Tranfused Human Umbilical Cord Blood Cells Prevent Progressive Renal Failure Induced by 5/6 Nephrectomy in Rats.

İnsan Umblikal Kord Kanı Verilmesinin 5/6 Oranında Nefrektomi Yapılan Sıçanlarda Progresif Böbrek Yetmezliğini Engelleyici Etkisi

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Abstract
Purpose: We attempted to assess the therapeutic effectiveness of human umbilical blood stem cells (HUCBSCs) in a 5/6 nephrectomy rat model.
Material and Methods: We used the 5/6 nephrectomy model in rats to induce chronic renal failure. Male Sprague-Dawley rats were allocated to 3 groups: Sham, 5/6 Nephrectomy and 5/6 nephrectomy plus human umbilical cord blood stem cells transfusion. We assessed blood pressure, renal blood flow, glomerular filtration rate, fractional excretion sodium percentage and histological examination.
Result: HUCBSCS-treated animals had significantly better renal function and better injury scores at day 10 after nephrectomy. HUCBSCs treatment prevented the progressive increment in plasma creatinin and blood urea nitrogen observed in the 5/6 nephrectomized rats. The decrement in glomerular filtration rate induced by renal ablation was modified by HUCBSCs treatment. Increments in systolic, diastolic and mean arterial blood pressures induced by 5/6 nephrectomy did not return to control levels in the HUCBSCs-treated group. There were remarkable tubular necrosis, protein cast, medullary congestion and fibrosis in 5/6 nephrectomized rats but HUCBSCs treatment significantly reduced all but fibrosis was not responding to treatment.
Conclusion: HUCBSCs ameliorated progressive renal damage and some renal function in the remnant kidney after 5/6 nephrectomy, whereas renal blood flow and systemic hypertension was unchanged.

Key words: **Blood pressure; Umbilical Cord Blood; stem cells; Glomerular filtration rate; Kidney Failure.**

Özet:
Amaç: Hasarlanmış böbrek dokusunu yenilemede, iyileşmeyi artırmada ve böbrek fonksiyonunu düzeltmede insan umblikal kord kanı kök hücreleri (HUCBSCs) verilmesinin alternatif bir tedavi olacağı hipotezinin araştırılması amaçlanmıştır.
Gereç ve Yöntemler:Sıçanlarda 5/6 nefrektomi modeli ile kronik renal yetmezlik oluşturulmuştur. Erkek Sprague-Dawley sıçanlar 3 gruba ayrılmıştır. Sham, 5/6 nefrektomili ve 5/6 nefrektomi yapılıp, HUCBCs verilen . Hayvanların kan basıncı, renal kan akımı, glomerüler filtrasyon hızı, fraksiyonel sodyum atılım yüzdesi değerlendirilmiştir.
Bulgular:HUCBCs, nefrektominin 10. gününde böbrek hasarı ve fonksiyonlarında düzelmeyi sağladı . HUCBCs, nefrektomi yapılan sıçanlarda artan serum kreatinin, kan üre nitrojen artışını engelledi. Azaltılmış böbrek dokusunun neden olduğu azalmış glomerüler filtrasyon hızı, HUCBSCs verilenlerde normale yaklaştı. Artmış sistolik, diyastolik, ve ortalama arteryal basınçlar, HUCBSCs verilenler ve verilmeyen nefrektomili sıçanlarda yüksekti. Nefrektomili sıçanlarda, önemli derecede artmış tubüler nekrozis, protein kast, medüller konjesyon ve fibrozis gelişti, fakat HUCBSCs verilenlerde fibrozis dışında gelişen histolojik değişimler azaldı.
Sonuç:HUCBSCs verilenlerde 5/6 nefrektomi ile ileri derecede artan böbrek hasarı, bazı böbrek fonksiyonları iyileşirken, renal kan akımı ve sistemik hipertansiyonda önemli düzelme sağlanamadı.

Anahtar kelimeler: **Kan basıncı; Glomerüler Filtrasyon Hızı; Böbrek yetmezliği.**

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Introduction

Renal failure is defined as a progressive, irreversible deterioration of kidney function caused by various kidney diseases (diabetes mellitus, hypertensive renal diseases renal diseases). Renal failure is a severe state which results in a considerable increase in mortality, reduced quality of life, and high costs for renal replacement therapy (1). Nephrons are gradually lost in renal failure. Remaining nephrons are hypertrophied to compensate for the loss of renal function, and hyperfiltration in these remaining nephrons leads to further nephron loss (2). These nephron losses induce the reduction of renal function via progressive proteinuria and a declining glomerular filtration rate. Nephron loss is caused by glomerulosclerosis, tubulointerstitial fibrosis, loss of glomerular cells and tubular atrophy. Although effective medications which ameliorate the tissue injury and functional losses in renal failure are desperately needed, there is as yet no established therapy (3).

Question about the mechanism of cellular repair and regeneration in the kidney after injury remain unanswered. In general, two major mechanisms may be discussed in repair and regeneration. The first mechanism is repair after ischemia or toxic damage. These cells may be replaced by either neighboring cells or by infiltrating cells most likely from the blood stream. An implicit assumption for such a repair process is the still existing integrity of the local "milieu" (i.e., structures such as basement membrane or other matrix components, neighboring cell membranes, and locally produced factors) which guarantee appropriate differentiation and proliferation of those "repair" cells. Lately, several researchers have investigated the possible role of "stem cells" in repair processes. The second mechanism is regeneration of lost tissue. Regeneration assumes that complete structures of the kidney can be replaced. In its purest form, regenerated tissue may come from stem cells which are capable of generating glomerular and tubular structures by an inborn program. As in embryonic development, those cells may be of mesenchymal origin and need appropriate stimulation for nephrogenesis. In mammals, nephrogenesis in an adult organ has not been observed so far; however, in amphibiae, formation of new nephrons in adult animals is not a rare phenomenon (4)

Physiologically, mesenchymal stem cells give rise to osteocytes, chondrocytes, and adipocytes, but they were recently found to differentiate into endothelial (5), myocardial (6) liver, renal and pulmonary epithelial cells (7). However, the use of adult stem cells for repair may be limited by low recovery of cells from any adult tissue thereby leading to difficulties in obtaining the appropriate number of stem cells in a reasonable period (8, 9). Mononuclear cells derived from human umbilical cord blood were also found to differentiate into cells that improve a variety of disease conditions in animals (5). They contain a relatively high number of CD133 and CD34 progenitor cells (5, 10, 11). Those cells have homing, myogenic potential those are relevant to myocardial repair (12, 13). They can be easily obtained, can be enhanced for self-renewal and differentiation, and can be stored for future use (14).

The aim of the present study is to test the hypothesis that human umbilical blood stem cells (HUCBSCs)-derived stem/progenitor cells can be used as an alternative cell source to rejuvenate injured kidney tissue, enhance healing, and improve kidney function.

Material and Methods

Design of The Study and Experimental Groups. Five-week-old male (250g to 300g in body weight) Sprague-Dawley rats were used. These animals were maintained under lighting conditions (from 6:00-20:00 hr), temperature (241 ^{o}C), humidity (555 %) and were given both a commercial diet and water ad libitum. The present study was performed in accordance with the guidelines for animal welfare and approved by the Institutional Review Board of the Faculty of Medicine, Erciyes University, Turkey.

Through a right dorsolateral incision, the right kidney was pulled out to expose the renal vessels and ureter, which were then ligated with cotton thread and cut between the hilus and the ligated portion to remove the kidney. After kidney removal, the incision was sutured. The left kidney was pulled out through a left dorsolateral incision to expose the renal vessels and ureter. The renal vessels were clipped by a clamp, and the cranial and caudal parts of the organ were cut so that one third of the kidney remained. Finally, the treated left kidney was returned to the abdominal cavity, and the incision was sutured. Sham operations consisted of laparotomy and manipulation of the kidneys and renal pedicle without destruction of renal tissue.

Four days after surgery, the surviving 5/6 nephrecromized rats were randomly divided into three groups.

1. Sham (iv saline infused group, Sham, n= 10)

2. Subtotal nephrectomy (Nx; iv saline infused group, n= 10)

3. Subtotal nephrectomy (iv human umbilical cord blood cells transfused group, Nx-HUCSCs, n=7)

Collection and Preparation of Human Umbilical Cord Blood Cells Stem Cells. Human umbilical blood stem cells were obtained from placentas of healthy full-term neonates. Volunteers signed consent for research and clinical use. Each cord blood sample was collected into a 50-100 mL sterile polypropylene tube containing citrate phosphate dextrose as an anticoagulant. According to the following method, erythrocytes were discharged by centrifuge at 900g 8 minutes, and plasma-containing nucleus cells were centrifuged again at 225g for 10 minutes. Nucleus cells were collected. Our cord blood mononuclear cells included 35-45% CD34+ by flow cytometry (15). An equal volume (0.5 ml) of HUCBSCs or saline was infused (0.5ml/dk) through a jugular vein day 4 after rats were nephrectomized or received sham operations. Immunosuppressive drugs were not used in HUCBSCs transfused rats.

Since this was a pilot study, we wanted to evaluate only renal functional and histological changes following HUCBSCS transfusion, therefore transfused CD34 cells in HUCBSCs group were not visualized by vital staining with fluorescence in the renal tissue. Similar studies have not visualized the CD34 cells (16-17).

Protocol for Experiments in Rats. This protocol was performed 10 days after HUCBCs or saline infusion. A. femoralis and V. femoralis catheterization were performed to record rats, direct blood pressure and to give p-aminohippuric acid (PAH) and inulin respectively. A combination of fentanyl citrate (125 g. kg^{-1} i.p.) and dormicum (5 mg/kg i.p.) which have minimal effects on blood pressure was used for anaesthesia during catheterization. A combination of 2% inulin and 0.1% PAH mixure was given at the rate of 60 L/min after catheterization of the vena. An incision was made at the inguinale region which allowed access to the bladder for uroflow rate measurement and for obtaining urine samples. The bladder was taken off catheterization by PE-90. 20 minutes after vena infusion was begun, blood pressure was recorded on a computer using the MP-30 system (Biopac Systems, Inc., CA). After recording the blood pressure, blood samples were collected in citrated tubes; the rats were then sacrificed. Plasma from the blood samples was separated at 3000 rpm for 5 minutes +4ºC by centrifugation. Plasma and urine samples were stored at -20 ºC until inulin and PAH measurements were performed. Kidney tissues were removed and stored in formalin. Left kidneys and hearts were weighed. Sodium (Na^+), potassium (K^+), creatinine (Cr) and blood urea nitrogen (BUN) measurements in plasma and sodium and creatinine amounts in urine were determined by routine methods employed in clinical biochemistry laboratories (Beckman Coulter Synchron-LX-20). The following equation for fractional extraction sodium (FE_{Na}; percentage) was used:

Fractional Extraction of Na^+ (%)= Urine Na^+ ($U_{Na}V$) x Plasma Cr / Urine Cr x Plasma Na^+ (P_{Na})

in which $U_{Na}V$ is the urinary extraction of sodium (mEq/L), and P_{Na} is the plasma sodium concentration (mEq/L).

Inulin and PAH concentrations in plasma and urine samples were measured using the methods of Bojesen E (18) and Smith HW et al. (19) respectively, by modifying a proportionate reduction of reagent volumes for the assay of small sample volumes. The glomerular filtration rate (GFR), effective renal plasma flow (ERPF), renal plasma flow (RPF) and renal blood flow (RBF) were calculated from the following equations:

$$(GFR)\ C_{in} = V_u \times U_{in} / P_{in}$$
$$(ERPF)\ C_{PAH} = V_u \times U_{PAH} / P_{PAH}$$
$$RPF = C_{PAH} / 0{,}9$$
$$RBF = RPF / 1\text{-}Hct$$

in which C_{in}= Inulin clearance, V_u = Uroflow rate (ml/min), U_{in} = Inulin concentration in urine, (µg/ mL), P_{in} = Inulin concentration in plasma (µg/ mL), C_{PAH} = PAH clearance, V_u= U_{PAH} = PAH concentration at urine (µg/ mL), P_{PAH} = PAH concentration at plasma (µg/ mL),Hct= Hematocrit.

Histological Examination. Excised left kidneys were processed for light microscopic observation. According to standard procedures, the kidneys were fixed in 10% formalin, after which they were chopped into small pieces, embedded in paraffin wax, cut at 4 µm, and stained with hematoxylen-eosine. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts, medullary congestion, as suggested by Nakajima et al (20). The results were rated from 0 to 4 (0; no change, 1; changes less than 25%, 2; change from 25% to 50%, 3; from 50% to 75%, 4; from 75% to 100%).

Statistical Analyses. Statistical analyses were made using Excel and SPSS version 11.0. In the assessment of differences between more than two groups; in the conditions that were apposite to normal distribution, a one-way ANOVA test and then post hoc Tukey's HSD test were used. In assessment of differences between more than two groups, in the conditions that were inapposite to normal distribution, Kruskal Wallis and then Mann-Whitney U tests were used. A value was adjusted according to the number of comparisons between groups. Values were shown as mean ± SEM. $p<0.05$ was accepted as significant.

Results

Arterial Blood Pressures and Heart Rate Values. Body, left kidney and heart weights of groups has shown into the Table I. The systolic, diastolic and mean arterial pressures increased significantly in the nephrectomy groups (Nx, Nx-HUCBSCs) compared to the Sham group. Human umbilical blood stem cells transfusion to the nephrectomized rats did not change blood pressure significantly. Heart rates were similar in all groups (Table II).

Table 1. Body, left kidney and heart weights of groups.

Groups	n	BW1 (g)	BW_2 (g)	Left kidney weight ($mg.100^{1}$ g)	Heart weight ($mg.100^{-1}$ g)
Sham	10	277 ± 5	265 ± 3	394±12	352±12
Nx	11	286 ± 4	$261 \pm 4_{a,b}$	388±19	349±11
Nx-HUCBC	7	279 ± 2	240 ± 5	379±24	362±32

a: $p<0,01$ vs Nx; b: $p<0,01$ vs Sham. BW1: Body weights before test began, BW2: Body weights measured ten days after sham operation or nephrectomy. Sham: Sham Group; Nx: Subtotal Nephrectomy Group; Nx-HUCBC: Subtotal Nephrectomy + Human Umbilical Cord Blood Cell infusion group. Values were mean ± SEM.

Renal Function. Plasma Cr and BUN values in the Nx group were higher than in the Sham group ($p<0,001$). Values in HUCBSCs transfused group were found close to the Sham group (Figure 1A, Figure 1B). Compared with the nephrectomy group, FE_{Na}% in the HUCBSCs transfused group improved significantly ($p<0.001$; Figure 1C). The RBF, EPRF, RPF values in the Nx group were significantly lower than in the Sham group ($p<0.01$). Values in the Nx-HUCBSCs transfused group were found close to normal values; however, there were still RBF, EPRF, RPF differences between the Nx-HUCBCs group and the Sham group ($p<0.05$). The GFR, which is an indicator for renal fuction in the nephrectomy group, was significantly lower than in the sham group. This parameter also improved significantly in the HUCBSCs transfused group ($p<0.05$; Figure 2).

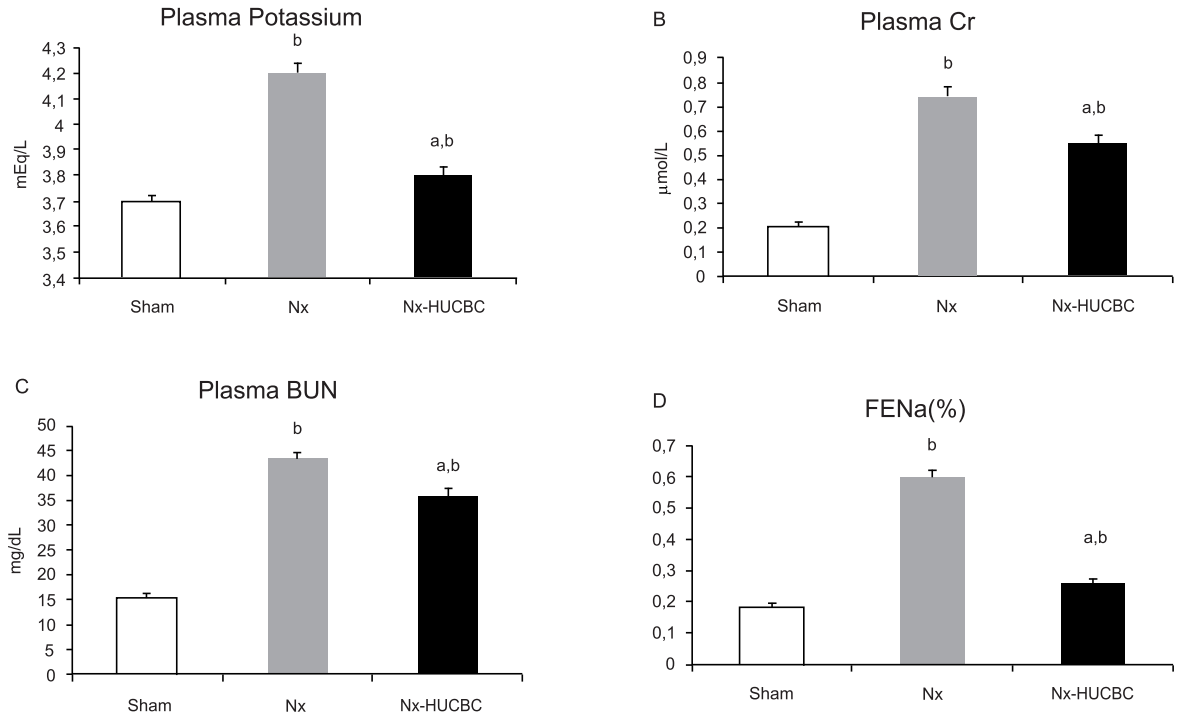

Figure 1. Plasma potassium (Figure A), Plasma creatinin (Figure B), Plasma BUN (Figure C) and % Fractional Excretion of Na^+ (Figure D) values of groups. a: p<0.001 vs Nx group; b: p<0.001 vs Sham groups. FENa(%): % Fractional Excretion of Na. Values were mean ± SEM

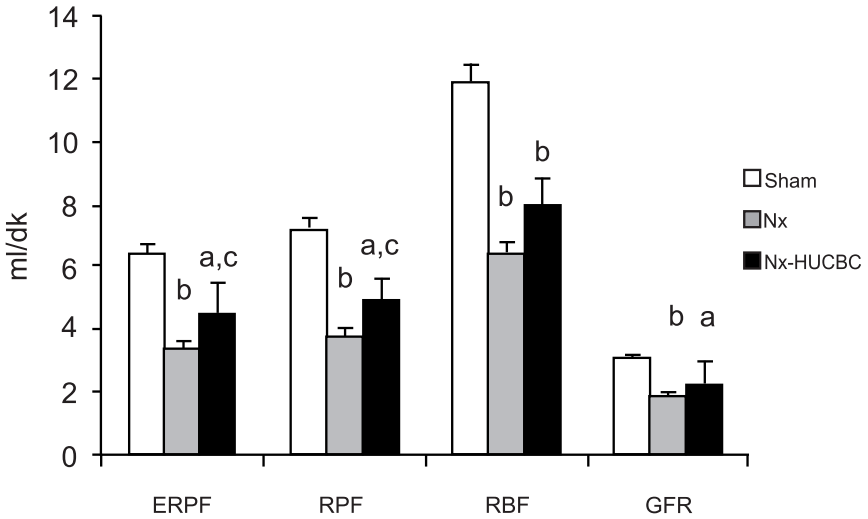

Figure 2. Effective renal plasma flow (ERPF), renal plasma flow (RPF), renal blood flow (RBF), glomerular filtration rate values of groups (GFR). a: p<0.05 vs Nx; b: p<0.01 vs Sham; c: p<0.05 vs Sham. Values were mean ± SEM

Histological Examination. Upon histological examination, the kidneys showed marked tubular necrosis, protein cast and medullary congestion in the Nx group (Figure 3C-D). These changes were significant compared to the Sham group ($p<0.05$ vs Sham). The degrees of tubular necrosis, protein cast and medullar congestion were reduced markedly in the Nx-HUCBSCs group compared to the Nx group ($p<0.05$ vs Nx; Table III; Figure 3E-F).

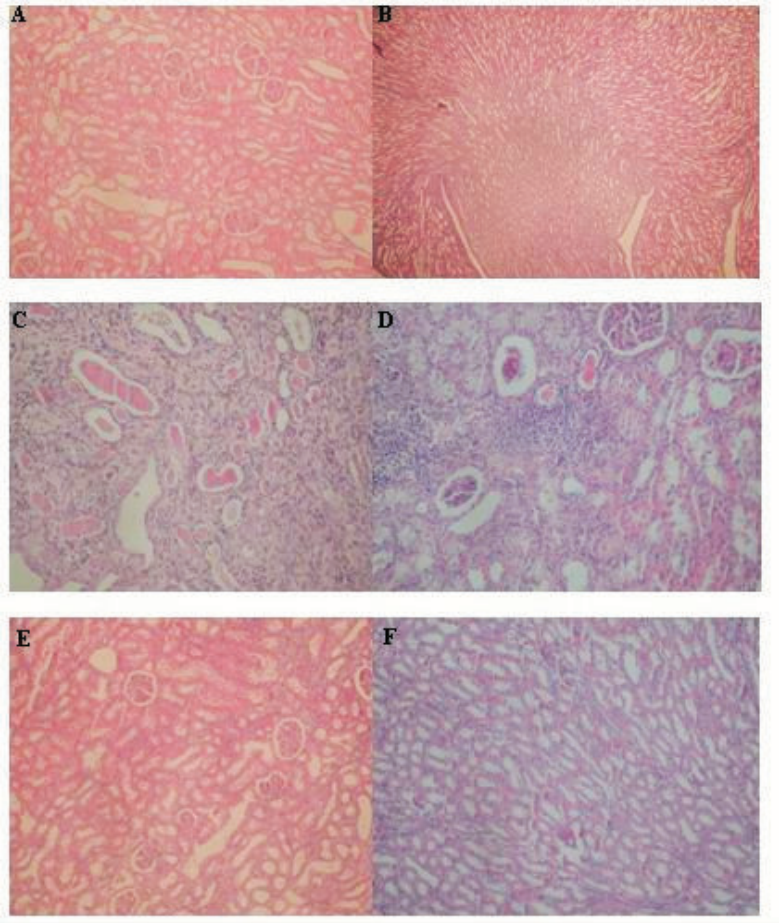

Figure 3. Light microscopy of the cortex (first column) and medulla (second column) of the kidney in groups (H&E staining, magnification, x100). Figure A-B is the Sham group, Figure C-D is the Nephrectomy group and Figure E-F is the Nephrectomy + Human Umbilical Cord Blood Cell infusion group.

Table II. Mean, systolic and diastolic blood pressures and heart rates of groups.

Groups	SBP(mmHg)	MBP (mmHg)	DBP(mmHg)	HR (beats.min^{-1})
Sham	123±10	102±14	80±9	359 ± 14
Nx	137±6[a]	119±12[a]	100±10[a]	345 ± 23
Nx-HUCBC	142±11[a]	118±15[a]	96±9[a]	354 ± 6

b: $p<0,001$ vs Sham. MBP: Mean blood pressure, SBP: Systolic blood pressure, DBP: Diastolic blood pressure and HR: Heart rate.

Table III. Histological examination of renal tissues in groups.

Groups	Tubular Necrosis	Protein Cast	Medullary Congestion
Sham	9,00	6,50	8,86
Nx	15,00[b]	15,36[b]	14,64[b]
Nx-HUCBC	9,00[a]	11,14	9,50

a: p<0,05 vs Nx; b: p<0,05 vs Sham. Values were average rank.

Discussion

Chronic renal failure is a disease group that is as prevalent as heart diseases such as coronary disease, cardiac failure, and peripheral vascular disease cerebrovascular disease. Subtotal (5/6) nephrectomy was performed on laboratory animals to achieve a model for chronic renal failure (21, 22). The remaining kidney of the (5/6) nephrectomized animals was utilized as a model for renal fibrosis (23-25) and a model for glomerular sclerosis (26, 27). In the remaining kidney, glomerular sclerosis was observed following increased proliferative activity in glomeruli (26), and renal fibrosis was accompanied with apoptotic cells (28).

Human umbilical cord blood stem cells were found differentiated into cells that improve a variety of disease conditions in animals (29).They contains significantly higher numbers of hemapoietic stem/progenitor cells compared with normal human peripheral blood (30). Transfusion of HUCBSCs has several advantages over other sources of stem/progenitor cells for hematopoietic rescue. These advantages include widespread availability; absence of donor risk; absence of donor attrition; low risk of transmissible infectious disease; decreased graft-versus-host disease without an increase incidence of relapse, even in mismatched situations; and increased precursors of immune effector cells (31, 32).

The purpose of this study was to determine the healer effect of HUCBSs on renal function by giving HUCBSCs to rats with damaged kidneys and, if possible, to explain the mechanism by planning more complex studies. Human umbilical blood stem cells, given on the fourth day after nephrectomy through stem cell infusion, should be more effective at the beginning of renal failure.

Upon histological examination, a significant increase was seen in tubular necrosis, protein cast, and medullar congestion which indicated renal failure in 5/6 nephrectomized rats. A decrease in tubular necrosis, protein cast and medullar congestion in the group that was nephrectomized and given HUCBSCs indicated the healer effect of HUCBSCs in injured renal tissue. This study examined the improvement in renal function of HUCBSCs transfusion in renal failure. After the healing effect on renal function was shown, we investigated its mechanism. When biochemical values of the groups were examined, increase in the plasma BUN and creatinine values in the nephrectomized group indicated impaired renal function as a result of 5/6 nephrectomy. Decreasing of these values in the group that was given HUCBSCs suggest that stem cells are effective in recovering impaired renal functions. The healer effect of HUCBSCs on renal failure was supported with many findings such as increased RBF, FE_{Na}% and GFR. To our knowledge, no study has addressed the implication of HUCBCs therapy on kidney disease but mesenchymal cells recovered renal functions by differentiating particularly to renal tubular cells (7). Ende and co-workers (17) showed that when $200x10^6$ HUCBSCs was given to diabetic rats, the glomerular hypertrophy and tubular dilatation improved. Kao and co-workers (33) demonstrated that systemic transfusion of CD34+ cells stimulated production of vascular endothelial growth factor (VEGF) in the injured spinal cord area. These findings support the hypothesis that the transplanted CD34+ cells promote an environment conducive to neovascularization in the injured area so that neuronal regeneration can proceed. In addition, trophic mediators produced by CD34+ cells could promote the survival and morphological differentiation. Kao and co-workers (33) have also demonstrated that systemic transfusion of CD34+ cells stimulated production of glial cell line-derived neurotrophic factor (GDNF) in the injured spinal cord area.

Blood pressure increased in 5/6 nephrectomized experienced rats as we expected. Systolic and diastolic blood pressures did not change when HUCBSCs was given to rats. We thought that many factors in cord blood can cause minimal vasocontraction.

In conclusion, intravenous transfusion of human umbilical cord blood cells in nephrectomized rats has a beneficial effect on renal dysfunction. Two major mechanisms of the HUCBSCs effect can be explained. Human umbilical blood stem cells transfusion might play an important role in the angiogenesis, nephron regeneration or nephron proliferation and apoptosis. Collection of HUCBSCs can take place at any hospital or birthing center. The procedure takes about 5 min and poses no risk to mother or baby. Human umblical cord blood banking provides enough CD34+ cells needed to clinically benefit humans. In addition, CD34+ cells are safe to use and are associated with few ethical issues. Thus, it seems that CD34+ cell therapy is one of the potentially useful strategies for treating renal failure.

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