Correlation Between Intestinal Colony Numbers and Inflammation Markers (TNF-Alpha, IL-6, High-Sensitive CRP) Following the Use of Sevelamer in Rats with Kidney Failure

Böbrek Yetmezlikli Ratlarda Sevelamer Kullanımı Sonrası İnflamasyon Belirteçleri (TNF-Alfa, IL-6, Hassasiyeti Yüksek CRP) ile Bağırsak Koloni Sayısı Arasındaki İlişki

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ABSTRACT Objective: Our aim was to investigate anti-inflammatory effect of sevelamer in experimentally induced renal failure (RF) and whether this effect was related to a decrease in *Escherichia coli* colony counts in feces. **Material and Methods:** Eighteen female Wistar albino rats weighted 200-250 g were divided into three groups. RF was induced by 5/6 nephrectomy. Group 1 was the control group. Group 2 rats were those with induced RF receiving a high phosphate diet. Group 3 contained rats with induced RF receiving a high phosphate diet + sevelamer. *E.coli* count in feces, plasma creatinine, high-sensitive C-reactive protein (hsCRP), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) concentrations were determined at the beginning and at the end of 1st, 3rd and 6th weeks in all rats. **Results:** In Group 2 and 3, creatinine concentrations were found to be increased (p=0.03, p=0.02) in the 1st week. In Group 2, there was an increase in number of *E. coli* colony count in the 3rd week (p=0.05). In group 3, *E. coli* colony count and levels of inflammatory markers (IL-6, CRP, TNF-alfa) significantly were increased in comparison to the basal values (p=0.042, p=0.021, p=0.042, respectively) in the 1st week. In group 3, in the sixth week, *E. coli* colony count (p=0.02) and levels of inflammatory markers (IL-6, hsCRP, TNF-alpha) ($p<0.05$) were found to be significantly decreased in comparison to the 1st week levels. **Conclusion:** Anti-inflammatory effects of sevelamer might be explained by its effects on the bacterial colonization in colon.

Key Words: *Escherichia coli* infections; inflammation; kidney failure, chronic; sevelamer; models, animal

ÖZET Amaç: Amacımız deneysel modelde böbrek yetmezliği (BY) durumunda sevelamerin antiinflamatuar etkisini araştırmak ve bunun gaitada *Escherichia coli* koloni sayısında azalma ile ilişkili olup olmadığını incelemektir. **Gereç ve Yöntemler:** Wistar albino, dişi, 200-250 g ağırlığında 18 rat üç gruba ayrıldı. BY, ratlarda 5/6 nefrektomi yapılarak sağlandı. Birinci grup kontrol grubu, 2. grup BY+ yüksek fosforlu (P) diyet alan grup, böbrek yetmezliği oluşturulan 3. grup yüksek fosforlu diyet + sevelamer alan gruptur. Gaitada *E. coli* sayımı yapıldı. Tüm ratlarda bazal, 1. hafta, 3. hafta ve 6. hafta kreatinin, kalsiyum, fosfor, interlökin-6 (IL-6), tömör nekrozis faktör-alfa (TNF-alfa) ve high-sensitif C-reactive protein (hsCRP) ölçümleri yapıldı. **Bulgular:** Grup 2 ve 3'ün kreatinin konsantrasyonları kontrol grubuna göre 1. haftada yüksek olarak bulundu (p=0,03, p=0,02), Grup 2'de, 3. haftada, *E. coli* sayısında artış vardı (p=0,05). Grup 3'te 1. haftada *E.coli* ve inflamasyon belirteçleri (IL-6, CRP, TNF-alfa), bazal değerlere göre belirgin artmıştı (sırasıyla p=0,042, p=0,021, p=0,042). Grup 3'te 1. haftaya göre 6. haftada *E. coli* sayısı (p= 0,02) ve inflamasyon belirteçlerinin değerleri (IL-6, hsCRP, TNF-alfa] ($p<0,05$) anlamlı düzeyde azalmış olarak bulundu. **Sonuç:** Sevelemerin antiinflamatuar etkileri bakteriyel kolonizasyon üzerine etkileri vasıtasıyla açıklanabilir.

Anahtar Kelimeler: *Escherichia coli* enfeksiyonları; inflamasyon; böbrek yetmezliği, kronik; sevelamer; modeller, hayvan

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According to the United States Renal Database, the mortality risk of endstage kidney disease (ESKD)is at least 10-20 times higher compared to the control group even after adjustments are made for the age and gender.[1] Approximately half of these deaths are due to cardiovascular causes.[1]

The correlation between chronic inflammation which is characterized by increased levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), high-sensitive C-reactive protein (hs-CRP) and oxidative stress-endothelial dysfunction has been demonstrated in patients with chronic kidney disease (CKD).[2] Furthermore, elevated serum levels of proinflammatory cytokines have been demonstrated to be associated with increased mortality in CKD.[3] Patient-specific processes, such as clotted access grafts, or persistent infections, such as *Chlamydia pneumoniae* and dental infections, may cause inflammation in ESKD patients. However, decreased renal clearance of proinflammatory cytokines, comorbidities (such as chronic heart failure), volume excess, accumulation of advanced glycation end-products (AGEs) and various factors associated with the dialysis procedure may also contribute to inflammation in CKD patients.[3] In these patients, the strong correlation between malnutrition, inflammation and atherosclerosis caused the definition of malnutrition-inflammation-atherosclerosis syndrome.[2,4] Sevelamer, a non-calcium based phosphorus binder was shown to decrease or slow the progression rate of cardiovascular calcification in patients with CKD. However, the mechanism of this calcification-inhibiting effect could not be completely clarified.[2] Decreased expression of serum fetuin-A was shown to be correlated with valvular calcification, atherosclerosis, malnutrition and inflammation. This decrease was also shown to be correlated with increased cardiovascular mortality and morbidity in patients with peritoneal dialysis through its close association with the florid expression of malnutrition, inflammation, atherosclerosis/cardiovascular syndrome.[5] In a short term study, it was shown that sevelamer treatment caused increased serum fetuin-A levels and improved flow-mediated vasodilation (which is an indicator of ameliorated endothelial dysfunction as shown by Doppler ultrasound[6]) in non-diabetic Stage 4 chronic kidney disease patients.[6] Endothelial dysfunction was shown to be a risk factor in apparently healthy patients for the development of atherosclerosis years before atheromatosis plaques were formed.[7] Furthermore, administration of the sevelamer to maintenance hemodialyis patients is associated with a significant decrease in hs-CRP, IL-6 and serum endotoxin levels. On the other hand, increased translocation of endotoxin to the systemic circulation was shown to be correlated with inflammation in various animal models.[8] Based on these facts, this study aims to clarify whether the anti-inflammatory effect of sevelamer, which occurred in RF-induced rats,[9] was accompanied by a decrease in *E. coli* colony count in feces.

MATERIAL AND METHODS

Female rats of Wistar albino species with a weight of 200-250 g were used in the study.[10] The rats were obtained from Dokuz Eylül University, Faculty of Medicine, Multidisciplinary laboratory, Experimental Animals Laboratory, and were sheltered there throughout the study. There were not any significant differences regarding the average weights of the rats between the groups. Photoperiod was administered to the study rats for 12 hours during the day and 12 hours at night.[11] RF was induced by executing 5/6 nephrectomy in Group 2 and 3 rats at the study initiation. 5/6 nephrectomy was performed under general anesthesia with ligation of one of the extrarenal branches of left renal artery and right nephrectomy.[7] The special food containing high phosphate (P) and high P+ sevelamer were manufactured in MBD Yem Ticaret in Gebze-Kocaeli. Eighteen rats were included in the study and were divided into three groups. Group 1, control rats (n=6) were fed with the standard rat food (pellet at a diameter of 10 mm) ad libitum. throughout the study. Group 2 rats were those with induced RF. They were fed with the standard rat food during the first week. At the end of the first week with the appearance of uremia, diet was changed to the high phosphate diet [0.9% P; 0.6%

Ca (calcium) diet] (n=6). Group 3 was the group (n=6) containing rats with induced renal failure. They were also fed with the normal diet during the first week, then their diet was switched to a high phosphate diet [0.9% P; 0.6% Ca (calcium) diet] ad libitum, but the following the appearance of hyperphosphatemia at the end of the 3rd week, these rats were fed with a high phosphate diet (0.9% P; 0.6% Ca diet) + (0.3%) sevelamer ad libitum.[12]

As the biochemical parameters; creatinine, Ca and P were studied by spectrophotometric method using autoanalyzer BeckmanCX800-with Beckman Coulter kits (Fullerton, USA). IL-6 (pg/ml) was measured using Invitrogen (Camarillo, USA) kit, TNF-alpha (ng/ml) with Invitrogen (Camarillo, USA) kit, and hs-CRP (pg/ml) using Immunology Consultans Lab inc. (Newberg, UAS) kit in Celal Bayar University, Faculty of Medicine, Department of Biochemistry. TNF values (9% in interassay Cv 135 pg/ml, 6.9% in intra assay Cv 130.7 pg/ml) and IL-6 values (7.2% in interassay Cv 62.5 pg/ml, 3.8% in intra assay Cv 59.4 pg/ml) were indicated in package leaflet of the kit.

E. coli count in feces was determined in Celal Bayar University, Faculty of Medicine, Department of Microbiology. *E.coli* count in feces was determined as follows:[13] Fecal samples, which were collected from each rat after defecation and placed directly into sterile containers, were sent to the laboratory within 1 hour. One gram (1 g/rat) feces were collected from each sample and mixed in 9 mL 0.9% saline by vortexing. The prepared suspension was diluted 10 times and then, 100 µl sample was inoculated onto three separate non-selective broths, McConkey agar, EMB agar and 5% sheep blood agar; plaques were incubated at 35 ^{0}C for 48 hours in aerobic media. During 24 hours of incubation, the plates were evaluated and the plates with growth were identified. Plates with no growth were re-incubated and re-evaluated after 48 hours. The growing colonies were quantified by counting *E. coli* colonies [colony forming unit/mL (CFU/mL)] and using classic microbiological methods as well as ready-to-use commercial kits (BBL Crystal GN; N/F ID, Becton Dickinson-USA).

Measurements of creatinine, Ca, P levels, hs-CRP, IL-6, TNF-alpha values and *E. coli* colony counts in feces were performed at the basal state and the end of the 1st, 3rd and 6th weeks.

Ethical approval of the study was obtained from Ethical Board of Dokuz Eylül University, Faculty of Medicine, Multidisciplinary laboratory, Experimental Animals Laboratory. The application form included a statement guarenteing strict observance to the animals' rights. Attention to this rule was paid throughout the study.

During calculation of creatinine clearance in rats, a standard formula was used. Creatinine clearance (ml/min)=($\text{Urinary}_{\text{creatinine}} \text{x} \text{Volume}_{\text{urine}}$)/ $\text{serum}_{\text{creatinine}}$ X 1440.[14]

STATISTICAL ANALYSIS

All data obtained in the study was analyzed using Wilcoxon Signed Ranks test, Friedman test, Kruskal-Wallis test and Mann Whitney U test [median(minimum-maximum)]. $p<0.05$ values were considered to be significant.

RESULTS

Levels of creatinine, Ca, P, IL-6, CRP, TNF-alpha and *E coli* count values of the rats are shown as mean± standard deviations according to the weeks in Table 1.

Basal values of each group were compared to 1st, 3rd and final week values using Wilcoxon Signed Ranks Test within the same group. With the exception of basal vs 3rd week creatinine and basal versus 6th week phosphate comparisons, no statistically significant change was observed in all parameters during 6 weeks in the control group (Table 1).

In Group 2 and 3, creatinine concentrations increased after renal failure induction (p=0.032 and p=0.021, respectively)$^{(I)}$ and remained significantly elevated in the 3rd (p=0.024, p=0.027 respectively)$^{(II)}$ and 6th weeks (p=0.038, p=0.027 respectively)$^{(II)}$ in comparison to their corresponding basal values (Table1).

When it comes to comparison among all groups (Friedman test), *E. coli* count correlated with renal failure was high in Group 2 (p= 0.004).$^{(III)}$ *E.coli* count, IL-6, CRP and TNF-alpha

TABLE 1: Mean± standard deviation values of creatinine, Ca, P, IL-6, CRP and TNF-alpha levels and *E. coli* counts of group 1, 2 and 3.

Rats	Basal	1st Week	3rd Week	6th Week
Creatinine (mg/dL)				
Group 1	0.3±0.05	0.3±0.05	0.4±0.01	0.3±0.04
Group 2	0.4±0.1	0.6±0.1(I)	0.6±0.07(II)	0.6±0.1(II)
Group 3	0.3±0.04	0.5±0.05(I)	0.6±0.04(II)	0.6±0.1(II)
Calcium (Ca) (mg/dL)				
Group 1	8.9±0.2	9.5±0.2	9.2±0.2	9.2±0.2
Group 2	9±0.3	9±0.2	9.1±0.3	8.7±0.2
Group 3	8.7±0.07	8.9±0.3	9.1±0.2	8.9±0.4
Phosphor (P) (mg/dL)				
Group 1	3.9±0.6	4.3±3.6	4.1±0.8	4.5±0.4
Group 2	4.1±0.3	5.1±0.3	6.3±0.7	6.5±0.5
Group 3	3.9±0.4	5.3±4.1	4.8±0.1	5.7±0.2
E. coli CFU/mL				
Group 1	$8.6x10^6$±$3.5x10^5$	$8.9x10^6$ ±$1.1x10^7$	$1x10^7$±$1.4x10^7$	$1x10^7$±$2.1x10^6$
Group 2	$2.8x10^7$±$1.7x10^7$	$9.6x10^{10}$±2x1011(III)	$9.3x10^9$±$2.3x10^8$	$1.1x10^{10}$±$1.8x10^9$
Group 3	$1.8x10^7$±$4.2x10^6$	$1.1x10^9$±$2x10^{10}$	$2.5x10^6$±$2.4x10^6$	$7x10^6$±$1.5x107^{(IV)}$
IL-6 (pg/mL)				
Group 1	12.4±7.2	13.5±6.5	16.1±8.3	15.5±25.4
Group 2	29.9±37.7	46.6±20.7	40.5±16.3	39.2±16.5
Group 3	23.3±23	49.3±34.4	8.7±4.8	14.1±10.6(IV)
CRP (ng/mL)				
Group 1	4.1±0.8	4±0.7	4±0.6	3.7±0.4
Group 2	4.4±0.3	5.3±1.2	3.5±0.7	3.5±0.6
Group 3	4.4±0.2	5.4±1.7	2.1±1.05	2.2± 0.5(IV)
TNF-alpha (pg/mL)				
Group 1	13.2±5.1	12.4±4.9	9± 4.8	5.9±6.1
Group 2	14±13.2	25±10.5	22.4± 9.1	23.7±5.5
Group 3	19.2±13.9	22.4±19.2(v)	9± 4.5	11.7±9.8(IV)

(I): p<0.05 (group 2 and 3-creatinine concentrations) 1st week compared to basal values;

(II): p<o.o5 (group 2 and 3-creatinine concentrations) 3rd and 6th week compared to basal values;

(III): p<0.05 (Group 2- *E.coli* count) 1st week compared to basal values;

(IV): p<0.05 (group 2 and 3- *E.coli* count, IL-6, CRP and TNF-alpha) 6th week compared to basal values;

(IV): p<0.05 (group 3- IL-6, CRP and TNF-alpha) 6th week compared to 1st week;

(v): p<0.05 (IL-6, CRP and TNF-alpha) 1st week compared to basal values.

values were low in Group 3 with the use of sevelamer compared to those in Group 2 (p= 0.004, p=0,009, p=0.002, p=0.002, respectively, Table 1).(IV)

In Group 3, IL-6, CRP and TNF-alpha levels in the first week were significantly increased in comparison to their basal values (p=0.042, p=0.021, p=0.042 respectively).(V) Again in Group 3, IL-6, CRP and TNF-alpha levels at the sixth week were found to be significantly decreased in comparison to those values of the 1st week (p=0.041, p=0.020 and p=0.041 respectively, Table 1).(IV)

Percent changes of the variables [(RD-basal)/basal, (3 week-basal)/basal, (6 week-basal)/basal] of group 3 were analyzed using Kruskal Wallis test. Statistically significant values were compared using Mann Whitney U test in a binary fashion. The results are shown in Table 2.

TABLE 2: % Changes in the variables of the group 3 at 1st, 3rd and 6th weeks were compared with basal values.

Variable Median (minimum-maximum)	% change in group 3 (Basal vs 1st week)	% change in group 3 (Basal vs 3rd week)	% change in group 3 (Basal vs 6th week)	P (Kruskal Wallis test)	Groups	P (Mann Whitney U test)
Creatinine	0.55(0.50-0.70)	0.6(0.6-0.7)	0.6(0.5-0.8)	0.258	A-B group	-
					A-C group	
					C-B group	
Ca	9.1(8.6-9.3)	9.15(8.9-9.5)	8.9(8.4—9.5)	0.471	A-B group	-
					A-C group	
					C-B group	
P	5.3(4.8-6)	4.8(4.7-5)	5.8(5.2-6)	0.002	A-B group	0.199
					A-C group	0.004
					C-B group	0.004
E.Coli	$2.2x10^8$	2 x 106	$7.9x10^5$	0.003	A-B group	0.004
	($3.1x10^7$-$5.2x10^9$)	($1x10^5$-$6.2x10^6$)	($2.8x10^5$-$3.8x10^7$)		A-C group	0.004
					C-B group	0.631
IL-6	36.2(28.1-117.2)	8.4(2.1-15.2)	12.4(2.1-31.5)	0.007	A-B group	0.004
					A-C group	0.025
					C-B group	0.262
CRP	4.5 (4.2-8.3)	2.1 (0.6-3.8)	2.2 (1.5-3.1)	0.003	A-B group	0.004
					A-C group	0.004
					C-B group	0.749
TNF-alfa	25.2 (15.2-67.2)	8.1 (4.2-14.5)	5.4 (3.4-9.1)	0.008	A-B group	0.164
					A-C group	0.006
					C-B group	0.394

(A= % change from basal to 1st week, B=% change from basal to 3rd week, C=% change from basal to 6th week).

Creatinine clearance was significantly lower (p=0.007) in Group 2 (0.26±0.07) and 3 (0.18±0.06) compared to control group (0.45±0.12).

DISCUSSION

Inflammation is correlated with increased cardiovascular mortality in hemodialysis patients.[2] Bergström et al. emphasized first in patients with hemodialysis that CRP could predict mortality.[15] Increased CRP levels, age, low body mass index and pre-existing cardiovascular diseases were defined as independent, strong predictors for all cause-related and cardiovascular mortalities in hemodialysis patients.[2] Recent studies have shown the correlation between general population which will experience coronary heart disease and acute phase response products such as CRP and sialic acid.[16,17] In dialysis patients treated with sevelamer, improvement in inflammatory parameters (increase in serum albumin levels, decrease in CRP, TNF-alpha, IL-10 levels) was observed.[18,19] In hemodialysis patients, it was determined that, with the use of sevelamer, CRP, LDL and total cholesterol levels further decreased in comparison to calcium-containing phosphorus binders.[20] In cases with ESKD with a very high cardiovascular mortality due to dyslipidemia and inflammation, the use of sevelamer has been a promising treatment method for improving hyperlipidemia and inflammation.[20] Similarly in our study, in rats with renal failure, inflammation markers increased (TNF-alpha, CRP, IL-10), but the use of sevelamer decreased these markers.

Garg et al. reported that sevelamer did not only decrease the phosphorus levels but also decreased low-molecular-weight uremic toxins such as uric acid in patients with ESKD.[21] Peres et al. suggested that the use of sevelamer as a phosphorus binder decreased reactive oxygen products thus contributed to the decrease in endothelial damage.[19]

In a study conducted by Nikolov et al. it was shown that the use of sevelamer decreased oxidative stress markers in uremic cases. The authors proved that in patients with end-stage renal failure, there was a decrease in nitrotyrosine, a local oxidative stress marker released from aorta as a response to sevelamer treatment.[22]

Peres et al. speculated that sevelamer prevented absorption of the substances which can stimulate the cells responsible for synthesis of proinflammatory cytokines with reactive oxygen types by forming chelates in intestinal lumen with these substances.[19] However, there is no literature information or study for the existence of such an effect of sevelamer.[19] It was considered that sevelamer decreased inflammation and oxidative stress in hemodialysis patients with the given chelation effect.[19] In another study, sevelamer was shown to be associated with decreased absorption of uremic toxin precursors in intestinal lumen.[23] Hauser et al. stated that in 5/6 nephrectomised rats sevelamer carbonate reduced systemic inflammation in association with reduced systemic endotoxin levels.[24] In a clinical study conducted by Sun et al. it has been shown that in chronic hemodialysis patients sevelamer hydrochloride is associated with a lower plasma endotoxin level supporting the hypothesis that it binds to endotoxin in the intestinal lumen.[25] In another study it has been demonstrated that in chronic hemodialysis patients sevelamer administered for 6 months decreased inflammatory parameters along with the paralel decreases in systemic endotoxin levels.[26]

In our study, we have shown that the fecal *E. coli* colony count, which increased with renal failure, decreased with the use of sevelamer and this was accompanied by decreases in inflammation markers. As far as we know, our study demonstrates for the first time that systemic endotoxin level-reducing effect of sevelamer might have been associated with the decreased colonic bacterial colonization. This effect might be considered as a more proximate one in comparison to the hypothesized endotoxin binding effect in the intestinal lumen or to the speculated decreased translocation of endotoxin to the systemic circulation, thus in our view giving more insight in to the mechanism of the systemic anti-inflammatory effects of the drug. Our study's major drawback is its small sample size. In our view, if it had been performed, the systemic endotoxin level measurement would have added little to the value of our study because decreased systemic endotoxin level secondary to sevelamer use in both animal and clinical studies can be regarded as an epiphenomenon due to decreased bacterial colonization, decreased endotoxin formation and thus decreased translocation of endotoxin to the systemic circulation. Another conclusion which can be derived from our study is that systemic inflammation in chronic renal failure might be associated with increased colonic bacterial colonization thus justifying the efforts aimed to find other novel antibacterial measures without precipitating the overgrowth of other pathogens.

In conclusion, systemic anti-inflammatory effects of sevelamer might be explained on the basis of its local effects on the colonic bacterial colonization. To elucidate the possible antibacterial-bactericidal effects of this drug, more elaborate studies are urgently needed.

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