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SOME STUDIES ON SPONTANEOUS *HYMENOLEPIS DIMINUTA* INFECTION IN LABORATORY RATS

By

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Abstract

Hymenolepis diminuta is a tapeworm that occurs worldwide. It is known to be found commonly in areas where large amounts of food grains or other dry feed products, which are the favorite foods for rats. Transmission of disease to human is uncommon; however, it may be a serious threat for population who are living in rural areas which are suffering from excessive rodents. Here, this study had done on spontaneous *H. diminuta* infection in laboratory rats as a model. Out of thirty five adult laboratory rats investigated for parasitic diseases only nine (25.71%) were diagnosed positive for spontaneous *H. diminuta* infection. Four of them (44.44%) were found losing of weight and lacking of motility, while the others were normal. On microscopic examination, *H. diminuta* eggs had been found in their stool. On autopsy, small intestines were found to contain from 5-6 multi-segmented tapeworms in each rat. Histopathologically, intestinal lumen showed varying sections of *H. diminuta* segments with serrated borders. *H. diminuta* infection caused multiple mucosal ulcers with absence of intestinal villi from the surface epithelium and excessive mucin. Moreover, inflammatory cells infiltration in the connective tissue core of the villi. Furthermore, the Toluidine blue stain showed that there are Mastiocytosis. Additionally, there were goblet cells hyperplasia on using PAS. Moreover, there were high expression of cyclooxygenase 2 (COX-2), tumor necrosis factor-α (TNF-α) and inducible Nitric-Oxide Synthase (iNOs). This implicate, strong correlation between COX-2, TNF-α and iNOs expression and inflammation induced by *H. diminuta*

Keywords: *Hymenolepis diminuta*, Spontaneous infection, Histopathology, COX-2, TNF-α & iNOs expression.

Introduction

Hymenolepis diminuta is a tapeworm that occurs worldwide. Rats and other rodents are the definitive host, while arthropods such as fleas, Lepidoptera and coleopteran act as intermediate host (De Carneri, 2004). Hymenolepiasis is a disease caused by either *H. diminuta* and/or *H. nana*. Both cestodes can infect humans, particularly children. However, *H. nana* has been mainly found in man, while *H. diminuta* occurs mainly in rats and mice. Though, it has been recorded in man particularly in children. The infection rate of *H. diminuta* in human beings ranges from 0.001%-5.5% in different parts of world (Tena *et al*, 1998; Watwe and Dardi, 2008). In Egypt (Mansoura City) the infection rate of *H. diminuta* in human was 1.4% (El-Shazly *et al*, 2006).

In the rat as well as other hosts, mucosal mast cells (MMC), eosinophil, and goblet cells have been associated with host responses to intestinal helminthes infection, changes in intestinal motility and clearance of intestinal-stage worms (Ishikawa *et al*, 1994; Ovington and Behm, 1997). Because the intestinal stage of tapeworm infection has been viewed as relatively benign and the parasite is not cleared from the intestine, we might expect that in the rat host the expansion of the population or effector response of these cell types is reduced and therefore ineffective; however, contrary to that hypothesis, MMC numbers are increased during *H. diminuta* infection of the rat (Featherston *et al*, 1992; Dwinell *et al*, 1998). Others researchers suggested various and highly selective effector/regulator cells are involved in worm expulsion depending largely

on the genus of intestinal helminthes (Horii *et al*, 1993).

Several physiological and morphological changes occur in the small intestine of the rat during the establishment of the chronic *H. diminuta* infection: smooth muscle hypertrophy, changes in smooth muscle contractile patterns, and deepening of mucosal crypts (Dwinell *et al*, 1994; Dwinell *et al*, 1998; Starke and Oaks, 1999). The host immune response to infections involves up-regulation of Th2 cytokines, pointed towards eliminating the parasite with increases in mast cells, immunoglobulin E, eosinophil and goblet cell mucin production (Mc Kay *et al*, 1990; Andreassen *et al*, 1999). However, how the host is recognizing helminthes antigens and responding to them is still unanswered issue (Mac Donald *et al*, 2002). Mast cells are important for protective immunity to intestinal helminthes infections and as mediators of allergic disease (Li *et al*, 2004). In this study, spontaneous chronic *H. diminuta* infection was a great opportunity to identify exactly host-parasite interaction during intestinal infection. Accordingly, *H. diminuta* is used as a model for a variety of histopathological and immunopathological studies on tape worm.

Here in this work, we aimed to study the histopathological changes induced by spontaneous *H. diminuta* infection in laboratory rats, and to define the contribution of some inflammatory mediators such as; cyclooxygenase 2 (Cox-2), Tumor Necrosis Factor alpha (TNF-a) and inducible Nitric-Oxide Synthase (iNOs) in the pathogenesis of *H. diminuta.* Furthermore, we emphasized on the description of the morphological changes and distribution of mast cells in relation to the lesions of small intestine.

Material and Methods

A total of 35 wild-type white-Albino adult laboratory rats were used in this study (all males). They were obtained from Laboratory Animal Research Section, Faculty of Medicine, Minia University. The animals had free access to standard rodent chow and water. All experimental procedures were conducted according to the ethical standards approved by the Institutional Animal Ethics Committee Guidelines for animal care and use, Minia University, Egypt.

Physical study

For weight and activity**.**

Helminthic examination

Fecal samples of rats were collected in 5% formal saline in air tight containers. Each sample examined macroscopically for presence of tapeworm segments and then, examined microscopically by direct smear method for eggs of *H. diminuta*.

Rats scarifying

Rats were euthanized humanely using chloroform anesthesia. Inspection for any gross lesions of pathological significance was observed in visceral organs. The small intestine was removed; slit open, inspection for the tapeworms inside. The worms had been removed and counted.

Histopathology

The small intestine was fixed flat in 10% buffered formalin. After fixation, the tissues were rinsed and stored in 70% ethanol until dehydrated by passage through a series of graded alcohol dilutions, followed by embedment in paraffin. Sections of these tissues were stained with H&E, toluidine blue and per-iodic acid, Schiff (PAS).

Immunohistochemistry

Tissue samples from walls of small intestine, of scarifying animals were fixed in 10% formalin, embedded in paraffin, sectioned, stained either with COX-2, TNF-α or iNOs. For detection of expression, Intestinal sections were deparaffinized and rehydrated. Non-specific binding of IgG was blocked using Ultra V block for 10 min at room temperature. The sections were then incubated with diluted primary antibodies (COX-2 1:1000, TNF-α 1:1000 and iNOs 1:1000) at 4°C overnight. After three washes, the sections were incubated for further 30 min with Ultra Vision One HRP polymer-conjugated anti-mice antibody. Color reaction was developed by incubation with diaminobenzi-

dine. The slides were counterstained with Meyer's hematoxylin and dehydrated in ethanol prior to mounting.

Result

Out of 35 adult laboratory rats investigated for parasitic diseases only 9 (25.71%) were diagnosed positive for spontaneous *H. diminuta* infection. Four of them (44.44%) were found losing of weight and lacking of motility. On direct smear examination, there were a large number of *H. diminuta* eggs: globular oval shaped eggs, the embryophore had thickening at the poles and hexcanth embryo was present within the oncosphere.

No gross lesions of pathological significance were observed in visceral organs. Small intestine had 5-6 multi-segmented tapeworms but the number was as high as up to ten. These worms were visible as whitish-yellow structures during gross examination of intestine. In few rats, intestinal lumens were dilated due to presence of multiple numbers of tapeworms.

Histomorphology: Some intestinal sections stained with H&E showed cross section of a mature segment with serrated cuticle (Pl. 1a). Other showed longitudinal section of gravid proglottids, eggs can be seen in the parenchyma (Pl. 1b). Segments of *H. diminuta* showed serrated borders and elliptical shaped uterus (Pl. 1c).

Histopathologically, lumen of small intestine contained tapeworm segments (Pl. a-c). Excessive mucin secretion and its presence were in luminal debris. There were multiple mucosal ulcers with absence of intestinal villi from the surface epithelium. Some intestinal villi appeared blunt and reduced in height. Infrequently, eosinophilia cellular infiltration was seen. Moreover, inflammatory cells infiltration in the connective tissue core of the villi (Pl. 1e, f). In response to infection, alterations in goblet cell and mucin responses included goblet cell hyperplasia, and increased (mucin) secretion. Goblet cells appeared as clear "bubbles" in crypt epithelium in H&E stain. They are crowded and so many in the infected sections of rates (Pl. 1h) compared to that in the control rate (Pl.1g). Furthermore, using PAS goblet cells appeared as crowded red-stained rounded spots in the lining of the villi and basal crypts (Pl.1k). Inflammatory cells infiltrate in the core connective tissue as shown in Pl. 1j. Additionally, infected sections of rates stained with toluidine blue showed that there are large numbers of mast cell in the ileum (Mastiocytosis). The metachromatic character of these mast cell granules appears purple in color (Pl. 2b). Some of these mast cells are degranulated (Pl. 2c).

Immunohistochemistry: Ileal immunostaining for COX-2, iNOS, and TNF-α were examined. COX-2, iNOS, and TNF-α expression were significantly enhanced. The COX-2 expression was significantly enhanced in the ileum during *H. diminuta* infection. All sections showed diffuse positivity staining of COX-2 in the epithelial cells and staining of lamina propria toward the tips of the villi (Pl. 2e). Also, iNOS was expressed in the ileum mucosal layer, while inflammatory cells (in the connective tissue core of the villi) show no iNOS expression (Pl. 2g). TNF-α showed that diffuse positivity staining as COX-2 and staining of lamina propria toward the tips of the villi (Pl. 2i). These results showed a strong correlation between expression of these factors (COX-2, TNF-α & iNOS) and inflammation induced by *H. diminuta*.

Discussion

H. diminuta infects the small intestine of several species, including the rats. The incidence of 25.71% of *H. diminuta* in present study is in accordance with reports of earlier workers (Webster and Mac Donald, 1995; Somvanshi, 1997; Sadjjadi and Massoud, 1999). Clinically, infection of *H. diminuta* is diagnosed by fecal examination and presence of its characteristics eggs. Arai, (1980) reported that concentrated stool samples revealed about 70 micron diameter, spherical eggs, with a striated outer membrane and a thin inner membrane and containing six central hooklets but no polar filaments, (of *H.*

diminuta eggs) and differentiated from *H. nana* eggs, which have a similar appearance but are smaller and have two evident polar thickenings, from each of which arise four to eight polar filaments.

The present histopathological findings are in accordance with (Somvanshi, 1997; Goswami *et al*, 2011). Excessive mucin secretion, desquamation of epithelial cells and ulceration were clearly in sections of small intestines stained with H&E. The pathology induced by irritation caused by serrated border of segments of tapeworms. Atrophy and absence of intestinal villi from the surface epithelium may constrain the parasites (Maizels and Holland, 1998).

Mucosal mast cells and goblet cells are the 2 cell types that show significant increase in the infected intestinal tissues. Furthermore, goblet cell hyperplasia was seen by PAS in accordance with others (Miller and Nawa, 1979; Ishikawa *et al*, 1997; Khan and Collins, 2004). During infection, pathogens can actively disrupt the mucus barrier, reach the epithelial cell surface, and subsequently create an opportunistic environment for commensal microbes. In response to infection, there are alterations in goblet cell and mucin responses including goblet cell hyperplasia, increased mucin secretion, and changes in mucin glycosylation. These changes, in addition to other components of the host immune response, will help to clear the infection (Kim and Khan, 2013). However, high levels of mucus production retain parasites in the lumen and decrease their ability to anchor in the gut (Maizels and Holland, 1998).

Moreover, there are large numbers of mucosal mast cells (MMC), and eosinophils were present in the connective tissues of both the sub-mucosa and the lamina propria in *H. diminuta*-infected ilea were shown by toluidine blue stain. However, there were small numbers of eosinophils, but not MMC, were also observed in the ileum's muscularis externa of infected animals. These data go with other researches (Castro, 1989; Starke and Oaks, 2001). The populations of MMC and eosinophils present in the lamina proprial connective tissue of the ileum were greater than that observed in control animals. These data match with (Starke and Oaks, 2001). However, mastocytosis does not led to expulsion of the infecting parasites. In other parasite species such as *Trichinella spiralis*, expulsion of the intestinal stage is clearly related to mastocytosis as well as to changes of intestinal physiology and motility (Castro, 1989). Thus, the tapeworm suppresses MMC participation in expulsion, or mast cell function in this host–parasite interaction was redirected, as for example to involvement in modifying intestinal functions or tissue changes. The present results agreed with Starke and Oaks (2001) and with Featherston *et al*. (1992) who found that the kinetics of enteric mastocytosis in response to *H. diminuta* is rat strain specific. As well, Ishih (1992) showed that tapeworm survival was not directly related to MMC numbers when MMC population size was compared between rat strains that either support chronic infections or those that do not support chronic tapeworm survival.

The present study showed a well-defined inflammatory response of the ileal mucosa caused by *H. diminuta* infection. This encouraged to study the role of COX-2 and TNF-α as pro-inflammatory mediator and NO as an antimicrobial for a wide range of bacterial and parasitic pathogens (Clark and Rockett, 1996; Fang, 1997).

COX-2 is an isoform of cyclooxygenase (COX) is a potent mediator in inflammation (Smith *et al*, 1996). However, its role in intestinal injury as a result of helminthic infection is still not clear. Here, in this study we assessed its expression to investigate whether the pathological lesions produced by *H. diminuta* in intestine were the result of over expression of COX-2 or not. In the present study, the COX-2 expression was significantly enhanced in the ileal mucosa during *H. diminuta* infection. All sections showed staining in the mucosal layer of the ileum.

Staining was most intense in epithelial cells at the villous tips. Also staining was seen in the lamina propria cells toward the tips of the villi diffuse. These results implicate that there was a strong correlation between COX-2 expression and inflammation induced by *H. diminuta* (Barbara *et al*, 2001; Akiho *et al*, 2005).

In the present study, correlation between iNOS expression and inflammation induced by *H. diminuta*. Nitric oxide (NO) is a free radical associated with multiple physiological functions. This molecule is synthesized from L-arginine by a group of isoenzymes called NO synthases (NOS). Inducible Nitric-Oxide Synthase (iNOS) was the major NOS isoform expressed by intestinal epithelial cells (Salzman *et al*, 1996; Witthöft *et al*, 1998).

This study revealed that iNOS was highly expressed in the mucosal layer of the ileum, While inflammatory cells (in the connective tissue core of the villi) show no iNOS expression. NO is important in host defense and homeostasis; however, it is also harmful and has been involved in the pathogenesis of many inflammatory and autoimmune diseases (Liew, 1995). Many evidences stated that NO has been contributed in the pathogenesis and pathophysiology of inflammatory bowel diseases, which includes Crohn's disease and ulcerative colitis (Guslandi, 1998). Moreover, NO has been associated with a variety of induced or spontaneous intestinal inflammation in animals (Miller *et al*, 1995; Matsumoto *et al*, 1998). Sustained release of NO, due to increase iNOS expression, may lead to cellular damage and gut barrier failure (Potoka *et al*, 2002). Results obtained here in this study (all the intestinal epithelial surface showed diffuse and marked iNOS expression) supported these evidences that obtained by Potoka *et al*. (2002).

Tumour necrosis factor alpha (TNF-α) is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation. TNF-α is responsible for a variety of signaling cascade inside cells leading to necrosis or apoptosis. Also it is important for protection against infection and cancers (Idriss and Naismith, 2000). TNF-α staining was confined to epithelial cells and in the lamina propria cells toward the tips of the villi. TNF increases the permeability of the intestinal mucosal barrier by inducing destruction and apoptosis of the epithelial tight junction and epithelial cells respectively. Binding of TNF to its receptors activates the IκB/NFκB signaling pathways led to the release of inflammatory cytokines (McGee *et al*, 1995; Awane *et al*, 1999).

The present study showed that location staining of TNF-α in the intestine was relatively similar to COX-2 staining but the intensity was higher in COX-2. The intestinal epithelium has barrier function responsible for secretion of mucins, antimicrobial molecules and absorbed toxins into the gut lumen. Intestinal epithelial cells also can secrete immunologically active substances that activate protective mucosal inflammatory and immune responses (Roediger and Babidge, 1997). In this study, TNF-α, COX-2 and iNOS were potentially involved in the injury of intestinal mucosa.

Conclusions

The spontaneous prevalence of hymenolepiasis was higher in laboratory rats. It caused sub-clinical infections without causing any mortality. Conventional fecal, gross, histopathological and immunohistochemistry methods were good tools in the study of pathogenesis and diagnosis of *H. diminuta* infections. Presence of zoonotic infection within human populated area is a threat that human might be infected. Intestinal epithelial cells are likely to be the most relevant COX-2, TNF-α and NO producing cells. Also, regulation of COX-2, TNF-α and NO productions could be helpful in controlling intestinal damage during *H. diminuta* infection.

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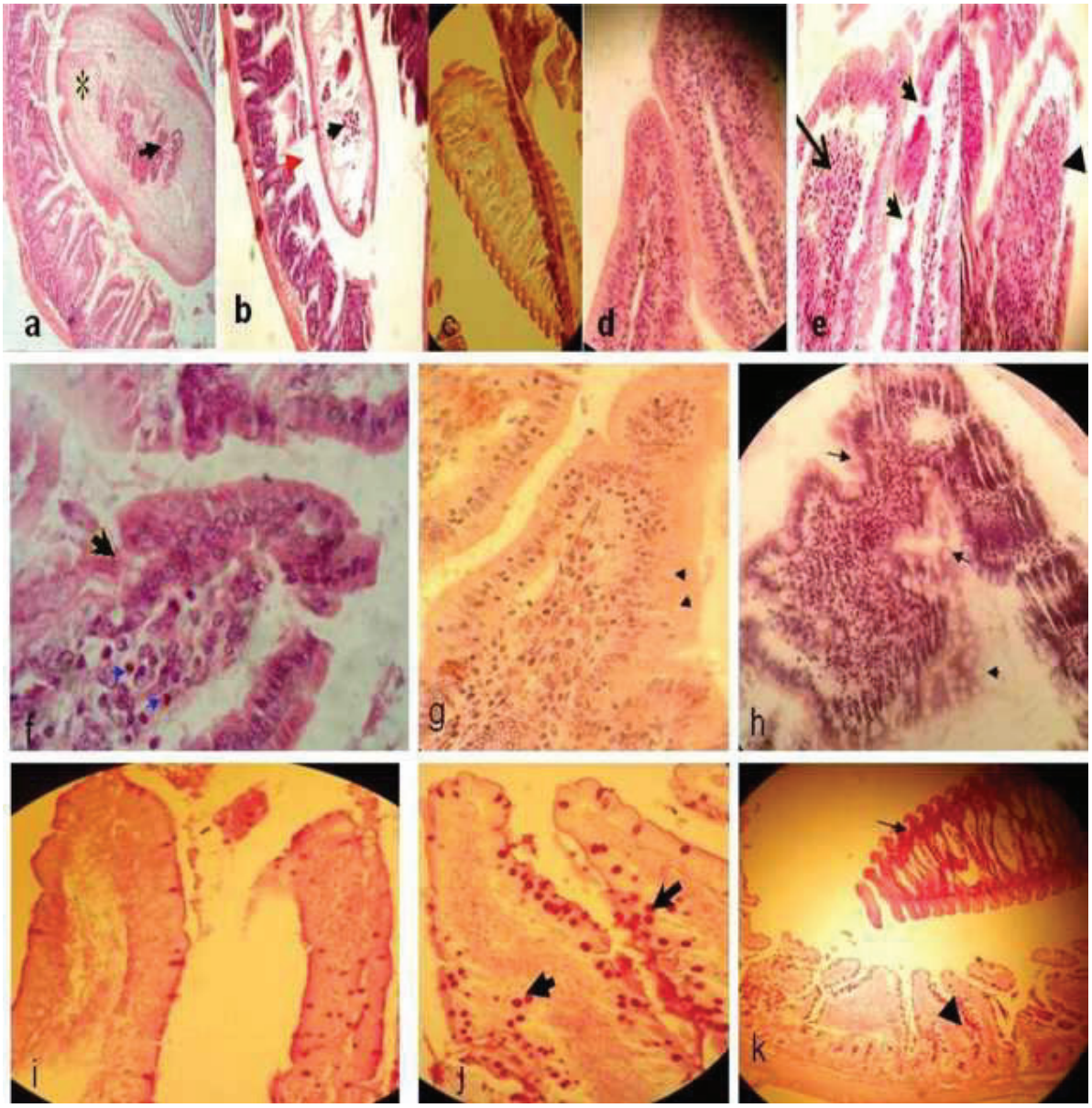
Explanation of Plates

Plate 1: Photomicrographs of Ileal sections of non-infected and infected rats stained with H&E &PSA:
a. Cross section of mature proglottid stained with H&E with median ovary (black arrow) and cirrus primordial (asterisks) (x10).
b. Longitudinal section of gravid proglottids stained with H&E in lumen with multiple eggs in body's parenchyma (black arrow, x 10), ileum stained with H&E showing mucosal ulcer (red arrow, x10).
c. Longitudinal section of gravid segments of *H diminuta* in lumen showing serrated borders and elliptical shaped uterus (x 10).
d. Ileal section of normal non-infected rat stained with H&E (x40).
e. Ileal section of *H. diminuta*-infected rat stained with H&E. showing blunted villous lined with flattened and some scattered vacuolated cells and inflammatory cells infiltrate in lamina properia (black arrow, x 40). Many mucosal ulcer (arrow head, x40).
f. Ileal section of *H. diminuta*-infected rat showing many mucosal ulcer (black arrow head, x100). Inflammatory cells in connective tissue core of villi (blue arrow head, x100).
g. Ileal section of normal non-infected rat stained with H&E showing very few goblet cells (black arrow, x100).

h. Ileal section of *H. diminuta*-infected rat stained with H&E showing the mucus in goblet cells poorly stained and crowded goblet cells appear as pale spots (black arrows) especially in crypts' bases (x40).
i. Ileal section of normal non-infected rat stained with PAS showing scattered red-stained goblet cells (x40).
j. Ileal section of *H. diminuta*-infected rat stained with PAS showing crowded red-stained goblet cells in lining of villi and basal crypts (x40).
k. Ileal section of *H. diminuta*-infected rat stained with PAS showing crowded red-stained goblet cells in lining of villi and basal crypts. Inflammatory cells infiltrate in core connective tissue. Notice longitudinal section of gravid segments of *H. diminuta* in lumen (x10).
Plate 2: photomicrographs of Ileal sections of non-infected and infected rats stained with toluidine blue, COX-2, iNOS, and TNF-α:
a. Ileal section of normal non-infected rat stained with toluidine blue showing lining columnar epithelium with basal located nuclei. Scattered few goblet cells in-between epithelial lining and small capillaries within loose connective tissues.
b. Ileal section of *H. diminuta*-infected rat stained with toluidine blue showing metachromatic character of mast cells granules appear purple in color (yellow arrowhead).
c. Ileal section of *H. diminuta*-infected rat stained with toluidine blue showing some of mast cells granules degranulated (black arrow).
d. Ileal section of normal non-infected rat stained with COX-2 showing undetectable expression of COX-2.
e. Ileal section of *H diminuta*-infected rat stained with COX-2 showing diffuse brown staining of COX-2 found in mucosal layer of the ileum, Staining intensive in epithelial cells at villous tips. Also staining was seen in lamina propria cells toward tips of the villi (x40) (black arrows).
f. Ileal section of normal non-infected rat stained with iNOS showing negative expression of iNOs.
g. Ileal section of *H diminuta*-infected rat stained with iNOs showing brown staining in mucosal layer of the ileum (x 100) (black arrows), While inflammatory cells (in connective tissue core of villi) show no iNOs expression (red arrows).
h. Ileal section of normal non-infected rat stained with TNF-α showing undetectable expression of TNF-α.
i. Ileal section of *H diminuta*-infected rat stained with TNF-α showing diffuse brown staining of TNF-α in mucosal layer of ileum, Staining confined to epithelial cells, Also staining seen in lamina propria cells toward villi tips (black arrows, x100).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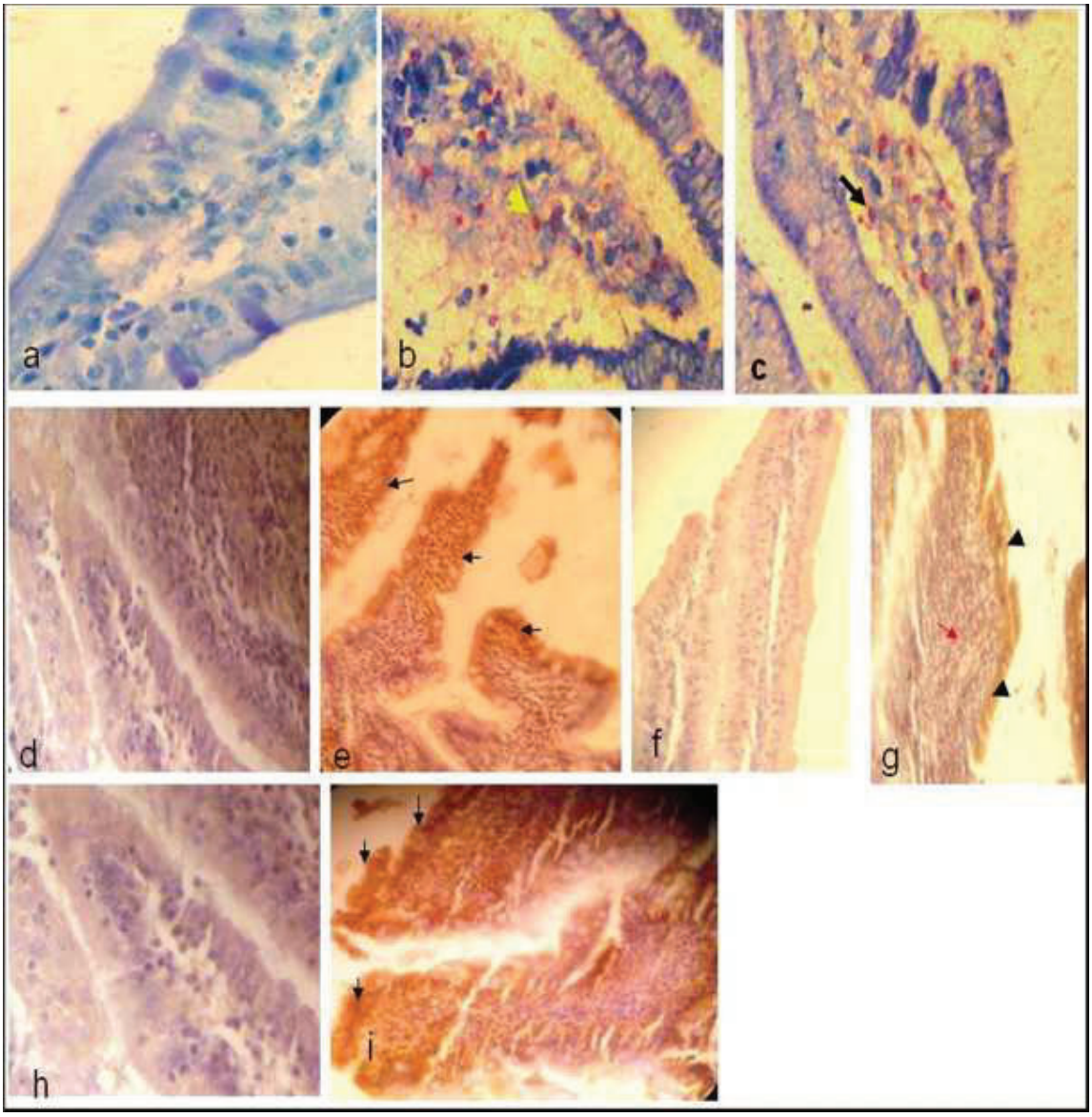
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