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The comparative effects of calcium channel blockers in an experimental colitis model in rats

Ratlarda oluşturulan deneysel kolit modelinde kalsiyum kanal blokerlerinin etkilerinin karşılaştırılması

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Background/aims: *In this study two calcium channel blockers (CCB), diltiazem and verapamil, which demonstrate their effects on two different receptor blockage mechanisms, were assessed comparatively in an experimental colitis model regarding the local and systemic effect spectrum.* ***Methods:*** *Eighty male Swiss albino rats were divided into eight groups (n:10 each): Group I) colitis was induced with 1 ml 4% acetic acid without any medication. Group II) Sham group. Group III) Intra-muscular (IM) diltiazem was administered daily for five days before inducing colitis. Group IV) IM verapamil was administered daily for five days before inducing colitis. Group V) Transrectal (TR) diltiazem was administered with enema daily for two days before inducing colitis. Group VI) TR saline was administered four hours before inducing colitis. Group VII) TR diltiazem was administered with enema four hours before inducing colitis. Group VIII) TR verapamil was administered with enema four hours before inducing colitis. All subjects were sacrified 48 hours after the colitis induction. The distal colon segment was assessed macroscopically and microscopically for the grade of damage, and myeloperoxidase (MPO) activity was measured.* ***Results:*** *All the data of the control colitis group (group I), including the microscopic, macroscopic and MPO activity measurements, were significantly higher than in the groups in which verapamil and diltiazem were administered over seven days (3.100±0.7379to 1.300+0.9487and 1.600±0.9661) ($p<0.05$). The data of the Sham group, group II, were less than the other groups in which colitis was induced ($p<0.05$). For the local effect spectrum, after the assessment of groups V-VIII, the control colitis group (group I) and group VI had significantly higher values than the others (3.300±0.4830 to 1.800±0.6325 and 1.700±0.8233 ($p<0.05$).* ***Conclusions:*** *Calcium channel blockage has systemic and local effects on the colitis model.*

Keywords: Diltiazem, verapamil, inflammatory bowel disease, acetic acid colitis

Amaç: *Bu çalışmada iki kalsiyum kanal blokerinin ratlarda oluşturulan deneysel kolit modelindeki etkileri lokal ve sistemik teropötik açıdan karşılıklı olarak araştırılmıştır. Yöntem: 80 erkek Swiss albino rat 8 gruba ayrılarak çalışma gerçekleştirilmiştir. 1) Herhangibir medikasyon uygulamaksızın 1 mlt. %4'lük Asetik asit uygulaması ile kolit oluşturulan grup. 2) Sham grubu 3) Kolit oluşturulmadan önce 5 gün sistemik olarak Diltiazem uygulanan grup. 4) Kolit öncesi 5 gün sistemik olarak Verapamil uygulanan grup. 5) Kolit oluşturulmadan önce 2 gün boyunca transrektal (TR) lavman olarak Diltiazem uygulanan grup. 6) Kolit oluşturulmadan 4 saat önce TR salin uygulanan grup. 7) Kolit oluşturulmadan 4 saat önce lavman olarak TR Diltiazem uygulanan grup. 8) Kolit oluşturulmadan 4 saat önce lavman olarak TR Verapamil uygulanan grup. Tüm tedavilere ratların sakrifiye edildiği 48. saate kadar devam edildi. Tüm gruplardaki ratlar asetik asit ile kolit oluşturulduktan 48 saat sonra sakrifiye edilerek distal kolon segmenti makroskopik ve mikroskopik olarak kolon hasarının değerlendirilmesi ve my eloper oksidaz ölçümleri amacıyla incelemeye alındı.* ***Bulgular:*** *Kontrol kolit grubu (Grup 1'nun tüm değerleri sistemik olarak Diltiazem ve Verapamil uygulanan gruplara göre yüksekti (3,100±0,7379 karşı 1,300±0,9487 ve 1,600±0,9661) ($p<0,05$). Sham grubunun değerleri kolit oluşturulan tüm gruplara göre düşüktü ($p<0,05$). Lokal etki spektrumu açısından değerlendirilen grup 5, 6, 7 ve 8 arasında, kontrol grubu oluşturan grup 6 değerleri istatiksel olarak daha yüksek bulundu (3,300±0,4830 karşı 1,800±0,6325 ve 1,700±0,8233) ($p<0,05$).* ***Sonuç:*** *Kalsiyum kanal blokerlerinin kolit modelinde hem sistemik, hem de lokal olarak olumlu etkileri vardır.*

Anahtar kelimeler: Diltiazem, verapamil, inflamatuar barsak hastalığı, asetik asit ile oluşturulan kolit

INTRODUCTION

A definitive cause of inflammatory bowel disease (IBD) is still unknown; however, there are increasing data disclosing the important roles of the arachidonic acid metabolites in the pathogenesis (1, 2). Recently, the protective effect of prostaglandins on the bowel mucosa has been suggested, in

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spite of the known harmful effects of another arachidonic acid metabolite, leukotrienes (3). Clinical progression is seen with prostaglandin inhibitors in patients with IBD. In some studies in the literature, a decrease in mucosal damage was also obtained with prostaglandin analogues in experimental models (4). The effect of calcium ions on Ieukotriene synthesis with the enzyme 5-lipooxygenase from arachidonic acid has been suggested in several studies (5). In many experimental studies, it has been suggested that the enzyme 5-lipooxygenase is calcium dependent and requires adenosine triphosphate (4, 6, 7, 8). When the leukocytes are stimulated under appropriate conditions, the 5-lipooxygenase enzyme in the cytosolic fraction translocates to the cell membrane and merges with the 5-lipooxygenase activating protein existing in the membrane, which then initiates the Ieukotriene synthesis in the cell, whether or not the calcium exists in the medium (9, 10). In the macrophages of the colon mucosa, arachidonic acid must be secreted from the membrane and the intracellular calcium level must be increased for the 5-lipooxygenase activity. The cyclooxygenase enzyme activity essential for the prostaglandin synthesis is not calcium dependent (11).

Recently the effect of calcium channel blockage (CCB) on the physiological and pathological changes in the gastrointestinal (GI) tract has become one of the most attractive topics (6, 7, 12, 13). In many experimental colitis models based on this theory, the effects of CCB in decreasing mucosal damage have been suggested in the prophylaxis of IBD (7). In different studies, verapamil and diltiazem have been assessed separately (7). A decrease was noted in the mucosal damage with the systemic effects in the experimental model, but the local effects and a comparison between local and systemic effects were not investigated.

The aim of our study was to assess the systemic and local effects of two calcium channel blockers, verapamil and diltiazem, in an experimental colitis model comparatively, and to show their effects on different mechanisms.

MATERIALS AND METHODS

Eighty Swiss albino male rats were used in the study, with a mean age of 4 months and mean weight of 150-200 g. All groups were fed with the standard nutrition feed track. Twelve hours before the transrectal administration of acetic acid or 0.9% NaCl, feeding was stopped and the rats were only allowed to drink water. According to the data obtained after a review of the literature about the induction of the colitis model, the clinical and histopathological initiation of the colitis occurs 24 hours after the 15-second contact of 4% acetic acid and regresses after four days (4, 6, 7, 13, 14, 15). The Ethical Committee of Ege University approved this study.

Each group consisted of 10 rats

Group I: Control colitis group. A 8 mm soft pediatric catheter was advanced 6 cm from the anus under low-dose ether anesthesia. Rats were in Trendelenburg position during this process and 1 ml of 4% acetic acid solution (pH:2.4) was slowly administered transrectally (TR). The rats were maintained in head-down position for 30 seconds to prevent a leakage, and the rest of the solution was aspirated. After this process, 2 ml of phosphate buffer solution with pH: 7 was administered TR.

Group II: Sham group. Neutral saline solution was administered TR in the same manner as above.

Group III: Systemic diltiazem treatment group. 2 mg/kg intramuscular (IM) diltiazem (Mustafa Nevzat Drug Industry, Istanbul) was administered to the rats every 12 hours during the five days before inducing colitis with acetic acid. At the end of five days the acute colitis model was induced with TR-administered 1 ml of 4% acetic acid with pH:2.4. After 48 hours, without sacrificing the rats, 2 mg/kg of IM diltiazem administration was continued every 12 hours.

Group IV: Systemic verapamil treatment group. 4 mg/kg IM Isoptin (Knoll, Switzerland) was administered to the rats every 12 hours during the five days before inducing colitis with acetic acid. At the end of five days the acute colitis model was induced with TR- administered 1 ml of 4% acetic acid with pH: 2.4. After 48 hours, without sacrificing the rats, 4 mg/kg of IM Isoptin administration was continued every 12 hours.

Group V: TR diltiazem treatment group. 2 mg/kg of diltiazem was administered TR per 12 hours by a soft pediatric catheter advanced through the anus under low-dose ether anesthesia during the 48 hours before inducing colitis with acetic acid. At the end of 48 hours, the acute colitis model was induced with 1 ml of 4% acetic acid with pH:2.4 administered TR. After 12 hours following acetic acid administration, 2 mg/kg TR diltiazem was administered for periods of 12 hours until the rats were sacrificed.

Group VI: Control colitis group with TR administered saline. 2 ml saline solution of neutral pH was administered TR under low-dose ether anesthesia four hours before inducing colitis with acetic acid. After four hours, acute colitis model was induced with TR- administered 1 ml 4% acetic acid with pH:2.4.

Group VII: TR diltiazem prophylaxis group. 2 mg/kg diltiazem was administered TR under low-dose ether anesthesia four hours before inducing colitis with acetic acid. Four hours after this administration, the acute colitis model was induced with 1 ml 4% acetic acid of pH:2.4 administered TR under low-dose anesthesia.

Group VIII: TR verapamil prophylaxis group. 4 mg/kg Isoptin was administrated TR under low-dose anesthesia four hours before inducing colitis with acetic acid. Four hours after this administration, the acute colitis model was induced with 1 ml 4% acetic acid of pH:2.4 administered TR under low-dose anesthesia.

All the rats in the eight groups were sacrified with overdose ether 48 hours after TR saline administration; during this time they were allowed normal feeding. After sacrifice of the rats, laparotomy was applied with scissors from umbilicus to pubis, and the rectum was excised as distally as possible. A 10 cm colon segment was then resected totally through proximal end (??). During this resection, the feces in the colon were purified by hand caressing and dried after washing with saline. The nearest 5 cm to the rectum was scored macroscopically by the same pathologist without knowledge of the group, and maintained in formalin for microscopic evaluation. The next 5 cm was taken for biochemical analysis for assessing myeloperoxidase (MPO) activity.

The assessment of colonic inflammation and damage: The colonic mucosal surface was assessed macroscopically according to the depth, width and distribution of the lesions occurring on the mucosal surface (7).

Macroscopic Score:

Grade 0: Normal mucosal pattern,

Grade 1: Scattered erosions,

Grade 2: Linear ulcerations,

Grade 3: Diffuse inflammatory tissue featuring small lesions of less than 5 mm,

Grade 4: Diffuse ulcerations, wide lesions.

Mucosal cell infiltration and inflammation were noted with routine hematoxylin and eosin on histopathological assessment.

Microscopic Score:

0. Normal mucosa,
1. Edema,
2. Congestion,
3. Focal erosion,
4. Ulceration.

After sacrifice of the rats with ether in all groups, the MPO activity was studied on the colon specimens obtained. It was assessed according to the method described by Krawisz et al. (16). The tissue protein amount was determined by the Brad ford method. 150-600 mg fresh tissue specimen in 1 ml hexadecyltrimethylammoniumbromide (HTBA) buffer was homogenized 30 sn in ice using polytron homogenizator (B. Braun Melsungen). The homogenizator was washed with 1 ml HTBA buffer two times and the homogenate was collected in a test tube, which was then mixed 10 seconds in a supersonic mixer. The supernatant, which was frozen three times, solved and centrifuged for 15 min at $+4^0$C at 40000 gravity, was then assessed. For this process 0.1 ml supernatant was mixed with 2.9 ml reaction mixture and the variation of the absorbance was measured for 3 min. on 430 mm. 1 unit of MPO was accounted as 1 (µmol H_2O_2 spent at 25^0C in one minute.

Solutions: Reaction mixture=50 mM phosphate buffer pH:6; 0.167 mg/ml o-dianiside hydrochloride; 0.0005% H_2O_2, HTAB=0.5% HTAB, 50 nM pH:6 phosphate buffer.

Statistical analysis

The statistical analysis of the groups was done in the Department of Statistics, University of Ege using SPSS for MS Windows program. One-way ANOVA for variance analysis, Levere test for variance homogeneity and Duncan test for multiple range evaluation were utilized.

RESULTS

The overall evaluation of the results revealed that only the rats of the Sham group, in which TR saline was administrated, showed no colitis or damage after macroscopic, microscopic and clinical assessment.

The statistical analyses were evaluated separately for the systemic medication and TR administration groups. Statistically, macroscopic scoring for MPO volumetric activity, MPO specific activity and total MPO activity were done comparatively.

All the data of the control colitis group, group I, were significantly higher than the groups in which verapamil and diltiazem were administrated over seven days ($p<0.05$). The data of the Sham group, group II, were less than in the other groups in which colitis was induced ($p<0.05$). The data of group III, in which diltiazem was administrated systemically, were significantly less than of group I ($p<0.05$). They were higher than the verapamil group, but the difference was not statistically significant. In group IV, in which verapamil was administrated systemically, the data were significantly less than in group I ($p<0.05$). The MPO activity levels of the verapamil-administrated group were lower than of the diltiazem group, but this difference was also not statistically significant.

In the enema and medication groups, which were formed according to the local effects of the CCB with the potential for calcium blockage and inhibition of inflammation on mucosa, the microscopic and macroscopic scores and MPO levels of group V were significantly lower than of group VI, the control group in which saline enema was applied before inducing colitis ($p<0.05$).

Statistically no significant difference was seen in macroscopic scoring and MPO activity between group VII and group VIII. Macroscopic scoring and MPO activity were higher than in the systemic medication groups.

The mean values of macroscopic scoring and MPO activity for all groups are assessed in (Table 1).

Regarding the local administration of CCBs, there were no statistically significant differences between the outcomes of administration either 48 h before or 4 h before colitis induction. For therapeutic effectiveness, local administration of CCB after colitis suggested similar results with systemic therapy regarding MPO activity. For all criteria, close mean values were obtained after the study. An alternative verapamil group for TR diltiazem administered 48 h before colitis induction was not formed. This group was included only in a pilot study for similar effect spectrum and for a comparison with the group administered 4 h before colitis.

There were no significant differences observed between verapamil and diltiazem in the grade of local effects ($p<0.05$).

DISCUSSION

Inflammatory bowel disease remains an important health problem today (17, 18). Clinically, the physician can encounter a group of diseases presenting colonic inflammation. In this group, we can observe ulcerative colitis (UC) with diffuse inflammation of the mucosa of the colon and the rectum, with an unclear etiology, characterized by mucosanguineous diarrhea and proceeding with remissions and exacerbations; amebic or bacterial-originated colitis secondary to the infectious agents; and colitis secondary to the irritation of the mucosal layers of the colon and the rectum. The medical approach to colitis has a wide time process and still requires investigation dependent on the etiology. The absence of a certain etiological factor and of an experimental model imitating the IBD make it difficult to understand the pathogenesis of the disease (13, 19, 20, 21). Although there are some potential responsible factors thought to effect the initiation of the inflammatory process, it is generally accepted that something is lacking in immune suppressing events (6, 22, 23, 24).

Table 1. The mean values for macroscopic scoring and myeloperoxidase activity for all groups

Group	Macroscopic Score (Mean +/- SD)	Volumetric Myeloperoxidase Activity Unit/ml (Mean +/- SD)	Specific Myeloperoxidase Activity Unit/mg.Protein (Mean +/- SD)	Total Myeloperoxidase Activity Total Unit/g mass (Mean +/- SD)
1	3.100±0.7379	0.1572±0.0903	0.0374±0.0168	0.3099±0.1578
2	0.000±0.000	0.0431±0.0129	0.0144±0.0250	0.1022±0.0278
3	1.300±0.9487	0.1184±0.0235	0.0284±0.0057	0.2478±0.0391
4	1.600±0.9661	0.0871±0.0407	0.0239±0.0046	0.1500±0.0413
5	2.500±0.8498	0.0948±0.0277	0.0373±0.0065	0.4020±0.0946
6	3.300±0.4830	0.2344±0.0543	0.0508±0.0086	0.8070±0.1649
7	1.800±0.6325	0.1032±0.0198	0.0289±0.0047	0.3920±0.0413
8	1.700±0.8233	0.1183±0.0350	0.0305±0.0058	0.3870±0.1306

Inflammatory bowel disease has an increasing potential for investigation, and accounts for 15-20% of the literature (1). The term IBD is generally used for idiopathic chronic bowel disease and differs from the other IBDs of known causes.

Ulcerative colitis is a chronic recurrent disease of the colon characterized by diffuse mucosal inflammation. The disease generally holds the rectum and the colon partially or entirely uninterrupted through the proximal. Crohn's disease is a chronic recurrent disease which can hold the GI tract interruptedly from the mouth to the anus (25). The treatment in IBD varies according to the limits and severity of the diseased mucosa. CD and UC are similar in many aspects, but the main difference is that while colectomy provides a cure in UC, recurrence is often seen in Crohn's disease after surgery.

Verapamil and diltiazem are the medical agents with investigation potential for IBD treatment via calcium blockage or by phospholipase A_2 inhibition. The treatment of IBD in the future will be established based on the studies of basic sciences as in many other diseases.

CCBs are the agents blocking the transmission of extracellular calcium into the cell during depolarization, and are effective both on the calcium canals of the smooth muscle cells of coronary vessels and on myocardial cell membranes. CCBs are separated into two groups as selective and nonselective. Verapamil and diltiazem are selective CCBs. The advantage of both for cardiologic usage is the equal blockage effect on heart muscle and vascular smooth muscle. Reducing the O_2 consumption of the myocardium hemodynamically occurs by decreasing the afterload with the dilatation effect on the peripheric arterioles. The coronary dilatation effect increases the arterial perfusion of the myocardium. The negative inotropic effect decreases O_2 need of the muscle by reducing the work. The noncardiac indications of the CCBs are migraine therapy, relaxation of lower esophageal sphincter in diffuse esophageal spasm, and relaxation of internal anal sphincter and myometrium (26, 27).

The definite cause of IBD is still unknown; however, there are increasing data revealing the important roles of the arachidonic acid metabolites in the pathogenesis. Recently, the effect of CCB on the physiological and pathological changes in the gastrointestinal tract has become one of the most attractive topics. In many experimental colitis models based on this theory, the effects of CCB on decreasing mucosal damage are suggested in the prophylaxis. In different studies, verapamil and diltiazem have been assessed separately (6, 7, 12, 13). A decrease is noted in the mucosal damage with the systemic effects in the experimental model, but the local effects and a comparison between local and systemic effects have not been investigated.

In the acute colitis model induced with acetic acid, the lesions seen in rats are very similar to the histopathological changes in human UC. However, this model has significant differences from the accustomed UC. Acetic acid colitis shows acute development, followed by rapid healing (13). While acute inflammation is dominant histopathologically in acetic acid colitis, the appearance and remissions of UC are slower. Again both acute and chronic inflammation cascades are activated histopathologically (8).

In the studies reported in the literature, diltiazem and verapamil have been investigated separately in the experimental colitis models for the systemic effects and therapeutic effect spectrum (6,7). But a comparative analysis and CCB treatment and prophylaxis on local conditions have not been investigated. Because the arachidonic acid metabolism in the colitis induced with acetic acid in rats is similar in many aspects with the studies about IBD, the colitis model induced in rats with acetic acid provided the ideal conditions for our study.

The 2 mg/kg dose of diltiazem was taken from the studies in the literature (4, 6, 7). The verapamil dose is the therapeutic dose reported by Fedorak et al. (7). For systemic administration, IM was preferred after pharmacokinetic assessment. However, there was no data for local effect spectrum, and the dose used for systemic effect was taken as a base. Considering the disappearance of the first passing elimination, the doses were decided as 2 mg/kg for diltiazem and 4 mg/kg for verapamil. The local administration periods were based on the principal of classic administration of suppository drugs in UC. For the determination of the therapeutic and prophylactic medication effectiveness, the macroscopic and microscopic scores and the measurements of tissue MPO activity were assessed as different entities.

After a general evaluation of the results, a limiting effect of CCB on the inflammatory process in

the colitis model was suggested, as reported before in the literature (6, 7, 14, 19, 28). The results of the systemic administrated CCB were affirmative. Verapamil and diltiazem are both CCBs, but they have different therapeutic responses, especially in cardiology. For instance, verapamil has a greater efficacy in calcium channel blockage in arrhythmia than diltiazem. When their pharmacological structures are analyzed, it has been established that they demonstrate their effects on different receptor groups.

In our study, verapamil and diltiazem did not show statistically significant differences for effect spectrum and grade in acute colitis. But when cardiologically higher blockage doses of verapamil are considered, not statistically proven in our study but based on the results obtained from systemic administration, lower scores and MPO tissue values are established for the verapamil group in which the blockage was relatively increased. No difference was seen in the results of local administration. The discovery of CCB is an important development in the treatment of cardiovascular diseases. Recently, in view of their effect in decreasing lower esophageal sphincter pressure, CCBs are accepted as an alternative treatment regimen in gastroenterology. Many different features of CCB, such as their efficacy in preventing intraperitoneal adhesions, have led to these drugs being the focus in many investigations (29). Recently, a protective effect of prostaglandins on bowel mucosa has been suggested, in spite of the harmful effects reported for another arachidonic acid metabolite, leukotrienes (3). Clinical progression is seen with prostaglandin inhibitors in patients with IBD. Also, in some studies in the literature, a decrease in mucosal damage was obtained with prostaglandin analogues in experimental models (4). The effect of calcium ions on leukotriene synthesis with the enzyme 5-lipooxygenase from arachidonic acid has been suggested in several studies (5). In many experimental studies, it has been suggested that the enzyme 5-lipooxygenase is calcium-dependent and requires adenosine triphosphate (4, 6, 7, 8). When the leukocytes are stimulated under appropriate conditions, the 5-lipooxygenase enzyme in the cytosolic fraction is translocated to the cell membrane and merges with the 5-lipooxygenase activating protein existing in the membrane, which then initiates the leukotriene synthesis in the cell, whether or not calcium exists in the medium (9, 10). In the macrophages of the colon mucosa, arachidonic acid must be secreted from the membrane and the intracellular calcium level must be increased for the 5-lipooxygenase activity. The cyclooxygenase enzyme activity essential for the prostaglandin synthesis is not calcium-dependent (11).

The effects of diltiazem and verapamil on the colitis model have been previously discussed in the literature. However, these two CCBs, which are effective on two different mechanisms, have not been assessed comparatively. These two CCBs, with well-known different cardiological and pharmacological indications and activity groups, demonstrate their effect on the inflammatory cascade by intracellular calcium blockage. Although not statistically proven, it may be interpreted that verapamil's efficacy is greater than that of diltiazem. We hope that the similar effect potential between the local administration of calcium channel blockage and systemic response will be the subject of more focus in future studies.

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