

Pathological and bacteriological studies on the effect of vitamin C On the small intestine of rabbit experimentally infected with E Coli O103:K-:H22 (E22)

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ABSTRACT

A well-established rabbit model infected with reference strain of enteropathogenic *E.coli* (EPEC) {*E coli* O103:K-:H22 (E22)} was used to examine whether vitamin C (VC) nutritional supplementation had an effect on the pathological changes induced in the bowel by EPEC{*E coli* O103:K-:H22 (E22)}.

In this study artificial infection model, weaned female rabbits were infected with *E coli* O103:K-:H22 (E22) and infected tissues were evaluated by light microscopy. Twenty-three rabbits were used in this study. They divided into five groups (first group of rabbits were used as control (3 rabbits), second group were treated with vitamin C (60mg/kg) in drinking water (5 rabbit), third group were infected with *E coli* {O103:K-:H22 (E22)} (5 rabbits), fourth group of rabbits infected with *E coli*{O103:K-:H22 (E22)} and treated with vitamin C (60mg/kg) in drinking water (5 rabbits), and fifth group were infected with *E coli*{O103:K-:H22 (E22)} and injected with antibiotic, ceftazidime) 5ml for each rabbit. and treated with vitamin C (60mg/kg) (5 rabbits). Rabbits were treated daily pre- experiment with an oral dose of vitamin C(60mg/kg) for ten days. The percentages of bacterial isolation of E22 from fecal samples of rabbits after inoculated with {*E coli* O103:K-:H22 (E22)} reference strain in four groups were (20,80,20 and 40%) in second, third, fourth and fifth group respectively. These results indicated that Vitamin C did not affect EPEC (REPEC) strain "E22". Whereas the pathological changes after inoculation with *E coli*_O103:K-:H22 (E22) reference strain in rabbit model were hyperplasia of ileal payer's patches, in some animal intestinal epithelial ulceration was found with lymphocytic infiltration. Liver showed hydropic degeneration, area of necrosis with hemorrhages and few lymphocytic infiltration. Vitamin C did not affect EPEC (REPEC) strain "E22" (O103:K-:H2, colonization and did not give significant protection against EPEC-induced changes and diarrhea. Although it had no effect on the EPEC-related increase of enterocyte apoptosis, it clearly contributed to an acceleration of epithelial cell proliferation in the ileal crypts. We showed that Vitamin C ameliorated somewhat the effects of EPEC on intestinal mucosal architecture. showing hyperplasia of jejunal villi, payer's patches, ileum and mucous gland. There are also leucocytic infiltration in lamina propria..

Keywords: bacterial infection; Enteropathogenic *E coli*; EPEC; *Escherichia coli*; rabbit; vitamin

INTRODUCTION

E.coli strains belonging to serovar O103:K-:H2 and to rhamnose-negative biovars are responsible of severe enteric diseases in weaned rabbits, with considerable economical involvement in industrial fattening farms. These strains could adhere in vitro to rabbit ileal villi and to HeLa cell line in a diffuse pattern by means a specific adhesin (1). They induce

attachment-effacement lesions in ileal enterocytes of infected rabbits (2), Feeding plays an important role on the immune system of the animals including rabbits. Enteric diseases frequently occur in rabbit breeding, more especially in young around weaning. These troubles cause mortality and reduced growth rates with important economic losses (3).

Heczko, et al., (4) studied that infection with enteropathogenic *E.coli* (EPEC) causes a diarrhea and in developing countries EPEC remains a major cause of pediatric mortality REPEC O103 is now the most common cause of severe diarrhea in weaned rabbits REPEC O103 shares virulence factors and mechanism with human EPEC (5). A family of animal pathogens, including enteropathogenic *E.coli* (EPEC), trigger formation of 'attaching and effacing' lesions on cultured and intestinal epithelial surfaces. EPEC is an extra cellular bacterium that causes diarrhea by binding to the surface of enterocytes in a multistep process that results in a characteristic ultra-structural apical membrane lesion (6). *E coli* and Enterococcus spp. colonize the gastrointestinal tract of many animals especially rabbits and are also commonly found in soil, plants and water (7). Infection with enteropathogenic *E coli* (EPEC) causes a diarrhea, with unclear mechanism. Due to inherent difficulties of studying EPEC diarrheal disease in natural animal models, including infection of rabbits with enteropathogenic *E coli* serogroup O103, have been used to study the disease. However, certain pathogenic mechanisms of the rabbit infection model remain unresolved, including the initial site of bacterial adherence in the intestine. Another poorly understood area concerns the role of goblet cells and mucus secretion during diarrheal disease (8).

Peeters et al., (5) showed that REPEC O103 is now the most common cause of severe diarrhea in weaned rabbits (9,10) reported that REPEC O103 (like RDEC-1) shares virulence factors and mechanisms with human EPEC. It produces the outer membrane protein intimin, and it secretes Tir, which is translocated into the host cell membrane where it functions as a receptor for intimin. The initial adherence of REPEC O103 has been shown to be due to a chromosomally encoded adhesin, named AF/R2 (Adhesive Factor /Rabbit 2) (1,11). Initial adherence of EPEC and RDEC-1 are mediated by plasmid encoded bundle forming pilus and AF/R1 (Adherence Factor /Rabbit 1), respectively. These findings imply the possibility of host and tissue specificity mediated by specific adhesions (12,13).

VonMoll et al., (14), found that REPEC O103 adherence to the domed villi within , Peyer's patches, was less than that seen with other rabbit EPEC strains. Several reports have shown that this pathogen adheres extensively to mucus isolated from both ileal and colonic goblet cells (15,16). They postulated that mucus could prevent bacterial adherence to enterocytes. However, mucus may also serve as a site for and replication colonization before bacterial adherence to the intestinal mucosa. These findings have not been examined for REPEC O103 infection. The aim of this study was to examine if Vitamin C nutritional supplementation by oral intake had an effect in bowel changes induced by EPEC(O103).

MATERIALS AND METHODS

Bacterial Strains

The Rabbit EPEC (REPEC) strain "E22" (O103:K-H2, rhamnose-negative, Adhesive Factor /Rabbit 2- positive) and the a pathogenic strain "BM21" (prototropic strain, nalidixic acid-resistant) used in this study were obtained from Animal Health Research Institute (Egypt) and were prepared kindly provided by (17). Bacteria stored in 30% (v/v) glycerol broth at -70° C were cultivated on eosin methylene blue agar (EMB) (BioMe L rieux, Marcy l'Etoile, France). For the preparation of the inoculum, *E. coli* cells were grown at 37°C over night in 10 ml of tryptone soya broth (Oxoid, Hampshire, England) without shaking, harvested by centrifugation and resuspended in 2 ml *E. coli* phosphate-buffered saline (PBS) 7.5x10. Faecal shedding of from all animals was determined daily throughout the experiment by growth of diluted faecal samples on (EMB) {eosin methylene blue agar (109 CFU/ml) (BioMe L rieux)} according to a previously published subculture scheme which permits distinction of E22 from resident *E. coli* flora (11). In particular, after selective enumeration, screening for the inoculated strains was attempted on six randomly selected isolates per sample on the basis of the following test results. For E22: slide agglutination positive, rhamnose

negative after growing on phenol red agar (Agar Parma, Italy) containing rhamnose 1%, and kanamycin- and chloramphenicol-sensitive after growing on nutrient agar (Oxoid) containing kanamycin 50 mg/ml and chloramphenicol 25 mg/ml. For BM21: rhamnose positive and nalidixic acid-resistant. Each result was given in colony-forming units (CFU)/g of faeces.

Rabbit Infection and Experimental Design

Animals and Maintenance (Diets and Feeding)

Twenty-three female New Zealand white rabbits were used in this study. Rabbits were collected from Animal House Center Faculty of Medicine Assiut University. Rabbits were classified into 5 groups and housed in cages every group individually and fed daily with antibiotic-free commercial feed, supplemented with the coccidiostatic agent Robenidine (Cycostat66 G; Alparma Belgium BVBA, Antwerp Belgium) for 10 days. Water was available. Faecal samples from all animals were confirmed to be free of coccidia and were also cultured on EMB {eosin methylene blue agar (BioMeL rieux)} to confirmed the absence of *E. coli* E22. Rabbits were weaned at the age of 28 days and inoculated with bacteria at the age of 30 days. Twenty-three rabbits were treated daily throughout the experiment with an oral dose of Vitamin C (60mg/kg) (manufactured by Arab Company for Medical products about city- Industrial Area.Cairo-Egypt-3652) commencing 10 days before the time of infection.

Experimental Design

The twenty three female New Zealand white rabbits were classified into five groups. Every group included five rabbits housed in cages every group individually except first group: act as a control (3 rabbits were Vitamin C (60mg/kg) treated) Second group treated daily throughout the experiment with an oral dose of Vitamin.C (60mg/kg) .Third group were infected intramuscular I/M with 2ml of a PBS suspension of the E22 strain (a single dose of strain E22).Fourth group were infected intramuscular I/M with 2ml of a PBS suspension of the E22 strain (a single dose of

strain E22) and treated daily throughout the experiment with an oral dose of Vit.C (60mg/kg). Fifth group It were infected intramuscular I/M with 2ml of a PBS suspension of the E22 strain (a single dose of strain E22),and treated daily throughout the experiment with an oral dose of antibiotic (ceftazidime) 5ml. and treated with vitamin C (60mg/kg). The vitamin C and *E. coli* Pathogenicity twenty three animals were weighed daily and monitored for loss of appetite, diarrhoea and dehydration Sampling From 1 to 7 days post inoculation. rabbits were humanely killed every 24 h. Priority was given to morbid and severely affected rabbits at each time point .The samples were taken from the caecum, the last 10 cm of distal ileum and the first 10 cm of proximal colon to be examined for pathological examinations.

Tissue preparation for light microscope

Specimens from the liver and intestines were collected from animals and prepared for histopathological examination. Samples of intestines were washed in PBS and all samples fixed in 10% neutral buffered formalin paraffin sections of 5µm thickness were prepared through passing the specimens in different dilutions of ethyl alcohol, then in xylon and embedding in paraffin. The sections were stained with Hematoxyline and Eosin, then examined microscopically for comparative studies, (18).

Statistical Analysis

Statistical analysis was carried out with statistical analysis system (SAS) software for windows (SAS Institute Cary, NC, USA). Percentage weight loss was analyzed cross-sectionally at given time points using an unpaired Student's t-test; a value of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical symptoms, and fecal shedding of REPEC O103

The clinical signs of infected rabbits began by shedding of bacteria in the stool between day 1 and 10 after inoculation. Group

(1)consisted of 3 rabbits(control) that shed no detectable REPEC O103 during the entire experimental period, and stool pellets did demonstrate a soft consistency. Five rabbits from each of the {*E coli* O103:K-:H22 (E22)} infected groups showed soft fecal pellets at first, progressing to diarrhea. Severe watery diarrhea, anorexia and dehydration were seen in animals from both the {*E coli* O103:K-:H22 (E22)} and {*E coli* O103:K-:H22 (E22)} + Vitamin C groups ,and rabbits became lethargic and later moribund. other hand, Five rabbits from each of the two groups {*E coli* O103:K-:H22 (E22)}+ antibiotic (ceftazidime) group and group fed on vitamin C only, transient passage of soft fecal pellets, remained clinically normal throughout the experiment. Vitamin C. treatment had no significant effect on body weight loss in -diseased animals or on weight gain in clinically healthy control animals .The growth rates of infected animals were impaired and most rabbits lost weight. Vitamin C treatment had no significant effect on body weight loss.

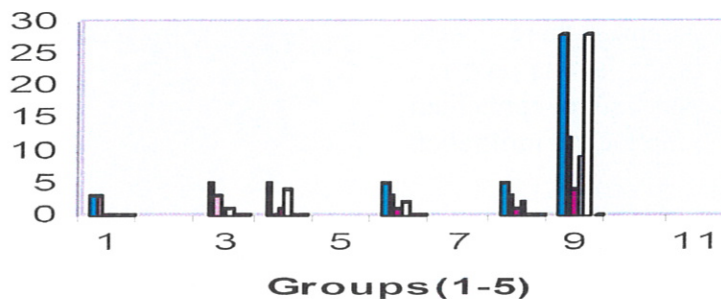
Bacterial isolation

The bacterial isolation of fecal samples after inoculated with{ *E coli* O103:K-:H22 (E22)}reference strain in four groups showed that group of rabbits treated with vitamin C included five female rabbit revealed to only one rabbit infected with *E coli* (20%),another rabbit was suspect and the remain three rabbits ,their feces free from *E coli* O103:K-:H22 (E22)}, this result accorded with these obtained by fourth group which infected intramuscular I/M with 2ml of a PBS suspension of the E22 strain and treated daily throughout the experiment with an oral dose of Vit.C (60mg/kg) whereas third group which inoculated only with *E coli* O103:K-:H22 (E22)},the strain were be isolated from four rabbits(80%)as shown in Table (1). In, the fifth group which infected I/M with 2ml of a PBS suspension of the E22 strain and treated oral dose of antibiotic (ceftazidime) 5ml) and treated with vitamin C (60mg/kg),the strain were be isolated from two rabbits(40%)as shown in Table (1).

Table 1. Results of bacteriological isolation post inoculated with {E Coli O103:K-:H22 (E22)}

Group	Number	+ve	susp	-ve	Total	Percentage (%)
1 st - control	3 female rabbit	-	-	3	3	0
2 nd Vit.C	5 female rabbit	1	1	3	5	20
3 rd E22	5 female rabbit	4	1	-	5	80
4 th E22+VC	5 female rabbit	1	1	3	5	20
5 th Ceft + E22+VitC	5 female rabbit	2	1	3	5	40
Total	28	9	4	12	28	

Fig(1) Bacteriological Isolation



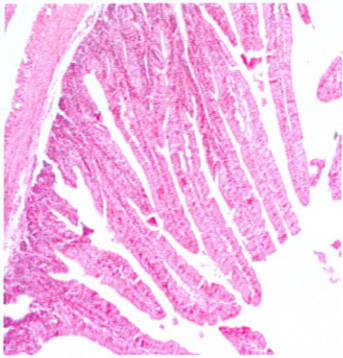
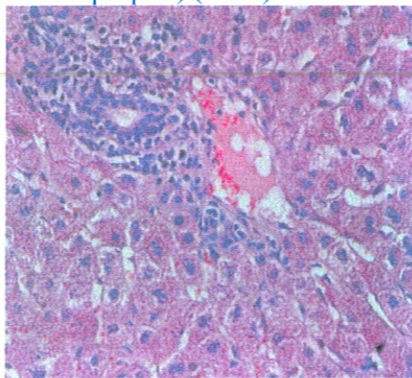
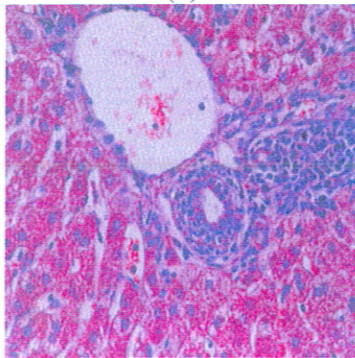


Fig 6b Jejunal villi showing hyperplasia of the epithelium with lymphocytic infiltration in lamina propria) (x250)



(a)



(b)

Fig. 7 a,b Liver showing hydropic degeneration with lymphocytic infiltration in portal area and around the bile duct (x400)

DISCUSSION AND CONCLUSION

In this study, nutritional supplementation of rabbits with VC did not give substantial protection against EPEC O103. The bacterial isolation from rabbits after inoculated with *E coli* O103:K-:H22 (E22) (REPEC O103), we observed that second group which treated with vitamin C give the same result of fourth group which treated with

vitamin C and inoculated with *E coli* O103:K-:H22 (E22) (REPEC O103), the strain were be isolated from one rabbits this results explained vitamin C did not give any protection against *E coli* O103:K-:H22 (E22) this result was agreed with that obtained by (6). The fifth group which inoculated with *E coli* O103:K-:H22 (E22) (REPEC O103) and treated with (ceftazidime) 5ml) and treated with vitamin C (60mg/kg), the strain were be isolated from two rabbits, this mean treated with vitamin c don't give best results with (ceftazidime). The third group which inoculated with *E coli* O103:K-:H22 (E22) (REPEC O103) only, the strain were isolated from four animals, that examined this serotype of *E coli* were specific for rabbits infection, this result was agreed with that obtained by other investigators (6,8). Nutritional supplementation of rabbits With VC did not give protection against EPEC O103 induced pathological changes and diarrhea; however, it ameliorated the effect of EPEC O103 on intestinal mucosal architecture through a mechanism related to the acceleration of intestinal crypt epithelial cell proliferation. *E coli* O103:K-:H22 rabbit infection and examined its histopathological features. We observed that REPEC O103 expressed fimbriae-like organelles on its surface only during early stages (24h) of infection. Villi of ileal peyer patches were the site of initial bacterial adherence and probably replication. We found that mucus secretion was increased in all examined intestinal segments in response to REPEC O103 infection. Mucus secretion resulted in intraluminal binding of the inoculated pathogen during early stages of infection, predominantly in the proximal colon. Three days after infection, lesions were observed in ileal PP, in the ileum, and the proximal colon. We demonstrated that pedestals showed striking variations in their appearance and length. Therefore, it could be speculated that excessive in intestinal contents would facilitate *E coli* survival and colonization. However, the EPEC colonization of rabbits in this study, as assessed by counts of the organism in faces, was not affected by nutritional supplementation. In addition, since bacterial

adherence is essential for EPEC to induce disease.

Uberos et al., (19) and Drumm et al. (20) showed that the decrease of surface hydrophobic of rabbit *E. coli* reduced the adherence of the bacterium.

Group of rabbits infected with *E. coli*

Postmortum examination revealed that livers were congested. The large intestine was distended with gases and small intestinal contents were also soft and foal smelling, these findings were also recorded by (6,21,22).

The small intestine represented in jejunum and ileum showed hyperplasia epithelial lining the villa of both. There were lymphocyte infiltration in the lamia propria. Hyperplasia in mucous glands of jejunum and in Peyer's patches and plenty of mucous secretion. Domed villi of ileal Peyer's patches were the site of initial bacterial and hercuse and probably replication this cleared why mucosal secretion increased in examined intestinal segments in response to REFE *E. coli* O103 infection. (8) recorded marked increase in mucous secretion which associated with separation of intercellular junctions between PP goblet cells and columnar epithelial cells. (6). agree with our results which were leucocytic infiltration and intestinal epithelial ulceration. Also recorded an increase in mucosal lamia propria cellularity mainly plasma cells and few heterophils. Livers of infected rabbits showed areas of hydropic degeneration of hepatocytes, focal areas of necrosis, multifocal areas of haemorrhages and leucocytic infiltration. Thrombus in central vein and congestion of sinusoids. In our opinion these lesions happened due to the route of injection, this lead to reach of microorganisms to Liver through portal circulations.

Group of rabbits infected with *E. coli* and treated with vitamin C

Intestinal villi showed better than that infected rabbits, the villi of jejunum showed hyperplasia with lymphocytic infiltration and the mucous glands were apparently normal (23). revealed that vitamin C supplementation

has been shown to increase lymphocyte proliferation in mice, while observed that there was no significant effect in immune function, although lymphocyte infiltration ended to be highest in vitamin C supplementation group. In our study vitamin C supplementation group appeared like normal. Livers of this group showed the same lesions except hydropic degeneration decreased Vitamin C improved slightly, the resistance of the body against *E. coli* infection. The effect of high doses of vitamin C supplementation and immune responsiveness in human are controversial. It has been suggested that this may be due to selective effects only in populations that are deficient or marginal in their vitamin C status, (23,24).

Group of rabbits infected with *E. coli* and treated with vitamin C in drinking water and injected with antibiotic

Intestinal lesions were hyperplasia in Peyer's patches of ileum and lymphocytic infiltration in the lamina propria. Liver showed areas of necrosis and hydropic degeneration in some areas, in addition to cellular infiltration in portal vein and around the bile duct., Congestion in central vein and hepatic sinusoids were also seen. These lesions were similar to those observation rabbits treated with vitamin C. Vitamin C increases white blood cells production and it is important to immune system balance. Low vitamin C level in the body increase the risk of infection (25). This explained why lesions in livers appeared even after treatment with antibiotic.

From the previous it is cleared that Vitamin C did not give substantial protection against EPEC O103:K:H22 (E22)-induced pathological changes and diarrhea and the presence of pathological lesions even after treatment with antibiotic.

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الملخص العربي

دراسات باثولوجية و بكتريولوجية على تأثير فيتامين ج علي الأمعاء الدقيقة للأرانب المعديّة صناعياً بالميكروب القولوني (E22) O103:K-:H22

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أجريت هذه الدراسة علي أرانب معملية موجودة في بيت الحيوان بكلية الطب جامعة أسيوط واستخدمت ثلاثة وعشرين أرنب في هذه الدراسة. وقسموا إلى خمس مجموعات واستخدمت المجموعة الأولى من الأرانب ضابطة للتجربة وتشمل ثلاثة أرانب والمجموعات الأربعة الأخرى تشمل خمسة أرانب في كل مجموعة تم تغذية الأرانب كلهم علي (فيتامين س ٦٠ mg / كجم) لمدة عشرة أيام قبل بدء التجربة. تم حقن المجموعة الثالثة والرابعة والخامسة بالسلالة المرجعية الممرضة للأمعاء القولونية الميكروب القولوني (E22) O103: K-: H22

وتمت معالجة المجموعة الثانية والرابعة والخامسة بفيتامين س في مياه الشرب أما المجموعة الخامسة حقنت عن طريق العضل إي كوالي (E22) O103: K-: H22 وتمت معالجة المجموعة بالمضاد الحيوي (السيفتازيديم) ٥ ملي مع فيتامين س (٦٠ mg / كجم) للأرنب واحد وجرى تقييم الأنسجة المصابة بواسطة المجهر الضوئي بعد ذبح الأرانب وأظهرت النتائج البكتريولوجية أن عترات السلالة المرجعية الممرضة للأمعاء القولونية الميكروب القولوني (E22) O103: K-: H22 تم عزلها من براز الحيوانات بنسبة (١) ٢٠، (٤) ٨٠، (١) ٢٠، (٢) ٢٠% من المجموعات الثانية والثالثة والرابعة والخامسة علي التوالي. وهذه النتيجة أوضحت تشابه بين المجموعتين الثانية (عولجت فيتامين س ٦٠ mg / كجم) والرابعة (عولجت فيتامين س ٦٠ mg / كجم + السلالة المرجعية الممرضة للأمعاء القولونية الميكروب القولوني (E22) O103: K-: H22) وعلي ذلك فان فيتامين س لم يؤثر علي السلالة المرجعية الممرضة للأمعاء القولونية الميكروب القولوني (E22) O103: K-: H22. أما بالنسبة إلى التغيرات الباثولوجية أثار طفيفة تتمثل في التأثير علي العصارة المخاطية المعوية. واطهر أيضا تضخم في اللفانفي ، والبقع دافع، والدقاق والغدة المخاطية و نزيف في الكبد وبعض الخلايا اللمفاوية واحتقان في الوريد المركزي وهذه النتائج تتوافق مع الفحص البكتري.