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The Effects of Aldosterone Inhibition on Peritoneal Membrane Dearrangements in Encapsulated Peritoneal Sclerosis Model

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Abstract

Background. Encapsulated peritoneal sclerosis (EPS) is the leading cause of long term peritoneal membrane failure. Epithelial to mesenchymal transition (EMT) via increased peritoneal inflammatory and fibrotic cytokine activity underlies the pathophysiology.
The effects of aldosterone, a well-known inflammatory and fibrotic mediator, inhibition with Aldactone® (ALDA) on EMT and membrane functions were investigated in our rat EPS model.
Methods. Forty non-uremic wistar albino rats were divided into four groups as fallow: 2 ml isotonic saline intra peritoneally (IP) daily, 3 weeks (w), (Control group); Daily IP 2 ml/200 g injection of chlorhexidine gluconate (0.1%) and ethanol (15%) dissolved in saline , 3 w, (CG group); CG + additional 3 w without any treatment, total 6 w, (Resting group); CG + additional 3 w 100 mg/kg daily ALDA in dirinking water, total 6 w (ALDA group).
At the end of the study, one hour PET was performed. Dialysate cytokine and osteopontin levels were measured. Functional parameters and morphological changes were examined.
Results. ALDA renewed ultrafiltration (UF) failure, D_1/D_0 glucose levels and dialysate protein loss (Dx prot). Peritoneal thickness, WBC count and inflammation of peritoneum were also decreased with ALDA treatment. ALDA improved over-expression of dialysate transforming groth factor (TGF-β1), monocyte chemoattractan protein (MCP-1), vascular endothelial growth factor (VEGF) and osteopontin levels as compared to resting.
Conclusion. In conclusion, ALDA may preserve membrane viability in long term peritoneal dialysis patients via inhibition of EMT through dialysate cytokine inhibition.

Keywords: Aldosterone inhibition, EPS, rat model

Introduction

Encapsulated peritoneal sclerosis (EPS) is the leading cause of long term peritoneal membrane disassembly characterized by thickening and fibrous band formation of peritoneum. Both inflammation and fibrosis underlies the pathophysiology of EPS. Epithelial to mesenchymal transition (EMT) through increased peritoneal inflammatory cytokine activity take place in the core of this mortal complication [1,2].
Bioincompatible dialysis solutions and recurrent peritonitis attacks with high glucose exposure have been implicated in the pathogenesis. Oxidative stress due to the uremic state with increased TGF-beta$_1$, fibronectin and vascular permeability factor expressions in peritoneal mesothelial cells have been demonstrated [3-5].
Classically we know that aldosterone interact with transport epithelia in distal convulated tubule in the nephron. The main effect is the sodium reabsorbtion and potassium excretion along the luminal surface of the last part of distal convulated tubule through the epithelial sodium channel and potassium channel [6]. However in recent years not only transport epithelia but also endothelial cells, fibrocytes and some other inflammatory cells have been identified in the concept of aldosterone targeting. It is clear that various organs, including heart, brain and vessels, and many tissues can express aldosterone receptors. This refers that aldosterone acts in a complexity of endocrine and paracrine fashion. It has been showed that aldosterone inhibition can prevent the activation of inflammatory mediators, such as NF-κβ, TGF-β1, PAI-1, ROS, ICAM and VCAM activation in many organ systems [7-11]. More recently, the potential benefits of aldosterone inhibition on peritoneal functions, peritoneal fibroblasts and mesothelial cells have been reported [12-14].
Here in the present study we determined the effects of aldosterone inhibition on the functional and structural parameters of peritoneal membrane which were disrupted due to EPS. Dialysate cytokine over-expression and osteopontin, an EMT marker, were also evaluated in experimental EPS model.

Materials and methods

Study protocol

Non-uremic Wistar-Albino male rats (n=40;weight 175-200g) which housed in polycarbonate cages under 24°C

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room temperature with 12 hour light/dark cycle and feeding with standard laboratory diet were divided into four groups. The Animal Ethics Committee of Ege University Hospital approved the study design.

The four groups of rats; (Group I, sham) Control rats (n=10) were used the characterized intact peritoneum received 2 ml isotonic saline intraperitoneally (IP) daily, 3 weeks without any other treatment. (Group II) Chlorhexidine Gluconate (CG) rats (n=10), received daily IP 2 ml/200 g injection of chlorhexidine gluconate (0.1%) and ethanol (%15) dissolved in saline, 3 weeks. (Group III) Resting rats (n=10), CG 3 weeks plus additional 3 weeks without any treatment, total 6 weeks. (Group IV) ALDA: Aldosterone inhibitor [(Aldactone®, Ali Raif Pharmaceuticals, İstanbul, Turkey); 100 mg/kg body weight (BW)] rats (n=10), CG 3 weeks with additional 3 weeks daily 100 mg/kg ALDA in drinking water, total 6 weeks.

At the end of study one-hour peritoneal equilibration test with 25 ml 3.86% PD solution (Dianeal 3.36%; Eczacibasi-Baxter Healthcare, Istanbul, Turkiye) was performed. After 1 hour, ketamine HCL anesthesia (60 mL/kg BW) was applied and immediately blood samples collected through direct cardiac puncture and dialysate samples through midline incision without any dialysate solution leakage via insertion of shortened dialysis catheter.

Functional Parameters

Dialysate protein was determined using an enzymetic kinetic method (Randox Laboratories, San Francisco, California, USA) to calculate dialysate protein level (g/L). The D_1/D_0 glucose ratio was measured according to ratio of glucose levels of drained dialysate to uninfused dialysate. The net ultrafiltration (UF) was calculated as the difference between the instilled and the drained dialysate volumes.

Structural Parameters

The peritoneal membrane samples were fixed in 10% formalin and embedded in paraffin wax. Paraffin blocks were divided into sections in size of 5 micrometer in thickness and then stained with hematoxylin-eosin and Masson trichrome. All samples were examined by the same pathologist who was unaware of the nature of the samples originated from groups. Peritoneal thickness, dialysate leukocyte count, inflammation and fibroblastic activity were evaluated. The former was measured with an ocular micrometer and the others were defined as counting capillaries, mononuclear cells and fibroblasts per high power field at 400x magnification

Dialysate cytokine profile (ELISA)

Dialysate cytokines; transforming growth factor beta-1(TGF-β1), vascular endothelial growth factor (VEGF) and monocyte chemotactic protein 1(JE/MCP-1) and osteopontin were measured by ELISA kit purchased from R&D Systems, Inc. Minneapolis, MN 55413 United States Of America. To avoid inter assay variation, plasma samples from all three rat groups were measured on a single ELISA plate.

Statistics

Study results are presented as mean ± standart error of the mean (SEM). The statistical analyses were performed using ANOVA, unpaired t-test, and the Mann-Whitney U test. A p value of less than 0.05 was considered significant

Table 1. Functional parameters of peritoneum

	Control n=10	**CG n=10**	**REST n=10**	**ALDA n=10**
UF, mL	7.3±0.3	0.3±0.8[a]	3.1±0.8[ab]	6.1±0.8[bc]
D1/D0 glucose	0.4±0.0	0.17±0.0[a]	0.3±0.0[b]	0.32±0.0[ab]
Dx prot g/L	0.6±0.0	3.23±0,3[a]	2.22±0.3[ab]	1.55±0.3[a]

ALDA= Aldactone, aldosterone inhibitor; Dx prot (g/L)= Dialysate protein level; D1/D0= ratio of dialysate concentration after 1 hour to initial dialysate concentration; UF=ultrafiltration;
(a) p<0.05 group compared with Group control; (b) with Group CG; (c) with Group Rest

Results

Peritoneal functional and structural changes were shown in Table 1 and 2. According to our results CG has an harmful effect on functional and structural assembly in peritoneal membrane. As mentioned in Table 1; CG resulted in significant UF failure as compared to control group (0.3±0.8ml vs 7.3±0.3ml, p<0.05). Resting has beneficial effects on UF failure (3.1±0.8 ml vs 0.3±0.8 ml, p<0.05) as compared to CG. Three weeks of Alda therapy significantly reversed UF failure as compared to resting (6.1±0.8 ml vs 3.1±0.8 ml, p<0.05). The Dialysate protein level which increased with CG (3.23±0,3 vs 0.6±0.0, p<0.05) as compared to control, has improved significantly with resting (2.22±0.3 vs 3.23±0,3, p<0.05) as compared to CG. Alda treatment also improved dialysate protein leakage as compared to resting (1.55±0.3 vs 2.22±0.3, p=NS). As the same manner, D1/D0 glucose ratio has been worsened with CG (0.17±0.0 vs 0.4±0.0, p<0.05) as compared to control. D1/D0 glucose ratio has been improved with resting of peritoneum (0.3±0.0 vs 0.17±0.0, p<0.05) as compared to CG. However Alda treatment has no beneficial effects (0.32±0.0 vs 0.3±0.0, p=NS) as compared to resting.

Table 2. Structural parameters of peritoneum

	Control n=10	CG n=10	REST n=10	ALDA n=10
Peritoneal thickness, µm	11.8±2.2	160.6±7.3[a]	120.7±9.8[ab]	116.5±13.9[ab]
WBC, mm3	739±47	1025±125	727±99	175±35[abc]
Inflammation	0.0±0.0	1.1±0.1[a]	1.5±0.1[ab]	1.4±0.2[a]
Fibrosis	0.1±0.0	1.2±0.1[a]	2.0±0.2[ab]	2.1±0.2[ab]

ALDA= Aldactone, aldosterone inhibitor; WBC, mm3= Dialysate leukocyte count; Thickness was measured with an ocular micrometer, fibroblastic activity was defined as counts of fibroblasts and inflammation was defined as counts of mononuclear cells per high power field at X400 magnification after peritoneal membrane samples were being fixed in 4% formalin and embedded in parafin waxes following cut into sections 5 µm in thickness and then stained with hematoxylin and eosin; CG, Chlorhexidine gluconate; Rest, peritoneal resting group; ALDA, Aldactone, aldosterone inhibitor group.
(a) p<0.05 group compared with Group control; (b) with Group CG; (c) with Group Rest

Structural changes were shown in Table 2. Increased peritoneal thickness was improved with octreotide treatment as compared to resting and CG (116.5±13.9µm vs 120.7±9.8 µm, p>0.05, vs 160.6±7.3 µm, p<0.05) although still high as compared to control (116.5±13.9µm vs 11.8±2.2µm, p<0.05). The other parameters; dialysate WBC cell count and inflammation but not fibrosis were also improved with OCT as compared to resting (175±35 vs 727±99, p<0.05; 1.4±0.2 vs 1.5±0.1, p=NS and 2.1±0.2 vs 2.0±0.2; p=NS).

Table 3. Dialysate cytokine measurement

	Control n=10	CG n=10	RESTING n=10	ALDA n=10
TGF-β1, pg/mL	4230±103	7133±539[a]	6870±852[a]	4451±159[b,c]
MCP-1, pg/mL	34.3±3.2	443.7±28.2[a]	223.9±82.3[a,b]	212.6±34.2
VEGF, pg/mL	22.0±2.4	62.6±2.4[a]	51.0±4.8	48.6±3.9
Osteopontin, pg/mL	352.7±23	619.0±34.7[a]	501.5±67	392.0±41.4

CG, Chlorhexidine gluconate; Rest, peritoneal resting group; ALDA, Aldactone, aldosterone inhibitor group.
(a) p<0.05 group compared with Group control; (b) with Group CG; (c) with Group Rest

Dialysate cytokine measurements by ELISA were shown in Table 3. CG, significantly increased the dialysate proinflammatory cytokines, TGF-β1 MCP-1 and VEGF, and osteopontin levels (7133±539pg/ml vs 4230±103, p<0.05; 443.7±28.2 vs 34.3±3.2, p<0.05; 62.6±2.4 vs 22.0±2.4, p<0.05 and 619.0±34.7 vs 352.7±23, p<0.05) as compared to control. Especially dialysate TGF-β1 and MCP-1, VEGF and Osteopontin concentrations clearly improved with OCT as compared to resting (4451 ±159 pg/ml vs 6870±852, p<0.05; 212.6±34.2 vs 223.39 ±82.3, p>0.05; 48.6±3.9 vs 51.0±4.883, p>0.05 and 392.0±41.4 vs 501.5±67, p>0.05).

Discussion

Aldosterone inhibition renewed ultrafiltration (UF) failure, and dialysate protein loss (Dx prot). Peritoneal thickness, WBC count and inflammation of peritoneum were also decreased with ALDA treatment. ALDA improved over-expression of dialysate transforming growth factor (TGF-β1), monocyte chemoattractan protein (MCP-1), vascular endothelial growth factor (VEGF) and osteopontin levels as compared to resting (Table 1, and figure 1).

Aldosterone is a novel fibrotic, inflammatory, proliferative and fibrotic agent. Multitude of effects of aldosterone has been appreciated in recent years. Kramer *et al.* recently reported that aldosterone inhibition has reduced glomerulosclerosis with combining diminished tubulointerstitial damage markers in experimental nephrosis model [15]. The effects of aldosterone inhibition on about endothelium and heart failure [16], prevention of ROS generation and CsA nephrotoxicity in uremic rat model [17,18] and finally increased peritoneal membrane permeability [12] have been showed.

In harmoniously with these trials, aldosterone inhibition significantly improved ultrafiltration failure, dialysate WBC cell count and TGF-β1 activity. Aldosteron inhibition also reversed deteriorated dialysate cytokine levels as seen in figure 1 that includes marked decreased in dialysate VEGF and MCP-1 levels. In addition, Aldosteron inhibition decreased osteopontin, a mesenchymal marker, level. Epithelial-to-mesenchymal transition (EMT), underlies the moleculer basis of EPS. In this process, mesothelial cells are transformed into myofibroblasts which plays central role in fibrogenesis. TGF-β1 and VEGF are the major responsible inducer cytokines of this differentiation. Both of them are responsible for the main steps of the development of fibrotic phenotype of peritoneal membrane and the main inducer molecules of eNOS [2]. If we assume that aldosterone inhibition may have capable of inhibiting dialysate TGF-β1 activity and osteopontin, we can speculate that Aldosteron inhibition may inhibit EMT.

EPS is the most dangerous long term complication of peritoneal dialysis firstly described by Gandhi *et al.* [19]. The main pathophysiological process that takes place in EPS is the loss of mesothelial cell layer and fibrosis of the submesothelial compact zone with increased neovascularization in submesothelial region. Fibro-

sis results in decreased effective area for dialysis and the neoangiogenesis leads to loss of ultrafiltration via disappearing of osmotic gradient. In order to produce EPS model, we injected 0.1% chlorhexidine gluconate and 15% ethanol dissolved in saline solution intraperitoneally totally three weeks. Io *et al*. reported that 3 weeks CH injection intraperitonealy is sufficient to induce marked neovascularization, mRNA expression of VEGF, angiotensin-1 and angiotensin-2 and accumulation of $CD_{34}(+)$ cells in peritoneum [20,21]. CG has severely disrupted ultrafiltration capacity, D1/D0 glucose ratios and dialysate protein leakage. Peritoneal membrane inflammatory activity, dialysate cell count and fibroblastic activity also were increased with CG. Possibly decreased effective dialysis area due to the fibrosis and increased inflammatory neo-angiogenesis ultimately resulted in UF failure (Table 1).

A peritoneal resting phenomenon about the prevention or regression of EPS is conflictive approach. Alvaro *et al*., Rodrigues *et al*. and Zareie *et al*. reported that peritoneal resting is beneficial in peritoneal hyperpermeability and ultrafiltration failure in vivo [22-24] and Tomo *et al*. in-vitro. Some of the authors suggest that intravenous hyperalimentation with bowel resting and transferred to patients into hemodialysis may be a treatment choice in patients with EPS [25]. On the other hand Kawanishi *et al*. reported that after cessation of peritoneal dialysis there is still continuing risk for developing for EPS [26]. Significant amount of patients with EPS can develop the syndrome an average of 4 months after withdrawal from PD therapy which may be as long as 4 years. These conflicting results have to been clarified before to constitute resting is beneficial. We believe that degree of injury correlated with longevity of PD is the major determinant of this topic.

Because of the EPS is a progressive process, after one point that has to be defined not yet, progression may be irreversible. Our results about resting of peritoneum have beneficial effects. On the other hand dialysate cell count and fibrosis during resting period continued to be increase. Aldosterone inhibition has no benefits on fibrosis. Decreased dialysate leukocyte count and significantly better TGF-β1 profile with decreased osteopontin in aldosterone inhibition carries more promosing challenge as compared to resting. However it is also important to point out that our experimental model composed of severe peritoneal injury made by CG.

We took tissue samples other than region of injection areas in peritoneal cavity. Results of our histological assessment were in parallel to results of structural parameters and dialysate cytokine counts. Our experimental EPS model may represent the irreversible injury that can not be reversed with resting. We believe that EPS process continues to progress during resting period. Aldosterone inhibition may be a future therapeutic agent long term peritoneal integrity.

Conclusion

Although the prevalence of EPS is low, it is progressive disease carries high mortality and morbidity rates. Aldosterone inhibition has beneficial effects in some clinical conditions including renoprotection and cardiovascular protection. If we assume that peritoneal dialysis patients are under the risk of cardiovascular complications and the residual renal function is important for the volume control in these populations, aldosterone inhibition may have survival advantage beyond its membrane protective properties. Preventive use of aldosterone inhibition at early phases of long term peritoneal dialysis vintage may be beneficial for both peritoneal membrane which is under deleterious effects of dialysis procedure and patients who are under cardiovascular complications.

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Conflict of interest statement. None declared.

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