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Cyclophosphamide Inhibits PI3K/AKT/mTOR Signaling Pathway in Mice Kidneys †

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Abstract: Cyclophosphamide (CTX), also known as cytophosphane among other, is a medication used as chemotherapy and to suppress the immune system. The PI3K/AKT/mTOR pathway is involved in the regulation of diverse cellular functions, including cell growth, protein synthesis, cell cycle regulation, glucose metabolism, and motility. In our study eight weeks old C57BL/6 female mice were divided into 3 groups as control (C), sham (S) and experimental group. The experimental group has been established with CTX treatment. No treatment was applied to the C group. The S group were given an equal amount of saline. CTX was administered intraperitoneally one every 2 days for 3 weeks; the first dose was 70 mg/kg, the ongoing doses were 30 mg/kg. At the end of 3 weeks mice were sacrificed and kidneys were taken for investigation. In order to show the effect of cyclophosphamide in kidney tissue, the tissues were stained via indirect immunohistochemistry with PI3K, AKT and mTOR primary antibodies. In our study, PI3K, AKT and mTOR expression levels were found to be significantly decreased in CTX-mediated mechanisms indicating that the mechanisms of CTX might involve in the inhibition of PI3K/AKT/mTOR signaling pathway.

Keywords: Cyclophosphamide (CTX); Kidney; PI3K; AKT; mTOR

1. Introduction

Cyclophosphamide (CTX), also known as cytophosphane among other, is a medication used as chemotherapy and to suppress the immune system. As chemotherapeutic it is used to treat lymphoma, multiple myeloma, leukemia, ovarian cancer, breast cancer, small cell lung cancer, neuroblastoma, and sarcoma. As an immune suppressor it is used in nephrotic syndrome, granulomatosis with polyangiitis, and following organ transplant, among other conditions [1]. Cyclophosphamide metabolites are primarily excreted in the urine unchanged, and drug dosing should be appropriately adjusted in the setting of renal dysfunction [2].

The PI3K/AKT/mTOR pathway is involved in the regulation of diverse cellular functions, including cell growth, protein synthesis, cell cycle regulation, glucose metabolism, and motility. Activation of this pathway begins with the PI3K family, which is lipid and serine/threonine kinases. A critical downstream mediator of PI3K is AKT, which acts on a number of different targets that affect cellular survival, activation of transcription/translation, apoptosis and proliferation through resistance to chemotherapy. Upstream regulation of mTOR with a downstream target of AKT and a serine/threonine kinase leads to activation of proteins that directly affect protein translation and growth progression through the cell cycle [3]. In this study, we aimed to demonstrate the effect of cyclophosphamide on PI3K/AKT/mTOR signaling pathway in kidney tissue.

2. Materials and Methods

2.1. Animals and Experimental Procedures

Adult C57BL/6 mice (female), weighing between 20–25 g were used in the experiment. Mice were kept in Manisa Celal Bayar University Experimental Animal Research and Application Center facility in standard polypropylene cages at 25 ± 2 °C temperature, 50–60% relative humidity, 12 h light-dark cycles and having ad libitum food and water. Eighteen female C57BL/6 mice at the age of 8 weeks randomly divided into 3 groups as C, S and experimental group (n:6, for each group). The experimental group has been established with CTX (sc-202117, Chem Cruz, USA) treatment. No treatment was applied to the C group. The S group were given an equal amount of saline. CTX was administered intraperitoneally one every 2 days for 3 weeks; the first dose was 70 mg/kg, the ongoing doses were 30 mg/kg. At the end of 3 weeks mice were sacrificed and kidneys were taken for investigation.

2.2. Histological Analysis

After sacrifice, kidneys were immediately excised and fixed in 10% formaldehyde at room temperature for 24 h. Routine paraffin process was applied to the tissues. The renal tissues were embedded in paraffin, cut into 5 μm sections, stained with hematoxylin and eosin (H&E), and examined using a light microscope.

2.3. Immunohistochemistry

Tissue sections were incubated at 60 °C overnight and then held in xylene and rehydrated through a series of ethanol solutions. Sections were washed with distilled water and phosphate-buffered saline (PBS- Biowhittaker, BE17-516F, Lonza, Belgium) for 10 min and then treated with 0.1 % trypsin (00-3008, Invitrogen, Camarillo, CA, USA) at 37 °C for 10 min and washed with PBS. Sections were incubated in a solution of 3% H_2O_2 (K31355100, Merck, Darmstadt, Germany) for 5 min to inhibit endogenous peroxidase activity. After washing with PBS, the tissue sections were incubated with non-immune serum (85-9043; Invitrogen, Camarillo, CA, USA) for 1 h. After incubation with appropriate primary antibodies mTOR (sc-8319 Santa Cruz Biotechnology, Texas, USA), AKT (sc-377457 Santa Cruz Biotechnology, Texas, USA) and PI3K (sc-376112, Santa Cruz Biotechnology, Texas, USA) overnight at 4 °C, the sections were incubated with biotinylated secondary antibody (85-9043; Invitrogen, Camarillo, CA, USA) and then with streptavidin conjugated to horseradish peroxidase (85-9043; Invitrogen, Camarillo, CA, USA) in PBS for 30 min each. After washing 3 times with PBS, sections were incubated with diaminobenzidine (ACK125, ScyTek DAB Chromogen, Logan Utah, USA) for 10 min for immunostaining. The sections were then counterstained with Mayer's hematoxylin (W0102090207, DDK Italia), dehydrated, cleared, and mounted with mounting solution (Aqueous-Mount SycTek, AML030, Logan Utah , USA). The negative control slices were treated with the same procedures described as above except the primary antibodies were replaced with PBS. All sections were evaluated using a light microscope (BX43, Olympus, Japan). Immunostaining intensities of antibodies were evaluated as mild (+), moderate (++), strong (+++) or very strong (++++). H-Score was used to compare the groups and ANOVA statistical test was used. $p < 0.05$ was considered as statistically significant.

3. Results

To understand the mechanisms used by the cyclophosphamide in kidney, we investigated the effects on PI3K/AKT/mTOR signaling pathways. The expression of PI3K, AKT, mTOR in the C57BL/6 mice treated with CTX decreased significantly compared to the control and sham groups as shown in Figure 1 ($p < 0.01$). These results indicate that CTX inhibited PI3K/AKT/mTOR signaling pathways.

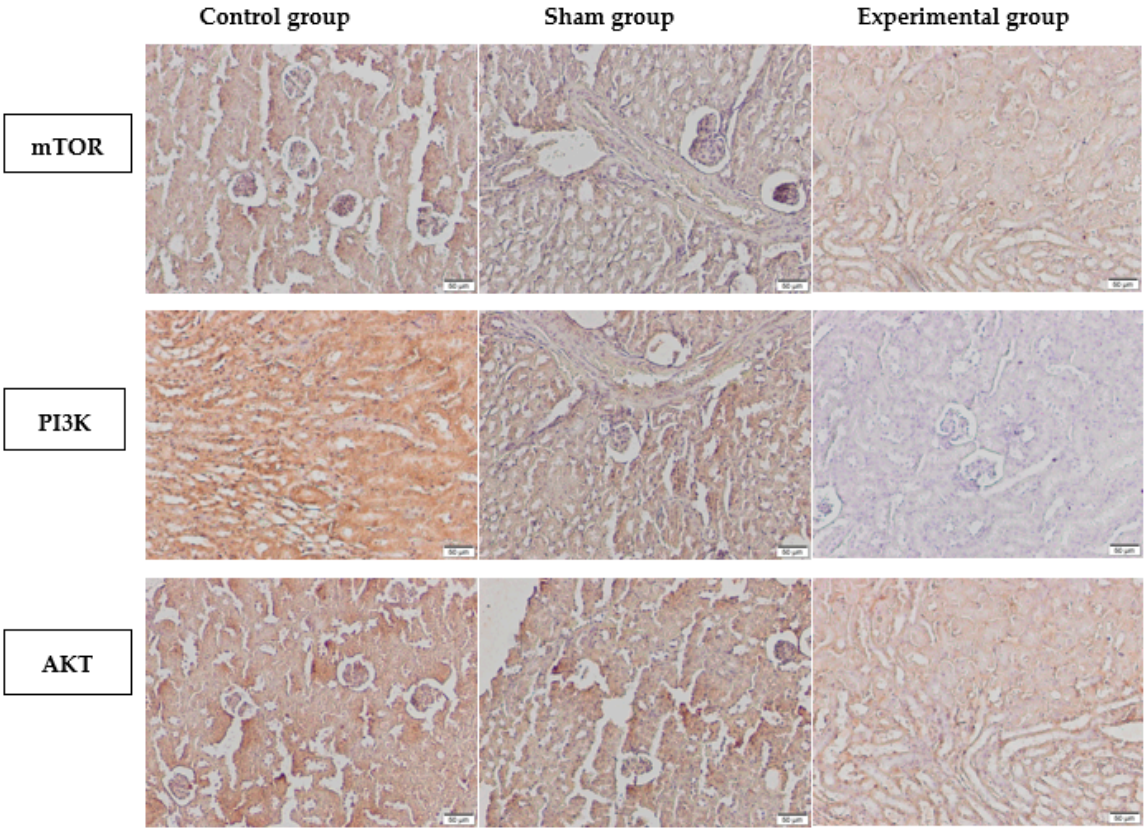

Figure 1. Distributions of PI3K, AKT and mTOR immunoreactivities in kidney samples from experimental, control and sham groups.

4. Discussion

Cyclophosphamide is an effective antineoplastic agent that can be used alone or simultaneously with other antineoplastic drugs. Cyclophosphamide is also a powerful immunosuppressive agent used in the treatment of nonneoplastic autoimmune disorders such as transplant rejection, Wegener's granulomatosis, rheumatoid arthritis, nephrotic syndrome and multiple sclerosis [4,5].

The PI3K/AKT /mTOR signaling pathway plays a central role in cell growth, proliferation and survival. The downstream targets of the PI3K/AKT/mTOR signaling pathway include protein kinase mTOR, the most important regulator of translation and autophagy. Despite the limited number of studies on the association of renal diseases with PI3K/AKT/mTOR activation, an increasing number of studies suggest that PI3K/AKT/mTOR may play an important role in many renal disorders [6,7].

In our study, PI3K, AKT and mTOR expression levels were found to be significantly decreased in CTX-mediated mechanisms indicating that the mechanisms of CTX might involve in the inhibition of PI3K signaling pathway.

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