

The Effect of stemcell treatment on nephrotoxicity developing after cyclophosphamide treatment

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Abstract

Objectives

This study examines the effects of cyclophosphamide, a cancer treatment that also functions as a cytotoxic agent, on the nephrotic system. The extent to which stem cell applications can be effective in preventing nephrotoxicity caused by agents is also a subject of investigation. The extent to which the nephrotoxic effects detected in the animal model treated with cyclophosphamide can be prevented by stem cell application will be investigated.

Material and Methods

A total of 18 Sprague Dawley rats were included in the study, divided into 3 groups. Group 1 consisted of the control group, which received intraperitoneal (IP) saline injection. Group 2-cyclophosphamide and Group 3-cyclophosphamide + stem cell was administered IP cyclophosphamide (50 mg/kg cyclophosphamide on the first day and then 8 mg/kg intraperitoneally for 14 days) to create a nephrotoxicity model. Group 3-cyclophosphamide + stem cell also received weekly hUCMSC 10*6 IP for 2 weeks. 4 weeks after the treatment, the animals were euthanized, their kidney tissues were histopathologically and immunohistochemically evaluated, and their blood values were biochemically evaluated.

Result

In histopathological examination, glomerulosclerosis and tubular damage were seen the most in Group 2, and this difference was found to be statistically significant ($p<0.001$ and $p<0.01$). However, no statistically significant difference was observed in terms of inflammation in the kidney tissues ($p=0.068$). No significant change was observed in the biochemically evaluated BUN, creatinine, or urea levels in all three groups ($p<0.8$; $p<0.141$; $p<0.8$).

Conclusion

In light of the current information, human Umbilical Cord Mesenchymal Stem Cell (hUC-MCS) has been demonstrated to reduce the nephrotoxicity caused by cyclophosphamide given for cytotoxic purposes on the kidney and exhibit regenerative effects. Our findings create new hope for the use of stem cell therapies in the field of kidney diseases.

Introduction

Cyclophosphamide (CTX) is widely used in cancer treatment and has many side effects, primarily nephrotoxicity and hepatotoxicity (1). Today, cyclophosphamide is widely used as a chemotherapeutic agent, alone or in combination. It is used as an immunosuppressant especially in acute and chronic leukemias; ovarian cancers; multiple myeloma; lymphoma; testicular, small cell lung, and breast cancers;

as well as organ transplantation; rheumatoid arthritis; systemic lupus erythematosus; and multiple sclerosis (2,3).

The two active metabolites of cyclophosphamide are phosphoramidate mustard and acrolein. The antineoplastic effects of cyclophosphamide are related to phosphoramidate mustard. It is thought that phosphoramidate mustard suppresses cell division by binding to DNA and mediating the immunosuppressive and antitumor effects of cyclophosphamide. The toxic effect of cyclophosphamide is related to its active metabolite, acrolein (4). Acrolein inhibits P-450 by alkylating sulfhydryl groups (5) and metabolizing glutathione via rapid modification of sulfhydryl groups to form mercapturic acid, which is excreted in urine (6). These metabolites cause both hepatic and renal damage. The renal pathological damage process caused by CTX is mediated by apoptosis and necrosis of renal tubular epithelial cells (7), release of inflammatory factors, and inflammatory response (8).

Stem cells are cells that can self-renew, differentiate into multiple cell types, and proliferate to produce and renew different types of tissues and organs. Stem cells are used in the treatment of many diseases (5) (6,7). In addition, stem cell transplantation is performed after CTX in some cancer treatment protocols (8). While research has explored strategies for the treatment of both hepatotoxicity (9,10,11) and nephrotoxicity (12,13,14), we have not come across any reports investigating the efficacy of stem cell therapies in cyclophosphamide treatment. Stem cell therapies are used after cyclophosphamide in the treatment protocol of some cancer diseases (15). In addition, stem cells have paracrine effects by promoting their differentiation capacity into other cells and generating immune regulatory, anti-inflammatory, anti-apoptotic, and anti-oxidative effects (16,17). Stem cells also have analgesic and angiogenic effects (18). Due to the regeneration properties of stem cells, damaged tissues can be renewed and repaired. In this study, we aimed to investigate the efficacy of umbilical cord-derived mesenchymal stem cells in animals with renal damage induced by CTX.

Materials and Methods

Formation of cyclophosphamide-induced kidney injury model and administration of h-USMSC.

A total of 18 female Sprague Dawley rats that were 9–10 weeks old and weighed 200–220 g were used in the study. The animals were obtained from the Gazi University Laboratory Animal Breeding and Experimental Research Centre and studied in the same laboratory. The study was approved by the Gazi University Animal Experiments Local Ethics Committee (Approval number: G.Ü.E.T-66332047-604.01-1015251). The animals were randomly divided into 3 groups. Group 1 (control group) received 2 cc 0.9% saline infusion intraperitoneally. For the liver and kidney injury models, Groups 2 and 3 were injected with 50 mg/kg cyclophosphamide (Endoxan, EIP ECZACIBAŞI, Istanbul, Turkey) on the first day, and then 8 mg/kg intraperitoneal cyclophosphamide (CTX) was injected into the animals for 14 days. Group 3 was then injected with $0.6\text{ml} \times 10^5/\mu\text{L}$ human Umbilical Cord Mesenchymal Stem Cell (hUC-MCS) intraperitoneally once a week for 2 weeks.

At the end of the 4th week of the experiment, animals were euthanized by intracardiac blood sampling under xylazine (5 mg/kg, i.m) and ketamine (45 mg/kg, i.m) anesthesia. Kidney and liver tissues were then removed and fixed in formaldehyde.

Histological examination of rat kidneys

Kidneys were fixed with 4% paraformaldehyde and embedded in paraffin for pathological analysis. Paraffin blocks were cut into 4- μ m thick sections and stained with hematoxylin and eosin (H&E) and caspase.

Elisa

Whole blood samples from the study groups were centrifuged at 3500 rpm for 10 minutes to separate the sera. Humor samples were stored in a -80°C deep freezer until the day of the study together with aqueous humor samples. Serum samples were measured using the BT Lab (Zhejiang, China) brand kit using the sandwich ELISA principle. The experiment was performed according to the instructions of the commercial kit. A standard graph was drawn according to the absorbance in the standard samples, and the absorbance values read in the samples were substituted into this graph to calculate the concentrations.

Kidney function evaluation

To evaluate kidney function, BUN (blood urea nitrogen), urea, and creatinine levels were checked at 6 weeks in Groups 1, 2 and 3. Histopathologically, the degree of tubular atrophy was examined at four stages (0–25%, 25–50%, 50–75%, and 75–100%) and scored between 0–3. The presence of glomerulosclerosis was divided into 4 stages (0-25%, 25-50%, 50-75%, and 75-100%) and scored between 0–3 according to the percentage of presence in the examined area. Those with and without inflammation were graded as 0 and 1, respectively.

Statistical Analysis

In this study, the studied data set was statistically compared among three different separate groups based on specific measurement criteria. The Kruskal-Wallis analysis of variance was done to evaluate differences in measurement parameters between group levels. If a difference existed, the Dunnett method was used to determine pairwise comparisons of group levels. The test's critical value was 0.05, and a p-value greater than 0.05 indicated that there was no significant difference between variables.

The variables examined in the study glomerulosclerosis, tubal damage, and inflammation are categorical in nature, and their distributions were compared across three different groups (Control, CTX, and CTX + stem cell). These variables typically have between 2 to 4 levels and exhibit ordinal categorical structure (e.g., scores ranging from 0 to 3). Due to the relatively small sample sizes in each group and the presence of expected cell frequencies below 5 in some contingency tables, the assumptions of the chi-square test could not be met. Therefore, the Fisher's Exact Test, a more appropriate and sensitive

alternative for small sample sizes, was used to assess group differences. Otherwise, a statistical significance was measured between the variables. All analyses were conducted using the R programming language.

Results

When BUN, creatinine, and urea levels were evaluated biochemically in the three groups, no significant change was observed (Table 1).

In histopathological evaluation, in the Group 1 control group, 3 samples (60%) showed Stage 0 and 2 samples (40%) showed Stage 1 glomerulosclerosis. In the Group 2 cyclophosphamide group, 4 samples (80%) showed Stage 2 and 1 sample (20%) showed Stage 3 glomerulosclerosis. In the Group 3 cyclophosphamide + stem cell therapy group, 5 samples (100%) showed Stage 1 glomerular sclerosis, and the difference was statistically significant ($p < 0.001$). When tubular atrophy was evaluated, in Group 1, 2 samples (40%) showed Stage 0 and 3 samples (60%) showed Stage 1. In Group 2, 3 samples (60%) showed Stage 2 and 2 samples (40%) showed Stage 3. In Group 3, 4 samples (80%) were detected as Stage 1 and 1 sample (20%) as Stage 2. The observed differences were statistically significant ($p = 0.010$).

The presence of inflammation was observed in 2 samples (40%) in Group 1, 5 samples (100%) in Group 2 and 1 sample (20%) in Group 3 and was significantly lower in Group 3. However, this difference was not statistically significant ($p = 0.068$) (Table 2).

Histopathological comparison was made between Group 1-control, Group 2-CTX and Group 3-CTX+ stem cell included in the study. In histopathological examination, normal shapes and structures were seen in all areas of the kidney tissues in Group 1. In Group 2, glomeruli shrinkage, Bowman space widening, dilatation, degeneration, and epithelial vacuolization in tubules were observed. In addition, moderate inflammation was observed throughout the entire section. In Group 3, serious regeneration was observed in all areas of the kidney tissue, including an improvement in Bowman spaces; tubular dilatation, epithelial degeneration and vacuolization were also observed to significantly decrease. In caspase-3 staining, normal findings were detected in Group 1, while increased staining was observed in glomeruli and tubular structures in Group 2. In Group 3, a high rate of tubular and glomerular regeneration was observed (Figure 1).

Glomerulosclerosis and tubular damage were seen most in Group 2, and this difference was found to be statistically significant ($p < 0.001$; $p < 0.01$) **Figure 2**.

Discussion

Since the metabolism enzymes of many drugs are located in the kidneys, these organs are where these agents are excreted from the body and can therefore show toxicity. Cyclophosphamide (CTX) is a drug generally used in chemotherapy, and its metabolites are primarily excreted in the urine. Therefore, it is

known that CTX can be toxic to the kidneys. CTX delays the growth of malignant cells due to its toxic metabolites phosphoramidate and acrolein. Acrolein inhibits the cellular antioxidant defense cycle and causes the formation of reactive oxygen radicals, including superoxide anions, hydroxyl radicals, and hydrogen peroxide, which cause physiological and morphological changes (19). The formation of free radicals causes the disruption of many signaling pathways, including the inflammation pathway and ultimately organ fibrosis (20). This situation has led to the hypothesis that the effects of CTX can be reduced using antioxidant agents. It is known that the mutagenic and cytotoxic effects of cyclophosphamide are specific to proliferating cells only, and that quiescent cells are relatively protected by damage detection mechanisms. However, cyclophosphamide is known to cause severe side effects in high doses and/or long-term use. In today's nephrology practice, the use of CTX has been significantly restricted. However, it is still used as a steroid-reducing drug in minimal change disease and focal segmental glomerulosclerosis, both in combination with steroids in membranous glomerulonephritis and as the first choice in rapidly progressive glomerulonephritis and certain lupus patients. It is generally recommended to avoid long-term use (21). Although there are restrictions on its use in nephrology practice, cyclophosphamide is used in the treatment of many malignancies. Since significant beneficial effects are observed in allogeneic or autologous stem cell transplantation applied after cyclophosphamide treatment, the question of whether it may have similar effects on damaged kidney tissues has emerged.

As in tumors, damaged tissues secrete various factors that attract stem cell migration via the CXCL12/CXCR4 axis. Factors that promote stem cell migration include interleukin 8 (IL-8) and transforming growth factor-beta 1 (TGF- β 1) (22), platelet-derived growth factor (PDGF) (23), fibroblast growth factor 2 (FGF-2) (24), vascular endothelial growth factor (VEGF) (24), and extracellular matrix molecules such as matrix metalloproteinase-2 (MMP-2) (25). The primary reparative mechanisms of stem cells include paracrine effects such as angiogenesis (26), anti-apoptosis (26), anti-fibrosis (27), and immunomodulation (28). It is known that exosomes, which are vesicles derived from stem cells that are 30 to 160 nm in size, play an important role in regulating intercellular communication (29). It is also known that stem cells have the potential to replace damaged mitochondria in damaged cells and increase cell quality (30), therefore their healing effects on damaged tissues were examined in our study.

The differences between kidney function tests in the samples were first evaluated. However, no significant difference was found between the groups in terms of BUN, urea, or creatinine levels. It is thought that this situation may be because these markers were measured at the 6th week. In similar studies, kidney functions were evaluated in rats given CTX in a shorter period of time. For example, in Uygun et al., kidney function tests were measured 24 hours after cyclophosphamide administration, and significant increases in creatinine and urea were observed in the CTX arm (31). In a similar study, the protective effects of tolcapten on CTX treatment were investigated; blood samples were taken one day after CTX administration, and significant increases in creatinine and urea were observed in the CTX arm (32). In a study aiming to reduce the effects of high-dose CTX treatment, the effects of Wuzhi capsules on treatment were investigated; once again, as blood samples were taken at an early stage, urea and

creatinine values were found to be significantly higher in the CTX group compared to the control group (33).

In many similar studies, urea and creatinine were observed to be significantly higher in the CTX groups compared to the control groups in the samples taken immediately after the completion of the experiment (34,35,36,37). In a study where creatinine, BUN, and urea were measured on the 25th day of cyclophosphamide administration, kidney function tests were found to be significantly higher in the CYS arm (38). In our study, taking the samples at the 6th week made a difference in terms of providing information on the late effects of cyclophosphamide on kidney functions. Although BUN, creatinine, and urea levels were higher in the cyclophosphamide arm at the end of the 6th week, this increase was not statistically significant. This situation can be explained by the fact that the acute kidney injury picture started to improve in rats that had experienced an acute kidney injury when there was no repeated dosing.

To observe organ integrity in the event of any damage, pathological indicators can be shown by the deterioration of the structural integrity and shape of the organ. It is known that cyclophosphamide directly induces oxidative stress, causes apoptosis and inflammation, and leads to histopathological conditions (39). In our study, via hematoxylin-eosin staining, renal integrity was seen to be impaired: There was shrinkage in the glomeruli, widening in the Bowman spaces, dilatation in the renal tubules, and vacuolization in the epithelium and degeneration, especially in the renal tubules. In addition, moderate inflammation was observed in all sections. The findings are also consistent with previous studies (19,33,34,35,36).

In the group that received stem cell application with the aim of repairing effects on damaged tissues, significant regeneration was observed in the hematoxylin and eosin-stained sections. There was an improvement in glomerular Bowman spaces; and tubular dilatation, epithelial degeneration, and vacuolization had significantly lessened.

Apoptosis, which is a cell collapse characterized by condensation of the cell membrane, shrinkage of the cell, condensation of chromatin and fragmentation of DNA, occurs as a result of reactive oxygen radicals (40, 41). Caspase-3 is considered the main mediator of apoptosis in cells because it initiates the apoptotic process by activating other caspase enzymes, causing fragmentation of DNA and protein structures and condensation of chromatin (42). In our study, while a normal appearance was detected with caspase staining in the control group, a significantly increased uptake in glomerular and tubular structures was observed in the CTX group, and tubular and glomerular regeneration was observed in the CTX+stem cell group. This situation was observed in our study, similar to existing studies (19, 42, 43).

Today, hUC-MCS is used in the treatment of diseases with nephropathy, such as diabetes mellitus and systemic lupus erythematosus. When applied in the treatment of diabetic nephropathy, it has been observed to reduce the expression of inflammatory cytokines; increase the number of Sertoli cells and rearrange their proteins; and increase the expression of anti-apoptotic proteins in the kidneys (44). In studies conducted in diabetic mice, it has been observed that BUN and 24-hour urine albumin excretion

significantly decrease after hUC-MCS application (45). In lupus nephritis, it is thought that hUC-MCS migration to the kidney is accelerated by CXCL10 synthesized by increased glomerular vascular endothelial cells via the FN- γ /IRF1-KPNA4 pathway. The negative effect of therapeutic effects on the blockade of the CXCL10-CXCR3 pathway also supports this view (46). It has also been shown that hUC-MCSs improve lupus nephritis by affecting T and B cell responses (47).

The use of hUC-MCS treatment in diseases with nephropathy and its beneficial effects shed light on its use in primary and secondary nephropathies. The waste material, a human umbilical cord, was obtained non-invasively; the lack of ethical issues provides an advantage over other sources of stem cells. In addition, the neonatal age of the donor creates a difference in terms of stem cell potency. In our study, it was observed that cyclophosphamide improved the pathological effects and provided significant morphological improvement. These findings suggest that its use in drug-related nephropathies may be beneficial, indicating that it may be worth trying in other nephrological diseases.

The factors affecting the results of the study include the reparative effects of the stem cells administered for therapeutic purposes on the kidney tissue, the duration and frequency of treatment, the number of cells administered for therapeutic purposes, the selected stem cell source, and even the donor selection. Another factor affecting the results is the time elapsed after administration of the stem cell treatment. Changes in kidney tissue after stem cell transplantation may also vary depending on the time of euthanasia. We believe that these factors explain the different results among studies to some extent. Therefore, more studies are needed.

Conclusion

Permanent organ damage due to cytotoxic agents used for different purposes has the potential to create serious problems in the future. In this context, the possible nephrotoxic effects of cyclophosphamide, which is used in many cancer treatments, were examined in this study and shown to cause pathologies resulting in glomerulosclerosis and tubular damage. Although no early deviations were detected in laboratory parameters considered as clinical indicators, it is possible that possible failures may occur in the long term. In this context, it has been shown that stem cell applications given together reduce the formation of glomerulosclerosis and tubular damage, and it is thought that this effect may be effective in long-term survival. In the future, it can be expected that these applications will be beneficial in terms of effectiveness within classical protocols in cost-effectiveness studies.

Declarations

Conflict of Interest: There is no conflict of interest between the authors. All authors read and approved the final version of the manuscript

Attestation Statement: Animal experiment study

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Compliance with Ethical Standards: Written approval from the Gazi University Ethics Committee, was obtained for the study. **The study was approved by Gazi University Animal Experiments Local Ethics Committee (Approval number: G.Ü.E.T-66332047-604.01-1015251).** All authors have approved the manuscript submission, and the manuscript has not been published. It is not being considered for publication elsewhere, in whole or in part, in any language.

All data generated or analysed during this study are included in this published article and its supplementary information files and the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for Publication : Not Applicable

Author contribution.

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

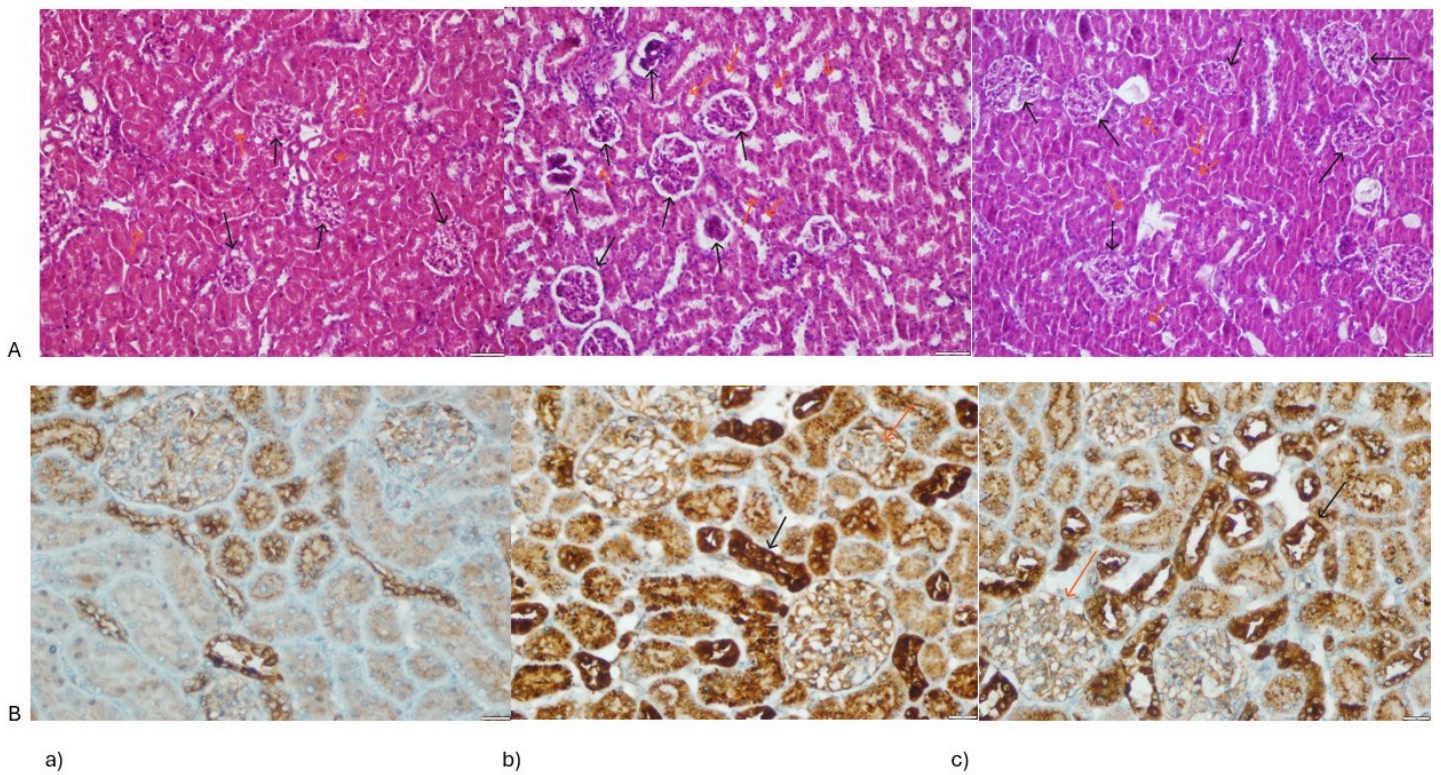


Figure 1

H&E staining of the kidney (orange arrows indicate renal tubules and black arrows glomeruli). 40x; scale bar = 40 μ m.

A- H&E. a- Control group, b-CTX group, c-CTX+ Stem cell group. H&E staining results showed that CTX group rats exhibited abnormal renal structure, including glomerular shrinkage, renal tubule vacuolisation and hydrated renal tubule degeneration. In the CTX + Stem cell group, damage to glomeruli and renal tubules was significantly attenuated and exhibited regenerating morphology.

B- Caspase-3. Active caspase-3 positive cells, which is a marker for apoptosis. Cells with apoptosis are stained brown. a- Control group is observed in normal appearance, a high rate of tubular and glomerular apoptosis is observed in the b-CTX group and in c-CTX+ Stem cell group high rate of tubular and glomerular regeneration is observed.

Image not available with this version

Figure 2

It was observed that there was increased glomerulosclerosis and tubular damage in Group 2, and this difference was statistically significant ($p < 0.001$; $p < 0.01$).

Supplementary Files

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