ORIGINAL ARTICLE

EFFECT OF RAPAMYCIN AND 3-METHYLADENINE ON HEAT SHOCK PROTEINS IN CISPLATIN-INDUCED HEPATOTOXICITY

Sisplatin Kaynaklı Hepatotoksisitede Rapamisin ve 3-Metiladenin'in Isı Şoku Proteinleri Üzerindeki Etkisi

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ABSTRACT

Objective: This study aimed to investigate the effects of autophagy activator (rapamycin) and autophagy inhibitor (3-methyladenine) on heat shock proteins (HSP) in cisplatin-induced hepatotoxicity.

Material and Methods: Wistar albino rats (28) in the study were divided into four equal groups. Control group, Cisplatin group; On day 7 of the experiment, a single dose of 8 mg/kg intraperitoneal (ip) cisplatin was administered, Rapamycin+Cisplatin (Rapamycin group); Starting from the 1st day of the experiment, rapamycin 2 mg/kg(ip) was administered once a day for 15 days, 8 mg/kg(ip) single-dose cisplatin was administered on the 7th day, 3-methyladenine+Cisplatin (3-Methyladenine group) 15 mg/kg(ip) was administered once a day for 15 days, 8 mg/kg(ip) single-dose of cisplatin was administered on the 7th day. Routine histological techniques were applied for liver tissues. Immunohistochemistry staining was performed to identify HSPs. TUNEL was performed to demonstrate apoptosis.

Results: All HSPs in the cisplatin group were statistically increased compared to the control. Rapamycin resulted in decreased expression of all HSPs, which was increased by cis. 3-Methyladenine was less effective in HSPs compared to Rapamycin. TUNEL-positive cells increased statistically in the Cisplatin group. Rapamycin was similar to the control group. TUNEL-positive cells did not change in the 3-methyladenine group.

Conclusion: We think that Rapamycin reduces both HSPs and related cell death by contributing to the reduction of Cisplatin-induced oxidative stress in the tissue. Inhibition of autophagy with 3-Methyladenine in tissue causes increased expression of Cisplatin-induced HSPs. As a result, Rapamycin is more effective than 3-Methyladenine on HSPs in Cisplatin toxicity.

Keywords: *Heat Shock Proteins; Cisplatin; Rapamycin; 3-methyladenine; Liver*

ÖZET

Amaç: Bu çalışmanın amacı, otofaji aktivatörü (rapamisin) ve otofaji inhibitörünün (3-metiladenin) sisplatin kaynaklı hepatotoksisitede ısı şoku proteinleri (HSP) üzerindeki etkilerini araştırmaktı.

Gereç ve Yöntemler: Çalışmadaki Wistar albino sıçanları (28) dört eşit gruba ayrıldı. Kontrol grubu, Sisplatin grubu; Deneyin 7. gününde, tek doz 8 mg/kg intraperitoneal (ip) sisplatin uygulandı, Rapamisin+Sisplatin (Rapamisin grubu); Deneyin 1. gününden itibaren, rapamisin 2 mg/kg(ip) günde bir kez 15 gün boyunca uygulandı, 8 mg/kg(ip) tek doz sisplatin 7. günde uygulandı, 3-metiladenin+Sisplatin (3-Metiladenin grubu) 15 mg/kg(ip) günde bir kez 15 gün boyunca uygulandı, 8 mg/kg(ip) tek doz sisplatin 7. günde uygulandı. Karaciğer dokuları için rutin histolojik teknikler uygulandı. HSP'leri tanımlamak için immünohistokimya boyama yapıldı. Apoptozu göstermek için TUNEL yapıldı.

Bulgular: Sisplatin grubundaki tüm HSP'ler kontrole kıyasla istatistiksel olarak arttı. Rapamisin, cis tarafından artırılan tüm HSP'lerin ekspresyonunun azalmasına neden oldu. 3-Metiladenin, Rapamisin ile karşılaştırıldığında HSP'lerde daha az etkiliydi. TUNEL pozitif hücreler Sisplatin grubunda istatistiksel olarak arttı. Rapamisin kontrol grubuna benzerdi. TUNEL-pozitif hücreler 3-metiladenin grubunda değişmedi.

Sonuç: Rapamisin'in dokuda Sisplatin kaynaklı oksidatif stresin azaltılmasına katkıda bulunarak hem HSP'leri hem de ilgili hücre ölümünü azalttığını düşünüyoruz. Dokuda 3-Metiladenin ile otofaji inhibisyonu Sisplatin kaynaklı HSP'lerin ekspresyonunun artmasına neden olur. Sonuç olarak Rapamisin, Sisplatin toksisitesinde HSP'ler üzerinde 3-Metiladenin'den daha etkilidir.

Anahtar Kelimeler: *Isı Şoku Proteinleri; Sisplatin; Rapamisin; 3-metiladenin; Karaciğer*

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INTRODUCTION

Any stress or exposure to agents that cause disruption of homeostasis in cells changes the protein folding that the cell continues as a normal process. Prolonged and irreversible excessive protein accumulation can cause cell death. Therefore, cells have developed a conserved defense mechanism to prevent this condition and reduce damage due to protein folding changes (1). These are called heat shock proteins (HSPs) and serve as molecular chaperones within the cell, with activities such as facilitating protein folding and limiting aggregation (2). HSPs are known as a fundamental and well-conserved cellular response to stress within the cell, and also function as excitatory stress signals that activate the immune system and other cells (3). HSPs are generally classified by their molecular weight; such as HSP47, HSP60, HSP70, HSP90 and major HSPs (4). Maintaining regular protein homeostasis is important for maintaining intracellular homeostasis. This process is regulated by various systems. Two of them; cellular molecular chaperones and autophagy (5). Although autophagy is defined as cellular self-digestion, it is an adaptation of the cell to starvation. In this process, the cell breaks down and uses its own reserves. However, in this process, an irregular degradation capacity above the basal level carries the risk of death for the cell (6). The autophagy mechanism is a highly complex process and is regulated by mammalian target of rapamycin (mTOR) signaling pathways known to control checkpoint cellular homeostasis (7). Rapamycin is a mammalian target of rapamycin complex 1 (mTORC1) inhibitor and is also widely used as an activator of autophagy (8). On the contrary, 3-Methyladenine, as a class I phosphoinositide 3-kinase (PI3Ks) inhibitor, triggers the mTOR signaling pathway and is used as an autophagy inhibitor (9). Although cisplatin is used as one of the metal-based chemotherapeutic drugs used in the treatment of various solid cancers, it does not show its highest potential due to side effects and drug resistance (10). Therefore, the combination of cisplatin with different types of drugs and reducing its toxicity are of great importance. When cisplatin enters the cell, it causes processes such as inhibition of DNA synthesis, production of reactive oxygen species, lipid peroxidation, and induction of apoptosis (11). In addition, it has been shown in studies on tissues such as kidney and ear that several intracellular HSPs are affected in cisplatin toxicity (12,13). The relationship of cisplatin with autophagy has mostly been studied on tumor cells and due to drug resistance. There is information that tumors gain resistance to cisplatin chemotherapy with the presence of autophagy (14,15). One of the most common effects of cisplatin is hepatotoxicity. It is known to affect gene expression and apoptosis biochemically as well as histological changes in tissue (16,17). In this study, we evaluated the effects of rapamycin and 3-Methyladenine, known as autophagy activators and inhibitors, on cisplatin toxicity in liver tissue through molecular chaperones HSPs. We also examined whether programmed cell death in tissue due to this change is affected by apoptosis. Thus, it will be possible to determine to what extent HSPs, which change depending on the activation and inhibition of autophagy with cisplatin, trigger intracellular homeostasis.

MATERIAL AND METHODS

Wistar albino rats were used in the experiment. These rats were obtained from the Experimental and Clinical Research Center of Erciyes Universtiy, Kayseri, Türkiye. The rats were housed in well-ventilated plastic cages in a room with a 12-hour light:dark cycle. Access to food and water was not restricted. Humane care was applied in accordance with standard guidelines. Ethical approval for the study was obtained from the Erciyes University Animal Research Local Ethics Committee (date: 05/05/2021, decision no: 21/121) and ethical regulations were followed in accordance with national and institutional guidelines. The control group (n=7) was the group that received physiological serum. Cisplatin group (n =7) was injected with a single dose of 8 mg/kg (ip) Cisplatin on the 7th day. Rapamycin+Cisplatin (Rapamycin group) (n=7) 2 mg/kg Rapamycin (CAS#53123-88-9, LC Laboratories) per day (dissolved in ethanol+dH_2O) throughout the experiment, also injected with a single dose of 8 mg/kg (ip) Cisplatin on 7th day of the experiment. 3-Methyladenine+Cisplatin (3-Methyladenine

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group) (n=7) 15 mg/kg 3-Methyladenine (Cat. No: m9281, Sigma Aldrich, USA) per day (dissolved in ethanol+dH_2O) throughout the experiment, also injected with a single dose of 8 mg/kg (ip) Cisplatin on 7th day of the experiment. The experiment thus lasted for 15 days, and at the end, the animals were anesthetized with 30 mg/kg ketamine and 4 mg/kg xylazine, and liver tissue was taken for examination. Liver tissues were taken and placed in formaldehyde. They were kept in alcohol, then xylene, and embedded in paraffin, respectively. 5 µm sections were taken. Immunohistochemistry staining and apoptosis (TUNEL) were applied. Sections were incubated in an oven. It was then washed with water by passing through xylene and alcohol. The sections was treated in 0.01 M citrate buffer for antigen retrieval in the microwave for 10 minutes. Phosphate buffered saline (PBS) was used for washing in all subsequent steps. The sections were washed and kept in 3% hydrogen peroxide (H_2O_2). Serum block included in the staining kit (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was applied to the rewashed sections. Heat shock proteins (HSP) HSP47 (bs-1538R, Bioss, USA), HSP60 (bs-0191R, Bioss, USA), HSP70 (sc-33575, Santa Cruz Biotechnology, USA) and HSP90 (bs-10100R, Bioss, USA) were applied as antibodies. After applying the other solutions in the kit, the washed sections were treated with diaminobenzidine (DAB) (TA-060-HDX, Thermo Fisher Scientific, Waltham, Ma, USA). Sections that were counterstained with Gill hematoxylin were closed after soaking in alcohol and xylene (18). HSPs immunoreactivities in the pictures were measured using Image j program. It was demonstrated with the TUNEL method (Roche, In Situ Cell Death Detection Kit) to show programmed cell death in liver tissue. First, 5 µm sections were left in the oven at 60 degrees to remove paraffin. Tissues were deparaffinized with xylene again. Then they were rehydrated with alcohol. Slides were washed with PBS three times for a total of fifteen minutes. Citrate buffer (0.01 M) was applied for antigen retrieval in liver sections. Then, they were washed with PBS again. The instructions of the kit manufacturer were applied for the following steps. Finally, they were treated with DAPI (4',6-diamidino-2-phenylindole) in the dark to make the nuclei visible (19). TUNEL-positive cells (105 fields in total) in fifteen randomly selected areas in animals from all groups were counted under a fluorescence microscope and statistically analyzed.

Statistical Analysis

In this study, the GraphPad Prism7.00 program was used for statistics. One-way analysis of variance was used for parametric tests, and the Tukey post-hoc test was used for differences between groups. Kruskal Wallis test was used for non-parametric data. $p<0.05$ was considered statistically significant.

Table 1. HSPs immunoreactivity results of all groups

Groups	Control	Cis	Rapa	3MA	p
HSP47	81.80±2.49^a	85.18±3.19^b	77.59±3.98^c	78.75±3.39^c	*0.001*
HSP60	79.11±3.10^a	81.57±4.13^b	78.11±3.99^a	78.23±3.64^a	*0.001*
HSP70	78.53±2.96^a	82.67±5.37^b	83.58±2.73^b	91.61±4.21^c	*0.001*
HSP90	78.72±3.78^a	88.36±3.87^b	86.06±3.24^c	90.08±4.04^b	*0.001*

Data are shown as ± standard deviation. $p<0.05$ was considered significant. There was no significant difference between the groups containing the same letter (a, b and c). HSPs: Heat shock proteins, Cis: Cisplatin, Rapa: Rapamycin, 3MA: 3-methyladenine

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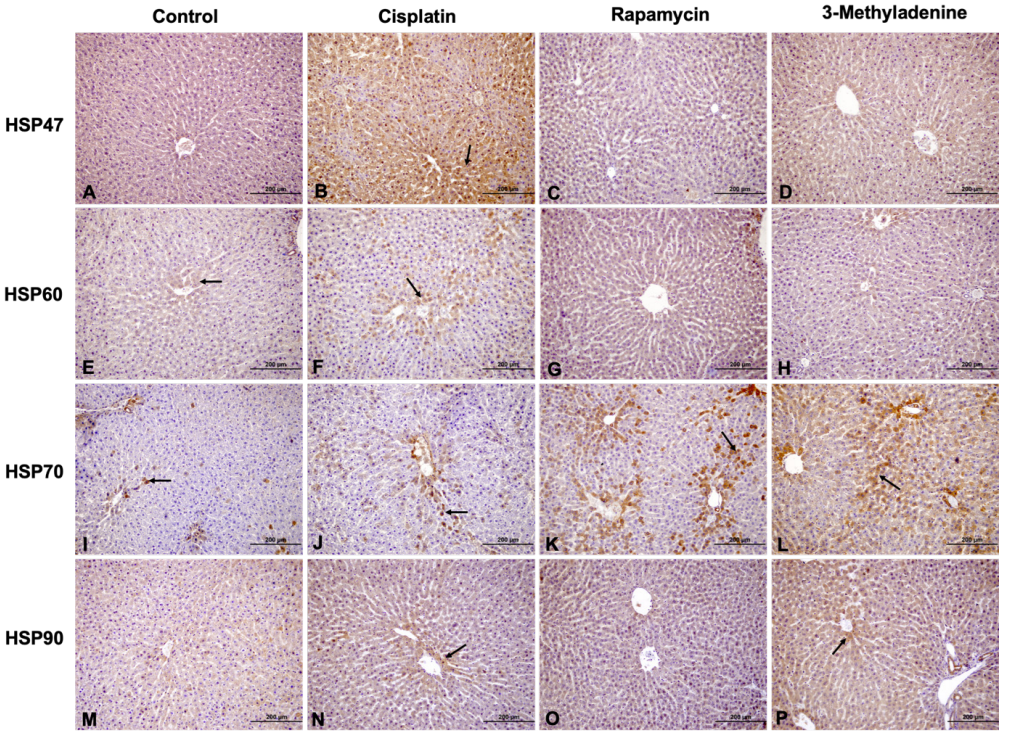

Figure 1. Liver tissue HSPs belonging to all groups were stained by immunohistochemistry. Black arrows indicate the stained areas corresponding to the proteins. HSP47 immunoreactivity of all groups (A, B, C, D). HSP60 immunoreactivity of all groups (E, F, G, H). HSP70 immunoreactivity of all groups (I, J, K, L). HSP90 immunoreactivity of all groups (M, N, O, P). Scale bar: 100 µm. Abbreviations: HSPs; Heat shock proteins

HSP47 was statistically increased in the cisplatin group compared to the control group. The reduction in the rapamycin and 3-Methyladenin group was statistically significant compared to both the control and cisplatin groups. HSP47 immunoreactivity was not statistically different between rapamycin and 3-Methyladenine groups. HSP60 immunoreactivity was increased in the cisplatin group compared to the control group. There was a statistically significant reduction in the Rapamycin and 3-Methyladenin groups compared to the cisplatin group. According to the results of HSP70 immunoreactivity, it showed a statistically significant increase in all other groups compared to the control group. The cisplatin group and the rapamycin group were similar. There was a statistically significant increase in the 3-Methyladenine group compared to both the cisplatin and rapamycin groups. HSP90 immunoreactivity was statistically significantly increased in the other groups compared to the control group. Compared to the cisplatin group, HSP90 immunoreactivity was decreased in the rapamycin group, which was statistically significant. The cisplatin group and the 3-Methyladenine groups were similar.

TUNEL-positive cells were observed in all groups. The results of TUNEL-positive cell counts in the groups are shown in Table 2, and their images are shown in Figure 2. According to Kruskal Wallis's results, there was a difference between Control and Cisplatin and 3-Methyladenine groups. The increased TUNEL+ cell count in the cisplatin group was similar to the 3-Methyladenine group. There was no statistical difference between control and rapamycin. There was a statistically significant decrease in the rapamycin group compared to the 3-methyladenine group.

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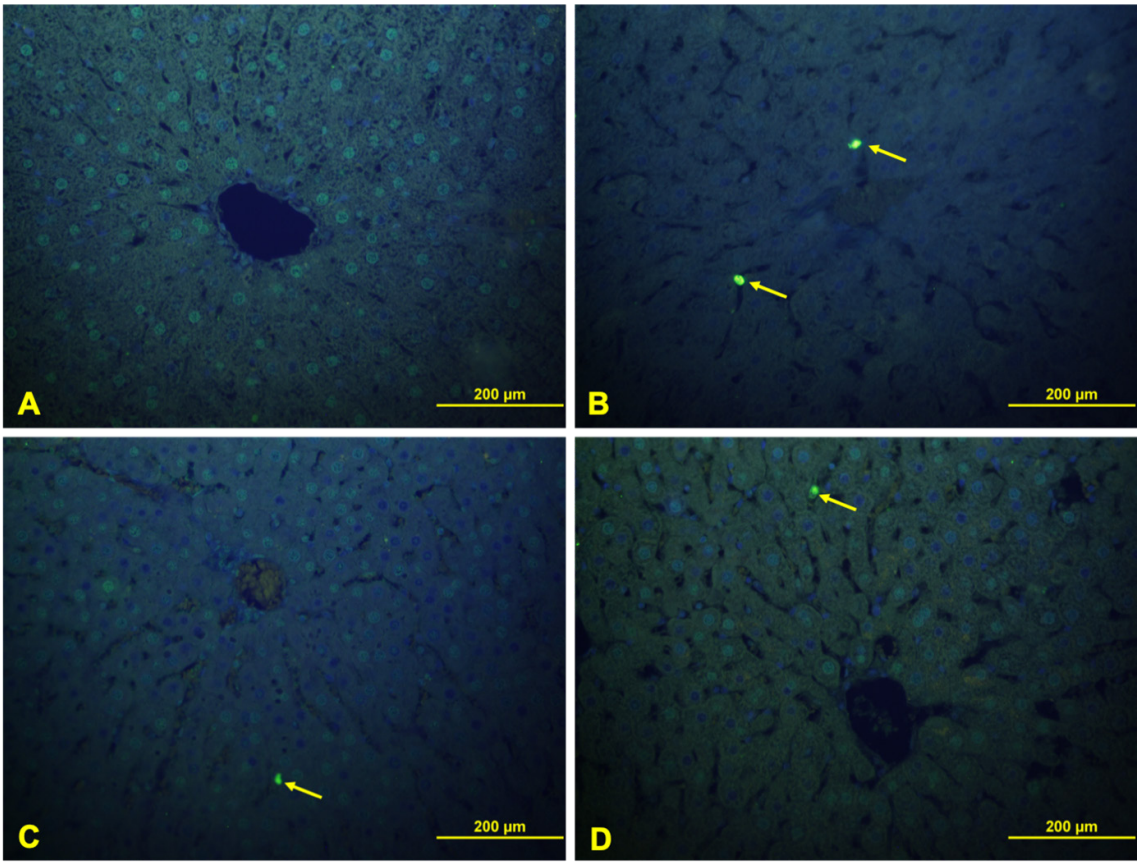

Figure 2. TUNEL staining of liver tissue. Images are given as FITC+DAPI. (A) Control group, (B) Cis group (C) Rapa group (D) 3MA group. The yellow arrow indicates TUNEL-positive cells. Scale bar 100 □m. Cis: Cisplatin, Rapa: Rapamycin, 3MA: 3-Methyladenine, FITC: Fluorescein isothiocyanate, DAPI: 4',6-diamidino-2-phenylindole

Table 2. Number of TUNEL-positive cells for all groups. It represents the number of TUNEL-positive cells per image of each animal. 105 fields were counted from each group

Groups	Control	Cis	Rapa	3MA	p
TUNEL-positive cells	0 (0-2)[a]	0 (0-4)[b]	0 (0-2)[a]	0 (0-4)[b]	0.001

Data are shown as ± standard deviation. p<0.05 was considered significant. There was no significant difference between the groups containing the same letter (a, b and c). Cis: Cisplatin, Rapa: Rapamycin, 3MA: 3-methyladenine

DISCUSSION

Although autophagy is an intracellular protective process that develops to break down its own reserves due to nutrient deficiency around the cell, it is a process that can cause the death of the cell (6). There are different types of functions and mechanisms defined as micro, macroautophagy and chaperone-mediated autophagy (CMA). CMA is the direct migration of substrate-specific proteins from the cytoplasm to the lysosomal membrane by the chaperone complex, which does not require vesicle formation or complex changes in the lysosomal membrane (20). In this process, the targeted protein is recognized by HSC70 (70 kDa heat shock-associated

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protein) and binds to LAMP2A (lysosome-associated membrane protein type 2A) located in the lysosomal membrane. Unfolding and translocation take place here and help degrade the HSC70 substrate protein in the lysosome lumen (21,22). Cytoplasmic and lysosomal HSC70 are very important for the cell. Because it cooperates with HSP90, HSP70, HSP40 and many more regulatory proteins (21). Of these, HSP70 and HSP90 are conserved ATP-dependent intracellular cytosolic chaperones that can cooperate with many co-chaperones to remodel and fold proteins (23,24). HSP60 is a versatile molecule that plays a role in physiological and pathological processes found in the cytosol, mitochondria and plasma membrane, as well as in the extracellular space, maintaining protein homeostasis (25). HSP47 is a molecular chaperone involved in the processing and secretion of collagen that binds to procollagen in the endoplasmic reticulum of the cell under normal conditions (26). Its expression and up-regulation in pathophysiological conditions such as fibrosis of the liver and other organs, and connective tissue diseases, which are closely related to collagen, are important (27). In this study, it was aimed to determine how these molecules are affected in the triangle of liver, cisplatin and autophagy.

Cisplatin increases DNA damage in the cell and causes hepatotoxicity with the release of reactive oxygen species (ROS) (17). Damaged organelles such as mitochondria, which cause ROS production in the cell, are eliminated by autophagy and oxidative stress is kept under control (28). It has been shown that cisplatin activates autophagy in hepatocyte cells both in vitro and in vivo, and this effect is further strengthened in the environment of ischemic cellular stress (29).

Cisplatin also induces mitochondrial damage in the cell and supports cancer cell and normal cell apoptosis (14, 30). In addition, in cisplatin toxicity, HSPs family forms are also transcriptionally up-regulated in response to stress. For example, altered expression of HSP70 has been reported in cisplatin ototoxicity and nephrotoxicity (12, 13). In our study, we found that HSP47, HSP60, HSP70 and HSP90 expressions from HSPs family increased in cisplatin toxicity. In addition, we observed that the expressions of HSPs changed in the groups in which we applied both rapamycin and 3-Methyladenin as autophagy activator/inhibitor. Against cisplatin toxicity, HSP47 and HSP60 expressions were well regulated by both rapamycin and 3-Methyladenine. Rapamycin regulated HSP70 and HSP90 expressions better than 3-Methyladenine. 3-Methyladenine was not as effective as rapamycin on HSPs expression. Rapamycin is key in the mTOR signaling pathway in processes associated with autophagy, apoptosis (31). Considering that it is also used as an autophagy activator, it can be said that it regulates the increased HSPs expressions in cisplatin toxicity. As soon as the fibrotic process begins to develop in the liver, markers such as HSP47 and alpha-smooth muscle actin (α-SMA) also differ (32). In addition to HSP47, it has been reported that HSP70 and HSP90 expressions are increased in diseases such as fibrotic liver, cirrhosis and hepatocellular carcinoma (33-35).

The increase of these molecules in hepatotoxic environments suggests that they may be a potential biomarker for liver diseases. According to the increased HSPs expression in our study, we can say that cisplatin toxicity is hepatotoxic. These increased HSPs expressions were best regulated by rapamycin. Suppression of autophagy ensures that while nutrients are abundant in the cell, autophagy does not accidentally degrade cellular components (36). 3-Methyladenine has been shown to improve liver fibrosis through inhibition of autophagy in hepatic stellate cell by investigating various signaling pathways (37). However, in this study, we found that 3-Methyladenine significantly increased the expression of HSPs against cisplatin toxicity.

The increase in HSPs is an indication that the physiological processes in the tissue are not going well. This suggests that rapamycin is more important in cisplatin toxicity based on its effect on HSPs, and therefore may be a more important hepatoprotective agent compared to 3-Methyladenine. It has been

reported that multitargeted drugs loaded with cisplatin and rapamycin may be a novel target in pancreatic cancer and liver metastasis (38). Rapamycin also showed this effect on apoptosis. According to our TUNEL results, rapamycin was also more effective on apoptosis than 3-Methyladenine and reduced cell death. In a study using the human liver cell line ($L0_2$), it was emphasized that the use of emodin, which is known to cause liver damage, together with 3-Methyladenin increased apoptosis while decreasing autophagy, and its use together with rapamycin increased autophagosome markers and reduced apoptosis (39). Administration of cisplatin with rapamycin and 3-Methyladenin shows the extent to which cisplatin-induced apoptosis changes. Rapamycin-induced autophagy attenuates cisplatin-induced increased apoptosis in the cell, suggesting that rapamycin may have a protective role against cellular apoptosis.

Study Limitations

In our study, our aim was to show liver HSP changes in cisplatin toxicity by using autophagy activators and inhibitors. For this purpose, we focused only on HSPs. However, the fact that biochemical parameters such as aspartate aminotransferase and alanine aminotransferase, which show how liver function changes, were not evaluated constitutes the limitation of the study.

CONCLUSION

Rapamycin and 3-Methyladenin are known as autophagy activators and inhibitors, respectively. In our study, we focused on the question of whether autophagy is activated or inhibited in cisplatin toxicity, and what is the role of HSPs. We found that HSPs increased in the cell in order to eliminate cisplatin-induced hepatotoxicity. Considering the contribution of HSP47 to fibrotic processes, the role of HSP60 in cellular homeostasis, and the structural presence of HSP70 and HSP90 in the cell, HSPs play an important role in maintaining the normal physiological process disrupted by cisplatin in the liver.

In this process, by activating autophagy, cisplatin-induced impaired protein-containing process takes place as HSPs mediator molecule and also contributes to the regulation of apoptosis. These results suggest that rapamycin may have hepatoprotective effects by regulating HSPs and reducing apoptosis in the treatment of cisplatin more effectively than 3-Methyladenine.

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