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# The Effect of Placental Mesenchymal Stem Cells on The Protection of Chronic Renal Failure: The Role of Adiponectin

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**ABSTRACT**

Chronic glomerular and tubulointerstitial fibrosis represents a major pathological process leading to end-stage renal failure. Mesenchymal stem cell (MSC) therapy has emerged as a promising approach for renal tissue repair. The identification of molecular markers associated with disease progression may contribute to the evaluation of therapeutic responses. Adiponectin has been suggested as a potential indicator in chronic kidney injury. The present study aimed to investigate adiponectin-related molecular changes following MSC therapy in a rat model of chronic renal injury. Mesenchymal stem cells were isolated from the amniotic membrane of term placentas. Chronic renal injury was induced in rats by subtotal (5/6) nephrectomy. MSCs were administered via the tail vein, and animals were evaluated at 15 and 30 days after transplantation. Protein and mRNA expression levels of adiponectin, adiponectin receptor 1, fibronectin, and phosphorylated AMP-activated protein kinase (AMPK) were assessed using Western blotting and real-time PCR. Serum and urinary adiponectin levels, as well as urinary albumin levels, were measured by rat-specific ELISA kits. Compared to control animals, nephrectomized rats exhibited increased expression levels of adiponectin, adiponectin receptor 1, fibronectin, and phosphorylated AMPK. Following MSC administration, these molecular alterations showed a declining trend. In parallel, urinary albumin levels were reduced in MSC-treated groups. The findings suggest that MSC therapy is associated with modulation of adiponectin-related molecular pathways in chronic renal injury. Adiponectin-associated changes may provide preliminary insight into molecular responses during disease progression; however, further studies are required to clarify its utility as a definitive biomarker.

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***Keywords:*** *Adiponectin, Chronic renal injury, Fibrosis, Mesenchymal stem cell, Placenta.*

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## 1. Introduction

Chronic kidney disease (CKD) is a progressive disorder affecting more than 800 million people worldwide, with a global prevalence exceeding 10% [1]. Progressive glomerular and tubulointerstitial fibrosis is a hallmark pathological feature driving CKD progression toward end-stage renal disease. Increasing evidence indicates that microvascular dysfunction, characterized by endothelial injury and capillary rarefaction, contributes to oxidative stress, inflammation, and progressive renal fibrosis [2].

Experimental studies have employed various pharmacological, genetic, and surgical models to investigate CKD pathogenesis and potential therapeutic strategies. Among these, the subtotal (5/6) nephrectomy model is one of the most widely used because it closely reproduces the structural and functional alterations observed in progressive human CKD [3].

Mesenchymal stem cells (MSCs) have emerged as promising therapeutic candidates because of their immunomodulatory, anti-inflammatory, and regenerative properties. Although MSCs possess multilineage differentiation potential, increasing evidence suggests that their beneficial effects in kidney injury are mediated predominantly through paracrine mechanisms rather than direct tissue replacement [4]. Among the various MSC sources, the human placenta offers several advantages, including non-invasive collection, minimal ethical concerns, high proliferative capacity, and potent immunomodulatory properties, making placenta-derived MSCs attractive candidates for regenerative therapies [5,6].

Adiponectin is a multifunctional adipokine with anti-inflammatory, insulin-sensitizing, and anti-fibrotic properties [7–9]. Its biological effects are primarily

mediated through adiponectin receptor 1 (AdipoR1), leading to activation of AMP-activated protein kinase (AMPK), a central regulator of cellular energy homeostasis [10–13]. Despite these protective functions, the role of adiponectin in CKD remains controversial. Elevated circulating adiponectin levels have paradoxically been associated with poor clinical outcomes and adiponectin resistance in patients with CKD, whereas experimental studies suggest that adiponectin attenuates oxidative stress and albuminuria and exerts renoprotective effects [14-16]. These apparently conflicting observations have led to the concept of the "adiponectin paradox," indicating a complex role for adiponectin signaling during CKD progression.

Previous studies, including our previous investigations using human placenta-derived MSCs in the same experimental CKD model, have demonstrated that MSC therapy attenuates renal inflammation, apoptosis, endoplasmic reticulum stress, and fibrosis through multiple paracrine mechanisms [17–19]. However, the relationship between MSC therapy and adiponectin-related molecular signaling during CKD progression remains insufficiently understood.

Therefore, the present study aimed to investigate adiponectin-related molecular changes following human placenta-derived MSC administration in a rat model of chronic renal injury induced by subtotal (5/6) nephrectomy. Rather than defining definitive molecular mechanisms, this study was designed to provide exploratory evidence regarding the potential association between MSC therapy and adiponectin-associated molecular responses during CKD progression.

## 2. Materials and Methods

### 2.1. Isolation of Mesenchymal Stem Cells from Amniotic Membranes of Term Placentas

All procedures involving human tissues were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Akdeniz University. Human term placentas obtained from uncomplicated pregnancies (38–42 weeks, n = 6) were collected following spontaneous or cesarean delivery after written informed consent.

Isolation of human amniotic mesenchymal stromal cells (hAMSCs) was performed according to our previously established protocol [18]. Briefly, placentas were processed under sterile conditions in a laminar flow cabinet. The amniotic membrane was separated from the chorionic membrane and washed with sterile physiological saline containing antibiotics and antifungal agents. Throughout the isolation and culture procedures, the growth medium was routinely supplemented with antibiotics and antifungal agents to minimize microbial contamination. The amniotic membrane was then cut into approximately 3 × 3 cm fragments and incubated in phosphate-buffered saline (PBS) containing 2.4 U/mL dispase at 37 °C for 7 minutes. The tissue fragments were transferred to RPMI-1640 medium supplemented with fetal bovine serum (FBS) and 2.2 mM L-glutamine and incubated at room temperature for 5–10 minutes. Subsequently, the tissues were digested with an enzyme solution containing 0.75 mg/mL collagenase and 20 µg/mL DNase at 37 °C for approximately 3 hours. The cell suspension was filtered through a 100 µm cell strainer and centrifuged at 200 × g for 10 minutes. The resulting pellet was resuspended in mesenchymal stem cell growth medium, seeded into culture flasks, and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

### 2.2. Characterization of Human Amniotic Mesenchymal Stromal Cells

Phenotypic characterization of hAMSCs was performed as previously described [19]. Cells were positive for CD44, CD90, CD73, and CD105 and negative for CD34, CD45, CD14, CD19, and HLA-DR. Flow cytometric analyses were conducted using a FACSAria III Cell Sorter (BD Biosciences, USA).

To assess multilineage differentiation capacity, hAMSCs were cultured in osteogenic, chondrogenic, and adipogenic differentiation media. After three weeks, cells were fixed with 4% paraformaldehyde and stained using Alizarin Red S and von Kossa/Safranin O for osteogenic differentiation, toluidine blue and Heidenhain's azan for chondrogenic differentiation, and Oil Red O for adipogenic differentiation.

### 2.3. Animals and Experimental Design

All animal procedures were approved by the Akdeniz University Animal Care and Use Committee and conducted in accordance with international guidelines for animal research. Forty-two male Wistar rats (250–300 g) were obtained from the Akdeniz University Experimental Animal Unit. Animals were housed under controlled conditions (22 ± 2 °C, 12-hour light/dark cycle) with free access to standard pellet chow and water.

Chronic kidney injury was induced using the subtotal 5/6 nephrectomy model. Initially, two of the three branches of the left renal artery were ligated, followed by complete right nephrectomy two weeks later. Chronic kidney injury was considered established eight weeks after completion of the surgical procedures.

Animals were randomly assigned to six experimental groups: Sham, 5/6 nephrectomy (5/6 Nx), 5/6 Nx + 15 days, 5/6 Nx + 30 days, 5/6 Nx + hAMSC + 15 days, and 5/6 Nx + hAMSC + 30 days.

For stem cell treatment, $1 \times 10^6$ hAMSCs suspended in 500 µL of DMEM (Lonza, Switzerland) were administered via tail vein injection. To reduce graft rejection, cyclosporine A (1 mg/day; Cell Signaling Technology, USA)

was administered starting one day prior to cell infusion and continued for seven days post-transplantation.

Urine samples were collected over 24 hours using metabolic cages. At the end of the experimental period, animals were anesthetized with ether and sacrificed by cervical dislocation.

### 2.4. Western Blot Analysis

Kidney tissues were homogenized in lysis buffer supplemented with protease inhibitor cocktail and centrifuged at 10,000 × g for 10 minutes to obtain protein extracts. Protein concentrations were determined using the Bradford assay. Equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond™ ECL, Amersham, UK) at 30 V overnight at 4 °C.

Membranes were blocked with 5% bovine serum albumin in TBS-T and incubated overnight at 4 °C with primary antibodies against adiponectin, AdipoR1, phosphorylated AMPK, total AMPK, fibronectin, and β-actin (Cell Signaling Technology, USA). After incubation with HRP-conjugated secondary antibodies (Bio-Rad, USA), protein bands were visualized using enhanced chemiluminescence (ECL) reagents and quantified by densitometric analysis. β-actin was used as the loading control.

### 2.5. Quantitative Real-Time PCR

Total RNA was isolated from kidney tissue samples using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The concentration and purity of RNA were assessed spectrophotometrically. Complementary DNA (cDNA) was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green chemistry (Power SYBR™ Green PCR Master Mix, Thermo Fisher Scientific) on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific). PCR amplification was performed according to the manufacturer's recommended cycling conditions.

The relative mRNA expression levels of adiponectin, adiponectin receptor 1 (AdipoR1), and fibronectin were normalized to β-actin as the internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

The primer sequences used for quantitative real-time PCR were as follows:

| | |
|---|---|
| Adiponectin: | |
| Forward | 5′-TCTCTTCACCTACGACCAGT-3′ |
| Reverse | 5′-GGTAGAGAAGGAAGCCTGTA-3′ |
| AdipoR1: | |
| Forward | 5′-GGAGAAGATGGAGGAGTTCG-3′ |
| Reverse | 5′-TGGCCATGTAGCAGGTAGTC-3′ |
| Fibronectin: | |
| Forward | 5′-CGTCTATGCTCTCAAGGACA-3′ |
| Reverse | 5′-CTGTCTTCGTTCTCCAGCTA-3′ |
| β-Actin: | |
| Forward | 5′-TATGCCAACACAGTGCTGTC-3′ |
| Reverse | 5′-GATAGAGCCACCAATCCACA-3′ |

### 2.6. ELISA

Serum and urinary adiponectin concentrations were measured using rat-specific ELISA kits (Millipore, USA). Urinary albumin levels were determined using a rat albumin ELISA kit (Elabscience, USA) according to the manufacturers' instructions.

### 2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Data were analyzed using non-parametric tests, and group comparisons were conducted using the Mann–Whitney U test with Bonferroni correction where appropriate. A p-value ≤ 0.05 was considered statistically significant.

## 3. Results

### 3.1. Characterization and Differentiation of Isolated Mesenchymal Stem Cells

Flow cytometric analysis demonstrated that the isolated human amniotic mesenchymal stromal cells (hAMSCs) expressed the mesenchymal stem cell markers CD44 (82.4%), CD73 (87.8%), CD90 (98.4%), and CD105 (85.5%), while exhibiting minimal expression of the hematopoietic markers CD34, CD45, CD14, CD19, and HLA-DR (1.1%), confirming their mesenchymal phenotype (Fig. 1).

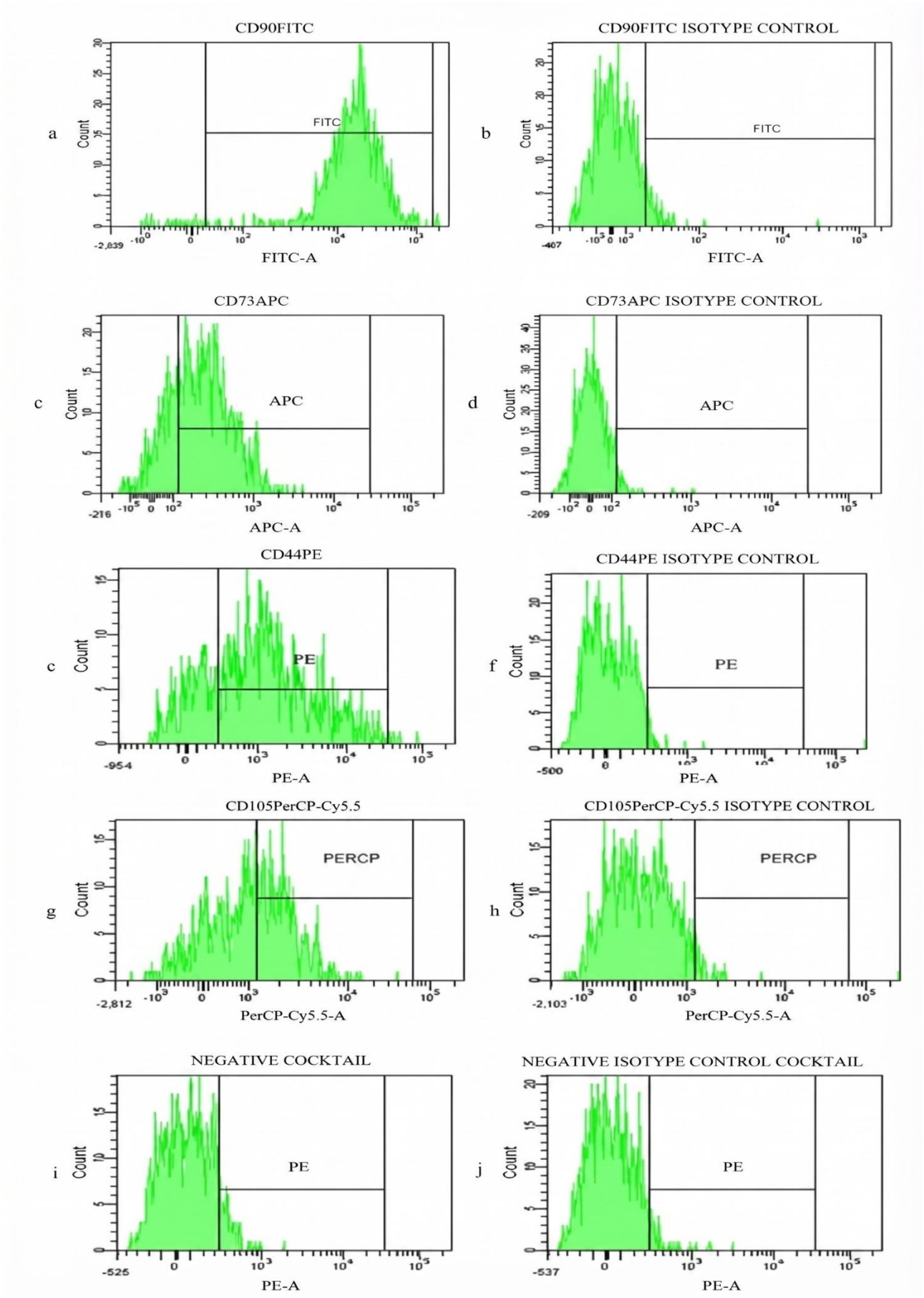


Figure 1. Flow cytometric characterization of isolated human amniotic mesenchymal stromal cells (hAMSCs). Cells showed positive expression of the mesenchymal stem cell markers CD90 (a, b), CD73 (c, d), CD44 (e, f), and CD105 (g, h). Negative marker cocktail and the corresponding isotype controls are presented in panels (i) and (j), confirming the mesenchymal immunophenotype of the isolated cells.

The multilineage differentiation potential of hAMSCs was confirmed in vitro. Adipogenic differentiation was demonstrated by intracellular lipid droplet formation detected with Oil Red O staining, osteogenic differentiation by calcium deposition visualized with Alizarin Red S staining, and chondrogenic differentiation by positive Prussian Blue and Sirius Red staining (Fig. 2a–d).

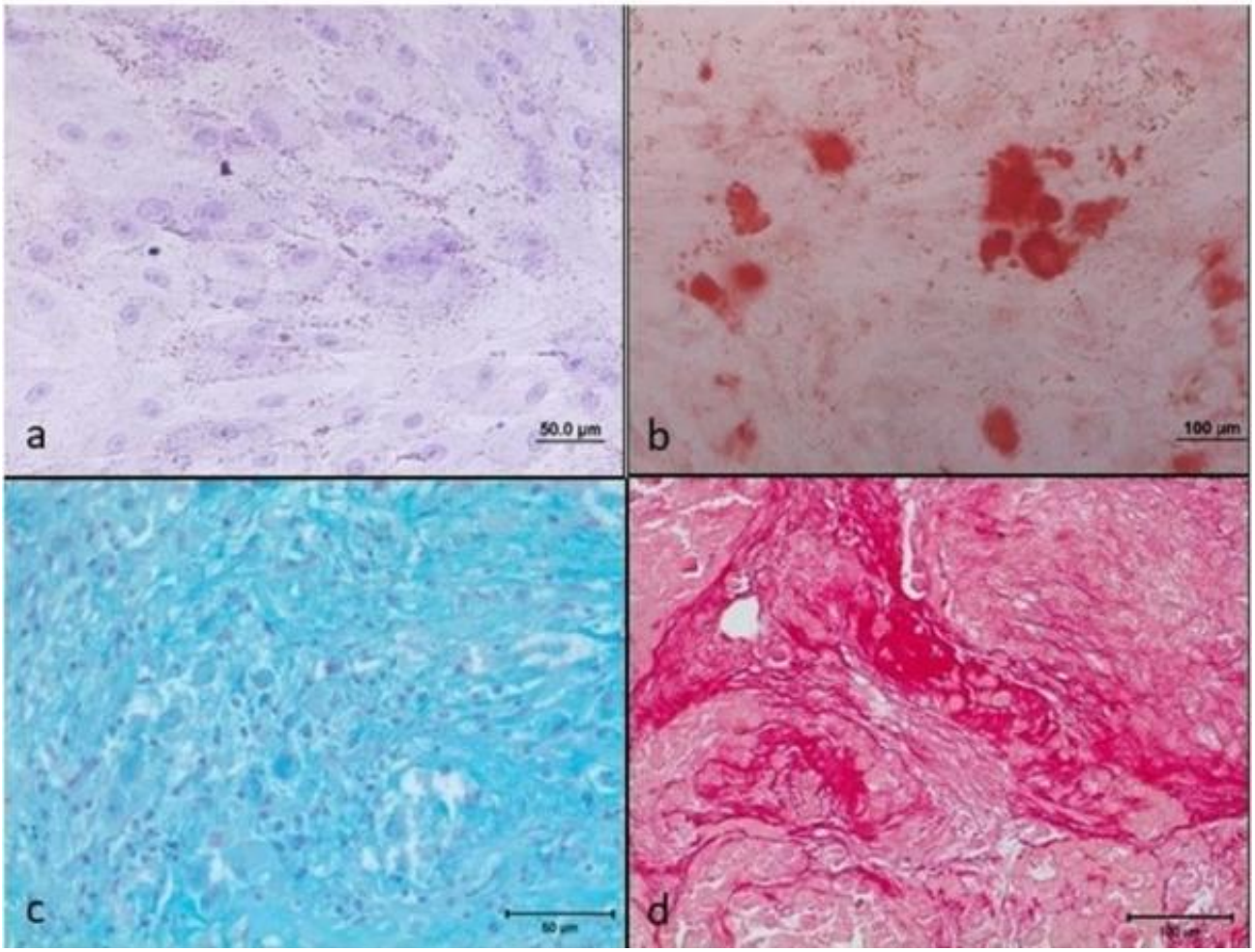


Figure 2. Multilineage differentiation potential of human amniotic mesenchymal stromal cells (hAMSCs). Adipogenic differentiation was confirmed by Oil Red O staining (a), osteogenic differentiation by Alizarin Red S staining (b), and chondrogenic differentiation by Prussian Blue (c) and Sirius Red (d) staining.

### 3.2. Western Blot Analysis

Western blot analysis demonstrated that adiponectin protein expression was significantly reduced in both the 5/6 Nx + hAMSC + 15 and 5/6 Nx + hAMSC + 30 groups compared with the untreated 5/6 Nx + 30 group ($p < 0.05$). No statistically significant difference was observed between the untreated 5/6 Nx and 5/6 Nx + 30 groups (Fig. 3a).

AdipoR1 protein expression was significantly reduced in both the 5/6 Nx + hAMSC + 15 and 5/6 Nx + hAMSC + 30 groups compared with the untreated 5/6 Nx + 30 group ($p < 0.005$). No statistically significant difference was detected between the untreated 5/6 Nx and 5/6 Nx + 30 groups (Fig. 3b).

Fibronectin protein expression did not differ significantly between the Sham and untreated 5/6 Nx + 30 groups. However, fibronectin protein expression was significantly reduced in the 5/6 Nx + hAMSC + 30 group compared with the untreated 5/6 Nx + 30 group ($p < 0.05$) (Fig. 3c).

The pAMPK/AMPK ratio was significantly reduced in the 5/6 Nx + hAMSC + 15 group compared with the untreated 5/6 Nx + 30 group ($p < 0.05$). A greater reduction was observed in the 5/6 Nx + hAMSC + 30 group compared with the untreated 5/6 Nx + 30 group ($p < 0.001$) (Fig. 3d).

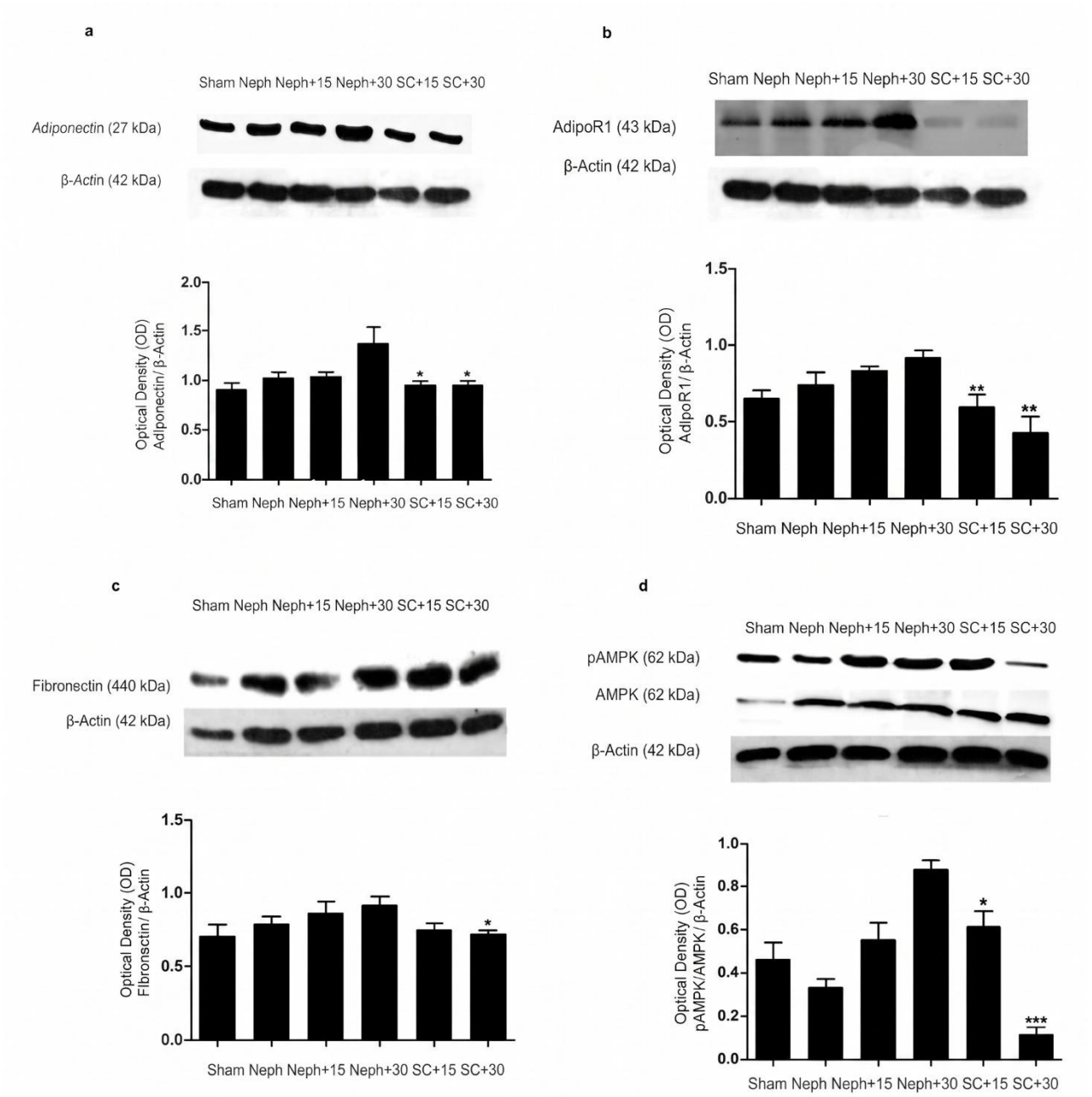


Figure 3. Representative Western blot bands and corresponding densitometric analyses of adiponectin (a), adiponectin receptor 1 (AdipoR1) (b), fibronectin (c), and phosphorylated AMP-activated protein kinase (pAMPK)/AMPK (d) protein expression in kidney tissues from the experimental groups. Protein expression levels were normalized to β-actin, whereas pAMPK expression was normalized to total AMPK. Data are presented as mean ± SEM (n = 6). Statistical significance was determined relative to the untreated 5/6 nephrectomy + 30 days (5/6 Nx + 30) group unless otherwise indicated (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

### 3.3. Quantitative Real-Time PCR

Quantitative real-time PCR analysis demonstrated that adiponectin mRNA expression increased progressively during disease progression, with a significant increase observed in the untreated 5/6 Nx + 30 group compared with the 5/6 Nx group ($p < 0.005$). Following hAMSC administration, adiponectin mRNA expression was significantly reduced in the 5/6 Nx + hAMSC + 15 group compared with the untreated 5/6 Nx + 30 group ($p < 0.005$). The greatest reduction was observed in the 5/6 Nx + hAMSC + 30 group compared with the 5/6 Nx group ($p < 0.001$) (Fig. 4a).

No statistically significant difference in AdipoR1 mRNA expression was observed between the Sham and untreated 5/6 Nx groups. However, AdipoR1 mRNA expression was significantly reduced in both the 5/6 Nx + hAMSC + 15 and 5/6 Nx + hAMSC + 30 groups compared with the untreated 5/6 Nx + 30 group ($p < 0.005$) (Fig. 4b).

Similarly, fibronectin mRNA expression did not differ significantly between the Sham group and the untreated nephrectomy groups. However, both the 5/6 Nx + hAMSC + 15 and 5/6 Nx + hAMSC + 30 groups exhibited significantly lower fibronectin mRNA expression than the untreated 5/6 Nx + 30 group ($p < 0.001$) (Fig. 4c).

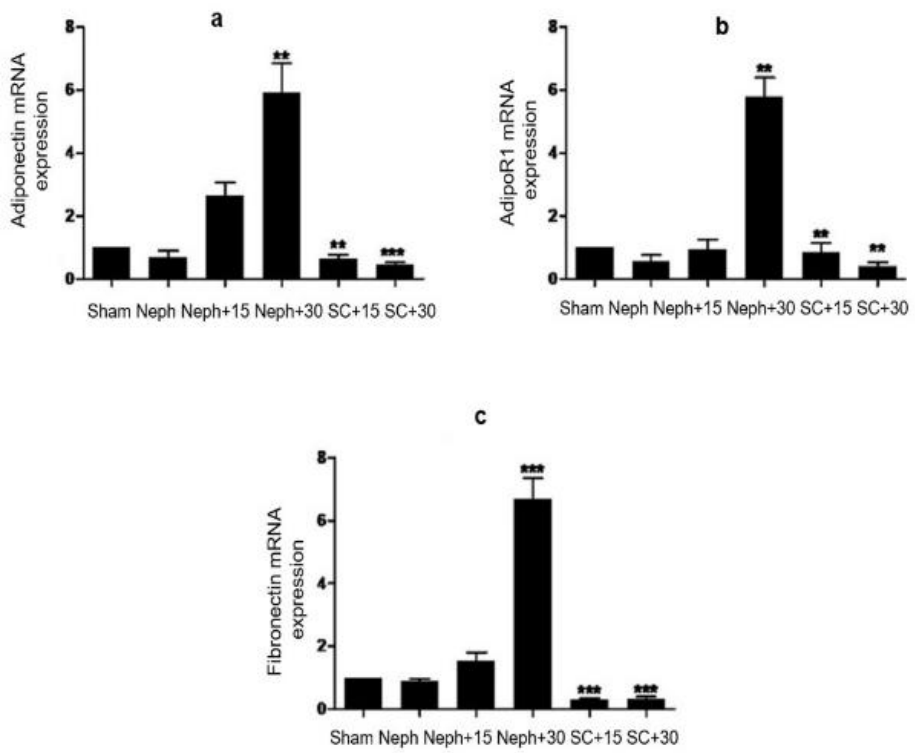


Figure 4. Quantitative real-time PCR analysis of adiponectin (a), adiponectin receptor 1 (AdipoR1) (b), and fibronectin (c) mRNA expression in kidney tissues from the experimental groups. Relative gene expression levels were normalized to β-actin and calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SEM (n = 6). Statistical significance was determined relative to the untreated 5/6 nephrectomy + 30 days (5/6 Nx + 30) group unless otherwise indicated (*p < 0.05, **p < 0.005, ***p < 0.001).

### 3.4. Serum and Urinary Adiponectin Levels

ELISA analysis demonstrated no statistically significant differences in serum adiponectin concentrations between the Sham group and the three untreated nephrectomy groups. However, serum adiponectin levels were significantly higher in the 5/6 Nx + hAMSC + 30 group than in the untreated 5/6 Nx + 30 group (p < 0.05) (Fig. 5a).

Urinary adiponectin concentrations were significantly increased in the untreated 5/6 Nx + 30 group compared with the Sham group (p < 0.005). Following hAMSC treatment, urinary adiponectin levels were significantly reduced in the 5/6 Nx + hAMSC + 30 group compared with the untreated 5/6 Nx + 30 group (p < 0.05) (Fig. 5b).

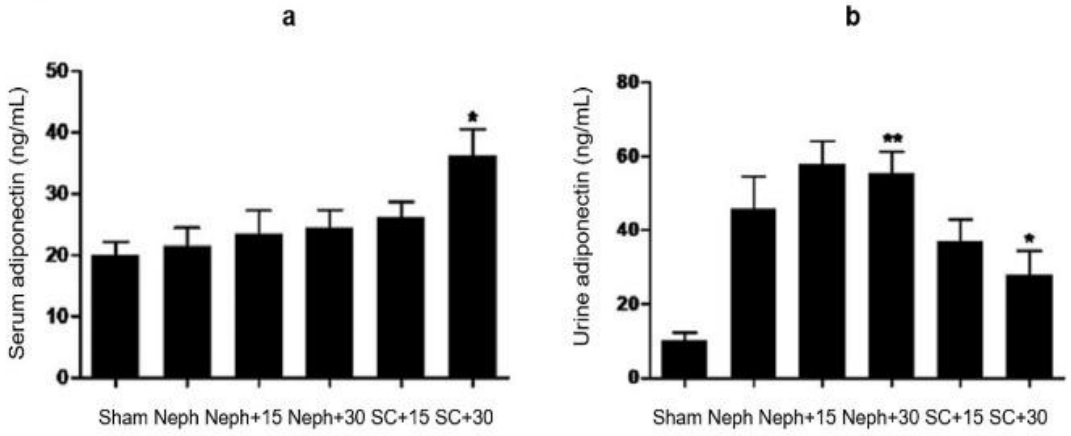


Figure 5. Serum (a) and urinary (b) adiponectin concentrations measured by ELISA in the experimental groups. Data are presented as mean ± SEM (n = 6). Statistical significance was determined relative to the untreated 5/6 nephrectomy + 30 days (5/6 Nx + 30) group unless otherwise indicated (*p < 0.05, **p < 0.005).

### 3.5. Urinary Albumin Levels

Urinary albumin levels were significantly increased in the untreated 5/6 nephrectomy group compared with the Sham group ($p < 0.005$). Following hAMSC administration, urinary albumin levels were significantly reduced. Both the 5/6 Nx + hAMSC + 15 and 5/6 Nx + hAMSC + 30 groups exhibited significantly lower urinary albumin concentrations than the untreated 5/6 Nx + 30 group ($p < 0.005$ and $p < 0.05$, respectively). No statistically significant difference was observed between the two MSC-treated groups (Fig. 6).

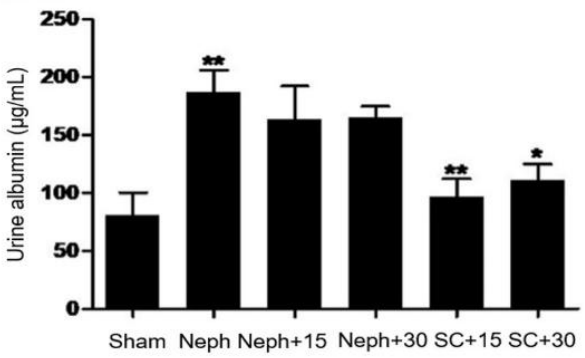


Figure 6. Urinary albumin concentrations measured by ELISA in the experimental groups. Data are presented as mean ± SEM (n = 6). Statistical significance was determined relative to the untreated 5/6 nephrectomy group or the untreated 5/6 nephrectomy + 30 days (5/6 Nx + 30) group, as indicated in the figure (*$p < 0.05$, **$p < 0.005$).

## 4.Discussion

The present study investigated the effects of human placenta-derived mesenchymal stromal cells (hAMSCs) on adiponectin-related molecular pathways in a 5/6 nephrectomy rat model of chronic kidney disease. The principal findings demonstrated that chronic renal injury was associated with increased adiponectin, AdipoR1, fibronectin expression, and AMPK phosphorylation, whereas hAMSC administration attenuated these molecular alterations. In addition, MSC treatment reduced urinary albumin excretion, suggesting a beneficial effect on renal injury and fibrosis-associated molecular responses.

Mesenchymal stem cells have emerged as promising therapeutic candidates for chronic kidney disease because of their immunomodulatory, anti-inflammatory, anti-fibrotic, and regenerative properties. Rather than replacing damaged renal cells through direct differentiation, MSCs are now believed to exert their therapeutic effects predominantly through paracrine mechanisms, including the secretion of cytokines, growth factors, extracellular vesicles, and exosomes that regulate inflammation, oxidative stress, apoptosis, and extracellular matrix remodeling [20–22]. Our findings are consistent with these proposed mechanisms and further support the therapeutic potential of placenta-derived MSCs in chronic kidney disease. Moreover, our previous studies using the same experimental model demonstrated that human amnion-derived MSCs attenuated renal apoptosis, proliferation abnormalities, and fibrosis, supporting the reproducibility of the beneficial effects observed in the present study [18,19].

In the present study, both protein and mRNA expression levels of adiponectin and AdipoR1 showed a declining trend following MSC administration, accompanied by reduced fibronectin expression and decreased AMPK phosphorylation. These findings suggest that MSC therapy modulates adiponectin-related molecular pathways during chronic renal injury. Previous experimental studies have reported increased adiponectin and adiponectin receptor expression in advanced stages of kidney disease, which has been interpreted as a compensatory response to persistent renal injury or as a consequence of adiponectin receptor resistance, a phenomenon commonly referred to as the "adiponectin paradox" [23-25]. Elevated circulating adiponectin concentrations in CKD may therefore reflect impaired receptor responsiveness and reduced renal clearance rather than enhanced biological activity.

Adiponectin has been shown to exert renoprotective effects by suppressing oxidative stress, reducing albuminuria, and activating AMPK-dependent signaling pathways in podocytes [26]. Previous studies have also demonstrated increased AdipoR1 expression and enhanced AMPK activation in fibrotic kidney tissue [27]. Our findings are in agreement with these observations and further suggest that the reduction in AMPK phosphorylation following MSC administration may reflect attenuation of injury-related metabolic stress rather than direct inhibition of adiponectin signaling. Collectively, these findings support the concept that normalization of adiponectin signaling may accompany improvement of the renal microenvironment following MSC therapy.

Fibronectin is one of the major extracellular matrix proteins deposited during renal fibrosis and represents an established molecular marker of extracellular matrix accumulation. Persistent activation of profibrotic pathways, particularly transforming growth factor-β-mediated signaling, promotes fibronectin synthesis and progressive tissue scarring during CKD. In the present study, fibronectin expression was reduced in MSC-treated groups, particularly at the later time point, supporting the anti-fibrotic effects of placenta-derived MSCs observed in previous experimental studies.

Analysis of serum and urinary adiponectin demonstrated differential regulation following MSC treatment. Although serum adiponectin concentrations changed only modestly, urinary adiponectin levels were significantly reduced after MSC administration. Previous studies have reported that urinary adiponectin correlates more closely with glomerular injury and albuminuria than circulating adiponectin concentrations [28,29]. Therefore, the reduction in urinary adiponectin observed in the present study may reflect attenuation of glomerular injury rather than simple alterations in systemic adiponectin

production. Nevertheless, adiponectin-associated changes should be interpreted as indicators of molecular responses to renal injury rather than definitive biomarkers of renal function.

## 5. Limitations

This study has several limitations. Renal function assessment was primarily based on molecular and urinary parameters, as the experimental design was specifically intended to investigate early molecular alterations associated with chronic renal injury. Consequently, conventional biochemical markers of renal function, including serum creatinine and blood urea nitrogen (BUN), as well as histopathological evaluation of renal fibrosis using Masson's trichrome staining, were not included. The incorporation of these complementary functional and histological assessments would have provided a more comprehensive evaluation of the therapeutic effects of hAMSC administration.

In addition, although alterations in adiponectin, AdipoR1, fibronectin, and AMPK signaling were demonstrated, the downstream molecular mechanisms underlying these changes were not directly investigated. Furthermore, the limited number of post-treatment time points restricted the evaluation of the temporal dynamics and long-term effects of MSC therapy. Therefore, the findings should be interpreted as exploratory and hypothesis-generating.

Future studies incorporating comprehensive renal function tests, biochemical and histopathological analyses, extended follow-up periods, and mechanistic investigations are warranted to further clarify the therapeutic potential of placenta-derived MSCs in chronic kidney disease.

## 6. Conclusion

In conclusion, the present study demonstrates that adiponectin-related molecular pathways are altered in a rat model of chronic kidney disease and are modulated following administration of human placenta-derived mesenchymal stromal cells. MSC treatment was associated with reduced adiponectin, AdipoR1, fibronectin, and pAMPK expression, together with decreased urinary albumin levels, suggesting attenuation of molecular processes associated with renal fibrosis and metabolic stress. These findings support the concept that placenta-derived MSCs may contribute to renal protection through modulation of adiponectin-related signaling pathways. Nevertheless, the present results should be regarded as exploratory, and further experimental and clinical studies are required to elucidate the underlying mechanisms and to determine the clinical relevance and biomarker potential of adiponectin in chronic kidney disease.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

This study was supported by the Research Foundation of Akdeniz University, Turkey (Project No: TYL-2017-2398).

## Ethical Approval Statement

All experimental procedures involving animals were reviewed and approved by the Local Ethics Committee for Animal Experiments (Approval No: 70, Protocol No: 2016/09/05, Date: 29 August 2016). The committee concluded that the proposed study did not pose any ethical concerns and approved the conduct of the project accordingly.

In addition, the study protocol was evaluated and approved by the Clinical Research Ethics Committee (Approval No: 464, Date: 18 August 2016). The committee confirmed that the study was scientifically and ethically appropriate and that there were no ethical objections to its implementation.

All procedures involving human-related data were conducted in accordance with the ethical principles of the Declaration of Helsinki, and all experimental procedures were carried out in compliance with relevant institutional and ethical guidelines.

## Declaration of Generative AI

The authors confirm that no generative AI technologies were used in the preparation, writing, or data analysis of this manuscript.

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