The Effect of Steroid-Sirolimus Combination on the Experimental Model of Encapsulating Peritoneal Sclerosis in Rats

Steroid-Sirolimus Kombinasyonunun Sıçanlarda Oluşturulan Deneysel Enkapsüle Periton Sklerozu Üzerine Etkisi

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ABSTRACT Objective: Encapsulating peritoneal sclerosis (EPS) is a devastating complication of peritoneal dialysis terminating with peritoneal sclerosis and coccooning of intestinal loops. The inhibitors of mammalian target of rapamycin (mTOR), everolimus and sirolimus, have attenuated EPS findings in experimental animal models. The effect of combination of sirolimus with steroid has not been documented so far. The aim of the study was to determine the effect of combination of sirolimus and steroid on experimental sclerosing peritonitis model. **Material and Methods:** 41 wistar albino male rats were divided into 6 groups : control group (C; isotonic saline injected intraperitoneally), chlorhexidine gluconate group (CG; model group), resting group (R; CG then peritoneal rest, prednisolone group (P; CG then prednisolone), sirolimus group (Sir; CG then sirolimus), and prednisolone-sirolimus group (P-Sir; CG then prednisolone plus sirolimus). Peritoneal specimens obtained after sacrification at the end of study were examined for peritoneal thickness, fibrosis, and vascular intensities under light microscopy. **Results:** In the CG and R groups there was a significant increase in peritoneal thickness, fibrosis score and vascular intensity compared to C, P, Sir, and P-Sir groups in both parietal and visceral peritoneum ($p<0.05$). The parameters at the end of the study were not different in C, P, Sir, and P-Sir groups. The difference between P, Sir, and P-Sir groups were not significant. Resting was shown to be ineffective in attenuating EPS parameters. **Conclusion:** In this study we observed that sirolimus-prednisolone combination was equally effective in experimental EPS model compared to prednisolone and sirolimus only regimens.

Key Words: Peritonitis; sirolimus; models, animal; adrenal cortex hormones; peritoneal dialysis

ÖZET Amaç: Enkapsüle periton sklerozu (EPS), periton sklerozu ve intestinal segmentlerin koza şeklinde sarılması ile sonuçlanan ciddi bir periton diyalizi komplikasyonudur. Memelilerde rapamisin hedefi inhibitörleri (mTOR), everolimus ve sirolimus, deneysel hayvan modellerinde EPS bulgularını azaltmışlardır. Steroid-sirolimus kombinasyonunun etkisine yönelik yapılan bir çalışma bulunmamaktadır. Bu çalışmada deneysel sklerozon peritorit modelinde sirolimus ile steroid kombinasyon tedavisinin etkisini belirlemek amaçlanmıştır. **Gereç ve Yöntemler:** 41 wistar albino erkek sıçan 6 gruba ayrıldı: kontrol grubu (C; intraperitoneal izotonik serum), klorheksidin glukonat grubu (CG; model grup), dinlenme grubu (R; CG sonrası periton dinlenme), prednizolon grubu (P; CG sonrası prednizolon), sirolimus grubu (Sir; CG sonrası sirolimus) ve prednizolon-sirolimus grubu (P-Sir; CG sonrası prednizolon ve sirolimus). Periton örnekleri ışık mikroskobisi ile; periton kalınlığı, fibrozis skoru ve damar yoğunluğu için değerlendirildi. **Bulgular:** CG ve R gruplarında C, P, Sir ve P-Sir gruplarına kıyasla periton kalınlıkları, vasküler yoğunluk ve fibrozis skorları anlamlı olarak artmıştı ($p<0.05$). C, P, Sir ve P-Sir grupları arasında fark yoktu. P, Sir ve P-Sir grupları arasında fark anlamlı bulunmadı. Dinlenme; EPS parametrelerini geriletmede etkisiz saptandı. **Sonuç:** Bu çalışmada deneysel EPS modelinde sirolimus-prednizolon kombinasyonunun tek başına sirolimus veya prednizolon tedavileri ile eşit etkinlikte olduğu gözlenmiştir.

Anahtar Kelimeler: Peritonit; sirolimus; modeller, hayvan; adrenal korteks hormonları; periton diyalizi

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Peritoneal dialysis (PD) is long established as an initial recommended choice of renal replacement therapy in patients with end-stage renal disease. Despite its clear advantages such as preserving residual renal functions better, affording better homeostatic stability, providing social independency, and low risk of infections, it has some serious complications that limits it for long-term use.[1,2] Major complications are infectious peritonitis episodes, and highly fatal encapsulating peritoneal sclerosis (EPS).[1-5]

EPS is a devastating clinical syndrome characterized by fibrosis of peritoneal membrane and is associated with adhesive obstruction of bowel loops (cocooning), weight loss, malnutrition, and hemorrhagic ascites. Overall the incidence of EPS in PD patients is stable at 1-3%.[3,6,7] Major risk factors are PD itself, duration of PD, exsposure to bioincompatible dialysis solutions, discontinuation of PD, infectious peritonitis attacks, and renal transplantation on probable genetic susceptibility.[3,5,8,9] The pathologic culprit is epithelial-to-mesenchymal transition (EMT) of mesothelial cells that results in tissue remodeling from an inflammatory to a fibrotic state.[4,10-12] Major stimulatory factors are proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-18), transforming growth factor-β1 (TGF-β1), and vascular endothelial growth factor (VEGF).[10-15] Fibrosis itself constitutes a hypoxic environment that also directly up-regulates TGF-β1 and induces angiogenesis via hypoxia-inducible factors (HIF). HIF1α has been linked to EMT by regulating metalloproteinases (MMPs) and the zinc finger regulatory proteins (Snail), also have significant fibrogenic and angiogenic effects.[16-18]

Inhibiton of the mammalian target of rapamycin (mTOR), which is a major controller of cell growth through phosphoinositine 3-kinase pathway (PI3K), has antiproliferative activity carried outby blocking clonal proliferation and expansion of stimulated lymphocytes. In addition to lymphocytes mTOR inhibiton acts antiprolifrolatively for vascular smooth muscle cells, mesangial cells, and endothelial cells as well.[19] Rapamycin, inhibitor of mTOR, has been shown to down-regulate HIF1α, and blocks the effects of TGFβ on EMT in mesothelial cell culture.[17] In a previous study by Duman et al, another m-TOR inhibitor everolimus was shown to decrease parietal peritoneal thickness, fibrosis, and neovascularization in experimental EPS model.[20]

Currently there is no well-established management strategy for EPS in PD patients. There is increasing evidence that in experienced hands surgery results in high rates of improvement in symptoms and survival.[1,3,5] So far the effectivity of a variety of immunosuppressives has been reported in both animal models of EPS and in clinical case reports.[19,21-23] Steroids alone were shown to be efficacious in the treatment of EPS, but in many cases combination of steroids were administered.[24]

The present study was designed to examine the effect of sirolimus-steroid combination on pathogenetic parameters in experimental EPS model developed in rats.

MATERIALS AND METHODS

The Animal Ethics Committee of Dokuz Eylul University Hospital approved the study design (no: 57/2010). The study was performed on 41 nonuremic Wistar albino male weighing between 200 g and 250 g. They were housed in polycarbonate cages under room temperature (24°C) with a 12-h light/dark cycle and fed ad libitum.

EPS model used intraperitoneal (ip) injection of 0.1% chlorhexidine gluconate (CG) and 15% ethanol dissolved in saline. The rats were divided into six groups as follows:

1) control group (C, n: 6), 2 ml isotonic saline injected ip daily for 6 weeks

2) chlorhexidine gluconate group (CG, n: 7), 2 ml 0.1% CG and 15% ethanol dissolved in saline injected ip daily for 3 weeks

3) resting group (R, n: 7) , CG for three weeks plus 3 weeks peritoneal rest

4) prednisolone group (P, n:7), CG for 3 weeks and then prednisolone 0.1 mg/kg (10 mg in 1 L tap water) for 3 weeks by feeding tube

5) sirolimus group (Sir, n:7), CG for 3 weeks and then oral sirolimus (Rapamune oral solution 1

mg/mL, Wyeth Europa Ltd, 1 mg /kg/day) for 3 weeks

6) prednisolone-sirolimus group (P-Sir, n: 7), CG for 3 weeks and then orally prednisolone and sirolimus for 3 weeks.

During the study the animals were weighed weekly. At the end of the third week the rats in the CG group, and all the rest at the end of the sixth week were sacrified humanely by ether anestesia. The specimens were collected from opposite site of injection for parieatal and from liver surface for visceral peritoneum. The specimens were fixed in 10% formalin in phosphate-buffered saline, and embedded in paraffin. The 5 μm paraffin blocks were stained by hematoxylen-eosin (HE) and Masson trichrome (MT). Peritoneal fibrosis and vascular intensity were examined under light microscopy by the same pathologist.

PATHOLOGICAL ANALYSIS

Peritoneal fibrois was measured as a mean thickness of submesothelial compact zone (between basal border of surface mesothelial cells and upper border of peritoneal adipose tissue, SMC) from ten different sites of HE stained specimens was measured by an image analysis microscope (Olympus BX50, Olympus Optical Co., Tokyo, Japan) linked to a compatible computer, and expressed as micrometers (μm).[4]

Vascular density was measured by counting vessels within SMC area of more 0.3 mm^2. The number was divided by total SMC (mm^2) and mesothelial surface length (mm).

STATISTICAL ANALYSIS

Data are presented as mean ±SD. The statistical analyses were performed using Kruskal-Wallis and Mann-Whitney-U test. A *p*- value of less than 0.05 was considered to be statistically significant. The difference between weights at beginning and end of the study was analyzed by Wilcoxon signed rank test. The statistical significance was accepted as p value less than 0.05.

RESULTS

There were no statistical difference in weights of the rats at the end of the study. The macroscopic findings are shown below (Figure 1). The structural parameters of peritoneum are demonstrated in the pathological specimens (Figure 2, Figure 3A and 3B). The pathological analysis comparing the data between groups were indicated in Table 1.

CG had detrimental effects on peritoneal membrane structure. It resulted in significant increase in both parietal and visceral peritoneal fibrosis thicknesses (μm) as compared to the control group (58,71±37 vs 6,33±0,51., 54,71±36,68 vs 7,00±0,89 respectively, $p<0.05$).

Peritoneal resting did not attenuate pathological findings (peritoneal thickness and vascularity) compared to C group, (peritoneal thickness; 57,00±31,44 vs 58,71±37,73 μm, and 67,00±57,00 vs 54,71±36,68 μm, vascularity; 5,57±3,69 vs. 7,00±5,06, and 2,95±2,09 vs. 3,08±1,63, for parietal and visceral peritoneum, respectively, $p>0.05$ for all.

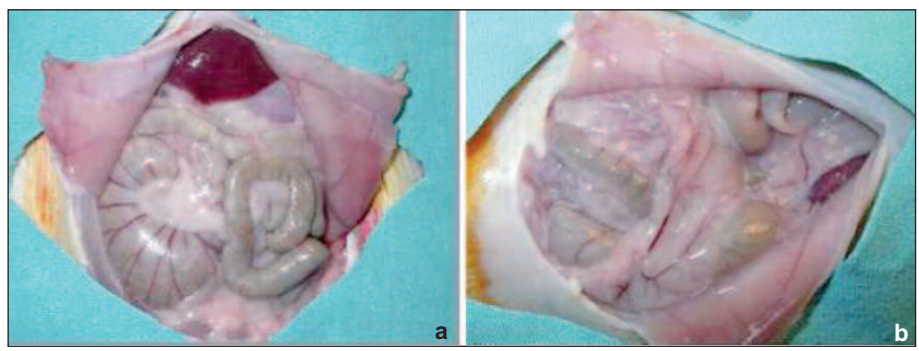

FIGURE 1: Macroscopic appearence of peritoneum. Normal appearence of viscera in the control group (a), and. CG group shows thickened peritoneum over intestinal loops and the liver (b).

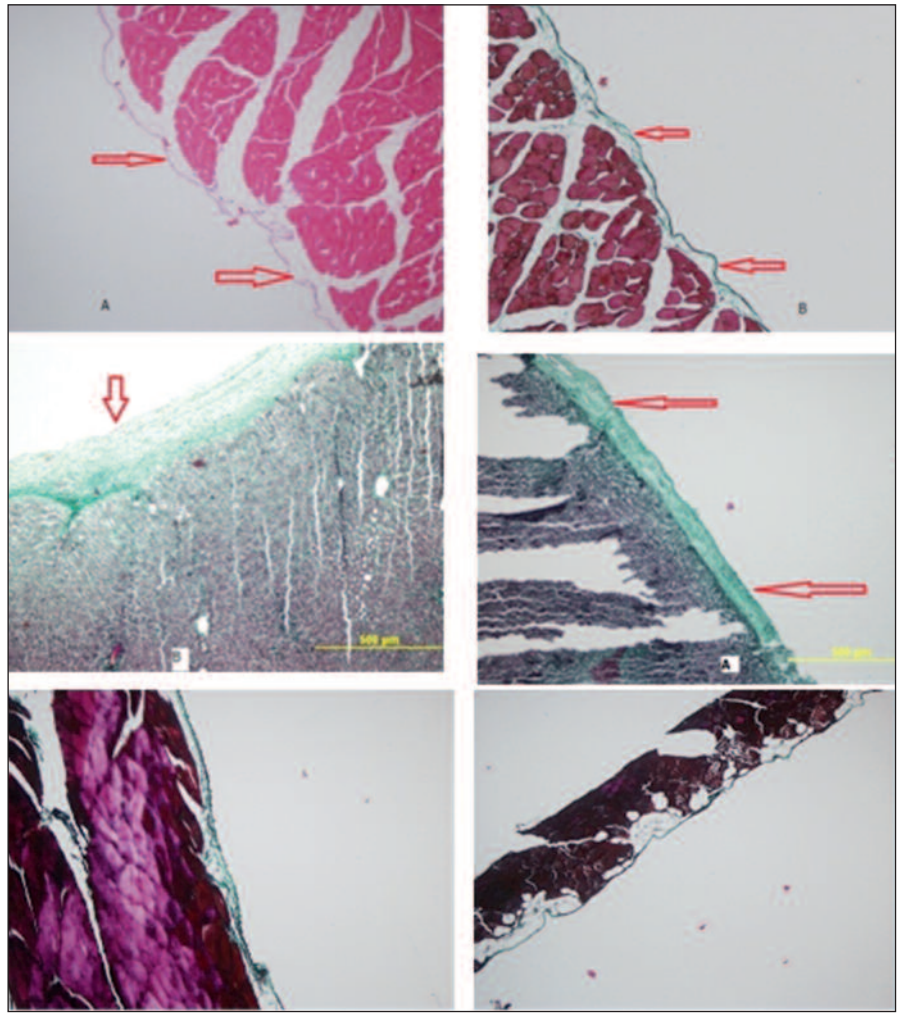

FIGURE 2: First line: normal peritoneal thickness in C group [(left; H&E, and right; MT stains (arrows)]; **second line**: peritoneal thickness increase in CG (left, arrow) and R groups (right; by MT, arrow); **third line**: the decrease in peritoneal thickness in Sir (left) and P-Sir groups (right, by MT).

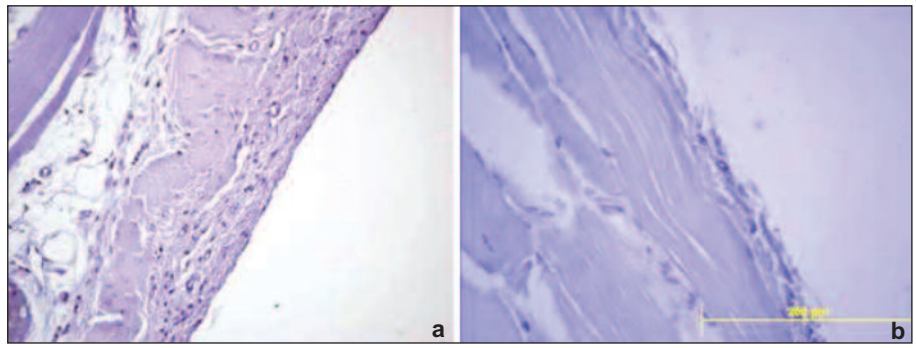

FIGURE 3: Vascular density increase in CG group (a), and decrease in vascularity in P group (right) (CD34 stain). (b).

Pathological findings improved significantly in P, Sir, and P-Sir groups compared to CG and R groups ($p< 0.05$). The vascularity decreased in P group whereas it apparently increased in C group (Figure 3B, Figure 3A, respectively). The combination of sirolimus and prednisolon, on the other hand, did not differ in attenuating structural changes than achieved by prednisolon or sirolimus alone.

TABLE 1: The comparisons of pathological data between groups.

Groups	Thickness (μm)	Fibrosis score (%)	Vascularity (number/mm^3)	p value*
C	6,33±0,51	0,51±0,27	1,16±0,40	$p<0.05^{a,b*}$ $p>0.05^{c,d,e}$
CG	58,71±37,73	4,31±1,67	7,00±5,06	$p<0.05^{f,g,h*}$ $p>0.05^{i}$
R	57,00±31,44	3,34±1,24	5,57±3,69	$p<0.05^{j,k,l*}$
P	7,71±4,99	0,61±0,31	1,85±1,86	
Sir	6,14±0,37	0,55±0,63	1,42±1,98	$p>0.05^{m,n,o}$
P-Sir	6,00±0,00	0,28±0,19	0,37±0,51	

*statistical significance in all parameters a,b: between groups C-CG, C-R, f,g,h: between groups CG-P, CG-Sir, CG-P+Sir, and j,k,l: between groups R-P, R-Sir, R-P+Sir
non significant difference in all parameters c,d,e: between groups C-P, C-Sir, C-P+Sir, i: between groups CG-R, and m,n,o: between groups P-Sir, P-P+Sir, Sir-P+Sir

DISCUSSION

This study showed that combined immunosuppression with steroid and mTOR inhibitor sirolimus had no additive beneficial effect on attenuating morphological changes in experimental EPS model. There had been reports so far that revealed beneficial effects of various therapeutic agents, like N-acetyl cystein, rosiglitazone, renin angiosystem blockers, monotherapies with mTOR inhibitors, and steroids alone on EPS models.[8,20,21,24,25] This report is the rare kind of its that underlines the beneficial effect of sirolimus or prednisolone alone on EPS model, but no further improvement with combination however.

A variety of associated factors have been identified, of which duration of PD therapy, the rate of peritonitis attacks, poor bioincompatibility of the dialysates and use of high glucose concentrations containing advanced glycation end products.[1,7,26] Animal models of EPS are used to define mechanisms, contributing factors and its developmental pathways, and most importantly to elucidate therapeutic strategies and preventive measures.[27,28]

In order to produce a model we injected 0.1% CG and 15% ethanol dissolved in saline solution intraperitoneally for three weeks. It was previously reported that this period of CG injection is enough to induce marked neovascularization, expression of vascular endothelial growth factor (VEGF), and stimulation of tissue growth factors (TGF-β1).[15,29] Figure 2 shows that CG model worked in our study as shown microscopically by increased peritoneal fibrosis measured by SMC thickness.

The exposure of the peritoneal membrane to various insults such as catheter itself, bioincompatible solutions, and infectious episodes causes secretion of various growth factors into the mileu resulting in decrease in or loss of mesothelial cells and gain of fibroblastic properties (EMT). This has been shown as loss of an adhesion protein (E-cadherin) of epithelial cells and emergence of mesenchymal markers (fibroblast specific protein-1, α-smooth muscle actin) on cells instead.[11,14] TGF-β1 and VEGF are the major cytokines responsible for this differentiation. The studies of mesothelial cell cultures demonstrated that an mTOR inhibitor, sirolimus, increased expression of E-cadherin which is associated with a reduction in α-smooth muscle actin and thus ameliorated fibrotic injury.[17,30] It also has been shown to inhibit TGF-β-induced peritoneal angiogenesis by blocking secondary hypoxic response mediated HIF1α.[17,18] Duman et al first demonstrated and recently Ceri et al confirmed the protective effect of mTOR inhibitors against peritoneal fibrosis and neovascularization in rat models induced by chlorhexidine.[20,25] The results of our study is compatible with their findings. In our study resting did not work in regression of fibrosis and vascularization. In fact peritoneal fibrosis continued to increase during this period. This finding suggested that cessation of PD is a risk factor for ongoing fibrotic process.

Several immunosuppressive drugs have so far shown to be effective in EPS, however, no uniformly accepted evidence-based therapy is available.[18,21,24,31]

In our study combination of steroid with an mTOR inhibitor failed to provide further protection against EPS than individual drugs achieved. This issue is especially important in managment of posttransplantation EPS in patients previously on PD. In calcineurin inhibitors era posttransplantation EPS may be a risk factor because of their profibrotic effects.[32,33] Bozkurt et al. reported that cyclosporine have a potent profibrotic and proangiogenic effects mediated by a variety of mechanisms operating on the expression of TGF-β and VEGF, and connective tissue growth factor (CTGF).[21] Based on these evidences of the association of posttransplantation EPS with calcineurin inhibitors an individualized immunosuppressive protocol for patients on PD ongoing to renal transplantation may be proposed, that includes early conversion to mTOR inhibitors from calcineurin inhibitors. However, we consider that adopting such a protocol for the selected patients needs large observational studies since the incidence of EPS after transplantation is rare. At other hand in patients who have not undergone transplantation, use of mTOR inhibitors is becoming popular for the treatment of EPS. Recently a study from our country reported an additive effect of everolimus plus tamoxifen therapy in EPS.[34]

The limitations of the study were lack of inflammatory scoring and cytokine expressions in pathological specimens, and of peritoneal equilibration test indicating the functional status of the peritoneum.

In conclusion although we failed to demonstrate additive effect of combination of sirolimus and steroid in ameliorating pathologic findings of EPS, both drugs may be tailored individually in other combinations for selected cases.

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