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THE EFFECTS OF IRBESARTAN AND SPIRONOLACTONE IN PREVENTION OF PERITONEAL FIBROSIS IN RATS

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♦ ***Background:*** **Bacterial peritonitis episodes may disturb the functional and histological integrity of the peritoneum in peritoneal dialysis patients. The renin–angiotensin–aldosterone system may have fibrotic effects on the peritoneum.**
♦ ***Objective:*** **To study the effects of an angiotensin II receptor antagonist (irbesartan) and an aldosterone antagonist (spironolactone) in the prevention of peritoneal fibrosis in a rat model of bacterial peritonitis.**
♦ ***Methods:*** **40 Wistar rats were randomized into 5 groups: bacteria (B), bacteria–irbesartan (BI), bacteria–spironolactone (BS), bacteria–irbesartan–spironolactone (BIS), and control (C) groups. The C group received only dextran beads (Cytodex; Sigma Chemicals, St Louis, Missouri, USA); the others were given bacteria and dextran beads intraperitoneally. Irbesartan and/or spironolactone were given to 3 groups: BI, BS, and BIS. On the eighth day, the rats were sacrificed, peritoneal adhesion was quantified, and peritoneal tissue sections were evaluated histologically.**
♦ ***Results:*** **The peritoneal total adhesion score was significantly higher in the B group than in the BI, BIS, and C groups ($p < 0.01$). Mean peritoneal thickness, mean inflammation score, and mean fibrosis score were significantly higher in the B group in comparison to the C group ($p < 0.05$). Mean peritoneal thickness of all treatment groups was significantly lower than the B group ($p < 0.05$). Serum transforming growth factor beta-1 level was significantly higher in the B group than in the BI, BS, and C groups ($p < 0.05$).**
♦ ***Conclusion:*** **Irbesartan and spironolactone seem to decrease the extent of peritoneal injury caused by bacterial peritonitis.**

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KEY WORDS: Peritonitis; peritoneal fibrosis; irbesartan; spironolactone; TGF-β1.

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A morphologically and functionally intact peritoneum is required for long-term and effective peritoneal dialysis (PD) treatment (1). Continuous exposure to bioincompatible components of dialysis solution and repeated episodes of bacterial peritonitis play major roles in long-term changes in peritoneal function (ultrafiltration loss and increased solute clearance) (2). Alterations in peritoneal function are related to structural changes in peritoneal membrane (3,4) that include loss or degeneration of the mesothelium, submesothelial thickening (described as fibrosis or sclerosis), changes in the structure and number of blood vessels, and vascular basement membrane reduplication (5). More recent studies of peritoneal changes with time show an increase in the number of peritoneal vessels that is correlated with the amount of peritoneal fibrosis (3,6). Peritonitis is a common complication of PD (7). The response of the peritoneum to infective organisms involves inflammatory cytokines and interaction between resident cell populations of macrophages, mesothelial cells, and fibroblasts (8,9). An inflammatory model using adenoviral-mediated gene transfer of the proinflammatory cytokines showed that, by day 7 there was an increase in submesothelial thickness and collagen deposition, markedly increased cellularity, and an associated increase in neovascularization in peritoneal tissue (10). Although the mechanisms underlying these morphologic alterations in the peritoneum are not understood completely, growth factors and cytokines secreted by mesothelial cells, macrophages, and fibroblasts have been shown to be associated with peritoneal fibrosis (11,12). Injurious stimuli induce reactivation of the local renin–angiotensin system (RAS), primarily angiotensin II (Ang II), and also aldosterone, which initiates a process mediated by the cytokine transforming growth factor beta (TGF-β), resulting in tissue repair (13). Failure to terminate this repair process, characterized by continued hyperexpression of TGF-β and the

pathologic accumulation of extracellular matrix, is the hallmark of all fibrotic disorders (13–15).

Efforts to minimize the damage that occurs during the accelerated phase, that is, peritonitis attack, may be of use in maintaining longevity of the peritoneal membrane. There has been an increasing number of reports focused on prevention of peritoneal fibrosis in PD patients (16). Pharmacological interventions that can inhibit the proliferation of peritoneal mesothelial cells and/or matrix formation may be of use in preventing or retarding peritoneal fibrosis; various drugs and chemicals have been tried (17–20). Although there are some reports of the favorable effect of irbesartan, an Ang II receptor antagonist, on peritoneal membrane functions (21), there are not enough data concerning the effects of irbesartan and spironolactone, an aldosterone antagonist, in preventing peritoneal fibrosis.

In the present study, we aimed to test the efficacy of irbesartan and spironolactone, alone and in combination, in preventing peritoneal fibrosis secondary to bacterial peritonitis in an experimental model of nonuremic rats.

MATERIALS AND METHODS

Forty nonuremic Wistar rats (weighing 180 – 240 g) were included in the study. The animals were housed at a constant room temperature with a 12-hour light/dark cycle and allowed free access to water and standard laboratory diet. The study was approved by the Animal Studies Ethics Committee of the Dokuz Eylül University Medical School.

In this study, Hung's experimental model of bacterial peritonitis was applied (18). The *Staphylococcus aureus* strain ATCC 25923 (American Type and Culture Collection, Rockville, Maryland, USA) was used as an infectious agent. Before the experiment, fresh 6 – 9 hour cultures in a brain–heart infusion broth (Oxoid, Basingstoke, UK) were prepared in an agitated waterbath at 37°C. Serial dilutions were prepared and plated to determine the actual inoculum sizes. To enhance local bacterial infection in the rat peritoneal cavity, dextran beads (Cytodex; Sigma Chemicals, St Louis, Missouri, USA) were added to the bacterial suspensions for easier inoculation into the peritoneum, as described previously (18). Dextran beads were prepared by suspending 1 g in 50 mL of phosphate-buffered saline as a stock solution and were autoclaved before use. Portions of the suspension equivalent to 8 mg of dextran beads were added to the broth with bacteria (3×10^9 culture forming units), for groups I – IV, or without bacteria, for group V (see below) and then broth was added to a final volume of 1 mL to be used for inoculation.

EXPERIMENTAL PROCEDURES

Forty rats were randomized into five groups of eight rats each: group I, bacteria (B); group II, bacteria + irbesartan (BI); group III, bacteria + spironolactone (BS); group IV, bacteria + irbesartan + spironolactone (BIS); and group V, control (C). Rats in groups I – IV were given bacteria mixed with dextran beads intraperitoneally via a tuberculin syringe. Group V was given only dextran beads via the same route. Irbesartan or spironolactone, alone or in combination, was given to groups II – IV as appropriate. Group I, bacteria (B), was not given irbesartan or spironolactone. Group II, bacteria–irbesartan (BI), received irbesartan (100 mg/kg/day dissolved in 0.5 mL distilled water) for 7 days via an oro-gastric tube. The dose of irbesartan was adjusted according to Fabiani *et al.*, who demonstrated that the maximal inhibition of AT-1 receptors was achieved with 100 mg/kg/day irbesartan orally (22). Group III, bacteria-spironolactone (BS), received spironolactone (50 mg/kg/day dissolved in 0.5 mL distilled water) for 7 days via an oro-gastric tube. The spironolactone dose was adjusted according to a previous study in rats (23). Group IV, bacteria–irbesartan–spironolactone (BIS), received irbesartan and spironolactone at the same doses and route as above for 7 days, beginning with the administration of *S. aureus*. Group V, control (C), was injected intraperitoneally with dextran beads only via a 1-mL tuberculin syringe.

The study was completed on day 8. Under ketamine anesthesia (80 mg/kg), as described by Ghellai *et al.* (24), 1 mL phosphate-buffered saline was injected intraperitoneally using a 22-gauge needle. Following a gentle 1-minute abdominal massage, a tube was inserted into the peritoneal cavity through a midline incision. Avoiding leakage, peritoneal fluid was aspirated through a syringe. The peritoneal lavage fluid was centrifuged immediately (+4°C, 4000 rpm for 10 minutes) and the supernatants were stored at –70°C. Afterwards, a colleague who was unaware of the groups to which the rats belonged performed laparotomy. The gross adhesion scoring system defined by Hung *et al.* was used to assess the extent of peritoneal adhesion (18). Blood samples of 2.5 – 3 mL were drawn from the vena cava caudalis and serum samples were stored –70°C. The rats were sacrificed by hypovolemia and, together with the peritoneum, tissue samples were taken from the liver, small intestine, and abdominal wall away from the injection site.

MORPHOLOGICAL AND HISTOCHEMICAL ANALYSIS OF THE PERITONEAL CAVITY

There is no standard method for assessing the degree of peritoneal adhesion. We used a gross visual adhesion scoring system (18,24), a quick and semiquantitative

assessment of the fibrotic response, and measurement of connective tissue thickness (17–19) as a quantitative analysis of the degree of peritoneal fibrosis.

Gross Visual Scoring System of Peritoneal Adhesions: Four separate sites with the most prominent adhesion, [used by Hung *et al.* (18)] were evaluated for scoring. These areas were between the liver and omentum, between the omentum and intestinal wall, between the intestine and the abdominal wall, and between the intestine and the stomach. The complete absence of adhesions is scored as zero; score 1 indicates from 1 to 3 separate adhesions, score 2 indicates 3 or more distinctly separate adhesions, and score 3 indicates diffuse sheet-like adhesions. The highest possible total score for the 4 sites is 12.

Histological Analysis: The tissue samples together with peritoneum were fixed immediately in 10% (v/v) formalin for histological analysis. The paraffin-embedded tissues, cut into 5-μ thick slices, were stained by hematoxylin & eosin and Masson's trichrome dyes. The sections were examined by light microscopy by the same pathologist, who was unaware of the group to which each rat belonged. The presence of inflammation was scored semiquantitatively as follows: score 0, no inflammation; score 1, mild inflammation (*i.e.*, only a few scattered inflammatory cells were identified); score 2, moderate inflammation (*i.e.*, small groups of inflammatory cells in many high-power fields); score 3, severe inflammation (*i.e.*, many inflammatory cells either in a diffuse pattern or in large groups). The presence of fibrosis was evaluated semiquantitatively from Masson's trichrome-stained sections: score 0, small scattered areas of green staining; score 1, thin bands of green staining; score 2, thicker, connected bands of green staining; score 3, thick and dense areas of green staining.

Peritoneal thickness was evaluated quantitatively. The thickness of the connective tissue lying between the mesothelium and the liver surface, the intestinal wall, or the abdominal wall was evaluated objectively, as described previously (17,18). Three random areas of each tissue section were measured and 9 tissue sections per rat (3 from the intestine, 3 from the liver surface, and 3 from the abdominal wall) were examined. Peritoneal thickness in 3 different locations was analyzed separately and also together, regardless of location.

Chemical Analysis: The TGF-β1 content of peritoneal fluids and serum samples was determined using enzyme-linked immunosorbent assay kits (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

STATISTICS

All data are expressed as mean ± SEM. Statistical analyses were carried out using SPSS 11.0 software (SPSS Inc., Chicago, Illinois, USA) for Windows operating system (Microsoft, Redmond, Washington, USA) on a personal computer. Continuous variables were analyzed with the Kruskal–Wallis test, and the Mann–Whitney test with Bonferroni correction was used for post hoc analysis. Correlation among the parameters was tested by Spearman's rank test. The statistical significance level was set at $p < 0.05$.

RESULTS

One rat from the B group was withdrawn from the study because of a subcutaneous abscess due to leakage of inoculum into the subcutaneous tissue. One rat in the C group died at the beginning of the study owing to ether anesthesia. One rat in the BI group and one rat in the BIS group died during the follow-up period.

PERITONEAL ADHESION SCORING SYSTEM

Total peritoneal adhesion score was zero in the control group. It was significantly higher in the B group than that in the control group (4.00 ± 0.72 vs 0, $p < 0.01$), the BI group (4.00 ± 0.72 vs 0.57 ± 0.30, $p < 0.01$), and the BIS group (4.00 ± 0.72 vs 0.43 ± 0.30, $p < 0.01$). However, the scores for the BS group and the B group were not significantly different (0.88 ± 0.30 vs 4.00 ± 0.72, $p = 0.06$). There was no significant difference between the scores for the BI and BS groups, the BI and BIS groups, or the BS and BIS groups ($p > 0.05$).

HISTOLOGICAL ANALYSIS

When the peritoneal thickness of three different sites was analyzed separately, mean peritoneal thickness of liver surface was significantly lower in the BI (13.19 ± 6.49 μm), the BS (9.50 ± 5.6 μm), the BIS (49.34 ± 19.92 μm), and the C (1.16 ± 1.06 μm) group compared to that of the B group (393.78 ± 246.12 μm) ($p < 0.05$).

Mean peritoneal thickness of intestinal wall was lower in all treatment groups (BI: 19.78 ± 3.16 μm; BS: 291.88 ± 281.61 μm; BIS: 19.75 ± 3.05 μm) than in the B group (835.50 ± 302.29 μm), but these differences were not significant ($p > 0.05$). It was significantly lower only in the control group (17.38 ± 4.87 μm) versus the B group ($p < 0.05$).

Mean peritoneal thickness of abdominal wall was lower in the BI (61.59 ± 11.80 μm), the BS (47.64 ± 5.40 μm), and the BIS (71.29 ± 8.32 μm) groups than in the B group (171.48 ± 69.66 μm), but these differences were not significant ($p > 0.05$). It was significantly lower

only in the control group (29.70 ± 3.46 μm) compared to the B group ($p < 0.05$).

When all three peritoneal location data were analyzed together, the values for mean peritoneal inflammation, mean peritoneal fibrosis, and mean peritoneal thickness were significantly higher in the B group in comparison to the control group (1.19 ± 0.34 vs 0.23 ± 0.06, 1.47 ± 0.32 vs 0.85 ± 0.06, 465.58 ± 119.14 μm vs 16.08 ± 2.02 μm, respectively; $p < 0.05$). Mean peritoneal thicknesses in the treatment groups (BI, BS, and BIS) were significantly lower compared to that of the B group (31.52 ± 4.59 μm, 114.72 ± 92.66 μm, 46.79 ± 8.86 vs 465.58 ± 119.14 μm, respectively; $p < 0.05$). Mean peritoneal thicknesses in all groups are shown in Figure 1. The peritoneum of liver surface in all the rat groups is shown in Figure 2(a–e).

For the mean inflammation and mean fibrosis scores, there was no significant difference between the B group and each of the treatment groups (Table 1). The following parameters were positively correlated: total

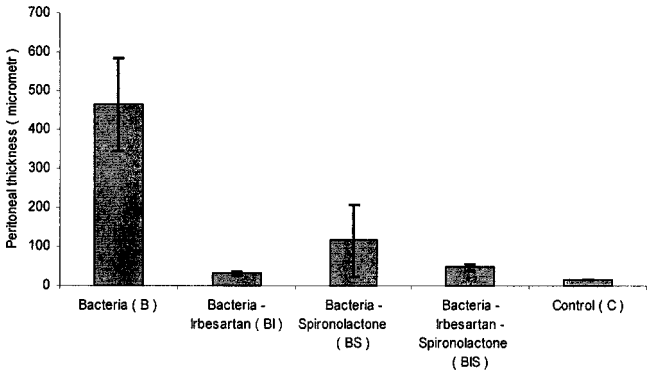


Figure 1 — Mean peritoneal thickness in all groups is shown (mean ± SEM). B versus C, B vs BI, B vs BS, B vs BIS, BS vs BIS: $p < 0.05$; BI vs BS, BI vs BIS: $p > 0.05$.

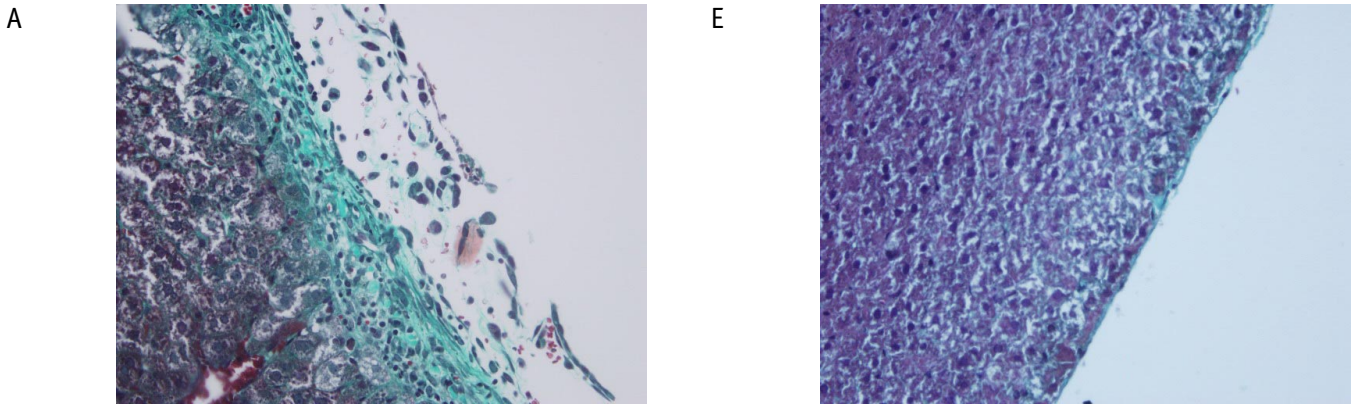


Figure 2 — Fibrotic reaction in the peritoneum of liver surface: Representative sections of Masson's trichrome-stained peritoneum demonstrate fibrotic thickening between the mesothelium and the liver surface in the bacteria group (A), the bacteria–irbesartan group (B), the bacteria–spironolactone group (C), the bacteria–irbesartan–spironolactone group (D), and the control group (E). (Original magnification ×40)

TABLE 1
Mean Peritoneal Inflammation and Mean Peritoneal Fibrosis Scores (Mean±SEM)

Group	Inflammation[a,b]	Fibrosis[a,b]
Bacteria (B)	1.19±0.34	1.47±0.32
Bacteria–irbesartan (BI)	0.66±0.19	1.28±0.15
Bacteria–spironolactone (BS)	0.79±0.22	1.12±0.20
Bacteria–irbesartan–spironolactone (BIS)	0.95±0.13	1.42±0.11
Control (C)	0.23±0.06	0.85±0.06

[a] B versus C: $p < 0.05$.
[b] B vs BI, B vs BS, B vs BIS, BI vs BS, BI vs BIS, BS vs BIS, BIS vs C: $p > 0.05$.

adhesion scores and inflammation scores ($r = 0.57$, $p < 0.01$); total adhesion scores and fibrosis scores ($r = 0.59$, $p < 0.01$); total adhesion scores and mean peritoneal thickness ($r = 0.52$, $p < 0.01$); mean peritoneal thickness and inflammation scores ($r = 0.70$, $p < 0.01$); mean peritoneal thickness and fibrosis scores ($r = 0.74$, $p < 0.01$); fibrosis scores and inflammation scores ($r = 0.70$, $p < 0.01$).

CHEMICAL ANALYSIS

Levels of TGF-β1 in the peritoneal washing fluid were similar in all groups. Levels of TGF-β1 in serum were significantly higher in the B group than in the control group; however, levels of TGF-β1 in serum were significantly lower in the BI and BS groups than in the B group. The difference between the B and BIS groups was not statistically significant. All the TGF-β1 data are shown in Table 2.

DISCUSSION

Episodes of peritonitis contribute to the development of peritoneal fibrosis in PD patients. In the present study, we used a rat model of peritoneal fibrosis and vehicle-enhanced staphylococcal peritonitis as described by Hung *et al.* (18).

When gross peritoneal adhesion scores were considered, as expected, no adhesion was observed in the control group and the score in the B group was highest of all and significantly different from the other groups, with the exception of the BS group ($p = 0.06$), which demonstrates the efficacy of the experimental model and the success of intervention to prevent deleterious effects of peritonitis. The lack of significant difference in gross peritoneal adhesion scores between the BS and B groups

TABLE 2
Mean Transforming Growth Factor Beta-1 (TGF-β1) Levels in Peritoneal Washing Fluids and Serum (Mean±SEM)

Group	TGF-β1 (ng/mL) Peritoneal washing fluid[a]	TGF-β1 (ng/mL) Serum[b]
Bacteria (B)	2.84±0.72	53.87±6.50
Bacteria–irbesartan (BI)	4.99±1.07	20.72±1.11
Bacteria–spironolactone (BS)	5.47±1.08	34.88±6.11
Bacteria–irbesartan–spironolactone (BIS)	3.15±0.99	38.46±7.42
Control (C)	2.30±0.43	17.15±2.04

[a] All comparisons: $p > 0.05$.
[b] B versus C, B vs BI: $p < 0.01$; B vs BS: $p < 0.05$; B vs BIS, BI vs BS, BI vs BIS, BS vs BIS: $p > 0.05$.

may reflect the limited number of rats in the study. Adhesion scores in all treatment groups were similar. The adhesion scores do not allow differentiation between the efficacies of different medications used in this study.

Mean peritoneal thickness was significantly higher in the B group and significantly lower in the control group compared to the other groups. The increased peritoneal adhesion and peritoneal thickness observed in the B group compared to the control group demonstrate the efficacy of the experimental model in damaging the peritoneal membrane. Additionally, the lower peritoneal adhesion scores and thinner peritoneum in rats given spironolactone and/or irbesartan than those of the B group demonstrate that these drugs were able to limit peritoneal injury due to acute bacterial peritonitis. This is the unique finding of our study.

As expected, in this study, mean peritoneal thickness was significantly lower in all treatment groups (BI, BS, and BIS) compared to that of the B group; similarly, when the thickness of each peritoneal site was analyzed separately, peritoneal thickness in all treatment groups was lower than that of the B group, but reached significant levels only in liver surfaces. Failure to get significant differences in intestinal wall and abdominal wall may be due to the low number of animals enrolled.

When peritoneal inflammation and fibrosis scores were considered, the only significant difference was between B group and the control group. With this method, the efficacy of either treatment was not documented. Mean fibrosis and inflammation scores for the groups administered drugs were numerically higher than those of the control group and lower than those of group B, but the differences did not reach statistical significance, which may reflect the semiquantitative nature of the

method and the short duration of the study. Additionally, peritoneal adhesion scores, mean peritoneal thickness, and inflammation and fibrosis scores were found positively correlated with each other. All these data show the efficacy of intervention in prevention of peritoneal fibrosis secondary to bacterial peritonitis in a rat model. Although Coronel *et al.* reported favorable effects of irbesartan on peritoneal membrane function (21), data are lacking concerning the efficacy of irbesartan and spironolactone with respect to peritoneal fibrosis directly. Duman *et al.*, in a rat model of PD, showed that peritoneal function and morphology could be partially preserved with enalapril, lisinopril, and valsartan thorough Ang II-related mechanisms (19,20).

Considering our data together with those of Coronel *et al.* and Duman *et al.*, we propose potential clinical benefits of an angiotensin-converting enzyme inhibitor and an angiotensin receptor blocker for protection of peritoneal integrity and function in PD patients. The use of an angiotensin receptor blocker and/or spironolactone during an episode of bacterial peritonitis may add further benefit.

Our study shows that the drugs used were able to limit peritoneal damage due to bacterial peritonitis; however, elucidation of the underlying mechanisms is beyond the scope of this study.

There is strong evidence that Ang II and/or aldosterone cause fibrosis directly by increasing collagen synthesis, and cellular proliferation by inducing growth factors (25,26). However, the effects of the same pathogenetic mechanism in peritoneal fibrosis have not been investigated thoroughly. Angiotensin II plays a role in the activation of TGF-β1 secretion in macrophages and fibroblasts (11,25). Some experimental studies show that Ang II blockade results in a decrease in TGF-β1 expression and matrix accumulation (27,28). According to those results, it is thought that locally increased Ang II may contribute to the pathogenesis of interstitial fibrosis via direct interaction with fibroblasts.

The mechanisms of the pathologic changes occurring at the peritoneum in PD are not understood completely. However, peritoneal mesothelial cells, macrophages, and fibroblasts are involved in inflammation of the peritoneum. They also take part in extracellular matrix formation and remodeling via growth factors and cytokines they secrete (11,12). Transforming growth factor-beta1 is a significant growth factor in extracellular matrix modulation (13). It is suggested that frequent attacks of peritonitis may cause the permanent presence of TGF-β1 in dialysate and TGF-β1 mRNA expression in peritoneal mononuclear cells, which may be a clue to peritoneal fibrosis development in patients with PD (29). In our study, we failed to observe any change in the level of TGF-β1 in the peritoneal washing fluid on day 8. Ghellai *et al.* reported that the total level of TGF-β1 in peritoneal fluid did not differ significantly in a peritonitis model. However, they demonstrated that the expression of TGF-β1 mRNA in adhesions peaked at day 4 (24). In addition, it was previously reported that, in patients with infrequent attacks of peritonitis, a high level of TGF-β1 in the dialysate was detectable in the early stages of peritonitis and that it decreased rapidly. However, it was observed that patients with recurrent attacks of peritonitis had persistently high levels of peritoneal TGF-β1, together with sustained TGF-β1 gene overexpression (29). In our model, the single and short episode of peritonitis may be the explanation for the stable level of TGF-β1 in the peritoneum. On the other hand, similar to Ghellai's study, we documented that the levels of serum TGF-β1 in the B group were highest and statistically different from the rest of the groups, and that it was found to be decreased significantly in the BI and BS groups. However, the decrease in the level of TGF-β1 in the BIS group did not reach significance, which may be due to the small number of rats in the study. Increased levels of TGF-β1 in the serum reflect systemic inflammatory response secondary to peritonitis attack and a considerably longer plasma half-life.

There is accumulating evidence showing that aldosterone has a direct role in fibrosis via a direct cellular effect on vascular smooth muscle cells and mesangial cells (30). Aldosterone perpetuates myocardial (31) and vascular fibrosis (32) and nephrosclerosis (26,33), whereas aldosterone receptor antagonist reduces these processes (34). In our study, peritoneal adhesion scores and mean peritoneal thickness in rats given aldosterone were decreased, demonstrating for the first time the beneficial effects of aldosterone in preserving the peritoneum. Failure to observe significant benefits in the BIS group necessitates further work. Angiotensin II-related mechanisms, as well as aldosterone, may play an important role in peritoneal injury related to bacterial peritonitis, and the mechanism of action is probably through these routes. On the other hand, potential antibacterial effects of the drugs are a matter of debate.

In conclusion, the histopathological data demonstrate that irbesartan and spironolactone decrease peritoneal injury due to bacterial peritonitis. The fact that the effects of irbesartan and spironolactone were not additive suggests that there may be a common mechanism of action. Therefore, irbesartan or spironolactone may be used for prevention of peritoneal injury during peritonitis attacks in peritoneal dialysis patients.

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