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Original Article

The effects of Cisplatin on Embrionic Renal Cell (Hek-293) Amino Acid Metabolism

Cisplatinin Embriyonik Renal Hücre (Hek-293) Amino Asit Metabolizması Üzerine Etkileri

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Abstract

Background: Cisplatin is one of the most widely used and most potent chemotherapy drug, due to its side effects in normal tissues and organs, especially its nephrotoxic effects in the kidneys, its use was limited. Therefore, new studies are being conducted to elucidate the mechanism of nephrotoxicity and to identify new protective agents in order to reduce the side effects of cisplatin. This study was conducted to investigate the effects of cisplatin on amino acid metabolism of embryonic kidney cells (HEK-293). **Materials and Methods:** In the study, HEK-293 were incubated with cisplatin at different doses (1-20µg) in appropriate medium for 24 hours, and then the cells were homogenized. The intracellular free amino acid profile in the samples was analyzed by LC-MS/MS method. **Results:** Compared to the control group, a significant decrease was observed in 1-methylhistidine, 3-methylhistidine, aspartic acid and cystine levels, while a significant increase was found in other amino acids. As a result of conducting advanced bioinformatic analyzes through Principal Component Analysis (PCA) and Variable Importance in Projection (VIP), it was determined that the most significant difference that was realized between the control and cisplatin applied groups was in glutamine, glutamic acid, glycine, arginine and lysine amino acids.
Conclusions: It was observed that high doses of cisplatin can inhibit protein synthesis in kidney cells and increase the amount of free amino acids by increasing the digestion of intracellular damaged proteins.
Keywords: Amino Acid, LC-MS/MS, Metabolomic, Prostate Cancer

ÖZ

Amaç: Sisplatin en yaygın kullanılan ve en güçlü kemoterapi ilaçlarından biridir, normal doku ve organlardaki yan etkileri, özellikle böbreklerdeki nefrotoksik etkileri nedeniyle kullanımı sınırlı kalmıştır. Bu nedenle, sisplatinin yan etkilerini azaltmak için nefrotoksisite mekanizmasını aydınlatmak ve yeni koruyucu ajanları belirlemek için yeni çalışmalar yürütülmektedir. Bu çalışma, sisplatinin embriyonik böbrek hücrelerinin (HEK-293) amino asit metabolizması üzerindeki etkilerini araştırmak amacıyla yapılmıştır.
Gereç ve Yöntem: Çalışmada HEK-293, sisplatin ile farklı dozlarda (1-20µg) uygun ortamda 24 saat inkübe edildikten sonra hücreler homojenize edildi. Numunelerdeki hücre içi serbest amino asit profili LC-MS/MS yöntemi ile analiz edildi.
Bulgular: Kontrol grubu ile karşılaştırıldığında, 1-metilhistidin, 3-metilhistidin, aspartik asit ve sistin düzeylerinde önemli bir düşüş gözlenirken, diğer amino asitlerde önemli bir artış tespit edildi. Temel Bileşen Analizi (PCA) ve Projeksiyonda Değişken Önem (VIP) ile ileri biyoinformatik analizler sonucunda, kontrol ve sisplatin uygulan gruplar arasında en önemli farkın glutamin, glutamik asit, glisin, arginin ve lisin amino asitlerinde olduğu belirlendi.
Sonuç: Yüksek dozlarda sisplatinin böbrek hücrelerinde protein sentezini engelleyebileceği ve hücre içi hasarlanan proteinlerin sindirimini artırarak serbest aminoasit miktarını artırabileceği görüldü.
Anahtar Kelimeler: Amino Asit, LC-MS/MS, Metabolomik, Prostat Kanseri

Highlits

- High doses of cisplatin alter amino acid and protein metabolism in kidney cells.
- Free amino acids significantly reflect nephrotoxicity.

Introduction

Cisplatin is one of the most remarkable achievements in the 'battle against cancer'. Although it was accidentally discovered forty years ago, cisplatin showed very highly effect as a chemotherapy treatment. Cisplatin nowadays, it is used in the treatment of many types of cancer such as non-small cell lung cancer, ovarian, cervical, testicular, head and neck cancer (1-5). It has a high cure rate of over 90% in testicular cancers. Although many of the drugs used in treatment of cancer are complex organic compounds, meanwhile, cisplatin is considered to be an inorganic and simple molecule. Cisplatin's anticancer activity and its mechanisimare not fully understood, but a widely accepted view of points declared that cisplatin binds to DNA, leading to the formation of interchain crosslinks. The cross-linking causes defective DNA templates and cessation of DNA synthesis and replication. This mechanism of action may cause cross-linking and DNA damage in rapidly dividing cells as well as in cancer cells (6-10). The success of cisplatin in the treatment of cancer has led to an increase in studies aimed at investigating such side effects. Although cisplatin is a mainstay for the treatment of cancer, its use is mainly limited to two factors: severe side effects in normal tissues and acquired resistance to cisplatin (2-5). The cisplatin's resistance mechanism has been studied; this mechanism may include increased out flowor decreased uptake of cisplatin, increased DNA repair, neutralization of cisplatin by glutathione and other sulfur-containing molecules, and defective apoptotic signaling in response to DNA damage (7-10). Another important limiting factor in the use of cisplatin is that its side effects on normal tissues, such as ototoxicity, nephrotoxicity, vomiting and nausea, and neurotoxicity. Various approaches and studies have been done for many years to reduce these side effects. One strategy was done to synthesize and screen for new cisplatin analogues with lower toxicity in normal tissues. In this way, several analogues of cisplatin, such as carboplatin, have been identified with less serious side effects (11-14). Another approach that has been successfully used to hydrate patients during cisplatin therapy. Although many efforts was made, the nephrotoxicity side effect of cisplatin, in particular, remain an important limiting factor in the use of cisplatin in cancer treatment. Understanding the cisplatin's nephrotoxicity mechanism may lead to new renoprotective interventions (15-17). In this study, the effects of cisplatin on the amino acid metabolism of normal kidney cells were examined for the first time.

Material and Method

Cell Culture Conditions

Normal human embryonic kidney epithelial (HEK-293) cells, that obtanied and stocked from American Type Culture Collection(ATCC), were used in the study. DMEM-F12 (Sigma-Aldrich Cat No: D9785,USA) fattening media, 10% FBS (Sigma-Aldrich Cat No: F7524, USA) and 1% L-glutamine (Sigma-Aldrich Cat No: 59202C, USA), 1% penicillin/streptomycin (Sigma-Aldrich Cat No: P4333, USA) were used for the nutrition and growth of the cells. The cells were taken from -80 °C and dissolved in a water bath at 37°C.The thawed cell solution was carefully transferred to the nutrient medium taken into the falcon tube and centrifuged at 5000 rpm for 5 minutes, 2 ml of medium was added to the formed pelleted cells, suspended with a pipette and homogenized, 8 ml of medium was added to a 25 cm^2 flask and the cell solution was added. It was incubated at 37 °C, 5% CO_2 and 95% humidity for 24 hours and cell proliferation was observed. After the cells reached 80% in 25 cm^2 flasksthe study was started. After the sample was taken from the medium after incubation, the remaining medium was removed. In order to remove the remaining medium waste, the cells were washed 2 times with cold 1x Dulbecco Phosphatebuffered saline (dPBS, Sigma-Aldrich Cat No: D8537, USA) and scraped with a cell scraper. After being taken into 1 ml cold 1x dPBS medium, the cell suspension was taken into a 15 ml sterile falcon tube. The pellet was obtained by centrifuging at 1200 rpm for 5 minutes and removing the supernatants. After the obtained pellet was washed 2 times with 1xdPBS, it was lysed with cold lysis buffer and homogenizer (Qiagen tissue lyser, Germany) at 4^{+}°C for 10 minutes. The resulting lysate was centrifuged at 14,000 rpm. Analyzes were made with the obtained supernatant.

Investigation of free amino acid profile by LC-MS/MS

Intracellular free amino acid profiling analysis was performed using LC-MS/MS (Shimadzu-8045) and applying commercial kit protocol (Bome Trivitron, Trimaris-BR130030, Turkey)(21). In this kit method, a derivative method is used in the analysis of free amino acids, after 100 µL of sample is taken, it is mixed with an internal standard mixture consisting of 20 amino acids with C13 and N15 labeled atoms prepared in 0.1 M HCl. In the second step, basic organic buffer components prepared in propanol are added tothe pH balance toenable the derivative reactionis taking place more efficiently. At this stage, the precipitation of the proteins in the sample also takes place. Then, a chloroform/isooctane mixture containing 5% alkyl chloroformate as an active ingredient is added to the mixture and left at room temperature for 3 minutes. Then, by centrifugation the derivatized amino acids are taken to the upper phase that contains organic solvents. 1 µL of this phase is injected into the LC-MS/MS system. Since the molecular weightafter the extraction and derivatization process of the esterified amino acids increases and becomes more volatile accordinglly, the signal given by the MS device also increases. The separation of chromatographic was performed on Trimaris Amino Acid LC-MS/MS column (250 mm x 2 mm, 3 µM) containing C18 reversed phase filler. Mobile phase A content was determined as Water: MeOH:1M Ammonium formate (85:14:1) and Mobile phase B content was determined as MeOH. Amino acid molecules were analyzed in MRM mode by ESI (+) ionization method.

Statistical Analysis

Data were statistically analyzed using SPSS version 22.0 (SPSS Inc.). Results were given as mean ± standard deviation. Differences between groups were analyzed using the Kruskal-Wallis test followed by the Tamhane test, and $p<0.05$ was considered significant. MetaboAnalyst 5.0 was used for multivariate statistical analysis. Segregation and clustering between groups were determined by Principal Component Analysis (PCA). Variable Importance in Projection (VIP) scores were found to detect amino acids that contributed to this dissociation and aggregation. In addition, a heat map was created to visualize the densities of amino acidsthat varied between groups.

RESULTS

Effects of cisplatin on the profile of the free amino acid of kidney cells

Different doses effects of cisplatin on the amino acid profile (40 amino acid) of HEK-293 cells, compared with the control group, were statistically examined by the classical SPSS method, and the results are given in Table-1. Compared with the control, it was observed that cisplatin caused a significant change in other amino acid except anserine and alpha-aminopimelic acid in kidney cells. When comparing with the control group, a significant decrease was observed in 1-methylhistidine, 3-methylhistidine, aspartic acid and cystine levels, while a significant increase was observed in other amino acids.

Table 1. Quantitative analysis of inside cell of amino acids composition of the groups.

Amino Acids Name	Group Means (µm)					*p*
	Control	Cisplatin 1µm	Cisplatin 5µm	Cisplatin 10µm	Cisplatin 20µm	
1-Methylhistidine	0.017	0.008	0.013	0.031	0.065	<0.01
3-Methylhistidine	0.008	0.007	0.002	0.004	0.018	<0.01
Alanine	1.523	3.205	3.025	4.318	11.682	<0.01
Alloisoleucine	0.001	0.004	0.004	0.013	0.021	<0.01
Alpha-Aminoadipic Acid	0.003	0.064	0.065	0.038	0.185	<0.01
Alpha-Aminobutyric Acid	0.003	0.004	0.008	0.004	0.114	<0.01
Arginine	0.517	1.459	1.282	5.756	9.911	<0.01
Argininosuccinic Acid	0.032	0.061	0.195	0.025	0.182	<0.01
Asparagine	0.353	0.711	0.351	1.216	2.583	<0.01
Aspartic Acid	3.729	4.222	2.277	2.757	3.700	<0.01
Beta-Alanine	0.136	0.235	0.176	0.163	0.707	<0.01
Beta-Aminoisobutyric Acid	0.025	0.058	0.071	0.217	0.436	<0.01
Citrulline	0.134	0.479	0.448	0.572	0.483	<0.01
Cystathionine	0.813	0.006	0.422	0.012	1.886	<0.01
Cystine	0.013	0.007	0.010	0.526	0.022	<0.01
Gamma-Aminobutyric Acid	0.017	0.150	0.096	0.313	0.554	<0.01
Glutamic Acid	11.454	17.025	12.837	11.655	34.274	<0.01
Glutamine	0.810	2.013	14.013	29.007	57.784	<0.01
Glycine	4.491	7.585	6.217	7.681	17.938	<0.01
Histamine	0.011	0.028	0.034	0.002	0.023	<0.01
Histidine	0.277	0.599	0.604	1.460	2.322	<0.01
Hydroxylysine	0.012	0.021	0.022	0.007	0.007	<0.01
Hydroxyproline	0.073	0.168	0.181	0.533	0.927	<0.01
Isoleucine	0.527	1.032	1.052	3.481	5.965	<0.01
Leucine	0.827	1.790	1.580	4.515	7.714	<0.01
Lysine	0.758	1.960	1.731	5.425	9.215	<0.01
Methionine	0.171	0.345	0.292	0.774	1.500	<0.01
Ornithine	1.097	2.570	2.101	3.959	4.510	<0.01
Phenylalanine	0.516	1.141	1.094	2.972	5.029	<0.01
Proline	1.632	3.338	2.640	3.481	11.900	<0.01
Serine	1.498	2.933	2.524	4.040	5.409	<0.01
Serotonin	0.007	0.004	0.008	0.025	0.004	<0.01
Taurine	12.792	17.293	19.488	14.290	22.729	<0.01
Thiaproline	0.011	0.004	0.005	0.031	0.066	<0.01
Threonine	1.259	2.591	2.608	5.168	11.150	<0.01
Tryptophan	0.086	0.180	0.162	0.440	0.811	<0.01
Tyrosine	0.379	0.862	0.848	2.456	4.326	<0.01
Valine	0.549	1.230	1.268	3.666	6.023	<0.01
Anserine	17.511	19.709	18.478	18.231	18.619	>0.05
Alpha-Aminopimelic Acid	0.857	0.863	0.907	0.854	0.891	>0.05

Different doses of cisplatin distinguished by PCA Analysis

PCA was performed to visualize the distribution of samples in the cisplatin-administered and control groups. Two-dimensional and three-dimensional score graphs of PCA analysis are shown in figure 1A and figure 1B, respectively. PC1, PC2 and PC3 accounted for 93.7% and 5% of the variation in the data, respectively. PCA analysis In the amino acid profile of different doses of cisplatin applied to HEK-293 cells, a significant clustering and separation was observed between the groups.

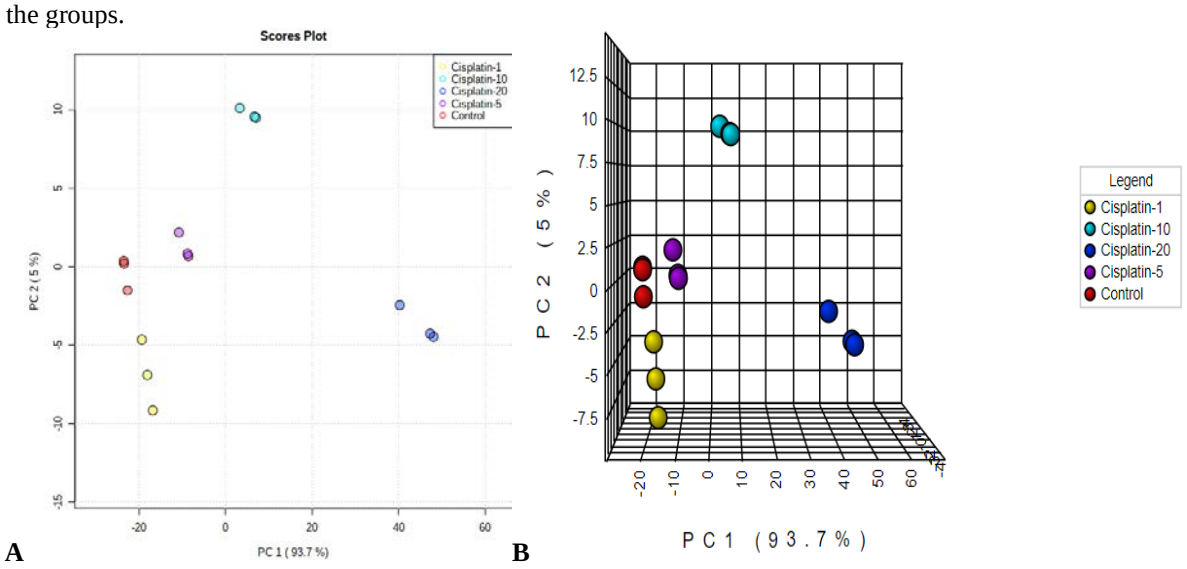

Figure 1. The amino acid profile of the Cisplatin groups applied at different doses and the control group. 2D (A) and 3D (B) score graphs of PCA.

Screening of Differential Metabolites

VIP analysis was performed to determine which amino acids changed significantly compared to the control group in HEK-293 cells treated with different doses of cisplatin (Figure 2.). The higher the VIP score (more than 1), the higher the probability of separation. Conssequantly, it was showed that the first three amino acids with the highest VIP score were glutamine, glutamic acid and glycine. An increase in the score of amino acids indicates that their contribution to the separation between the groups increases.

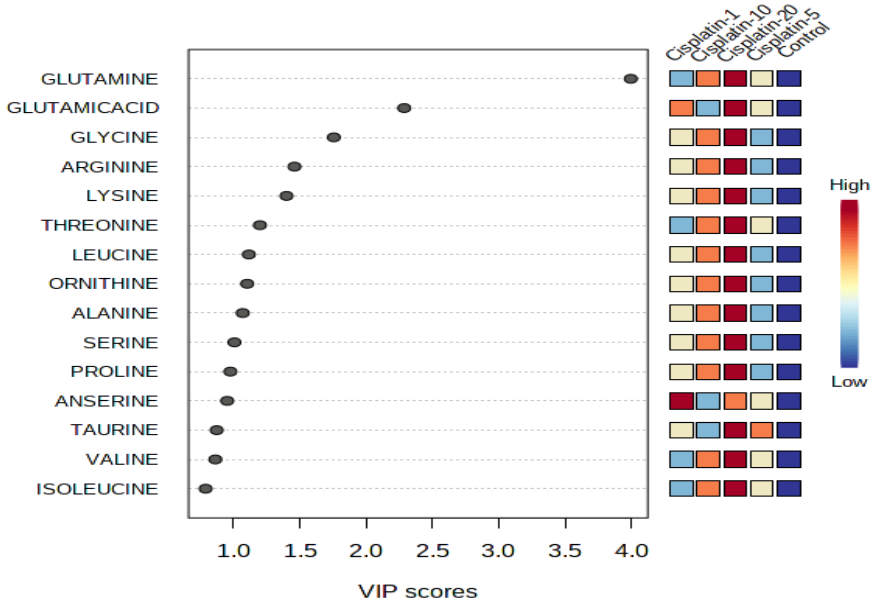

Figure 2. VIP score analysis graph.

A heat map was drawn to show the concentrations of the 40 amino acids analyzed in groups (Figure 3). In the heatmap, the columns represent cellular samples and the rows represent amino acids. Blue bands indicate that amino acids are down-regulated and brown bands are up-regulated. In addition, the increase in the depth of blue and brown colors indicates that the difference in amino acids between the groups increases. Dose increase was generally positively correlated with amino acid density. There is a significant difference in density compared to the control group, especially in the 20µm and 10µm Cisplatin applied groups.

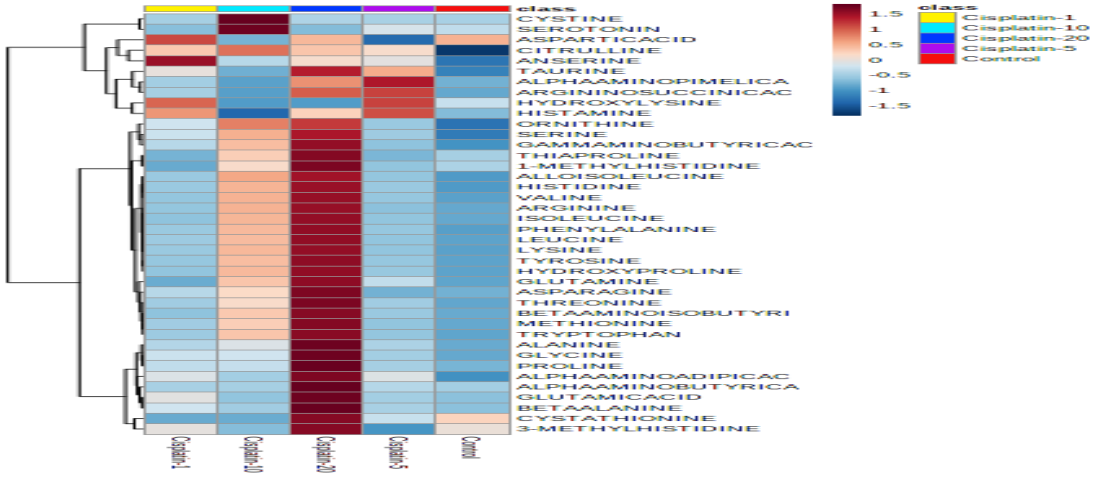

Figure 3. Hierarchical clustering heatmap of amino acids. Each group was shown in a different color code. Dendrograms were generated with Ward's clustering method and Euclidean distance measure.

Metabolic Pathway Analysis

To identify potential amino acid metabolism pathways affected by cisplatin, the pathway analyzes of amino acids identified by comparisons of groups (Cisplatin-1µm/Control, Cisplatin-5µm/Control, Cisplatin-10µm/Control and Cisplatin-20µm/Control) were investigated using MetaboAnalyst 5.0. The metabolic pathway analysis results in four comparisons of differential amino acids are shown in Figure 4. A total of 31 pathways were affected in all comparisons. Affected paths are common to all comparisons and have the same impact value. These pathways, Cisplatin-1µm/Control;11, for Cisplatin-5µm/Control; 10, for Cisplatin-10µm/Control; 9, for Cisplatin-20µm/Control; The effect value of 11 ways is >0.2 and p<0.05. Pathways with an effect value greater than 0.2 and $p < 0.05$ are considered to vary significantly. The most affected metabolic pathways that were detected are the phenylalanine, tyrosine and tryptophan biosynthesis pathway; aspartate, alanine and glutamate metabolism; D-glutamine, D-glutamate metabolism and arginine biosynthesis. In the comparisons shown in Figure 4, the match status, impact, *p*-value and -log(p) values of the altered metabolic pathways indicated by the arrow are shown in Table 2.

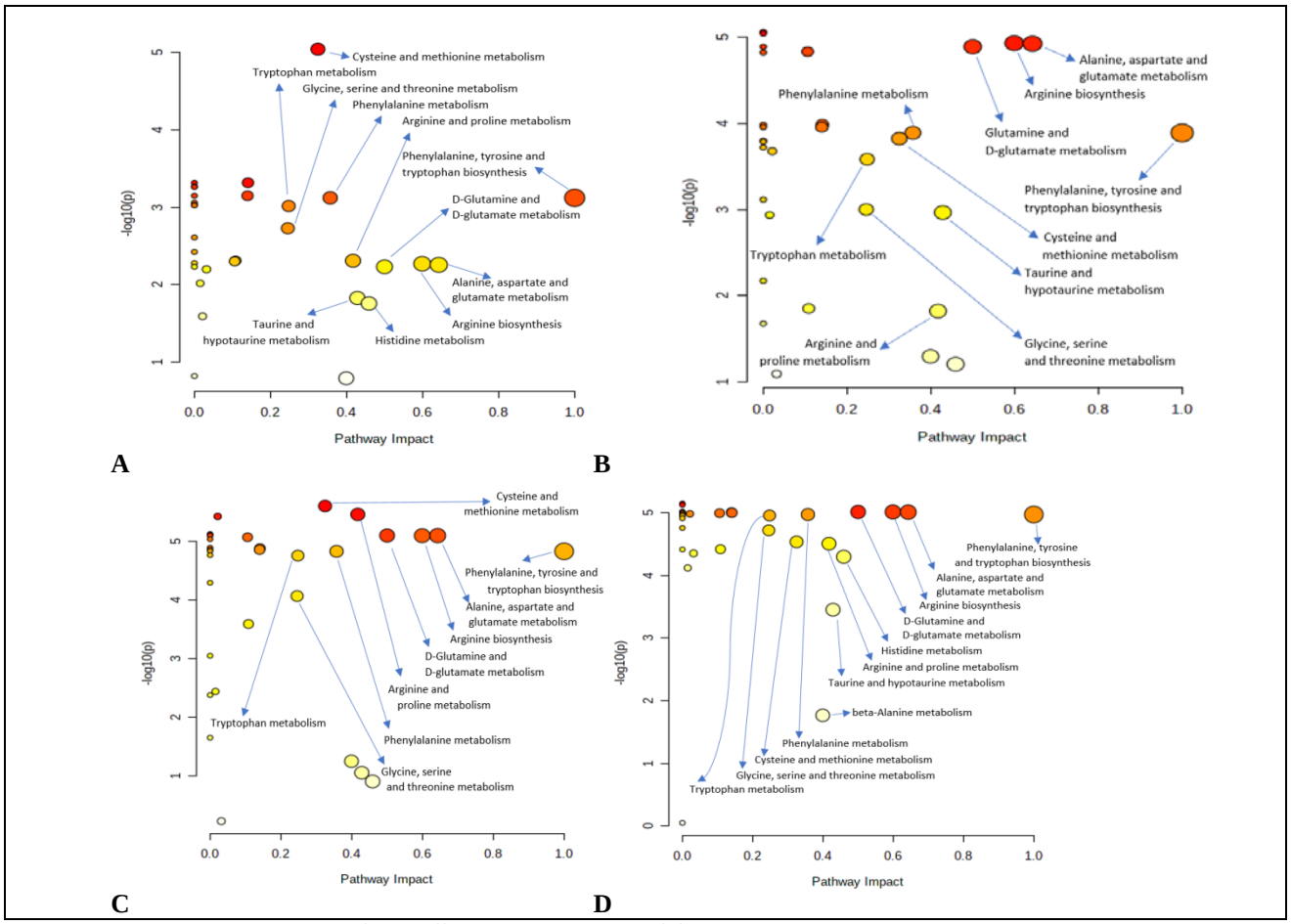

Figure 4. Metabolic pathway results from differential amino acids of four group comparisons, (A) Cisplatin-1µm/Control**, (B)** Cisplatin-5µm/Control, **(C)** Cisplatin-10µm/Control, and **(D)** Cisplatin-20µm/Control**. The color and size of the sphere depend on the p-value and the effect value, respectively. Shown with arrows are those with an effect value > 0.2 and p < 0.05.**

Discussion

Although cisplatin is an effective cancer drug that is frequently used in the treatment of various types of cancer, its various side effects, especially nephrotoxicity, limit its use. Acute kidney injury occurs in 20-35% of patients after cisplatin administration. This damage is dose-related and often results in dose reduction or discontinuation of therapy. In addition to DNA damage in the kidney proximal tubules, cisplatin causes nephrotoxicity through different cytotoxic mechanisms such as oxidative stress and inflammation (17, 18). As a result of studies carried out to date, it has been determined that increased apoptosis as a result of increased oxidative stress and DNA damage plays a fundamental role in the pathophysiology of cisplatin. However, it is still not clear how different cisplatin-induced pathways integrate and lead to kidney damage (17).

The protective efficacy of different antioxidant substances (curcumin, naringenin, Vit-E, etc.) to reduce nephrotoxicity caused by cisplatin has been investigated by many researchers. As a result of these studies, it was concluded that these substances, which have antioxidant and anti-inflammatory properties, can be used as supplements to alleviate cisplatin-induced nephrotoxicity (19-20). However, it is unclear whether such natural compounds with antioxidant activity interfere with cancer treatment while preventing cisplatin-induced kidney damage. Therefore, besides the protective effect of such natural compounds, it is necessary to investigate whether they have effects on tumor growth. Such studies may help to break the limited clinical use of cisplatin (21). Despite the studies on such protective agents, the pathogenesis of the toxic effect of cisplatin on kidney cells is still not clear. Therefore, new studies are needed. In this study, the effects of cisplatin on amino acid metabolism of embryonic kidney cells were investigated for the first time. In this way, it was aimed to predict the possible efficacy of amino acids both in the pathophysiology and in the treatment of nephrotoxicity.

Amino acids are the building blocks of proteins and are known to play a fundamental role in mediating epigenetic regulation and post-transcriptional modification as well as regulating the energetic regulation, biosynthetic support and homeostatic balance, as well as the regulation of redox balance. An example is the regulation of the methionine cycle, which plays an important role in DNA and histone methylation, by methionine, serine and glycine, and the derivation of acetyl-CoA, which plays an important role in tumor growth, from branched-chain amino acids and lysine (21-22).

Amino acids are also critical for the growth of normal and cancer cells as a building material of nucleotides biosynthesis, the regulation of lipid biosynthesis, methylation and acetylation in the regulation of ROS homeostasis by providing specific metastasis and tumor cells through epigenetic regulation. In addition, certain metabolic intermediates of certain amino acids may contribute to both tumorigenic and anti-tumorigenic activities. For example; While nitric oxide (NO), a product of arginine metabolism, promotes tumor growth by promoting angiogenesis, it can also act as a tumor suppressor by upregulating p53 (22-24).

In this study, the free amino acid profile of different doses of cisplatin in normal kidney cells was investigated. As a result of the SPSS analysis of the data obtained, it was observed that there was an increase in almost all free amino acids in the cisplatin administered groups. As a result of PCA, PLS-DA and VIP analyzes performed using the metaboanalyst program, it was determined that the most significant difference between the control and cisplatin applied groups was in glutamine, glutamic acid, glycine, arginine and lysine amino acids. It was observed that these amino acids increased with the increasing in the dose in the cisplatin administered groups, while the greatest increase was observed in the highest dose.

Conclusion

Cisplatin, a chemotherapeutic agent, is used in many types of cancer. However, the most important dose-dependent limitation of cancer treatment is that it causes nephrotoxicity. The science of metabolomics has become important in elucidating this pathophysiology. The spread of this science, which examines metabolites such as amino acids, may make it clear by which cellular mechanisms cisplatin causes nephrotoxicity. According to our data, it can be concluded that high dose cisplatin significantly inhibits intracellular protein synthesis and causes protein degradation. More scientific studies are needed to understand the effect on amino acid and protein metabolism. In this way, successful results can be obtained in the treatment by providing the use of cisplatin in the required doses to people with cancer.

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