The effect of anakinra to nephrotoxicity with cisplatin induced in rats: Biochemical, gene expression and histopathological evaluation

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Abstract

Background. Oxidative stress and interleukin-1 beta (IL-1β) have been reported to play a role in the pathogenesis of nephrotoxicity induced by cisplatin.

Objectives. The objective of this study was to investigate the effect of anakinra, which is an IL-1β receptor antagonist, on cisplatin-induced nephrotoxicity in rats, through biochemical, gene expression and histopathological analyses.

Material and methods. The study was designed with 4 groups. For 1 week, the control group (C) and the cisplatin (Cis) group received distilled water, while the cisplatin + anakinra 50 (Cis + ANA50) group and the cisplatin + anakinra 100 (Cis + ANA100) group were intraperitoneally administered 50 mg/kg and 100 mg/kg of anakinra, respectively. The Cis, Cis + ANA50 and Cis + ANA100 groups were intraperitoneally injected with a 2.5 mg/kg dose of cisplatin for 7 days. After sacrifice, the kidney tissue of each rat was extracted for the assessment of the malondialdehyde (MDA) and total glutathione (tGSH) levels, and for gene expression analyses of IL-1β. The kidney tissues were histopathologically evaluated. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA).

Results. The administration of cisplatin (the Cis group) yielded a higher level of MDA (4.75 ±0.25 nmol/mL; p < 0.001) and lower levels of tGSH (1.80 ±0.35 mg/L; p < 0.001) compared to other groups. Cisplatin also increased IL-1β gene expression (6.33 ±0.27 gene expression levels; p < 0.001) compared to other groups. The impact of anakinra on the MDA and tGSH levels, and on IL-1β gene expression induced by cisplatin was observed as a reversal of these findings (p < 0.05). Anakinra better prevented an increase of the levels of MDA and IL-1β at a dose of 100 mg/kg compared to a 50 mg/kg dose.

Conclusions. Anakinra prevents oxidative kidney damage induced by cisplatin in a dose-dependent manner. This result suggests that anakinra may be useful in the treatment of cisplatin-induced kidney damage.

Key words: rats, cisplatin-nephrotoxicity, anakinra
Introduction

Cisplatin is a platinum-derived anticancer drug which is widely used in chemotherapy. Since cisplatin is a non-cell-cycle specific chemotherapeutic agent, it is a broad-spectrum drug, commonly used in the treatment of various solid cancer types (stomach, testicular, ovarian, bladder, kidney, uterocervical, head and neck). However, nephrotoxicity during cisplatin chemotherapy makes it necessary to use cisplatin in limited doses, and sometimes even to discontinue the treatment. While the administration of cisplatin at low doses causes necrosis in the tubule cells of the kidney, high doses lead to apoptosis. Cisplatin has been reported to cause severe damage, especially in the epithelial cells of the proximal tubule of the kidney. Free oxygen radicals have been demonstrated to play a role in cellular death due to the use of cisplatin.

On the other hand, interleukin-1 beta (IL-1β) has been reported to play a crucial role in the pathogenesis of nephrotoxicity induced by cisplatin. These results suggest that antioxidants and IL-1β antagonists may be beneficial in the prevention of cisplatin nephrotoxicity. Anakinra, which we tested against cisplatin nephrotoxicity in the present study, is a recombinant human IL-1β receptor antagonist and the first biological agent which has been demonstrated to block pro-inflammatory effects in patients with rheumatoid arthritis. It has also been shown to be beneficial in the prevention of cisplatin nephrotoxicity.

Anakinra, cisplatin at a dose of 2.5 mg/kg for 1 week resulted in significant nephrotoxicity in the animals. These procedures were repeated once a day for 7 days. At the end of this period, all the rats were sacrificed with high-dose ketamine hydrochloride anesthesia and their kidney tissues were removed. Following the macroscopic evaluation of the kidney tissues, the MDA, tGSH and IL-1β gene expression levels were determined. In addition, the kidney tissues were histopathologically evaluated. The results obtained from the Cis + ANA50, Cis + ANA100 and C groups were evaluated in comparison with the Cis group.

Material and methods

Animals

A total of 40 male albino Wistar rats, each weighing 220–230 g, were randomly chosen. Prior to the experiment, the rats were divided into 4 groups, with 10 rats per group. The rats were kept and fed in the pharmacology laboratory at normal room temperature (22°C). The animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals, and approved by the local animal ethics committee (No. 179, November 27, 2015).

Chemical substances

Cisplatin, ketamine hydrochloride and anakinra were purchased from Koçak Farma Drug Industry (Istanbul, Turkey), Pfizer Drugs, Ltd. (Istanbul, Turkey) and Swedish Orphan Biovitrum AB (Stockholm, Sweden), respectively.

Experimental groups

The experimental animals were divided into control (C), cisplatin (Cis), cisplatin + anakinra 50 (Cis + ANA50), and cisplatin + anakinra 100 (Cis + ANA100) groups.

Experimental procedure

According to the method defined by Ohkawa et al., MDA forms a pink complex with thiobarbituric acid (TBA) at 95°C, which can be measured using spectrophotometry at a wavelength of 532 nm. A sample of 25 mg of tissue was homogenized using a solution of 1.15% KCl. Homogenates were centrifuged at 5000 g for 20 min, and the supernatants were used to determine the amount of MDA; 250 μL of homogenate, 100 μL of 8% sodium dodecyl sulfate (SDS), 750 μL of 20% acetic acid, 750 μL of 0.08% TBA, and 150 μL of purified water were pipetted into capped test tubes and
vortexed. The mixture was left for incubation at 100°C for 60 min before 2.5 mL of n-butanol was added to it, and then spectrophotometric measurement was conducted. The amounts of red color formed were read at 532 nm, using cuvettes of 3 mL, and, taking into account dilution coefficients, the MDA amounts in the samples were determined, using the standard chart. The standard chart created using MDA stock solution was prepared before.

**Total glutathione analysis**

The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications.14 The sample was weighed and homogenized in 2 mL of 50 mmol/L Tris–HCl buffer, containing 20 mmol/L of ethylenediaminetetraacetic acid (EDTA) and 0.2 mmol/L of sucrose at pH 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid (TCAA), the precipitate was removed after centrifugation at 4200 rpm for 40 min at 4°C, and the supernatant was used to determine the GSH level. A total of 1500 μL of measurement buffer (200 mmol/L of Tris–HCl buffer, containing 0.2 mmol/L of EDTA at pH 7.5), 500 μL of the supernatant, 100 μL of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), and 7900 μL of methanol were added to a tube and vortexed, and incubated for 30 min at 37°C. DTNB was used as a chromogen and it formed a yellow-colored complex with sulphydryl groups. The absorbance was measured at 412 nm, using a spectrophotometer (Beckman DU 500; Beckman Coulter, Inc., Brea, USA). The standard curve was obtained by using reduced glutathione.

**Superoxide dismutase analysis**

The superoxide dismutase (SOD) activity was based on the generation of superoxide radicals, produced by xanthine and xanthine oxidase, which reacts with nitro blue tetrazolium to form formazan dye. The SOD activity was thus measured at 560 nm by the degree of inhibition of this reaction.

**Interleukin-1 beta quantity measurement**

The tissue homogenate IL-1β concentrations were measured using a rat-specific sandwich enzyme-linked immunosorbent assay Rat Interleukin-1β ELISA Kit (Cat. No. YHB0616Ra; Shanghai LZ Biotech Co., Ltd., Shanghai, China). The analyses were performed according to the manufacturers’ instructions. Briefly, a monoclonal antibody specific for rat IL-1β was coated onto the wells of microwells. The tissue homogenate, standard solutions, biotinylated specific monoclonal antibody and streptavidin-HRP were pipetted into these wells, and then incubated at 37°C for 60 min. After washing, chromogen reagent A and chromogen reagent B were added, which were acted upon by the bound enzyme to produce a color. The mixture was incubated at 37°C for 10 min. Then, a stop solution was added. The intensity of this colored product was directly proportional to the concentration of rat IL-1β present in the original specimen. At the end of the course, the well plates were read at 450 nm via a microplate reader (BioTek, Winooski, USA). The absorbance of the samples was estimated with formulas that used standard graphics.

**Gene expression of IL-1β**

**RNA isolation**

RNA was isolated from the homogenized kidney tissue samples using a Roche Magna Pure Compact LC device with a MagnA Pure LC RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany). The quantity and quality of the isolated RNA was assessed with a nucleic acid measurement device (MaestroNano; Nucleotest Bio Ltd., Budapest, Hungary). The 50 μL RNA samples were stored at −80°C.

**Complementary DNA synthesis**

Complementary DNA (cDNA) was synthesized from the isolated RNA samples using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH). For each subject, 1 μL of ddH2O, 10 μL of RNA and 2 μL of random primer were combined and incubated in a thermal cycler for 10 min at 65°C. After incubation, 4 μL of reaction buffer, 0.5 μL of RNAase, 2 μL of deoxynucleotide mix, and 0.5 μL of reverse transcriptase were added. The reactions were incubated for 10 min at 25°C, for 30 min at 55°C, for 5 min at 85°C, and then they were held at 4°C. At the end of incubation, 1 μL of RNase H was added. The reaction was stopped by allowing the polymerase chain reaction (PCR) device to stand for 20 min at 37°C. The prepared product was stored at −80°C.

**Quantitative gene expression evaluation with real-time polymerase chain reaction**

For each cDNA sample, the gene expression of IL-1β and the reference gene (G6PD) was analyzed, using a Roche LightCycler 480 II Real-Time PCR instrument (Roche Diagnostics GmbH). The PCR reactions were recorded in a final volume of 20 μL, including 5 μL of cDNA, 3 μL of distilled water, 10 μL of LightCycler 480 Probes Master (Roche Diagnostics GmbH), and 2 μL of primer-probe set (Real-Time Ready single assay; Roche Diagnostics GmbH). The cycle conditions of the relative quantitative PCR (qPCR) were preincubation at 95°C for 10 min, followed by 45 amplification cycles of 95°C for 10 s, at 6°C for 30 s and at 72°C for 1 s, followed by cooling at 40°C for 30 s. The qPCR analysis and the calculation of quantification cycle (Cq) values for relative quantification were performed with the LightCycler 480 Software, v. 1.5 (Roche Diagnostics GmbH).
Diagnostics GmbH). Relative quantitative amounts were calculated by dividing the target genes by the expression level of the reference gene. The reference gene was used for the normalization of the target gene expression.

**Histopathological examination**

The renal tissues taken from the rats were fixed in 10% formalin for 24 h. Following the routine processing of tissue-embedded paraffin sections, 4 µm slices were obtained from the paraffin blocks. After deparaffinization and rehydration, the slices were stained with hematoxylin and eosin (H&E). The stained slices were evaluated under a light microscope (Olympus BX 52; Olympus, Tokyo, Japan) by a pathologist who did not know the applied treatment protocol. To assess inflammation and histopathological damage, some symptoms were examined, such as glomerular and tubular necrosis, dilatation and congestion in the blood vessels, and edema and hemorrhage in the interstitial area.

**Statistical analysis**

Statistical analyses were performed using the Statistical Package for Social Sciences, Windows v. 19.0 (SPSS Inc., Chicago, USA). Descriptive statistics for each variable were determined. The normality of data distribution was assessed by the Kolmogorov-Smirnov test. The results for continuous variables were demonstrated as mean ± standard error of the mean (mean ±SEM). The significance of differences between the groups was determined using the one-way analysis of variance (ANOVA) test followed by Tukey’s analysis. The results obtained from the drug-treated groups were evaluated in comparison with the Cis and C groups. A p-value <0.05 was considered significant.

**Results**

**Biochemical results**

The MDA level was significantly higher (p < 0.001) in the Cis group (4.75 ±0.25 nmol/mL) than that of the Cis + ANA50, Cis + ANA100 and C groups (2.93 ±0.08 nmol/mL, 1.93 ±0.14 nmol/mL and 2.03 ±0.39 nmol/mL, respectively). As seen in Fig. 1, the amount of MDA was significantly higher in the kidney tissue of the Cis group rats than in the C group (p < 0.001). The level of MDA was significantly lower in the kidney tissue of the Cis + ANA50 and Cis + ANA100 groups compared to the Cis group (p < 0.001). There was a significant difference between the MDA levels between the Cis + ANA50 and C groups (p < 0.01), while the levels of MDA were almost the same between the Cis + ANA100 and C groups (p > 0.05).

The tGSH level in the Cis group (1.80 ±0.14 mg/L) was significantly lower than that of the Cis + ANA50, Cis + ANA100 and C groups (3.15 ±0.18 mg/L; p < 0.005, 5.03 ±0.29 mg/L; p < 0.001 and 5.47 ±0.27 mg/L; p < 0.001, respectively). The level of tGSH was significantly higher in the kidney tissue of the Cis + ANA50 and Cis + ANA100 groups than in the Cis group (p < 0.001). There was a significant difference between the tGSH levels in the Cis + ANA50 and C groups, while the levels of tGSH were almost the same between the Cis + ANA100 and C groups (Fig. 2).

The SOD activity measured in the kidney tissue of the Cis group rats was 8.57 ±0.26 U/g protein, and the SOD activity in the Cis + ANA50, Cis + ANA100 and C groups were 5.55 ±0.34 U/g protein, 3.8 ±0.3 U/g protein and 3.4 ±0.22 U/g protein, respectively (Fig. 3).

The quantity of IL-1β was measured as 5.3 ±0.19 pg/mL in the kidney tissue of the Cis group. However, the amount of IL-1β was calculated as 3.1 ±0.16 pg/mL, 1.9 ±0.09 pg/mL and 1.6 ±0.13 pg/mL in the Cis + ANA50, Cis + ANA100 and C groups, respectively (Fig. 4).

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**Fig. 1. The MDA levels in the study groups**

MDA – malondialdehyde; Cis – group receiving cisplatin only; Cis + ANA50 – group receiving cisplatin and 50 mg/kg of anakinra; Cis + ANA100 – group receiving cisplatin and 100 mg/kg of anakinra; C – control group, receiving no drugs; * p < 0.001 compared with the C group; ** p < 0.01 compared with the C group.

**Fig. 2. The tGSH levels in the study groups**

tGSH – total glutathione; Cis – group receiving cisplatin only; Cis + ANA50 – group receiving cisplatin and 50 mg/kg of anakinra; Cis + ANA100 – group receiving cisplatin and 100 mg/kg of anakinra; C – control group, receiving no drugs; * p < 0.01 compared with the C group.
Interleukin-1 beta gene expression results

The IL-1β gene expression level was significantly higher (p < 0.001) in the Cis group (6.33 ±0.27) than in the Cis + ANA50, Cis + ANA100 and C groups (3.63 ±0.20, 2.62 ±0.18, and 2.22 ±0.21, respectively). As seen in Fig. 5, the gene expression level of IL-1β was significantly higher in the kidney tissue of the Cis group rats compared to other groups (p < 0.001). There was no statistically significant difference between the Cis + ANA100 and C groups regarding the IL-1β gene expression levels (p > 0.05).

Histopathological results

Histopathological examination of the renal tissue of the C group showed normal glomerular structure, Bowman’s capsule and Bowman’s space (Fig. 6A). However, a wide hemorrhagic area, dilated and congested blood vessels, and interstitial inflammation were observed in the Cis group (Fig. 6B). Moreover, in the renal tissue of the Cis group, hemorrhage was accompanied by glomerular and tubular damage, edema (circle arrow), and interstitial hemorrhage areas (Fig. 6C). Tubular necrosis, interstitial infiltration, and dilated and congested blood vessels were observed in the renal tissue of the Cis group (Fig. 6D). A near-normal appearance, mildly persistent tubule irregularities, and hemorrhage were found in the kidney glomeruli of the Cis + ANA50 group rats (Fig. 6E), whereas protected near-normal glomerulus, proximal and distal tubules were observed in the renal tissue of the group administered 100 mg/kg of anakinra (Fig. 6F).

Discussion

In this study, the effect of anakinra on cisplatin-induced nephrotoxicity in rats was investigated through biochemical and histopathological findings, and through gene expression analysis. Cisplatin increased the levels of MDA and IL-1β, and decreased the levels of tGSH in the renal tissue of rats. Anakinra prevented an increase of MDA and IL-1β, and a decrease of tGSH due to cisplatin. Additionally, the histopathological examination of anakinra used at a dosage of 100 mg/kg was found to improve renal damage which occurred due to cisplatin.

The pathogenesis of the nephrotoxic effect of cisplatin has not been fully elucidated; however, it has been argued in previous studies that the increase in the production of reactive oxygen species (ROS) leads to nephrotoxicity.16 As is known, ROS play a key role in the pathogenesis of cellular damage. As it is understood from the results of our biochemical experiment, the amount of MDA was increased and the amount of tGSH was decreased in the renal tissue of the rats administered cisplatin. It has also been reported that cisplatin causes oxidative damage to the kidneys by increasing the amount of MDA.17 The increase in the MDA level, the end product of lipid peroxidation...
in the tissues, is indicative of the increased ROS.\textsuperscript{18} It has also been documented that oxidative stress and inflammation are important factors in the development of cisplatin-induced nephrotoxicity.\textsuperscript{19} In our study, significant increases in the amount of MDA, as well as in the pro-inflammatory $IL-1\beta$ gene expression and amounts, were noted in the kidneys of animals receiving cisplatin.

Interleukin-1 beta has a number of functions, including the oxidative burst of neutrophils via the signaling molecules of inflammation and the release of free radicals.\textsuperscript{20} The increased expression and amount of IL-1$\beta$ we found supports the direct association between IL-1$\beta$ and oxidative stress in the renal tissue with high MDA and low tGSH and SOD levels. Additionally, it has been stated...
with histopathological examinations that cisplatin leads to renal damage by increasing the levels of oxidants and of IL-1β. Cisplatin is known to cause interstitial inflammation, dilated and congested blood vessels, hemorrhage, and edema in the kidneys. Furthermore, it has been argued that the serious side effects of cisplatin, such as tubular necrosis, are caused by the induction of cytokine production. In our experimental results, we also found that there was evidence of inflammatory markers (hemorrhage, dilated and congested blood vessels, interstitial inflammation, and edema), glomerular damage and tubular necrosis in the kidney tissues of the cisplatin group, whose MDA and IL-1β gene expression levels increased significantly. These biochemical and histopathological findings suggest that oxidative stress develops in the kidney tissue when we administer cisplatin.

There are endogenous antioxidant defense systems against ROS in living tissues. However, an overproduction of ROS leads to the consumption of the antioxidant defense system and also to oxidative stress. In our current study, the levels of non-enzymatic and enzymatic antioxidants, such as tGSH and SOD, in the kidney tissue of the cisplatin group (in which the above-mentioned histopathological damage was observed) was decreased. There are studies showing that a significant reduction in the amount of tGSH is associated with oxidative stress in cisplatin nephrotoxicity. It has also been reported that the SOD activity decreases in the kidney tissue with tubular damage, apoptosis and inflammation due to cisplatin treatment. These results support the hypothesis that cisplatin nephrotoxicity is associated with oxidative stress.

This information has led to the testing of anti-inflammatory and antioxidant drugs against cisplatin nephrotoxicity. The IL-1β receptor antagonist anakinra, which is used against the nephrotoxicity of cisplatin, has been found to prevent the increase in MDA and IL-1β, caused by cisplatin, and also to decrease the tGSH and SOD levels in the kidney tissue. The antioxidant and anti-inflammatory features of anakinra are believed to protect the renal tissue against the oxidative damage of cisplatin. In addition, there are studies associating the protective effect of anakinra with the antioxidant activity resulting from the blockage of IL-1β receptors. Severe pathological findings were observed in the renal tissue of the Cis group with significantly increased levels of oxidants and IL-1β, such as wide hemorrhage areas, dilated and congested blood vessels, interstitial inflammation, glomerular and tubular damage, edema, and tubular necrosis. However, while mildly persistent tubule irregularities and hemorrhage were seen in the Cis + ANA50 group with high amounts of tGSH identified, the glomerulus, proximal tubule and distal tubule were evaluated as healthy in the Cis + ANA100 group, in which the amount of tGSH was found to be higher. There are studies in the literature which histopathologically demonstrate that hemorrhage areas were developed in the renal tissues of animals treated with cisplatin.

The formation of congested blood vessels due to cisplatin has also been shown in previous studies. In addition, cisplatin leads to interstitial inflammation and edema in the kidneys. It has been stated that cisplatin causes more severe damage in the kidney tubules, such as necrosis. The role of free oxygen radicals has also been demonstrated in cellular death due to cisplatin.
As a result, severe histopathological damage was developed in the kidney tissue of the cisplatin group, which had high levels of the oxidant MDA and of gene expression, and high amount of pro-inflammatory IL-1β, but also had a low amount of the antioxidant tGSH. It was found that the increase in MDA and IL-1β gene expression and amount as well as the decrease in tGSH due to cisplatin in the kidney tissue were significantly inhibited by anakinra at a dose of 100 mg/kg compared to a dose of 50 mg/kg. It was observed that histopathologically, anakinra at a dose of 100 mg/kg better protects the renal tissue against the oxidative damage of cisplatin and more efficiently suppresses the production of MDA and IL-1β. This information suggests that anakinra may be useful in clinically reducing the toxic effect of cisplatin on the kidneys.

References