ILEAL MUCOSAL EFFECTS OF VITAMIN E IN EXPERIMENTALLY INFECTED RABBITS WITH ENTEROPATHOGENIC *ESCHERICHIA COLI* O103

TSALIE E.[1], KALDRYMIDOY E.[1], KOUZI K.[2], POUTAHIDIS TH. [1], XYLOURI E.[3], ILIADIS N.[4], SARRIS K.[4], ABAS Z.[5]

[1]Lab. of Pathology, Veterinary Medicine, Aristotle University of Thessaloniki, Greece
[2]Lab. of Histology-Embryology, Medicine school,
Aristotle University of Thessaloniki, Greece
[3]Dep. of Anatomy & Physiology of Farm Animals,
Agricultural University of Athens, Greece
[4]Lab. or Microbiology, Veterinary Medicine,
Aristotle University of Thessaloniki, Greece
[5] Dep. Of Agricultural Development, Democritos Universtity of Thrace, Greece

ABSTACT

Vitamin E effect on intestinal mucosal lesions caused by *E. coli* infection, was examined. Sixty rabbits were challenged with the highly pathogenic strain E22 and additionally thirty of them daily administered 60 mg/kg b.w. of Vitamin E. The lesions were evaluated histologically and computer-aided morphometry was used for the following measurements: Total mucosal thickness, Villous height, Crypt depth, Villous height/crypt depth ratio, Mononuclear and Polymorphonuclear cells at the submucosa and mucosa (tip of the villous and base of the crypt). The morphometric analysis showed significant differences, indicating that vitamin E may have some beneficial effects against REPEC E22 intestinal infection.

Key words: E.coli, enteritis, vitamin E.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is an extracellular bacterial pathogen that infects the human (HECZKO U. *et al*, 2000 and VALLANCE B. and FINLAY B., 2000), and animal (DE RYCKE J. *et al*, 1997 and BOULLIER S. *et al*, 2003) intestinal epithelium inducing diarrhea. EPEC strains belonging to serotype O103:K-:H2 and to rhamnose negative biotypes are responsible for severe diarrheas in weaned rabbits (FIEDERLING F. *et al*, 1997), with considerable economical involvement in industrial fattening farms (BLANCO *et al*, 1994). Vitamin E, a fat-soluble vitamin, is a minor component present among the lipids constituents of cell membranes and lipoproteins. The deficiency syndrome associated with lack of vitamin E in the diet of animals has been well known for many years (WANG X. and QUINN P., 1999). The beneficial effects of vitamin E dietary supplementation on the immune response of animals against a variety of infectious agents have been reported. However, the influence of vitamin E on the resistance of animals to disease is questionable (FINCH J. and TURNER R., 1996).

We sought to study the pathology induced by the E22 REPEC strain in experimentally infected rabbits and test whether dietary supplementation with vitamin E had any influence at the first stages of disease progression.

MATERIALS AND METHODS

Bacterial strain

The bacterial strains used in this investigation are: REPEC strain E22 (O103:K-:H2, rhamnose negative, AF/R2 positive strain), (CANGUILEM and MILON, 1989) and apathogenic strain BM21 (Prototropic strain, nalidixic acid resistant). Both strains were kindly provided by Prof. A. Milon, *Institut National de la Recherche Agronomique, Ecole Nationale Veterinaire de Toulouse.* Bacteria were stored in 30% (vol/vol) glycerol broth at -70^{o}C. For the preparation of the inoculum, they were grown in 10 ml of Tryptose soya broth without shaking at 37^{o}C.

Experimental infection of rabbits

Ninety (90) thirty-day old New Zealand weaned rabbits were used for experimental infection. They were housed individually and fed daily with antibiotic-free commercial feed, supplemented with a coccidiostatic agent (Robenidine). Water was ab libitum.
Rabbits were divided into four groups:*Group I*: thirty (30) rabbits infected with 2x10^{6} CFU of REPEC strain E22. *Group II*: thirty (30) rabbits infected with 2x10^{6} CFU of REPEC strain E22 and daily administered 60 mg/kg b.w. of Vitamin E *p.o.*, throughout the experiment starting 10 days prior to infection. *Group III*: fifteen (15) rabbits orally inoculated with 10^{9} CFU of the apathogenic strain BM21. *Group IV*: fifteen (15) rabbits orally inoculated with 10^{9} CFU of the apathogenic strain BM21 and daily administered 60 mg/kg b.w. of Vitamin E *p.o.*, throughout the experiment starting 10 days prior to infection.
For each group of rabbits, fecal shedding of E.coli was determined daily by dilution of fecal samples on Eosin Methylene Blue agar.
Animals were monitored daily for loss of appetite, diarrhea and mortality. Rabbits were sacrificed 24h, 48h, 3 days, 4 days, 5 days, 6 days, 7 days after infection. The most affected animals were sacrificed per day, and in lack of clinical signs, the sacrifice was carried out randomly.

Collection and processing of Material

Tissue from the distal ileum (10cm above the sacculus rotundus) was removed, while the animal was under anesthesia with ketamine and xylazine, and was fixed in 10% neutral formalin solution. About fifteen transverse sections of each ileum segment were processed by routine methods and embedded in four paraffin wax samples.
From each sample serial tissue sections were cutted at 4μm and stained with haematoxylin-eosin (H-E), and special stains (Giemsa, Gram, PAS). Immunohistochemistry method was applied on ileum sections by using as primary antibody, a serum prepared in the laboratory *Microbiologie Moleculaire, Institut National de la Recherche Agronomique, Ecole Nationale Veterinaire de Toulouse*, by ID injection of a sheep with formalin-fixed whole E22 (BOULLIER S. *et al*, 2003). This serum was kindly provided by Prof. A. Milon. After overnight incubation at 4 ^{o}C the slides were

washed and incubated with biotinylated donkey anti-sheep antibody (Abcam). After 30 min the slides were washed and incubated for 30 min with the complex extravidin-peroxidase (DAKO). For color development, 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) with substrate H_2O_2 was applied to tissue sections.

Morphometric analysis

All sections were examined by Axionplus microscope and images used for morphometric analysis were captured using a Sony Exawave digital videocamera. Subjective analysis of the histological lesions and the distribution of inflammatory cells in the lamina propria and submucosa were initially made with X10, X40 objectives. Images were transferred to an IBM-compatible computer by means of Image-pro Plus software (version 4,1).

For the morphometric analysis, the middle of the mucosal folds of well-orientated sections, cut perpendicularly from villous enterocytes to the muscularis mucosa, were measured.

The measurements carried out are:

- Total mucosal thickness (TMT) was measured from the tip of the villous to the lower end of the muscularis mucosa.
- Villous height (VH) was estimated by measuring ten (10) villi per section, from the villous tip to the villous-crypt junction.
- Crypt depth (CD) was measured from the villous-crypt junction to the lower limit of crypt.
- Villous height/ crypt depth ratio.
- The counts of mononuclear and polymorphonuclear cells were determined for standardized areas at the submucosa (area 1), base of the crypts (area 2), and tip of the villous (area 3). Five appropriate areas were chosen randomly and the results were expressed per 10.000 μm².The above areas were delineated on the computer screen (excluding, for example, the epithelium, lymphatics and larger blood vessels).

Statistical analysis

Statistical tests were performed using the SPSS statistical software package and the procedures nonparametric test K independent samples for the cell count and GLM for the measurements. The probability level of $p<0.05$ was set for statistical significance.

RESULTS AND DISCUSSION

Clinical signs and gross lesions

All rabbits of groups I and II had reduced feed consumption, were depressed but animals of group II were more vital in comparison with those of group I. Twenty two animals of group I and twenty animals of group II showed diarrhea from two to seven day after inoculation. Feces were mucoid in one animal from group I. No clinical signs were observed in group III and group IV.

Rabbits sacrificed 24h post infection (p.i.) showed moderate intestinal hyperhaemia and swelling. From 2nd day p.i. and onwards there was progressively increased catarrhal to mucus exudation on small intestinal and caecum mucosal surface. The sacculus rotundus and the mesenteric lymph nodes were edematous. Occasional mesenteric

lymph nodes showed diffuse haemorrhages. Peyer's patches were also markedly enlarged. The caecal serosa showed petechiae and extensive haemorrhages. The contents of the caecum were foul-smelling, watery and brown.

Group III and IV revealed no gross lesions.

Microscopic lesions of the ileum

Adherent bacteria were found (group I and II) on the enterocytes of the tips of ileal villi at 24 h p.i. From 24 h p.i. onwards there was edema in the intestinal lamina propria and the submucosa.

Microvilli of □olonized epithelial cells by REPEC E22 were effaced. Affected enterocytes were flattened, round, and shrunken. Also, enterocytes were found in different stages of exfoliation bulging into the lumen. Shortening and fusion of the villous were observed and polymorphonuclear as well as mononuclear cells infiltrated the mucosa and the submucosa of the ileum. Heavy neutrophil infiltrations correlated with areas of heavy bacterial colonization. The inflammatory reaction appeared to be more prominent in the intestine of rabbits which had received vitamin E and the lesion's intensity varied between group I and II at different days p.i.

The avirulent strain BM21 (group III and IV) did not induce any histopathological changes and immunohistochemically no bacteria were detected.

Morphometric and statistic analysis

The values (mean) of total mucosal thickness, villous height, crypt depth and villous height/crypt depth ratio for groups I and II are shown in **Table 1**.

Group II revealed the highest values for total mucosal thickness, villous height and villous height/crypt depth ratio in comparison with group I. Total mucosal thickness differ significantly at 2nd and 6th day p.i. while villous height differ significantly at 4th and 6th day p.i. As much as concerning villous height/crypt depth ratio significant difference was found at 1st, 2nd, 4th, 5th, and 6th day p.i.

Table 1. Comparisons of Total mucosal thickness, Villous height, Crypt depth and Villous height/Crypt depth ratio in groups I and II.

	Means							
	Group I				Group II			
Day p. i	TMT	VH	CD	VH/CD ratio	TMT	VH	CD	VH/CD ratio
1	691	436	180	2,36*	755	582	141	4,2*
2	592*	391	198*	2,3*	723*	537	144*	3,43*
3	511	337	194	1,8	682	499	177	2,76
4	480	265*	194*	1,43*	531	374*	136*	2,66*
5	526	292	232*	1,27*	502	324	131*	2,57*
6	427*	217*	196	1,23*	713*	473*	173	2,83*
7	644	387	244	1,62	750	497	186	2,79

*= significant, $p<0,05$

An overall view of the crypt depth measurements of group I showed totally higher values than those of group II and they were significantly different at 2nd, 4th and 5th day p.i .
The accumulations of polymorphonuclear and mononuclear cells present in the submucosa were higher in group II and showed significant difference at days 4th, 5th p.i., and days 3rd, 7th p.i. respectively (**Table 2**).

Table 2. Number of cell types in the submucosa (area 1)

	Means of cells/10.000 µm^2			
	Group I		Group II	
Day p.i	Polymorphonuclear cells	Mononuclear cells	Polymorphonuclear cells	Mononuclear cells
1	7,9	5,7	41,4	19
2	6,6	4,9	43,4	10,8
3	10,4	2,8*	36,2	6,9*
4	2,5*	6,2	12,3*	14,6
5	3,2*	4,1	13,3*	14,9
6	5,1	4,8	24,7	11,9
7	7	3,7*	17	17*

*= significant, p<0,05

Lamina propria polymorphonuclear and mononuclear cells counts were larger in group II. The number of polymorphonuclear cells differ significantly in area 2 (base of crypts) at 3th and 5th day p.i., and in area 3 (tip of villous) at 3rd, 4th, 5th and 7th day p.i.
Also, there was a significant difference in number of mononuclear cells at 5th day p.i (area 3) and at 7th day p.i (area 2) (**Table 3**).

Table 3. Number of cell types in the lamina propria (area 2 and 3)

		Means of cells/10.000 µm^2			
		Group I		Group II	
Day p.i	Area	Polymorphonuclear cells	Mononuclear cells	Polymorphonuclear cells	Mononuclear cells
1	2	4	8	16	13,9
1	3	17	5,7	28,2	21
2	2	3,2	2,9	24,1	12,3
2	3	7,4	3,5	29,9	6,3
3	2	3,2*	6	19,2*	16,3
3	3	15,7*	2,6	49,8*	32,8
4	2	2,6	4,9	14,5	7,7
4	3	1,1*	5,5	14,8*	8,5
5	2	2,1*	4,9	22,8*	7,8
5	3	4,1*	3,2*	12,5*	6,4*
6	2	2,5	4,5	10,6	12,6
6	3	6,6	5,4	25,4	18,2
7	2	2,4	4*	9,2	18*
7	3	4*	16,8	14,8*	10,8

*= significant, p<0,05

Total mucosal thickness, Villous height, Villous height/Crypt depth ratio (group III and IV) were greater than those of group I and II and there was significant difference at all days p.i (Table 4). Also, significant differences of Crypt depth measurements among groups III and IV and groups I and II were observed. The accumulations of polymorphonuclear and mononuclear cells at different areas for groups III and IV was much fewer than those of groups I and II.

Table 4. Data of Total Mucosal thickness(TMT), Villous height(VH), Crypt depth(CD), Villous height(VH)/crypt depth(CD) ratio, number of polymorphonuclear (PMN) and mononuclear(MON) cells

	Means									
	TMT	VH	CD	VH/CD ratio	PMN (area1)	PMN (area2)	PMN (area3)	MON (area1)	MON (area2)	MON (area3)
Group III	901±15	687±6	165±3	4,2±0,04	2,6±2,8	4±0,3	4,2±0,4	0,9±0,4	0,8±1,1	2,5±0,3
Group IV	857±11	646±5	146±2	4,5±0,06	9,8±1,6	3,2±0,6	11,3±0,3	11,5±0,4	3,7±0,3	4,7±0,2

CONCLUSIONS

- There are not differences in the morphometric analysis between group III and IV.
- The same measurements have been done in the groups I and II, and significant differences have been revealed.
- Also, the values of the examined morphometric parametrs of groups III and IV showed significant differences in comparison with those of groups I and II.
- Group I revealed the lowest values for Total mucosal thickness, Villous height and Villous height/Crypt depth ratio.
- Group II showed decrease of Total mucosal thickness, Villous height and Villous height/Crypt depth ratio, which is significantly lower than this of group I.
- The accumulations of mononuclear and polymorphonuclear cells were higher in group II in comparison with group I.
- Morphometric analysis showed that vitamin E administration improves the morphology of intestinal mucosa.

REFERENCES

BLANCO J. E., BLANCO M., BLANCO J., RIOJA L. AND DUCHA J., 1994. Serotypes, toxins and antibiotic resistance of *Escherichia coli* strains isolated from diarrhoeic and healthy rabbits in Spain. Vet. Microbiol., **vol 38**: 193-201

BOULLIER S., NOGAYEREDE J., MARCHES O., TASCA C., BOURY M.,OSWALD E., DE RYCKE J., MILON A., 2003. Genetically engineered enteropathogenic *Escherichia coli* strain elicits a specific immune response and protects against a virulent challenge. Microbes and Infection, **vol 5**:857-867.

CANGUILEM R., MILON A., 1989. Biotypes and O serogroups of *Escherichia coli* involved in intestinal infections of weaned rabbits: clues to diagnosis of pathogenic strains. J. Clin. Microbiol, **vol 27**:743-747.

DE RYCKE J., COMTET C., CHALARENG C., BOURY M., TASCA C., MILON A., 1997. Enteropathogenic *Eshcerichia coli* O103 from rabbit elicits actin stress fibers and focal adhesions in HeLa epithelial cells, cytopathic effects that are linked to an analog of the locus of enterocyte effacement. Infection and Immunity,**vol 65**:2555-2563.

FIEDERLING F., MICHELE B., PETIT C. AND MILON A., 1997. Adhesive Factor/Rabbit 2, a new fimbrial adhesin and a virulence factor from *Esherichia coli* O103, a serogroup Enteropathogenic for rabbits. Infection and Immunity, **Feb**:847-851.

FINCH J. AND TURNER R., 1996. Effects of selenium and vitamin E on the influence responses of domestic animals. Research in Veterinary Science, **vol 6**:7-106.

HECZKO U., ABE A., FINLAY B., 2000. In vivo interactions of rabbit enteropathogenic *Escherichia coli* O103 with its host: an electron microscopic and histopathologic study. Microbes and Infection, **vol 2**:5-16.

VALLANCE B. AND FINLAY B., 2000. Exploitation of host cells by enteropathogenic *Escherichia coli*. PNAS, **vol 97**:8799-8806.

WANG X. AND QUINN P., 1999. Vitamin E and its function in membranes. Progress in Lipid Research, **vol 38**:309-336.