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Original article

The effect of L-Arginine in experimental colitis

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SUMMARY

Inflammatory bowel diseases (IBD) include a variety of intestinal disorders, widely distributed throughout the human population. In recent years Nitric Oxide (NO) has been shown to play a role in the pathophysiology of IBD both in patients and in animal models. The aim of this study was to examine the effect of L-arginine on the inflammatory response ischemic colitis in an experimental rat model. The animals used in the experiment were male Wistar rats, weighing between 240g and 310g For the induction of ischemic colitis, a solution of TNBS was instilled into the rectum. Blood samples were taken and the mice were subjected to total colectomy. To assess the severity of colitis, haematological, biochemical and histopathological criteria were used. For the statistical analysis of the results obtained we used Student's t-test, or Welch test and Mann-Whitney test. Statistical significance between the two groups was proved in complete blood count parameters: WBC, Hgb, MCV, MCH and in biochemical parameters: Gluc., SGOT, SGPT, CPK and especially MDA (Malonaldehyde), which is a critical parameter in experimental colitis. Histological examination of the control group (No 1) revealed epithelial necrosis, acute infiltration of the Lamina propria consisting of polymorphonuclear leukocytes, lymphocytes, eosinophiles and macrophages, submucosal edema and development of granulation and fibrous tissue of the muscular layer. On the other hand in the L-Arginine group (No 2), the alterations were less and not widespread in all areas as in the control group. Some samples were almost intact and others revealed atrophy of mucosal surface of the gastrointestinal villae and minimal inflammation of the lamina propria. We concluded that L-Arginine and nitric oxide inhibitors may have a role in the treatment of inflammatory bowel diseases in humans.

Key words: inflammatory bowel disease, IBD, L-Arginine, ischemic colitis, nitric oxide

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INTRODUCTION

Inflammatory bowel diseases (IBD), (Crohn's disease, Ulcerative colitis and undetermined colitides) comprise a group of multifactorial intestinal disorders of unknown etiology and of high incidence, widely distributed throughout the human population.[1] The IBD is characterized by cycles of clinical exacerbation and remission, with periods of improvement followed by relapse. Factors such as microorganic infection, gastroenteritis, upper respiratory tract infection, emotional reactions[2] and seasonal changes[3] have all been implicated in relapses. Histological features of IBD include chronic inflammation with tissue infiltration by macrophages, lymphocytes, plasma cells and an unregulated B-cell response.

Over the last decade the pathophysiological role of Nitric Oxide (NO) has been well documented, both in patients with inflammatory bowel disease[4] and in animal models with experimental colitis.[5] The usual model of experimental colitis is the intrarectal administration of trinitrobenzine-sulphonic acid (TNBS) into rat colon. This agent induces focal inflammation and alterations in the colon similar to those found in chronic inflammatory bowel diseases in humans.[6]

L-Arginine is a semi-essential nitrogen rich amino acid, which becomes essential during periods of growth, illness or stress.[7] Supplemental administration of arginine in experimental models improves wound healing, has a trophic effect on the thymus gland, promotes T-lymphocyte proliferation and function and increases survival

following sepsis, burn injury, trauma and malignancy[8-13]. L-Arginine is the biosynthetic precursor of nitric oxide[14]. This molecule is highly lipophilic and has physiological functions as a vasodilator and neurotransmitter. Nitric Oxide is produced in large quantities by activated neutrophils, macrophages and T-lymphocytes[14-18] and may be the effector molecule by which arginine exerts many of its immune effects. There is also D-Arginine which is the inactive form of arginine.

The aim of this study was to investigate the effect of L-arginine on the inflammatory response in experimental colitis.

MATERIALS AND METHODS

Animals

All animals in these studies were male Wistar rats (from institute Pasteur-Athens), weighting between 240g – 310g.

Housing and Food

Seven days before induction of colitis, the animals were housed in groups (maximum 4 per cage – minimum 2 per cage) in wire mesh cages in controlled conditions (temperature 22C and 12hr.dark/light cycle). They were divided into two big groups of eight mice each: the control group and the L-arginine group. We used the L form of arginine instead of D form because L-arginine is the active form. Both groups had free access to tap water and standard laboratory food. Plain food and water were used for the control group and the L-arginine group were given 2mg/100gr of body weight L-arginine diluted in 5ml water to drink with a plastic syringe.

Induction of Colitis

Animals were fasted for 20 hours before induction of colitis and anesthetized by intramuscular injection of an anesthetic solution (0,3ml Ketamine/0,3ml Midazolame).[18-20] A 18G polypropylene catheter coated with paraffin oil was inserted into the rectum until the tip was 6-8 cm. above the anus, approximately at the splenic flexure. A solution of TNBS (trinitrobenzine-sulphonic-acid) (SIGMA pharmaceuticals-P2297) (10ml in 4,15ml ethanol 50%) was instilled with an insuline syringe in each animal (0,85ml in 50% ethanol). During the instillation the catheter was slowly withdrawn to allow distribution of the TNBS over an area of the colon. The syringe was then disconnected from the catheter and any remaining instillate allowed to drain into the colon under gravity. The catheter was finally cleared of induction agent by injection of 0,4ml air equivalent to the dead space within the catheter. Until recovery from the anesthesia, animals were maintained in a 30* supine Trendelenberg position to prevent immediate trans anal leakage of the induction agent. On recovery from anesthesia, tap water (with L-arginine in the 2nd group) and standard laboratory food were freely available.

Blood Sampling and Colectomy

Under general anesthesia on the 7th post induction day, 6-7ml of blood was taken from the inferior vena cava for haematological and biochemical tests. For haematological tests we measured: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, LYMPH, PDW, MPV and for biochemical tests we measured: Glucose, SGOT, SGPT, AF, γ-GT, Albumine, CPK and Malonaldehyde (MDA).

On the 7th day all mice were sacrificed and total colectomy was performed after abdominal incision, and all colon specimens were kept in tubes with a solution of 10% phormaldeyde and transferred to the pathology department where they were sectioned, fixed and stained with hematoxyline-eosine. They were then examined by a pathologist.

Histological Evaluation of Inflammation

Macroscopical examination of the 1st group (control) revealed extended damage more than 2cm along the rat colon in cases 1, 4, 6 and 8. Also in cases 5 and 7, major sites of damage extending more than 1cm along length of colon were observed. Finally, cases 2 and 3 exhibited signs of extended ulceration and inflammation.

From the 2nd group (L-Arginine) only case no.9 had similarities with cases no. 2 and 3 from the control group. Cases 13, 15 and 16 revealed ulceration without hyperemia and case no.10 revealed ulceration and inflammation locally. The rest of the cases were normal, with no pathological signs.

Microscopical examination of the 1st group revealed severe fibrosis of sub mucosal, muscular and serosa layers and acute infiltration of the lamina propria, accompanied by extensive epithelial necrosis in cases 1, 4, 5, 6, 7 and 8 (Figures 1 and 2). Cases 2 and 3 revealed minimal necrosis of the epithelium and moderate infiltration of the lamina propria. From the 2nd group cases 11, 12 and 14 were normal. Cases 13, 15 and 16 revealed milder alterations especially in epithelium and in the lamina propria (Figures 3 and 4). Only cases 9 and 10 exhibited severe infiltration of the lamina propria and extensive surface epithelial necrosis.

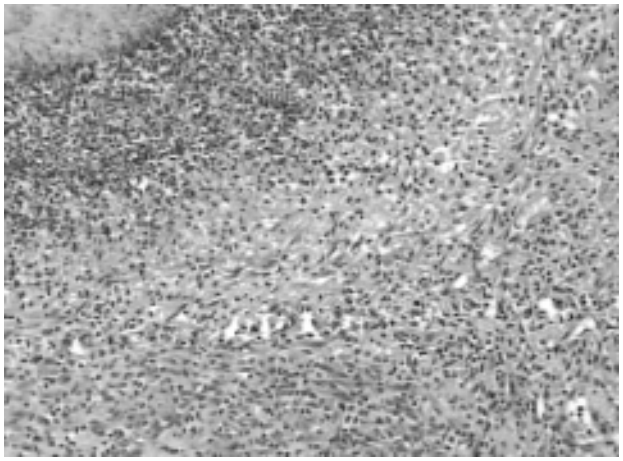

Figure 1. *Comments:* 1) Surface epithelial necrosis. 2) Acute infiltration of lamina propria. 3) Submucosal edema. 4) Development of granulation tissue in muscular layer.

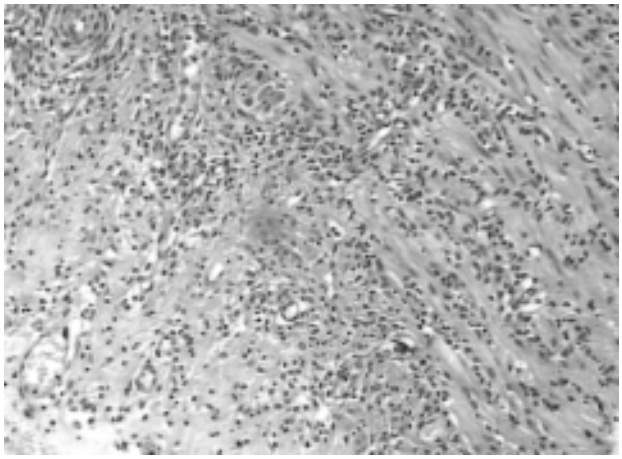

Figure 2. *Comments:* Development of fibrous tissue in muscular layer.

STATISTICAL ANALYSIS

The values of the variables are presented by using the number of the experimental models (N), the mean values (MV), the standard deviations (SD) and the medians for the continuous variables. The comparisons of the haematological, biochemical and histological parameters between the two groups were done parametrically, using the Student's t-test or Welch-test (in case we have unequal fluctuations) and non-parametricaly by using the Mann-Whitney test.

All the tests were two sided, with the level of significance a=0,05. For statistical analysis the statistical package SPSS vr 8.00 (Statistical Package for the Social Sciences) was used.

In order to assess mucosal disease activity we used the colon macroscopic score from the British Journal of Surgery 1995,82, p.1189 and for the histological damage scores we used the score table of Clinical Nutrition (2001) 20(5): 415-422.

RESULTS

Hematological results for Control and L-Arginine group are presented in table 1.

Biochemical results for Control and L-Arginine group are presented in table 2.

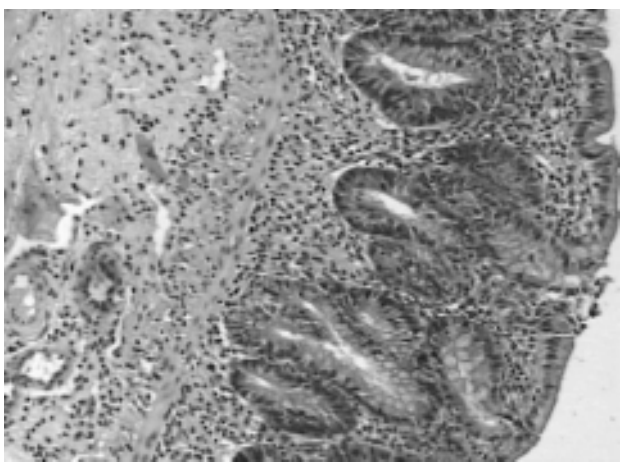

Figure 3. C*omments:* Atrophy of mucosal surface in the gastrointestinal villae.

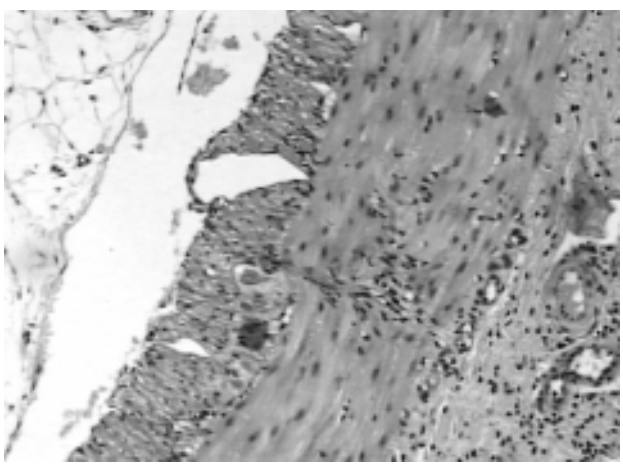

Figure 4. *Comments:* Minimal inflammation of the lamina propria.

Histological scores for the Control and L- Arginine group are presented in table No 3.

There is a statistically significant difference between groups for all histologic variables.

Histological examination of the control group revealed a monotonous picture among all cases with epithelial necrosis and ulcerations locally of different range and concentration of neutrophils leucocytes with nuclear dust in the lamina propria. Vasodilatation and edema in the submucosal layer was also observed. The muscular layer was elamaged and thickened, with development of granulation and fibrous tissue of different intensity among the cases. Many cases from the control group also revealed development of inflammatory elements, mainly eosinophils and neutrophils in all intestinal layers and in the subcutaneous layer. Finally, in some cases, the existence of mast cells in groups, was observed.

In L-Arginine group, the cases were normal apart from some mild alterations in the normal mucosa layer or with localized ulcerations but no necrosis. Few inflammatory elements (eosinophils and neutrophils)

Table 1. Hematological results for Control and L-Arginine group.

	CONTROL N=8	l-ARG N=7	P-VALUE
	median	median	
WEIGHT	265,00	285,00	N.S
WBCx10	12,40	6,80	0,003
RBC	6,91	7,02	N.S
HGB	12,15	11,00	0,024
HCT%	34,80	32,90	N.S
MCV	50,60	49,20	0,009
MCH	17,50	16,80	0,020
MCHC	34,30	34,10	N.S
PLT	1079,50	1106,00	N.S
LYMPH%	56,45	67,50	0,06
LYMPHx10	4,30	4,50	N.S
RDW-CV%	14,80	14,50	N.S
PDW	8,10	8,00	N.S
MPV	6,70	6,60	N.S
P-LCR%	5,50	5,30	N.S

Table 2. Biochemical results for Control and L-Arginine group.

	CONTROL N=8	l-ARG N=8	P-VALUE
	median	median	
GLUCOSE	237,500	310,000	0,009
SGOT	94,000	69,000	N.S
SGPT	14,500	4,500	0,002
AF	1,000	2,000	N.S
Γ-GT	1,000	1,500	N.S
ALBUMINE	2,600	2,750	N.S
CPK	271,000	164,000	0,009
MDA	3,430	2,180	0,010

Table 3. Histological scores for the Control and L- Arginine group.

		N	Median score	p-value
INFLAMMATION	Control	8	2	0,08
Surface epithelial necrosis	L-ARG	8	1	
INFLAMMATION	Control	8	2	0,050
Acute infiltration of lamina propria	L-ARG	8	1	
FIBROSIS				
Sumucosa	Control	8	2	0,050
L-ARG	8	1		
FIBROSIS				
Muscular	Control	8	1	0,001
L-ARG	8	0		
FIBROSIS				
Serosa	Control	8	1	0,005
L-ARG	8	0		
MACROSCOPIC	Control	8	5,50	<0,0005
L-ARG	8	2,00		

were observed below the ulcerated epithelium. Only in one case was the muscular layer infiltrated and in another case there was the observed localized mucosal atrophy.

DISCUSSION

Colitis produced by TNBS has been widely used as an experimental model because of its similarities with human inflammatory bowel diseases.[6,21,22] Earlier studies have centered on the evaluation of new therapeutic agents[23] and on immunological aspects of the disease.[24] However, severity of the disease is usually graded on the basis of features that are difficult to quantify, eg,number and location of areas with transmural necrosis and presence of ulcers. Areas of ulceration with loss of epithelium and infiltration of the lamina propria are features of human IBD that were also noted in the present experimental model of TNBS-induced colitis. Studies of the histopathological and ultrastructural alterations that appear in this model may help to more accurately characterize human disease.

Several ultrastructural alterations were seen at all levels of rat colon mucosa. The loss of intracellular junctions in the epithelium appeared to reflect progression of the inflammatory process from basal toward apical regions, eventually affecting the full thickness of the mucosa.

The polymorphonuclear cell infiltrate in the lamina propria, the accumulation of extracellular matrix elements, the transmural inflammation and edema in the affected areas, the appearance of ulcerations and desquamated areas in the epithelium, and the presence of lymphocytes in different stages of activation suggest a specific autoimmune response to TNBS.

The histological findings of the 1st group (control) were more obvious and present than in the 2nd group (L-Arginine). The purpose of the present study was to investigate whether the content of L-Arginine in the diet influences the inflammatory and repair processes in TNBS colitis. In a previous study, Neily et al. showed that L-Arginine supplementation induced a significant increase in inflammatory damage at day 7 after TNBS colitis induction.[20,25] However, the amount of L-Arginine administered (2% in drinking water;=3.3g/kg of body weight/day) was well over the highest dose used in the present study. We select the doses of 30 and 100mg of L-Arginine (=0.15-0.5g/kg body weight/day) on the basis of the amount of this amino acid administered with enteral diet formulas in human inflammatory bowel disease (range, 2-3g/1000kcal;=0.08-1.2g/kg body weight/day; data based on manufacturer's information). Therefore, data from the present study may be more relevant for the management of human disease.

In wound healing, a self-limiting process, L-Arginine intake may support repair mechanisms. However, chronic fibrotic states, such as human inflammatory bowel disease (particularly, Crohn's disease), involve unterminated wound repair characterized by progressive extracellular matrix accumulation. In this situation, L-Arginine intake might contribute to the development of fibrosis. Further studies are needed to investigate if L-Arginine might also contribute to progressive extracellular matrix accumulation after repeated episodes of inflammation and repair, which characterize the clinical course of human inflammatory bowel disease.

There is evidence that NO may be toxic to the intestinal mucosa in a number of ways. The molecule is eventually converted to the peroxynitrite ion, which is a highly reactive species and has been used on its own to produce a model of colitis.[26] NO can bind to iron sulphydryl groups, disrupting the electron transport chain and uncoupling oxidative phosphorylation, resulting cell death. NO also increases cGMP concentrations and may thus interfere with the regulation of normal cellular proliferation by reducing DNA synthesis.[27]

However, there is also evidence that NO may have a protective role in the intestinal mucosa. NO prevents vasoconstriction and the potentially damaging effects of mucosal ischaemia, which has been postulated as a major pathogenic mechanism in Crohn's disease. NO also prevents neutrophil aggregation and there is also some evidence that iNOS (inhibitor) may be of importance in the repair of damaged intestinal epithelium.[28]

These results have shown that the L-Arginine – Nitric Oxide pathway contributes to the inflammatory response in this model. As increased intestinal nitric oxide synthesis is also a feature of ulcerative colitis, L-Arginine and nitric oxide inhibitors may have a role in the treatment of inflammatory bowel diseases in humans.

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