Claudin is an Immunohistochemical Changes Associated with Experimental Infection of White Mice with 0111 – of non – 0157 Shiga Toxin Producing Escherichia Coli

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Abstract

Immunohistochemical stain for the tissue sections prepared from experimental infection of mice with 0111 – of non 0157 of shiga toxin producing Escherichia coli .The immunohistochemical changes revealed that the shiga toxin produced by 0111 –STEC effect on epithelial tight Junction ,The IHC stain was directed to detect the claudin (the structural protein of tight Junction) revealed absence of this protein in the paracellular sites with discohesion of the epithelial cells in the intestine and endothelial cells in the brain blood vessels which was responsible for induction of diarrhea and nervous signs

Key words *: infection ; health; Escherichia coli ; Immunohistochemical*

Introduction

The intestinal epithelial barrier is established by a single layer of epithelial cells and the space between these cells is sealed by Tight Junctions (TJS) and Adherence Junctions (AJs) which regulate permeability of the intestinal barrier .TJS are complex proteins structures comprised of transmembrane proteins .A major component of TJ strands is the integral proteins ,claudin .Claudin are transmembrane proteins and there are 24 members of claudin family [1, 2] Differential expression of properties of claudin are believed to determine tissue – specific variation in electrical resistance and paracellular ionic selectivity among epithelia .Alteration in TJ and AJ protein expression and localization were reported in human and animals in IBD [2]. In previous studies increased expression of claudin -2 and decreased expression of claudin 3,4,7 and 8 were demonstrated in the colonic mucosa of human IBD [2,3] . Other previous study .E- cadherin expression was significantly decreased in the villus epithelium in duodenal mucosa samples obtained from animals with IBDC [4] From the importance of shigatoxin effect on cellular tight Junction , This study aimed to detect the effect of shigatoxin of 0111 of non 0157 STEC on both epithelial and endothelial Junction in intestine and brain by immunohistochemistry .

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Materials and Methods

Immunohistochemistry :

The principle of immunohistochemical techniques use of an antigen-antibody reaction coupled with a reaction that produces a chromogen (colored product) or attachment of a fluorescein dye to identify specific components in tissues .Most methods are " indirect " because two antibodies are utilized [5]

A- Primary antibody : an antibody is raised to a specific tissue component that acts as an antigen . The primary antibody is typically from a non – human mammalian source such as mouse or goat or rabbit .

B- Secondary antibody : an antibody is raised to the Fc portion (the constant part) of the primary antibody .It is raised via another mammalian species which will consider the primary antibody as a foreign antigen and make antibody to it .It is this secondary antibody that has a marker attached to it that will provide visibility [5]

Dilution of the primary antibody and selection of the suitable concentration :

According to manufacturer instruction stock solution of antibody (for detection of Claudin) which contain $\theta\ \mu l$ was thawed and divided into aliquots $5\mu l$ each to avoid repeated freezing – thawing of the antibody then one of the aliguots was diluted to the following dilutions (1:10 ,1:25, 1:50,1:50,1:100) , two slides was stained by each dilution and then the dilution 1:100 was choose as a working solution for staining of the slides , because it gave the better results .

Preparation of the tissue for IHC staining :

$5\mu l$ Tissue sectioning and immunohistochemistry staining were performed in the pathology laboratory / college of medicine / Missan university .Paraffin embedded tissue were cut into thickness using microtome .The sections were applied to Leica positively charged slides .

Sections were prepared for IHC staining by follow the manufacturer instructions which include the following steps .

Deparaffinization and rehydration

1. Slides were immersed in Xylene I and Xylene II successively for 10 minutes respectively .

2. Slides were immersed in anhydrous ethanol II,95% ethanol , 80% ethanol ,and 70% ethanol successively for 5 minutes respectively , and then slides were washed for twice , each time for 2 minutes by using deionized water

Antigen retrieval (optional)

3. Slides were put into the repair box , and then 0.01 M citric acid buffer (PH6.0) was added to make the tissue immersed .

4. The antigen was repaired with medium power microwave for 10 minutes (Start timing when the liquid boils) , care taken to avoid tissue drying during the process .

5. Then repair box was taken out from microwave oven , and naturally cooled down to room temperature , then slides was took and washed 3 times with PBS(pH7.4) each time for 3 times (care taken to avoid flushing of the tissue directly during washing process to avoid breaking of the tissue) .

Inactivation

6. (3%) H2O2 that was prepared well in advance was added to the slides drop by drop to block endogenous peroxidase , and incubated at the room temperature for 15 minutes (ionized water was used to dilute 30% H2O2) , then PBS (PH 7.4) was used to wash the slides for 3 times , each time for 3 minutes .

Antibody incubation

7. PBS was bloted up with absorbent paper ,and 5% normal serum (Sharing the same or similar species with secondary antibodies) was added drop by drop on the sections , then blocked at 37° C for 30 minutes

8. Liquid around the tissue on the slides was wipe dried with absorbent paper , and circle was drew oil pen around the tissue , and then the diluted primary antibodies was added drop by drop After adding primary antibodies , the slides were put into wet box to be incubated at 4° C overnight .

9. Then slide were washed with PBS for 3 times , each time for 2 minutes , slides were dried and then HRP- conjugated secondary antibodies were added , finally slides were incubated 37° C for 30 minutes .

Signal detection

10. Sections were washed with PBS for 4 times , each time for 3 minutes , and wipe dried with absorbent paper , then DAB substrate reagent that was prepared freshly was added drop by drop to each section ,and observed under a microscope .The positive signal appears brown - yellow or brown in color .The time should be well controlled avoid the color appears too deep .Sections were washed with tap water to terminate the reaction

11. Hamatoxylin coutrstaining slides were immersed in Harris hematoxylin solution for about 30 seconds to 1 minute , and then slides transferred into ethanol solution with 1% HCI after washing with water .

Dehydration and mounting

12. Firstly slides were immersed in water and washed , then slides were put into the following reagents in order to dehydrate and permeate .70% ethanol ,80% ethanol , 90% ethanol ,95% ethanol , anhydrous ethanol I, anhydrous ethanol II,xylene I and Xylene II, for 2 minutes each , finally sections were left to dry.

13. Then slides were covered with the cover glass .

The scoring system of the sections in the present study was done according to the [6]

Statistical Analysis

Statistical analysis was performed to estimate the statistical differences among the groups of the study .T test was used for this purpose by ready to use statistical design .IBM statistical package for social science (IBM SPSS Statistics version 23)

The Results

Immunohistochemistry study for Claudin of intestinal epithelial and cerebral vascular endothelial tight junctions :

The results of immunohistochemistry of claudin of tight junction for both intestinal epithelial and cerebral vascular endiothelial cells was revealed .

In the Ist inoculation there was no alteration in the Claudin expression of intestinal epithelial cells of both small and large intestine Fig-1 and Fig-2 Immunohistochemical stain for the Claudine reveal the descohesion between epithelial cells was started at 3rd day post inoculation in which there were destruction of interpithelial Claudin in both small and large intestine Fig-3 the sloughing of the epithelium along small and large intestinal parts(Fig-4) at 14th and 21st day post inoculation were also revealed by DAB stain for the Claudin in which there were marked colonic epithelail cells discohesion ,sloughing with superficial inflammatory infiltrate forming pseudomembrane indicating pseudomembranous colitis .Immunohistochemical study of the Claudine in the brain vessels revealed that there were complete intact cerebral vascular endothelial cells in the control group, while there were sites of absence of Claudine staining in the cerebral vessels with breakage of the endothelail cells Fig (Fig-5) . Table (1)

Table (1) Score of Claudin down regulation

Period	Duodenum	Colon	Brain
1st day	-	-	-
3rd day	+	+	-
7th day	+++	++	+
14th day	++++	++++	+
21st day	++++	++++	++

❖

(-) No change (+) refer to Claudin down regulation where (+) in 25 cells , (++) in 50 cells , (+++) in 75 cells , (++++) in 100 cells / field

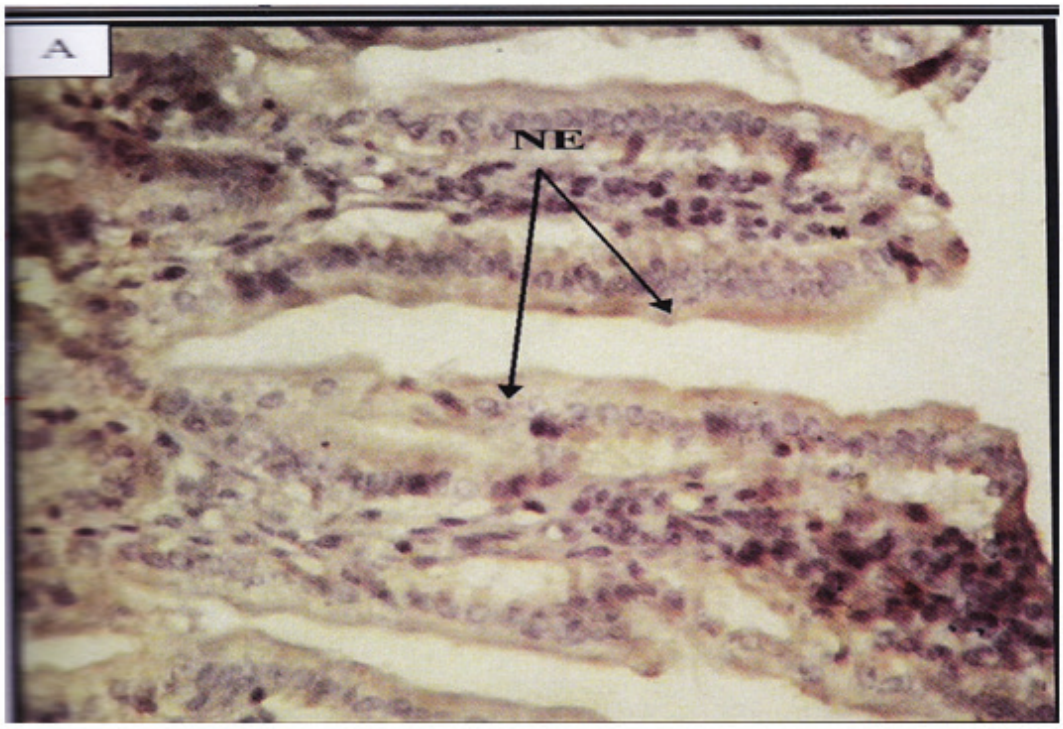
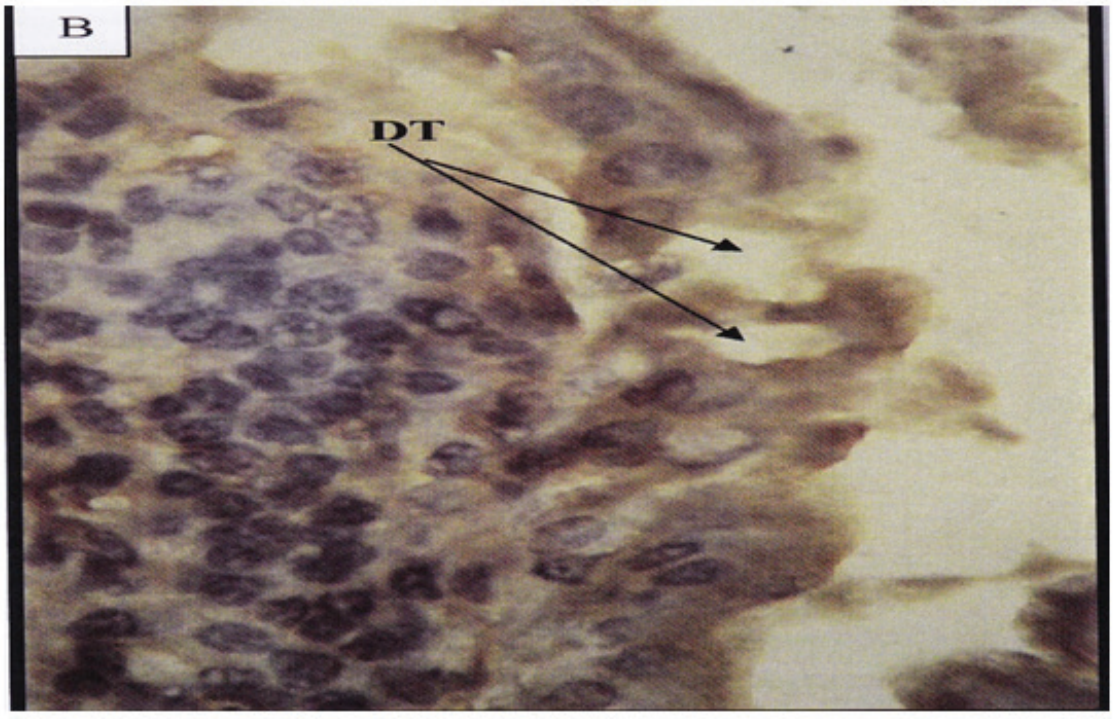
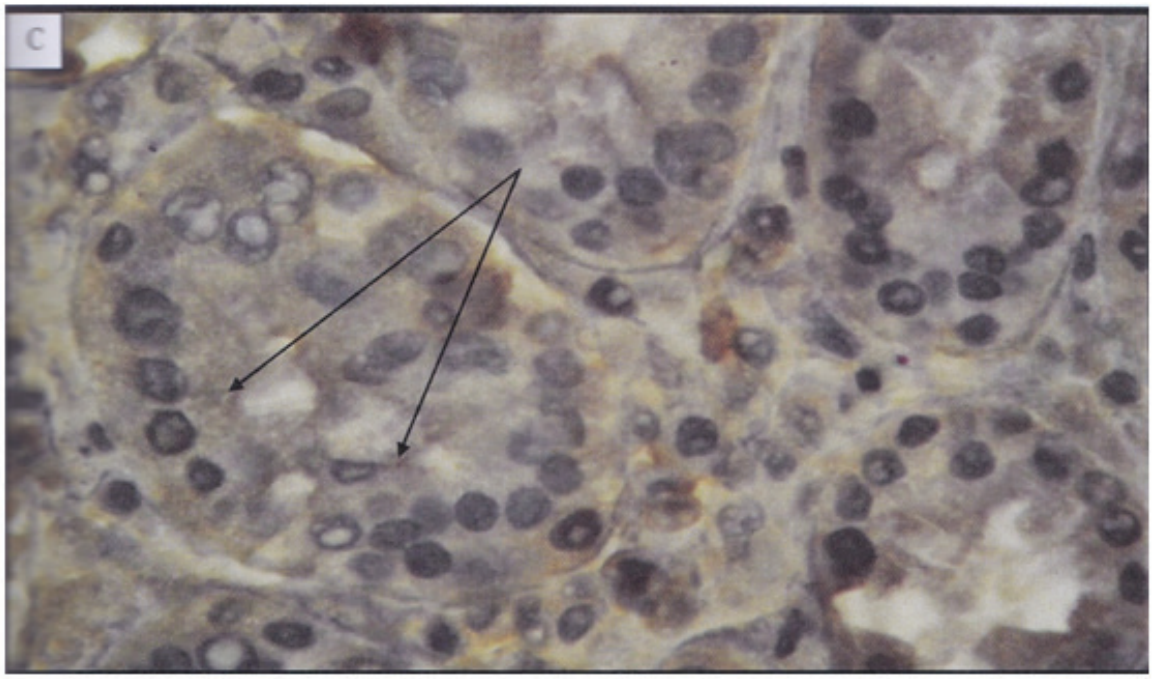


Figure (1) duodenum of the infected animals at 1st day post infection showed Normal interepithelial tight junction (NE) indicated by normal Claudin (NC) DAB stain (dark brown intercellular deposits) 500X



Figure(2) : duodenum of the infected animals at 3rd day post infection showed superficial sloughing (S) of the epithelial cells due to destruction of inter-epithelial tight junction (DT) from both lateral sides of the cells (L) and the basal site (B) DAB stain 1250 X .



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Figure(3) section of colon at 1st day post infection shows intact epithelial cells in both superficial mucosa and colonic glands revealed by intact epithelial tight junction (T) DAB 1250X

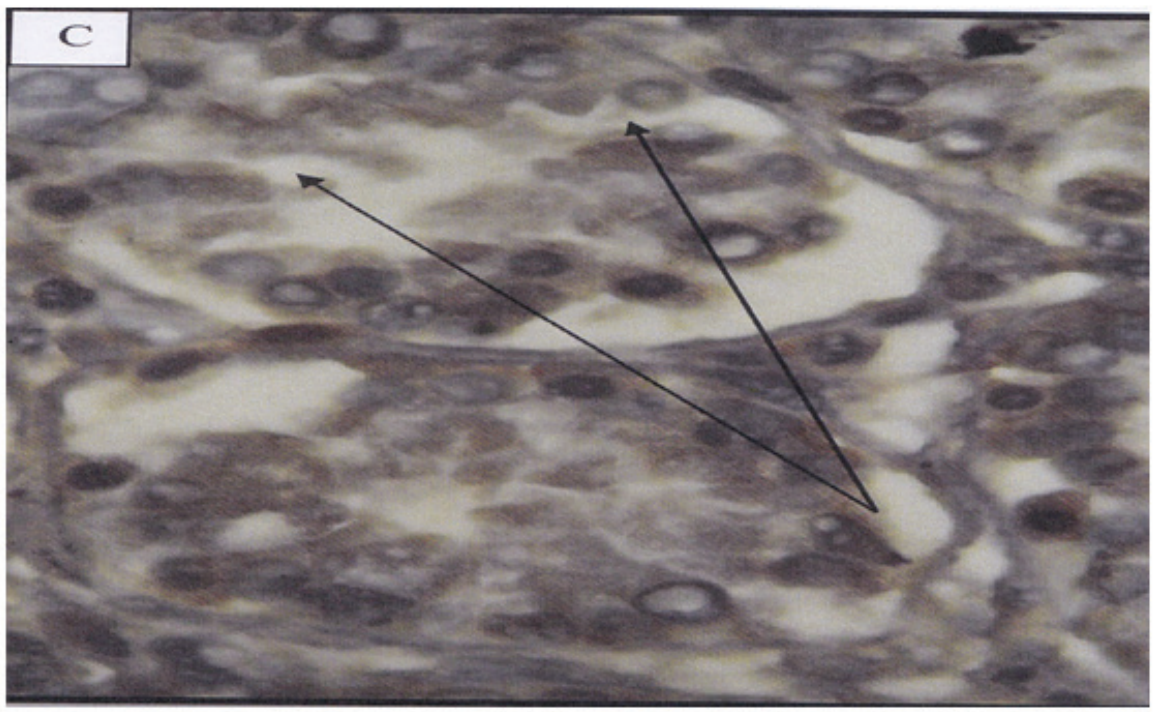

Figure (4) A section of colon at 14th day post infection shows that the down regulation of epithelial tight junction was both in the superficial epithelium and the colonic glands .DAB stain 1250X

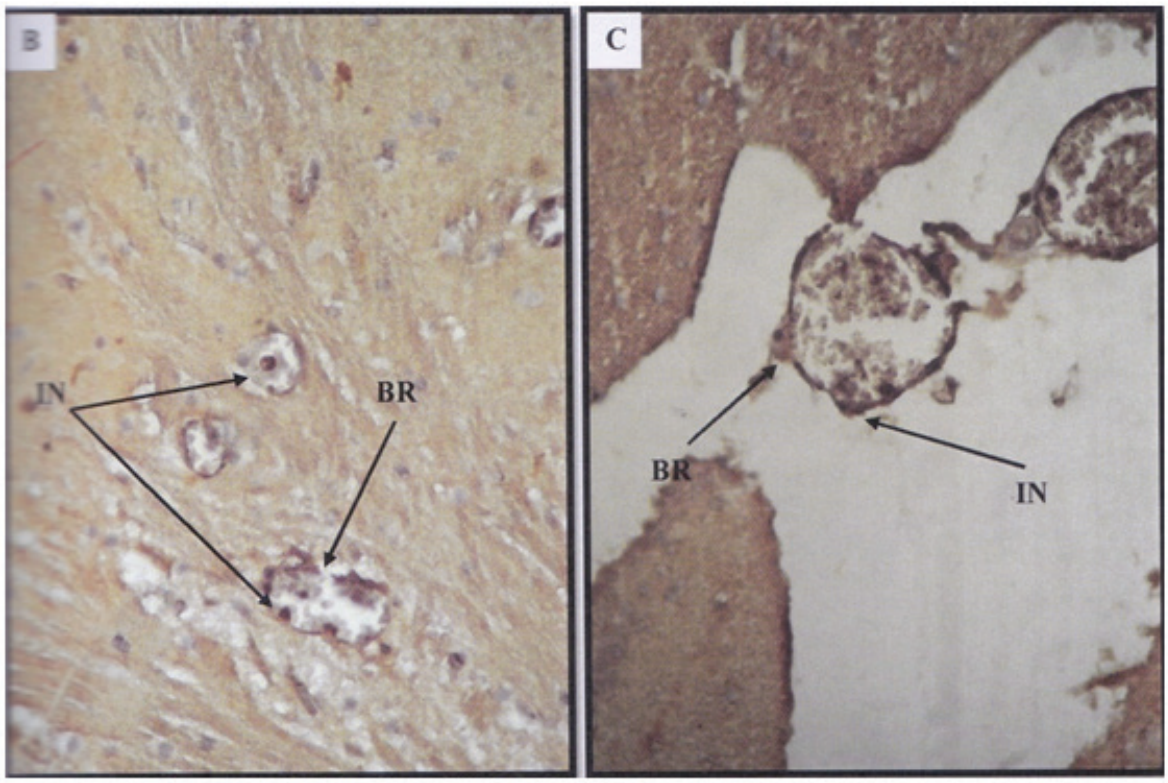

Figure (5) cerebrovascular endothelial tight junction which reveal (A) Normal endothelial cells (EN) of cerebral capillaries,sites of breakage between endothelial cells (BR) and extravasation of inflammatory cells (IN) to the brain parenchyma (B) &(C) DAB stain for Claudin of showed the 500X .

Discussion

The results of the present , study revealed that sloughing and ulceration of epithelia lining intestinal epithelial in multiple sites along small and large intestine occur from destraction of intestinal epithelial junction proteins particularly claudin , similar in previous studies of [7, 8] reported that the immune – staining of the small intestinal and clonic Claudine revealed that there were no alteration in claudin of intestinal tight junction in mice experimentally infected with STEC in the ist day post infection but the main alteration and redistrbution of claudin start in 3rd post infection and continue diminished in the intercellular area but its expersion was increased intracellularly experiment .

Results of the present study was also in consistent with [9] who reported that the immune fluorescent staining of the T48 cell culture for the tight junction proteins revealed reorganization of the claudin was observed as a complete fragmentation of the cell boundaries and focus formation also they reported that claudin -1 was lost from cell – cell contact sites , this change could be observed as the obsence of the brightly stained cell boundaries , indicating loss and / or redistrbution of claudin -1 [9,10] reported that the TJ protein alteration caused by STb may be due to actin rearrangement as a loss of organization in the perijunctional F-actin ring has been identified to be acritical event in the mechanism controlling transepithelial electrical transport (TER) and paracellular permcabillity [10-12] decreasing (TER) and increase permeability will be the cause of diarrhea and in the some time for systemic distribution of bacterial toxin with blood circulation reaching other organs in the body

Results of immunohistochemistry in the present study revealed that separation of endothelial cells of the cerebral capillaries due to down regulation of the claudin in the cerebral endothelial tight junction ,these results in line with [13] who reported that animal models treated with stx -1 and Stx -2, [14] also develop neurological

symptoms and exhibit similar neuropathology .Stx -1 , Stx-2 binding sites was immunohistochemically demonstrated in small vessels and / or the subsets of some cells in the lesions , but not in the neurous [15] .A similar findings reported by [16] whom they found neurological manifestation have been occurred in rabbits experimentally infected with stx-2 in which the signs resulted from necrotic infarction , acute neuronal damage aggravated by sever endothelial injury and microvascular thrombosis .

Conclusion

Shiga toxin cause marked down regulation of the claudin and disturb the structure and function of the tight junction in both intestinal cells and cerebral vascular endothelial cells

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

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