantica* infected	
		No.	%
Male	38	7	18.4
Female	63	30	47.6
Total	100	37	37.0

Examination of collagen accumulation using rabbit anti-collagen I antibody showed an immunoreactive reaction (brown color) around the bile duct in the portal area with strong intensity, weak intensity was found in the interlobular area, and in bridging fibrosis. The immunoreactive reaction of rabbit anti-collagen I antibodies on liver tissue of Bali cattle infected with *F. gigantica* is shown in Fig. 2.

DISCUSSION

Fasciolosis is one of the neglected but important parasitic diseases because it can cause death, liver failure, decreased milk and meat production and decreased reproductive performance (Keiser and Utzinger 2007). This disease usually runs chronically characterized by fibrosis in the portal, interlobular and surrounding sinusoids. Various etiologies of liver disease cause fibrosis to form through integrated signals that regulate extracellular matrix (ECM) deposition. This sequence of responses promotes the activation of hepatic stellate cells (HSCs) into myofibroblast-like phenotypes that are proliferative, fibrogenic, and contractile. The distribution of fibrosis in liver tissue is closely related to the number of worm larvae found (Marcos et al. 2007). This study found that the incidence of fibrosis in Bali cattle found at slaughterhouses in the Badung district was 37%. Various fibrosis stages can be found starting from deposition in the portal area, interlobular, and bridging fibrosis. An increase in collagen fiber deposition characterized the portal area fibrosis found in this study. The fibers are thick bundles surrounding the biliary tract in a strong brown color stained with rabbit anti-collagen I antibody. Immunoreactive reactions with brown color were also found in interlobular fibrosis and bridging fibrosis but with different intensities. The results of this study follow the findings of Mark and Isseroff (1983), who said that collagen I and III deposits occurred in the bile ducts of rats infected with *F. hepatica*. Similar results were also found by Trivilin et al. (2014) who said that there was periportal fibrosis formation in cows naturally infected by *F. hepatica*. Kolodziejczyk et al. (2015) also said that there were collagen fibers around the bile duct and cirrhosis with foci of necrosis in experimental animals that were successively infected with *Fasciola hepatica*. Alvarez et al. (2015) also reported the massive infiltration of inflammatory cells and collagen deposition in sheep liver eight weeks after infection with *Fasciola spp*. Marcos et al. (2007) reported that liver fibrosis in cattle infected by *Fasciola spp.* was around 67.7%. This study

also follows the theory of Gressner et al. (2008), which states that liver fibrosis is indicated by the progressive accumulation of extracellular matrix (ECM) fibrils in the structure of liver tissue. Shiba et al. (2008) also found that the accumulation of type 1 collagen was higher than that of type III in the portal area of rats induced by dimethylnitrosamine.

Inflammation persistence causes changes in the collagen profile accompanied by an increase in the relative amount of type I and III collagen and modification of extracellular protein cross-links. ECM protein cross-linking in persistent fibrosis makes it more resistant to degradation attempts (Issa et al. 2004). In the fibrotic liver, the total collagen content is 3-10 times higher than normal. In normal liver conditions, the collagen that makes up collagen fibrils types I, III, V, and type XI collagen are contained in the portal areas, capsule, and large blood vessels (Friedman 2007). However, relatively small amounts of type I and III collagen are found in the subendothelial space. The activation of myofibroblast precursor cells, which results in the progressive deposition of ECM proteins, is thought to be the main mechanism of fibrogenesis. Hepatic stellate cells (HSCs, Ito cells), hepatic resident fibroblasts (portal or centrolobular), epithelial cells transitioning from epithelium to mesenchyme, bone marrow-derived fibrocytes, and muscle cells are sources of ECM production during hepatic fibrosis and plain surrounding blood vessels (Pinzani and Rombout. 2004; Gressner et al. 2008). In chronic hepatitis, HSCs are the primary source of myofibroblasts, while portal fibroblasts have an essential fibrogenic role in cholestatic liver disease (Crosas-Molist and Febregat 2015). Perisinusoidal HSCs appear to play a role in the pathogenesis of liver fibrosis in dogs (Boisclair et al. 2001). Periportal fibrosis is often also found in areas of necrosis, especially in idiopathic chronic hepatitis (Poldervaart et al. 2009). A retrospective study on dogs found as much as 36% copper accumulation as a cause of chronic hepatitis (Smedley et al. 2009).

Collagen accumulation in liver fibrosis can increase vascular resistance and portal hypertension, both of which are critical factors for complicating in advanced fibrosis (Konigshofer et al. 2021). Clinical ascites in cattle with chronic fasciolosis are closely related to the presence of collagen fibril deposits and portal hypertension (Buob et al. 2011). In addition, ascites can result from decreased cardiac output, splanchnic arterial vasodilation, and activation of renin-angiotensin system, RAS, which causes water and sodium retention (Sanyal et al. 2008). Hepatic encephalopathy is frequently caused by portosystemic shunting, in which abnormal ammonia metabolism interacts with further factors such as inflammatory mediators and neurosteroids to cause neurological dysfunction and astrocyte swelling (Lidbury et al. 2016). In vivo and case studies reporting cirrhosis as a condition accompanying fasciolosis include two additional studies describing the sequential progression from liver fibrosis to cirrhosis (Marcos et al. 2007; Kolodziejczyk et al. 2015). Liver damage was also reported in animals infected for 6 months with fibrotic nodules (stage IV liver fibrosis or cirrhosis) in most of the lobes (Perez et al. 1999). Collagen accumulation in liver fibrosis can increase vascular resistance and portal hypertension, both of which are critical factors for complicating in advanced fibrosis (Konigshofer et al. 2021). Indart et al. (2019) also reported that fluid accumulation in the abdominal cavity in calves was found due to widespread liver fibrosis.

In rodent models and human patients, liver fibrosis is extensively studied. Even though the pathogenesis appears to be similar to canine fibrosis, more research is needed to confirm or refute these findings. For the diagnosis of liver fibrosis in various animal species, histological examination of specimens is required. Future studies should develop, serum markers of liver fibrosis shown to have some discriminatory abilities to a limited degree in dogs (Eulenberg and Lidbury 2018). The elastography technique is useful for diagnosing liver fibrosis in humans and deserves evaluation in dogs as well (Eulenberg and Lidbury 2018). Even if such a non-invasive liver fibrosis test is developed it could fully be used in dogs (Eulenberg and Lidbury 2018). Li et al (2022) said that herbal therapy using periplaneta americana (EPA) extract could reduce the formation of fibrous tissue in the liver of rats induced by porcine serum. Liver fibrosis can also be reduced in animal models given CCl4 for 3 weeks post-treatment using nintedanib (Wollin et al. 2020). Susutlertpanya et al, (2019) also stated that there was a reduction in fibrotic lesions on microscopic examination of the liver in mice with NASH given nintedanib for two weeks. Liver fibrosis that does not get proper treatment will lead to cirrhosis, the final condition of liver damage in which the liver is no longer functioning at all. In the Western world alone, this disease is the 8th leading cause of death. The only way to survive is a liver transplant. However, this method has many drawbacks, among others, it is very dependent on the availability of donors and is very expensive.

Conclusion

Fibrosis and accumulation of collagen I in Bali cattle infected with *Fasciola gigantica* at the traditional abattoir of Badung district, Bali Indonesia was found in the portal, interlobular, and bridging fibrosis areas.

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Authors Contribution

IBOW designed the study and was major contributor in writing the manuscript. IBOM and IBWA conducted and judged the pathological examination. PHS collected field samples and data. All authors read and approved the final manuscript.

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