Original Article

Inflammatory cells' role in acetic acid-induced colitis

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Abstract

Background: Free radicals are the known mechanisms responsible for inducing colitis with two origins: Inflammatory cells and tissues. Only the inflammatory cells can be controlled by corticosteroids. Our aim was to assess the importance of neutrophils as one of the inflammatory cells in inducing colitis and to evaluate the efficacy of corticosteroids in the treatment of inflammatory bowel disease (IBD).

Materials and Methods: Thirty-six mice were divided into six groups of six mice each. Colitis was induced in three groups by exposing them to acetic acid through enema (group 1), *ex vivo* (group 3), and enema after immune suppression (group 5). Each group had one control group that was exposed to water injection instead of acetic acid. Tissue samples were evaluated and compared based on macroscopic damages and biochemical and pathological results.

Results: Considering neutrophilic infiltration, there were significant differences between groups 1, 3, 5, and the control of group 1. Groups 3, 5, and their controls, and group 1 and the control of group 3 had significant differences in terms of goblet depletion. Based on tissue originated H_2O_2, we found significant differences between group 1 and its control and group 3, and also between groups 5 and the control of group 3. All the three groups were significantly different from their controls based on Ferric Reducing Ability of Plasma (FRAP) and such differences were also seen between group 1 with two other groups.

Conclusion: Neutrophils may not be the only cause of oxidation process in colitis, and also makes the effectiveness of corticosteroids in the treatment of this disease doubtful.

Key Words: Acetic acid-induced colitis, corticosteroid, inflammatory bowel disease, neutrophil infiltration

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease commonly seen in all ages from infancy to adulthood.[1,2] This disease is clinically presented with abdominal pain, diarrhea, and sometimes accompanied by mucus, blood, or pus secretion.[3] The disease occurs worldwide,[4] and there is no difference in incidence between males and females.[1] Results of different investigations show that incidence of the disease is more among South Europeans, Asians, and in many other developing countries, especially among children.[5,6]

The etiology of this disease is still unknown.[4,7-11] Current treatments such as corticosteroids[12] are not completely promising[13-15] and their side effects

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remain a major clinical problem.[16,17] Many researches are under way to control the disease, and one of the suggested approaches in this regard is focusing on free radicals [Reactive Oxygen Species (ROS)].[13,18-21] Reactive oxygen metabolites which are generated via inflammatory and also oxidation–reduction reactions (in normal physiologic processes or enzymatic metabolism of endogenous materials) play a crucial role in the development and persistence of this disease.[18,21-23] In addition to the free radicals that are generated in the injured tissue, inflammatory cells which are recalled through chemotaxis to the target tissue play a pivotal role in the pathogenesis of the disease.[24] Neutrophils may play a key role in this regard by producing reactive peroxides that are involved significantly in the tissue necrosis and mucosal dysfunction.[17]

Although we can control inflammatory cells by using anti-inflammatory drugs such as corticosteroids, tissue oxidant formation is not affected by these agents.[25,26] Defining the role of each of the two parts (inflammatory cells and oxidant formation) can be of benefit in the management of IBD. If the main role in the pathogeneses is assigned to inflammatory cells, the treatment strategy should be focused on preventing chemotaxis and inflammatory mediator secretion, and if not, it should be focused on antioxidants to reach treatment goals. Various animal models of experimental colitis have been designed and used to define the mechanisms of IBD and to evaluate effective drugs for its treatment. Acetic acid-induced colitis, which is our model in this investigation, is an animal model that imitates some of the acute inflammatory responses of ulcerative colitis and is used wildly in this regard.[17]

The aim of this study was to investigate the role of neutrophils as one of the inflammatory cells in the pathogenesis of IBD and also to assess the efficacy of corticosteroids which are one of the widely used drugs for IBD in the treatment of this disease, in the acetic acid-induced colitis model.

MATERIALS AND METHODS

Animals

Thirty-six NMRI male mice of weight 25-30 g and age 5 weeks were divided into six groups of six members each. Mice were normal without any prominent gastrointestinal problems such as diarrhea or mucus secretion. Presenting any disease, especially gastrointestinal problems, was the exclusion criterion of the study. Mice were kept in antirust cages at a temperature of 24 ± 1°C and in 12-h light-dark cycle, moisture of 65-70%, and with standard food and water.[26]

Induction of colitis

Following 24 h fasting (receiving nothing except water) and under light ether anesthesia, colonic inflammation was induced by acetic acid. After mechanical anal lavage with normal saline (in order to clean their colons), group 1, named "exposed group," was treated with 0.1 ml acetic acid 4% enema, through a polypropylene trocar cannula which was inserted into the colon via the anus. Group 2 (named "exposed control group") was treated with 0.1 ml distilled water instead of acetic acid with the same method to serve as the control of the exposed group. In a sterile environment, we resected the distal end of the colon of six mice in group 3 and added 0.1 ml of acetic acid 4% on them. This group was designed to evaluate the direct chemical effect of acetic acid on tissue and is called as "*ex vivo* group." Tissues were kept for 15 sec with acetic acid[27] and then washed with warm normal saline.

In order to assess the effect of resection, after resection of the distal colon of group 4 mice (called "*ex vivo* control group"), they were treated with 0.1 ml distilled water instead of acetic acid for at least 15 sec.

In order to suppress neutrophil infiltration, group 5 mice were immune suppressed with daily intra peritoneal injection of 20 mg/kg hydrocortisone.[28-30] After 7 days, the mice of this group, called "prevention group," were exposed to acetic acid, like those in exposed group. Mice of group 6 (prevention control group) were immune suppressed like group 5 mice and were treated with distilled water similar to those in exposed control group. The groupings and the aim of designing them are presented in Table 1.

In order to prevent liquid excretion, all mice were positioned supine in trendelenburg position,[14] and at least 15 sec later, their colons were processed with warm normal saline.[7] After 4 days, the mice were sacrificed, and through laparotomy, 10 cm of each terminal colon was removed,[7] the tissue borders were detached, and all the tissues were treated with phosphate buffered saline.[14] After that, all the tissue samples were compared based on their tissue macroscopic damages and pathological and biochemical results.

Macroscopic analysis

Macroscopic mucosal damages were evaluated, scored, and quantified by Morris *et al.* scoring system.[31] One centimeter of each damaged tissue was extracted and the extent of damage was evaluated by the light microscope.

Table 1: The names and the aim of the six study groups

Treatment process	The aim of designing the group	Given name
Treating by 0.1 ml acetic acid 4% enema	This group was designed as the prototype of acetic acid-induced colitis model	Exposed group
Treating by 0.1 ml distilled water enema (instead of acetic acid)	This group was designed as a control group and specially to assess the effect of enema	Exposed control group
The distal colon was resected and exposed to 0.1 ml of acetic acid 4% *ex vivo*	This group was designed to evaluate the direct chemical effect of acetic acid on tissue	*Ex vivo* group
The distal colon was resected and exposed to 0.1 ml of distilled water *ex vivo*	This group was designed as a control group and specially to assess the effect of resection	*Ex vivo* control group
Daily intraperitoneal injection of 20 mg/kg hydrocortisone for 7 days and then treated by 0.1 ml acetic acid 4% enema	This group was designed to evaluate the therapeutic effect of hydrocortisone	Prevention group
Daily intraperitoneal injection of 20 mg/kg hydrocortisone for 7 days and then treated by 0.1 ml distilled water enema	This group was designed as a control group and specially to assess the probable effects of multiple injections	Prevention control group

Pathological analysis

Samples were stained with H and E, and neutrophil infiltration, goblet depletion, and crypt abscess of sampled tissues were assessed.[32] Neutrophil infiltration assessment was based on a qualitative scoring of 0-3 (absence of neutrophil = 0 and presence of it in all histological layers = 3). Regarding goblet depletion, 0 was for samples with normal goblet and 1 for samples with pathologically depleted goblet. And finally, crypt abscess was assessed based on the presence and absence of it, wherein cases with crypt abscess were scored 1 and the ones without them were scored 0. All evaluations and scorings were done by a blinded pathologist.

Biochemical analysis

In a blinded manner, the rest of each colon was dissected longitudinally into small pieces and a homogenized tissue section was obtained from each colon part.[14]

The total antioxidant capacity of each sample was assessed by Ferric Reducing Ability of Plasma (FRAP) method[33-35] and by evaluating tissue H_2O_2 level with ferrous ion oxidation xylenol orange (FOX) method.[36,37]

To equalize homogenized tissue samples, tissue protein level was measured in all samples. Also, the amounts were counted and compared per sample protein unit by FRAP and H_2O_2. All measurements were done by two laboratory technicians in a blinded manner.

Data analysis

To assess each two groups based on their FRAP and H_2O_2 averages, we used *t*-test, and to assess and compare them based on goblet depletion and grade of neutrophil infiltration, we used χ^2 and Fisher's exact tests. All data were analyzed through SPSS 14 and $P < 0.05$ was considered as statistical significance.

RESULTS

We had 36 mice without diarrhea and bloody feces, until the end of the study.

Macroscopic results

Among all cases, only three samples showed stage 1 damage and there were no significant differences between groups with respect to macroscopic results ($P = 0.69$).

Pathological results

Regarding neutrophil infiltration, exposed group was significantly different from its control group ($P = 0.007$), prevention group ($P = 0.025$), and also *ex vivo* group ($P = 0.007$). We did not find any statistically significant differences in neutrophil infiltration among other groups [Table 2].

Considering goblet depletion, we found significant differences between *ex vivo* group and its control ($P = 0.015$), between prevention group and its control ($P = 0.015$), and also between prevention group and *ex vivo* control group ($P = 0.002$). There were also significant differences on comparing exposed group with *ex vivo* control group ($P = 0.015$) and prevention control group ($P = 0.021$). We did not find any other significant differences [Table 3].

Also, we found crypt abscess in only two samples without any significant difference among the groups ($P = 0.56$).

Biochemical results

With respect to FRAP concentration averages, all the three acetic acid treated groups (exposed group, *ex vivo* group, and prevention group) were significantly different from their controls [($P = 0.03$), ($P = 0.02$), and ($P = 0.04$), respectively]. Also, exposed control group was significantly different from prevention ($P = 0.04$) and *ex vivo* groups ($P = 0.01$). Besides, there were significant differences between *ex vivo* group and

two immunosuppressed groups [prevention group (P = 0.03) and its control (P = 0.02)]. There was no other significant difference among the other groups [Table 4].

Regarding tissue H_2O_2 concentration, we found significant difference between exposed group and its control (P = 0.01). Also, we found significant difference between two groups treated only with acetic acid (exposed group and *ex vivo* group) (P = 0.01) and also between prevention group and *ex vivo* control group (P = 0.009). Finally, among the three control groups, prevention control group showed significant differences from exposed control group (P = 0.048) and *ex vivo* control group (P = 0.02) [Table 5].

Table 2: The comparison of all groups based on grade of neutrophil infiltration

Grade of neutrophil infiltration								
6 Mice in each group						*P* value	Groups	Set
Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6			
2	3	3	3	2	3	*P*=0.025	Exposed group	A
0	2	0	0	0	1		Prevention group	
2	3	3	3	2	3	*P*=0.007	Exposed group	B
0	0	0	0	0	1		*Ex vivo* group	
2	3	3	3	2	3	*P*=0.007	Exposed group	C
0	1	0	0	0	0		Exposed control group	
0	2	0	0	0	1	*P*=0.51	Prevention group	D
0	1	0	1	0	0		Prevention control group	
0	2	0	0	0	1	*P*=0.57	Prevention group	E
0	0	0	0	0	1		*Ex vivo* group	
0	0	0	0	0	0	*P*=1.00	*Ex vivo* control group	F
0	0	0	0	0	1		*Ex vivo* group	

DISCUSSION

Results of this study show that activated neutrophils may not be the main cause of oxidation process in tissue and makes the effectiveness of the corticosteroids in the treatment of this disease doubtful. Acetic acid-induced colitis is one of the commonly used experimental models of colitis that allows many studies to be done on this disease.[17,38] The significant differences in neutrophil infiltration between exposed group and its control, prevention group and *ex vivo* group, and also the goblet depletion difference between prevention control group and *ex vivo* control group showed that the model used was successful in

Table 3: The comparison of all groups based on goblet depletion

Number of mice in each category				
Goblet depletion		*P* value	Groups	Set
0[1]	1[2]			
1	5	*P*=1.00	Exposed group	A
0	6		Prevention group	
1	5	*P*=1.00	Exposed group	B
1	5		*Ex vivo* group	
1	5	*P*=0.24	Exposed group	C
4	2		Exposed control group	
0	6	*P*=0.015	Prevention group	D
5	1		Prevention control group	
0	6	*P*=0.002	Prevention group	E
6	0		*Ex vivo* control group	
1	5	*P*=0.015	*Ex vivo* group	F
6	0		*Ex vivo* control group	
1	5	*P*=0.015	Exposed group	G
6	0		*Ex vivo* control group	
1	5	*P*=0.021	Exposed group	H
5	1		Prevention control group	

0: Without goblet depletion, 1: With goblet depletion

Table 4: The comparison of all groups based on their FRAP averages

Set	A		B		C		E		F		G	
Groups	Exposed group	Exposed control group	*Ex vivo* group	*Ex vivo* control group	Prevention group	Prevention control group	Exposed group	Prevention group	Exposed group	Prevention control group	Prevention group	Exposed control group
FRAP (mean)	73.8	49.8	37.33	65	52.8	65.4	73.8	52.8	73.8	65.4	52.8	49.8
FRAP (SD)	18.83	9.9	12.7	22.83	6.30	8.79	18.83	6.30	18.83	8.79	6.30	9.9
P value	*P*=0.03		*P*=0.02		*P*=0.045		*P*=0.04		*P*=0.39		*P*=0.58	

Table 5: The comparison of all groups based on their H_2O_2 averages

Set	A		B		C		E		F		G		H	
Groups	Exposed group	Exposed control group	*Ex vivo* group	*Ex vivo* control group	Prevention group	Prevention control group	Exposed group	Prevention group	Exposed group	*Ex vivo* group	Prevention group	Exposed control group	Prevention group	*Ex vivo* control group
H_2O_2 (mean)	81.2	40.8	34.16	14.83	63.4	50.6	81.2	63.4	81.2	34.16	63.4	40.8	63.4	14.83
H_2O_2 (SD)	14.53	24.81	31.6	12.87	34.07	34.79	14.53	34.07	14.53	31.6	34.07	24.81	34.07	12.87
P value	*P*=0.01		*P*=0.19		*P*=0.57		*P*=0.31		*P*=0.01		*P*=0.09		*P*=0.009	

inducing mild acute colitis in this study. Also, as there was a significant difference in goblet depletion of the prevention group with prevention control group and *ex vivo* control group, and also a significant difference between the *ex vivo* group and its control, we concluded that acetic acid had an acceptable impact on samples, and this would be confirmed when considering the non-significant differences among the exposed, prevention, and *ex vivo* groups. Besides, regarding macroscopic view, hyperemia, which was only seen in exposed group and prevention group, may help to reaffirm the presence of induced colitis in samples.

On the other hand, significant difference in neutrophil infiltration between exposed group and prevention group, and nonsignificant differences between prevention group and its control group, exposed control group, *ex vivo* group, and *ex vivo* control group may confirm the effect of corticosteroid on the prevention of neutrophil infiltration in this animal model.

Nonsignificant difference in the variable of goblet depletion between exposed group and its control may be justified as due to the colon mechanical lavage before enema, which may result in goblet depletion.

On comparing H_2O_2 concentration between *ex vivo* group and its control, it showed no significant difference, and thus may reveal that the impact of acetic acid on tissue H_2O_2 concentration is not simply due to its local chemical effect on colon. Significant difference between exposed and *ex vivo* groups also can confirm this conclusion. Considering significant difference between exposed group and its control, it can be concluded that colitis may increase tissue H_2O_2 concentration. Lack of significant difference between exposed group and prevention group shows that although corticosteroid decreases neutrophil count, it does not decrease tissue H_2O_2 concentration in colitis model.

We derived two conclusions from the comparison between the prevention group and its control group, which did not show a significant difference based on H_2O_2 concentrations:

- Corticosteroid may prevent the effect of acetic acid and cause decrease in H_2O_2, or
- Corticosteroid may spontaneously increase H_2O_2

Regarding the fact that prevention group showed significant differences from *ex vivo* and exposed control groups, it can reinforce the second conclusion.

On the other hand, as H_2O_2 production in prevention group is significantly different from its production in exposed group but there is no difference between the two aforementioned groups with regard to neutrophil infiltration, we may criticize the role of neutrophil in the oxidation process which induces colitis.

By considering the significant difference in FRAP concentration between exposed group and its control, we can conclude that colitis is accompanied by increased FRAP in tissue. Increasing FRAP results in reducing H_2O_2 to H_2O and consequently decreasing tissue H_2O_2 concentration (may be in order to balance the increased H_2O_2 level). Comparing *ex vivo* group with its control based on FRAP concentration, it showed significant difference, but by considering the fact that exposing *ex vivo* tissue to acetic acid may generate a permanent acidic environment and because the isolated tissue is not able to neutralize it and subsequently may lead to acid–base equation imbalance, we cannot have any discussion on this pair. In this regard, comparing exposed group with prevention group showed statistical difference as a result of FRAP average decreasing in the prevention group. As there was also a significant difference between prevention group and its control based on a decline in the FRAP concentration average in the former group, we can hypothesize that in colitis model, corticosteroid may decrease FRAP or prevent it from increasing. Nonsignificant difference between *ex vivo* group and prevention group may help to confirm this hypothesis.

As colitis may increase H_2O_2 in tissue, if something decreases FRAP or prevents it from increasing, it can result in increasing H_2O_2 and exacerbates the inflammation process and its consequences and complications.

So, based on all aforementioned discussions, we should take this possibility into account that corticosteroids may increase free radical formation and oxidant factors and also decrease antioxidants in this model.

IBD has still saved its importance as a subject of many investigations to discover its exact mechanisms and treatments. The results of the study published by Bilsela and colleagues showed that inflammatory cells are not the sole factors in inducing and exacerbating colitis.[14] In 2011, a study considering the main roles of increased free-radical production, decreased antioxidant capacity, and excessive inflammation in the pathogenesis of IBD proposed that melatonin as a powerful antioxidant may be a hopeful therapeutic agent for ulcerative colitis.[23]

The acetic acid-induced colitis model which is used in this study is a commonly used model, and in recent

years, some studies have accomplished to investigate the effects of new proposed drugs and treatments like royal jelly,[39] budesonide-succinate-dextran,[38] and angiotensin converting enzyme inhibitors[40] on this disease through this (acetic acid-induced colitis) model.

This study had some limitations such as small sample size and confined available techniques, which hampered researchers from using miscellaneous confirmatory techniques and methods. Also, all of these may end in some kinds of biases and misconceptions in deriving the conclusions.

CONCLUSION

In summary, the results of this study make suspicious the pivotal role of neutrophils as the main cause of oxidative reactions and free radical formation in colitis. Also, due to increased H_2O_2 concentration in the presence of corticosteroid and decrease or no change in samples' FRAP concentration averages, it may be possible to doubt the confirmed role of corticosteroids in the management of colitis. Further studies with greater sample size and the possibility of taking advantages of more advanced techniques are warranted to support this idea. Also, it is suggested to repeat this study with other animal models for colitis and to run some more studies for evaluating the effectiveness of antioxidant drugs on the treatment of this disease.

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